Universidade Estadual de São Paulo (UNESP) – Campus Jaboticabal

Centro de Aquicultura (CAUNESP)

RELATÓRIO CIENTÍFICO DE PÓS DOUTORADO

Título: Determinação de Florfenicol e Metabólitos em tilápia-do-Nilo por LC-UHPLC-MS/MS: Desenvolvimento, Validação e Aplicação

Bolsista: Gabriela Freitas Pereira de Souza

Orientadora: Fabiana Pilarski

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RESUMO

Este estudo teve como objetivo desenvolver e validar um método analítico para a quantificação de florfenicol (FF) e seus principais metabólitos, florfenicol amina (FFA), florfenicol álcool (FFOH) e florfenicol monoclorado (FFCl), em filés de tilápia-do-Nilo (*Oreochromis niloticus*), utilizando extração em fase sólida on-line (SPE on-line) acoplada à cromatografia líquida de ultra-alta eficiência com espectrometria de massas sequencial (UPLC-MS/MS).

Para tal, foi realizada a síntese de padrões analíticos dos metabólitos e um método analítico foi desenvolvido e validado de acordo com os critérios estabelecidos por guias internacionais, sendo avaliada a seletividade, linearidade, exatidão, precisão, recuperação, efeito matriz e estabilidade. Foi realizado um experimento de administração de ração medicada com florfenicol para tilápia-do-Nilo e os tecidos desses animais foram analisados utilizando a metodologia desenvolvida. Os resultados demonstraram que a metodologia proposta é robusta e adequada para fins de monitoramento de resíduos de florfenicol em produtos de origem aquática, sendo aplicável em contextos regulatórios e de segurança alimentar. Esses resultados foram compilados em um artigo cientifico publicado no período.

1. INTRODUÇÃO

O florfenicol (FF) é um antimicrobiano sintético de amplo espectro pertencente à classe dos anfenicóis, cuja ação baseia-se na inibição da síntese proteica bacteriana por meio da ligação à subunidade 50S do ribossomo (Guo et al. 2024). Na aquicultura, o FF é amplamente empregado no tratamento de infecções bacterianas em peixes, como a septicemia hemorrágica causada por *Aeromonas spp*. e a estreptococose provocada por *Streptococcus agalactiae* em tilápias. Atualmente, seu uso está aprovado para diversas espécies aquícolas em mais de 25 países (Mallik et al. 2023).

Estudos demonstram que após administração oral, o FF é extensivamente metabolizado no organismo animal, originando florfenicol amina (FFA), florfenicol álcool (FFOH) e florfenicol monocloro (FFCl). Há evidências de que essas vias metabólicas podem ocorrer com ou sem a formação intermediária de florfenicol ácido oxâmico (FCOOH) (Anadon et al., 2008).

A maioria dos estudos sobre o metabolismo do FF em peixes concentra-se na detecção do FFA, metabólito frequentemente identificado em espécies como truta arco-íris, salmão-do-atlântico, bagre-coreano, pacu e tilápia (Hormazabal et al. 1993; Horsberg et al. 1996; Park et al. 2006; Valim et al. 2018). No entanto, diversos autores apontam limitações dessa abordagem, especialmente em espécies tropicais como a tilápia, cujo metabolismo apresenta características distintas. De fato, o FFA não foi detectado em linguado-oliva (Lim et al. 2010) nem em bacalhau (Samuelsen et al 2003), reforçando a necessidade de uma investigação mais abrangente dos metabólitos formados.

A biotransformação do FF é influenciada por fatores como espécie, temperatura e salinidade da água (Gaikowski et al 2013), os quais podem impactar de forma significativa o perfil de resíduos nos tecidos. Apesar da relevância científica e regulatória da caracterização completa desses metabólitos, não há relatos na literatura sobre estudos farmacocinéticos envolvendo os derivados do FF em peixes, lacuna que se deve, em grande parte, à ausência de métodos analíticos apropriados para sua quantificação.

O resíduo marcador para o florfenicol (FFC) compreende a soma do fármaco e seus principais metabólitos — FFCl, FFCOOH e FFOH — expressos como florfenicol amina (FFA) segundo estabelecido pelo Comitê de Produtos Veterinários (CVMP) da Agência Europeia de Medicamentos (EMA). Para viabilizar essa conversão, uma etapa de hidrólise deve preceder a extração com solventes orgânicos a partir da matriz biológica. Entretanto, a inclusão dessa etapa torna o preparo da amostra mais demorado e complexo, aumenta os efeitos de matriz e inviabiliza a obtenção de um

perfil metabólico detalhado, já que após a hidrólise não é possível distinguir os metabólitos. Além disso, a indisponibilidade comercial de padrões analíticos dos metabólitos limita consideravelmente o desenvolvimento de métodos multicomponentes. Estudos clássicos, como o de Horsberg et al. (1994), recorreram ao uso de compostos radiomarcados, técnica que, embora eficaz, apresenta custos elevados e requer infraestrutura especializada.

Diante desse cenário, o presente estudo propôs o desenvolvimento e a validação de um método analítico robusto, baseado em SPE on-line acoplada à UPLC-MS/MS, com capacidade para quantificar simultaneamente o FF e três de seus principais metabólitos (FFA, FFOH e FFCl), previamente sintetizados e caracterizados por RMN e HRMS. A aplicação deste método em filés de tilápia tratados experimentalmente visou gerar dados inéditos sobre o perfil de resíduos de FF sob condições tropicais, contribuindo para o aprimoramento da segurança alimentar e o fortalecimento de estratégias regulatórias na aquicultura.

2. OBJETIVOS

- Desenvolver e validar um método analítico baseado em SPE on-line acoplado à UPLC-MS/MS para quantificação simultânea de florfenicol e seus principais metabólitos em filés de tilápia-do-Nilo;
- Realizar a síntese e caracterização estrutural de padrões analíticos puros dos metabólitos FFA, FFOH e FFCl;
- Realizar o tratamento de tilápia com florfenicol através de ração medicada segundo as recomendações comerciais;
- Aplicar o método desenvolvido as amostras de filés de tilápia tratadas experimentalmente com florfenicol.

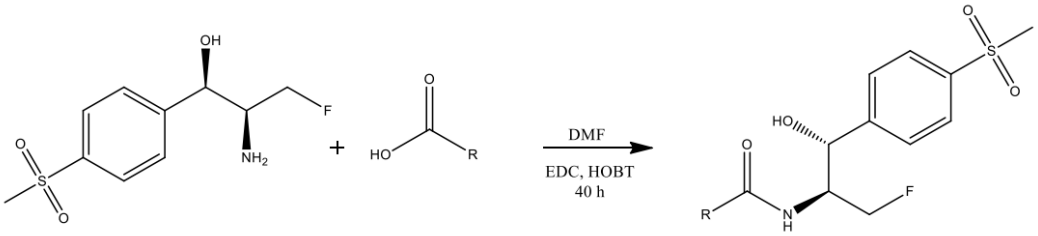
3. MATERIAIS E MÉTODOS

3.1. Síntese dos metabólitos

Os três principais metabólitos intermediários do florfenicol (FF) — florfenicol álcool (FFOH), florfenicol ácido oxâmico (FFCOOH) e florfenicol monocloro (FFCl) — são compostos do tipo amida. Assim, uma estratégia sintética viável consiste na reação entre a florfenicol amina (FFA) e diferentes ácidos carboxílicos: ácido glicólico, ácido oxálico e ácido 2-cloroacético, respectivamente. Considerando o elevado custo do padrão analítico de FFA e o menor custo de formulações veterinárias injetáveis contendo FF, optou-se por extrair o fármaco de um produto comercial e convertê-lo em FFA.

A extração do FF foi realizada a partir da formulação injetável utilizando acetato de etila como solvente. Em seguida, o composto extraído foi purificado por cromatografia líquida preparativa, empregando uma mistura de hexano:acetato de etila (1:4, v/v) como fase móvel. O material purificado foi então submetido à hidrólise ácida com ácido clorídrico concentrado a 100 °C por 2 horas, resultando na conversão do FF em FFA. Após a reação, o pH da mistura foi ajustado para valores acima de 10 com hidróxido de sódio, permitindo a extração do FFA com acetato de etila.

A síntese dos metabólitos FFCl e FFOH seguiu a mesma abordagem geral, diferenciando-se apenas quanto ao ácido carboxílico empregado e às condições de purificação. Para isso, 100 mg de FFA (0,40 mmol) foram dissolvidos em dimetilformamida (DMF), seguidos da adição dos ácidos cloroacético (para FFCl) ou glicólico (para FFOH), além dos reagentes de acoplamento 1-etil-3-(3-dimetilaminopropil) carbodiimida (EDC) e hidroxibenzotriazol (HOBT), ambos adicionados em proporções de 1,2 equivalentes em relação ao FFA. As reações foram conduzidas sob agitação por 40 horas à temperatura ambiente. Após esse período, o solvente foi removido por evaporação. Os produtos obtidos foram reconstituídos em pequenas quantidades de metanol e purificados por cromatografia líquida preparativa, utilizando-se acetato de etila:hexano (4:1, v/v) como fase eluente para o FFCl e acetato de etila puro para o FFOH. Os rendimentos obtidos foram de 95% para FFA, 72% para FFOH e 83% para FFCl. A Figura 1 apresenta o esquema geral da reação do FFA com os ácidos carboxílicos para a obtenção dos respectivos metabólitos.



Metabólitos	R
Monocloro florfenicol	CH_2Cl
Florfenicol álcool	CH_2OH
Florfenicol ácido oxâmico	COOH

Figura 1. Esquema geral da reação do FFA com ácidos carboxílicos para formação dos metabólitos.

A caracterização estrutural dos compostos sintetizados foi realizada por espectroscopia de Ressonância Magnética Nuclear (RMN) de ^{1}H (500 MHz) e ^{13}C (125 MHz) em soluções de DMSO-d_6, à temperatura ambiente. Também foram obtidos

espectros de massas de alta resolução por espectrometria de massas utilizando um sistema Q-Exactive Orbitrap, com ionização por electrospray (ESI) em modo negativo. As purezas dos compostos foram determinadas por RMN quantitativo, utilizando 4-(dimetilamino)benzoato de etila como padrão interno. Os valores de pureza obtidos foram de 98% para o FF e de 94% para o FFA, FFOH e FFCl, considerados adequados para uso como padrões analíticos no desenvolvimento e validação do método proposto.

No caso do metabólito FFCOOH, a tentativa de síntese pela mesma rota não foi bem-sucedida, uma vez que se observou a decomposição do ácido oxálico na presença do reagente de acoplamento EDC. Rotas alternativas foram avaliadas, mas não apresentaram resultados satisfatórios. A literatura especializada também sugere que esse metabólito pode não estar consistentemente envolvido na via metabólica do FF (Anadón et al., 2008). Em estudo de depleção radiométrica conduzido com ^{14}C-florfenicol administrado a salmões mantidos a 10 ºC, ao longo de 10 dias com dose de 10 mg/kg peso corporal, o FFCOOH representou apenas 0,70% do total de resíduos radioativos no músculo e entre 0,4% e 1,2% na pele (EMEA, 2000). Esses achados indicam tratar-se de um metabólito de baixa relevância (<10%), cuja caracterização não é considerada essencial em avaliações de risco, conforme critérios estabelecidos pelo Comitê Conjunto FAO/OMS de Especialistas em Aditivos Alimentares (JECFA), vinculado ao Codex Alimentarius. Dessa forma, considerando sua baixa ocorrência e o escopo deste estudo, decidiu-se não prosseguir com esforços adicionais para a síntese de FFCOOH.

A síntese dos metabólitos foi realizada em colaboração com o Instituto de Química da Unicamp.

3.2 Desenvolvimento e validação de método analítico para a determinação de resíduos de FFC e metabólitos utilizando LC-UHPLC-MS/MS

3.2.1 Preparo das Amostras

Amostras de 3,0 g de filé de peixe, previamente trituradas com gelo seco, foram adicionadas a tubos de polipropileno juntamente com os padrões internos isotopicamente marcados FFA-d_3 e FFC-d_3 (500 ng g^{-1} cada). Em seguida, foram adicionados 10 mL de acetonitrila, com agitação em vórtex por 1 minuto. Após a adição de 8,0 g de sulfato de magnésio ($MgSO_4$) e 2,0 g de acetato de sódio, a mistura foi novamente agitada por 1 minuto e então centrifugada a 1056 g por 5 minutos. Uma alíquota de 5 mL do sobrenadante foi transferida para outro tubo contendo 750 mg de $MgSO_4$ e 125 mg de fase C18. Após agitação e nova centrifugação, o sobrenadante foi

evaporado sob fluxo de nitrogênio, reconstituído em 1 mL de água/metanol (65:35, v/v), filtrado com filtro PVDF de 0,22 µm e submetido à análise por LC-UHPLC-MS/MS.

3.2.2 Desenvolvimento do Método LC-UHPLC-MS/MS

Para a quantificação dos analitos, foi utilizado um sistema LC-UHPLC acoplado a espectrômetro de massas com analisador triplo quadrupolo (QqQ) operando em modo de ionização electrospray (ESI). A separação cromatográfica foi realizada em duas dimensões (LC-LC). A primeira dimensão empregou uma coluna OASIS HLB (30 × 2,1 mm, 20 µm) e a segunda, uma coluna analítica Acquity UPLC BEH C18 (50 × 2,1 mm, 1,7 µm). Este arranjo permitiu a concentração dos analitos, limpeza da matriz, injeção de maiores volumes e fracionamento seletivo da amostra.

O sistema conta com duas bombas — uma binária (BSM) e uma quaternária (QSM) — e é operado por meio de duas válvulas comutáveis. Inicialmente, a amostra é carregada na coluna 1D via QSM (posição 1). Após a eluição da faixa de interesse, a válvula comuta para a posição 2, transferindo os analitos para a coluna 2D via BSM. A detecção ocorre no espectrômetro de massas operando em modo de reações selecionadas (SRM), com uma transição m/z para quantificação e outra para confirmação. A padronização foi realizada por calibração interna, utilizando padrões marcados isotopicamente.

Durante o desenvolvimento do método, foram avaliados parâmetros como: volume e solvente de carregamento da amostra, volume de transferência entre dimensões, uso de aditivos na fase móvel, regeneração da coluna 1D e volume de injeção.

Os parâmetros empregados no espectrômetro de massas sequencial, bem como, os parâmetros para as transições de quantificação monitoradas no modo SRM (do inglês - Selective Reaction Monitoring) para o FFA, FFC e FFC-d3, foram baseados em trabalho anterior (Marques, 2018). Enquanto para os demais analitos (FFOH, FFCl e FFA-d3), as condições foram estabelecidas a partir de infusão direta dos compostos na concentração de 1,0 µg mL^{-1} no espectrômetro de massas (Tabela 1).

Tabela 1. Parâmetros para transições monitoradas no espectrômetro de massas

Analito	ESI	Íon precursor m/z	Íon produto m/z	Transição	Voltagem do cone (V)	Energia de colisão (eV)	Dwell time (s)
Florfenicol amina	+	248	130	Quantificação	30	20	0,06
		248	230	Confirmação			
Florfenicol amina-d_3	+	251	233	Quantificação	28	23	0,03
		251	130	Confirmação			
Florfenicol	-	356	185	Quantificação	30	20	0,5
		356	339	Confirmação			
Florfenicol-d3	-	359	188	Quantificação	30	20	0,05
		359	339	Confirmação			
Monocloro florfenicol	-	322	185	Quantificação	34	34	0,05
		322	119	Confirmação			
Florfenicol álcool	-	304	100	Quantificação	34	26	0,05
		304	284	Confirmação			

3.2.3. Validação do método

O método foi validado segundo VICH GL49 (VICH 49). Os parâmetros avaliados foram: seletividade, linearidade (20 a 4000 µg/kg), exatidão, precisão intra e interdia (n = 6), recuperação, efeito matriz (comparação entre curva em solvente e matriz) e estabilidade (congelamento/descongelamento, armazenamento a longo prazo).

O desenvolvimento e validação do método analítico foram realizados em colaboração com o Instituto de Química da Unicamp.

3.3. Administração de florfenicol em modelo animal

Neste ensaio experimental, foram utilizados exemplares machos de tilápia-do-Nilo (*Oreochromis niloticus*) com peso médio de 600 g. Os animais foram distribuídos aleatoriamente em tanques de fibra de vidro com capacidade de 450 litros. O sistema foi mantido em fluxo contínuo com água de poço e aeração constante. Durante o período de aclimatação de 30 dias, os peixes receberam uma dieta comercial não medicada (Aqua Pirá Acabamento 4–5 mm, Guabi).

Após esse período, os peixes foram alimentados com ração contendo florfenicol (FFC), na dose nominal de 10 mg kg^{-1} de peso corporal, por períodos consecutivos de sete e dez dias. A ração medicada foi preparada em laboratório por meio de revestimento superficial (*top coating*), seguindo o protocolo descrito por

Marques (2018). A concentração de FFC na ração foi determinada por UHPLC-DAD, obtendo-se um valor médio de 1280 ± 21 mg kg^{-1} (n = 4). A quantidade de ração medicada fornecida foi calculada com base na biomassa total de cada tanque e administrada sempre pela manhã, considerando uma taxa alimentar de 1% do peso vivo. A biometria dos animais foi realizada 13 dias antes do início da administração da ração medicada.

A coleta de amostras foi realizada em pontos específicos ao longo do tempo (sete e dez dias de tratamento), com o filé de cinco peixes sendo obtido em cada tempo de abate. Todos os animais sacrificados foram pesados imediatamente, e as medidas de comprimento total e padrão foram registradas. Um grupo controle, mantido sob as mesmas condições, não recebeu a dieta medicamentosa.

Os filés foram coletados 3 horas após a última administração e armazenados a –20 °C, até análise, seguindo o método validado descrito anteriormente.

4. RESULTADOS E DISCUSSÃO

Os resultados completos obtidos estão apresentados no **Anexo 1**, que corresponde ao artigo científico publicado com os dados experimentais detalhados. A seguir, apresentam-se de forma resumida os principais achados obtidos durante a validação do método e sua aplicação em amostras reais.

O método desenvolvido demonstrou desempenho analítico dentro dos parâmetros de aceitação. A linearidade foi comprovada com coeficientes de determinação (r) iguais ou superiores a 0,996 em toda a faixa de concentração avaliada para os quatro compostos-alvo. Os limites de quantificação (LOQ) foram estabelecidos entre 20 e 50 µg/kg. A precisão, avaliada por meio da repetibilidade (intra-dia) e da reprodutibilidade (inter-dia), apresentou valores de desvio padrão relativo (RSD) inferiores a 13%, demonstrando robustez metodológica. A exatidão oscilou entre 94% e 109% nos três níveis de fortificação (50, 500 e 4000 µg/kg), enquanto os percentuais de recuperação variaram de 81,5% a 102,6%, dependendo do metabólito e da concentração avaliada. Um efeito matriz considerável foi observado, especialmente para FFA e FFCl, com supressão de sinal superior a 40%. No entanto, tal interferência foi devidamente compensada pela utilização de curvas matrizadas, garantindo a confiabilidade dos resultados. A estabilidade dos analitos foi confirmada mediante análises após ciclos sucessivos de congelamento/descongelamento e armazenamento prolongado a –20 °C.

A aplicação do método em amostras *incurred* evidenciou o FF como principal composto residual detectado, seguido pelo metabólito FFOH, que se destacou como o derivado mais persistente nos tecidos comestíveis. Essa distribuição metabólica

diverge dos dados obtidos em espécies de clima temperado, como o salmão, nas quais a FFA foi reportada como metabólito predominante (Horsberg et al., 1994). Essas discrepâncias reforçam a importância de estudos farmacocinéticos específicos para diferentes espécies e condições ambientais, como também apontado por Gaikowski et al. (2010).

Do ponto de vista regulatório, os achados deste estudo são relevantes ao evidenciar a viabilidade de monitoramento direto dos metabólitos mais representativos sob a ótica toxicológica e farmacocinética, superando a limitação dos métodos baseados exclusivamente na hidrólise e quantificação indireta de FFA. A implementação bem-sucedida do método em amostras reais obtidas em condições experimentais controladas possibilita, ainda, estimativas mais precisas dos perfis de depleção de cada composto, aspecto fundamental para a determinação de períodos de carência.

Em síntese, este estudo representa um avanço metodológico importante no monitoramento de resíduos de antimicrobianos na aquicultura. A abordagem analítica desenvolvida amplia o conjunto de ferramentas disponíveis para o controle de resíduos em peixes cultivados, além de abrir caminhos para métodos futuros que considerem múltiplos marcadores. Adicionalmente, a metodologia proposta pode ser adaptada a outras espécies e sistemas produtivos, contribuindo para a segurança alimentar e o uso racional de antibióticos em ambientes aquícolas.

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Anexo 1

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Quantification of florfenicol and its metabolites in fillets of Nile tilapia: Synthesis of metabolites and validation of an on-line solid-phase extraction-ultra high-performance liquid chromatography-tandem mass spectrometry method

Anna Paula Rocha de Queiroga [a,b], Gabriela Freitas Pereira de Souza [a,c], Inácio Mateus Assane [c], Thiago Messias [a], Fabiana Pilarski [c,d], Michael Schloter [e], Airton Gonçalves Salles Jr. [b,*], Susanne Rath [a]

[a] Department of Analytical Chemistry, Institute of Chemistry, University of Campinas, Rua Monteiro Lobato, 270, Cidade Universitária, Campinas, SP 13083-862, Brazil
[b] Department of Organic Chemistry, Institute of Chemistry, University of Campinas, Rua Monteiro Lobato, 270, Cidade Universitária, Campinas, SP 13083-862, Brazil
[c] Laboratory of Microbiology and Parasitology of Aquatic Organisms, São Paulo State University (UNESP), Aquaculture Center of UNESP, Jaboticabal, SP 14884-900, Brazil
[d] Graduate Program in Agricultural and Livestock Microbiology, São Paulo State University (UNESP), School of Agricultural and Veterinary Sciences, Jaboticabal, SP 14884-900, Brazil
[e] Helmholtz Zentrum München, Comparative Microbiome Analysis Research Unit, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany

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On-line solid-phase extraction-ultra high-performance liquid chromatography-tandem mass spectrometry

ABSTRACT

This study concerns the synthesis of the florfenicol (FF) metabolites florfenicol amine (FFA), florfenicol alcohol (FFOH), and monochloroflorfenicol (FFCl), for their subsequent use as reference standards in On-line solid-phase extraction-ultra high-performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS) analysis. The metabolites were characterized using ^{1}H and ^{13}C NMR, as well as HRMS, and their purities were confirmed by quantitative NMR to ensure analytical reliability. Validation of the developed analytical method showed that it presented acceptable performance, with linearity >0.99 for all the target analytes, accuracies within ±10 % of nominal concentrations, and intra- and inter-day precisions within 15 %. Application of this method to fillets from fish that had been treated with florfenicol (dose of 10 mg/kg bw daily) demonstrated its effectiveness in consistently detecting FF and its metabolites throughout the treatment. The results emphasized the utility of the method for enhancing pharmacokinetic and residue depletion research. The ability to precisely monitor the drug and its metabolites in treated fish provides important insights into florfenicol metabolism, laying the groundwork for further comprehensive profiling studies of metabolites in fish tissue.

1. Introduction

Florfenicol (FF) is a broad-spectrum antimicrobial belonging to the amphenicol family, whose action involves the inhibition of protein synthesis by binding to the ribosomal subunits of bacteria. In aquaculture, FF is indicated for the treatment of hemorrhagic septicemia caused by *Aeromonas* sp. and streptococcosis caused by *Streptococcus agalactiae* in tilapia species and their hybrids. It is also used to treat red mouth disease caused by *Yersinia ruckeri* in rainbow trout. Its use is approved in 25 countries for a wide variety of fish species [1], with a recommended dosage of 10 mg/kg of body weight for 10 consecutive days. The literature reports the absorption, distribution, metabolism, and excretion of FF in animals such as veal calves [2], cattle [3], sheep [4], swine [5], poultry [6], crucian carp [7], rainbow trout [8] and tilapia [9]. After administration, FF is transformed into florfenicol amine (FFA), according to two possible pathways [6]. In the first pathway, FF is metabolized to FFA via florfenicol alcohol (FFOH), with or without florfenicol oxamic acid (FCOOH) as an additional intermediate. In the second pathway, the conversion involves monochloroflorfenicol (FFCl) as an intermediate. These pathways are summarized in Fig. 1.

The European Union has established maximum residue limits for florfenicol in fish (muscle plus skin, in natural proportions), considering

* Corresponding author.
E-mail address: hoffman@unicamp.br (A. Gonçalves Salles).

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the sum of florfenicol (FF) and its metabolites, measured as florfenicol amine (FFA) with a limit set at 1000 μg kg^{-1}. To adhere to this specific definition, it is recommended to perform a hydrolysis step prior to any solvent extraction, to convert FF and all its metabolites into FFA.

Various methodologies employing liquid chromatography for detecting and quantifying FF and FFA have been described in the literature, some of them incorporating an acid hydrolysis step [10–22]. However, none of these methods enable the detection of all the FF metabolites. In their 1994 metabolism profiling study, Horsberg et al. [23] used radiolabeled florfenicol ([^{14}C]-florfenicol) to detect and quantify florfenicol (FF), its amine and alcohol derivatives in Atlantic salmon muscle tissue. They found that 90 % of the radioactivity could be extracted, and noted that water temperature during fish harvesting influenced the ratios of the metabolites. The experiments, however, were costly and required infrastructure not available in all laboratories for daily use.

Fish metabolism is known to vary between species and can also be influenced by environmental temperature [24]. As a result, the distribution and relative proportions of different metabolites can differ under various conditions. To gain a more detailed understanding of how antimicrobials are metabolized in different fish species and which metabolites are present over time, pharmacokinetic and metabolism studies must be designed to distinguish between the parent antimicrobial compound and its metabolites at various time intervals. This distinction is crucial because each molecule may have unique microbicidal properties. Achieving this level of detail requires an analytical method that can identify and quantify each of these molecular species. These studies are vital for accurately determining drug metabolism pathways, understanding all molecular species in consumable tissues, establishing safe withdrawal periods, and ensuring compliance with strict food safety regulations. However, the feasibility of such studies depends critically on the availability of an analytical method capable of quantifying all present metabolites. Hence, it is highly desirable to introduce novel chromatographic methodologies capable of detecting and quantifying all the florfenicol metabolites.

Developing such methods necessitates the acquisition of costly commercial analytical standards for the metabolites, which are not readily accessible in every location in a timely manner. Alternatively, synthesizing the metabolites is a viable option. However, no synthesis pathways for florfenicol metabolites have been reported in the literature to date. Therefore, the present study focused on three main objectives: (1) Establishment of a synthesis pathway for florfenicol metabolites, specifically florfenicol amine, florfenicol alcohol, and monochloroflorfenicol; (2) Development and validation of an on-line solid-phase extraction-ultra high-performance liquid chromatography-tandem mass spectrometry method (SPE-UHPLC-MS/MS) for the quantification of florfenicol and its metabolites; (3) Application of the developed method to analyze samples from florfenicol-treated fish (tilapia), demonstrating its suitability and applicability for this purpose.

2. Materials and methods

2.1. Chemicals and reagents

The analytical standards florfenicol-d_3 and florfenicol amine-d_3 were sourced from Toronto Research Chemicals Inc. (Canada). The standards florfenicol (FF), florfenicol amine (FFA), and 4-(dimethylamino)benzoate (99.69 % purity) were obtained from Sigma-Aldrich (Germany). The analytical grade reagents hydrochloric acid, sodium hydroxide, and sodium acetate were purchased from Synth (Brazil). Other reagents including glycolic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 1-hydroxybenzotriazole hydrate, chloroacetic acid, and magnesium sulfate were purchased from Sigma-Aldrich. Di-potassium hydrogen phosphate and formic acid were obtained from Merck

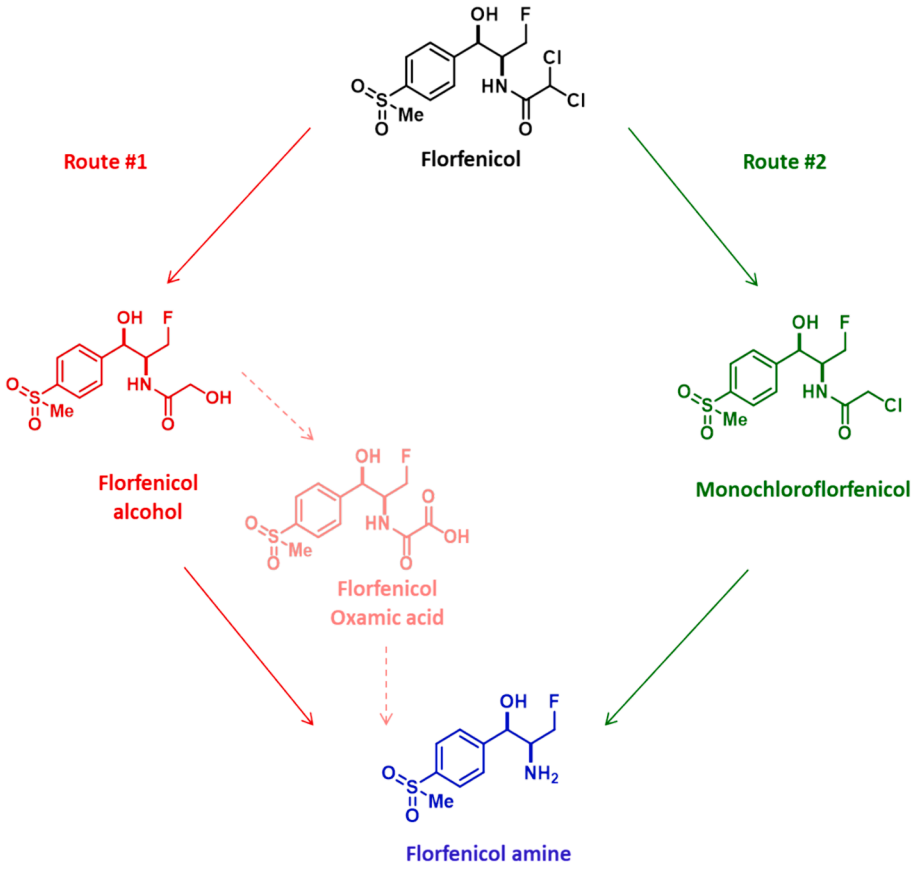


Fig. 1. Florfenicol biotransformation pathways. Based on Anadón et al. (2008).

(Germany). Discovery DSC-18 was obtained from Supelco (USA) and hydrochloric acid from Vetec (Brazil). Acetonitrile was obtained from J. T. Baker (China), methanol from Supelco (Germany), hexane from UltimAR, and ethyl acetate from AppliChem (Germany). All the solvents were of HPLC grade.

Flash column chromatographic separations were performed using silica gel (Sigma-Aldrich) with pore size of 60 Å and particle sizes of 230–400 mesh (40–63 μm). Thin layer chromatography (TLC) was performed using pre-coated TLC sheets (GF_{254}, 0.20 mm thickness, Macherey-Nagel, Germany).

The water used throughout the work was obtained from a Milli-Q purification system (Millipore, USA).

Animal experiments were approved by the Ethics Committee for the Use of Animals (CEUA) at São Paulo State University (UNESP), under certificate of approval No. 1115/2023, in accordance with the regulations issued by the Brazilian National Council for the Control of Animal Experimentation (CONCEA). Additionally, the experiments received approval from the Ethics Committee for the Use of Animals at the University of Campinas, Brazil.

2.2. Stock and working solutions

Stock standard solutions of each analyte and internal standard were prepared in methanol, at concentrations of 1000 μg mL^{-1} (FF, FFA, FFCl, and FFOH) or 100 μg mL^{-1} (FFA-d_3 and FF-d_3). Intermediate standard solutions at concentrations of 100 and 10 μg mL^{-1} were prepared by mixing all the compounds and using appropriate dilutions of stock solutions in methanol. All the solutions were stored at 4 °C for no longer than six months. Working standard solutions were prepared daily by diluting the intermediate standard solutions with ultrapure water.

2.3. Characterization and analytical techniques

Thin layer chromatography (TLC) was performed using TLC sheets (Macherey-Nagel, Germany), with visualization using short wavelength UV light (254 nm) or $KMnO_4$ staining solution followed by heating.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained at 500 MHz, using an Avance III instrument (Bruker, Germany) and DMSO-d_6 solutions, at ambient temperature. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were obtained at 125 MHz, using DMSO-d_6 solutions, at ambient temperature. Chemical shifts (δ) were given in ppm and the residual solvent signals were used as references for the ^{1}H and ^{13}C NMR spectra (DMSO-d_6: δH=2.50 ppm, δC=39.50 ppm).

The purities of the synthesized FF, FFA, FFOH, and FFCl were determined using quantitative NMR, with ethyl 4-(dimethylamino) benzoate as the internal standard. For each sample, 16 scans were acquired, totaling 32,768 data points. A spectral width of 30 ppm was used and a 60-second delay between pulses ensured full T1 relaxation of protons.

High-resolution mass spectra were recorded using a Q Exactive Orbitrap spectrometer (Thermo Scientific, USA) equipped with an electrospray ionization source (ESI) operating in negative mode.

Analyte quantification employed an SPE-UHPLC-MS/MS system (Waters, USA) featuring an autosampler with a single injection capacity of 250 μL, binary (BSM) and quaternary (QSM) solvent pumps, a column manager for SPE-UHPLC mode operation, and a triple quadrupole mass detector (Xevo TQD Zspray, Waters) with an electrospray ionization source (ESI) operating in positive mode. Data acquisition and equipment control were managed with MassLynx v.4.1 software (Waters Laboratory Informatics), while chromatogram analysis was performed using TargetLynx software (Waters Laboratory Informatics).

2.4. Synthesis of florfenicol metabolites

2.4.1. Quantitative ^{1}H NMR

An exact mass (between 10–15 mg) of both the sample and the reference standard (4-(dimethylamino) ethyl benzoate, 99.47 %) were weighed and dissolved in DMSO-d within an NMR tube. This tube was then subjected to analysis using the following parameters: relaxation time of 60 s, pulse sequence: zg, receiver gain: 32, SW (cyclical): 12019.23 Hz, sweep width: 12018.86 Hz and 64 scans. The area of the peaks at 6.7 ppm (2H, for the standard), at 3.19 ppm (3H, for FFOH) and at 3.18 ppm (3H, for FFCl) were used to perform quantitative calculations based on the equation: $P_a=(I_a/I_s)\times(M_a/X_a)\times(P_s.X_s/M_s)\times(N_s/N_a)$, where P_a and P_s represent the purity of the analyte and standard, respectively. I_a and I_s denote the integrals of the analyte and standard. M_a and M_s are the molecular weights of the analyte and standard, while X_a and X_s refer to the masses of the analyte and standard. N_a and N_s indicate the number of protons in the analyte and standard. All measurements were conducted in triplicate to ensure reliability.

2.4.2. Starting material: Florfenicol (FF)

Florfenicol was isolated and purified starting with a commercially available injectable veterinary drug that had a nominal FF concentration of 0.3 g mL^{-1}. The extraction process was performed using ethyl acetate (3 × 10 mL). The organic fractions were combined and magnesium sulfate was added to remove any residual moisture, followed by removal of the solvent using a rotary evaporator at a bath temperature of 40 °C. Further purification of the extract was performed by silica flash column chromatography, employing an eluent consisting of acetate:hexane (80:20 v/v). The process yielded FF as a white solid with a 92 % yield. The purity of the resulting FF was quantitatively assessed using quantitative ^{1}H NMR, with 4-(dimethylamino) ethyl benzoate as a reference standard. The calculated purity was 98 %. The retention factor for florfenicol in acetate:hexane (80:20 v/v) solution was determined to be 0.53.

2.4.3. Florfenicol amine (FFA)

A 250 mg sample of florfenicol was weighed and transferred to a 5 mL glass vial with a screw cap, followed by addition of 1 mL of water and 0.5 mL of concentrated HCl. The vial was sealed under air atmosphere and agitated overnight at a temperature of 100 °C. Subsequently, the vial was cooled to room temperature and addition was made of 1 mL of water and solid NaOH until reaching pH 14. Subsequently, liquid–liquid extractions were carried out using ethyl acetate (3 × 5 mL) and dichloromethane (3 × 5 mL). The organic phase was dried by the addition of magnesium sulfate, followed by filtration and subsequent removal of the solvent using a rotary evaporator at a bath temperature of 40 °C. The resulting florfenicol amine was obtained in the form of a white solid, with a 95 % yield. The purity (94 %) of the FFA was determined by quantitative ^{1}H NMR, using 4-(dimethylamino) ethyl benzoate as a reference standard. The retention factor for FFA in methanol:ethyl acetate (10:90 v/v) solution was determined to be 0.37.

2.4.4. Florfenicol alcohol (FFOH)

In a glass reaction tube with a screw cap, a 100 mg mass of FFA was mixed sequentially with 1 mL of dimethylformamide, 38 mg of glycolic acid (1.2 eq.), 92 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.2 eq.), and 64.8 mg of hydroxybenzotriazole (1.2 eq.). The tube was sealed under air atmosphere and the reaction mixture was agitated at room temperature for approximately 40 h. The solvent was then removed using a Speedvac (Thermo Fisher Scientific, USA) for 2 h. The material obtained was resuspended in a small amount of methanol and purified using a silica flash column with ethyl acetate as solvent. Florfenicol alcohol was isolated as a white solid, with a yield of 72 % and purity of 95 %, determined by quantitative ^{1}H NMR with 4-(dimethylamino) ethyl benzoate as a reference standard. The retention factor for florfenicol alcohol in ethyl acetate was determined to be 0.30.

2.4.5. Monochloroflorfenicol (FFCl)

In a glass reaction tube with a screw cap, a 100 mg mass of FFA was mixed sequentially with 1 mL of dimethylformamide, 38 mg of

chloroacetic acid (1.2 eq.), 92 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1.2 eq.), and 64.8 mg of hydroxybenzotriazole (1.2 eq.). The tube was sealed under air atmosphere and the reaction mixture was agitated at room temperature for approximately 40 h. The solvent was then removed using a Speedvac for 2 h. The resulting product was resuspended in a small amount of methanol and purified using a silica flash column with acetate:hexane (80:20 v/v). Monochloroflorfenicol was obtained as a white solid, with a yield of 83 % and purity of 94 %, as determined by quantitative ^{1}H NMR using 4-(dimethylamino) ethyl benzoate as a reference standard. The retention factor for monochloroflorfenicol in acetate:hexane (80:20 v/v) was determined to be 0.43.

2.5. Fish fillet samples

Healthy male Nile tilapias with average weight of 600 g were randomly stocked in three 450-L fiberglass tanks, at 26 °C, with constant aeration and open water flow. After an acclimation period of eight days, the fish from two tanks (10 fish per tank) received medicated feed containing florfenicol, at a daily dose of 10 mg/kg bw. The treatment was administered for either 7 or 10 days consecutively, and the fish were euthanized 3 h after the last administration of the medicated feed. One tank (10 fish) was used as the control, where the fish only received feed without florfenicol.

The medicated feed was prepared by top coating using vegetable oil and a 50 % v/v premix, as described in previous work [25]. The fish were euthanized by immersion in a saturated solution of benzocaine (500 mg L^{-1}), seven and ten days after the start of the florfenicol treatment. Fillet samples were collected, packed in identified plastic bags, and stored in a freezer until analysis. During acclimation and the experimental period, the fish were fed twice a day with commercial feed appropriate for their developmental stage.

2.6. Sample preparation (QuEChERS approach)

Before the extraction of analytes, the entire tilapia fillet (muscle plus skin, in natural proportions) was ground and homogenized, in the presence of dry ice, using an Ultra Turrax® system. The QuEChERS approach used in the sample preparation step was described in a previous work [25]. Briefly, a 3.0 g portion of ground and homogenized fish fillet was transferred to a polypropylene tube and the surrogates FFA-d_3 and FF-d_3 (500 μg kg^{-1}) were added. For the calibration curves, 3.0 g portions of ground blank fish fillet samples from the control group (see Section 2.5) were transferred to polypropylene tubes and fortified with FF, FFA, FFOH, and FFCl, at concentrations from 50 to 4000 μg kg^{-1} for FF and from 20 to 4000 μg kg^{-1} for FFA, FFOH, and FFCl. The internal standards used were FFA-d_3 (for FFA) and FF-d_3 (for FF, FFOH and FFCl), each at a concentration of 500 μg kg^{-1}. Subsequently, 10 mL volumes of acetonitrile were added and the mixtures were vortexed for 1 min. Following this step, 8.0 g of $MgSO_4$ and 2.0 g of CH_3COONa were added, followed by vortexing for 1 min and then centrifuging at 1,056 *g* for 5 min. Aliquots (5 mL) of the resulting supernatants were then transferred to Falcon tubes containing 750 mg of $MgSO_4$ and 125 mg of C18. The mixtures were immediately vortexed for 1 min and subsequently centrifuged at 1,056 *g* for another 5 min. The supernatants were collected, dried using a gentle flow of nitrogen gas, and resuspended in 750 μL volumes of mobile phase. Finally, the extracts were filtered through 0.22 μm PVDF syringe filters before analysis by SPE-LC-MS/MS.

2.7. SPE-UHPLC-MS/MS method

Quantitation of FF, FFA, FFOH, and FFCl employed on-line solid-phase extraction-ultra high-performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS). The technique was based on previous methods used by the same research group for the determination of antimicrobials and antiparasitics in fish and environmental samples [26,27]. Fortified blank fish fillet samples were used in this analysis. The first chromatographic dimension (^{1}D) used an OASIS HLB column (30 mm × 2.1 mm; 20 μm), while the second dimension (^{2}D) used an Acquity^{TM} UPLC BEH C18 analytical column (50 mm × 2.1 mm; 1.7 μm). The operational conditions are shown in Fig. 2.

Tables S1 and S2 (Supplementary Information) provide the sequential mass spectrometer settings, together with the quantification and confirmation transitions monitored in selective reaction mode (SRM), for all the analytes and the isotopically labelled standards (FF-d_3 and FFA-d_3).

Fig. 3 shows representative chromatograms for each molecule under investigation, determined in fortified samples.

2.8. Method validation

The reliability of the analytical method for determining FF, FFA, FFOH, and FFCl in fish fillet was assessed, considering key parameters including linearity, linear range, accuracy, precision, matrix effect, limit of quantification (LOQ) and stability in the matrix.

The equation and linearity of each calibration curve were obtained using the linear least squares regression method, with the Levene test to confirm homoscedasticity of variances. The limit of quantification (LOQ) was estimated individually for each analyte, with the signal-to-noise ratio set at 10. Intra-day precision was assessed by analyzing five replicates of blank fillet samples fortified with FF, FFA, FFCl and FFOH at three concentration levels (50, 500, and 4000 μg kg^{-1}) and internal standards FF-d_3 and FFA-d_3 (500 μg kg^{-1}). Inter-day precision was evaluated by analyzing fortified blank samples at the same three concentration levels in triplicate over a additional day, by the same analyst and using the same equipment. Both intra-day and inter-day variability were expressed as the relative standard deviation. Accuracy was determined in recovery tests performed in quintuplicate at the same concentration levels used in the precision evaluation.

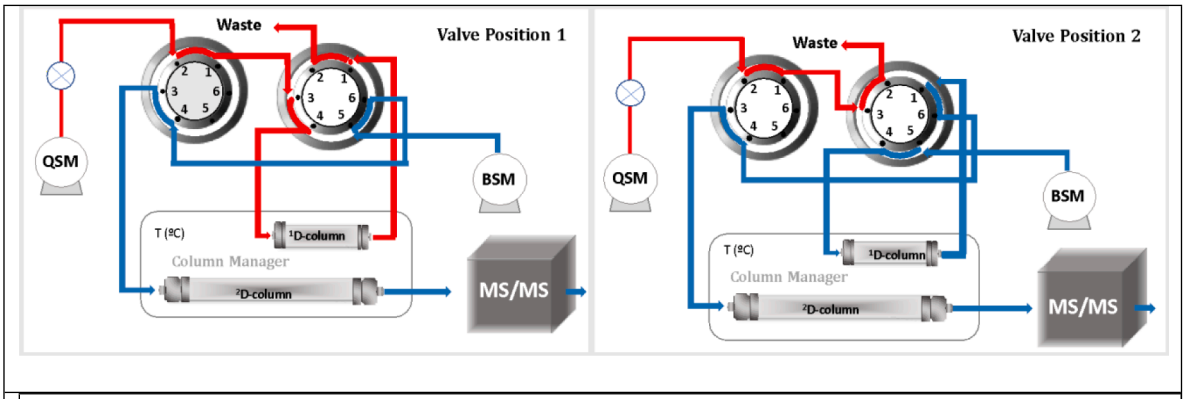
The matrix effect was evaluated by comparison of the areas of each analyte in solvent and in the post-extraction matrix, at two concentration levels (equivalent to 50 and 500 μg/kg) in triplicate. The matrix effect was calculated as the mean ratio of the areas of the analyte in solvent and in the post-extraction matrix, expressed as percentage.

Stability tests were conducted to assess the stability of the FF and its metabolites in the fish fillet. Freeze-thaw stability was evaluated by subjecting fortified blank samples to three cycles of freezing and thawing. Each cycle consisted of freezing at −20 °C for at least 12 h, followed by thawing at room temperature. Long-term stability was assessed by storing fortified blank samples at −20 °C for two weeks. For both stability tests, fortified samples at a concentration of 100 μg kg^{-1} were used in triplicate. The concentration of the fortified samples after the stability period was compared with freshly prepared samples.

3. Results and discussion

3.1. Preparation of florfenicol and synthesis of its metabolites

Analytical-grade reference standards are a crucial requirement for the validation of robust quantitative analytical methods suitable for application in pharmacokinetic and depletion studies. In this work, it was necessary to obtain such standards for four compounds (FF, FFA, FFOH, and FFCl), selected based on the florfenicol metabolic pathways shown in Fig. 1. There are two potential pathways by which FF can be metabolized to FFA, requiring monitoring and quantification of the intermediate metabolites FFOH and FFCl. Although there could be the possible presence of a fifth metabolite, florfenicol oxamic acid (FFCOOH), which could participate in the biotransformation of FFOH to FFA, it has been suggested that FFCOOH might not be consistently present in the metabolic pathway (Anadon et al., 2008). In a radiometric depletion study using [^{14}C]-florfenicol in Atlantic salmon kept at 10 °C, with administration of the compound at 10 mg/kg bw for 10 consecutive days, FF represented 33 %, FFA 50 %, FFOH 5 %, and FFCOOH 0.70 % of



SPE-UHPLC conditions

BSM, 0.40 mL min^{-1}			QSM, 1.00 mL min^{-1}				Valves	
Time (min)	Formic acid, 0.01% v/v in water	B	Time (min)	A	B	C	Time (min)	Position
0	85	15	0	85	15	0	0	1
1.3	85	15	1.3	85	15	0	1.3	2
3.0	0	100	3.0	85	15	0	3.0	1
5.0	0	100	3.2[a]	5	0	95		
6.0	85	15	7.8[a]	5	0	95		
			8.0[a]	85	15	0		
			10.0	85	15	0		

A: water; B: methanol; C: acetonitrile; *a*: flow rate of 1.25 mL min^{-1}.

Sample injection volume: 150 µL.

Column temperature in the BSM step set at 40 ºC.

Fig. 2. Schematic illustration of the SPE-UHPLC-MS/MS system, with a table showing the operational conditions. The red line indicates the solvent delivered by the QSM pump (first chromatographic dimension, valve position 1). The blue line (valve position 2) indicates the elution of the analytes from the ^{1}D column to the ^{2}D column. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the total radioactive residue in muscle, after a 24 h withdrawal period. In skin, irrespective of the water temperature (10 or 5 °C), FF represented 65–70 % of the radioactivity extracted, FFA 11–14 %, FFOH 2.5 %, and FFCOOH 0.4–1.2 % (EMA, 1997). Therefore, given the objective of the present research, it was deemed unnecessary to invest further effort into synthesizing FFCOOH, considering its negligible concentrations reported in fish.

The goal was to develop a cost-effective and reproducible method for synthesizing these standards, which would allow easy replication in other laboratories, avoiding the high costs of commercial standards. Rather than directly buying an analytical standard of FF, a method was developed to extract FF from readily available veterinary drugs. FF was isolated by liquid–liquid extraction (LLE) of an injectable veterinary formulation, employing ethyl acetate as the extraction solvent. The isolated FF was then further purified by preparative liquid chromatography, which facilitated the removal of additional excipients. A florfenicol amine (FFA) standard was synthesized by acid hydrolysis of florfenicol, followed by basification to pH 14 and extraction with ethyl acetate and dichloromethane. This method effectively eliminated the need for further purification steps. Due to the amide characteristics of the residual FF intermediates, namely florfenicol alcohol (FFOH) and monochloroflorfenicol (FFCl), their synthesis was achieved by peptide bond coupling between FFA and the corresponding carboxylic acids (glycolic acid and 2-chloroacetic acid). Subsequent purification was performed using preparative flash column chromatography. The synthesis pathway is detailed in Fig. 4. To the best of our knowledge, this is the first study describing the synthesis of florfenicol metabolites.

Characterization of all the synthesized molecules was performed using ^{1}H and ^{13}C NMR, together with the two-dimensional NMR techniques of heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) for FFOH and FFCl. Figs. 5 and 6 show the results of the ^{1}H NMR and HSQC analyses of FFOH and FFCl, respectively. Additional NMR characterization details are provided in the Supplementary Information.

High-resolution mass spectrometry analysis was also applied for FFOH and FFCl (the results are available in the Supplementary Information), to confirm their identities. Purity assessment of all the compounds employed quantitative nuclear magnetic resonance (q-NMR), resulting in values of 98 % (FF), 94 % (FFA), 95 % (FFOH), and 94 % (FFCl), which indicated that the compounds were of sufficient purity for use as analytical standards. Quantitative nuclear magnetic resonance (q-NMR) is an effective single-point replacement for preliminary

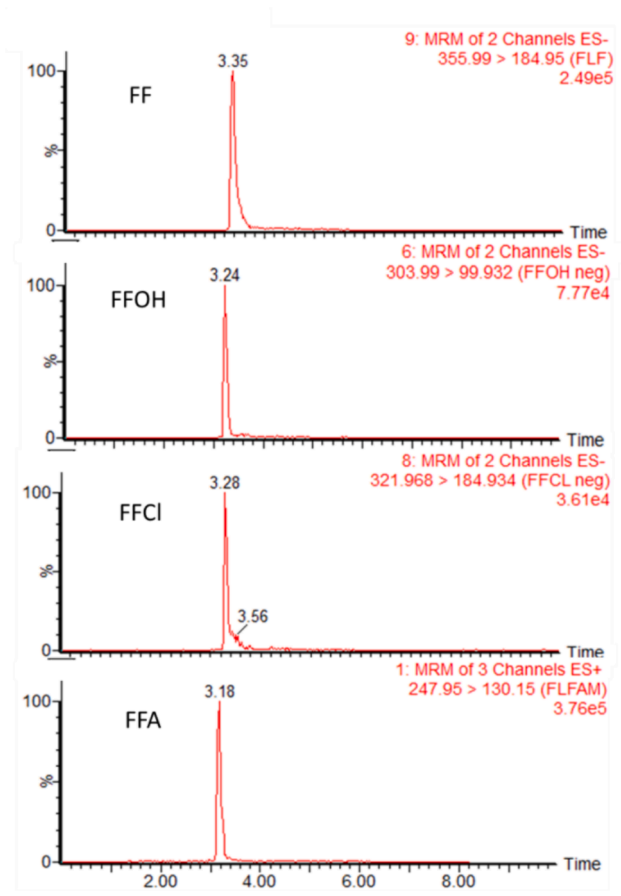


Fig. 3. Representative chromatograms were obtained for a sample fortified with florfenicol (FF), florfenicol alcohol (FFOH), monochloroflorfenicol (FFCl), and florfenicol amine (FFA) at 50 μg kg^{-1}.

developmental testing, traditionally encompassing identity verification, chromatographic assays, moisture content analysis, residual solvent evaluation, and elemental analysis [28].

3.2. Quantification of florfenicol and its metabolites using SPE-UHPLC-MS/MS

To ensure that precise quantification of the target analytes could be achieved, the analytical method was validated for the quantification of FF, FFCl, FFOH, and FFA in tilapia fillets. While one-dimensional LC is capable of fulfilling the objective, the adoption of SPE-UHPLC was chosen to mitigate matrix effects and enhance sensitivity, thus achieving a lower quantification limit. This approach yielded peaks with satisfactory symmetry. Table 1 presents the validation parameters of the method for quantifying florfenicol and its metabolites in tilapia fillet, utilizing the QuEChERS approach for sample preparation and SPE-UHPLC-MS/MS for quantification. The results demonstrated that the developed method complied with the criteria specified in the guideline "VICH GL49: Studies to evaluate the metabolism and residue kinetics of veterinary drugs in human food-producing animals: validation of analytical methods used in residue depletion studies" [29], Stability tests confirmed that the analytes remained stable after three cycles of freezing and thawing, and after two weeks of storage at −20 °C. The matrix effect evaluations revealed higher matrix effects for FFA and FFCl (Table 1). For the internal standards FF-d_3 and FFA-d_3 the matrix effect was +6 % and −56 %. These findings highlight the necessity of using matrix-matched calibration curves for the quantitation of florfenicol and its metabolites in fish fillet.

3.3. Sample analysis

Following method validation, fillet samples from fish treated for seven and ten days (see Section 2.5) were analyzed. It is important to highlight that the purpose of analyzing these samples was to demonstrate that the method is applicable to incurred samples. However, this study does not intend to offer a comprehensive analysis of metabolite metabolism or distribution. We anticipate that future research will use this method to investigate these aspects with different fish species across various environments. For both sets of samples, the SPE-UHPLC-MS/MS method was able to successfully obtain the florfenicol metabolite profiles in the tissues, as shown in Fig. 7. The method also enabled the quantification of florfenicol and the three metabolites (FFA, FFOH, and FFCl) at varying concentrations in the samples, indicating their persistence throughout the treatment. Florfenicol alcohol was the major metabolite identified in the samples collected on days 7 and 10 during the oral application of florfenicol at a dose of 10 mg/kg bw.

It should be highlighted that it would not be possible to obtain these

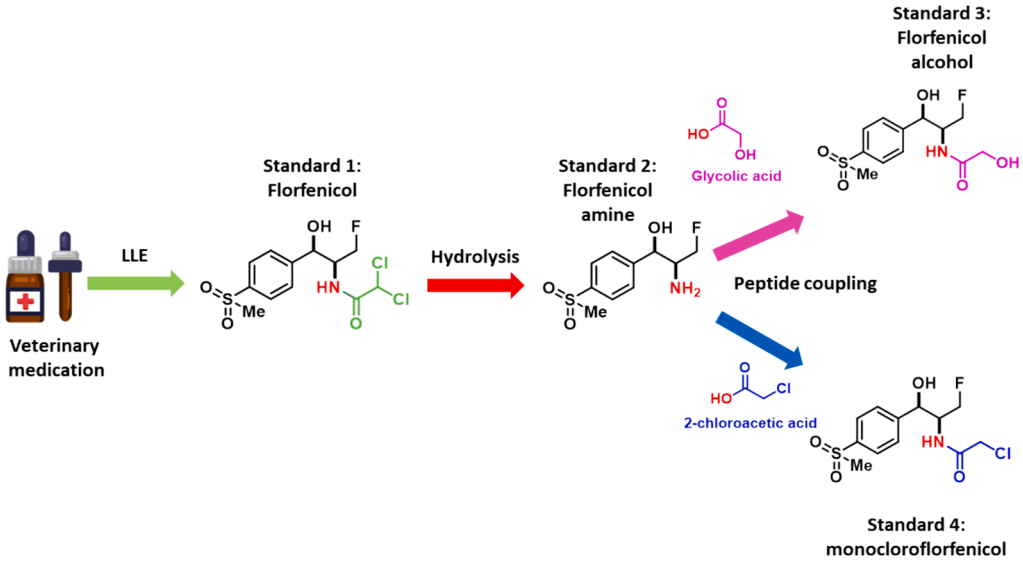


Fig. 4. Synthesis routes for the florfenicol metabolites.

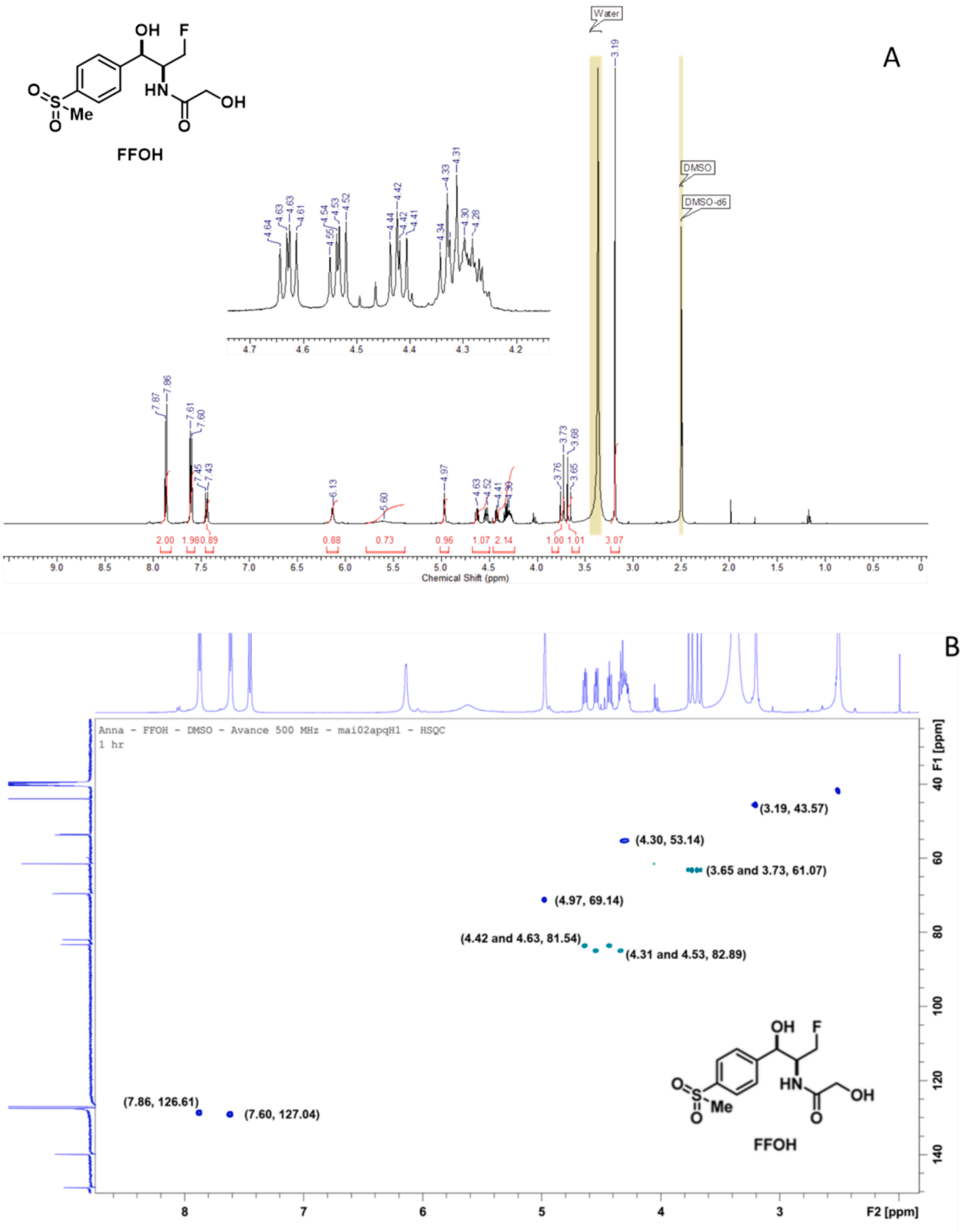


Fig. 5. Spectra obtained for the ^{1}H NMR (A) and HSQC (B) analyses of FFOH.

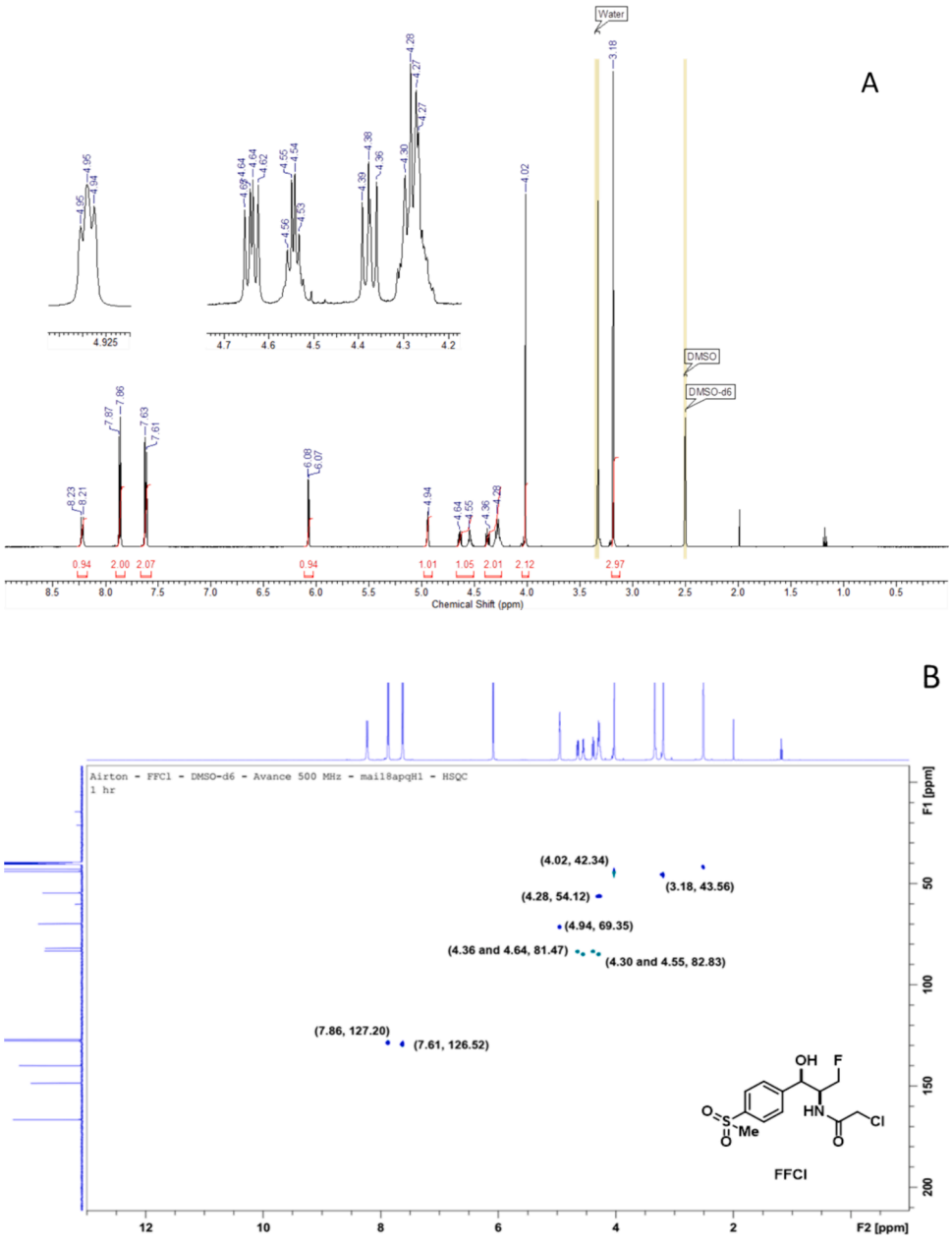


Fig. 6. Spectra obtained for the ^{1}H NMR (A) and HSQC (B) analyses of FFCl.

data using traditional methods that rely on the conversion of FF and its metabolites into FFA by hydrolysis.

4. Conclusions

Florfenicol metabolites were synthesized at high yields and were characterized by ^{1}H NMR, ^{13}C NMR, two-dimensional NMR techniques (HSQC and HMBC), and HRMS. The purities of the compounds were determined by quantitative NMR (q-NMR), with the results showing that the molecules were suitable for use as standards in the SPE-UHPLC-MS/MS method. The method proved to be effective for the detection and quantification of florfenicol and its metabolites. Validation of the method indicated its suitability for the determination of FF and its metabolites in tilapia fillet, and importantly, it complied with the parameters established in the VICH GL49 guidelines. Analysis of fillet samples from fish undergoing treatment revealed the continuous presence of FF and its metabolites, confirming the significance of the metabolites in the process of metabolization of FF in the fish.

The ability to identify all the metabolites using a simplified method represents a notable advancement in the quantification techniques available for use in pharmacokinetic and residue depletion research. This progress has the potential to provide deeper insights into the dynamics of drug metabolism in fish. Studies are under way to develop a detailed profile of the FF metabolites in fish tissue.

CRediT authorship contribution statement

Anna Paula Rocha de Queiroga: Formal analysis, Data curation. **Gabriela Freitas Pereira de Souza:** Formal analysis, Data curation. **Inácio Mateus Assane:** Formal analysis, Data curation. **Thiago Messias:** Formal analysis. **Fabiana Pilarski:** Data curation, Conceptualization. **Michael Schloter:** Conceptualization. **Airton Gonçalves Salles:** Conceptualization. **Susanne Rath:** Conceptualization.

Table 1
Validation parameters for the determination of florfenicol and its metabolites in tilapia fillet.

Parameter	Unit	Fortification level	FF	FFOH	FFCl	FFA
Linear range	μg kg^{-1}	–	50–4000	20–4000	20–4000	20–4000
Linearity (r)		–	0.997	0.997	0.998	0.996
Intra-day precision (n = 5)	RSD (%)	50 μg kg^{-1}				7.1
		500 μg kg^{-1}	6.4	12.1	13.3	5.6
		4000 μg kg^{-1}	5.5	9.3	8.4	6.0
			1.74	7.2	8.7	
Inter-day precision (n = 8)	RSD (%)	50 μg kg^{-1}			12.5	9.5
		500 μg kg^{-1}	9.5	9.7	7.8	10.0
		4000 μg kg^{-1}	10.2	12.3	7.8	5.8
			1.7	6.6		
	%	50 μg kg^{-1}				
Accuracy		500 μg kg^{-1}	98.2	99.4	108.9	108.4
(n = 5)		4000 μg kg^{-1}	102.6	98.4	107.3	95.0
			98.1	102.1	100.8	102.2
LOQ	μg kg^{-1}	–	50	20	20	20
Matrix effect	%		−9	−14	−42	−59
		50 μg kg^{-1}	−14	−20	−43	−66
		500 μg kg^{-1}				
Stability in matrix[a]	%		95.9	107.3	95.6	110.9
		100 μg kg^{-1}				
Freeze-thaw stability	%		101.1	96.6	96.6	112.0
		100 μg kg^{-1}				

[a] For 14 days, at −20 °C.

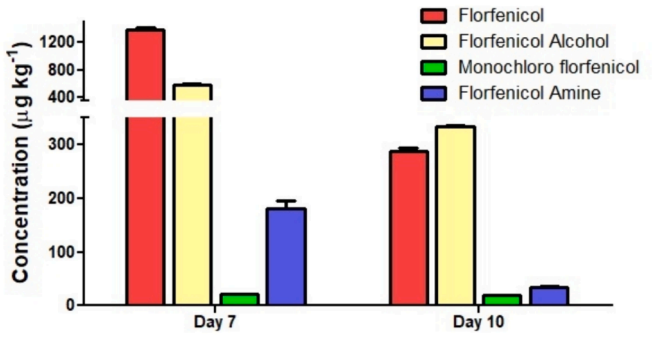


Fig. 7. Concentrations of florfenicol and its metabolites in Nile tilapia fillet samples collected on days 7 and 10, following oral application of florfenicol at a daily dose of 10 mg/kg bw. The analytical runs were performed in quadruplicate.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jchromb.2024.124282.

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