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IDENTIFICAÇÃO E CARACTERIZAÇÃO DE METABÓLITOS DE SULFAQUINOXALINA

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“A Ciência, quando não humaniza, deprava.”

Mikhail Bakunin

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ABREVIATURAS

ACN: acetonitrila.

AF: ácido fórmico.

LC: cromatografia líquida.

LC-ESI-MS/MS: cromatografia líquida acoplada à espectrometria de massas em modo *tandem* com ionização por *electrospray.*

LC-MS/MS: cromatografia líquida acoplada à espectrometria de massas em modo *tandem.*

LC-MS: cromatografia líquida acoplada à espectrometria de massas.

LC-QqLIT: cromatografia líquida acoplada à espectrometria de massas com sistema de *ion trap* linear.

LC-Qq-TOF-MS: cromatografia líquida acoplada à espectrometria de massas com detector de tempo de vôo.

LD: limite de detecção.

LMR: limite máximo de resíduo.

LQ: limite de quantificação.

MeOH: metanol.

MRM: do inglês "*multiple reaction monitoring*".

PLE: do inglês "*pressurized liquid extraction*", extração por líquido pressurizado

RMVs: resíduos de medicamentos veterinários.

SFAs: sulfonamidas.

SPE: do inglês "*solid phase extraction*", extração em fase sólida.

SQX: sulfaquinoxalina.

SQX-OH: sulfaquinoxalina hidroxilada.

TOF: do inglês "*time of flight*", detector de tempo de vôo.

RESUMO

A presença de resíduos de medicamentos antibacterianos em alimentos é um importante problema de saúde pública. Estas substâncias podem estar presentes nos alimentos em níveis inaceitáveis como resultados de práticas produtivas inadequadas. Devido a estas preocupações, são estabelecidos limites máximos de resíduos para estas substâncias (LMRs). No caso das sulfonamidas, este valor de LMR refere-se à soma do princípio ativo e de todos seus metabólitos. Neste trabalho, identificam-se e caracterizam-se metabólitos de sulfaquinoxalina (SQX) em diversas espécies animais. Dentro do processo investigativo, foram realizados estudos comparativos de métodos de extração, processos de validação e determinação de efeito de matriz. Foi elaborado e proposto um modelo para a priorização de fármacos baseado em análise de risco e discutiu-se o panorama atual da presença de resíduos de sulfonamidas em amostras ambientais. A investigação da formação de metabólitos de SQX *in vitro* e *in vivo* levaram à identificação de três compostos, dois deles ainda não descritos na literatura: N^4-acetil-SQX, SQX-OH e N^4-acetil-SQX-OH. O perfil de formação destes compostos em diversas espécies animais foi analisado e discutido.

Palavras-chaves: LC-MS/MS, metabólitos, sulfaquinoxalina, sulfonamidas, elucidação estrutural.

ABSTRACT

The presence of antibacterial drugs residues in food is an important public health issue. These substances can be present in food at unacceptable levels due to inappropriate veterinary practices. Because of that, maximum residue levels (MRL) are established for these compounds. In the sulfonamide drugs case, this value corresponds to the sum of parent drug and their metabolites. In the present work, sulfaquinoxaline (SQX) metabolites were identified and characterized in several animal species. Inside that investigation process, several studies were developed about extraction methods, validation processes and matrix effects determination. A model for drugs residues prioritization based on risk analysis was proposed. Also, the state-of-art of sulfonamides residues analysis in environmental samples was discussed. The in vivo and in vitro investigation of SQX metabolites formation lead us to the identification of 3 compounds, 2 of them previously unreported: N^4-acetyl-SQX, SQX-OH and N^4-acetyl-SQX-OH. The formation profile of these compounds in several animal species was analyzed and discussed.

Keywords: LC-MS/MS, metabolites, sulfaquinoxaline, sulfonamides, structural elucidation.

1. INTRODUÇÃO

O cenário mundial da produção de alimentos revela o grande interesse da sociedade quanto à segurança do produto a ser consumido. O uso de pesticidas e de medicamentos na produção animal e vegetal tem sido altamente questionado pelos consumidores e é uma fonte crescente de preocupação no que se refere à segurança alimentar.

Medicamentos veterinários são utilizados no mundo todo para promover a saúde animal, propiciar ganhos econômicos e aumento da produtividade da indústria de alimentos de origem animal (1,2). No entanto, se as boas práticas veterinárias não forem rigorosamente observadas, e os períodos de carência pré-abate ou coleta não forem cumpridos, poderá ocorrer permanência de resíduos destes medicamentos nos animais destinados à produção de alimentos (3,4). A problemática dos resíduos de medicamentos veterinários (RMVs) levou ao desenvolvimento de legislações e regulamentações próprias tanto no âmbito nacional como entre blocos econômicos e órgãos internacionais como o *Codex Alimentarius*, com o propósito de propor e, posteriormente, harmonizar valores de limites máximos de resíduo (LMR) para as diversas combinações fármaco/matriz. O estudo dos potenciais efeitos da ingestão de alimentos contendo quantidades acima do LMR foi e segue sendo uma área de pesquisa de grande relevância, já que diz respeito diretamente à saúde pública bem como às relações comerciais internacionais. A análise de resíduos de fármacos veterinários é uma fração vital dos programas de monitoramento estabelecidos pelas agências reguladoras em praticamente todos os países envolvidos com exportação e importação de alimentos. A demanda pelo controle regulatório de contaminantes químicos em alimentos expandiu-se dramaticamente nas últimas décadas, fazendo da área de análise destes resíduos um importante fator a ser considerado no comércio internacional de *commodities* (5–8).

As sulfonamidas constituem a primeira classe de agentes anti-infecciosos descobertos na terapêutica para o tratamento de doenças infecciosas, antecedendo até mesmo o advento da introdução da penicilina. Esta classe de fármacos teve uso

intenso na era pré-penicilina e ainda durante muitas décadas após a introdução das penicilinas e demais classes de antibióticos. Embora ainda hoje sejam fármacos de eleição para algumas situações clínicas muito bem determinadas, os mecanismos de surgimento de resistência fizeram com que as sulfonamidas - ou simplesmente sulfas - caíssem em desuso na medicina humana. Não obstante, seu uso ainda é muito frequente na medicina veterinária, na área de produção animal, como profilático de infecções na produção em larga escala de aves e suínos, principalmente (9–11). Uma das principais vias de utilização é através de rações medicamentosas, que incluem, além das sulfas, diversos outros compostos em associação, mais comumente as penicilinas, tetraciclinas e agentes coccidiostáticos, principalmente do grupo dos ionóforos. As sulfonamidas são comercializadas na forma isolada ou formuladas em associações com outras sulfonamidas e/ou antibióticos e outros agentes antibacterianos. As sulfas possuem um amplo espectro de atividade bacteriostática, afetando bactérias *gram* negativas, *gram* positivas e alguns organismos protozoários (12).

A utilização destes fármacos na produção animal torna-se um grave problema de saúde pública quando não são observadas boas práticas de produção e os animais e os produtos originados destes, como ovos e leite, são destinados ao consumo humano sem a devida observação do tempo de retirada destes fármacos da dieta dos animais, antes de seu abate ou coleta de produtos derivados. A presença de resíduos de sulfas e de outros princípios ativos em alimentos pode gerar uma série de consequências de diferentes graus de risco, desde reações alérgicas em pessoas hipersensíveis até o surgimento de resistência microbiana passível de ser transferida para a microbiota humana normal. A dose sub-terapêutica, que é bastante comum quando se almeja o simples aumento da taxa de conversão alimentar, pode ainda afetar a homeostase entre microbiota e hospedeiro, balanço que vem sendo sugerido como um dos fatores que regula e modula a resposta imune (13–15).

2. JUSTIFICATIVAS

O presente trabalho busca identificar e caracterizar metabólitos de sulfaquinoxalina (4-amino-*N*-2-quinoxalinilbenzenosulfonamida; CAS 59-40-5). A sulfaquinoxalina é um dos antimicrobianos mais utilizados em animais produtores de

alimentos, especialmente aves (16,17). Este fármaco foi responsável pela expansão das criações industriais de aves e pela redução do valor da carne de frango nos últimos 80 anos, por ter sido a primeira substância a mitigar os danos causados pela coccidiose nos aviários comerciais. Embora seja um dos mais conhecidos medicamentos veterinários, sua metabolização não é bem conhecida. Sabe-se que a SQX possui processo de metabolização espécie-dependente, variando tanto quali como quantitativamente na produção de metabólitos, dependendo da espécie em que é administrada. É sabido que, como praticamente todos os compostos oriundos do grupo das sulfonamidas, a SQX sofre acetilação no nitrogênio da posição 4 (18). Outros prováveis metabólitos tiveram sua estrutura proposta teoricamente nos anos 1940, mas até o momento não foram objetivamente elucidados (19). O presente trabalho visa isolar, elucidar estruturalmente e obter as características físico-químicas de metabólitos ainda não descritos oriundos da SQX. Do ponto de vista farmacológico, serão realizados os testes de toxicidade e de espectro de ação bactericida e bacteriostático em comparação com a droga-base.

3. OBJETIVO GERAL

Identificação dos principais metabólitos da sulfaquinoxalina e implementação de metodologia analítica para quantificação dos mesmos em amostras ambientais e de alimentos.

4. OBJETIVOS ESPECÍFICOS

- Identificar os principais metabólitos de sulfaquinoxalina produzidos em diferentes espécies animais.
- Revisar o estado-da-arte em relação à análise de resíduos de sulfonamidas em amostras ambientais, com enfoque nas técnicas de espectrometria de massas.
- Propor um modelo de priorização de fármacos a serem monitorados em amostras ambientais e de alimentos, baseados em análise de risco destas substâncias.
- Propor novas abordagens analíticas para a validação de extensão de escopo para a análise de resíduos de medicamentos veterinários em alimentos.

- Avaliar métodos de determinação de efeito de matriz em amostras ambientais e de alimentos para métodos de análise de resíduos de fármacos usando espectrometria de massas.
- Implementar metodologia analítica por LC-ESI-MS/MS, LC-Qq-TOF-MS e LC-QqLIT-MS para a determinação de sulfaquinoxalina e seus metabólitos em urina e tecidos de aves, suínos, bovinos, ovinos e eqüinos.

4. REVISÃO BIBLIOGRÁFICA

4.1 Histórico das sulfonamidas

As sulfas foram sintetizadas pela primeira vez em 1908 por Gelmo et al. como produto da busca por novos azocorantes (20). Seguindo a linha deste trabalho, Hoerlein et al. descobriram que corantes contendo o grupamento sulfanil apresentavam afinidade por proteínas da seda e da lã (19). Tal achado levou a descoberta por Eisenberg, em 1913, de que a crisolidina, um dos azocorantes estudados, possuía pronunciada ação bactericida (21). Entretanto, somente em 1932 as propriedades terapêuticas das sulfas foram determinadas: Dogmagk et al. ao ensaiar vários corantes, encontraram uma considerável atividade antibacteriana *in vitro* no Prontisil, ((p-[2,4-diaminofenil) azo]sulfanilamida). Logo em seguida, descobriu-se que tal atividade se devia a liberação, *in vivo*, da sulfanilamida, sendo esta portanto a fração ativa da molécula do Prontisil (22). O trabalho de Dogmagk levou a uma intensa atividade de pesquisa com a sulfanilamida e diversas sulfas foram sintetizadas a partir dela nos anos 1930. Um grande número destes novos compostos demonstrou possuir atividade antibacteriana contra uma variedade de streptococci e pneumonococci. Várias sulfapirimidinas introduzidas a partir de 1941 aliavam uma potente atividade antibacteriana com toxicidade inferior às sulfas sintetizadas previamente. A partir deste ponto, muitas novas sulfas foram sintetizadas. Atualmente, cerca de 5000 diferentes compostos desta classe são conhecidos, mas somente pouco mais de 30 tem efetivo emprego, seja na medicina humana como nas ciências veterinárias (23). A partir do núcleo químico formado pelos grupamentos anilina e ácido sulfônico, várias outras classes de fármacos foram sintetizadas, desde antimaláricos até agentes hipoglicemiantes.

4.2 Propriedades físico-químicas das sulfonamidas

Sulfas, como resultado das propriedades indutivas do grupamento SO_2, são compostos que exibem comportamento anfotérico, por possuírem grupamentos químicos com caráter ácido e básico, o que permite que em determinadas faixas de pH estas moléculas se comportem como *zwitteríons*, ou seja, são capazes de manter carga formal positiva e negativa. É bem estabelecido que este comportamento desempenha um importante papel na atividade antibacteriana das sulfas. Sugere-se que a relação entre a constante de dissociação ácida e a atividade bacteriostática descreve um arco parabólico que apresenta um máximo entre pK_a 6 e 7,5 (24). Estes autores concluem que a forma iônica do fármaco é mais ativa do que forma neutra, mas que sulfas demasiadamente ácidas sofrem diminuição da atividade pelo fato do grupo SO_2 ser menos eletronegativo do que nas sulfas moderadamente ácidas (24).

Um grave problema no uso das sulfonamidas é a mudança de sua solubilidade com a variação do pH. Este fato pode acarretar deposição de cristais de sulfas no tecido renal e presença de cristalúria em pacientes sob tratamento com estes compostos; problema este que está fundamentado em princípios físico-químicos simples. A solubilidade de uma sulfonamida em uma solução tamponada ou em urina pode ser prevista por duas constantes, a solubilidade da forma não-dissociada e a constante de dissociação ácida do composto. Para fins fisiológicos, muitas sulfas podem ser administradas na forma de ácidos monobásicos, cuja forma não-dissociada é fracamente solúvel, mas cujos sais básicos são altamente solúveis (25).

O grupamento p-NH_2 é essencial para a atividade. Quando substituído, deve regenerar *in vivo* o NH_2 para ser ativo. É o caso de pró-fármacos como o ftalilsulfatiazol e a sulfassalazina que liberam, *in vivo*, o sulfatiazol e a sulfapiridina, respectivamente. Em geral, as sulfas são pós cristalinos brancos, geralmente pouco solúveis em H_2O, mas seus sais sódicos são facilmente hidrossolúveis (24).

4.3 Metabolização de fármacos

Os tecidos biológicos são diariamente expostos a xenobióticos, ou seja, substâncias estranhas que não são encontradas naturalmente no organismo. Os fármacos são, em sua maioria, xenobióticos que são utilizados para modular funções corporais com fins terapêuticos. Fármacos e outras substâncias químicas que venham a ser introduzidas no organismo sofrem diversos processos modificadores por ação de enzimas endógenas. As transformações biológicas efetuadas por essas enzimas alteram o composto e, consequentemente, suas propriedades físico-químicas. Os processos pelos quais os fármacos são alterados por reações bioquímicas dentro de um organismo específico são designados, em seu conjunto, como metabolismo ou biotransformação.

O resultado da biotransformação de um fármaco pode gerar quatro importantes alterações:

- Um fármaco ativo pode ser convertido em metabólito inativo.
- Um fármaco ativo pode ser convertido em um metabólito ativo ou tóxico.
- Um pró-fármaco inativo pode ser convertido em fármaco ativo.
- Um fármaco não-excretável pode ser convertido em metabólito passível de excreção (por exemplo, aumentando a depuração renal ou biliar).

4.3.1 Principais mecanismos de metabolização

4.3.1.1 N-Glicuronização

Compostos contendo grupos funcionais nitrogenados de caráter nucleófilo, como aminas aromáticas primárias, hidroxilaminas, amidas, sulfonamidas, aminas alifáticas terciárias e N-heterociclos aromáticos são suscetíveis à N-glicuronização. A N-glicuronização representa a principal rota de eliminação para muitas drogas. Conjugados de ácido glicurônico são geralmente metabólitos hidrossolúveis pouco tóxicos, mas em alguns casos podem ampliar carcinogenicidade de algumas moléculas, como é o caso das arilaminas primárias (26,27).

Entre as diferentes espécies animais foram observadas diferenças na habilidade de catalisar distintas reações de N-glicuronizações (28). Por exemplo, a habilidade de formar glicuronídeos de amônio quaternário (N^+-glicuronídeos) a partir de aminas alifáticas terciárias parece ser restrita à humanos e primatas superiores (29).

O processo de biotransformação muitas vezes denominado de reações sequenciais, de onde surge a denominação ainda comumente encontrada em farmacologia de reações de fase I e reações de fase II. Esta terminologia pode levar a determinados equívocos de interpretação por conferir um sentido cronológico implícito. Mais corretamente, podemos definir dois grandes grupos de reações de metabolização: reações de oxidação/redução e reações de hidrólise/conjugação. Estas reações, embora possam ocorrer ambas em um mesmo fármaco, são independentes entre si, podendo as enzimas responsáveis pelos dois tipos de reação competir em entre si pelo substrato (30).

A maioria dos processos de biotransformação ocorre no fígado, embora todos tecidos possam, em menor ou maior grau, metabolizar fármacos, especialmente pele, pulmões e trato gastrintestinal (31). No caso dos fármacos administrados por via oral, após a absorção dos compostos pelo trato gastrintestinal, os mesmos são transferidos ao fígado pela circulação porta. Ou seja, antes mesmo da chegada dos fármacos aos tecidos-alvo, uma fração da dose já é metabolizada. Esta característica, denominada de efeito de primeira passagem ou eliminação pré-sistêmica, é tão importante que alguns fármacos não podem ser administrados por via oral, devido à grande fração que é eliminada pela ação hepática (31).

A maioria dos fármacos necessita apresentar certa lipossolubilidade, de modo que seja capaz de atravessar as barreiras celulares. Esta característica, indispensável para a ação farmacológica, torna-se obstáculo para a eliminação destas substâncias, uma vez que a depuração renal é a via majoritária e requer que os fármacos ou seus metabólitos sejam solúveis na urina (32).

Portanto, em síntese, a metabolização é, em termos gerais, um processo de incremento da hidrofilicidade das moléculas, para que possam ser eliminadas do sistema (33).

Assim, nas reações de fase I, ou de oxidação/reação, o que geralmente se verifica é a adição ou exposição de grupos funcionais hidrofílicos, que confiram à substância uma maior hidrossolubilidade, como hidroxila (-OH), tiol (-SH) e amina (-NH_2). Este processo produz majoritariamente substâncias farmacologicamente inativas e que não necessitam alterações posteriores para serem eliminadas na urina (34).

Em alguns casos, entretanto, é necessário que ocorra a conjugação destes metabólitos com outras substâncias capazes de propiciarem maior polaridade à molécula, como ácido glicurônico, por exemplo (35).

4.3.1.2 Reações de oxidação / redução

As reações de oxidação/redução envolvem enzimas associadas a membranas, expressas no retículo endoplasmático (RE) dos hepatócitos e, em menor grau, das células de outros tecidos. As enzimas que catalisam essas reações de fase I são tipicamente oxidases; essas enzimas são, em sua maioria, hemoproteínas monooxigenases da classe do citocromo P450 (CYP P450). As enzimas P450 são também conhecidas como oxidases de função mista microssômicas (35).

A reação tem início quando o fármaco liga-se ao citocromo P450 oxidado (Fe^{3+}), formando um complexo que, a seguir, é reduzido através de duas etapas de oxidação/redução consecutivas. O fosfato de nicotinamida adenina dinucleotídeo (NADPH) é o doador dos elétrons em ambas as etapas. Na primeira etapa, o elétron doado reduz o complexo citocromo P450-fármaco. Na segunda etapa, o elétron reduz o oxigênio molecular, formando um complexo de oxigênio ativado–citocromo P450–fármaco. Por fim, à medida que o complexo torna-se mais ativo através de rearranjo, o átomo de oxigênio reativo é transferido para o fármaco, resultando na

formação do produto oxidado do fármaco, com reciclagem do citocromo P450 oxidado no processo (35).

As oxidases hepáticas do citocromo P450 exibem, em sua maioria, uma ampla especificidade de substrato. Entretanto, muitas enzimas P450 exibem especificidades parcialmente superpostas que, em seu conjunto, permitem ao fígado reconhecer e metabolizar uma ampla série de xenobióticos. Em seu conjunto, as reações mediadas pelo P450 respondem por mais de 95% das biotransformações oxidativas (36).

4.3.1.3 Reações de conjugação e hidrólise

As reações de conjugação e de hidrólise proporcionam um segundo conjunto de mecanismos destinados a modificar os compostos para sua excreção. Embora a hidrólise de fármacos que contêm éster e amida seja algumas vezes incluída entre as reações de fase I na antiga terminologia, a bioquímica da hidrólise está mais estreitamente relacionada com a conjugação do que com a oxidação/redução (32,34). Os substratos dessas reações incluem tanto metabólitos de reações de oxidação (por exemplo, epóxidos) quanto compostos que já contêm grupos químicos apropriados para conjugação, como hidroxila (-OH), amina ($-NH_2$) ou carboxila (-COOH). Esses substratos são acoplados a metabólitos endógenos (por exemplo, ácido glicurônico e seus derivados, ácido sulfúrico, ácido acético, aminoácidos e o tripeptídio glutationa) por enzimas de transferência, em reações que frequentemente envolvem intermediários de alta energia. As enzimas de conjugação e de hidrólise localizam-se tanto no citosol quanto no retículo endoplasmático dos hepatócitos (e de outros tecidos). Na maioria dos casos, o processo de conjugação torna o fármaco mais polar. Praticamente todos os produtos conjugados são farmacologicamente inativos, com algumas exceções importantes (por exemplo, glicuronídeo de morfina) (37).

4.4 Metabolismo geral das sulfonamidas

A duração de um efeito quimioterapêutico é geralmente determinada pela especificidade do agente a certos mecanismos enzimáticos e atividade metabólica

próprias de cada espécie. Muitas substâncias com ação farmacológica estão sujeitas a modificações bioquímicas antes de serem excretadas pelo organismo. Somente uma pequena quantidade da dose total aplicada permanece indiferenciada. O metabolismo dos compostos farmacológicos, bem como de xenobióticos em geral, é determinante para seus perfis farmacodinâmico e farmacocinético. Décadas de investigação nas áreas de bioquímica, farmacologia e toxicologia revelam que, a despeito do imenso número de compostos com ação farmacológica, um número relativamente pequeno de mecanismos enzimáticos parece estar envolvido (17,38,39).

Reimerdes e Thumim revisaram as principais vias metabólicas que agem sobre as sulfonamidas (40). As sulfas, como grupo, estão suscetíveis a muitas modificações metabólicas. Quatro principais mecanismos enzimáticos podem ser listados:

a. Acetilação
b. Hidroxilação
c. Glicuronação
d. Formação de sulfato éster

A acetilação do grupamento amina ligado ao anel aromático das sulfas foi uma das primeiras reações do metabolismo destes compostos que foi estudado e elucidado. Esta forma de metabolização é comum para todas as sulfas, mas é extremamente dependente da espécie alvo bem como do substrato. Não obstante, é sem dúvida o sistema majoritário de metabolização destes fármacos. O estudo da acetilação das SFAs levou à eventual identificação da acetil coenzima A. Investigações em diferentes órgãos mostram que a acetilação das sulfas se dá majoritariamente no fígado, mas também ocorre em quantidade significativa nos rins (26).

Esta via de metabolização tem um relevante papel por ser a via de formação dos derivados N^4-acetilados das sulfas, os quais apresentam efeitos indesejados no sistema renal, como albuminúria, oligúria e anúria (40).

4.5 Metabólitos de sulfaquinoxalina

A estrutura de alguns metabólitos foi proposta inicialmente por Scudi e Silber, de modo teórico (41). A detecção de metabólitos deve ser incluída em métodos de análises de resíduos de sulfas, pois o LMR é considerado como a soma da droga mãe e de seus metabólitos. Em trabalhos prévios, foi detectada a interferência da formação de um metabólito de SQX *in vitro* quando da análise de amostras de rotina de fígado de eqüinos (17,42). A formação deste metabólito foi validada e diversos parâmetros foram investigados. Esta série de experimentos levou ao isolamento e caracterização parcial de um metabólito de SQX, proposto como sendo a estrutura sugerida por Scudi e Silber (41). Todo o trabalho experimental realizado nesta etapa, bem como a análise dos dados obtidos, foi publicada na forma de artigo científico no periódico *Analytical Methods*, cuja íntegra compõe o Anexo I. No referido artigo, também encontra-se a revisão bibliográfica do metabolismo da SQX em diversas espécies animais (17,41).

4.6 Análise de resíduos de sulfonamidas

Resíduos de sulfonamidas são determinados em uma série de matrizes. A presença indesejada de resíduos de SFAs em alimentos é geralmente decorrente da não observação das boas práticas de produção. Alimentos como carne, leite, mel, ovos, pescado e camarão são matrizes de frequente interesse para a pesquisa de resíduos de SFAs (1). Na análise de carne, geralmente se utilizam os tecidos musculares, entretanto outros órgãos e tecidos, como rins e fígado são também considerados como tecidos-alvo (42). As rações para alimentação animal são também um foco de grande interesse para a análise de SFAs, uma vez que é o meio preferencial de veiculação destes fármacos na administração aos animais (43). Mais recentemente, a partir de 2000, cresceu enormemente o interesse pela presença de resíduos de fármacos em geral, com certa ênfase nas SFAs e demais antimicrobianos, em amostras ambientais, como solos e água (44). Tem-se estudado as rotas de transporte e vias de degradação deste grupo de moléculas em amostras ambientais dos mais diversos tipos, desde água do mar até efluentes de estações de tratamento de esgoto (45).

Tratando-se em geral de amostras complexas e com enorme quantidade de substâncias potencialmente interferentes ou amostras em que os analitos estão em concentrações muito baixas, exigindo um fator de concentração adequado (águas superficiais, por exemplo), um dos principais desafios na análise de resíduos de SFAs é a preparação da amostra (46). Técnicas das mais diversas são empregadas com o propósito múltiplo de eliminar compostos interferentes e concentrar os analitos de interesse. Métodos convencionais como extração simples com solvente orgânico (extração sólido-líquido) e extração líquido-líquido são utilizados isoladamente ou em conjunto com técnicas mais complexas como dispersão de matriz em fase sólida, extração em fase sólida (SPE) ou extração por liquido pressurizado (PLE) (47–51). Técnicas mais recentes, como *single-drop* e micro-extração líquido-líquido dispersiva tem ganho espaço, porém ainda com aplicabilidade restrita (52–54). Em termos gerais, o modelo mais usual de preparação de amostras para a análise de resíduos de SFAs envolve uma etapa extrativa inicial (para o caso de amostras sólidas), geralmente baseada em extração simples (extração sólido-líquido) por solvente orgânico (ACN, por exemplo), seguido por uso de SPE e finalizando com uma etapa de evaporação.

As técnicas analíticas utilizadas para a análise de SFAs são bastante variadas. No caso de amostras em que o nível de concentração de resíduos de SFAs é relativamente mais alto, orbitando em valores acima de 25 μg kg^{-1} ou μg L^{-1}, são empregadas técnicas cromatográficas mais usuais, como HPLC-UV, HPLC-DAD ou HPLC-FD, sendo este último modo envolvendo derivatização prévia das amostras com um reagente fluorogênico (geralmente fluorescamina) (3,12,46,55). Modos clássicos de cromatografia também são utilizados, como cromatografia em camada delgada (10,56). Na literatura, são ainda descritos métodos em cromatografia gasosa para a detecção de SFAs, com a necessidade de derivatização dos analitos previamente à introdução das amostras no sistema analítico (57). Dentre os métodos de separação não cromatográficos aplicados para a análise de SFAs, a eletroforese capilar é a mais frequentemente relatada, usualmente com detecção por UV ou ainda por fluorescência induzida a *laser* (EC-LIF) (12,22,58,59). Finalmente, há um grande número de técnicas microbiológicas e/ou imunológicas de análise de SFAs, sendo que a maioria destas provê resultados qualitativos ou semi-quantitativos (60–64).

Todavia, quando tratamos de amostras ambientais ou biológicas, em que o nível de concentração de analitos pode chegar ao nível de ultra-traços (abaixo de 0,1 $\mu g\ kg^{-1}$), a técnica analítica majoritariamente dominante é a cromatografia liquida de alta eficiência acoplada a espectrometria de massas (17,45,49,50). Esta técnica apresenta diversas modalidades de análise e praticamente todas elas já foram relatadas como ferramentas para a análise de SFAs.

Com o propósito de expor o estado-da-arte na análise de resíduos de SFAs, uma breve revisão foi realizada focando na utilização de métodos espectrométricos desenvolvidos e aplicados para amostras biológicas e/ou ambientais. Esta revisão compõe o Anexo II.

A extração por líquido pressurizado (PLE, do inglês *pressurized liquid extraction*) é uma técnica extrativa relativamente recente. A vantagem principal desta técnica é o aumento da solubilidade dos analitos e da cinética da extração através do uso associado de alta temperatura e pressão (65) . Em geral, a técnica apresenta um consumo menor de solventes quando comparada a técnicas tradicionais. Entretanto, a técnica ainda não é popular na química analítica. Alguns motivos para isso podem ser o custo de aquisição do equipamento, bem como a falta de publicações relacionadas à otimização dos métodos, conforme apontado em uma revisão recente sobre o tema (66). A técnica de PLE permite, por exemplo, que limpeza e extração da amostra sejam realizados de modo sequencial e automático, embora esse tipo de aplicação ainda não tenha sido descrito na literatura (66).

Outra técnica relativamente subestimada para a análise de resíduos de medicamentos em alimentos é o uso de ultrassom. O uso de ultrassom como método extrativo apresenta diversas vantagens. Tem baixo custo de aquisição, pois podem ser utilizados estes equipamentos simples como, por exemplo, os banhos de ultrassom utilizados para limpeza de materiais odontológicos (67,68). Além disso, podem ser processadas dezenas de amostras simultaneamente. Apenas um artigo utilizando este tipo de extração para a análise de sufonamidas em matrizes de origem animal foi publicado nos últimos anos (69).

Em geral, a PLE é utilizada para extração de amostras ambientais, como plantas, sedimentos e solo (70–73). Apenas alguns trabalhos que utilizaram PLE para extração de fármacos em tecidos animais foram publicados nos últimos anos (74–78). Recentemente, dois métodos para a análise de sulfas em amostras biológicas e ambientais foram publicados (79,80). Ambos trabalhos utilizaram PLE seguido de SPE. Em geral, após o uso de PLE se faz necessário o uso de uma ou mais técnicas complementares para obter extratos com purificação adequada.

No anexo VII, encontra-se a publicação científica onde se relata o processo de desenvolvimento, otimização e validação de um método de PLE sem necessidade de uso posterior de SPE para a determinação de 16 sulfonamidas e metabólitos em tecidos animais. Neste mesmo trabalho, faz-se a comparação do método de PLE com um método de extração usando ultrassom, o qual também foi desenvolvido e validado durante a execução das etapas experimentais do presente trabalho.

4.7 Cromatografia líquida de alta eficiência (HPLC) acoplada à espectrometria de massas em *tandem* (LC-MS/MS)

Atualmente, a cromatografia líquida acoplada com espectrometria de massas no modo *tandem* (LC-tandem MS, LC-MS^2 ou LC-MS/MS) é um sistema que apresenta excelente sensibilidade e seletividade na análise de traços, para amostras como alimentos, determinação de contaminantes ambientais e indústria farmacêutica (81).

A espectrometria de massas é uma técnica analítica usada para identificar compostos desconhecidos, quantificar compostos conhecidos, e para elucidar a estrutura e propriedades químicas das moléculas. Seu uso requer quantidades bastante pequenas de amostras, sendo uma técnica destrutiva (82).

A técnica é baseada na obtenção de íons a partir de moléculas orgânicas em fase gasosa; uma vez obtidos estes íons, os mesmos se separam de acordo com sua massa e sua carga e são por fim detectados por meio de um dispositivo adequado (83). Um espectro de massas será, como conseqüência, uma informação bidimensional que representa um parâmetro relacionado com a abundância dos diferentes tipos de íons em função da relação massa / carga de cada um deles (*m/z*) (84).

O requisito fundamental para a análise de um composto utilizando a espectrometria de massas é a ionização prévia do composto. O analito deve estar também em fase gasosa. Deste modo, a parte inicial do sistema de um espectrômetro de massas é a fonte de ionização. Na fonte de ionização, a amostra sofre a ionização antes de ingressar dentro do sistema analítico. Existem diversos tipos distintos de ionização e de fontes de ionização. Uma das primeiras técnicas de ionização desenvolvidas na espectrometria de massas é a técnica denominada de Impacto Eletrônico (EI) que consiste no bombardeio da amostra (previamente vaporizada mediante uso de alto vácuo e uma fonte de calor) com uma corrente de elétrons em alta velocidade (84).

Mediante este processo, a substância perde alguns elétrons e se fragmenta gerando diferentes íons, radicais e moléculas neutras. Os íons (moléculas ou fragmentos carregados) são então conduzidos mediante um acelerador de íons até um tubo analisador sobre o qual opera um forte campo magnético. Em seguida, os íons são conduzidos a um coletor/analisador sobre o qual incide o impacto dos referidos íons em função da relação massa/carga dos mesmos (82).

Cada composto é único, e cada substância se ionizará e se fragmentará de uma determinada maneira, em um padrão único e é nesse princípio que se baseia a espectrometria de massas para identificar cada analito.

Um espectrômetro de massas deve ser capaz de, sequencialmente, vaporizar amostras de volatilidades distintas; originar íons a partir de moléculas neutras em fase gasosa; separar estes íons em função de sua razão massa/carga (*m/z*); detectar os íons formados e registrar esta informação de modo adequado. Assim,

podemos intuir as principais partes de um sistema de espectrometria de massas: sistema de introdução de amostras; fonte de ionização, analisador para separação dos íons e sistema detector/registrador (82).

A introdução de amostras é uma parte critica do espectrômetro de massas. Quando a amostra está na fase líquida, como no caso de sistemas de LC-MS, a amostra que elui da coluna cromatográfica deve ser dessolvatada. Esse processo se dá pela ação combinada de temperatura, fluxo de gás inerte e diferença de potencial. A aplicação de uma diferença de potencial, através de um capilar, como ocorre nas fontes de ionização por *electrospray* (ESI), gera cargas nas moléculas de solvente e de soluto. As gotículas de solvente, contendo agora cargas positivas e negativas, tem seu tamanho diminuído pela ação da temperatura até que a repulsão entre as partículas com cargas opostas promove a denominada explosão de Coulomb. A gotícula se desfaz, as moléculas carregadas passam para a fase gasosa e são atraídas para o interior do sistema. Moléculas com carga oposta bem como moléculas neutras e solvente não vaporizado são removidas do orifício de introdução de amostras por ação dos gases inertes (geralmente nitrogênio) (82). No caso da análise de sulfonamidas, por exemplo, a absoluta maioria das aplicações utiliza a fonte de ionização por *electrospray* em modo positivo, ou seja, com o capilar da fonte provendo voltagem positiva, o que promove a ionização positiva das moléculas do analito, gerando íons moleculares de massa $[M + H]^+$ (84). Outros sistemas de ionização utilizados são a ionização química em pressão atmosférica (APCI), onde o solvente sofre a ionização e, em uma etapa seguinte, a carga do solvente é transferida para os analitos (85). Esta técnica é usada para substâncias de difícil ionização nas condições do *electrospray*. Há ainda a fotoionização em pressão atmosférica (APPI), onde uma lâmpada de kriptônio emite fótons de luz UV que promovem a ionização (86). É uma técnica comumente aplicada para a determinação de hormônios (86).

Uma vez introduzidos dentro do sistema, é necessário a aplicação de um processo físico-químico que promova a separação dos íons de modo a tornar possível a análise. Este processo é denominado de seleção de massa. A seleção de massa é obtida pela escolha adequada da combinação radiofrequência (RF) e voltagem de corrente (DC), de tal forma que os íons pertencentes a uma estreita

faixa de massa/carga, possam ser estáveis dentro do trajeto do quadrupolo, em uma determinada radiofrequência (RF) e voltagem de corrente (DC). Em termos físicos, esse processo se dá no chamado quadrupolo, que consiste em 4 (ou 6) barras metálicas magnéticas e paralelas que constituem o quadrupolo propriamente dito (ou hexapolo, no caso de 6 barras) (82). Quando a análise é realizada apenas com a ionização e posterior seleção dos íons, a análise é convencionalmente chamada de MS. Quando se produz a fragmentação dos íons e posterior seleção dos fragmentos, temos a análise de tipo MS/MS ou *tandem* MS. Para este tipo de análise, geralmente são utilizados os sistemas denominados de triplo quadrupolo, também denominados "*tandem-in-space*" o que significa que cada etapa dos experimentos MS/MS é conduzida em zonas espacialmente distinta do equipamento. Nestes sistemas, teremos um quadrupolo inicial (Q1) onde se dá a seleção dos íons de interesse dentro de uma faixa de massas (seleção de massa). Todos os demais íons fora desta faixa são desviados. No segundo quadrupolo (Q2), também chamado de câmara de colisão, os íons selecionados em Q1 são submetidos ao gás de colisão (geralmente argônio ou nitrogênio) e se fragmentam gerando os chamados íons-filho. A seguir, estes fragmentos entram no último quadrupolo (Q3), onde uma nova seleção de massa irá remover os íons indesejados (83,84). A figura 1 mostra a estrutura usual de um sistema de espectrometria de massas com triplo quadrupolo.

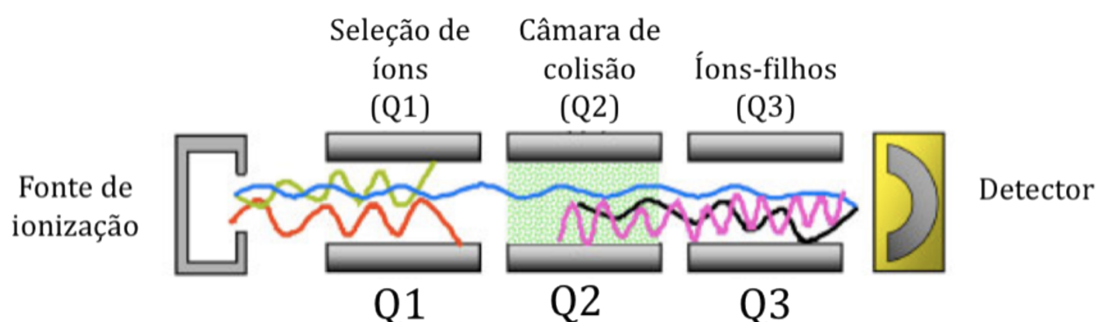

Figura 1. Representação em forma de esquema de um sistema de espectrometria de massas de triplo quadrupolo.

Esta configuração permite diversos modos de análise. Para fins quantitativos, o modo mais usado é o de *multiple reaction monitoring* (MRM). Em análises de tipo MRM, se conhece a estrutura do íon pai (íon molecular) e dos íons-filhos. Logo, o sistema pode ser otimizado para selecionar valores específicos de *m/z* em Q1 e Q3.

Esta técnica possui a maior sensibilidade, capaz de realizar análises em nível de traço e ultra-traço (87). Outro modo de análise é o *product ion scan*, onde se faz uma seleção de massa em Q1 e se geram todos os fragmentos possíveis, podendo ser aplicado um gradiente de energia de colisão. O espectro produzido é o espectro MS/MS ou MS^2 (84). O triplo quadrupolo também possui dois tipos de *scan* MS/MS de alta seletividade, o *precursor ion scan* e o *neutral loss scan*, que são particularmente úteis para identificação estrutural e para a quantificação de analitos em matrizes complexas. No caso do *precursor ion scan*, a lógica é inversa ao do *product ion scan*: conhece-se um ou mais fragmentos (íons-filhos) e se faz a varredura em Q1 para selecionar aqueles íons-pai que quando submetidos à câmara de colisão, geram os íons-filho selecionados. Este modo de análise é particularmente útil para elucidação de metabólitos e produtos de degradação, especialmente para grupos de compostos como as sulfonamidas, onde o padrão de fragmentação é bastante homogêneo em toda a família destas substâncias (88). Já o *neutral loss scan* permite observar os íons que sofrem uma perda neutra específica. A perda neutra é aquela parte da molécula que, quando fragmentada na câmara de colisão, fica sem carga nominal. Este tipo de análise é bastante útil no monitoramento de pequenas modificações moleculares como a perda de uma molécula de água, por exemplo. Outra aplicação é na elucidação estrutural de metabólitos, onde pode se monitorar a perda neutra em conjugados como, por exemplo, ácido glicurônico e glutationa (89).

Finalmente, os íons chegam ao sistema de detecção. O detector mede a abundância de elétrons gerados pelos íons, para cada relação *m/z*. A maioria dos sistemas de MS usa algum tipo de multiplicador de elétrons como detector, combinado a um amplificador de sinal. O registro de todas as cargas detectadas durante a varredura constitui o espectro de massas (83).

Por si só, a espectrometria de massas pode identificar de uma maneira quase inequívoca qualquer substância pura, porém normalmente não é capaz de identificar os componentes individuais de uma mistura sem separar previamente seus componentes, devido a extrema complexidade do espectro obtido pela superposição dos espectros particulares de cada componente (82).

A grande versatilidade atualmente atribuída a espectrometria de massas se deu pelo acoplamento desta técnica com a cromatografia. São os chamados métodos hifenados: GC-MS (ou GC-MS/MS) quando acoplada à cromatografia gasosa e LC-MS (ou LC-MS/MS) quando acoplado à cromatografia liquida. A utilização de um método de separação anterior ao processo de ionização e introdução no espectrômetro de massas permite a resolução de misturas e amostras complexas (84).

A técnica atualmente mais difundida é, sem dúvida, LC-MS/MS. A cromatografia liquida, com suas enorme variedade de polaridades de colunas, de combinações de solventes para compor a fase móvel e diversos parâmetros que podem ser otimizados, tem uma versatilidade analítica que permite desde a análise de pequenas moléculas até grandes polímeros, passando por fármacos, pesticidas, peptídeos, proteínas, etc. As interfaces desenvolvidas para permitir o uso das duas técnicas promovem a remoção eficiente da fase móvel e a ionização dos analitos. Mesmo grandes moléculas de polaridade relativamente baixa podem ser perfeitamente ionizadas e levadas a fase gasosa (84).

Essa versatilidade, associada a grande sensibilidade e especificidade da técnica, fez com que LC-MS/MS se tornasse método de escolha para estudos farmacocinéticos e bioanálises em geral. O método é largamente aplicado para a determinação de traços em matrizes biológicas complexas. Estas aplicações compreendem resíduos de medicamentos, resíduos de pesticidas, bem como metabólitos e produtos de degradação destes compostos (82).

Mais recentemente, novos sistemas com analisadores de massas de alta resolução estão disponíveis comercialmente. Dentre as tecnologias recentemente introduzidas, temos o detector de tempo de vôo (TOF), o *ion trap* (IT) e sua variedade linear, *linear ion trap* (LIT).

O analisador de massas tipo tempo de vôo (TOF, *time of flight*) é, como o próprio nome indica, um modo de análise em que os íons se diferenciam uns dos outros pelo tempo de vôo. Todos os íons que ingressam no sistema dotado de um analisador TOF recebem um pulso de energia igual (pulso de extração), mas são

acelerados de maneiras diferentes devido à sua *m/z* e chegam ao detector em tempos diferentes. Os íons com menor m/z terão maior velocidade e chegarão primeiro ao detector, e assim por diante. Desta forma, pela medida do tempo de vôo dos íons, pode-se deduzir sua *m/z*, podendo analisar compostos de massa baixa até macromoléculas. Alguns sistemas permitem a fragmentação dos íons selecionados em Q1 (qTOF/MS) (90).

Em teoria, os analisadores TOF, não tem limite máximo de massa. Portanto são especialmente adequados para acoplar com técnicas suaves de ionização como ESI, que podem ionizar macromoléculas sem induzir fragmentação. Estes analisadores também têm alta taxa de transmissão de íons, fazendo que tenham alta sensibilidade. Se os íons são formados de forma pulsada, o pulso de extração do TOF pode ser coordenado com a fonte de ionização e todos os íons formados podem ser detectados. Já com ionização contínua (ESI, APCI), se não houver um trapeamento inicial haverá perda de íons. Isto pode ser resolvido, em grande parte, por extração ortogonal e lentes eletrostáticas que controlam a entrada dos íons no TOF (90).

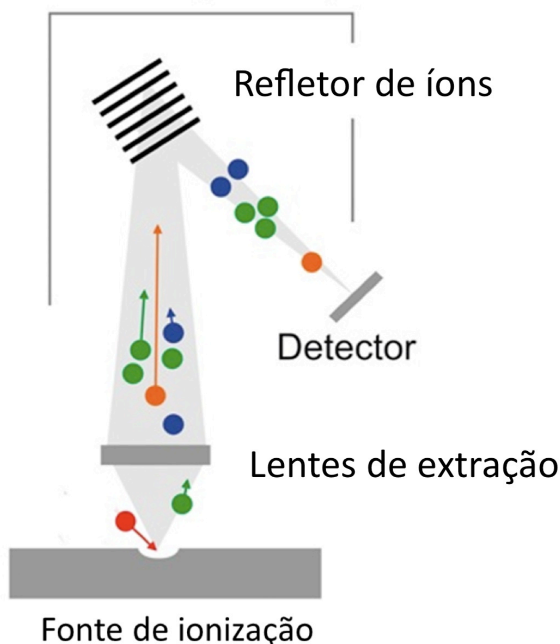

Figura 2. Representação em forma de esquema de um sistema de espectrometria de massas com detector de tempo de vôo

O sistema *ion trap* usam um eletrodo esférico para produzir um campo elétrico que captura os íons à medida que entram. Os íons capturados são processados em um campo oscilante tridimensional, a partir do qual são liberados seletivamente. Esses sistemas são conhecidos como 3D *ion trap* (91).

No *ion trap linear* (LIT), os íons são confinados radialmente por um campo de radiofreqüência (RF) bidimensional (2D). O LIT faz uso da estrutura básica de um quadrupolo, ou seja, um arranjo de quatro superfícies elétricas. No entanto, em vez de ser usado para filtrar íons de todos os valores *m/z*, eles são utilizados para captura, manipulação da trajetória de íons, e ejeção do íon *m/z* selecionado. Os íons num LIT estão confinados radialmente (direções x e y) por um campo RF bidimensional, semelhante ao empregado em um analisador quadrupolo; e axialmente (direção z) por potenciais aplicados aos eletrodos, que limitam o fluxo de íons longitudinalmente. Isso aumenta a capacidade de captura e a sensibilidade do sistema em comparação com *ion trap* convencional (91).

Todas as técnicas aqui descritas foram utilizadas em modo combinatório para a investigação de SQX e de metabólitos no decorrer de todo projeto. Para análises qualitativas e quantitativas com resolução de massas relativamente baixa, foram usados sistemas de LC-MS/MS de tipo triplo quadrupolo com ionização por electrospray. Para análises de elucidação estrutural, utilizaram-se sistemas de tipo LC-Qq-TOF-MS e LC-QqLIT.

4.8 Elucidação estrutural

O conhecimento do arranjo dos átomos em uma molécula (estrutura molecular) e da posição relativa de todas as moléculas em um cristal (estrutura cristalina), para substâncias obtidas tanto sinteticamente como isolados de fontes naturais, é extremamente útil para o entendimento das propriedades químicas, físico-químicas e biológicas dos compostos para os mais variados ramos da ciência (92). Na Física e nas Ciências Moleculares, é fundamental a relação entre propriedades físicas e a estrutura interna dos sólidos. Em Química as características

estéricas de novos compostos ou complexos só podem, em muitos casos, serem conhecidas através da determinação de sua estrutura. Em Biologia e Bioquímica a atividade funcional de uma biomolécula está intimamente relacionada com sua estrutura tridimensional. Em Medicina e Farmacologia a ação de certos fármacos envolve interações entre fármaco-receptor e a maneira como isto acontece é determinada pela estrutura de ambos componentes.

Após a obtenção dos metabólitos de SQX em quantidade adequada e com o grau de purificação necessário, a elucidação estrutural foi obtida com o uso associado de diversas técnicas de espectrometria de massas de alta resolução.

5. RESULTADOS E DISCUSSÃO

5.1 Análise de SQX e metabólitos em tecidos animais

A análise de resíduos de SQX foi realizada utilizando LC-MS/MS. Os detalhes e parâmetros do método estão descritos no Anexo I. O método utilizado foi publicado anteriormente (12). A partir de seu desenvolvimento e validação inicial, o escopo deste método foi estendido para abranger diversas novas matrizes e analitos. O artigo que trata desta extensão de escopo e das abordagens utilizadas para validar tais extensões compõe o Anexo III deste trabalho, publicado na revista *Food Additivies and Contaminants: Part A*.

5.2 Elucidação estrutural de metabólitos de SQX

O uso de técnicas associadas de espectrometria de massas e a produção de extratos semi-purificados de SQX e seus metabólitos a partir de amostras biológicas foi crucial para a elucidação da estrutura molecular dos metabólitos SQX-OH e N^4-acetil-SQX-OH. A investigação completa desta etapa de desenvolvimento da tese foi publicada na revista *Analytical Chemistry* (anexo V).

5.3 Validação e desenvolvimento de métodos de análise

A aplicação de métodos extrativos mais eficientes, em termos de custos, velocidade e facilidade de execução, é uma busca permanente para métodos de análise de rotina. No campo de análise de resíduos de fármacos e pesticidas, estes temas têm grande importância. Durante o desenvolvimento desta tese, foram considerados tópicos de grande relevância para a área bem como de aplicação prática nas demandas analíticas da rede de laboratórios do Ministério da Agricultura, Pecuária e Abastecimento. Os resultados obtidos são apresentados nos anexos. O anexo II apresenta uma revisão dos métodos mais recentes para análises de sulfonamidas em amostras ambientais, bem como o perfil dos níveis de concentração encontrados para estas substâncias em diversos países. O anexo III apresenta um trabalho publicado a respeito de validação de métodos, com ênfase em extensões de escopo e apresenta propostas práticas e concisas de realizar validações de modificações em métodos já estabelecidos, sem que se tenha a necessidade de fazer uma validação completa ou revalidação da técnica. O anexo IV apresenta o resultado de trabalhos experimentais que investigaram quais seriam as melhores abordagens analíticas para a determinação do efeito de matriz em métodos de LC-MS/MS. Para este estudo, foram utilizados os dados experimentais obtidos no desenvolvimento de diversos métodos para a análise de resíduos de sulfonamidas. No anexo VI, apresenta-se modelo baseado em análise de risco para elencar ou priorizar fármacos que apresentem potencial tóxico, seja por sua presença residual em alimentos ou em amostras ambientais. O modelo foi avaliado utilizando como exemplo o grupo das sulfonamidas, onde foi traçado o perfil de todas as apresentações comerciais contendo sulfonamidas disponíveis no Brasil. Finalmente, o anexo VII apresenta o desenvolvimento, otimização e validação de dois métodos de análise de resíduos de sulfas e metabólitos em amostras biológicas, bem como a comparação das duas metodologias.

6. Relação das etapas desenvolvidas neste trabalho

6.1 Laboratório Nacional Agropecuário de Porto Alegre (Lanagro/RS):

- Desenvolvimento e validação de método para análise de sulfaquinoxalina (SQX) e metabólitos em tecidos e fluidos biológicos de bovinos, eqüinos, aves, suínos e ovinos.
- Análise das diferenças de metabolismo de SQX nas espécies estudadas.

- Obtenção de metabólitos de SQX a partir de experimentos *in vitro* (fígado equino) e *in vivo* (urina equina).
- Estudos *in vivo* (ovinos e/ou aves) para verificação da formação de metabólitos conjugados com ácido glicurônico e investigação de outras vias de metabolização. Desenvolvimento e validação de método para análise de sulfaquinoxalina (SQX) e metabólitos em tecidos e fluidos biológicos de bovinos, equinos, aves, suínos e ovinos.
- Purificação e isolamento de metabólitos.
- Análise de metabólitos en LC-qTOF e LC-LIT-MS/MS.

6.2 *Instituto de Diagnóstico Ambiental y Estudios del Água* (IDAEA-CSIC), em Barcelona, Espanha, no período de setembro a dezembro de 2013.

- Análise das diferenças de metabolismo de SQX nas espécies estudadas.
- Obtenção de metabólitos de SQX a partir de experimentos *in vitro* (fígado equino) e *in vivo* (urina equina).
- Purificação e isolamento de metabólitos.
- Análise estrutural de metabólitos semi-purificados e purificados por LC-Qq-TOF-MS e LC-QqLIT-MS.
- Estudos de fotodegradação e outros estudos experimentais auxiliares à elucidação estrutural dos metabólitos de SQX.
- Uso de extração com líquido pressurizado (PLE) e ultra-som (US) como técnicas alternativas de extração para SQX e metabólitos.
- Determinação de SQX e metabólitos em peixes de rios e cursos de água próximos à granjas aviárias.

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Anexo I – Artigo publicado em *Analytical Methods:* **Characterization and estimation of sulfaquinoxaline metabolites in animal tissues using liquid chromatography coupled to tandem mass spectrometry.**

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PAPER

Characterization and estimation of sulfaquinoxaline metabolites in animal tissues using liquid chromatography coupled to tandem mass spectrometry

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Sulfaquinoxaline (SQX) is a sulfonamide that is widely used in veterinary medicine, with a maximum residue limit (MRL) established for several food matrices. In Brazil, the MRL for liver and muscle is 100 μg kg^{-1} for equine, bovine, poultry and swine. This value includes not only free drug but also the sum of all metabolites. Several reports showed limitations for SQX residue analysis, especially when mass spectrometry methods were used. These limitations include poor recoveries and unacceptable accuracy responses. In this work a metabolite of SQX, present in liver and kidney samples, was identified. The structure proposed was a hydroxylated form of SQX, called SQX-OH, with an *m*/*z* of 317. SQX-OH is also produced *in vitro* in equine, swine and bovine liver samples. The influence of time, temperature, solvent and dehydration was evaluated in the formation of SQX-OH. Different degrees of hydroxylation were observed in matrices. The N_4-acetyl derivates for both SQX and SQX-OH were also detected. In equines, the metabolism of SQX is complete. The mass spectrometry analysis of SQX-OH was determined *in vitro* using equine liver microsomal fraction. The characterization of this compound was performed using liquid chromatography coupled to mass spectrometry in tandem mode. The fragmentation profile of SQX-OH was seen to be similar to that of the sulfonamides group, producing two high abundant daughter ions: 317 > 156 and 317 > 108, common to most sulfonamides. The main conclusion of this work is that the residue analysis of SQX needs to consider the presence of the SQX-OH in order to give more realistic results, especially when using MRM transitions.

Introduction

Sulfonamides are a class of antibacterial compounds widely used in human and veterinary medicine. Metabolites of this class of drugs were very well studied in the human species.[1] However, some sulfonamides, specific for the treatment of animals, are less well known. Sulfaquinoxaline (SQX), for example, is one of the most used sulfonamides in poultry and swine treatment. The use of SQX as a coccidiostat agent was responsible for mass poultry production in the last century.[2,3] To our knowledge, there are only a few studies on SQX metabolism in animal species. Several reports deal with other sulfonamides, showing that N_4-acetyl metabolite formation is one of the most common ways to eliminate these drugs from the organism. The major routes for sulfonamide metabolism are conjugations with acetyl, hydroxyl or glucuronic acid groups, in order to obtain more polar compounds which are eliminated in urine.[4,5] Generally, just few works deal with other routes of biotransformation of sulfonamides.[6–13] Kishida and Furusawa report a HPLC analysis of four hydroxylated metabolites and also acetylated metabolites of sulfamonomethoxine and sulfadimethoxine.[14] Vree and co-workers have also discussed the metabolism of sulfonamides by hydroxylation and acetylation in chicken.[15] Sulfadimethoxine metabolism by hydroxylation in positions 2, 6 and both 2 and 6 of the pyrimidine ring, generating 3 hydroxylated metabolites, produced *in vivo* in poultry were investigated by Nagata and Fukuda.[16] The same hydroxylation patterns were observed for sulfamonomethoxine in turtles.[17] OH-metabolites, when the hydroxylation occurs in the radical moiety, still possess a free *para*-aminophenyl group which interferes with *para*-aminobenzoic acid synthesis in bacteria.[18] Moreover, acetylated metabolites of sulfonamides have no bacterial activity, a lower solubility in physiological pH, which may lead to kidney precipitation, de-acetylation of the parent drug both *in vivo* and *in vitro* and higher plasma protein binding than the parent drug.[19–24] Considering the differences found among animal species in sulfonamide metabolization, especially for liver mediated biotransformation, further knowledge of these processes can be considered relevant not only for pharmacological studies, but also for the analysis of sulfonamide residues in food.[25]

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Like other sulfonamides, SQX has a maximum residue limit (MRL) established for several food matrices. In Brazil, a value of 100 μg kg^{-1} is adopted for liver and muscle.[25] This value is extended not only to the parent compound but also to the sum of all sulfonamide metabolites. Sulfonamide residues are routinely monitored at various government and private laboratories around the world.[26] Considering the amphoteric behaviour of these compounds, sulfonamides can be analyzed using distinct methods, such as liquid chromatography,[27–31] capillary electrophoresis or microbiological and immunological assays.[32]

SQX analysis presents some difficulties, especially in analytical methods using HPLC with UV or fluorescence detection. Generally, SQX is analyzed together with other sulfonamides. For instance, sulfadimethoxine, a sulfonamide that is also common in veterinary medicine and has an octanol–water partition coefficient very similar to the SQX, co-elutes with SQX in reverse phase chromatography.[28]

Limitations for SQX residue analysis, when mass spectrometry methods using multiple reaction monitoring (MRM) mode have been reported.[33] These limitations include poor recoveries and unacceptable accuracy responses, even when extraction losses are negligible. Degradation, extraction losses and SQX metabolization were suggested as possible reasons. Some authors proposed that liver enzyme activity might continue postmortem.[34]

In the present work, some SQX metabolites were detected and investigated. The influence of several factors in SQX metabolites formation and distribution between distinct species was evaluated. Also, a procedure for the production of an SQX metabolite *in vitro* were developed and applied to metabolite quantitative analysis in real samples.

Experimental

Chemicals and reagents

Except when indicated, all reagents were HPLC grade. HPLC purity water was obtained from a Milli-Q purification unit (Millipore, Bedford, MA, USA). For the mobile phase, solvents were filtered through a 0.22 μm nylon membrane filter (Millipore) and sonicated before use.

Analytical standards of SQX (99.0%) and sulfapyridine (SPY, 99.5%) were obtained from Fluka. Magnesium chloride, sucrose, sodium acetate, ethyl acetate, chloroform, methanol and *tert*-butanol were purchased from Merck. Fluorescamine and potassium cyanide were from Across Organics. Acetonitrile, acetone and ammonium acetate were obtained from J.T. Baker. Stock solutions were made by dilution of solid standards with methanol to a concentration of 1 mg mL^{-1}. Work solutions were made by dilution of the stock solutions with ammonium acetate 10 mM/methanol (75 : 25) to the appropriate concentrations. Stock solutions were stable for 6 months at −20 °C. The working standard solutions were stable for 1 month at 4 °C.[13]

Instrumentation

Liquid chromatography tandem mass spectrometry. Liquid chromatography coupled to tandem mass spectrometry was applied according to a previously developed and validated method for sulfonamide analysis published elsewhere.[27] The LC-MS/MS system used was an API 5000 mass spectrometer (Applied Biosystems, Foster City, CA). The analytical column was a Luna C18 150 × 2.1 mm (Phenomenex, Torrance, CA). The pre-column used was a guard cartridge system consisting of a C18 cartridge with 4.0 × 3.0 mm (Phenomenex) inserted in a holder. The mobile phase consisted of ammonium acetate 10 mM with 0.1% acetic acid (solvent A) and methanol (solvent B) in gradient mode, starting with 25% of solvent B and 75% of solvent A, and held for 3 minutes. Next, solvent B concentration was increased to 90% in 1 minute and decreased again to 25% in 2 minutes, for a total duration of 6 minutes for each run with an equilibrium time of 3 minutes under the same initial conditions. The mobile phase flow was 800 μL per minute; the injection volume was 20 μL. Analytes were introduced into the mass spectrometer through an electrospray probe operating in positive mode. All data were processed by software Analyst version 1.4.2 (Applied Biosystems). Mass spectrometry parameters were used according to the sulfonamide residue analysis method used currently as a routine in our laboratory[27] and are shown in Table 1.

Samples

Blank samples of bovine, equine, swine and poultry liver were obtained from Brazilian Federal Inspection Services (SIF), the national food inspection service managed by the Brazilian Ministry of Agriculture, collected in several slaughterhouses and meat plants.

Extraction procedure

For extraction, 2.5 g of chopped and homogenized liver tissue were weighed in a 50 mL polypropylene centrifuge tube. Internal standard (SPY) was added to a concentration of 100 ng g^{-1}. Approximately 3.0 g of anhydride sodium sulphate were added to tissue and mixed with a glass stick. An aliquot of 10 mL of acetonitrile was added and the mixture was placed in a head-to-head shaker for 30 min. The mixture was then centrifuged for 20 min at 4000 rpm. The supernatant was transferred to an empty, clean glass tube. Solid residue was submitted to an additional acetonitrile extraction (5 mL) and extracts were combined before the evaporation step. Organic extract was evaporated in a water bath (40–45 °C) under a gentle flow of nitrogen until dryness. Dry residue was reconstituted in 2 mL of the mobile phase mixture (10 mM ammonium acetate : methanol, 75 : 25, v/v) and mixed vigorously in a tube shaker for 30 s and then tubes were centrifuged for 5 min at 2000 rpm. A 300 μL aliquot of supernatant was transferred to an empty HPLC vial in which a volume of the mobile phase mixture was added to a final volume of 1.5 mL. Aliquots of 20 μL of this diluted extract were analysed.

Animal study 1 – rats

Three adult male Wistar rats were treated with an aqueous solution of sodium SQX. Approximately 0.5 mL of a solution containing 150 mg mL^{-1} of SQX was administered orally using a sterile plastic syringe. After 4 hours, the animals were sacrificed and tissues (liver and kidney) were analyzed by routine analysis protocol.[27] All animal studies were executed under surveillance and previous approval of Lanagro/RS Biosafety Internal Commission (CIBio) (MET RPM 01/05).

Table 1 Mass spectrometry analysis parameters

Compound	Precursor ion $[M + H]^+$ *m*/*z*	Transitions observed	Collision voltage (V)	Cone voltage (V)
SPY[a]	250	**250 > 156**[b]	25	46
		250 > 108	35	46
SQX	301	**301 > 156**	25	71
		301 > 108	37	71

[a] Internal standard. [b] Bold transitions are used for quantitative analysis.

Animal study 2 – poultry

For metabolomic evaluation of SQX, farmed chickens were treated with commercial feed spiked with SQX. A negative control group was analyzed simultaneously. NeoSulmetina SM® was used to medicate the feed. This pharmaceutical form presents 2% of SQX associated with 0.2% of neomycin. Administration of the medicated feed was carried out using the supplier's instructions. Animals were sacrificed according to a humanitarian protocol and their tissues (liver, kidneys and muscle) were analyzed for sulfonamide metabolites. Tissues were collected after 2 hours and 8 hours of administration. Sulfonamide extraction was performed with 5.0 mL of acetonitrile. This procedure was repeated twice and supernatants were combined in a clean tube. Organic extract was evaporated to dryness in a water bath under nitrogen stream at 40–45 °C. Residues were reconstituted with 2.0 mL of ammonium acetate 10 mM : methanol (75 : 25).

Animal study 3 – horse

A mare weighing approximately 300 kg was treated with SQX in the feed. The SQX concentration in the medicated feed was calculated to obtain a therapeutic dosage of 10 mg kg^{-1}. Blood samples (5 mL) were collected 0.0, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 hours after ingestion of the medicated feed. Urine samples were collected approximately 2.5 and 7.0 hours after ingestion. Blood samples were immediately placed in test tubes containing 1.67 mL of sodium citrate solution, as an anticoagulant agent. Plasma and urine were centrifuged at 3000 rpm for 10 minutes. 100 μL of supernatant were transferred to microcentrifuge tubes (1.5 mL) and 280 μL of acetonitrile and 20 μL of sulfapyridine solution (internal standard) at 2.5 μg mL^{-1} were added to each sample. Tubes were manually and vigorously mixed and were kept in a refrigerator for 2 hours. After that, tubes were centrifuged at 10 000 rpm for 5 minutes. An aliquot of 150 μL of supernatant was placed in an HPLC vial and diluted to 1.5 mL with ammonium acetate 10 mM : methanol (75 : 25).

Horse microsomal liver assay

A blank liver sample from equines was chopped and homogenized. 25 g of tissue was weighed. Then, microsomal enzymatic fraction was isolated using extraction with 50 mL of 5 mM magnesium chloride and 250 mM sucrose (pH 7.4).[35] The sample was mixed for 30 minutes and allowed to stand for 1 hour. After that, the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant pH, which contain microsomal fraction, was adjusted to 5.25 using acetate buffer. Then, the extract was spiked with SQX standard solution to obtain a concentration of 40 μg mL^{-1}. The solution was stirred for 30 minutes and after 15 minutes SQX metabolites were extracted by liquid–liquid extraction using ethyl acetate. Organic fractions were collected and evaporated to dryness. The extract was redissolved in methanol and submitted to the following chromatographic analysis.

Thin layer chromatography (TLC) isolation

Methanol extracts obtained in horse microsomal liver assay were applied to TLC plates. Each sample application consisted of 20 μL aliquots of methanolic extract. A pool solution of sulfonamides was used as positive control. Whatman LK6D TLC glass plates with 20 × 20 cm with a silica layer were used (Whatman). Samples were applied at 1 cm above the plate bottom and plates were developed in a cube containing 100 mL of methanol up to 1 cm. Then, plates were gently dried (~40 °C) and eluted in methanol again up to 7 cm. After that, plates were dried once more and developed in 60 mL of chloroform : *tert*-butanol (80 : 20) up to 14 cm. Samples were revealed spraying the plates with fluorescamine solution (250 mM in acetone). Spots were analyzed under UV light (410 nm).

High performance liquid chromatography with diode array detection (HPLC-DAD) isolation

HPLC-DAD was used to collect the sample fractions containing SQX-OH. Horse liver was spiked with SQX solution in order to obtain a concentration of 40 μg g^{-1}, before the extraction with acetonitrile. Then, samples were analyzed using the same protocol described before for mass spectrometry analysis. HPLC-DAD analysis was performed using the same mobile phase, gradient mode and column used for LC-MS/MS analysis. However, the injection volume was 50 μL and the mobile phase flow was 0.5 mL min^{-1}. Fraction collection was performed manually monitoring the peaks at 254 and 270 nm. Fractions were pooled and analyzed by direct infusion in the MS system.

Results and discussion

Poor SQX recoveries were reported in several studies. Bogialli *et al.* report a very poor recovery for SQX, especially when extracting it from bovine liver.[33] Using HPLC with a DAD detector, they verified that loss of SQX was accompanied by a peak that was eluted about 4 min before SQX. They also investigated and discarded thermal degradation of SQX occurring during extraction by observing that no significant loss occurred on extracting it from both chicken liver and kidney or from bovine muscle. In conclusion, these authors proposed that this compound, characterized by a molecular mass 16 Da larger than that of the parent compound was formed during the sample treatment, presumably by enzymatic oxidation. Bogialli and coworkers detected a compound resulting from SQX modification, with *m*/*z* 317, but in that report, they did not investigate the structure of this compound or possible mechanisms involved.[33] In our laboratory, sulfonamide residues were routinely monitored in liver, egg and milk samples.[27] For SQX three *m*/*z* transitions are monitored: 301 > 156, 301 > 108 and 301 > 92. Each analysis batch was routinely composed of 3 types of quality

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control (QC) samples: (1) a matrix-matched calibration curve spiked with the analytes before extraction; (2) recovery samples, in which analytes are added before extraction and (3) "tissue standard" samples, in which analytes are added after extraction. From these QC samples, data about batch performance were obtained by the following procedure: (1) recovery samples give the accuracy data for the batch and were calculated using the calibration curve. As both were spiked before the extraction, no correction factor is applied. (2) Comparison between QC samples types 2 and 3 gives the recovery rate for a particular batch. QC samples types 2 and 3 were always spiked at the same concentration, but type 2 is spiked before extraction and type 3 after the extraction. So, the difference in peak area between these samples provides the recovery of the extraction procedure. To prepare these quality control samples, a previously analyzed sample is defined as a blank sample. When equine liver blank sample was used, no sign of MRM transition for SQX appeared in quality control samples types 1 and 2, but normal peak shape and signal were shown for samples type 3. Considering that tissue standard samples are spiked after extraction, we conclude that some SQX alteration occurred during the extraction process. During routine analysis, loss and/or absence of SQX were observed in spiked samples of equine, bovine and porcine liver.

Firstly, the total absence of SQX in spiked samples of equine liver previously spiked with this analyte led us to consider a possible chemical degradation. To evaluate this possibility, a critical analysis of sample preparation was performed. Evaporation of acetonitrile extract was considered a critical point. In order to investigate the influence of evaporation temperature on SQX, six analyses were performed without a matrix. Water bath temperatures of 40 and 50 °C were investigated. Although the highest temperature provokes a loss of 10% in SQX recovery, this does not explain a total absence of analyte.

Reviewing the literature, the post-mortem enzymatic activity of the liver was considered. Liver contains very active metabolic enzyme systems such as the cytochrome P450 complex and reductase activity.[34] This enzymatic activity may lead to post-mortem *in vitro* drug metabolism, as is the case in the rapid and complete inactivation of chloramphenicol and carbadox in the liver and kidney. This hypothesis was experimentally evaluated in a fast experiment in which 200 mM of a well-known microsomal inhibitor, potassium cyanide, was added to horse liver samples spiked with SQX before organic solvent extraction. When cyanide was present, SQX showed normal recovery and accuracy values. Samples without cyanide addition showed complete absence of SQX.

Animal specie specificity for SQX metabolism

Horse liver presents physiologic differences compared to bovines, swine and other food-producing species. Like rats, horses do not have a gallbladder. To evaluate whether this

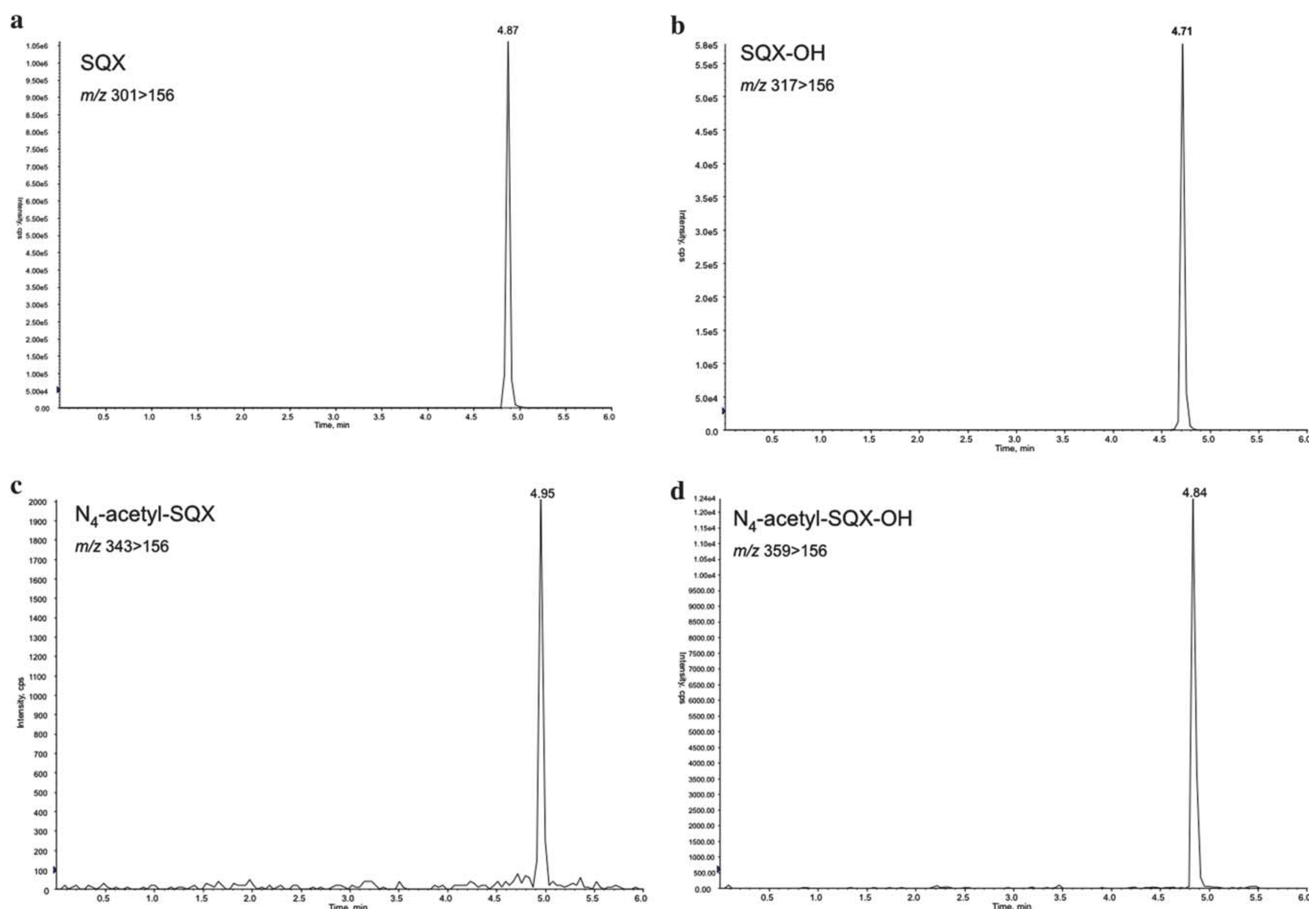

Fig. 1 MRM extracted chromatograms of rat liver extract. From top to bottom: SQX-OH. SQX. N_4-SQX and N_4-SQX-OH.

characteristic was correlated with *in vitro* enzymatic activity, experiments were performed with Wistar rats. Results show that after 4 hours of SQX administration, SQX and three different metabolites can be found in liver extracts (Fig. 1). SQX, SQX-OH, N_4-acetyl-sulfaquinoxaline (N_4-AC-SQX) and N_4-acetyl-hydroxilsulfaquinoxaline (N_4-AC-SQX-OH) were present in the liver and kidney at several degrees of concentration (Table 2).

To evaluate specie-specificity of enzymatic activity, farmed chicken was treated with a commercial formulation containing SQX. Animals were divided into 2 groups of 3 animals each: an untreated negative control group and a test group. Two and 8 hours after administration, no detectable trace of SQX-OH was found in liver, muscle and kidney of the control or test group.

Bovines and swine show a variable enzymatic conversion *in vitro*. Swine liver (n = 10) and bovine liver (n = 7) were analyzed individually. All 17 samples had been previously analyzed and no trace of sulfonamides was detected. Therefore, another portion of each sample was then placed in contact with SQX standard solution (to obtain 100 ng.g^{-1}) for 15 minutes before the extraction procedure. Two matrix-matched calibration curves were used to calculate sulfonamide residue in these samples. The first one was performed in poultry blank liver, spiked with SPY and SQX. The second curve was performed in equine blank liver and likewise spiked with SPY and SQX. The "equine curve" was used to estimate SQX-OH concentration. All samples, including calibration samples, were placed in contact with the standards for 15 minutes before extraction. As shown in Table 3, SQX to SQX-OH conversion was similar in all swine samples, with a mean value of 11.6 ng.g^{-1} and a relative standard

Table 2 SQX and metabolites distribution in rat liver and kidney after 4 hours of SQX single dose (150 mg) administration

	4 hours			
Compound	Liver[a]	Kidney[a]	% in liver[b]	% in kidney[b]
SQX	1.1×10^6	1.5×10^6	45.76	92.34
SQX-OH	1.3×10^6	1.2×10^5	54.08	7.39
N_4-AC-SQX	2010	4020	0.08	0.25
N_4-AC-SQX-OH	1910	380	0.08	0.02

[a] Values for peak intensity. [b] Percentage of sum of SQX and the 3 metabolites.

Table 3 SQX and SQX-OH concentration in swine and bovine liver samples

	Calculated concentration (ng g^{-1})			Calculated concentration (ng g^{-1})	
Sample name	SQX-OH	SQX	Sample name	SQX-OH	SQX
Swine 1	18.1	83.2	Bovine 1	62.8	36.9
Swine 2	9.17	85.3	Bovine 2	18.9	78.6
Swine 3	13.7	84.4	Bovine 3	21.8	83.2
Swine 4	14.6	84.9	Bovine 4	41.1	59.6
Swine 5	8.32	83.9	Bovine 5	83.9	4.35
Swine 6	14.6	66.9	Bovine 6	11.1	71.1
Swine 7	8.62	89.6	Bovine 7	36.7	59.4
Swine 8	7.79	80.4	—	—	—
Swine 9	11.6	91.4	—	—	—
Swine 10	9.06	85.9	—	—	—
Average swine	11.6	83.6	Average bovine	39.5	56.2
SD swine	3.5	6.6	SD bovine	26.0	27.5
RSD swine (%)	30.4	7.9	RSD bovine (%)	66.0	49.0

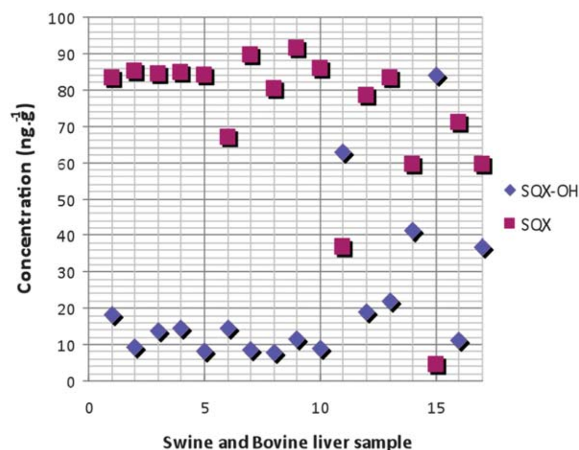

Fig. 2 Plot of SQX and SQX-OH concentration in swine and bovine liver. Samples 1 to 10 = swine liver. Samples 11 to 17 = bovine liver.

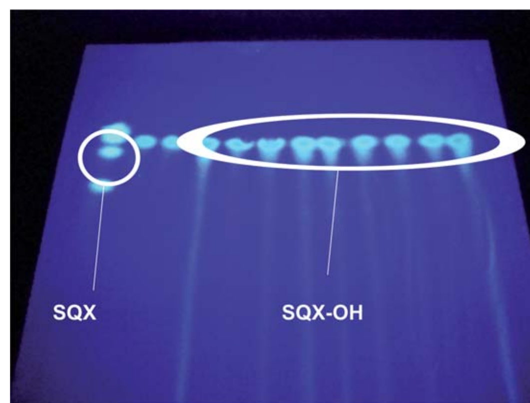

Fig. 3 TLC purification of SQX-OH. First channel from left to right is sulfonamide pool application with SQX highlighted. Other spots correspond to SQX-OH obtained from microsomal liver fraction.

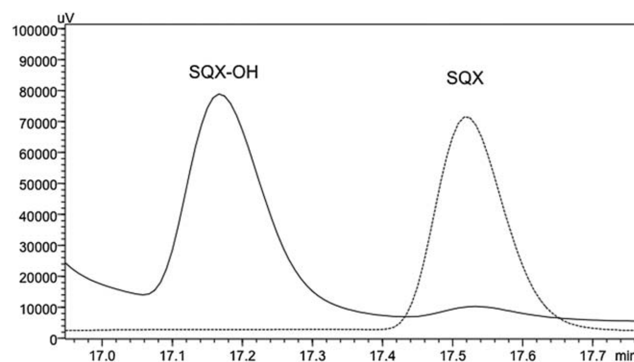

Fig. 4 HPLC-DAD chromatograms overlay. The first peak (SQX-OH) corresponds to the TLC spot obtained from microsomal fraction of equine liver spiked with SQX. The second peak (SQX, in dot line) corresponds to a poultry liver sample spiked with SQX before extraction.

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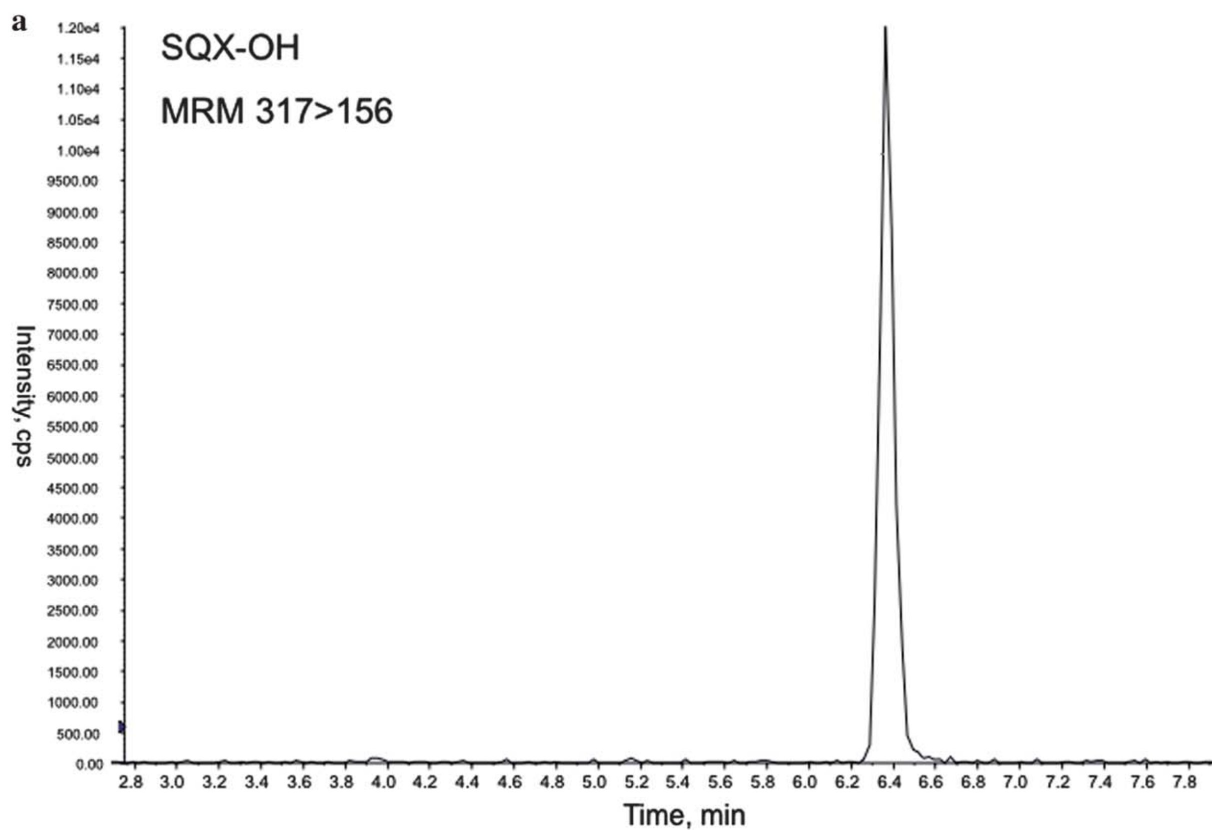

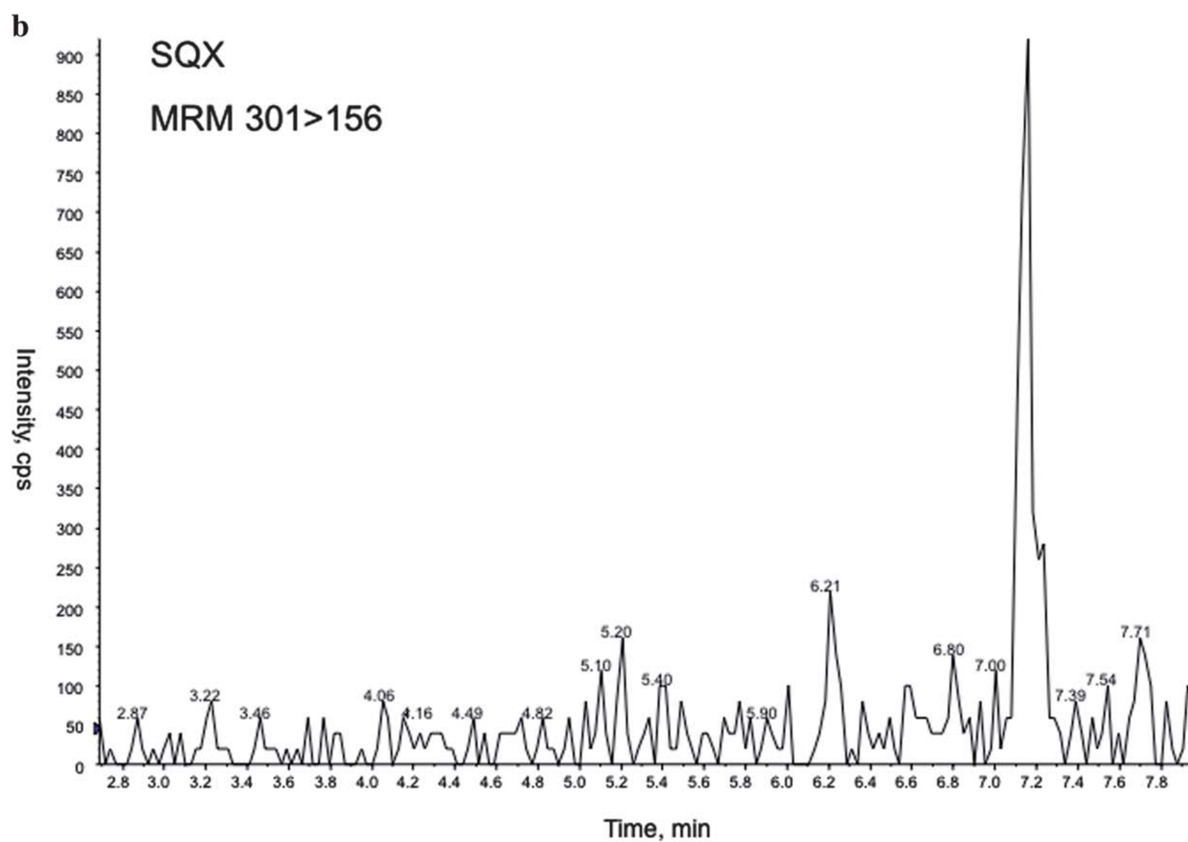

Fig. 5 MRM extracted chromatogram of an equine liver sample spiked with SQX (100 ng g^{-1}). SQX-OH was present in a high extent, while only trace amounts of original SQX were detected.

deviation (RSD) of 30%. Bovine samples had a more heterogeneous degree of enzymatic activity, with a mean value of 39.5 ng.g^{-1}, but with a high RSD (66%). Actually, SQX-OH concentration in bovine liver varied in a range from 11.1 to 83.9 ng.g^{-1}. Fig. 2 present a plot for SQX and SQX-OH distribution in swine and bovine liver samples.

Three metabolites were identified with a molecular mass corresponding to hydroxylated-SQX (SQX-OH), N_4-acetyl-SQX and N_4-acetyl-SQX-OH. These metabolites were found in rat liver and kidney, varying concentrations depending on time of ingestion of the medicated feed. SQX-OH proved to be the metabolite with the greatest intensity in all species studied. In horses, the *in vitro* conversion of SQX to SQX-OH is quantitative and occurs in less than 5 minutes for liver samples with concentrations of 100 ng g^{-1}.

In the most conclusive field experiment, a 12 month old mare was treated with SQX as described above and plasma and urine were analyzed. Besides base SQX drug, the same 3 metabolites found in rat tissues were detected in horse urine and plasma.

SQX-OH *in vitro* production and characterization

In order to obtain a reasonable amount of metabolite, a microsomal fraction of equine liver was separated and used to convert SQX into SQX-OH. Equine liver sample was obtained from a slaughterhouse. A process was conducted to obtain a purified extract that was applied to the TLC separation system. Spots were compared with a pool of sulfonamides (Fig. 3). Spots of presumable SQX-OH were cut from TLC plate and reconstituted in methanol. After dilution with the mobile phase, samples were submitted to HPLC-DAD and LC-MS/MS analysis (Fig. 4). HPLC-DAD analysis was used to verify the absence of other peaks before the analysis in LC-MS/MS. Using DAD in a range from 190 to 400 nm, no other peak was found in chromatograms. In LC-MS/MS analysis, data confirmed that conversion of SQX to SQX-OH was total no trace of SQX was found in the samples.

SQX-OH quantitative analysis. However, for routine analysis, it is not feasible in-lab production of metabolites. For this reason, an approach based on *in vitro* production of SQX-OH was adopted to estimate this metabolite in equine liver samples. Further investigations that are still being developed show that *in vitro* SQX-OH formation is also detected in bovine and swine liver. In bovine and swine tissues, the conversion is partial (25–60%), ranging between samples. Fig. 5 show an extracted ion chromatogram for SQX and SQX-OH in equine liver, in which SQX was added as spike. For SQX MRM transition (301 > 156) just traces were detected. The SQX-OH MRM transition (317 > 156) shows an intense signal. In the case of a bovine sample, conversion is variable. Fig. 6 show a scan of a bovine liver extract obtained in a precursor ion experiment (precursor of *m/z* 156 and 108, characteristics of sulfonamide moiety) when SQX and SQX-OH were simultaneously detected. Precursor ion mass spectra of SQX-OH are presented in the secondary box.

Considering that the MRL of sulfonamides was expressed as the sum of all sulfonamides and their metabolites, it is necessary to use a method capable of estimating these metabolites. As our routine method is based on matrix-matched calibration curves, equine blank liver was used to produce a matrix-matched curve for SQX-OH estimation in real samples. LC-MS/MS analyses

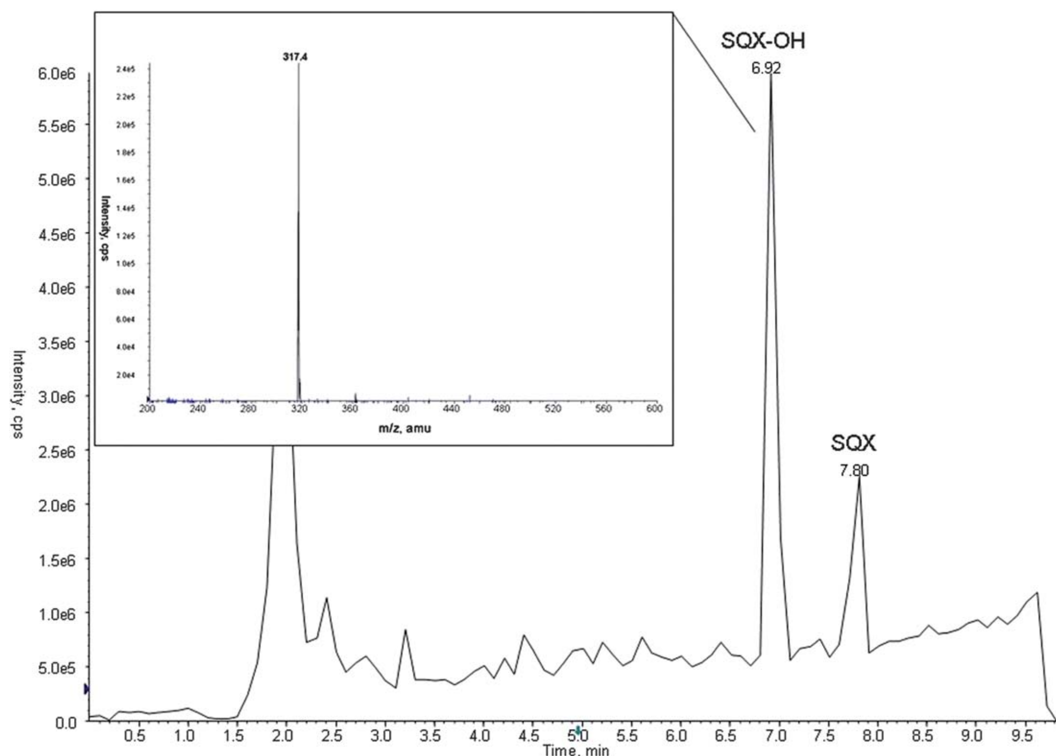

Fig. 6 Precursor ion MS experiment for precursor of daughter ions with *m/z* 156. The secondary box shows a mass spectrum for SQX-OH (*m/z* 317). The sample was a bovine liver sample spiked with SQX (100 ng g^{-1}). SQX and SQX-OH were present.

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show that there is total SQX to SQX-OH conversion. Thus, a curve produced *in vitro* is able to quantify SQX-OH with satisfactory parameters. Calibration curves were performed using horse liver as a matrix. Samples were spiked, extracted and analyzed according to the routine method. The curve covered a range between 20 and 200 ng g^{-1}, with a detection limit and quantification limit of 2.5 and 10 ng g^{-1}, respectively. Results were quantitative and correlation values were satisfactory, as demonstrated in Table 4.

The hypothesis of Bogialli and group was in total agreement with our findings, considering that our further investigation shows the same enzymatic conversion in bovine and swine although in several degrees. Meanwhile, SQX conversion to SQX-OH is quantitative in equine liver samples.

Mass spectrometric analysis shows a characteristic profile of SQX-OH, considering that this compound presents typical sulfonamide group fragments, as 156, 108 and 92. An *m*/*z* ratio of 317 confirms the same finding as Bogialli *et al.* and matched with an OH addition.[33,36] This compound is proposed as a hydroxylated form of SQX, called SQX-OH, with a $[M + H]^+$ of 317 (Fig. 6). This metabolite was theoretically proposed in a report of 1944, including the position of the OH in the amino substituent.[36] Likewise, the N_4-acetylated form of SQX-OH was also characterized in the present work. The N_4-acetylated form of SQX is a well known metabolite which was previously detected and determined in chicken tissues by other authors.[37,38] However, from the best of our knowledge, SQX-OH and N_4-acetyl-SQX-OH were analyzed by LC-MS/MS for the first time.

Sulfonamides presented a very regular fragmentation pattern. Product ions common to most sulfonamides include the *p*-aminobenzene sulfonic acid moiety, $[M–RNH_2]^+$ (*m*/*z* 156), $[M–RNH_2–SO]^+$ (*m*/*z* 108) and $[M–RNH_2–SO_2]^+$ (*m*/*z* 92).[39] These fragments were used to perform analysis firstly in precursor ion mode, in order to detect any compound which produces fragments with *m*/*z* 156 and 108. Following, the precursor ions corresponding to $[M + H]^+$ of 301 (SQX), 317 (SQX-OH), 343 (N_4-acetyl-SQX) and 359 (N_4-acetyl-SQX-OH) were optimized to be analyzed using MRM mode, in which these four molecular ions produce fragments with *m*/*z* 156 and 108. The structures are showed in Fig. 7.

Table 4 SQX-OH calibration curve with internal standardization (SPY). Regression equation: $y = 1.01x + -0.00551$ ($r = 0.9958$), where y is the internal standard (IS)/analyte response ratio and x is the IS/analyte concentration ratio

Expected concentration (ng g^{-1})	Mean calculated concentration	Accuracy (%)	Std deviation
0.00	1.05	N/A	0.96
30.00	30.16	100.5	0.47
50.00	52.38	104.8	0.14
100.00	91.46	91.5	4.95
150.00	152.04	101.4	5.88
200.00	202.12	101.1	17.77

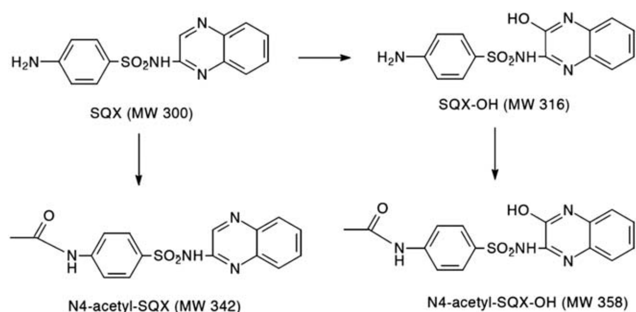

Fig. 7 Proposed structure for SQX metabolites included in the present study.

Conclusions

In conclusion, SQX residue analysis should take into account the formation of SQX-OH and other metabolites, especially when using MRM transitions. Samples of various species with SQX-OH formation could be adequately quantified using a metabolite calibration curve produced *in vitro*. SQX-OH quantitations and confirmation were included in the method and SQX residues are now estimated as the sum of SQX and SQX-OH. Moreover, N_4-acetylated derivatives of both SQX and SQX-OH could be qualitatively monitored. Further studies must be carried out in order to also include the N_4-acetylated derivatives in quantitative analysis. Scheduled future studies include characterization of SQX-OH and their derivatives using high resolution MS and NMR.

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Anexo II – Artigo a ser submetido para *Trends in Analytical Chemistry*: **Sulfonamides and their by-products analysis in environmental samples using mass spectrometry techniques: a review**

Sulfonamides and their by-products analysis in environmental samples using mass spectrometry techniques: a review

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Abstract

The methods of analysis for sulfonamides (SFAs) in environmental samples using mass spectrometry techniques are reviewed. Sulfonamides were the first antimicrobial group of drugs used in the therapy. These compounds are still used today in the medicine and widely used in the veterinary medicine, also for the growth promoter effects. Through the waste or manure utilization, SFAs could migrate to soil and water. Trace and ultra-trace levels of SFAs were detected in several kinds of matrices and for achievement of this level of detection. Advanced mass spectrometry techniques as linear ion trap and time of flight mass detection associated with post-run strategies were currently applied to SFAs monitoring. In this work, a review of published reports in the period 2003-2013 is presented.

I. Introduction
II. Fragmentation pathways of sulfonamides
III. Methods for SFAs analysis in environmental samples
IV. Conclusions and outlook

I. Introduction

The discovery of antibacterial activity of sulfas was achieved in 1935 with the publication of the work "A Contribution to Chemotherapy of Bacterial Infections" [1], in which was described the biological activity of *p*-sulfamidocrisoidine (Prontosil Rubrum), by the german pathologist and bacteriologist Gerhardt Domagk. This

47 substance had been synthesized in 1932 by Mietsch and Klarer (Bayer), based on
48 the classic chemistry of textile dyes, specifically to be tested as antibacterial [2, 3].
49 The credit for this discovery gave Domagk the Nobel Prize for Medicine in 1939 [4].
50
51 After these years, other emerging antimicrobial substances were obtained, some
52 synthetically and others isolated from microorganisms such as penicillin,
53 discovered by Fleming in 1928, which showed severe bactericide action [3].
54
55 Forneau and co-workers, analyzing metabolites in blood and urine of patients
56 treated with Prontosil, detected the presence of sulfanilamide (substance known
57 since 1908) [3]. Through this study they finding that the active part of the molecule
58 was the sulfanilamide and that the various chemotherapeutic antibacterial hitherto
59 well known, only acted because of the presence of the sulfonamidic
60 pharmacophoric group, whose mechanism of action was subsequently clarified and
61 related to the inhibition of the bacterial enzyme diidropteroate synthase.
62
63 From the end of the decade of 40 antibiotics tended to replace the sulfas in
64 chemotherapy because of their lower toxicity and broader spectrum of action [5].
65
66 After the observation that certain bacteria bought resistance caused by antibiotics,
67 sprang anew interest for sulfas and search of new sulfonamidic derivatives,
68 encouraged by the ease of obtaining and low cost.
69

70 Today, some sulfas are used as associations, e.g. sulfamethoxazole and
71 trimethoprim to enhance its effects [6]. Moreover, new drugs have been derived
72 from sulfa rather promising as anticancer and antiviral drugs [7].
73 Recently, new applications have been demonstrated to the sulfas, as well as new
74 discoveries about its mechanism of action [8].

75

76 Despite the relative loss of importance in human medicine, sulfonamides are
77 widely used in developing countries as basic medications, especially for urinary
78 infections. Also to defeat *Plasmodium falciparum*, the causative agent of malaria,
79 an association between sulfadoxine and pyrimethamine are widely used in affected
80 zones [9].

81

82 Commonly, sulfonamides are used in food-producing animals to prevent diseases,
83 to promote growth, to increase the weight gain and to reduce the amount of feed
84 per animal, that means enlarge the food conversion rate. They are administered in
85 feed in sub-therapeutic doses during growth. The incorrect administration of
86 antibiotics in veterinary medicine has great potential risk that residues of these
87 drugs may be present in edible tissue [10].

88

89 The main risk to human health of using antibiotics on animals is the fact that animal
90 bacteria can develop resistance to drugs, mainly by using sub-therapeutic doses.
91 This resistance can develop by several pathways such as mutation, acquisition of
92 resistant genes or a combination of both [11].

93

94 Another major concern about the massive use of sulfonamides and other drugs in
95 intensive animal production is the residue amount that is transferred to the
96 environment through the waste of these animals [12]. This subject has received
97 increasing attention recently due to the fact that can be involved with the selective
98 up-regulation of the so-called resistome of soil microorganisms [13, 14] .
99
100 In several countries, consumer exposition to residues was estimated through
101 studies of the normal diet of an individual, by international agencies as WHO
102 (World Health Organization) or FAO (Food and Agriculture Organization of the
103 United Nations). These studies measured how much the population is exposed to
104 certain residues in food. These data allowed calculate the average exposure of
105 consumers to a range of chemical compounds present in diet and interpret the
106 average exposure in terms of an acceptable daily intake (ADI) to these substances.
107 ADI is an estimate of the quantity of the substance that can be ingested daily
108 throughout the life without appreciable risk to health [15] . To international boards
109 as Codex Alimentarius, all active compounds - new or existing - used in veterinary
110 medicines for food-producing animals requires the establishment of a maximum
111 residue limit (MRL). MRLs are based on the concept of acceptable diary intake
112 (ADI), but are determined taking into account the depletion of the drug in the target
113 species, so its values can be established for tissues and animal products [16]. Due
114 to pharmacokinetic differences between species, the MRL is specific to each
115 species, while the ADI for a substance is universal.
116

117 Residues analysis is an important fraction of food safety and public health, by
118 establishing parameters that tell if a food is safe or not for human consumption. For
119 drugs used in animal production, the analysis of their residues is a vital fraction of
120 programs and monitoring plans of regulatory agencies in virtually the entire world.
121 The tests are aimed to determining whether residues are within acceptable levels
122 for consumption, which means levels below MRL. Hence there is the importance of
123 developing analytical procedures to determine sulfonamides in biological matrices
124 in low levels, as the MRLs.
125
126 Residues analysis methods had enormous progress since 1980 decade. There
127 were many scientific advances in the area of analytical equipment, computerization
128 and automation of all method stages. Many of these advances have been directed
129 to increase the sensitivity and specificity of techniques. The demand for regulatory
130 control of chemical contaminants in food is expanded dramatically in the last
131 decade, making the residues regulation an important factor to be considered in
132 international trade of commodities. Here, the chromatographic techniques have
133 had the role of prominence, especially in high performance liquid chromatography
134 (HPLC). This versatile tool was widely studied and is commonly used to
135 sulfonamides analysis in several types of matrices [17-23]. HPLC is capable to
136 detect low concentration levels, which is the key in residues analysis. However,
137 HPLC is a technique sometimes limited by the low efficiency of separation
138 associated with non-adequate limits of detection for trace level analysis. Capillary
139 electrophoresis (CE) is also used for sulfonamides analysis but generally this
140 technique shows even more low detection capability than HPLC [24-26].

141

142 All this has generated a great demand for analytical methods for detection of
143 veterinary drugs residues in food and environmental matrices. The need to
144 evaluate and assessment of the risk of drugs residues came together with the
145 powerful analytical techniques able to detect and correctly identified this
146 compounds and their by-products in ultra-trace levels [27]. Metabolites formed in
147 vivo or degradation products of drugs are also a point of concern. SFAs group had
148 relatively high water solubility. Ally with that, these compounds have a low ability to
149 chelation. These characteristics associated with their amphoteric behaviour,
150 provide high locomotion capability for SFAs in the environmental [28]. The use of
151 SFAs in human medicine or in animal production provokes a continuous input of
152 these drugs in the wastewaters and waterbeds. Regarding with this issue, several
153 methods has been developed in order to monitor SFAs presence and metabolism /
154 degradation process in samples as food matrices, soil, wastewaters, superficial
155 waters, sludge, manure, etc [29]. For these methods, a use of an analytical
156 technique with high sensibility and specificity is required. The mass spectrometry
157 techniques, specially the hyphenated modes, are very useful to this purpose and
158 several reports had been published in recent years. Initially, systems with triple
159 quadrupole (QqQ) were preferred for quantitative purposes. Actually, with the
160 development of MS engineering, systems previously dedicated for qualitatitve
161 analysis and able to provide high resolution, as triple quadruple–time of flight
162 detection (QqTOF), triple quadrupole-linear ion trap (QqLIT) and Orbitrap are
163 applied for quantitative analysis too. The aim of the present work is review the
164 methods for SFAs analysis in environmental samples using MS techniques

165 published in the last ten years, covering the 2003-2013 period. In figure 1,
166 structures of the most common sulfonamides are showed and in table 1,
167 parameters of the reviewed methods are summarized.
168

169 **II. Fragmentation pathways of sulfonamides**

170 Despite the fact that sulfonamides are a large group of compounds and have
171 hundreds or even thousands of molecules, their fragmentation pathways are very
172 homogeneous and uniform [30]. Generally, the sulfonamides can be considered as
173 the product of the condensation between aniline and sulfonic acid. From this core,
174 thousands of radicals were attached in order to obtain sulfonamides with the more
175 diverse characteristics.
176

177 When analyzed by tandem mass spectrometry, the sulfonamides typically produce
178 ions at *m/z* 156, 108 and 92, independent from the molecular ion mass value.
179 Additional fragment ions were observed consistent with the neutral losses of 66, 93
180 and 155 Da. In the case of acetylated metabolites of sulfonamides, the same was
181 observed; however, the common fragment ions appeared at higher *m/z* values due
182 to the presence of the acetyl group, e.g. *m/z* 198 (156 + 42) [30].
183

184 These fragments were at least partially elucidated. The ion at *m/z* 156 is due to
185 cleavage of the sulfonamide bond. Further loss of SO_2 leads to the ion at *m/z* 92,
186 while it is know that *m/z* 108 is formed via a rearrangement. The losses of 93 and
187 155 Da produce fragments ions corresponding to the each substituent X, such as
188 $X\text{-}NH\text{-}SO_2$ and $X\text{-}NH_3$, while the loss of 66 Da is the loss of H_2SO_2 [30]. Due to the

189 regular fragmentation pattern, which is observed even for metabolites and
190 degradation products, precursor ion monitoring mode is a useful tool for
191 sulfonamides studies. E.g., for metabolites identification, the use of a precursor ion
192 monitoring for fragments at *m/z* 156 will produce a chromatogram with all quasi-
193 molecular ions which when fragmented in collision cell produce a fragment at *m/z*
194 156. Thus, full scan analysis associated with precursor ion and product ion
195 experiments and followed by multiple reaction monitoring (MRM) analysis were
196 commonly applied for sulfonamides investigation in biological and environmental
197 matrices [31].
198
199 Regarding to the chromatographic separation of sulfonamides, they contain one
200 basic amine group (-NH_2) and one acidic sulfonamide group (-SO_2NH). They are
201 ampholytes with weakly basic and acidic characteristics, having two pKa values,
202 pKa_1 (2–2.5) and pKa_2 (5–8), respectively. Thus, sulfonamides are positively
203 charged at pH 2 and 5, and negatively charged at alkaline conditions above pH 5,
204 explaining their good retention under all conditions tested.
205
206 **III. Methods for SFAs analysis in environmental samples**
207 For environmental samples, as soil, manure and water, extractions procedures are
208 designed with the purpose of concentrate the analytes and promote the clean-up of
209 the extracts, in order to avoid damage to the analytical systems through the
210 introduction of debris from the matrix into the internal part of equipments.
211 Generally, the most frequent extraction scheme is based in SPE using Oasis HLB
212 columns. SFAs contain one basic amine group (–NH_2) and one acidic sulfonamide

213 group ($–SO_2NH–$). They are ampholytes with weakly basic and acidic
214 characteristics. It is explained by the charge state of the SFAs at the particular pH
215 values because of their pKa values. The pKa_1 (2–2.5) and pKa_2 (5–8) correspond
216 to the protonation of the aniline group and deprotonation of the sulfonylamide
217 group, respectively. Weakly basic characteristics arise from the nitrogen of the
218 anilinic substituent which is able to gain a proton, designated for protonation during
219 ionization step of mass spectrometric detection, whereas the acidic characteristics
220 arise from the N–H linkage of the sulfoamidic group which is able to release proton
221 under specific pH conditions. Thus SFAs are positively charged at acidic conditions
222 at pH 2, neutral between pH 2 and 5, and negatively charged at alkaline conditions
223 at pH above 5. However, pH adjustment is quite rare in environmental samples for
224 SFAs analysis: pH of sample adjusted to pH 2.5 was the condition with more
225 higher recoveries compared with no pH adjustment in a SPE protocol [37] and in
226 the rest of studies pH adjustment was not reported. This is not usual when SPE
227 and the interaction between the analytes and the sorbent of SPE columns are pH
228 dependent. The interaction with the cartridge material is stronger for analytes in
229 uncharged forms. Mostly, the sample pH was adjusted to value about 3.0, in range
230 2.0–4.0 in multiresidue methods. This step led to good recovery rates that showed
231 that pH adjustment of sample was very important and it was in agreement with
232 their pKa values. As was already said above, majority of studies for the
233 determination of SFAs used Oasis HLB columns for their extraction from water
234 samples. All methods referred washing step after sample percolation through the
235 SPE columns which was suitable in the environmental analysis to remove

236 interferences. In most cases water was used. Elution of cartridges was generally
237 done by organic solvent.
238
239 In the case of soil, manure and sludge samples, SPE with Oasis HLB were also the
240 most frequent protocol but this matrices required one or more previous purification
241 steps to be able to SPE process. For that purpose, one of the most adequate
242 techniques in terms of efficiency is the use of pressurized liquid extraction (PLE) in
243 which the matrix is commonly mixed with a dispersion agent (e.g. Hydromatrix) and
244 submitted to extraction in stainless steel cells using aqueous, organic or aqueous-
245 organic mixtures solvents under pressure [27, 38-41].
246
247 García-Galán et al describes a study about the removal of sulfamethazine from
248 sewage sludge per action of one fungus (*Trametes versicolor*). In that report, the
249 products of SMZ by the action of fungus metabolism were identified and
250 characterized using UPLC-QqTOF-MS. Samples with SMZ level of 9 mg L^{-1} show
251 undetectable results for SMZ after 20 hs of incubation with the fungus. Four
252 degradation intermediates were identified and confirmed: desulfo-SMZ, N^4-formyl-
253 SMZ, N^4-hidroxy-SMZ and desamino-SMZ. Sludge samples were extracted using
254 PLE followed by SPE (with Oasis HLB cartridges) [42].
255
256 The use of linear ion trap permits the utilization of powerful techniques for
257 screening purposes. The IDA experiments (Information-dependent acquisition).
258 IDA is an artificial intelligence-based product ion scan mode that provide automatic
259 switching from MS to MS/MS. Performing IDA, two collision-induced dissociation

260 product ion spectra are generated for each detected compound above a pre-
261 determined signal in the initial MS scan. This approach has been increasing in the
262 last years and several applications for environmental analysis were published.
263
264 Gros et al report a method using LC-qLIT-MS with IDA experiment for analysis of
265 81 compounds in several water samples, including wastewater, seawater and
266 drinking water. SMA was included in the analytes scope. The instrumental
267 detection limit for SMA was 0.1 pg of injected substance. With the exception one of
268 the two studied WWTP, SMA was detected in all samples, including seawater (9.0
269 ng L^{-1}) [43].
270
271 In a more recent report, Gros et al describe a multi-residue method for 53
272 antibiotics residues, comprehending 12 SFAs and 4 N-acetylated SFAs
273 metabolites. In the same way of the previous work, they used LC-qLIT-MS to
274 monitoring hospital, urban wastewater and river water. For identification and
275 confirmation, two Selected Reaction Monitoring (SRM) transitions were monitored
276 per compound and quantification analysis was performed by the internal standard
277 approach using isotopically labeled antibiotics. As in other works, water samples
278 were automatically extracted by a GX-271 ASPEC^{TM} system using Oasis HLB SPE
279 cartridges. Just SMA are present and hospital and urban wastewater while SPY
280 was also identified in WWTP samples only [37].
281
282 In order to investigate the correlation between occurrence of antimicrobial residues
283 and antibiotic resistant bacteria in the sewage, Novo et al studied raw and treated

284 wastewater samples collected from an urban WWTP. Samples were characterized
285 for the occurrence of emergent pollutants including SMZ and STZ associated with
286 antibiotic resistance percentages for tetracycline, SMA, ciprofloxacin and
287 amoxicillin. Variations on the bacterial community structure of the final effluent
288 were significantly correlated with the occurrence of tetracyclines, penicillins,
289 sulfonamides, quinolones and triclosan in the raw inflow. Values so high as 13,100
290 ng L^{-1} of sulfonamides residues in raw wastewater collected in spring are
291 described. The authors demonstrate a relationship between antibiotic residues,
292 bacterial community structure and composition and antibiotic resistance, but further
293 studies must be accomplished to elucidate in more details this relationship [44].
294
295 Gros et al report a method for 73 compounds detection in surface and wastewaters
296 based in a LC-QqLIT-MS/MS analysis using an Information Dependent Acquisition
297 (IDA) experiment, with SRM as the survey scan and an enhanced product ion (EPI)
298 scan, at three different collision energies, as dependent scan. Compound
299 identification was carried out by library search with a developed library, created by
300 the infusion of standards, based on EPI spectra at the three collision energies. The
301 method shows limits of detection ranging from 0.1-55 ng L^{-1} [45].
302
303 The pH adjustment of the sample prior to SPE step was optimized for SFAs and
304 other classes of compounds in a work published by Tong et al. They analyzed 4
305 SFAs in the wastewater generated in pig's production farms. Samples pH was
306 adjusted to 2.0, 4.0 and 7.0 units. For SFAs recoveries, neutral pH shows the

307 higher values. However, the weak acidic condition was chosen by exhibit the best
308 compromise between recoveries of all compounds included in the method [46].
309
310 Tso and Aga published a report dealing with the simultaneous analysis of
311 estrogens and antibiotics (SFAs and tetracyclines). Those compound classes
312 analysis are typically performed in two separate methods because estrogen
313 analysis requires electrospray with negative ionization, while sulfonamide and
314 tetracycline antibiotics are analyzed under positive ionization. The authors
315 developed a method using wrong-way-round (WWR) ionization to demonstrate that
316 SFAs and tetracyclines can be analyzed at a high pH (10.4), allowing simultaneous
317 analysis with free and conjugated estrogens. Mass spectral data suggest that gas-
318 phase chemical ionization induced by ammonium ions to form adducts $[M + NH^4]^+$
319 occurred, with the subsequent dissociation to the molecular ion $[M + H]^+$ [47].
320
321 Commonly, the SPE extraction based on Oasis HLB is the most frequent protocol.
322 One exception is the method proposed by Yudthavorasit et al. In this report, the
323 authors used carrier-mediated hollow-fiber liquid-phase microextraction (HF-LPME)
324 for enrichment of multiple classes of antibiotics in water samples. With this
325 technique, an enrichment factor varying from 6 to 10.7 was obtained for the
326 analysis of STZ, SMZ and SMR. However, only analysis of spiked samples was
327 reported in this work [48]
328
329 Although HPLC or UPLC are the main used analytical tools associated with MS for
330 the detection of sulfonamides in environmental samples, other techniques are used

331 for the investigation of degradative processes of these compounds. For instance,
332 electrochemistry coupled with MS (EC-MS) was described by Hoffmann et al to
333 investigate oxidative behaviour of xenobiotics using SFAs as model. Results with
334 SDZ showed strong evidences for the elucidation of the oxidative degradation
335 mechanism. A EC-qLIT-MS system with Fourier transform ion cyclotron resonance
336 (FTICR) was utilized in the work [48, 49].

337

338 IV. Conclusions and outlook

339

340 Mass spectrometry-based methods for sulfonamides residues analysis in
341 environmental matrices have been reviewed within the period 2003-2013.
342 Currently, the residues analysis based on MS techniques are strongly exhibiting a
343 tendency to encompass two or more classes of compounds in multi-residues
344 approaches. Another tendency of great interest for environmental analysis is the
345 development of a multi-class compounds, i.e, methods able to analyze
346 pharmaceuticals, pesticides and chemicals contaminants.

347

348 In terms of analysis mode, the untargeted analysis has been gaining ground. The
349 use of mass spectrometers with high mass resolution - as TOF and Orbitrap series
350 – which currently are able to perform quantitative analysis with a similar
351 effectiveness than triple quadrupole based mass spectrometry has been increasing
352 year by year. Probably, with the software improvement, the post-run target
353 screening strategies will be one of the most used tools in residues laboratories.

354

355 Increasingly, metabolites and degradation products of sulfonamides had been
356 elucidated and even included in routine methods for monitoring and even
357 regulatory purposes as in the case of N_4-acetylated derivatives monitored in some
358 food and environmental matrices.

359

360 With the continuous development of mass spectrometry and the software tools
361 capable to analyze the high and complex data obtained in the most modern mass
362 spectrometers, one of the major challenges relies in the sample preparation.
363 Although the on-line extraction procedures was increased in last decade for
364 sulfonamides residues analysis, none newly technique for sample extraction can
365 be highlighted in the reviewed period. Thus, sample preparation is a topic still
366 highly fertile for research and development of more easy, cheap and fast
367 techniques.

368

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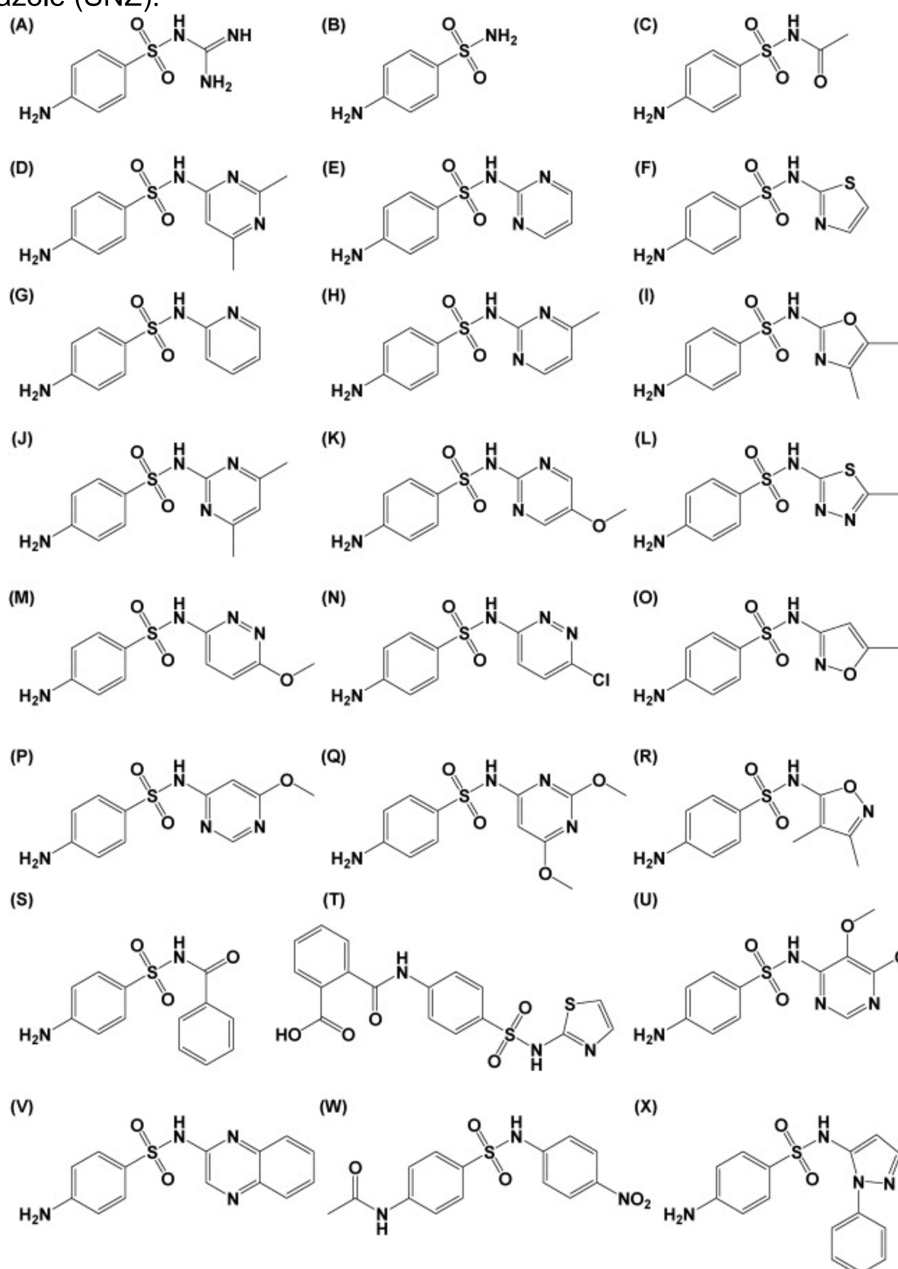
454 .

455

455 **Figure 1.** Chemical structures of most common sulfonamides: (A) sulfaguanidine (SGD),
456 (B) sulfanilamide (SA), (C) sulfacetamide (SAA), (D) sulfisomidin (SIM), (E) sulfadiazine
457 (SDZ), (F) sulfathiazole (STZ), (G) sulfapyridine (SPY), (H) sulfamerazine (SM), (I)
458 sulfamoxole (SMO), (J) sulfamethazine (SMZ), (K) sulfameter (SME), (L) sulfamethizole
459 (SMT), (M) sulfamethoxypyridazine (SMP), (N) sulfachloropyridazine (SCP), (O)
460 sulfamethoxazole (SMA), (P) sulfamonomethoxine (SMM), (Q) sulfadimethoxine (SDMX),
461 (R) sulfisoxazole (SSA), (S) sulfabenzamide (SB), (T) N^4-phthalylsulfathiazole (PST), (U)
462 sulfadoxin (SDO), (V) sulfaquinoxaline (SQX), (W) sulfanitran (SNT) and (X)
463 sulfaphenazole (SNZ).



464

Table 1. Summary of the methods for sulfonamides analysis in environmental samples using mass spectrometry methods

Sulfonamides	Sample	MS method summary	Sample Pretreatment	Detection/LOD achieved	Real samples analysis	Reference
SMA	Water (sea, river, tap, reservoir, WWTP)	UPLC-qLIT-MS in IDA experiments and using an Acquity HSS T3 colum (50 mm × 2.1 mm i.d., 1.8 µm particle size)	SPE (Oasis HLB) automatically extracted by a ASPEC^{TM} system.	0.1 (in drinking water) to 7.1 ng L^{-1} (wastewater)	0.5 ng L^{-1} (drinking water) 4.0 ng L^{-1} (reservoir water) 9.0 ng L^{-1} (seawater) 10.0-79.0 ng L^{-1} (river water) 768 ng L^{-1} (influent WWTP) 222 ng L^{-1} (effluent WWTP)	[43]
SMA, SDZ, SIM, STZ, SDMX, SPY, SMR, SMT, SMP, SSA, SNT, SB, N-acetylSDZ, N-acetylSMZ and N-acetylSMR.	Wastewater (hospital and urban)	UPLC-qLIT-MS, with 2 SIM transitions for each analyte, using an Acquity HSS T3 colum (50 mm × 2.1 mm i.d., 1.8 µm particle size)	SPE (Oasis HLB) automatically extracted by a ASPEC^{TM} system	1.39 (SMA) to 34.48 ng L^{-1} (N-acetylSDZ)	65-200.0 ng L^{-1} (SMA in hospital wastewater) 19-198.0 ng L^{-1} (SMA in WWTP) 32-159.0 ng L^{-1} (SPY in WWTP)	[37]
STZ SMZ	Wastewater	LC-ESI-MS/MS with 2 SIM transitions for each analyte, using an Purosphper Star RP-18 endcapped column (125 mm × 2.0 mm i.d., 5 µm particle size)	SPE (Oasis HLB) automatically extracted by a ASPEC^{TM} system	Not described	800-13,100.0 ng L^{-1} (not discriminate between STZ and SMZ)	[44]
SMZ, SMR, SAA, SDX, SB, STZ, SQX, SDZ, SMDX, SMZ SMT, SMA, SMTP, SPY, SSA, SNT, AcSMZ, AcSMA, AcSPY,	Soil and sludge	LCI-QqLIT-MS/MS in MRM mode, using a Atlantis C18 LC-column (Waters, 150 mm × 2.1 mm, 3 µm of particle size)	PLE with Hydromatrix dispersion agent followed by SPE (Oasis HLB)	0.03-2.23 ng g^{-1} (sludge) 0.01-4.19 ng g^{-1} (soil)	Higher level: 139.2 ng g^{-1} (SDZ/sludge) 8.53 ng g^{-1} (SDZ/soil) Metabolites: Not higher than 9.8 ng g^{-1}	[27]

AcSDZ, AcSMR						
SDZ, STZ, SMR, SMZ	Swine wastewater	LC-ESI-MS/MS in MRM mode using a Dionex Acclaim C18 reversed phase column (150 × 2.1 mm, 4.6 µm of particle size)	SPE (Oasis HLB) with sample pH adjustment to 4.0	2.1-4.1 ng L^{-1} (groundwater) 2.7-5.5 ng L^{-1} (lake water) 6.4-12.9 ng L^{-1} (WW influent) 2.2-5.9 ng L^{-1} (WW effluent)	±6.0 ng L^{-1} (SMR/groundwater) ±11.0 ng L^{-1} (SMR/lake water) Higher level: 21,692.7 ng L^{-1} (SMR/WW effluent)	[46]
SDZ, SMZ, SMA	Surface water and WWTP influent and effluent	LC-QqLIT-MS/MS in a IDA experiment using a Purospher Star RP-18 endcapped column (125 mm × 2.0 mm, particle size 5 µm)	SPE (Oasis HLB)	0.4-0.8 ng L^{-1} (surface water) 1.0-2.0 ng L^{-1} (WW effluent) 2.0-3.0 ng L^{-1} (WW influent)	Higher levels: 50.0 ng L^{-1} (SMA/surface water) 448.0 ng L^{-1} (SMA/WW effluent) 909.0 ng L^{-1} (SMA/WW influent)	[45]
SMA, SCP, STZ, SMZ, SDMX	Surface water, WWTP influent and effluent	LC-ESI-MS/MS in MRM mode, using a Phenomenex Luna C8 column (100×4.6 mm, particle size 3 µm)	SPE (Oasis HLB)	5.0-30.0 ng L^{-1}	Higher levels: 82.0 ng L^{-1} (SMA/surface water) 492.0 ng L^{-1} (SMA/WW effluent) 984.0 ng L^{-1} (SMA/WW influent)	[50]
SMA, SMZ, SDMX, SMT, SMI, SMR, SCP, SDZ	Surface waters	LC-ESI-MS/MS in MRM mode, using a Betabasic C18 column (100 × 2.1 mm, particle size 3 µm)	SPE (Oasis HLB) with sample pH adjustment to 4.0	2.0-20.0 ng L^{-1}	610.0 ng L^{-1} (SMA)	[47]
STZ, SMZ, SMR	Surface waters	LC-ESI-MS/MS in MRM mode, using a Acquity UPLC BEH C18 column (100 × 2.1 mm, particle size 1.7 µm)	Hollow-fiber liquid-phase microextraction (HF-LPME)	10.0-70.0 ng L^{-1}	The method was only applied for spiked samples	[51]

Anexo III – Artigo publicado em *Food Additives and Contaminants Part A:* **Scope extension validation protocol: inclusion of analytes and matrices in a LC-MS/MS sulfonamides residues method**

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Scope extension validation protocol: inclusion of analytes and matrices in an LC-MS/MS sulfonamide residues method

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Scope extension validation protocol: inclusion of analytes and matrices in an LC-MS/MS sulfonamide residues method

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Validation is a required process for analytical methods. However, scope extension, i.e. inclusion of more analytes, other matrices and/or minor changes in extraction procedures, can be achieved without a full validation protocol, which requires time and is laborious to the laboratory. This paper presents a simple and rugged protocol for validation in the case of extension of scope. Based on a previously reported method for analysis of sulfonamide residues using LC-MS/MS, inclusion of more analytes, metabolites, matrices and optimisation for the extraction procedure are presented in detail. Initially, the method was applied only to liver samples. In this work, milk, eggs and feed were also added to the scope. Several case-specific validation protocols are proposed for extension of scope.

Keywords: sulfonamides; tandem mass spectrometry; validation; residue analysis; scope extension; 2002/657/EC

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Introduction

Sulfonamides were the first antimicrobial group of drugs used in antimicrobial therapy (Gerhard Domagk 1965; Van Miert 1994). These compounds are still used today in medicine and in veterinary medicine for growth-promoting effects as well as for treatment (Wassenaar 2005; Acar & Moulin 2006). The possible presence of sulfonamide residues in animal products is a public health concern. Improved methods of sulfonamides analysis are a constant challenge for researchers. Several different analytical methods for sulfonamide analyses have been developed, including HPLC, GC, TLC, ELISA and others (Furusawa 2003; Kishida & Furusawa 2003; Alaburda et al. 2007; Gong et al. 2007; Adrian et al. 2008; de Keizer et al. 2008).

Method validation is a necessary tool for residue analysis because it plays an important role in statutory programmes involved in international trade of commodities. The European Union has issued a specific regulation decision (2002/657/EC Decision) concerning the performance of methods and the interpretation of results in the official control of residues in products of animal origin (Commission of the European Communities 2002). Several parameters must be calculated such as decision limit (CCα) and detection capability (CCβ). For this study we applied a validation process based on this regulation. Moreover, other validation parameters were considered, such as LOD and LOQ since we must also observe other validation guidelines such as those proposed by the Brazilian Metrology Institute (INMETRO) and the validation guideline of our laboratory network (Al-Masri & Amin 2005; Damin et al. 2013; Nogueira & Soares 2013).

In Brazil, the surveillance programme for sulfonamide residues in food is performed by the laboratory network of the Ministry of Agriculture, Livestock and Food Supply (MAPA). The official method applied in our laboratory for sulfonamide residues analysis was firstly developed and validated for the analysis of sulfadiazine (SDZ), sulfathiazole (STZ), sulfamethazine (SMZ), sulfamethoxazole (SMA), sulfadimethoxine (SDMX) and sulfaquinoxaline (SQX). The method was only validated in poultry liver, swine, bovine and equine species (Hoff et al. 2009). The method was based on LC-MS/MS analysis for quantitative and confirmatory purposes and the method was fully validated in compliance with the guidelines proposed in Decision 2002/657/EC.

However, during the application of that method for routine analysis, distinct aims were introduced by the laboratory, i.e. the inclusion of more analytes, the inclusion of other matrices and/or species as well as other minor changes in the method. The extension of scope usually requires undertaking several full validation processes in order to demonstrate the fitness of the method for the inclusions in the method. Generally, any of these alterations suggest a full validation process to verify the ability of the assay to obtain satisfactory results. Herein, we use simpler and faster validation protocols specifically designed for validation of the extension of scope, combining the Decision 2002/657/EC approach with the use of

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quality control data and control charts obtained in routine analysis.

In this work we show the validation protocols designed and proposed for extensions of scope cases, i.e. analytes, matrices and species inclusion, without the need to apply a full validation protocol.

Materials and methods

LC-MS/MS

The LC-MS/MS system used was an API 5000 mass spectrometer (Applied Biosystems, Foster City, CA, USA). The analytical column was a Zorbax® XDB C18 column 150 × 4.6 mm, 5 µm (Agilent Technologies, Palo Alto, CA, USA). The pre-column used was a guard cartridge system composed of a C18 cartridge 4.0 × 3.0 mm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of ammonium acetate 10 mM with 0.1% acetic acid (solvent A) and methanol (solvent B). The initial conditions used 25% of solvent B in solvent A; then hold for 3 min. In the following step, the solvent B concentration was increased to 90% in 1 min and decreased again to 25% in 2 min, for a total time of 6 min for each run with an equilibrium time of 3 min in the same initial conditions. The mobile phase flow was 0.8 mL min^{-1}; the volume of injection was 20 µl. Analytes were introduced into the MS through an electrospray probe operating in positive mode. The cone voltage (eV) was 71; source temperature was 700°C; and dwell time (ms) was 100. All data were processed by software Analyst v.1.4.2 (Applied Biosystems). The identification of analytes was achieved by using MRM. Quantifier and qualifier transitions are shown in Table 1.

Table 1. Mass spectrometry analysis parameters for sulfonamides.

Analyte	Protonated molecule (*m*/*z*)	Quantifier transition	Qualifier transition
SDZ	251	251 > 156	*251 > 108*
STZ	256	256 > 156	*256 > 108*
SPY	250	250 > 156	*250 > 108*
SMZ	279	279 > 156	*279 > 108*
SMA	254	254 > 156	*254 > 92*
SDMX	311	311 > 156	*311 > 108*
SQX	301	301 > 156	*301 > 108*
SDX	311	311 > 156	*311 > 108*
SCP	285	285 > 156	*285 > 108*
SMR	*265*	*265 > 108*	265 > 156

Note: SDZ, sulfadiazine; STZ, sulfathiazole; SPY, sulfapirydine; SMZ, sulfamethazine; SMA, sulfamethoxazole; SDMX, sulfadimethoxine; SQX, sulfaquinoxaline; SDX, sulfadoxine; SCP, sulfachlorpyridazine; SMR, sulfamerazine; *m*/*z*, mass-to-charge ratio.

Materials

Except when indicated, all reagents were of HPLC grade. HPLC purity water was obtained from a Milli-Q purification unit (Millipore, Bedford, MA, USA). For the mobile phase, solvents were filtered through a 0.22-µm nylon membrane filter (Millipore) and sonicated before use.

Analytical standards of sulfadiazine (SDZ), sulfathiazole (STZ), sulfamethazine (SMZ), sulfamethoxazole (SMA), sulfadimethoxine (SDMX), sulfadoxine (SDX), sulfamerazine (SMR), sulfachlorpyridazine (SCP) and sulfapirydine (SPY) were obtained from Sigma (St. Louis, MO, USA); and sulfaquinoxaline (SQX) was obtained from Fluka (Seelze, Germany). Acetonitrile, acetone and ammonium acetate was obtained from J. T. Baker (Phillipsburg, NJ, USA). Methanol was obtained from Merck (Darmstadt, Germany). Stock solutions were made by diluting the standards with methanol to a concentration of 1 mg ml^{-1}. Work solutions were made by diluting the stock solutions with methanol or ammonium acetate 10 mM/methanol (75/25) to the appropriate concentrations.

Sample preparation

Liver

In the protocol initially validated, 2.5 g of chopped and homogenised liver tissue were weighted into a 50-ml glass beaker (Hoff et al. 2009). Internal standard (SPY) was added to a concentration of 100 ng g^{-1}. Approximately 3.0 g of anhydride sodium sulphate were added to the tissue and mixed with a glass stick. An aliquot of 10 ml of acetonitrile was added and the mixture was placed in a head-to-head agitator for 30 min. The mixture was then centrifuged for 20 min at 4000*g*. The supernatant was transferred into an empty and clean glass tube. The solid residue was submitted to an additional acetonitrile extraction (5 ml) and the extracts were combined before the evaporation step. Organic extract was evaporated in a water bath (40–45°C) under a gentle flow of nitrogen until dryness. The dry residue was reconstituted in 2 ml of mobile phase mixture (10 mM ammonium acetate–methanol, 75:25, v/v) and mixed vigorously in a tube shaker for 30 s. The tubes were then centrifuged for 5 min at 2000*g*. An aliquot of 300 µl of the supernatant was transferred into an empty autosampler HPLC vial in which a volume of mobile phase mixture was added to a final volume of 1.5 ml to obtain a dilution factor of 20. Aliquots of 20 µl of this diluted extract were injected into the LC-MS/MS system.

The use of SPY as an internal standard resulted from the fact that this sulfonamide is absent from the national pharmaceutical market. The capacity of SPY as an internal standard was evaluated by a comparison of the responses obtained by both SPY and the analytes (Hoff et al. 2009).

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The calibration curves were constructed using the ratio of the peak area of the analyte/area of the internal standard peak versus the concentration of analyte. To evaluate this compound as an eligible internal standard, a correlation between SPY concentration increases and each other sulfonamide included in the method was performed. A value of $R^2 \geq 0.999$ was considered satisfactory.

Milk

An aliquot of 500 µl of milk was placed in a microcentrifuge tube (2.0 ml) and 200 µl of acidified ethanol (acetic acid 3%) were added. Samples were mixed (15 s) and centrifuged at 10 min at 12,000 rpm. An aliquot of supernatant (350 µl) was diluted with water (650 µl) in an HPLC vial and submitted to LC-MS/MS analysis.

Currently adopted protocol for liver samples

An aliquot of 2.5 g of chopped and homogenised liver tissue was weighed into a 50-ml glass beaker. Internal standard (SPY) was added to a concentration of 100 ng g^{-1}. A total of 2 ml of acetonitrile was added and the tubes were mixed vigorously for 10–15 s using a vortex. This step was repeated twice more, with 3 ml of acetonitrile in the second time and 5 ml in the last addition. Approximately 3.0 g of anhydride sodium sulphate were then added to the each tube using a spatula. The tubes were mixed in an orbital mixer for 20 min. The mixture was then centrifuged for 10 min at 4000*g*. The tubes were placed in a freezer for 1 h and then the centrifugation step was repeated. An aliquot of 1 ml of the supernatant was transferred into an empty tube. This extract was evaporated in a water bath (40–45°C) under a gentle flow of nitrogen until dryness. The dry residue was reconstituted in 1 ml of mobile phase mixture (10 mM ammonium acetate–methanol, 75:25, v/v) and transferred into an empty autosampler HPLC vial.

Feed

Feed samples were homogenised, crushed and allowed to dry at room temperature until the moment of analysis. Feed samples were weighed (1 g) into a 50-ml polypropylene centrifuge tube. Extraction was achieved using 3 ml of methanol–water (70:30) with formic acid 0.1% and 10 min of shaking. After extraction the samples were centrifuged at 3000 rpm (at 5ºC) for 10 min. Following that the extracts were maintained in a freezer for 30 min. After that 1 ml of the extract was transferred into a microtube and centrifuged at 12 000 rpm (at 5ºC) for 20 min. An aliquot of 500 µl was diluted with 1000 µl of mobile phase in another microtube, repeating the centrifugation. The clean supernatant was transferred into a vial and injected into the LC-MS/MS system.

Eggs

Eggs samples were initially prepared by mixing egg white and yolk with a manual mixer. Aliquots of 2.5 g were weighed into 50-ml conical tubes, and then were spiked with internal standard solution (sulfapyridine) in order to produce a concentration level of 10 ng g^{-1}. The samples were then extracted in the same way as described for liver. The only difference was that total supernatant volume (approximately 10 ml) was evaporated and not just to 1 ml as in the case of liver samples.

Liver extraction with sand

For extraction, 25 g of chopped and homogenised bovine liver tissue were weighed into a 50-ml glass beaker. Internal standard (SPY) was added to a concentration of 100 ng g^{-1}. Approximately 15 g of sand were added to the tissue and mixed with a glass stick. The mixture was placed in an ultrasonic bath for 30 min. The mixture was then transferred into a plastic reservoir with a fritted glass filter in the bottom (Varian, Palo Alto, CA, USA). The reservoir was placed inside polypropilene tubes and centrifuged for 30 min at 4000*g*. The bottom elute was collected and 4 ml of this extract were transferred into a glass tube. Acetonitrile (2 ml) was added and the tube was centrifuged for 5 min at 3000*g*. This extract was diluted 100-fold: an aliquot of 15 µl of the supernatant was diluted with ammonium acetate 10 mM–methanol (85:15) to a final volume of 1.5 ml. The vial was submitted for analysis in the LC-MS/MS system.

Results and discussion

Scope extension validation protocols

The development and validation of an analytical method is a complex task. When a laboratory deals with food scares it must be able to provide scientific data to support legal actions or even policy decisions regarding food safety and/or public health. A compromise needs to be made between a rapid response to urgent needs and the technical quality of the data produced in the laboratory (Gamba et al. 2009).

At this point some terms, which are frequent used in this paper, must be correctly explained: (1) R – samples spiked before the extraction; (2) TS – 'tissue standard', samples spiked after the extraction, i.e. a blank extract spiked in the last step of the sample preparation procedure; (3) B – blank sample, without any analyte; and (4) S – standard solution.

For the purpose of this paper, absolute or 'raw' recovery is considered the overall recovery of the method, including the losses that normally occur in the extraction procedure plus the matrix effect (ion suppression caused by matrix interferences when electrospray ionisation-mass spectrometry methods were used). Relative recovery is the

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recovery without the losses caused by the extraction, determined using blank extracts in which the analytes were added at a concentration equal to 100% of the initial spike. In routine analysis, these samples are denominated 'tissue standards' to differentiate them from samples spiked before extraction. These samples eliminate the losses caused by extraction and measure only the loss caused by any matrix effect.

Generally, before starting a validation procedure, much time is spent in the development and optimisation of extraction, concentration and clean-up procedures. A simple and fast method to evaluate a sample preparation procedure is a very useful tool in a residue laboratory. To deal with this need, a strategy was developed for use in our laboratory. Once a sample preparation process is chosen, its effectiveness can be assessed by the analysis of a single batch composed of eight to 12 samples: three R samples, three TS samples, one to three B samples, and one to three standards in solvent (S). All spiked samples (R and TS) and standards are prepared at the same level, preferentially at the MRL level if applicable. Generally, the validations levels are centralised at 0.5 × MRL, 1.0 × MRL and 1.5 × MRL.

With the results, data interpretation will provide all the necessary information about the effectiveness of the method. We used a modification of the approach proposed by Matuszewski et al. (2003). The data analysis is based in simple quantitative comparisons:

$$R_{abs}(\%) = A_R/A_S \times 100 \quad (1)$$

$$R_{rel}(\%) = A_{TS}/A_S \times 100 \quad (2)$$

$$\text{Matrix effect}\,(\%) = R_{abs} - R_{rel} \quad (3)$$

where R_{abs} is absolute recovery; R_{rel} is relative recovery; A_R is analyte signal in R samples; A_S is the analyte signal in standards in solvent (S); and A_{TS} is the analyte signal in TS samples.

The analysis of B samples provides the information about the ability of the clean-up procedures to provide properly purified extracts. If samples are analysed by MS, it is recommended that B samples are analysed not just in MRM mode but also using full-scan mode. Thus, the analyst can observe the presence of interfering peaks at the retention times of analytes, for example.

As any method has its specific characteristics, the application of this evaluation procedure is very flexible, with the necessary adaptations depending on the analytical technique. Generally, the presented protocol is more able for MS methods, but it can be also applied to conventional HPLC or GC methods, for instance.

Case study: liver extraction using sand

For the extraction of sulfonamide residues from liver using just sand and 2 ml of acetonitrile, a batch for extraction effectiveness was performed as described above. Recoveries of the analytes spiked at MRL levels are summarised in Table 2. Data show an average recovery for all analytes ranging from 23% to 100%. The causes of the poor recovery values for SQX (40%) and SMA (23%) remain undetermined. For calculation, a six-point matrix-matched calibration curve was used with $r^2 > 0.990$. The low recoveries values for SMA and SQX lead us to discard this extraction procedure as a potential method.

Validation steps

Once the sample preparation process is defined, validation can be performed. Each laboratory has its own internal procedure in agreement with one or more harmonised guidelines. In our protocol, the validation process starts with the linearity evaluation and the determination of the working range, LOD, LOQ and matrix effect. By using calibration curves prepared in the matrix, these five parameters can be determined in a single experiment. Generally, a calibration curve with six points (0 + 5) is used, having at its centre at the MRL or another adopted target concentration level.

A calibration curve was prepared using standard solutions diluted in pure solvent or in mobile phase (standard calibration curve, or 'S'). A second calibration curve was prepared spiking a blank matrix and followed by the extraction and/or clean-up procedure (recovery calibration curve, or 'R'). Finally, a third calibration curve was made by using a extract of a blank sample, which was submitted to the whole extraction and/or clean-up procedure and was spiked with standard solution at the end of the protocol, generally in the final dilution, immediately before injection (tissue standard calibration curve, or 'TS'). These calibration curves were prepared with the same number of points or replicates to obtain the same expected concentration in the three kinds of curve. Usually the MRL was the central point. Ideally, all

Table 2. Average recoveries for sulfonamides in liver at MRL level using extraction with sand.

Analyte	Average recovery (%)
SDZ	93
STZ	98
SPY	97
SMZ	100
SMA	23
SDMX	91
SQX	40

Note: SDZ, sulfadiazine; STZ, sulfathiazole; SPY, sulfapyridine; SMZ, sulfamethazine; SMA, sulfamethoxazole; SDMX, sulfadimethoxine; SQX, sulfaquinoxaline.

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curves were prepared and analysed in the same batch. After analysis, the curves were plotted together and inspected visually and/or statistically (in pairs).

After the analysis, the three calibration curves (S, R and TS types) were plotted and analysed. For matrix effect estimation, the following situations could be perceived:

- Situation 1: A similar slope, but with non-similar intercepts. Similarity between slopes shows that the matrix does not interfere in the linearity of the responses. The difference between intercepts is given by the losses caused by the sample preparation process. A lower response for the R curve is expected. If the TS and S curves overlap, there is no matrix effect. If those curves have non-similar behaviour, the matrix effect is present, but it affects just the signal, not the linearity. Any kind of curve can be used in this method if the appropriate corrections are applied to adjust response losses.
- Situation 2: Non-similar slopes: curve linearity is distinct between curves. If the TS and R curves were similar in the slopes this means that the presence of the matrix itself change the responses. In this case, just TS or R calibration curves may be used in this method.
- Situation 3: TS and S curves are totally overlapped. There is no matrix effect. However, if the R curve shows differences in the intercept and slope this means that the sample preparation process changed the response significantly. Thus, R curves may be used.
- Situation 4: All curves are perfectly overlapped. No matrix effects and recovery equals or is very close to 100%.

In some cases it is not possible to perform this analysis by just visualising the plot. In those cases a statistical Student's *t*-test may be performed for each pair of curves (S versus R, S versus TS, R versus TS).

For linearity and work range evaluation, one of the curves can be expanded with more calibration points (four to six points) at the desired higher concentration level. Generally, if this is the aim the solvent calibration curve is used.

LOD and LOQ may also be calculated based on the SD of the response and the slope of the calibration curve (S) at levels approximating the LOD according to the formula: LOD = 3.3(SD/S). The SD of the response can be determined based on the SD of the y-intercepts of regression lines and it shows how much variation or dispersion from the y-intercept values exist. A minimum of three independent curves is necessary to calculate this parameter. In the LOD equation, the number 3.3 is related to the signal-to-noise ratio.

For LOQ, the calculation method is again based on the SD of the response and S according to the formula: LOQ = 10(SD/S). Again, the SD of the response can be determined based on the SD of the y-intercept of the regression lines. In the LOQ equation, a signal-to-noise ratio of 10:1 was applied.

The SD and slope can be easily obtained from the LINEST function when creating a calibration curve in MS Excel software. The SD of y is that used for the calculation of both LOD and LOQ. Figure 1 demonstrates the parameters that can be obtained with the data from the calibration curve. Again, the same expanded calibration curve used for linearity evaluation can be used for the estimation of both LOD and LOQ.

Major validation parameters

The overall aim of the validation procedure is to demonstrate that the method can correctly determine, with an acceptable error limit, the amount of analyte in a sample.

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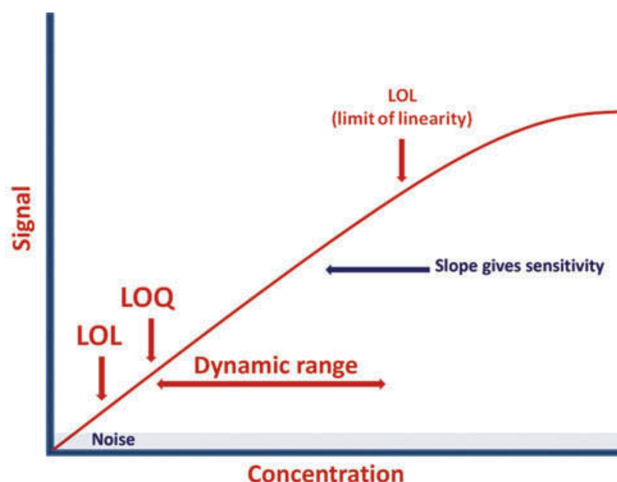

Figure 1. Schematic of a calibration curve plot showing the LOD, LOQ, dynamic range and limit of linearity (LOL).

For the determination of repeatability (in terms of intra- and inter-day precisions) and parameters as decision limit and detection capability, a procedure based on Decision 657/2002 is applied. The main parameters are obtained with the analysis of three independent batches, performed on 3 distinct days, executed by the same analyst. Each batch is composed of 31 samples, according to the following scheme:

- Seven samples spiked at 0.5 × MRL concentration level.
- Seven samples spiked at 1.0 × MRL concentration level.
- Seven samples spiked at 1.5 × MRL concentration level.
- Three TS samples spiked at the MRL concentration level (for recovery calculation).
- One blank sample.
- Calibration curve (in matrix, spiked before extraction).

With the exception of the TS samples, all samples are spiked before the extraction and clean-up procedure.

For a full validation procedure, several other studies are included: reproducibility, selectivity/specificity (through the analysis of 20 blank samples), stability (for standard solutions, extracts, samples), robustness, uncertainty measurement, and other complementary experiments.

Scope extension – inclusion of a new matrix

When a method was developed and validated for a specific matrix and it needs to be extended to include another matrix with close similarity, e.g. bovine muscle and swine muscle or poultry liver and poultry kidney, the proposed protocol is based in two analysis batches. The first is composed of seven distinct blank samples and the other by seven samples spiked at the MRL or action level (AL, as defined below). Each batch is performed together with the regular quality control (QC) samples (R, TS, standards). With the data obtained in the first batch, chromatograms of the blank samples of the new matrix can be superimposed with the original matrix data for the evaluation of interfering peaks, the signal-to-noise ratio, etc. The data obtained with the second batch are used to calculate recovery, RSD and CV. If these values are equal to or lower than that obtained for the original matrix, the extension of the scope is considered to be a validation. Until complete data (which are obtained from routine performance of the method) are not available, the CCα and CCβ values are considered to be the same as those obtained for the target analyte in the original matrix.

The degree of similarity between matrices is a subjective issue, depending on the previous experience of the analyst with the extraction procedures and some knowledge about the behaviour of the matrix in laboratory terms. For instance, a scope extension from liver to honey is an obvious case of low similarity between samples. In this case, we adopted a more detailed procedure. This includes the analysis of a complete validation batch (with three levels and seven replicates for each level) besides the analysis of a batch composed of 20 distinct blank samples.

Case study: milk and eggs

Although the administration of sulfonamides is not authorised for laying hens, extra-label use of these drugs can be a possible way for sulfonamide residues to occur in eggs for human consumption. Thus, it is strongly recommended that the analysis of sulfonamide residues in eggs must be included in the scope of the National Residues and Contaminants Control Plan.

Currently, no MRL has been adopted for sulfonamide residues in eggs in Brazil. In this case, the Ministry of Agriculture, Livestock and Supply (MAPA) adopts a value of 10 ng g^{-1} (the AL) for substances whose MRLs have not yet been established (Mauricio et al. 2009). For this reason, during all experiments 10 ng g^{-1} was assumed as the AL (Tabassum et al. 2007; Zheng et al. 2008).

For milk analysis, the same MRL adopted in Brazil for liver is extended for bovine milk (Mauricio et al. 2009; de Queiroz Mauricio & Lins 2012; Jank et al. 2012). For both milk and eggs matrices, the procedure was based on the analysis of a batch of 20 samples spiked at the MRL or AL together with the analysis of 20 blank samples. A summary of the major validation parameters is shown in Tables 3 and 4. For milk and liver, the MRL is 100 ng g^{-1} (or ng ml^{-1} for milk), LOD is 10 ng g^{-1} and LOQ was established at 25 ng g^{-1}.

Case study: feed

Some matrices were very different from one another, such as liver and feed. In such a case, a new full validation procedure must be performed. For feed samples, the method was validated in accordance with Decision 2002/657/EC. Method performance parameters were determined and evaluated according to the considerations proposed in the decision as linearity, accuracy, precision, specificity, selectivity, stability of standards and matrix interference, besides the parameters CCα and CCβ.

In Brazil, as the addition of antibiotics to animal feed is forbidden, it is also necessary to have analytical methods for monitoring this issue in veterinary production (Lopes et al. 2012). For the analysis of sulfonamides in feed, a very simple method was developed and validated as an extension of scope. However, for feed analysis a totally new extraction procedure was developed and a full

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Table 3. Summary of validation data for sulfonamides in eggs.

Parameter	Linearity	Accuracy (%)	Precision (%)	Recovery (%)	LOD (ng g^{-1})	LOQ (ng g^{-1})	CCα (ng g^{-1})	CCβ (ng g^{-1})
Criteria	$R^2 \geq 0.95$	–20% to 10%	≥ 15	–	–	–	–	–
SDZ	0.9737	0.56	10.3	81.7 ± 1.3	2.5	5.0	12.5	14.0
STZ	0.9770	2.39	13.4	70.3 ± 4.5	2.5	5.0	13.0	15.5
SMZ	0.9770	2.58	15.0	73.8 ± 2.3	2.5	5.0	13.8	16.9
SMA	0.9746	3.11	10.5	84.2 ± 2.0	2.5	5.0	12.2	14.1
SDMX	0.9851	3.15	7.5	75.9 ± 1.9	2.5	5.0	14.2	18.0
SQX	0.9789	1.58	9.9	74.4 ± 3.4	2.5	5.0	15.1	19.3

Note: SDZ, sulfadiazine; STZ, sulfathiazole; SMZ, sulfamethazine; SMA, sulfamethoxazole; SDMX, sulfadimethoxine; SQX, sulfaquinoxaline; CCα, decision limit; CCβ, detection capability; R^2 = coefficient of determination.

Table 4. Sulfonamide residues in liver and milk major validation parameters.

	Liver		Milk	
Analyte	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	CCα (µg l^{-1})	CCβ (µg l^{-1})
SDZ	113.0	126.0	120.0	149.0
STZ	115.0	128.0	122.0	150.0
SMZ	121.0	138.0	124.0	162.0
SMA	119.0	134.0	124.0	155.0
SDMX	112.0	125.0	123.0	153.0
SQX	115.0	130.0	123.0	153.0
SDX	107.4	114.8	106.6	113.2
SCP	108.1	116.2	112.9	125.8
SMR	106.6	113.2	106.2	112.4

Note: SDZ, sulfadiazine; STZ, sulfathiazole; SMZ, sulfamethazine; SMA, sulfamethoxazole; SDMX, sulfadimethoxine; SQX, sulfaquinoxaline; SDX, sulfadoxine; SCP, sulfachlorpyridazine; SMR, sulfamerazine; CCα, decision limit; CCβ, detection capability.

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validation performed. A summary of the major validation parameters is shown in Table 5.

Scope extension – inclusion of a new analyte

In the case of inclusion of one or more analytes, a batch of 20 spiked samples at the MRL (or AL) level was performed. The data allow the determination of CCα and precision.

Table 5. Feed validation major parameters.

Analyte	Usual level in feed/premix (mg kg^{-1})	MRPL (mg kg^{-1})	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
SDZ	150–300	1.5	0.005	0.075
SMZ	200–500	2.0	0.005	0.075
SMA	75–500	0.75	0.005	0.075
SQX	62–1000	0.62	0.020	0.075
SCP	185–700	1.85	0.005	0.075

Note: MRPL, minimum required performance limit; SDZ, sulfadiazine; SMZ, sulfamethazine; SMA, sulfamethoxazole; SQX, sulfaquinoxaline; SCP, sulfachlorpyridazine.

Case study: SMR, SCP and SDX inclusion in milk and liver analysis

SDX, SMR and SCP were included in the sulfonamide residues method for the matrices liver and milk. The scope extension procedure was based on a batch comprising 20 milk and 20 liver samples spiked at the MRL level (here it was 100 ng g^{-1} or ng ml^{-1}). Each batch was accompanied by the appropriate QC samples. Determination of CCα was based in the RSD from the dataset. CCβ was only estimated through multiplication of the combined RSD values obtained by a coverage factor k (k = 2). The k value was elected based on the confidence level required. Generally, it is between 2 and 3. The results and the acceptance criteria are described in Table 6.

Uncertainty measurement

For uncertainty measurement, a so-called top-down approach is based on trueness data from the control chart (Pecorelli et al. 2005; Dabalus Islam et al. 2008). These data were obtained from each routine batch of analysis. From each group of 20 batches or each year (whichever happened first), the variance of each group (three replicates) of control quality samples was calculated. The sum

Table 6. Data obtained for SDX, SCP and SMR inclusion in liver and milk analysis.

	Liver						Milk					
Analyte	Average calculated concentration (ng g^{-1})	SD (ng g^{-1})	CV (%)	Trueness (%)	CCα (μg kg^{-1})	CCβ (μg kg^{-1})	Average calculated concentration (ng ml^{-1})	SD (ng ml^{-1})	CV (%)	Trueness (%)	CCα (μg l^{-1})	CCβ (μg l^{-1})
SDX	88.9	4.5	5.1	89	107.4	114.8	101.7	4.0	4.0	102	106.6	113.2
SCP	83.4	4.9	5.9	83	108.1	116.2	105.4	7.9	7.4	105	112.8	125.8
SMR	75.3	4.0	5.3	81	106.5	113.2	100.9	3.8	3.7	101	106.2	112.4

Note: SDX, sulfadoxine; SCP, sulfachlorpyridazine; SMR, sulfamerazine; CCα, decision limit; CCβ, detection capability.

of the 20 variance data was used to obtain the overall SD, which was multiplied by the coverage factor (k = 2). The product is the expanded uncertainty for the concentration level of QC samples. An example of this approach is shown in Table 7.

Scope extension verification

The present scope extension protocols do not mean the methods are free of verification. The most indicated test to be applied just after the validation is the participation in proficiency test schemes (PT). Unfortunately, only a few combinations of matrix/analyte are available. For sulfonamide residue analysis, generally PT is performed for liver, muscle or kidney. Our laboratory has taken part in PT for sulfonamides since 2007, at a minimum of one PT for a group of analytes per year. When PT for new matrices are not available, the method verification is performed using QC samples associated with control charts. Additionally, the laboratory has an internal system of checking samples that are prepared monthly in a double-blind scheme and distributed to the analysts.

Conclusions

Extension of scope is a common issue for routine testing laboratories. Frequently, the need for a rapid response in the face of urgent problems does not permit the

Table 7. Uncertainty measurement for sulfaquinoxaline in liver using top-down approach and control chart data.

Data	QC sample 1	QC sample 2	QC sample 3	Average	SD	Variance
1	105	104	122	110.3	10.1	102.33
2	92	90	95	92.0	2.4	5.88
3	116	110	124	116.6	7.0	49.33
4	133	129	99	120.0	18.5	342.35
5	115	113	117	115.0	2.0	4.00
6	111	114	112	112.3	1.5	2.33
7	112	97	106	104.8	7.8	61.08
8	114	109	121	114.6	6.0	36.33
9	115	103	90	102.5	12.7	162.75
10	121	116	100	112.2	10.7	115.01
11	99	101	124	107.9	14.1	201.01
12	101	107	103	103.6	3.0	9.33
13	112	111	111	111.3	0.5	0.33
14	128	139	130	132.2	5.6	32.16
15	99	95	94	95.8	2.4	5.76
16	102	99	103	101.2	2.2	5.06
17	104	86	92	93.9	9.1	83.29
18	136	180	174	163.3	23.8	569.33
19	87	111	81	93.2	15.7	247.24
20	100	90	102	97.1	6.5	42.76
				SD_c =	16.18	10.192
				SD_k =	32.37	20.385
Expanded uncertainty (ng g^{-1})			20.38			
Expanded uncertainty (%)			20.4			

Note: QC, quality control; SD, standard deviation obtained from the three QC replicates analysed in each batch; SD_c, combined standard deviation from 20 batches; SD_k, coverage factor (here it is 2).

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development of a full validation process. For the extension of the scope of a method, i.e., the inclusion of more analytes, matrices or major changes in the sample preparation process, a more compact validation process is more adequate. Generally, the most adopted validation guidelines, as 2002/657/EC Decision or Eurachem, do not include more practical approaches for validation of the extension of scope. Even in the technical literature reports about this topic are scarce. In this work we have suggested several protocols for the validation of the extension of scope in a compromise between data quality and rapid response. Based on our previously published validation procedure for sulfonamide residue analysis, several improvements in the original method are presented, together with the validation protocols. Currently, all these scope extensions are applied in routine analysis and the method has been accredited by ISO 17025.

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Anexo IV – Artigo a ser submetido para *Talanta*: **Analytical quality assurance in veterinary drug residues analysis methods: matrix effects determination and monitoring**

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Abstract: In residue analysis of veterinary drugs in foodstuff, matrix effects are one of the most critical points. This work present a discuss considering approaches used to estimate, minimize and monitoring matrix effects in bioanalytical methods. All techniques were applied in a Brazilian government laboratory that deal with veterinary drugs and pesticide residue analysis (Lanagro/RS). Methods for qualitative and quantitative estimation of matrix effects such as post-column infusion, slopes ratios analysis, calibration curves (mathematical and statistical analysis) and control chart monitoring are discussed using real data. Advantages and drawbacks are also discussed considering a workflow for matrix effects assessment proposed and applied to real data from sulfonamides residues analysis in liver samples.

Opposed Reviewers:

Novelty Statement

The present work provides a comparison between analytical approaches to estimate, minimize and monitor matrix effects in mass spectrometry methods. Real data are applied to several strategies for matrix effect assessment. A walkthrough guide is presented to be used in methods development and validation.

Highlights

- Strategies for matrix effects assessment in mass spectrometry methods are discussed.
- Approaches for matrix effects estimations are compared using real analytical data.
- Advantages and drawbacks of each strategy were discussed
- A walkthrough guide for matrix effects assessment is proposed.
- Tools for estimation, minimization and monitoring of matrix effects are demonstrated.

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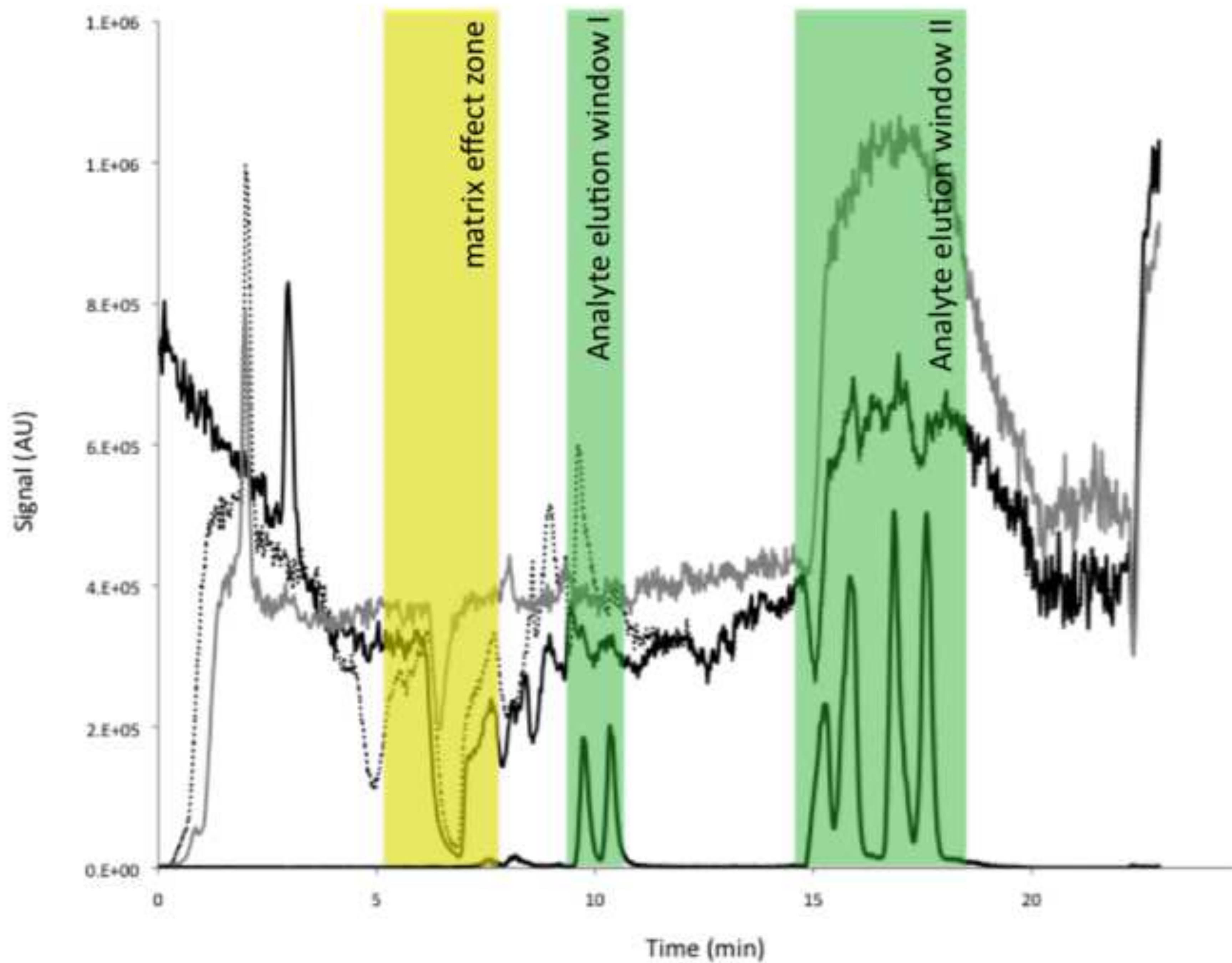

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Analytical quality assurance in veterinary drug residue analysis methods: matrix effects determination and monitoring

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Abstract

In residue analysis of veterinary drugs in foodstuff, matrix effects are one of the most critical points. This work present a discuss considering approaches used to estimate, minimize and monitoring matrix effects in bioanalytical methods. All techniques were applied in a Brazilian government laboratory that deal with veterinary drugs and pesticide residue analysis (Lanagro/RS). Methods for

qualitative and quantitative estimation of matrix effects such as post-column infusion, slopes ratios analysis, calibration curves (mathematical and statistical analysis) and control chart monitoring are discussed using real data. Advantages and drawbacks are also discussed considering a workflow for matrix effects assessment proposed and applied to real data from sulfonamides residues analysis in liver samples.

Keywords: Quality control; Veterinary drug residues; Matrix effects; Validation; Ion suppression; Control charts; Antibiotics; Sulfonamides.

Introduction

Farmers use veterinary drugs worldwide to promote animal health and welfare. Notwithstanding, the secondary aspects arising from this practice are mostly directed toward the enhancement of farm productivity through growth promotion, which is important to supply the growing global demand for food and can provide economic gains, and, on the other hand, the occurrence of residues in food of animal origin [1–4]. Food containing drug residues above maximum residue limit (MRL) is of major concern, since it is related directly to public health as well as international trade relationships. Veterinary drug residue matter has its own laws and regulation development, both within countries and among economic blocks and international bodies, in order to propose and to harmonize MRL values for various drug-matrix combinations. Veterinary drug residue analysis is the major component of monitoring programs established by regulatory agencies in virtually all countries involved in global food market. Its demand in food regulatory control has expanded dramatically in recent decades, and residues surveillance became an important factor to be considered in international trade of commodities [5,6].

In Brazil, veterinary drug and pesticide residues analysis in animal (and also in vegetable) products are under the Ministry of Agriculture, Livestock and Supply (MAPA) management [7]. Routine analysis and methods development and

validation are attributed to MAPA official laboratories network – National Agricultural Laboratories (Lanagro) – and MAPA accredited private laboratories [8].

MAPA's demand on method development and validation in residue analysis has been increased in the last decade due to the increased role of the Brazilian livestock products in national and international markets and meanly to ensure that the products traded are compliant with the safety and quality criteria required by consumers [8,9]. Wherefore, our laboratory has absorbed one important fraction of this demand in developing, validating, and submitting for accreditation methods for analysis of antimicrobial and non-antimicrobial residues in different matrices, such as milk and edible tissues of different animal species including cattle, pork, poultry, and even fish [10–14]. For these purposes, international guidelines, such as Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results, and others from the US Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH), are used in order to obtain methods validated according to the most stringent international criteria [15]. Within this issue, especial attention is paid to matrix effect (ME), which is a fundamental parameter to be determined, assessed and minimized especially when liquid chromatography- mass spectrometry (LC-MS) and/or tandem mass spectrometry (LC-MS/MS) methods are used [16,17]. The conceptualization of this phenomenon has been comprehensively reviewed by a number of authors [16–19]. Briefly, ME is related to the alteration of ionization efficiency in the ionization source by the presence of coeluting substances: the occurrence of endogenous substances originally present in the sample itself and that remains in the final extract, are appointed as the major source. A wide scope of molecules can lead to signal suppression or enhancement, especially when occurs in high concentration in the extract and elute in the same retention time window than the analyte [20]. A secondary cause are substances not originally present in the samples but able to migrate to extracts during sample preparation process as polymer and phthalates or material released by stationary phases, in bulk or in solid phase extraction (SPE) cartridges, for instance [18]. Normally, this

alteration affects dramatically the method accuracy and precision and has been regarded as a critical validation item by most guidelines consulted. However, there is no consensus on how this phenomenon should be assessed during method validation. Beside, different experienced approaches of ME evaluation, based on procedures published in the scientific literature such as post-column infusion, calibration curves comparison, quantitative estimation based in standards, spiked samples and matrix-matched control comparison and control charts evaluation, has been experienced [21–25].

Although the knowledge on ME in mass spectrometry analysis has been improved in recent years, only few practical approaches has been reported for routine analysis [26–29]. In the present work, practical approaches to detect and estimate the occurrence of ME in qualitative and quantitative terms in LC-MS/MS methods for veterinary drugs residues analysis are presented and discussed. Tools for monitoring ME along the execution of routine methods are also reported.

Materials and methods

Analytical standards and reagents

Analytical standards with high purity (≥99%) were obtained from Sigma-Aldrich (St Louis, MO, USA) namely sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxazole (SMA), sulfamethoxypyridazine (SMPZ), sulfadiazine (SDZ), sulfapyridine (SPY), sulfadimethoxine (SDMX), sulfaguanidine (SGA), sulfacetamide (SCA), sulfabenzamide (SBZ), sulfisomidin (SIM), sulfamethizole (SMTZ), sulfaquinoxaline (SQX), sulfathiazole (STZ), sulfaisoxazole (SIX) and sulfadoxin (SDX). The metabolite N^4-acetyl-sulfamerazine (AcSMR) and the isotopically labelled compounds d^4-sulfamethoxazole (d^4-SMA), d^4-sulfamethazine (d^4-SMZ) and d^4-sulfadiazine (d^4-SDZ), used as surrogate and/or internal standards, were purchased from Toronto Research Chemicals (North York, Ontario, Canada).

MeOH, acetonitrile (ACN), hexane and acetone of HPLC-grade were supplied by J. T. Baker (Deventer, The Netherlands). Diatomaceous earth was supplied by Agilent Technologies. Acetic acid and water (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Individual stock standard solutions were prepared in MeOH: acetone (50:50) at 1 mg mL^{-1} and stored at -4°C until use. Standard solutions of the mixtures of all compounds at appropriate concentrations were prepared by stock solutions dilutions using MeOH or acetone. Aliquots of each stock standard solution were diluted to obtain final concentrations of 10 µg mL^{-1} and 1 µg mL^{-1} and were stored at -20 ºC.

Samples and sample preparation

Liver of different food production animals, chicken eggs, and fish muscle were obtained from Federal Inspection Service (SIF) or collected from treated animals in a farm. Liver and muscle samples were manual and finely chopped and homogenized in order to avoid slurring. Egg samples were manual and gently homogenized in order to avoid protein denaturation. After these processes, all samples were stored at – 20 °C before extraction step.

Liver and fish samples were extracted by two different methods based on pressurized liquid extraction (PLE) and by ultrasounds-assisted extraction (US). For PLE, an ASE 350 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA) was used. Prior to extraction, d^4-SMA, d^4-SMZ and d^4-SDZ were added as surrogate standards at a concentration of 100 ng g^{-1}. Samples (0.5 g) were mixed into the PLE cells with diatomaceous earth as dispersing agent. Prior to extraction, the cells were submitted to a clean up method in order to remove lipids from the samples using hexane as solvent. PLE parameters were as follows: temperature 60ºC, 4 cycles of 5 minutes each one. Total flush volume of 80% and 300 s of purge with nitrogen flow were applied.

After that, the same PLE cells were submitted to a second extraction process using ACN with 0.2% acetic acid as extraction solvent. In this case, the extraction temperature was optimized at 90ºC. A preheating period of 8 min was selected and 3 cycles of 7 minutes each were carried out. A total flush volume of 80% and 60 s of purge with nitrogen flow were applied. Pressure was set at 1,500 psi as it has been demonstrated that this parameter is not decisive in PLE.

The extracts were maintained in freezer by one hour (at -18ºC) in order to promote protein precipitation. Following, samples were centrifuged at 3500 rpm for 10 min in a 5810 R centrifuge (Eppendorf). The supernatant was evaporated at 40°C under nitrogen flow using a Turbo-Vap system (Zymark) until dryness. Extracts were redissolved in 1.0 mL of mobile phase mixture (water-ACN, 85:15) and transferred to a HPLC vial.

In ultrasound-assisted extraction, samples (0.5 g) were weighted in 15 mL polypropylene tubes and spiked as described for the PLE method. Following, 10 mL ACN were added and tubes were mixed in a mechanical vortex by 10 s. Afterwards all samples were placed into an ultrasonic bath for 1 h. and then stored in freezer (-18°C) for 1 h. to promote protein precipitation. Then, samples were centrifuged at 3500 rpm for 10 min. Supernatant was brought to dryness at 40°C under a gentle nitrogen stream. The extracts were redissolved in 2.0 mL of the mobile phase mixture. An aliquot of 2 mL of hexane was added to remove the fat content. Afterwards, tubes were mixed in a vortex for 5 s followed by centrifugation (3500 rpm for 10 min). The lower layer was carefully transferred to a HPLC vial.

Sulfonamides analysis in eggs samples was performed as described elsewhere [30]. Briefly, samples were extracted with ACN and concentrated before reconstitution with mobile phase.

Instrumentation

LC analysis was performed with a Symbiosis™ Pico System (Spark Holland, Emmen, The Netherlands), equipped with a HPLC system consisting of an Alias™ autosampler, a loop injector and two binary pumps with a four-channel solvent selector for each one. Chromatographic separation was performed using a HPLC column Purospher® STAR (C18, ec, 150 x 4.6 mm, 5 µm) preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL min^{-1}, being eluent (A) HPLC grade water acidified with 10 mM of formic acid, and eluent (B) ACN with 10 mM of formic acid. The elution gradient started with 25% of eluent (B), increasing to 80% in 10 min and to 100% in 11 min. During the next 2 min the column was kept at 100% (B), readjusted to the initial conditions in 3 min and equilibrated for 7 min. MS/MS analyses were carried out in a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray ionization source (ESI) working in the positive mode (ESI+).

For fish and egg analysis, the LC-MS/MS system was an Agilent 1100 series LC (Santa Clara, CA, USA) with a quaternary pump, a vacuum degasser, and an auto sampler, coupled with an API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization source (ESI).

The optimization of the MS/MS experimental conditions was performed in previous studies [31]. For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring (SRM) mode, recording the two most intense transitions from the precursor ions to the product ions.

Post-column infusion method

Post-column infusions of individual standards into the MS system were performed to verify the ME of the extracts obtained for all methods, in order to verify if the whole extract or some elution fraction of the extract cause signal

suppression/enhancement. This procedure was based on the experiments described by Bonfiglio et al. [21] . Briefly, blank samples extracted by the above-mentioned methods were injected into the LC–MS/MS system under the chromatographic conditions optimized for each methodology. For each injection, a standard solution of individual compound was infused into the MS system using an infusion pump, at a flow rate of 10 μL min^{-1}, through a tee-joint installed post-column. ME were evaluated observing signal attenuation or signal enhancement on the response of the infused analyte.

Calibration curves evaluation method

A calibration curve was prepared using standard solutions diluted in pure solvent or in mobile phase (external Standard calibration curve or “S”). A second calibration curve was prepared spiking a blank matrix and following with the extraction and/or cleanup procedure (Recovery calibration curve or “R”). Finally, a third calibration curve was made using a extract of a blank sample, which was submitted to the whole extraction and/or cleanup procedure and was spiked with standard solution at the end of the protocol, generally in the final dilution, immediately before injection (Tissue Standard calibration curve (matrix-matched) or “TS”). These calibration curves were prepared with the same number of points or replicates to obtain the same expected concentration in the three kinds of curve. Usually, the MRL is the central point. All curves were prepared and analyzed in the same batch for a more accurate comparison. After analysis, the curves were plotted and inspected visually and statistically.

Alternatively, ME was evaluated using slope ratios comparison according to the approach proposed by Romero-Gonzáles et al [32] and Sulyok et al [33] in a modified application of the quantitative approach of Matuszewski et al [22]. Slopes are compared between each pair of curves obtained in the linear calibration curves prepared by spiking mobile phase (S), blank sample (R), and extract of blank sample (TS). Slope ratios below 0.9 or above 1.1 were associated with ion

suppression and ion enhancement, respectively. For values inside that range, ME was considered negligible.

To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated to yield the recovery (RE), the signal suppression/enhancement due to ME and the relative recovery, i.e. the recovery of the extraction step (RE_R) as follows:

$$RE\ (\%) = 100 \times slope_{spiked\ samples} / slope_{liquid\ standards} \quad \text{(Eq. 1)}$$

$$ME\ (\%) = 100 \times slope_{matrix-matched\ standard} / slope_{liquid\ standards} \quad \text{(Eq. 2)}$$

$$RE_R\ (\%) = 100 \times slope_{spiked\ samples} / slope_{matrix\ -\ matched\ standard} \quad \text{(Eq. 3)}$$

Matrix effect quantitative estimation

The quantitative estimation of a ME, when present, was performed using a modification of the equations previously proposed by Matuszewski et al [22]. This procedure allows determination of the ME along with the RE_R and overall “process efficiency” or method overall recovery (RE) by comparing the absolute peak areas of 3 sets of samples. Set A is composed by standard solutions (S). Set B is composed by samples spiked after extraction (TS) and set C is prepared with samples spiked before extraction (R). Since the values have been obtained, ME, RE_R, and RE values can be calculated as follows:

$$ME\ (\%) = (B/A \times 100) - 100 \quad \text{(Eq. 4)}$$

$$RE_R\ (\%) = C/B \times 100 \quad \text{(Eq. 5)}$$

$$RE\ (\%) = C/A \times 100 = (ME \times RE_R)/100 \quad \text{(Eq. 6)}$$

Control chart

Control charts are a useful tool for the monitoring of the analytical method behavior along with-in-batch and batch-to-batch variations including those due to ME. Within this purpose, for each analysis batch, 6 quality control samples (QC) spiked at the MRL concentration level for all compounds analyzed in each method were obtained. These QC samples, as described above, are composed by 3 samples spiked after (TS) and 3 samples spiked before extraction (R). Analyte peak area of each QC sample plus standards in pure solvent are plotted in a spreadsheet using Excel software. The cells include a formula to provide average, relative standard deviation and the upper and lower limits for ME, calculated according to the control chart parameters.

Results and discussion

As aforementioned, there is no consensus on how ME should be evaluated during method validation, neither on the criteria that should be adopted in establish when these effects are or not occurring [34]. However, according to recent literature, two main procedures have been used to determine ME on LC–ESI-MS/MS analysis: post-column infusion, which is a dynamic technique that provides qualitative information on where ME occur along the chromatographic run; and post extraction addition, which is a static technique that quantitatively provides the ME degree at the analyte retention time [18,35]. The last technique has been preferentially used to evaluate and compare ME of different matrices in terms of relative ME. In order to evaluate the most reported approaches to ME estimation, data of two in-house developed and validated methods were used as an example.

Case study: determination of sulfonamides (SAs) in liver, muscle and fish using two extraction procedures

Two extraction methods were developed and validated for analysis of SAs residues in liver, muscle and fish. The complete development, optimization and validation data for both methods were recently submitted to publication. Both, the PLE and

the US methods were previously evaluated by their potential ME using all the described approaches.

Post-column infusion method

Firstly, blank extracts from each extraction method were injected in a post-column apparatus for comparison with pure mobile phase in order to evaluate the variation of the standards mixture signal.

The chromatographic separation of SAs was achieved using a modification of the method published elsewhere. Some SAs had very similar chromatographic retention time (coeluting), but were well rsolved as individual peaks in the MS/MS SRM mode.

To evaluate ME, firstly we investigated if the extraction methods could contribute with co-extractive substances that might suppress selectively the different temporal regions of the chromatogram.

As can be observed in Figure 1, both PLE and US extracts presented signal suppression in some regions of the chromatogram. This suppression effect is more intense in the 5-6 min region. Mobile phase signal (MP, grey line) shows a standard solution infused in a post-column "T" connection, over a mobile phase injection. PLE (black line) and US (dashed black line) represent the signal of the respective blank extracts. The standard solution was a mixture of all analytes at a concentration level of 100 ng mL^{-1}. Any line represents a TIC signal for all monitored SRM transitions (>36). TS signal is a TIC chromatogram for a blank sample spiked at 100 ng mL^{-1} injected in normal mode. The signal was multiplied by a factor of 20 times for a better comparison with the post-column infusion chromatograms. PLE and US showed very similar matrix effects over the standard signal. In the region 11-22 min both signals were virtually overlaid. In summary, analytes do not elute in the most critical suppression zones. Thus, the chromatographic conditions could be used without modifications.

Figure 1. Total ion chromatograms of post-column experiments. Continuous black line is a pressurized liquid extraction (PLE) blank extract; dashed black line is a ultrasound-assisted extraction (US) extract; grey line is mobile phase injection (MP) and the lower chromatogram in bold black line is a spiked tissue extract injected without post-column infusion in order to identify the elution window of the target analytes (TS).

Rübensam et al used a post-column infusion to evaluate the ME variation in determination of macrocyclic lactones (as ivermectin) in milk, using several extraction protocols [10]. Through this procedure, a more intense ME was observed for moxidectin. ME of milk blank extracts obtained by solid phase extraction, liquid–liquid extraction with low temperature purification, precipitation at low temperature, and liquid–liquid extraction were evaluated by post-column infusion of moxidectin on LC–MS/MS. Although all macrocyclic lactones showed similar ME, these effects were more pronounced for moxidectin. The signal of the analyte was suppressed along the chromatographic analysis in an extract prepared with liquid-liquid extraction. In low temperature purification extracts, an enhancement of the signal was observed. In addition, an interfering compound of these extracts co-eluted with moxidectin peak, causing a “dip” in the base line. The ME was eliminated along the chromatographic runs when the samples were extracted by solid phase extraction or liquid-liquid extraction associated with protocols, which is an indication that the co-eluting compounds were removed by these extraction procedures.

Calibration curves approach

As explained before, the use of calibration curves to estimate ME can be performed in many ways. Herein, examples of each interpretation mode are demonstrated.

Graphical plot – visual and statistical analysis

Once the 3 curves are analyzed and plotted, the following situations are considered:

Situation 1. A similar slope, non-similar intercepts. Similarity between slopes show that matrix do not interfere in the linearity of the responses. The difference between intercepts is given by the losses caused by the sample preparation process. It is expected a lower response for R curve. If TS and S curve could be overlapped, there is no ME. If those curves have non-similar behaviour, ME is present, but it is affecting only the signal, not the linearity. Any kind of curve can be used in this method, if an appropriate correction is applied to adjust response losses. The Figure 2 shows an example of this situation.

Figure 2. Calibration curves comparison for ME evaluation: similar slopes, non-similar intercepts. Continuous line is S curve; dashed line is a TS curve and grey line represents an R curve.

Situation 2. Non-similar slopes: linearity is distinct between curves. If TS and R curves had similarity in the slopes, this means that the presence of the matrix itself change the responses. In this case, only TS or R calibrations curves may be used in this method. Figure 3 gives an example.

Figure 3. Calibration curves comparison for ME evaluation: non-similar slopes. Continuous line is S curve; dashed line is a TS curve and grey line represents an R curve.

Situation 3. TS and S curves are totally overlapped. There is no ME. However, if R curve shows differences in intercept and slope, this means that sample preparation process change significantly the response. Thus, R curves may be used. See Figure 4 for an experimental data example.

Figure 4. Calibration curves comparison for ME evaluation: absence of ME. Continuous line is S curve; dashed line is a TS curve and dotted line represents an R curve.

Situations 4. All curves are perfectly overlapped. No ME and recovery equals or very closely to 100%. Presumably this is just a theoretical possibility.

In some cases, a matrix can exhibit high heterogeneity from sample to sample, which can cause significant alterations in ME [36]. This situation must be also evaluated in method development and validation. The simple superposition of plot is useful to distinguish between each calibration curve. But in some cases statistical analysis must be performed to elucidate the variation. For slope variation values, a F-test is applied. If $F_{cal} < F_{tab}$, the F-test is not significant (5% significance level), and it can be considered that the variances are similar.

From statistical comparison for sulfamerazine analysis in liver using matrix-matched calibration curves prepared by 2 distinct extraction methods (PLE and US) we obtained $F_{cal} < F_{tab}$ (F_{cal} = 0.10 and F_{tab} = 12.22 (0.05, 1, 4)) which means that the variation difference between extraction methods (PLE and US) is not significant. The slope, intercept and respective variances of both curves were calculated by the ordinary least squares method. Based on the results it was possible to conclude that PLE and US extraction methods gave equivalent responses. In other words, matrix effects between these sample preparation methods are similar. In practical terms, it is possible to use a calibration curve prepared by US to quantify samples prepared by both methods or conversely.

Slope ratio and mathematical model for slopes comparison

Using the equations (1), (2) and (3), quantitative values for ME and recovery were obtained (see Table 1). The slopes obtained in the calibration curve using matrix-matched samples were compared with the values obtained with standards in pure solvent. Besides the equations, slope ratio was calculated for each pair of curves

to the 17 SAs included in the experiment. In this case, an acceptable range must be previously established. In the present study, a range from 0.9 to 1.1 was selected as lower and upper limits, respectively. The data were also demonstrated in Table 1.

As can be observed, using the equations (1), (2) and (3), ME was very high varying from 9.4 to 73.5 % to PLE method and from 8.5 to 76.2% when US method was applied. That means an ion suppression extension as high as 91.6% in the case of SIZ, for instance. In general terms, both PLE and US methods presented very intense and highly similar ME. The use of slope ratio with acceptance limits of 0.9-1.1 shows agreement with the data obtained using the equations: a extremely intense ME for both PLE and US methods and a high degree of agreement between ME produced by PLE and US methods. When slopes of R and TS curves, some analytes showed a ratio value between tolerance range indicating no significant difference between those curves. The only analyte that showed a selective behaviour was SCA, which was the sulfonamide that suffered less effects of the matrix.

Table 1. Relative and absolute recoveries (RE), matrix effect (ME) estimated using slopes data for PLE and US extraction methods for sulfonamides analysis in liver.

Quantitative estimation

Several degrees of ME were demonstrated, highlighting the huge variability among matrices. Depending on matrix nature, co-extractives can produce ion suppression or enhancement. For instance, Table 2 shows the quantitative ME data for some sulfonamides in fish and eggs. In the case of fish method, ME is present in the range of 30 to 40% of signal losses. RE and RE_R were in the ranges 25-41% and 46-79%, respectively. In other words, only ME can be responsible for approximately half of losses, if recovery will be considered as losses of extraction method plus losses by ion suppression. In the case of eggs, matrix is characterized by the intense presence of phospholipids, which are also related with a highly

intense ME, causing analytes losses from 95 to 97% [37]. However, in the case of sulfonamides in eggs, recoveries were satisfactory, from 80 to 102%. The selected cases demonstrate a common profile of ME (fish) besides an extreme case (eggs), but are useful to exemplify the co-existence between intense ME and high recovery values (>80%). The data corroborate that ME is independent of the recovery. For the eggs case, another extraction protocols should be evaluated, in order to remove the co-extractives.

Table 2. ME quantitative estimation for sulfonamides residues analysis in fish and eggs (n=3 for each value).

ME continuous monitoring using control charts

The estimation of the ME during a validation procedure is mandatory, because it is used for several matrices more or less distinct those used during validation. A method for ME behavior monitoring intra-batch and inter-batch will be always necessary. Moreover, monitoring itself is not the complete task: if ME are changing, QC samples must reflect this change and procedures to assess these modifications must be available to the analyst.

Using the same kind of QC samples (S, R and TS) in every batch, all necessary information about the method accuracy will be available at any moment, for any batch. Accuracy data for QC samples will provide data to build a control chart to monitor ME, recovery and method accuracy.

Control charts are very useful tools to monitor analytical methods behaviour. Over time, minor and major changes were naturally occurring in routine methods. Thus, ME could be suffering changes and method adjustments will be necessary to guarantee the adequate fitness to purpose. For ME monitoring, quality control samples were inserted in each analysis batch and the results were calculated according to the quantitative ME estimation.

Acceptance criteria follow those described in 2002/657/EC Commission Decision and Brazilian analytical quality assurance guidelines [15,38]. To evaluate accuracy obtained by routine data, a critical analysis of QC samples results is performed for every batch in accordance with limits showed in Table 3.

Table 3. Accuracy acceptability criteria.

QC samples data (n=3 by batch) were plotted on control charts and critically analyzed. Results for each routine analysis should be reviewed, and in case of non-compliance with criteria, it must be recorded. For a batch, if the review of QC samples shows non-compliances in relation to acceptance criteria, appropriate corrective actions must be taken. Table 4 and Figure 5 show an example for ME monitoring to a sulfonamide residue analysis in liver samples.

Table 4. Control chart for matrix effects monitoring along routine analysis for sulfadiazine determination in liver. Matrix effects were calculated using quality control samples results for each batch according to the equation (4).

Figure 5. Plot for matrix effects monitoring for sulfadiazine determination in liver. CL = central limit; UCL = upper control limit; LCL = lower control limit.

General remarks: matrix effect assessment in routine methods

Concisely, in our laboratory, the following workflow is used to evaluate, minimize and monitor ME:

i. In method development, qualitative and/or quantitative approaches for ME determination can be used for analytes and surrogates / internal standards.
ii. Extraction protocols can be modified or improved to avoid co-extraction of matrix compounds.
iii. Once ME is observed, the post-infusion protocol is used to determine in what chromatogram region the suppression occurs.

iv. Mobile phase gradient and/or additives can be modified in order to provide analytes elution in a region with absence of suppression.
v. Extract injection volume can be reduced in order to decrease ME, except when the analyte responses does reduce in the same extent.
vi. Evaluate the effect of surrogate/internal standards in the correction of ME.
vii. If even with these changes, ME still remain relevant, the magnitude of the effect can be monitored during routine analysis, using an accuracy control chart.

In validation data, a differentiation between recovery and ME must be clear. In mass spectrometry analysis, recovery can be deeply affected by ME although the sample preparation process showed high efficiency. As ME directly affects the yield of analytes ionization, method overall recovery have a correlation with the ME extension. In the present work, we refer to the IUPAC's recovery concept, which is the analyte yield obtained after the extraction procedure [39]. The "apparent recovery", according to IUPAC, is the degree of agreement between the nominated and calculated concentration. We use the term "relative recovery" to express the recovery value discounting the ME. Relative recovery represents the analyte losses caused only by the extraction procedure. Thus, this term should not be confused with the term "apparent recovery".

Summarizing, recovery includes losses of target compounds throughout the whole sample preparation process (extraction, concentration, derivatization, etc) plus the eventual ME. RE_R is the loss of analytes caused by the sample preparation but not include the ME.

Several approaches were considered to ME evaluation. Clearly, the methods that can be used before the method validation are more useful. The obtained data can be used to make changes or adjustments in the extraction and/or chromatography conditions to avoid or minimize the impact of ion suppression/enhancement. Once adequate conditions were established, remaining ME could be estimate using a

simple approach as those based in calibration curves or QC samples. If ME is relevant in a routine method, ME could be monitored using a control chart in order to detect advance changes in method behaviour.

Conclusions

ME is a very frequent issue in bioanalytical methods, especially in LC-MS and LC-MS/MS based methods. Despite the fact that currently there are no established acceptable limits for ME, it is a consensus that their magnitude must be estimated and, if possible, minimized. Thus, analytes extraction procedures and/or chromatographic conditions changes could be carried out. In literature, several approaches to estimate ME were reported. Herein, we report our experience with ME estimation, minimization and continuous monitoring, applying several ME estimation strategies for analytical methods which are used in routine analysis in our laboratory. Each technique was discussed and their advantages and/or drawbacks were appointed, in order to provide a practical guide for researchers interested in assessment of ME.

Acknowledgements

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Table

Table 1. Relative (RE_R) and absolute recoveries (RE), matrix effect (ME) estimated using slopes data for PLE and USE extraction methods for sulfonamides analysis in liver.

	SMR	SMZ	SMA	SMPZ	SDZ	SPY	SDMX	SGA	SCA	SBZ	SIM	SMTZ	SQX	STZ	SIZ	SDX	N4-SMR
PLE																	
RE (%)	8.8	8.1	4.7	7.6	6.0	7.8	8.3	4.2	25.2	1.5	9.2	3.0	5.0	6.7	3.0	11.7	13.9
ME (%)	23.9	25.7	13.5	28.7	16.4	24.1	29.3	12.9	73.5	6.7	30.8	19.2	20.0	11.3	9.4	34.7	24.5
RE_R (%)	36.7	31.5	35.1	26.5	36.5	32.6	28.2	32.7	34.4	23.0	29.8	15.5	25.3	59.2	31.5	33.7	56.8
USE																	
RE (%)	13.0	14.2	7.7	13.2	11.6	15.8	12.8	5.1	33.5	2.4	15.1	4.5	8.7	8.1	3.9	19.6	16.6
ME (%)	21.7	23.6	13.5	23.9	20.3	28.0	24.8	16.5	76.2	4.4	25.0	20.8	17.8	23.0	8.5	32.2	25.8
RE_R (%)	59.9	60.1	57.2	55.3	57.4	56.5	51.6	31.2	43.9	54.1	60.3	21.7	48.9	35.4	46.3	60.7	64.3
PLE slope ratio																	
TS/S	0.24	0.26	0.13	0.29	0.16	0.24	0.29	0.13	0.73	0.07	0.31	0.19	0.20	0.11	0.09	0.35	0.25
R/S	0.09	0.08	0.05	0.08	0.06	0.08	0.08	0.04	0.25	0.02	0.09	0.03	0.05	0.07	0.03	0.12	0.14
R/TS	0.37	0.31	0.35	0.26	0.37	0.33	0.28	0.33	0.34	0.23	0.30	0.16	0.25	0.59	0.32	0.34	0.57
USE slope ratio																	
TS/S	0.22	0.24	0.13	0.24	0.20	0.28	0.25	0.16	0.76	0.04	0.25	0.21	0.18	0.23	0.09	0.32	0.26
R/S	0.13	0.14	0.08	0.13	0.12	0.16	0.13	0.05	0.33	0.02	0.15	0.05	0.09	0.08	0.04	0.20	0.17
R/TS	0.60	0.60	0.57	0.55	0.57	0.56	0.52	0.31	0.44	0.54	0.60	0.22	0.49	0.35	0.46	0.61	0.64
Ratio USE/PLE																	
R/R	1.49	1.75	1.63	1.74	1.94	2.02	1.55	1.22	1.33	1.55	1.64	1.51	1.73	1.22	1.33	1.67	1.19
TS/TS	0.91	0.92	1.00	0.83	1.24	1.16	0.85	1.28	1.04	0.66	0.81	1.08	0.89	2.04	0.91	0.93	1.05

Table

Table 2. ME quantitative estimation for sulfonamides residue analysis in fish and eggs (n=3 for each value).

		Sulfonamides in fish (peak area)			Sulfonamides in eggs (peak area)		
	Sample type	SMR	SMZ	SMA	STZ	SMZ	SQX
A	Standard in solvent	6,43E+05	4,09E+05	4,88E+05	7.01E+06	3.39E+06	7.56E+06
B	Matrix-matched	3,46E+05	2,56E+05	2,53E+05	2.56E+05	1.29E+05	2.49E+05
C	Spiked sample	1,59E+05	1,66E+05	2,00E+05	2.14E+05	1.22E+05	2.19E+05
	Equation						
	ME(%) = (B/A x 100)-100	-46	-37	-48	-96	-96	-97
	RE_R(%) = C/B x 100	46	65	79	84	95	88
	RE(%) = (ME x R_R)/100	25	41	41	3	4	3

ME = matrix effects; RE = Recovery; RE_R = Relative recovery.

Table

Table 3. Accuracy acceptability criteria.

Concentration level	Range
≤ 1 μg Kg^{-1}	-50 a +20%
> 1 μg Kg^{-1} a < 10 μg Kg^{-1}	-30 a +10%
≥ 10 μg Kg^{-1}	-20 a +10%

Table 4. Example of a control chart for matrix effects (ME) monitoring along routine analysis for sulfadiazine determination in liver. ME were calculated using quality control samples results for each batch according to the equation (4).

Average (n = 20 batches) ME data for SDZ (in %)	
Central limit (CL)	46.4
Average standard deviation	2.6
Lower control limit (LCL)	38.5
Upper control limit (UCL)	54.3

Figure

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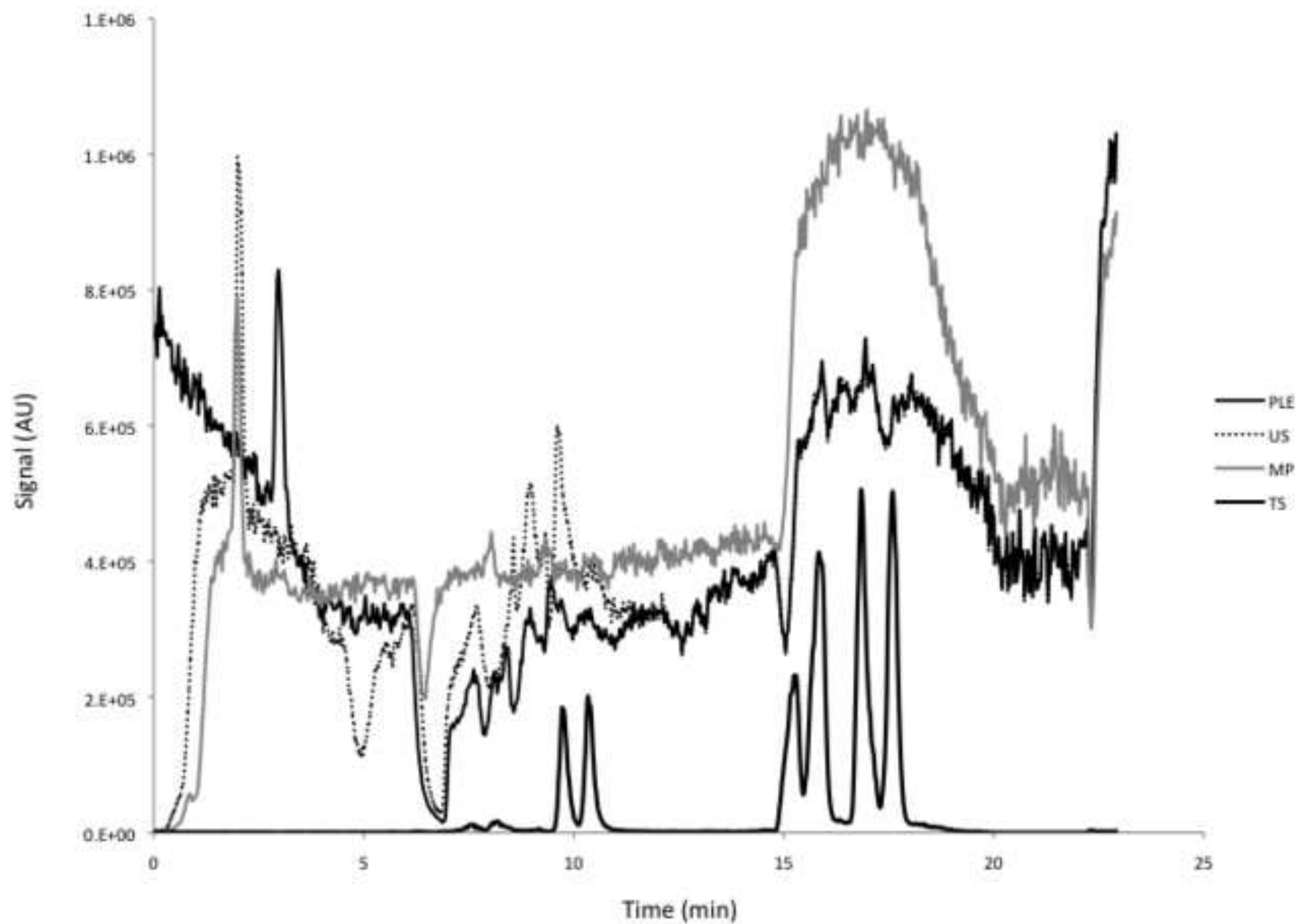

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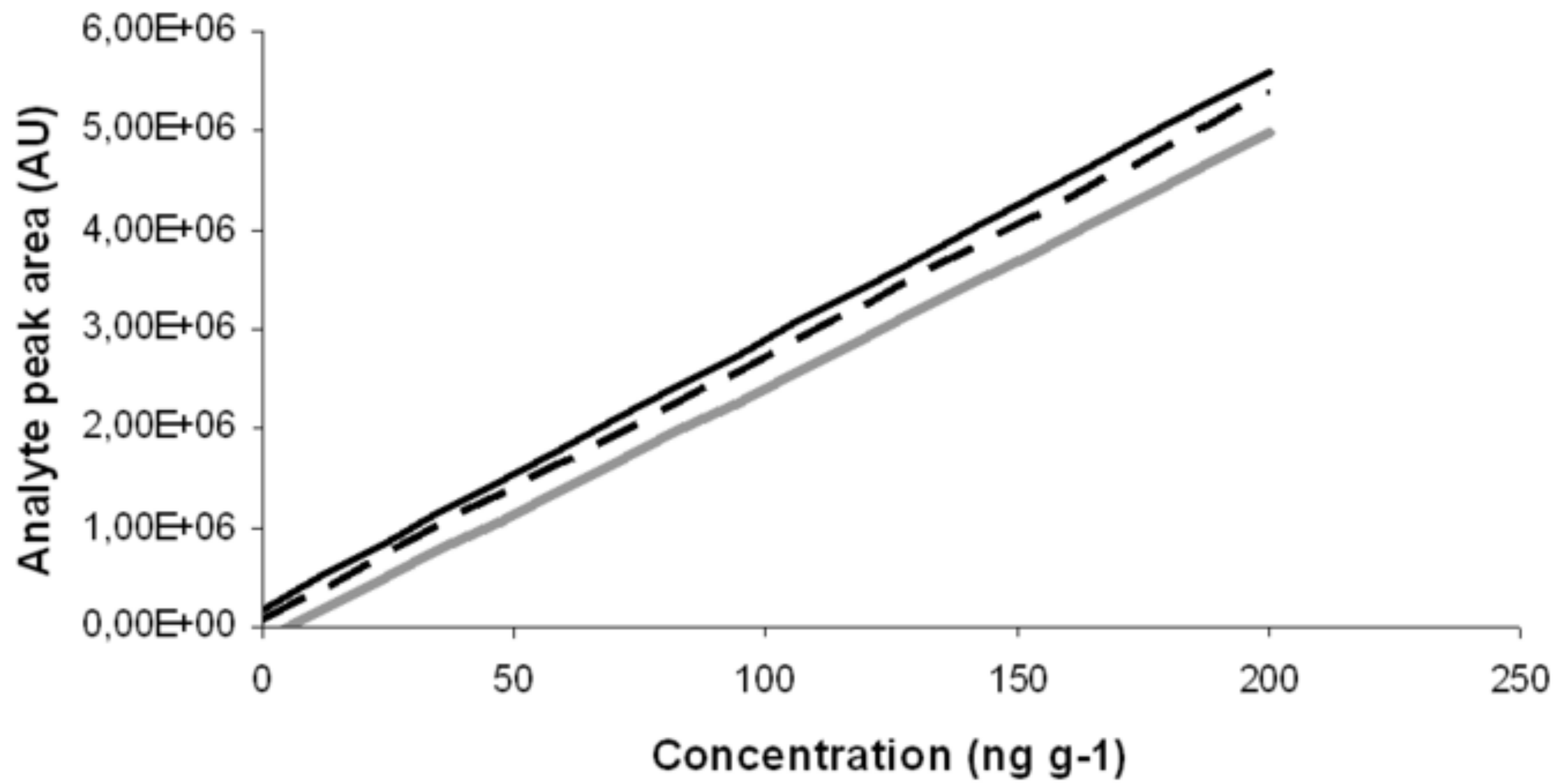

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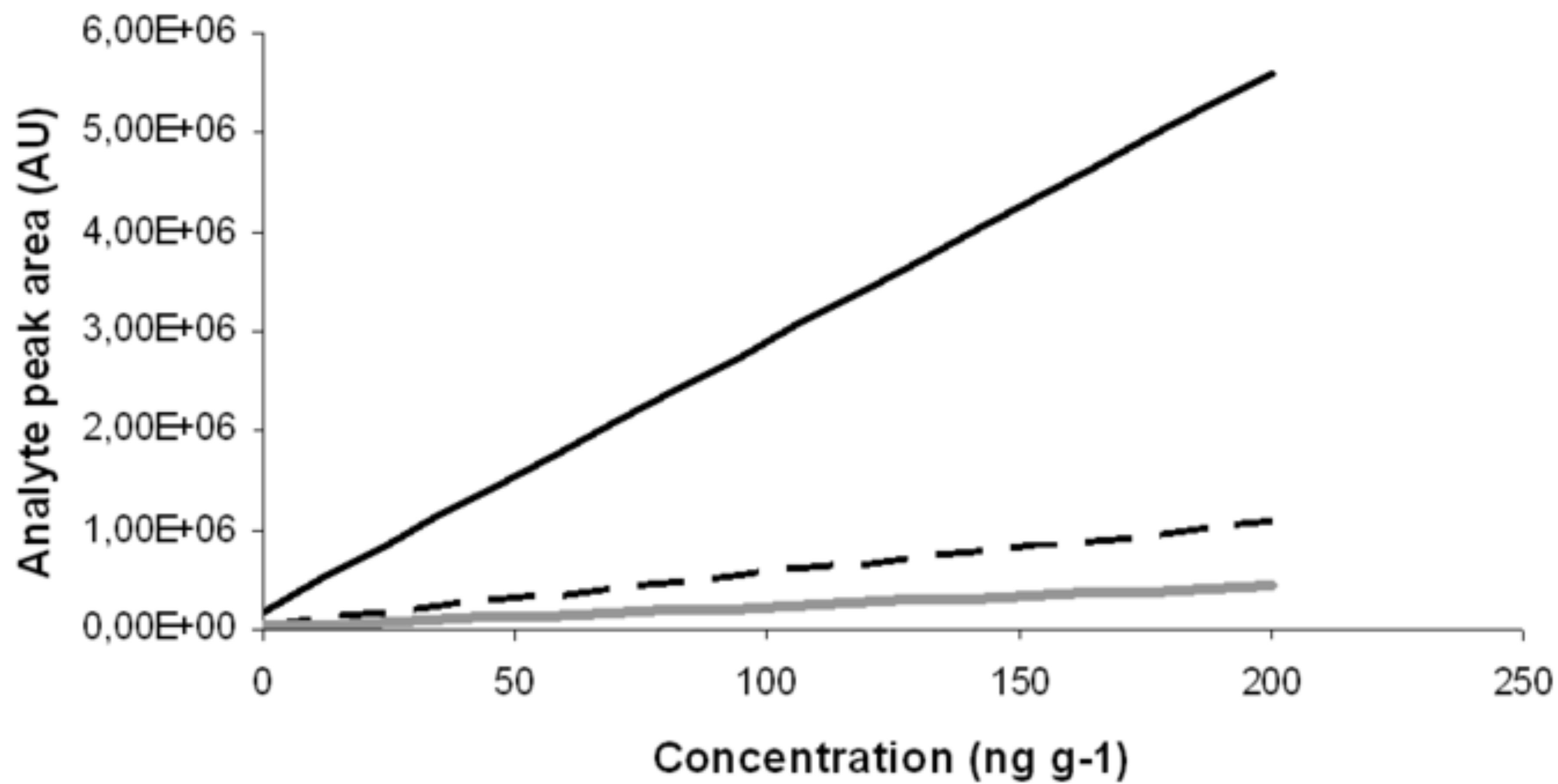

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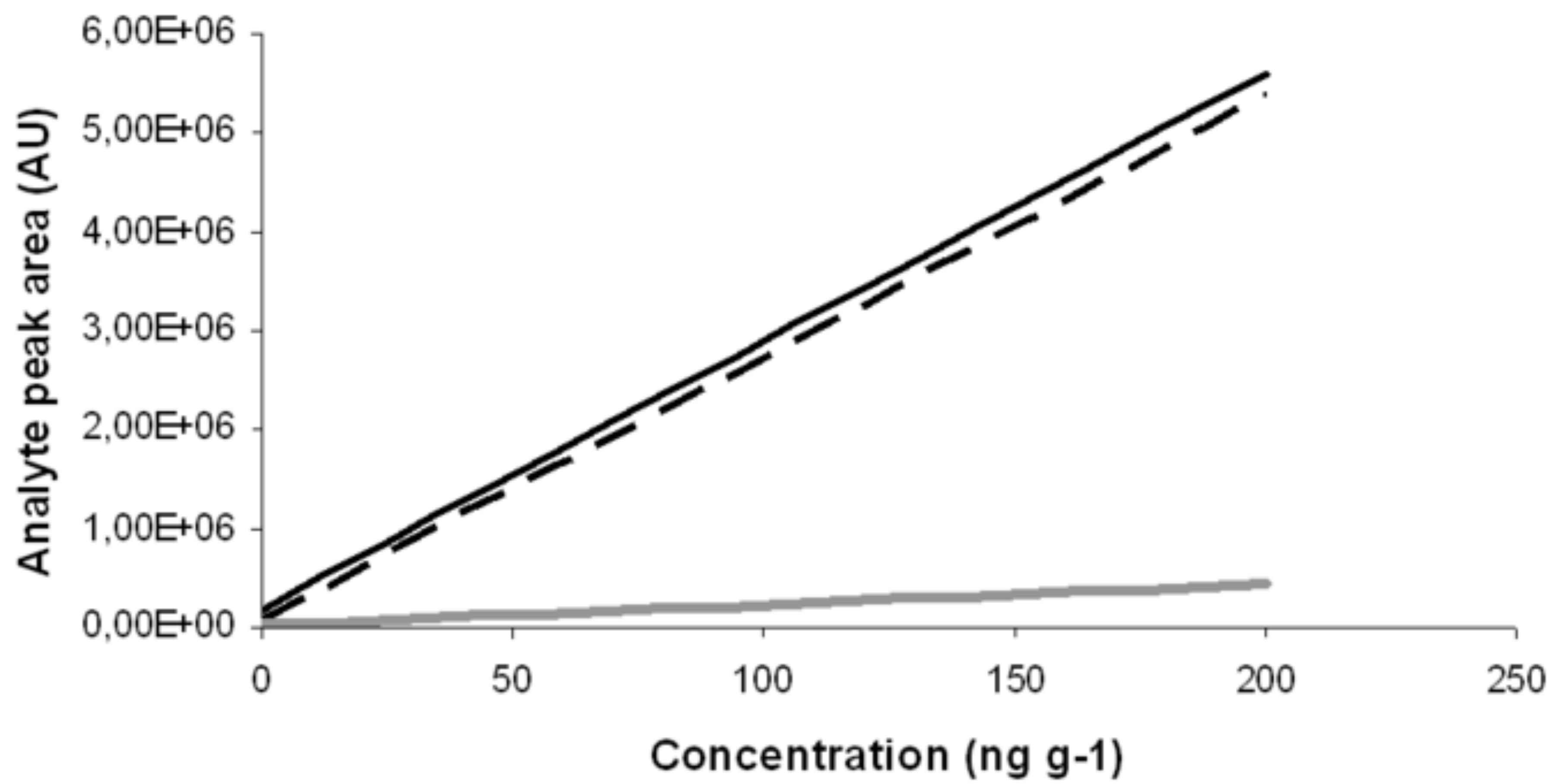

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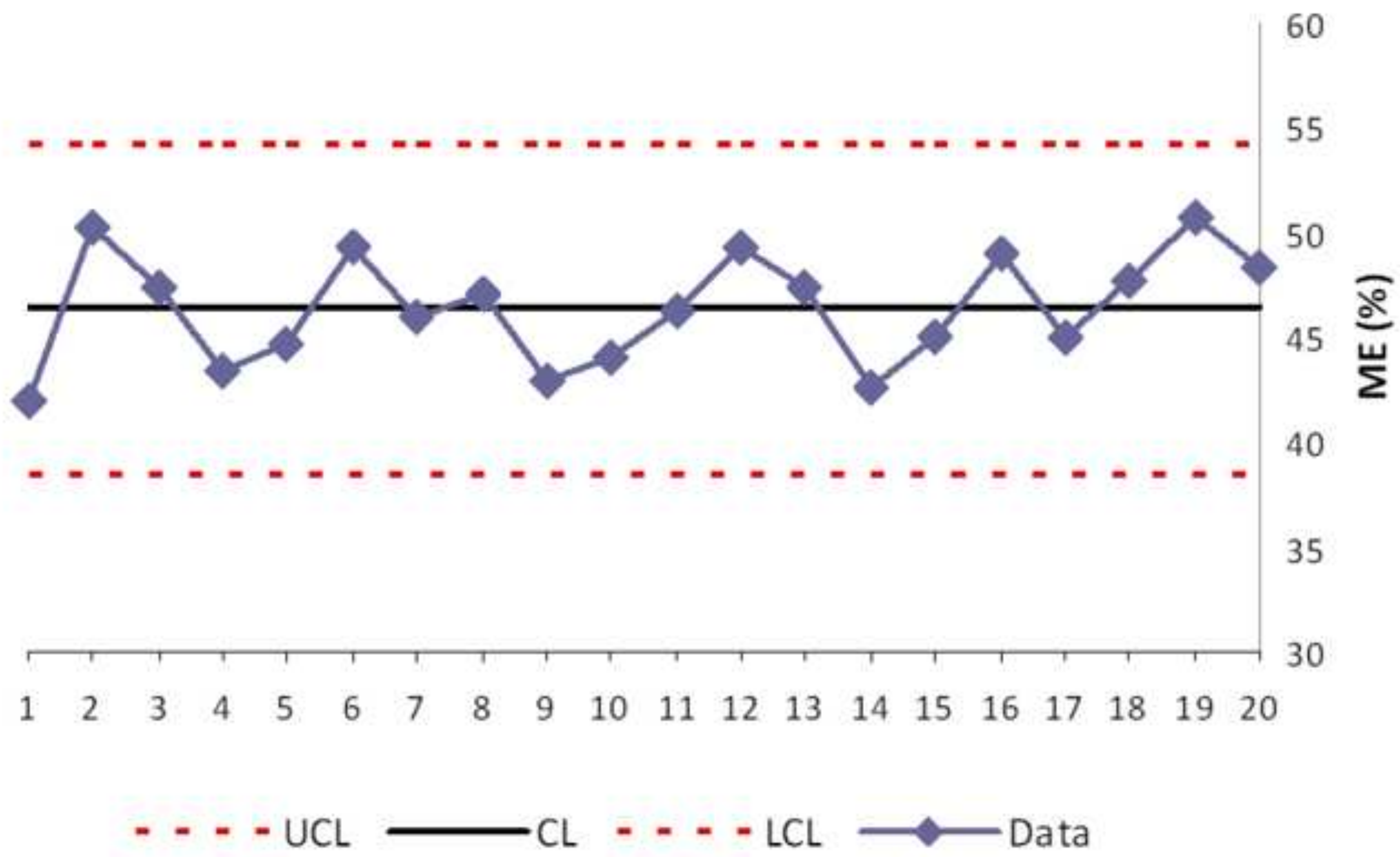

Anexo V – Artigo publicado em *Analytical Chemistry*: **Structural elucidation of sulfaquinoxaline metabolism products in poultry, swine, bovine, equine and fish using high-resolution Orbitrap mass spectrometry**

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Structural Elucidation of Sulfaquinoxaline Metabolism Products and Their Occurrence in Biological Samples Using High-Resolution Orbitrap Mass Spectrometry

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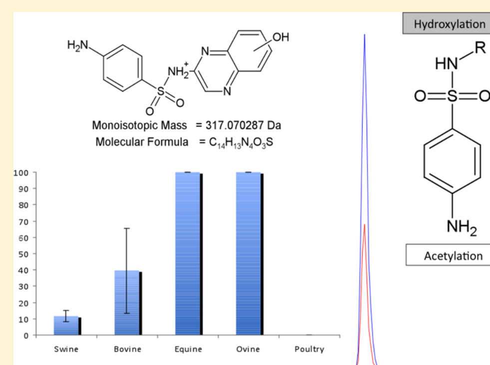
Supporting Information

ABSTRACT: Four previously unreported metabolism products of sulfaquinoxaline (SQX), a widely used veterinary medicine, were isolated and analyzed using liquid chromatography coupled to high-resolution Orbitrap mass spectrometry. Metabolites were structurally elucidated, and a fragmentation pathway was proposed. The combination of high-resolution MS^2 spectra, linear ion trap MS^2, in-source collision-induced dissociation (CID) fragmentation, and photolysis were used to analyze SQX and its metabolites. All metabolism products identified showed a similar fragmentation pattern to that of the original drug. Differential product ions were produced at *m*/*z* 162 and 253 which contain the radical moiety with more 16 Da units than sulfaquinoxaline. This occurs by a hydroxyl attachment to the quinoxaline moiety. With the exception of two low-intensity compounds, all the mass errors were below 5.0 ppm. The distribution of these metabolites in some animal species are also presented and discussed.



Sulfonamides are a widely used class of antibacterial compounds. Since their introduction in human and veterinary medicine in the 1940s, a thousand of sulfonamides were synthesized and evaluated.[1] One of the first compounds introduced to prevent and treat coccidiosis, an important disease for poultry, swine, and ovine, was sulfaquinoxaline (SQX).[2] By decades, SQX was the most important coccidiostatic drug, and its use promoted an impressive decline in the poultry production costs.[3] However, the incorrect use of SQX, as other sulfonamides, can lead to serious problems as permanence of residues of the drug and their metabolism products in food or the spread of bacterial resistance.[4−7] For this reason, residues of sulfonamides have maximum residues limits (MRL) established in several countries. In Brazil, SQX is still commonly used to prevent and treat poultry, swine, and ovine coccidiosis.[8] As Brazil is one of the most important poultry producers in the world, the continuous monitoring of sulfonamide residues in food of animal origin is a great concern.[9] For sulfonamides, Brazil adopts an MRL of 100 mg kg^{-1}. That value comprehends the sum of sulfonamides and their metabolism products.[10,11] Thus, the knowledge about the SQX metabolism is an issue of concern for regulatory limits and public health. More recently, the concern about the fate of veterinary drugs into environment has gained importance.[12−16] Commonly, manure of medicated animals is used in agriculture as fertilizer. Therefore, the metabolism and degradation process of these compounds should be investigated in order to determine their possible impacts over the environment.[17−19]

Generally, residues of sulfonamides can be analyzed in biological and environmental samples using several techniques, as liquid chromatography, bioactivity-based assays, and capillary electrophoresis among others.[20−25] However, due to high specificity and selectivity, hyphenated methods based in mass spectrometry detection are the most applied approach to determine sulfonamides residues in low concentrations in biological or environmental matrixes.[26]

Mass spectrometry is a very useful tool, not just for qualitative and quantitative analysis, but also for elucidating metabolism and/or degradation product structures.[27,28] Some

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sulfonamides have a well-known metabolism, as sulfamethoxazole and sulfadiazine.[29−31] In sulfamethoxazole metabolism, the two major generated pathways are acetylation and oxidation in the N^4 nitrogen, which produces N^4-acetyl-SMA and N^4-hydroxy-SMA. This last is responsible by the undesirable effects that occur in long-term treatment for pneumonia caused by *Pneumocystis carinii* in HIV-infected patients. Hydroxylation also occurs in the methyl group C^5, producing the 5-methylhydroxy-SMA and the corresponding acetylated derivative. Glucuronic conjugation in N^1 is also observed.[32,33]

Sulfonamides metabolism is highly species-dependent. For instance, pigs are unable to form N^1-glucuronides of sulfadimethoxine and sulfamethomidine, while humans do. The double-conjugate N^4-acetylsulphapyridine-*O*-glucuronide is produced by human metabolism but not by pigs. Sulfadiazine is normally acetylated in both human and pig metabolism.[33] Figure 1 summarizes the major described pathways of sulfonamides metabolism.

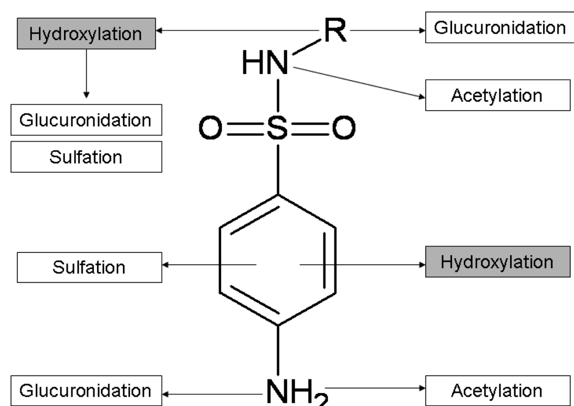

Figure 1. Major metabolism pathways reported for sulfonamides. Gray boxes represent redox metabolism, and white boxes represent conjugation metabolism.

Although some sulfonamide metabolisms were deeply studied, just few reports about SQX metabolism products have been reported.[34−37] Just after its introduction in therapeutics in 1944, SQX was discarded for human use due to the precipitation of metabolism products in primate kidneys.[2] Generally, it was established that one of the major metabolism pathways to SQX elimination is the acetylation.[36] On the other hand, in an experiment with rabbits performed by Eppel and Thiessen, in which SQX and N^4-acetyl-SQX were administered to those animals, results show that rabbits are able to promote deacetylation of the metabolite, reverting back to SQX.[38]

In a previous work, we demonstrated the in vivo and in vitro formation of a hydroxylated metabolite, probably mediated by microsomal CYP P450 enzyme complex. This compound, tentatively named hydroxyl-SQX (SQX−OH), shows a very distinct production profile between the species.[39] Equine liver is able to promote hydroxylation of SQX in quantitative proportion while poultry liver demonstrates an absence of that hydroxylation activity. Pigs and cattle show a highly heterogeneous profile of SQX hydroxylation, with a range varying from 8% to 84%.[39]

In the present work, several mass spectrometry techniques were associated with elucidating the molecular structure of the SQX−OH and derivative metabolites. Four previously nondescribed metabolism products of SQX were identified and characterized. Occurrence of these metabolites in distinct species, including species potentially exposed to SQX at environmental levels, is investigated and discussed.

■ EXPERIMENTAL SECTION

Chemical and Reagents. 4-Amino-*N*-2-quinoxalinyl-benzenesulfonamide or sulfaquinoxaline (SQX, CAS 59-40-5) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Water, acetonitrile (ACN), methanol (MeOH), hexane, and acetone of HPLC-grade were supplied by J. T. Baker (Deventer, The Netherlands). Ethyl acetate was from Merck (Darmstadt, Germany). Formic and acetic acid were obtained from Sigma-Aldrich.

SQX stock solution was prepared in MeOH/acetone (50:50) at 1 mg mL^{-1} and stored at −4 °C until use. Working solutions at several concentration levels were prepared by stock solution dilutions using MeOH or water/ACN (85:15).

Photodegradation Experiments. The photodegradation experiments were conducted under simulated solar irradiation conditions in a Suntest CPS simulator (Heraeus, Hanau, Germany). The system was equipped with a xenon arc lamp and appropriate glass filters to restrict the transmission of irradiation wavelengths below 290 nm, giving a wavelength spectrum closely resembling solar light. The lamp intensity was adjusted to an irradiance of 500 W m^{-2} corresponding to a light dose of 1800 kJ m^{-2} h^{-1}. The samples irradiated in the Suntest apparatus were contained in crimp-cap 20 mL quartz vials. After 2 h the solutions were withdrawn and frozen immediately.

HPLC−QqLIT-MS Analysis. High-performance liquid chromatography (HPLC) separation was performed with a Symbiosis Pico System (Spark Holland, Emmen, The Netherlands), equipped with an HPLC system consisting of an Alias autosampler, a loop injector, and two binary pumps with a four-channel solvent selector for each one. Chromatographic separation was performed using a Purospher STAR LC column (C18, end-capped, 150 mm × 4.6 mm, 5 μm) preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL min^{-1}, eluent A being HPLC grade water acidified with 10 mM of formic acid, and eluent B ACN with 10 mM of formic acid. The elution gradient started with 25% of eluent B, increasing to 80% in 10 min, and to 100% in 11 min. During the next 2 min the column was kept at 100% B, readjusted to the initial conditions in 3 min, and equilibrated for 7 min. Tandem mass spectrometry (MS/MS) analyses were carried out in a 4000 QTRAP hybrid triple quadrupole−linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) equipped with a turbospray ionization source (ESI) working in the positive mode (ESI+). The injection volume was 10 μL. All data were processed by the software Analyst version 1.4.2 (Applied Biosystems). For parent ion scans, precursor ion scans, and analysis in single reaction monitoring mode (SRM), a liquid chromatograph coupled to a tandem mass spectrometer with electrospray ionization source was used, according to a previously developed and validated method for sulfonamide analysis published elsewhere.[20,39]

UPLC−Orbitrap-MS. Exact mass determination experiments were performed in an Orbitrap Q Exactive (Thermo-Fisher, San Jose, CA) coupled to a Waters Acquity ultra-performance LC (UPLC) system (Waters, Manchester, U.K.). The parameters of the electrospray ionization source were adjusted as follows: polarity (+), spray voltage +4.0 kV, heater temperature 300 °C, and capillary temperature 350 °C. The

selected ion monitoring (SIM) resolution was 70 000, and the dd-MS^2 resolution was 35 000. Chromatographic separation was performed on a Merck Hibar HR 30-2.1 UPLC column with a Purospher STAR RP-18 end-capped cartridge (C18 end-capped, 30 mm × 2.1 mm, 2.0 μm) (Merck). The mobile phase was composed by (A) acetonitrile with 0.1% formic acid and (B) water with 0.1% formic acid. A gradient mode was used as following: 0 min (15% A) to 2 min (15% A) to 6 min (90% A), to 9 min (90% A) to 10 min (15% A) and stabilizing until 12 min. The flow rate was 200 μL min^{-1} with the column at room temperature. The injection volume was 10 μL.

Sample Preparation. Samples of ovine, equine, and poultry liver were obtained from Brazilian Federal Inspection Services (SIF), the national food inspection service managed by the Brazilian Ministry of Agriculture, collected in several slaughterhouses and meat plants. Fish samples (*Astyanax eigenmanniorum*) were collected in a creek located inside a poultry farm in southern Brazil. Samplings were performed at the place of the creek closest to the broilers' production houses (≅200 m). Fish (n = 7) showed an average length of 13.0 cm. Scales were removed, and whole fishes were chopped and homogenized in a pool. All samples were freeze-dried (−50 °C, 0.044 bar vacuum) and kept at −30 °C until analysis. Extraction was performed with ACN. To an aliquot of 0.5 g of sample, 10 mL of ACN was added and tubes were mixed in a mechanical vortex by approximately 10 s. After that, all samples were placed into an ultrasonic bath by 60 min. After the extraction time, samples were stored in freezer (−18 °C) by 1 h to promote protein precipitation. Then, samples were centrifuged at 3500 rpm for 10 min. Supernatants were evaporated at 40 °C under nitrogen flow until dryness. The extracts were dissolved in 2.0 mL of mobile phase mixture (water/ACN, 85:15). An aliquot of 2 mL of hexane was added to remove the fat content, and the tubes were shaken in a vortex by approximately 5 s followed by centrifugation (3500 rpm for 10 min). The lower layers were carefully transferred to an HPLC vial.

In Vitro Production of Hydroxylated Metabolite. A whole equine liver (≅700 g) was chopped and homogenized using a mixer. A commercial SQX preparation (Coccifin, one sachet with 100 g with 25% of SQX) was dissolved in sterile saline solution to a concentration of approximately 25 mg mL^{-1}, and this solution (≅1000 mL) was added to the tissue. After homogenization (30 min in an orbital mixer) the mixture was placed in room temperature by 4 h. Following, the liver extract was centrifuged (3500 rpm for 10 min) and the supernatant was transferred to a clean flask. The pH was adjusted to 5.2 using hydrochloric acid 0.1 M. At this pH value, sulfonamides are generally neutral molecules, enabling the extraction with organic solvents. This extract was submitted to a liquid−liquid extraction with ethyl acetate, and the organic phase was dried through filtration with anhydrous sodium sulfate. SQX and its possible metabolites were precipitated from this solution by slow addition of hexane. The extract was centrifuged, and the precipitate was freeze-dried.

■ RESULTS AND DISCUSSION

Sulfonamides Fragmentation Profile. Under MS/MS conditions sulfonamides gave generic product ions at m/z 156, 108, and 92. The exceptions are compounds with radicals attached to the aniline moiety. Generally, sulfonamides differ only in the heterocyclic base attached to the sulfonamide moiety. Ions at m/z 108 and 92 are most likely formed via m/z 156 ions and/or directly from the protonated molecule.[40] Formation of these ions involves rearrangements and more energetic C−S cleavages. Thus, the necessary collision energies are higher but virtually identical for all sulfonamides.[41]

Similarly, N^4-acetylated sulfonamide metabolites show a number of group-specific product ions which indicates an identical fragmentation pattern as compared to those of the compounds, i.e., at m/z 134 (92 + 42) and 198 (156 + 42), together with characteristic compound-specific product ions.[41] For instance, the m/z 145 could be chosen for confirmation of SQX and N^4-acetylsulfaquinoxaline as compound-specific ion and m/z 134 as the corresponding N^4-acetylated group-specific fragment.[40]

Thus, structural information could be obtained by monitoring the radical-specific fragments ions (R-NH3) formed from the N-heterocyclic base moieties. Volmer showed that the intensity of the R-NH$_3$ ions increased with the basicity of the N-heterocyclic moiety, probably due to the increasing proton affinity of the protonated amine fragments with increasing number of electron-donating methyl groups.[41]

On the basis of our previous report and in the fragmentation pattern for sulfonamides, expected fragments could be indicated. Figure 2 shows the expected fragmentation profile for SQX. See the Supporting Information (Figures S-1−S-5 to fragmentation profile for all metabolites included in the present study).

Metabolite Prediction. A database for 98 possible SQX metabolites was elaborated and applied for all tissues extracts (see the Supporting Information). In the case of glucuronic conjugates, a neutral loss experiment was associated with the full scan, product ion, and precursor ion experiments. When a compound is conjugated with glucuronic acid, a mass shift of 176 Da can be verified producing [M + H + 176]$^+$. Sodium adducts of glucuronic conjugates are also common producing [M + H + 176 + 23]$^+$. The neutral losses of 176 and 199 were monitored. Gluc−SQX, Gluc−SQX−Na, Gluc−SQX−OH, and Gluc−SQX−OH−Na were expected to present m/z values of 477, 500, 493, and 516, respectively. However, no detectable amounts of any glucuronic conjugates were confirmed.

Metabolite Structure Elucidation. The previously proposed structure for SQX−OH was not accompanied for sufficient data to appoint the exactly site of hydroxyl addition in quinoxaline moiety.[39] Taking this into consideration, Orbitrap and multiple mass spectrometry techniques have been used for predicted structural characterization for SQX−OH. The detected SQX metabolites were elucidated on the basis of (1) the mass shifts from the parent molecule, (2) molecular formulas derived from the accurate mass measurements, (3) interpretation of accurate MS/MS spectra, and (4) fragmentation of the radical moiety, produced by photolysis and confirmed using in-source fragmentation. Five compounds were detected and identified. The first one (SQX−OH) with experimental exact mass measurement of m/z 317.0699 indicated a protonated molecule of $C_{14}H_{13}N_4O_3S$, showing that one oxygen atom had been introduced into SQX molecule ($C_{14}H_{13}N_4O_2S$, protonated molecular ion) by forming a hydroxyl derivative. The identity of this metabolite was further confirmed by the appearance of the characteristic fragment at m/z 162.0663 ($C_8H_8N_3O$, protonated molecule) with −0.7 ppm mass accuracy. Double-bond equivalent (DBE) data (see Table 1) is also consistent with the proposed structure. The second one (N^4-acetyl-SQX, m/z 343; $C_{16}H_{15}N_4O_3S$, protonated molecule) is a well-known metabolite of SQX described elsewhere.

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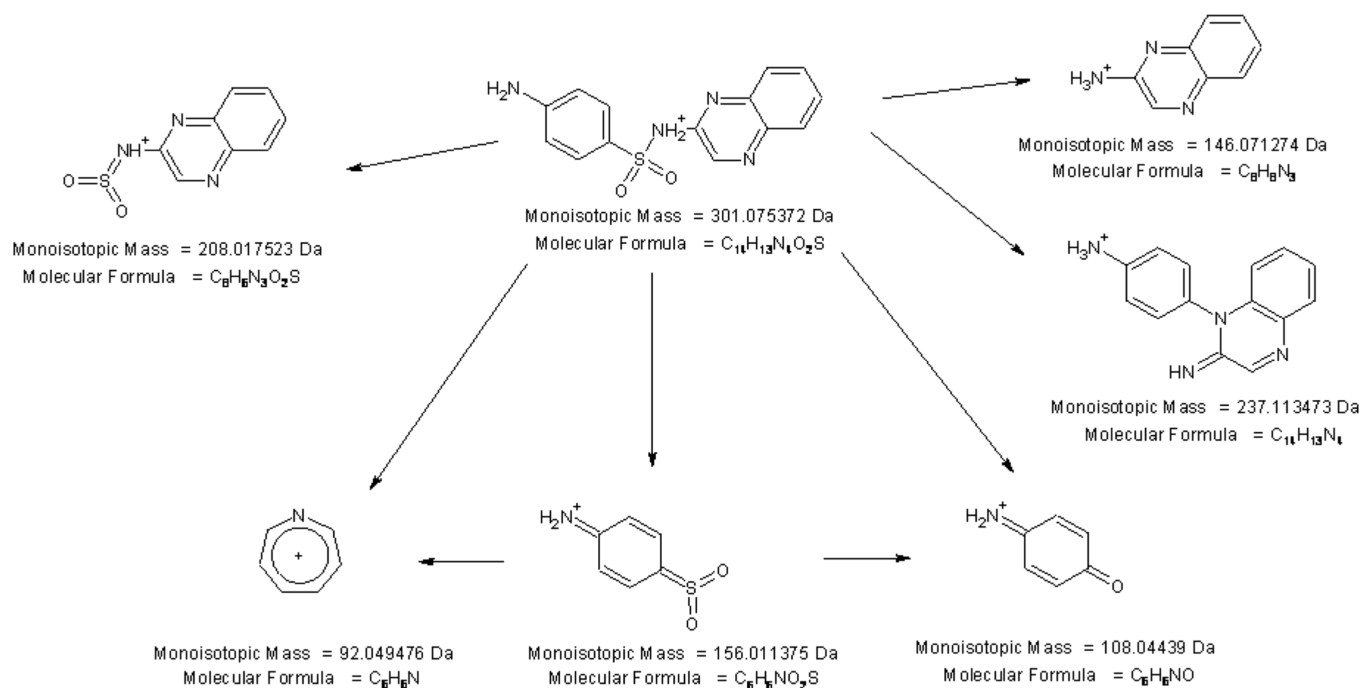

Figure 2. Fragmentation pattern for SQX.

Table 1. SQX and Transformation Products Exact Mass Measurement Parameters

compd	theor exact mass	exptl exact mass	error ppm	DBE[a]	molecular formula
SQX	301.075 372	301.0745	−2.9	10	$C_{14}H_{13}N_4O_2S$
N^4-acetyl-SQX	343.085 937	343.0853	−1.8	11	$C_{16}H_{15}N_4O_3S$
SQX−OH	317.070 287	317.0699	−1.2	10	$C_{14}H_{13}N_4O_3S$
N^4-acetyl-SQX−OH	359.080 851	359.0805	−1.0	11	$C_{16}H_{15}N_4O_4S$
N^4-formyl-SQX	329.070 287	329.0648	−16	11	$C_{15}H_{13}N_4O_3S$
N^4-formyl-SQX−OH	345.065 201	345.0781	37	11	$C_{15}H_{13}N_4O_4S$
SQX−OH fragment	162.066 188	162.0663	−0.7	6.5	$C_8H_8N_3O$

[a]DBE: double-bond equivalent.

The third compound was assigned as the N^4-acetyl derivative of SQX−OH. This compound shows the fragment m/z 162, assigned as the 2-aminoquinoxaline moiety with a hydroxyl added, and also shows the common fragments predicted to N^4-acetyl-sulfonamides (m/z 134 and 198). Other two possible N^4-formyl metabolites of SQX and SQX−OH were also considered. However, for these compounds, the mass error appears to be unsatisfactorily high to establish these structures. Although N^4-formyl derivatives of sulfonamides have been described in literature, the intensity of these two compounds was very low and only detected in poultry liver samples.[42,43] We suggested that these presumable N^4-formyl derivatives could be also transformation products of N^4-acetyl metabolites. Supporting Information Figure S-6 shows the isotope pattern simulation of SQX−OH and the experimental isotope pattern, respectively, which are in total agreement. In Supporting Information Figure S-7 the experimental isotope pattern for the SQX−OH specific fragment m/z 162 is represented.

Substructure-Specific Fragmentation. Substructure-specific fragmentation is a very useful tool to elucidate the site of modifications in sulfonamides.[43,44] In order to investigate the exact position of the hydroxyl added to the 2-aminoquinoxaline radical of SQX, the radical-dependent fragments (m/z 162 and 253) were tentatively obtained based on described SQX photolysis products.[45] A solution of the semipurified metabolite was submitted to a photolysis experiment. A solution of 1 mg mL^{-1} of SQX + SQX−OH was diluted with water/acetone (1:1) to produce a solution of 100 μg mL^{-1}. This solution (10−15 mL) was submitted to photodegradation experiments as described before. After that, aliquots of 100 μL were taken, diluted with a mixture of water/ACN (75:25) to a volume of 1.5 mL and analyzed by LC−MS/MS. The experiments were carried out in triplicate. A standard solution of SQX (analytical standard) was analyzed in parallel as a control. SQX standard showed a peak corresponding to m/z 145 and 237, as expected. For the semipurified metabolite, peaks corresponding to m/z 145, 162, 237, and 253 were observed. The SQX−OH specific molecular ions were fragmented, and the result spectrum was obtained. In order to confirm the substructure-specific fragment m/z 162, a method including in-source collision-induced dissociation (CID) application was designed. A voltage of 35.0 eV was applied, and the semipurified metabolite was injected. This procedure permits the fragmentation of compounds before their introduction in the mass spectrometer. Thus, it is possible to use Orbitrap to emulate MS^3 experiments but with the advantage of the high-resolution mass detection. As expected, an intense fragment for 2-hydroxy-aminoquinoxaline was observed. The fragmentation pattern was in agreement with the photolysis products analysis. The MS/MS spectrum of this

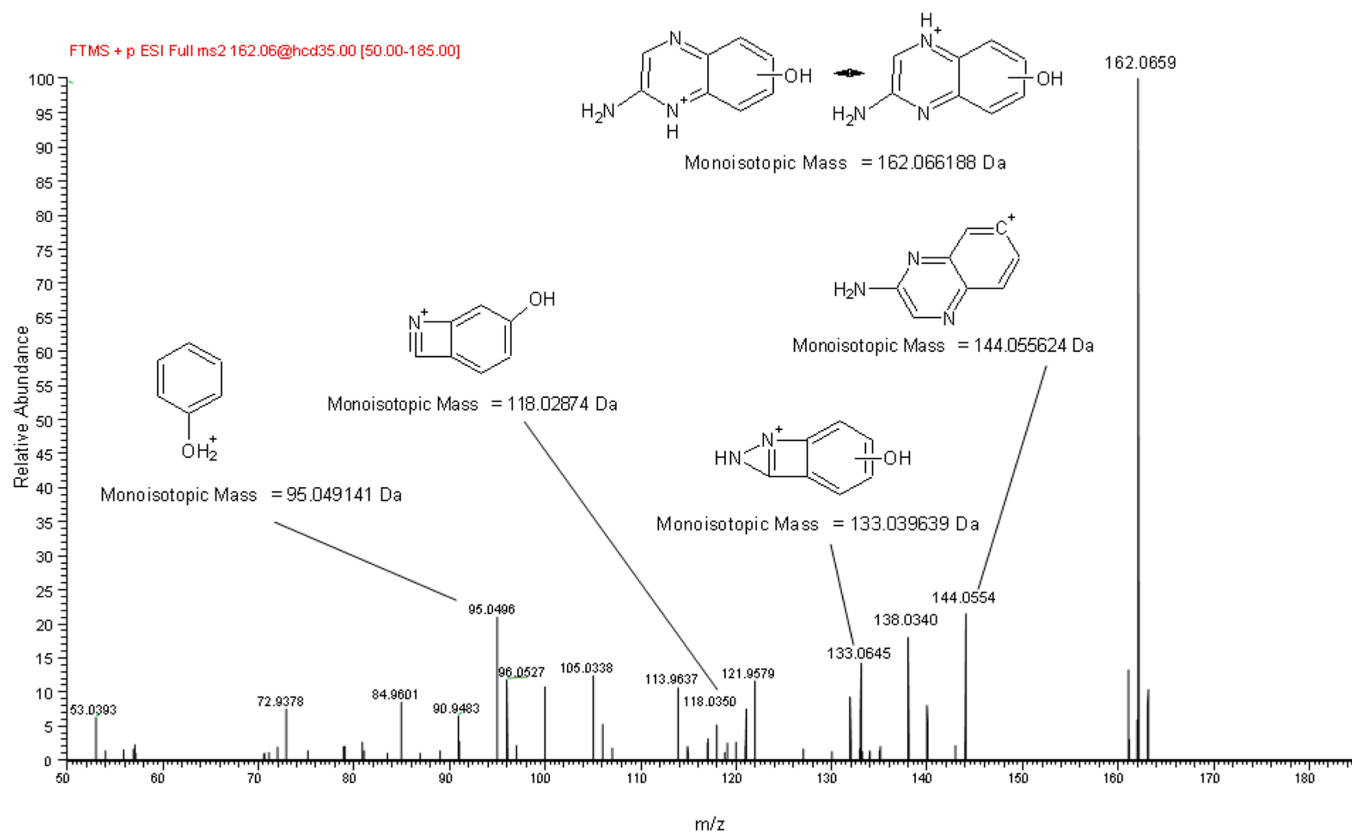

Figure 3. MS/MS spectrum for substructure-specific fragment m/z 162.

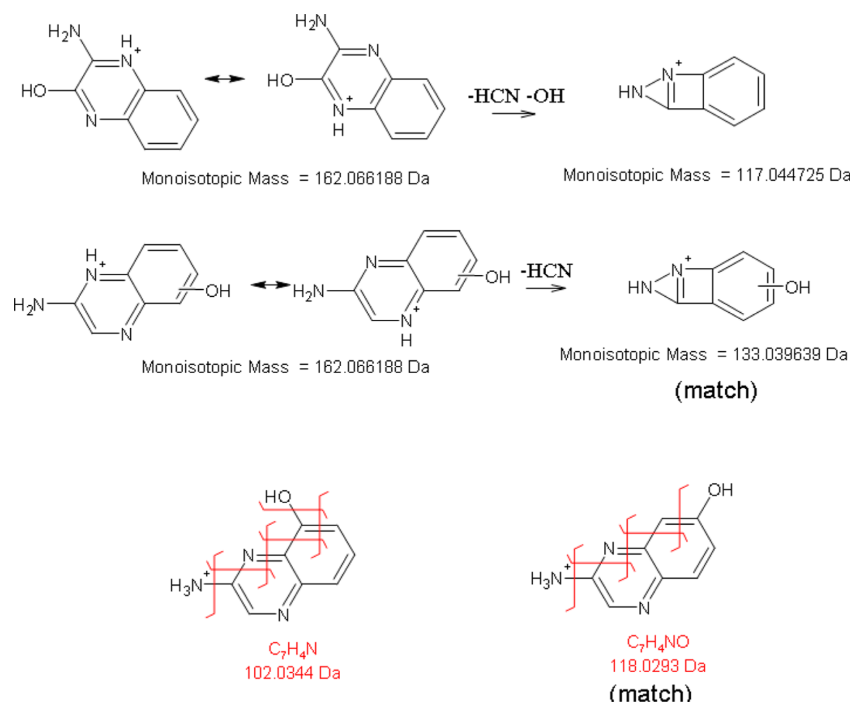

Figure 4. Theoretical fragmentation patterns used to support the structure hypothesis.

compound and the proposed fragment structures are shown in Figure 3.

The fragments observed in the m/z 162 spectrum were tentatively assigned and lead to assume the hydroxyl position at C^5 of the quinoxaline ring. The fragment with m/z 133.0646 corresponds to a fragment of quinoxaline ring in agreement with other report.[46] The same fragment was observed when negative ionization was also applied to the substructure-specific fragment, supporting the hypothesis that the hydroxyl is phenolic. This fragment leads us to discard the OH addition to position C^3 or in amine linked to quinoxaline moiety. The position of the hydroxyl linked to the benzene moiety of

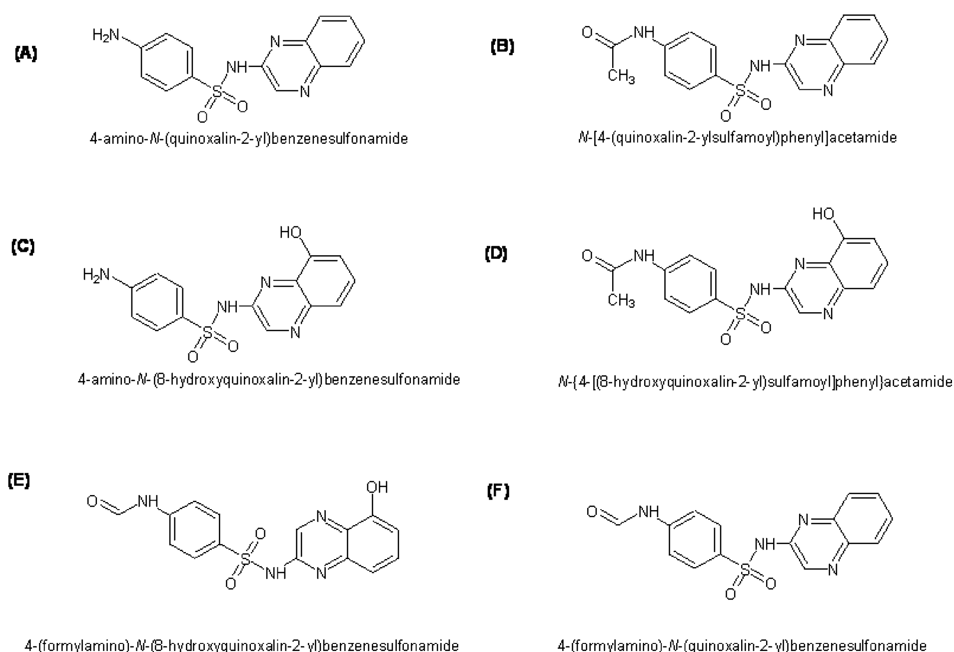

Figure 5. Proposed structures for SQX metabolites: (A) SQX; (B) N^4-acetyl-SQX; (C) SQX−OH; (D) N^4-acetyl-SQX−OH; (E) N^4-formyl-SQX−OH; (F) N^4-formyl-SQX.

Figure 6. (A) Extracted ion chromatogram of an ovine liver sample without traces of SQX. Monitored transitions are 301 > 156 and 301 > 108. (B) Extracted ion chromatogram of an ovine muscle sample with SQX−OH. Monitored transitions are 317 > 156 and 317 > 108. (C) Extracted ion chromatogram of fish sample (*Astyanax* sp.) with SQX. Monitored transitions are 301 > 156 and 301 > 108. (D) Extracted ion chromatogram of poultry liver sample with N^4-acetyl-SQX. Monitored transitions are 343 > 198, 343 > 134, and 343 > 92.

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quinoxaline ring was assigned based on the interpretation of fragments. Presence of fragments *m*/*z* 118 and 108 shows agreement with the structures represented in Figure 4.

On the basis of the best fit formula and MS/MS data, this metabolite would be assigned as 4-amino-*N*-(8-hydroxyquinoxalin-2-yl) benzenosulfonamide. The experimental findings from photolysis were compared with the results obtained from the in-source fragmentation of SQX metabolites, and the data showed correlation. Similarly, once the structure of SQX−OH was established, the formyl and acetyl derivatives of SQX−OH were also inferred. The assigned structures are given in Figure 5.

Although some reports of hydroxylated metabolites of sulfonamides analysis in biological and food samples were published, to the best of our knowledge, just two reports about sulfonamide hydroxylated metabolites' antimicrobial activity were issued.[47−49] In the first study, hydroxyl and N^4-acetyl derivatives of sulfamethazine, sulfadiazine, sulfamethoxazole, and sulfamerazine have their activity against *Escherichia coli* compared with the parent drugs. For acetylated metabolites, no activity was observed. For hydroxylated metabolites, activity (given as percent of parent drug activity) range from 2.5% to 39.5%.[50] In a more recent work, 4-hydroxy-sulfadiazine showed 10% of activity against *Geobacillus stearothermophilus* in comparison with the parent drug.[51]

Distribution of SQX Metabolites in Biological and Environmental Samples. In order to evaluate the presence and distribution of SQX and metabolites in animal tissues, samples of medicated animals were analyzed. These samples were obtained from routine analysis samples positives to SQX. Also, fish samples were collected in a creek located inside a poultry farm in southern Brazil, in which a recent case of coccidiosis was treated with SQX administered by oral feeding.

One sample of poultry liver, which was previously analyzed in routine analysis showing an SQX concentration level 10-fold above the MRL (≅1000 mg kg^{-1}), was evaluated to search for SQX metabolites. This sample revealed, in order of intensity, the presence of SQX, N^4-acetyl-SQX, SQX−OH, N^4-acetyl-SQX−OH, and presumable presence of N^4-formyl-SQX and N^4-formyl-SQX−OH. Our previous in vitro experiments with poultry liver have showed that no hydroxylation occurred. However, the present data show that, in high concentration, chicken liver can present hydroxylase activity. Again, we highlight our hypothesis that formyl derivatives could be the degradation products of acetyl derivatives.

Samples of muscle, liver, and kidney of an ovine medicated with SQX showed a variable profile of SQX and metabolites. The data lead us to compare the SQX metabolism in ovine in a very similar way with horse metabolism, in which hydroxylase activity is the major pathway, followed by acetylation. In ovine muscle, just SQX−OH was detected. In liver, SQX−OH and N^4-SQX−OH were observed in similar intensities. In ovine kidney, the original drug SQX could be observed together with SQX−OH and N^4-SQX−OH. Figure 6 show chromatograms in SRM mode for ovine, poultry, and fish samples.

Samples were analyzed under the SRM method, and all compounds were confirmed by analysis in the Orbitrap mass spectrometry system. The high-resolution MS/MS spectra are shown in the Supporting Information (Figures S-8−S-14). The fragmentation pattern of SQX−OH-specific fragment (*m*/*z* 162) was also confirmed using negative ionization (Supporting Information Figure S-15).

In fish samples (*Astyanax* sp., *n* = 7) with a high potential exposure risk to SQX residues and transformation products, intact SQX was detected (see Figure 6C and Supporting Information Figure S-11). No amount of N^4-acetyl-SQX, the major SQX metabolite in poultry, was detected, which is in agreement with the fact that N^4-acetyl-derivatives could be deacetylated in environmental conditions. Moreover, deacetylation restores the antimicrobial activity, increasing the impact of these compounds in the environment. Fish samples were obtained in a creek, in a point very close to the broilers' production house, approximately 200−300 m. These houses were recently under SQX intensive treatment to avoid coccidiosis. As the houses are placed in an elevated site in relation with the creek, it was expected that SQX would percolate into water and soil and reach the superficial water.

Ovine liver shows the same activity previously observed for equine liver.[39] Liver samples (2.5 g) collected from nine ovine were spiked with 100 ng g^{-1} of SQX. After 15 min, samples were extracted and analyzed using a matrix-matched calibration curve made using a blank sample of equine liver, and SQX−OH was quantified as described elsewhere.[39] Poultry (*n* = 10) and equine liver samples (*n* = 10) were also analyzed. Results show a high degree of homogeneity for each species. Both ovine and equine liver were able to convert quantitatively SQX to SQX−OH. Furthermore, poultry liver shows no detectable activity for SQX > SQX−OH conversion. Figure 7 shows a summary for SQX > SQX−OH in vitro conversion to equine, ovine, poultry, bovine, and swine species.

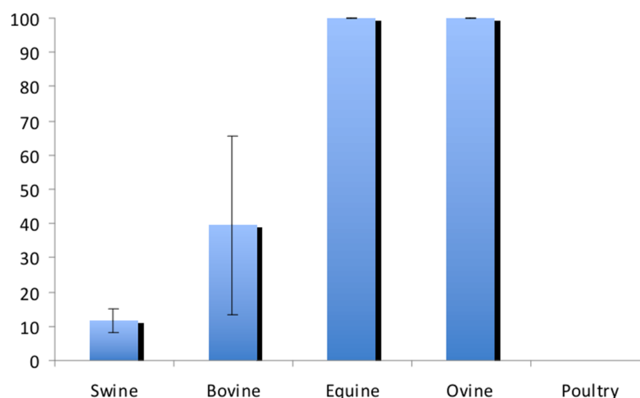

Figure 7. In vitro conversion of SQX to SQX−OH. Results are given in percentage (%). Bovine and swine data are from ref 39.

CONCLUSIONS

For the first time, hydroxylated SQX metabolites were reported and elucidated based on multiple mass spectrometry techniques. MS, MS^2, and MS^3 analysis were used in order to establish the metabolite structures. Structure-specific fragments were produced using photodegradation and confirmed by in-source CID fragmentation. Fragments produced under positive ionization support the proposed structural formulas. SQX and its metabolites were detected in animal tissues and environmental samples. Results confirm the co-occurrence of several SQX metabolites and their interspecies variability and emphasize the urgent need for further research in order to improve our knowledge and explore the impact of these metabolites on the food chain and the environment.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Supporting Information

Structural elucidation of sulfaquinoxaline metabolism products and their occurrence in biological samples using high-resolution Orbitrap mass spectrometry

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Abstract

This Supporting Information section come experimental data obtained with mass spectrometry techniques used to support the proposed molecular structure of SQX metabolites and to ensure the presence of SQX and their metabolism products in real samples.

Figure S-1. Fragmentation pattern for SQX-OH.

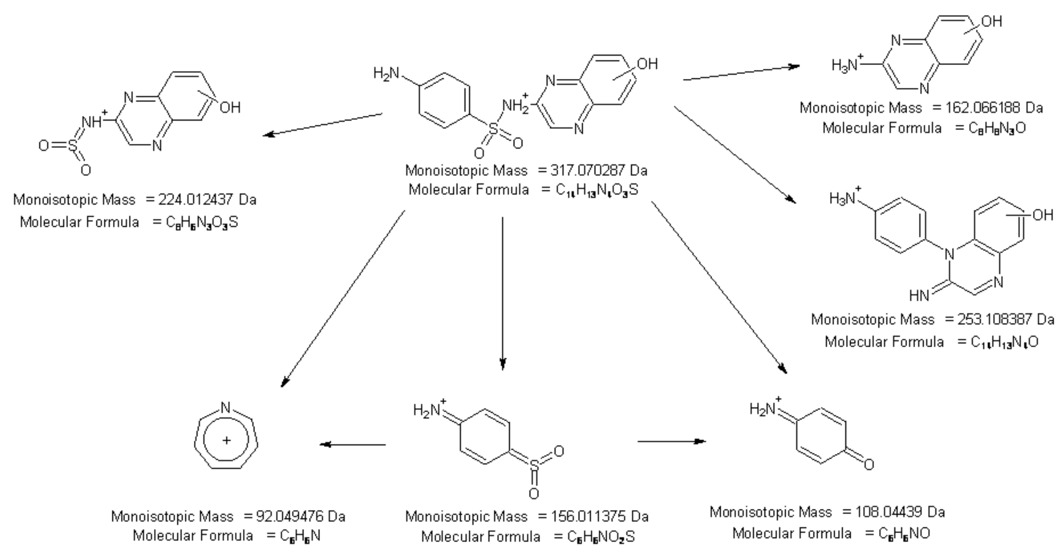


Figure S-2. Fragmentation pattern for N[4]-acetyl-SQX.

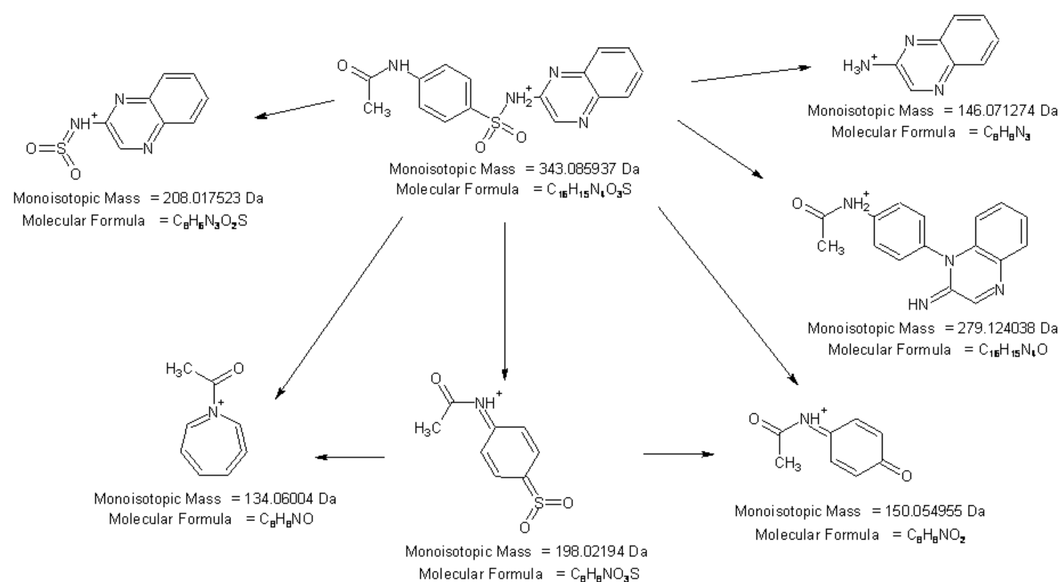


Figure S-3. Fragmentation pattern for N⁴-acetyl-SQX-OH.

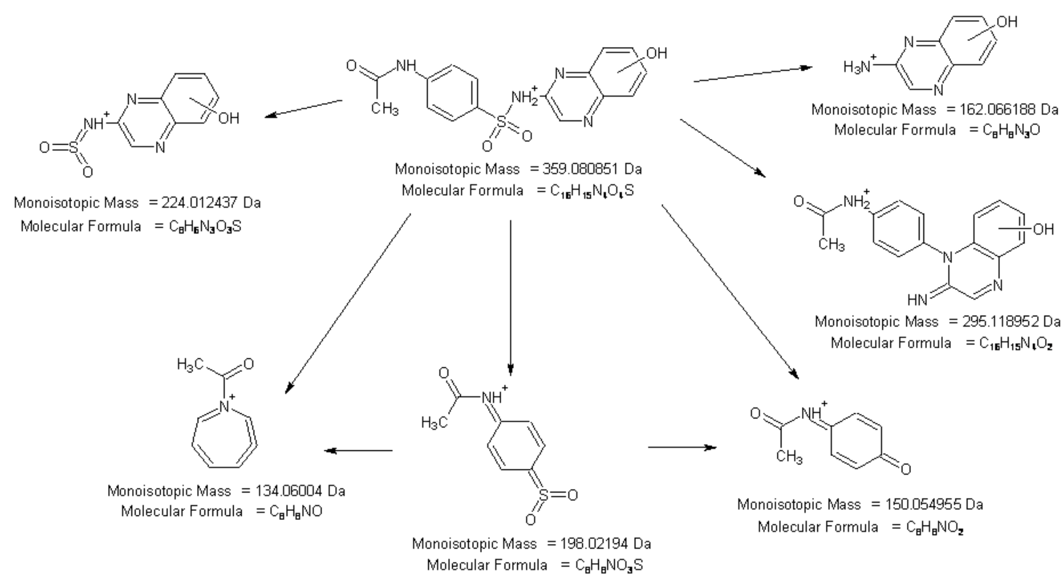


Figure S-4. Fragmentation pattern for N^4-formyl-SQX.

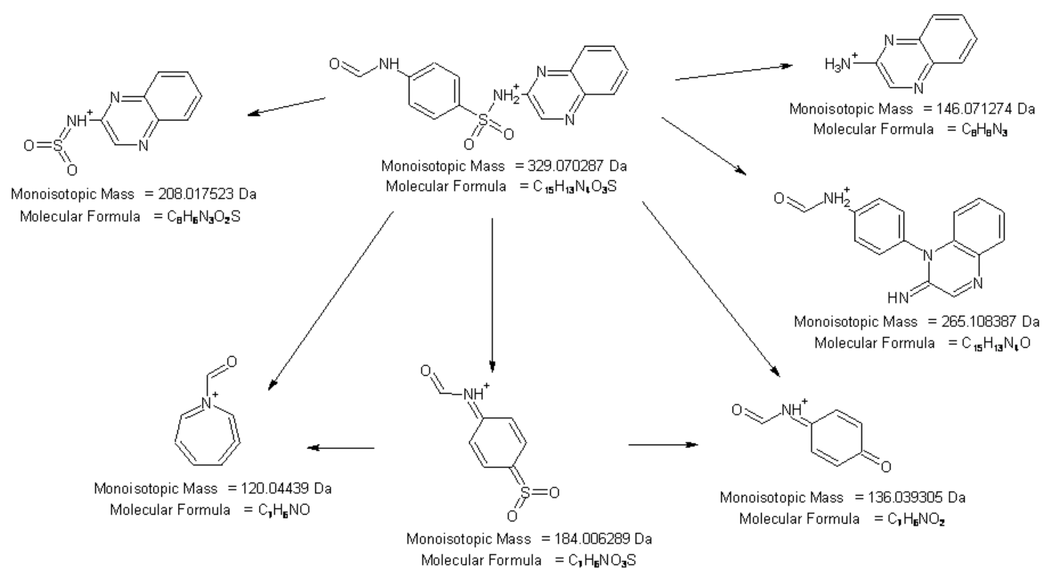


Figure S-5. Fragmentation pattern for N[4]-formyl-SQX-OH.

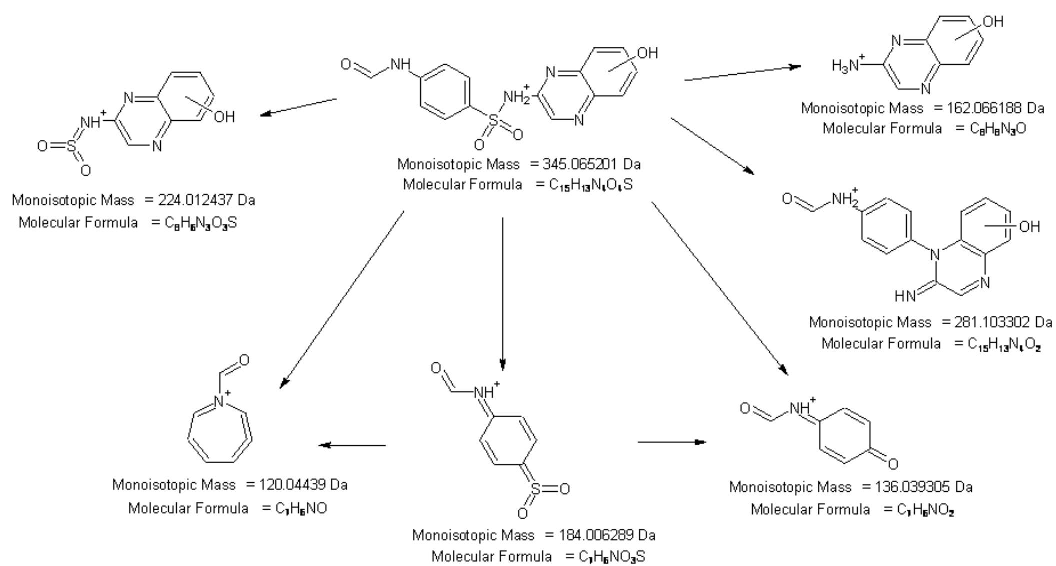
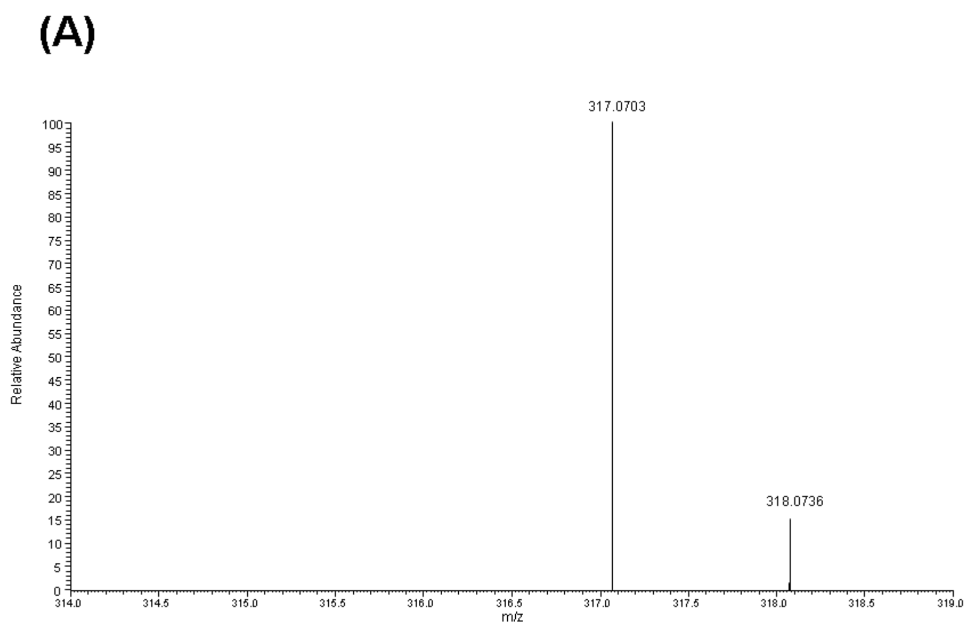


Figure S-6. SQX-OH isotope pattern simulation (A) and experimental isotope pattern (B) ($C_{14}H_{12}N_4O_3S$)

(A)



(B)

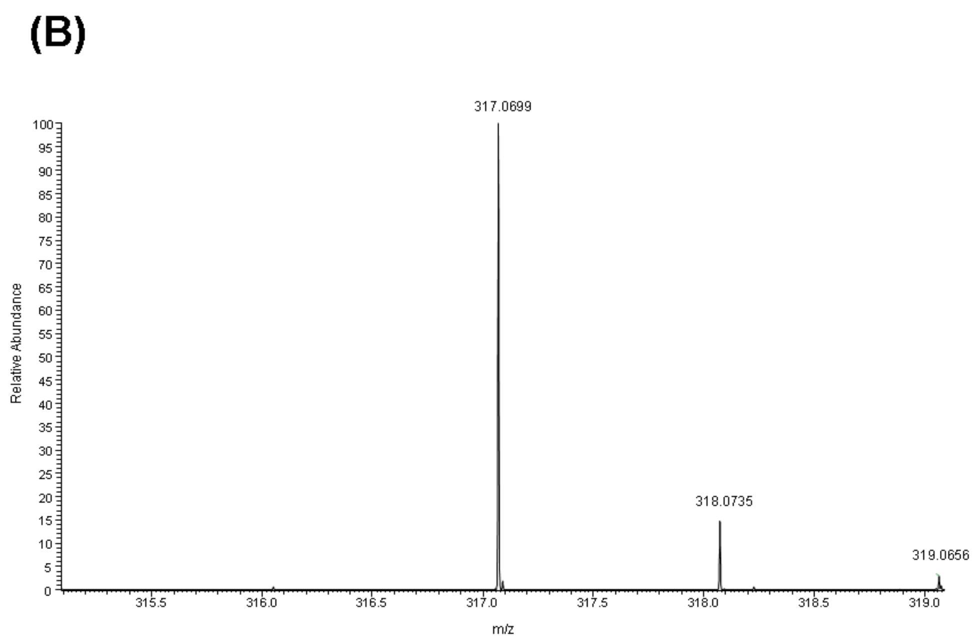


Figure S-7. Substructure-specific fragment for SQX-OH formed at in-source CID fragmentation: experimental isotope pattern ($C_8H_8N_3O$).

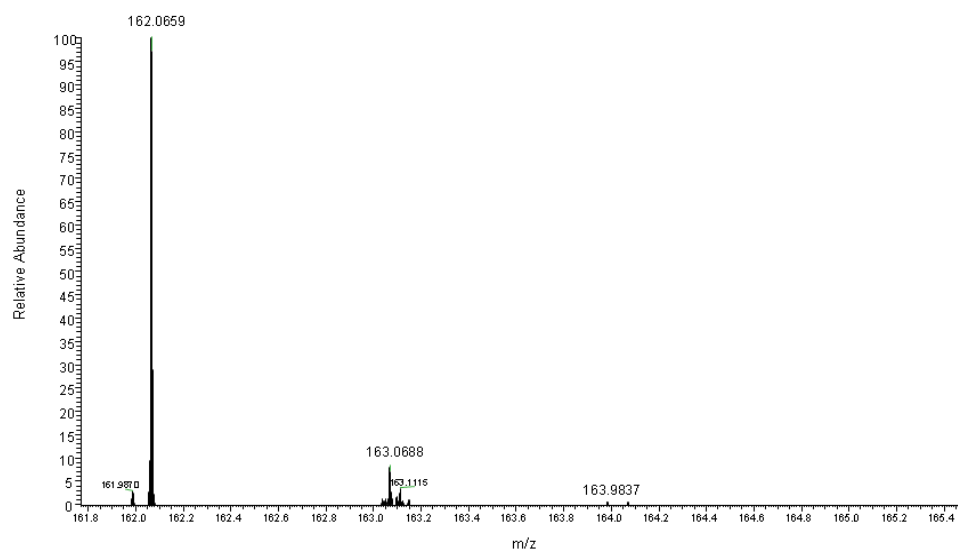


Figure S-8. MS/MS spectrum of SQX in poultry liver

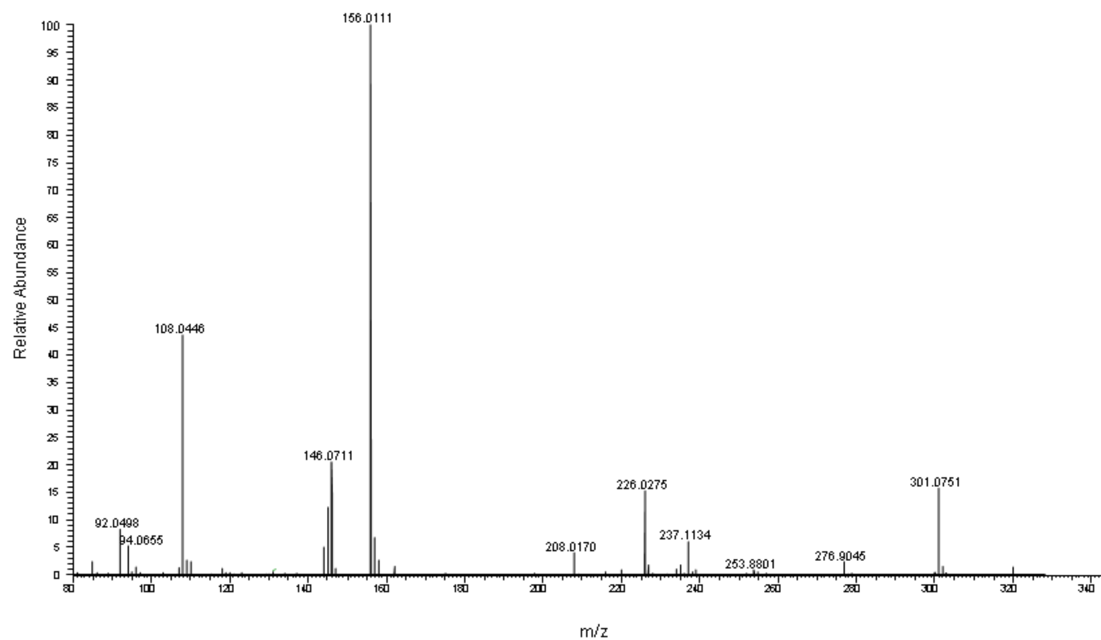


Figure S-9. MS/MS spectrum of SQX-OH in ovine liver

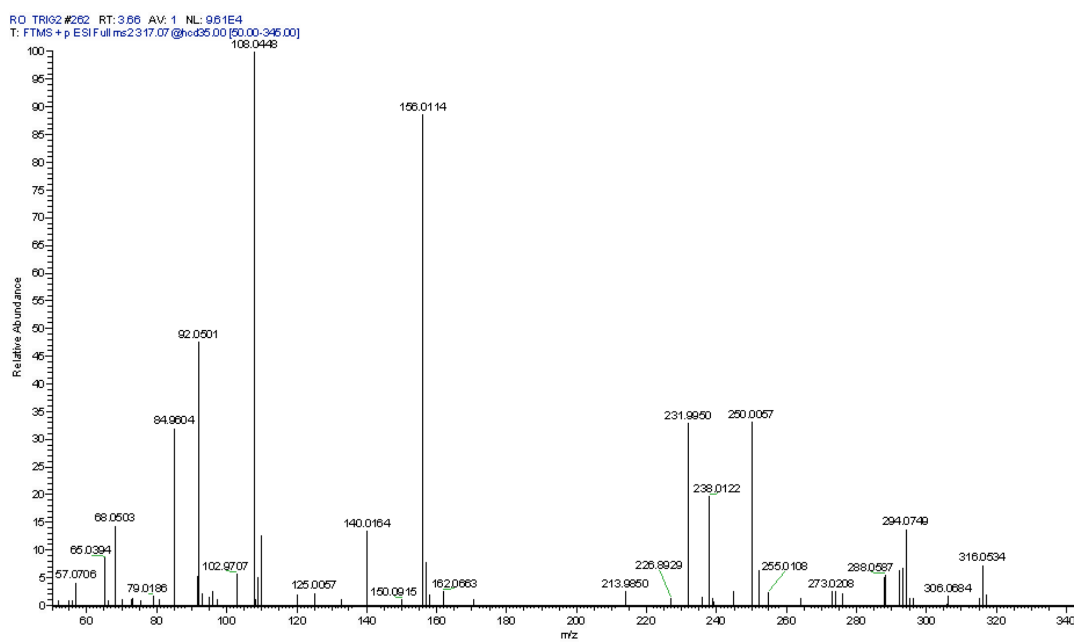


Figure S-10. MS/MS spectrum of N^4-acetyl-SQX-OH in poultry liver

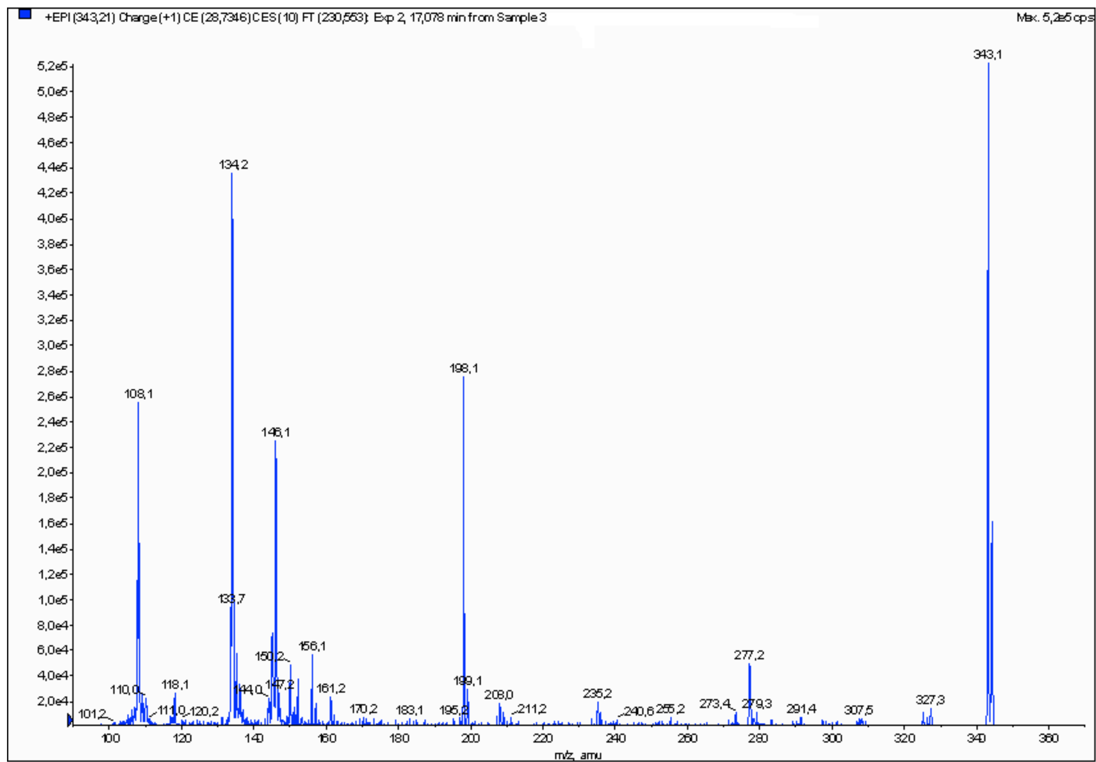


Figure S-11. Total ion chromatogram of fish sample and MS/MS spectrum of SQX.

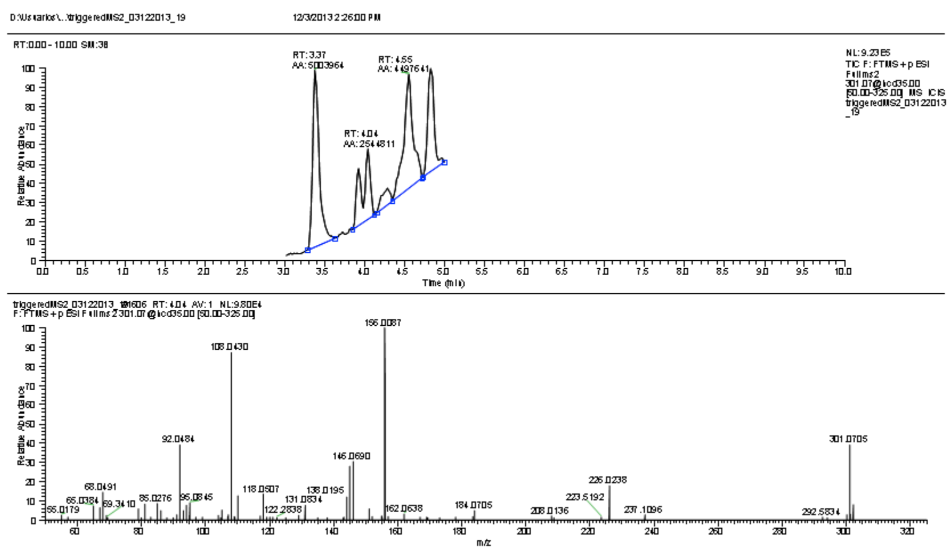


Figure S-12. Total ion chromatogram of poultry liver sample and MS/MS spectrum of presumable N^4-formyl-SQX.

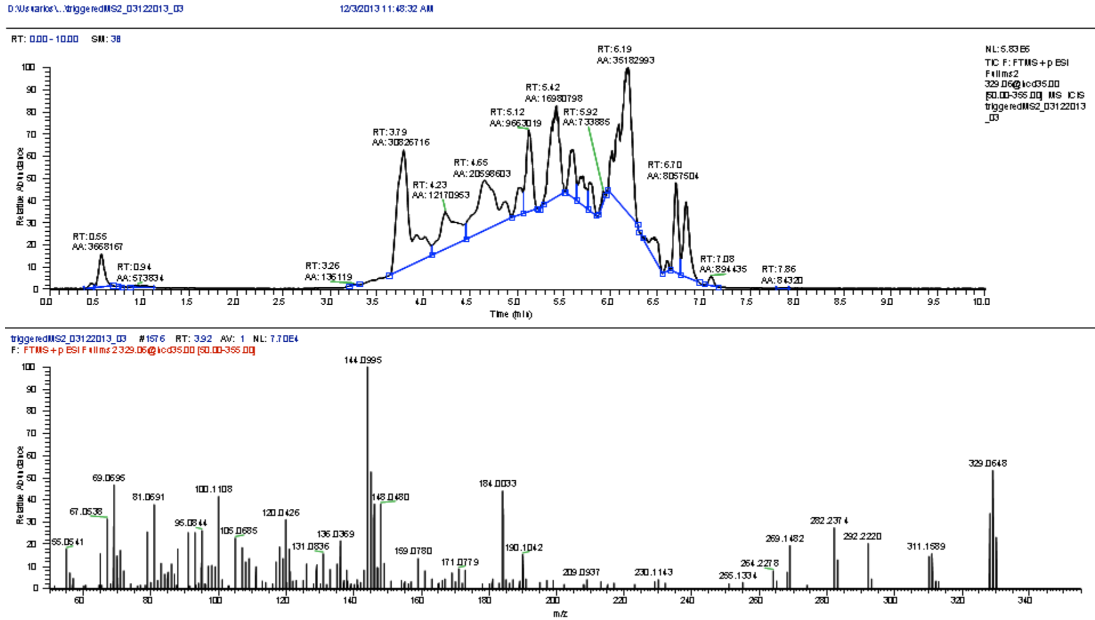


Figure S-13. Total ion chromatogram of poultry liver sample and MS/MS spectrum of N^4-acetyl-SQX.

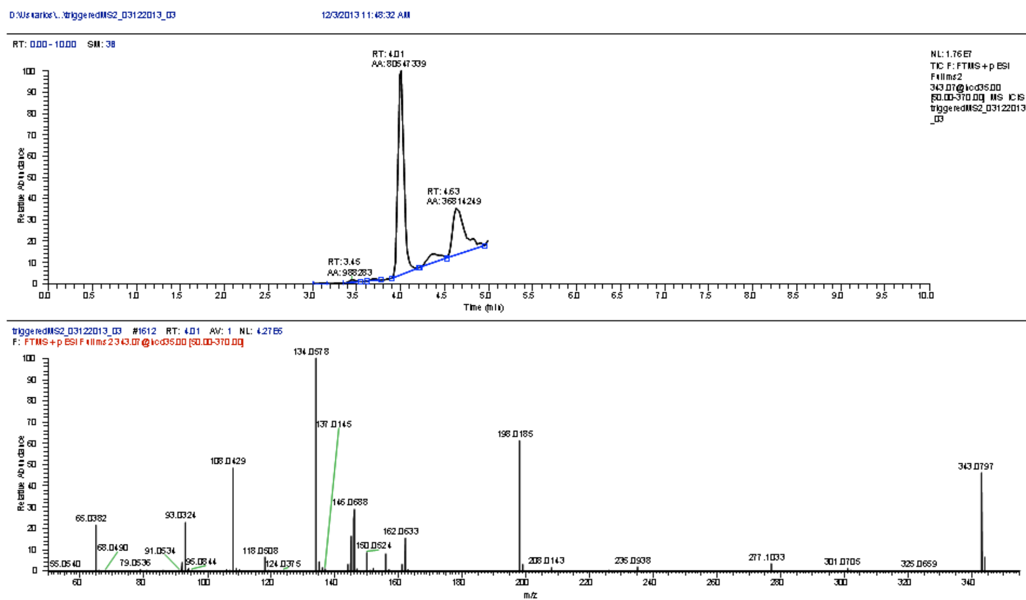


Figure S-14. Total ion chromatogram of poultry liver sample and MS/MS spectrum of presumable N^4-formyl-SQX-OH.

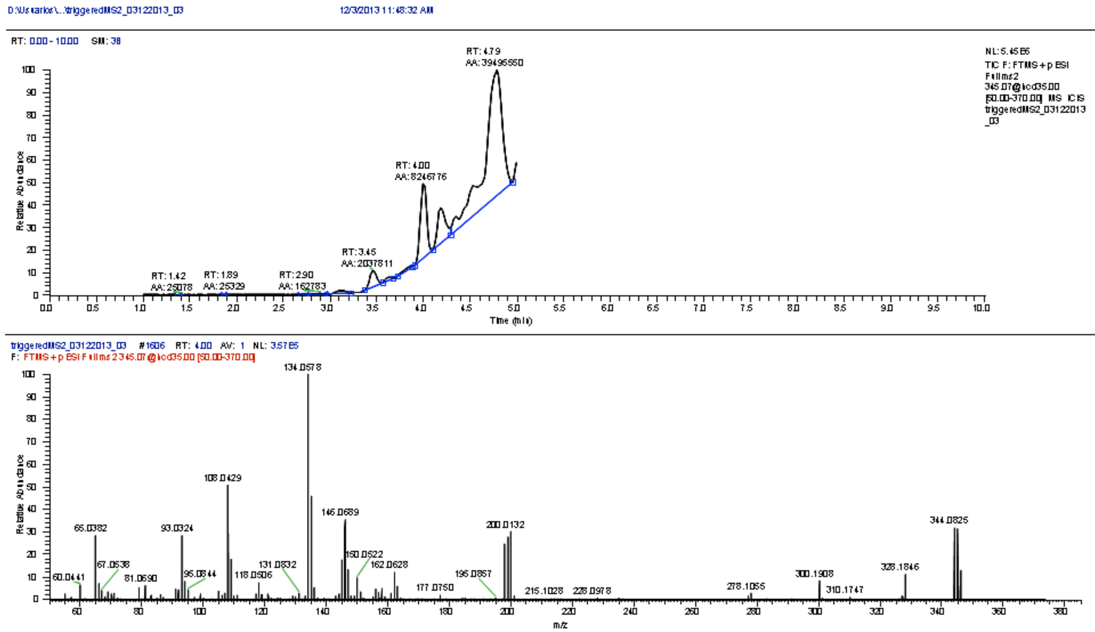


Figure S-15. Mass spectrum in negative ionization of photodegradation product m/z 160.0516.

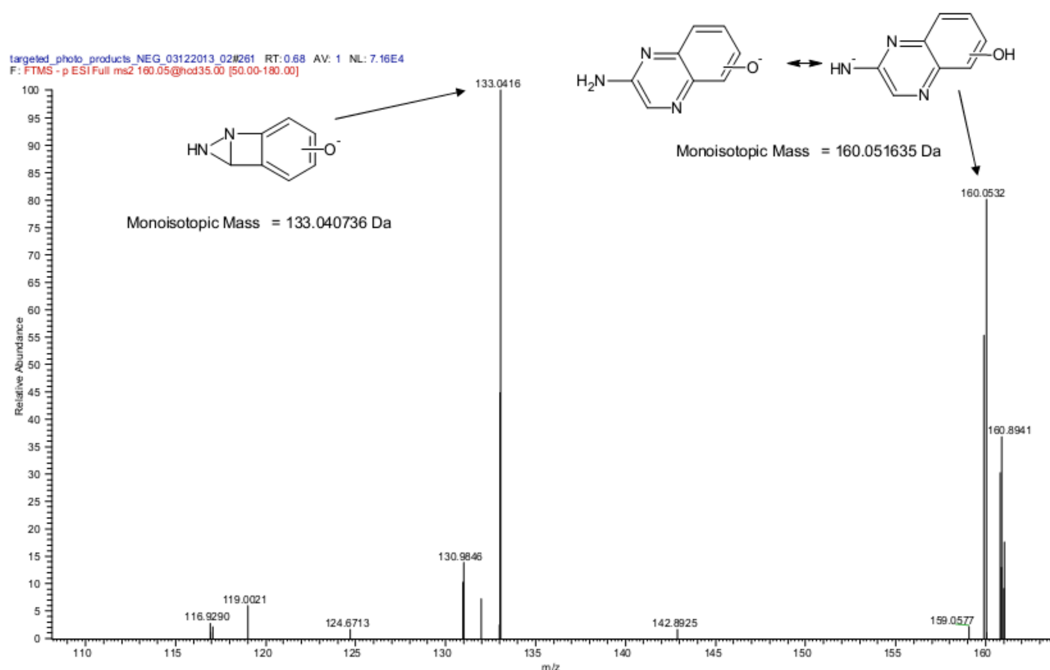


Figure S-16. SQX-OH MS/MS spectrum obtained in HPLC-LIT-MS/MS system.

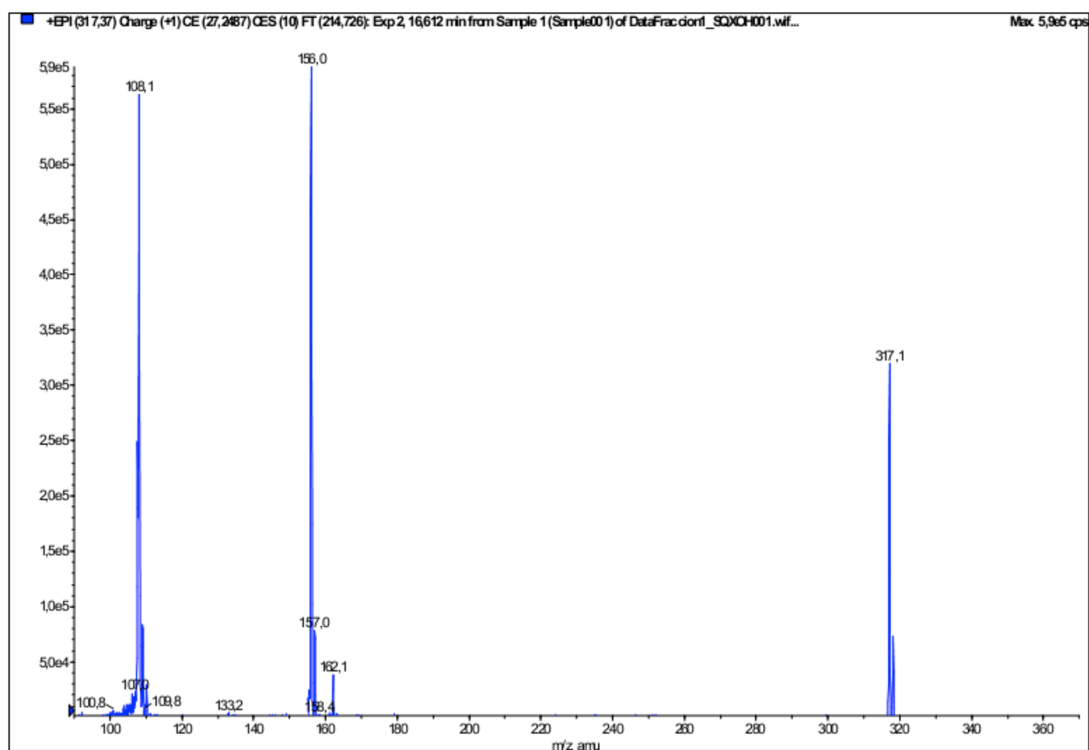


Figure S-17. SQX-OH MS spectrum obtained in HPLC-LIT-MS/MS system.

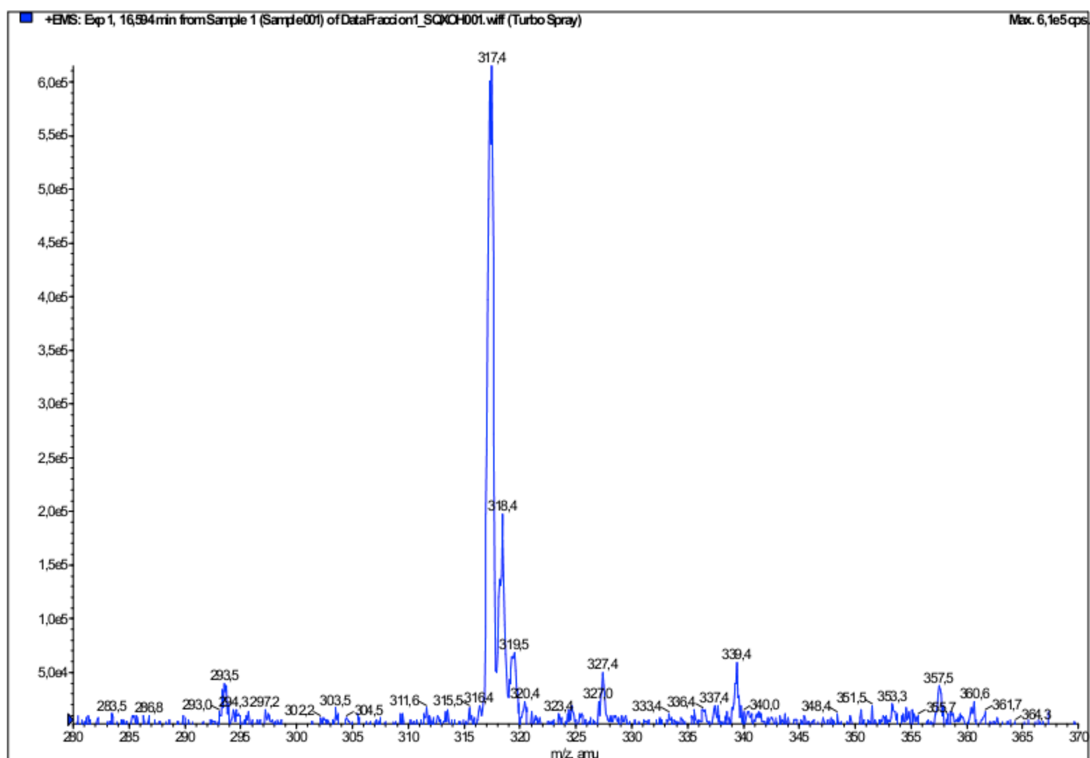
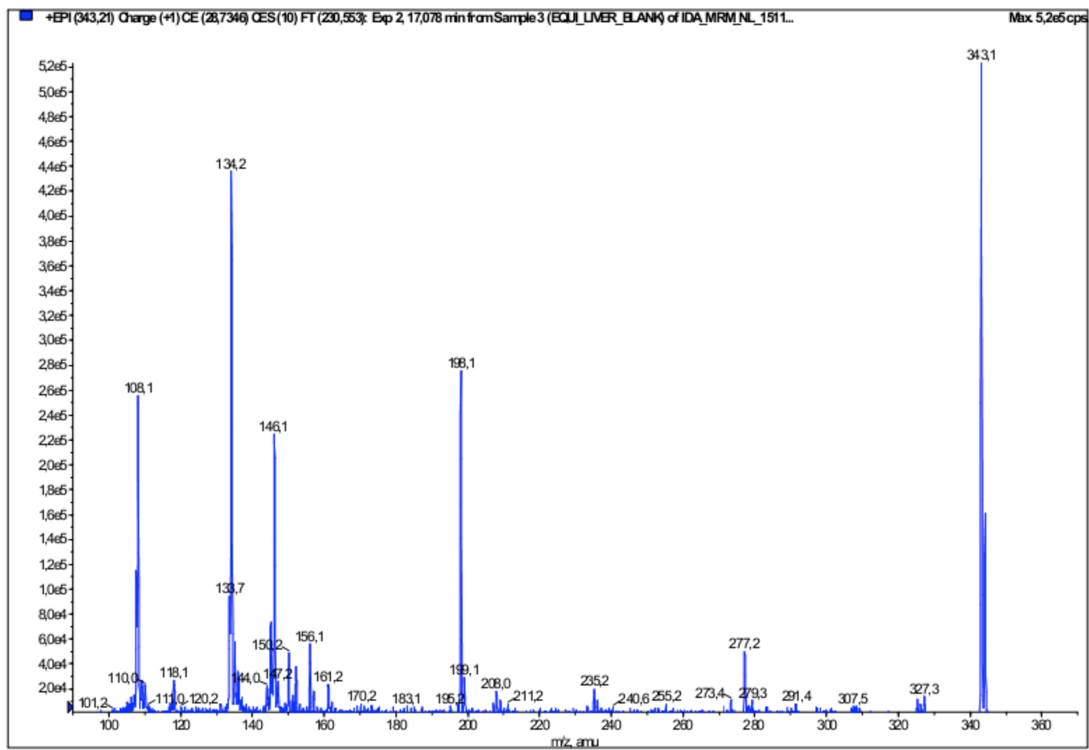


Figure S-18. N^4-acetyl-SQX MS/MS spectrum obtained in HPLC-LIT-MS/MS system.



Anexo VI – Artigo submetido para *Ciência & Saúde Coletiva*: **Modelo para priorização no monitoramento de resíduos de medicamentos veterinários em alimentos e no ambiente**

Ciência & Saúde Coletiva

Modelo para priorização no monitoramento de resíduos de medicamentos veterinários em alimentos e no ambiente

Journal:	*Ciência & Saúde Coletiva*
Manuscript ID:	Draft
Manuscript Type:	Free Theme Article
Keywords:	resíduos de medicamentos, meio-ambiente, toxicologia ambiental, análise de risco

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Introdução

Medicamentos veterinários são mundialmente utilizados para promover a saúde animal, propiciar ganhos econômicos e aumentar a produtividade da indústria de alimentos de origem animal [1,2]. Quando o período de carência não é cumprido, ocorre a permanência de resíduos destes medicamentos nos tecidos destinados à produção de alimentos. A questão dos resíduos de medicamentos veterinários (RMVs) acarretou o desenvolvimento de legislações e regulamentações próprias tanto no âmbito nacional e internacional, com o propósito de propor e, posteriormente, harmonizar valores de limites máximos de resíduo (LMR) para as diversas combinações fármaco / matriz. O estudo dos potenciais efeitos da ingestão de alimentos contendo quantidades acima do LMR foi e segue sendo uma área de pesquisa de grande relevância, já que diz respeito diretamente à saúde pública bem como às relações comerciais internacionais. O controle regulatório de contaminantes químicos em alimentos expandiu-se dramaticamente nas últimas décadas, fazendo da área de análise destes resíduos um importante fator a ser considerado no comércio internacional de *commodities* [3].

Mais recentemente o foco de estudo a respeito dos RMVs tem se voltado para o impacto destes compostos sobre o ambiente [4–8]. Após a administração, estes fármacos são liberados no ambiente através de processos de excreção dos animais, sendo que estas substâncias podem migrar para distintos recursos hídricos. Vários estudos têm citados a ocorrência de RMVs em águas superficiais, lençóis freáticos e efluentes de estações de tratamento [9–12]. Muitos trabalhos têm relatado ações sobre espécies não-alvo que incluem disrupção endócrina, inibição reprodutiva e até mesmo respostas em nível de ecossistema [6,13–17].

Considerando as lacunas existentes no que se refere aos dados de utilização e frequência de

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utilização de fármacos veterinários, faz-se necessário propor modos de classificação e priorização destes compostos baseados em seu uso, distribuição e perfil toxicológico.

As sulfonamidas constituem a primeira classe de agentes anti-infecciosos descobertos na terapêutica. Seu uso ainda é muito frequente na medicina veterinária, na área de produção animal e principalmente como profilático de infecções na produção em larga escala de aves e suínos. O Brasil adota um valor de LMR de 100 mg kg^{-1} para resíduos de sulfonamidas.

No presente estudo, utilizamos o exemplo das sulfonamidas, uma classe de compostos antibacterianos amplamente usada na medicina veterinária, para propor a construção de um modelo preditivo para elencar prioridades na área de RMVs, seja sob o ponto de vista da presença de resíduos em alimentos como também no ambiente. Para tanto, traçamos o perfil das sulfonamidas disponíveis no mercado veterinário brasileiro em dois períodos distintos (2007 e 2013) e aplicou-se o modelo sobre este cenário.

Material e Métodos

A pesquisa dos medicamentos de uso veterinário contendo sulfas foi realizada através de revisão bibliográfica, análise de bulas e rótulos, pesquisa através da Internet e em bancos de dados oficiais do MAPA (Ministério da Agricultura, Pecuária e Abastecimento). Os dados estatísticos foram obtidos através do uso de software comercial (Excel®).

Resultados e Discussão

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O mercado de medicamentos veterinários no Brasil é um dos maiores do mundo e encontra-se em expansão. A regulamentação deste mercado, desde os aspectos de registro e licença para produção até a análise fiscal, é atribuição do MAPA. O faturamento deste segmento no Brasil evolui de cerca de 0,5 bilhões de dólares em 2004 para mais de 3,5 bilhões de dólares em 2012.

O uso de fármacos veterinários no Brasil é de difícil mensuramento, dado que não são encontradas informações sobre quantidades comercializadas. Somente dados de volume de vendas são disponibilizados pelo Sindicato das Indústrias para a Saúde Animal (Sindan).

No ano de 2007, estavam disponíveis no Brasil 136 medicamentos de uso veterinário contendo sulfonamidas (Tabela 1). Em 2013, foram encontrados 93 produtos disponíveis contendo uma ou mais sulfonamidas como princípios ativos (Tabela 2). Foram analisados os seguintes fatores: sulfonamida(s) presente(s) na formulação; concentração dos princípios ativos; outros fármacos associados; forma farmacêutica; espécies animais com indicação de uso; período de carência para uso em animais produtores de alimentos.

Ocorreu uma significativa dimuição no número de produtos, aproximadamente 32%, sendo que algumas sulfas foram retiradas do mercado e hoje já não se encontram disponíveis para comercialização, como é o caso de sulfatiazol, sulfafurazol, sulfaisoxazol e sulfametilpirimidina. A Tabela 3 mostra um quadro comparativo entre os dois cenários avaliados.

No caso do sulfatiazol, cabe esclarecer que este fármaco segue sendo disponiblizado na forma de ftalilsulfatiazol, com 5 apresentações disponíveis em 2013. Em 2007, haviam 16 produtos

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contendo sulfatiazol ou ftalilsulfatiazol. O ftalilsulfatiazol é um pró-fármaco, elaborado para que não sofra absorção em nível estomacal ou nas primeias porções intestinais. O radical ftalil é liberado por clivagem mediada pela microbiota intestinal, liberando o sulfatiazol.

As concentrações do princípio ativo na forma farmacêutica variaram desde 0.1% (sulfadiazina de prata em spray) até 80% (sulfadiazina em pó para uso oral). As formas farmacêuticas são variadas, com predominância de pós para uso oral, soluções de uso oral e injetáveis.

As associações medicamentosas representaram 89% do total em 2007 e 82% em 2013. Dentre os fármacos de outras classes, a associação mais utilizada é com trimetoprima. Mais da metade (54% em 2007 e 63% em 2013) das associações contém trimetoprima. Há ocorrência de associações de duas, três e quatro sulfas em uma mesma forma farmacêutica.

As indicações de uso das apresentações abrangem praticamente todas as espécies animais produtoras de alimento (com exceção de pescado) bem como de animais de estimação (*pets*). O período de carência para retirada da medicação em animais produtores de alimentos foi analisado com base em estudo de bulas. Os períodos variam enormemente, mesmo para formas farmacêuticas semelhantes. Muitas bulas não apresentam nenhuma informação referente ao período de carência, mesmo em casos onde o medicamento seja indicado para espécies produtoras de alimentos.

Para estabelecer um modelo para priorização de sulfas a serem monitoradas em alimentos e no meio-ambiente, se propõe à adoção do modelo exposto na Tabela 4. Critérios de pontuação numérica simples foram utilizados, atribuindo valores a determinados parâmetros que devem ser observados para elencar, em escala de prioridades, os fármacos que devem ser

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monitorados dentro do Plano Nacional de Controle de Resíduos em Alimentos, e avaliados quanto ao seu impacto na microbiota de solo e mananciais de água.

Para as sulfas, o potencial de permanência de resíduos em alimentos é proporcional ao período de permanência do fármaco no organismo (1/2 vida) e inversamente proporcional à extensão do processo de metabolização do fármaco. Deste modo, para o sulfatiazol, que geralmente é utilizado na forma de pró-fármaco (ftalilsulfatiazol) e que é excretado de forma inalterada e com absorção desprezível, o potencial de permanência de resíduos em alimentos é bastante baixo. Não obstante, para avaliações do impacto ambiental de resíduos de sulfas, estas relações devem ser invertidas. Assim, o sulfatiazol, sendo amplamente excretado em curto intervalo de tempo, possui preocupante papel na migração de fármacos ao ambiente.

Baseado na proposta de pontuação que consta na tabela 5, as sulfas foram elencadas de acordo com sua pontuação para estudos de permanência de resíduos em alimentos. Estes resultados estão computados na Tabela 5.

Para o critério de número de apresentações, adotamos faixas de valores aos quais são atribuidos valores de 1 a 5. Este critério se baseia na inferência de que o número de apresentações disponíveis é diretamente proporcional ao volume utilizado. Esta correlação nem sempre é verdadeira, como por exemplo, no caso de medicamentos inovadores detentores de patente. No entanto, face a ausência de dados de comercialização, pode ser considerada como uma aproximação. Nos demais critérios, foram utilizados dados obtidos pelas próprias bulas dos RMVs, bem como dados farmacológicos e toxicológicos [18–26] .

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De acordo com os critérios propostos e o peso de cada um na pontuaçao geral, elencou-se a sulfametazina como a sulfa de maior impacto. Este modelo apresenta concordância com os resultados de monitoramento de resíduos de sulfas em alimentos, onde a sulfametazina apresenta a maior número de resultados não-conformes [27]. Em segundo lugar, esta o sulfatiazol. Conforme comentado anteriormente, este fármaco tem grande potencial de contaminação ambiental e aspectos de sua toxicidade e do risco ambiental associado estão bem documentados [28,29]. A sulfaquinoxalina aparece na terceira pontuação mais alta. Este fármaco, ao lado da sulfametazina, apresenta uma das maiores incidências em amostras não-conformes analisadas pelo MAPA [3,27]. Entretanto, em geral são amostras de fígado de aves. Uma investigaçao anterior publicada por nosso grupo mostra que as aves não apresentam um mecanismo de metabolização para SQX que é muito signifcativo para outras espécies [30]. Deste modo, não somente se deve levar em conta a molécula original, bem como seus metabólitos e produtos de degradação.

Embora se disponha de dados farmacológicos consistentes para muitas sulfas, como sulfametoxazol e sulfadiazina, ainda restam muitas lacunas de informações. Dados de comercialização, por exemplo, não puderam ser utilizados por serem virtualmente inexistentes. Informações toxicológicas também existem somente para alguns compostos. A ferramenta proposta necessita de diversos dados para que possa fornecer predições mais robustas e confiáveis. Conclui-se que a comunidade científica brasileira e o público em geral necessitam com urgência de acesso transparente a informações sobre o real cenário da utilização de fármacos de uso veterinário no Brasil, de modo a poder avaliar corretamente os impactos ambientais e alimentares de RMVs.

Conclusões

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Não estão disponíveis dados sobre o consumo de fármacos de uso veterinário no Brasil, que permitam estabelecer critérios técnicos objetivos para subsidiar a regulamentação e a pesquisa científica. As sulfonamidas disponíveis no mercado veterinário brasileiro tem apresentado perfil mais homogêneo nos últimos anos, focando-se em produtos destinados para suínos e aves.

1. O modelo proposto para priorização será adequado uma vez que os dados necessários sejam disponibilizados.

2. Evidencia-se a necessidade urgente de maiores pesquisas e criação de bases de dados sobre o uso RMVs no Brasil.

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Tabela 1. Perfil das apresentações de sulfas no Brasil em 2007. INJ (injetável), SOL (solução de uso oral), SUS (suspensão de uso oral), PÓ (pó para uso oral), POM (pomada), CMP (comprimido), INF (infusão intramamária), ERR (errino), PRE (premix), OUT (outros).

		FORMAS FARMACÊUTICAS											
SULFONAMIDA	APRESENTAÇÕES	INJ	SOL	SUS	PÓ	POM	CMP	INF	ERR	PRE	OUT	total	%
Sulfadiazina	32	7	2	6	9	1	1	1	---	3	2	32	23,5
Sulfametazina	16	3	3	1	2	---	1	---	1	5	---	16	11,7
Sulfametoxazol	15	3	4	1	5	---	2	---	---	---	---	15	11,0
Ftalilsulfatiazol	13	---	2	1	9	---	---	---	---	---	1	13	9,6
Sulfaquinoxalina	13	---	8	---	4	---	---	---	---	1	---	13	9,6
Sulfanilamida	12	---	---	---	---	9	---	1	1	---	1	12	8,8
Sulfaguanidina	9	---	1	1	6	---	1	---	---	---	---	9	6,6
Sulfaclorpiridazina	5	---	2	---	3	---	---	---	---	---	---	5	3,7
Sulfadimetoxina	5	2	1	---	1	---	1	---	---	---	---	5	3,7
Sulfacetamida	4	2	1	---	---	---	---	---	---	---	1	4	2,9
Sulfamerazina	3	---	---	---	3	---	---	---	---	---	---	3	2,2
Sulfatiazol	3	1	---	1	---	---	1	---	---	---	---	3	2,2
Sulfadoxina	2	1	1	---	---	---	---	---	---	---	---	2	1,5
Sulfafurazol	1	---	---	---	1	---	---	---	---	---	---	1	0,7
Sulfaisoxazol	1	---	---	---	1	---	---	---	---	---	---	1	0,7
Sulfametilpirimidina	1	1	---	---	---	---	---	---	---	---	---	1	0,7
Sulfametoxipiridazina	1	1	---	---	---	---	---	---	---	---	---	1	0,7
Total	136	21	25	11	44	10	7	2	2	9	5	136	
Porcentagem	100	15	18	8	32	7	5	2	2	7	4		

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Tabela 2. Perfil das apresentações de sulfas no Brasil em 2013. INJ (injetável), SOL (solução de uso oral), SUS (suspensão de uso oral), PÓ (pó para uso oral), POM (pomada), CMP (comprimido), INF (infusão intramamária), ERR (errino), PRE (premix), OUT (outros).

		FORMAS FARMACÊUTICAS											
SULFONAMIDA	APRESENTAÇÕES	INJ	SOL	SUS	PÓ	POM	CMP	INF	ERR	PRE	OUT	total	%
Sulfadiazina	24	9	3	---	4	---	---	2	---	---	6	24	17,6
Sulfametazina	16	1	4	---	10	---	---	---	1	---	---	16	11,8
Sulfametoxazol	16	3	4	2	5	---	2	---	---	---	---	16	11,8
Ftalilsulfatiazol	5	---	---	---	4	---	1	---	---	---	---	5	3,7
Sulfaquinoxalina	14	---	4	---	10	---	---	---	---	---	---	14	10,3
Sulfanilamida	4	---	---	---	---	2	---	1	1	---	---	4	2,9
Sulfaguanidina	2	---	---	---	1	---	1	---	---	---	---	2	1,5
Sulfaclorpiridazina	3	---	---	---	3	---	---	---	---	---	---	3	2,2
Sulfadimetoxina	4	2	1	---	1	---	---	---	---	---	---	4	2,9
Sulfacetamida	1	1	---	---	---	---	---	---	---	---	---	1	0,7
Sulfamerazina	1	---	---	---	---	---	1	---	---	---	---	1	0,7
Sulfatiazol	0	---	---	---	---	---	---	---	---	---	---	0	0
Sulfadoxina	2	2	---	---	---	---	---	---	---	---	---	2	1,5
Sulfafurazol	0	---	---	---	---	---	---	---	---	---	---	0	0
Sulfaisoxazol	0	---	---	---	---	---	---	---	---	---	---	0	0
Sulfametilpirimidina	0	---	---	---	---	---	---	---	---	---	---	0	0
Sulfametoxipiridazina	1	---	---	---	1	---	---	---	---	---	---	1	0,7
Total	93	18	16	2	39	2	5	3	2	0	6	93	
Porcentagem	100	13	12	1,5	29	1,5	3,7	2,2	1,5	0	4,4		

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Tabela 3. Comparação das apresentações contendo sulfonamidas disponíveis em 2007 e em 2013.

Sulfa	2007	2013	2007(%)	2013(%)
Sulfadiazina	32	24	23,5	25,8
Sulfametazina	16	16	11,8	17,2
Sulfametoxazol	15	16	11,0	17,2
Ftalilsulfatiazol	13	5	9,6	5,4
Sulfaquinoxalina	13	14	9,6	15,1
Sulfanilamida	12	4	8,8	4,3
Sulfaguanidina	9	2	6,6	2,2
Sulfaclorpiridazina	5	3	3,7	3,2
Sulfadimetoxina	5	4	3,7	4,3
Sulfacetamida	4	1	2,9	1,1
Sulfamerazina	3	1	2,2	1,1
Sulfatiazol	3	0	2,2	0,0
Sulfadoxina	2	2	1,5	2,2
Sulfafurazol	1	0	0,7	0,0
Sulfaisoxazol	1	0	0,7	0,0
Sulfametilpirimidina	1	0	0,7	0,0
Sulfametoxipiridazina	1	1	0,7	1,1
Total	136	93		

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Tabela 4. Proposta de critérios de pontuação para prioritização de sulfonamidas

Critério	Pontuações	Alimentos	Ambiente	Peso
Número de apresentações disponíveis: um grande número de apresentações pode ser levado em conta como um indicador subjetivo de "popularidade" do fármaco, embora sejam necessários dados de uso e volume de vendas por substância para racionalizar devidamente o processo	>30	5	5	1
	20-30	4	4	
	10-20	3	3	
	05-10	2	2	
	<05	1	1	
Espécies indicadas: de acordo com a(s) espécie(s) em que o fármaco é utilizado, tem-se um perfil de manejo e administração que é de extrema relevância para o potencial de resíduos ou impacto ambiental	Aquacultura	5	5	3
	Tratamento coletivo	4	4	
	Tratamento individual	3	3	
	Animais de companhia	3	3	
Dados farmacológicos: substâncias de baixa absorção, destinadas à efeito na luz intestinal, como o sulfatiazol, por exemplo, tem baixo potencial de resíduos em alimentos, mas alto índice para avaliação de impacto ambiental	Baixa metabolização	3	3	2
	Média metabolização	2	2	
	Alta metabolização	1	1	
	Ação curta	1	4	2
	Ação intermediária	2	3	
	Ação longa	3	2	
	Ação ultra-longa	4	1	
Forma farmacêutica: dependendo da forma farmacêutica, há maior ou menor probabilidade de permanência do fármaco em tecidos	Injetáveis	5	2	1
	Rações	4	3	
	Solução oral	4	3	
	Errinos, pomadas	1	2	
Genotoxicidade e teratogenicidade	Alto ou desconhecido	5	5	3

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	Médio	4	4	
	Baixo	3	3	
	Insignificante	1	1	
Potencial alergênico	Alto ou desconhecido	5	5	
	Médio	4	4	2
	Baixo	3	3	
	Insignificante	1	1	

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Tabela 5. Pontuação das sulfonamidas de acordo com os critérios propostos. SDZ (sulfadiazina), STZ (sulfatiazol), SMZ (sulfametazina), SMA (sulfametoxazol), SQX (sulfaquinoxalina), SFA (sulfanilamida), SGD (sulfaguanidina), SCP (sulfaclorpiridazina), SDMX (sulfadimetoxina), SCA (sulfacetamida), SMR (sulfamerazina), SDX (sulfadoxina),.

Critério	SDZ	STZ	SMZ	SMA	SQX	SFA	SGD	SCP	SDMX	SCA	SMR	SDX	*PESO*
Apresentações	5	3	3	3	3	3	2	2	2	1	1	1	*1*
Indicações	4	4	4	4	4	4	4	4	4	4	4	4	*3*
Farmacologia (metabolizaçao)	3	3	3	3	3	3	3	3	1	3	2	3	*2*
Farmacologia (1/2 vida)	2	1	3	1	2	1	1	2	3	2	2	4	*2*
Forma farmacêutica	4	4	4	4	4	1	4	4	4	4	4	5	*1*
Genotoxicidade Teratogenicidade	1	5	5	1	3	1	1	1	1	1	3	1	*3*
Alergenicidade	3	3	3	3	3	3	3	3	3	3	3	3	*2*
Total	40	48	52	36	44	33	35	37	35	36	40	41	

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Anexo VII – Artigo a ser submetido para *Talanta:* **Determination of sulfonamides and their metabolites in muscle, kidney and liver samples by pressurized liquid extraction (PLE) followed by liquid chromatography –quadrupole linear ion trap mass spectrometry (QqLIT-MS/MS)**

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Manuscript Draft

Manuscript Number: TAL-D-14-01458

Title: Determination of sulfonamide antibiotics and metabolites in liver, muscle and kidney samples by pressurized liquid extraction or ultrasound-assisted extraction followed by liquid chromatography-quadrupole linear ion trap-tandem mass spectrometry (HPLC-QqLIT-MS/MS)

Article Type: Research Paper

Keywords: Sulfonamides; Veterinary drugs; Metabolites; Food analysis; Ultrasound-assisted extraction; Pressurized liquid extraction; Central composite design.

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Abstract: Sulfonamides are widely used in human and veterinary medicine. The presence of sulfonamides residues in food is an issue of great concern. In the present work, we developed and validated a method for 16 sulfonamides and metabolites residue analysis in several matrices i.e. liver, muscle and kidney samples of poultry, ovine, equine, swine, and fish. Extraction and clean-up was statistically optimized using central composite design experiments. Two extraction methods were developed, validated and compared: i) pressurized liquid extraction, in which samples were defatted with hexane and subsequently extracted with acetonitrile and ii) ultrasound-assisted extraction with acetonitrile and further liquid-liquid extraction with hexane. Extracts were analyzed by liquid chromatography - quadrupole linear ion trap-tandem mass spectrometry. Validation procedure was based on the Commission Decision 2002/657/EC and included the assessment of parameters such as decision limit (CCα), detection capability (CCβ), sensitivity, selectivity, accuracy and precision. Method performance was satisfactory, with CCα values in the range 111.2 -161.4 µg kg-1, limits of detection of 10 µg kg-1 and accuracy for all compounds at three concentrations levels around 100%.

Opposed Reviewers:

Novelty Statement

The present work presents the development, optimization and validation of two sample preparation methods. Only few reports of pressurized liquid extraction (PLE) and ultrasound-assisted extraction (USE) for sulfonamides residues analysis were published until now. Chemometric tools were used to optimize PLE parameters. Finally, data were used to perform sample preparation methods comparison in terms of recovery, accuracy, reproducibility and other figures of merit.

Highlights

- Two sample preparation methods for sulfonamides residues analysis were developed, optimized and validated.
- Pressurized liquid extraction and ultrasound-assisted extraction were compared in terms of performance.
- Both methods were fully validated according to Commission Decision 2002/657/EC.
- Methods were applied to real incurred samples.
- Results were compared with a reference method.

*Graphical Abstract (for review)
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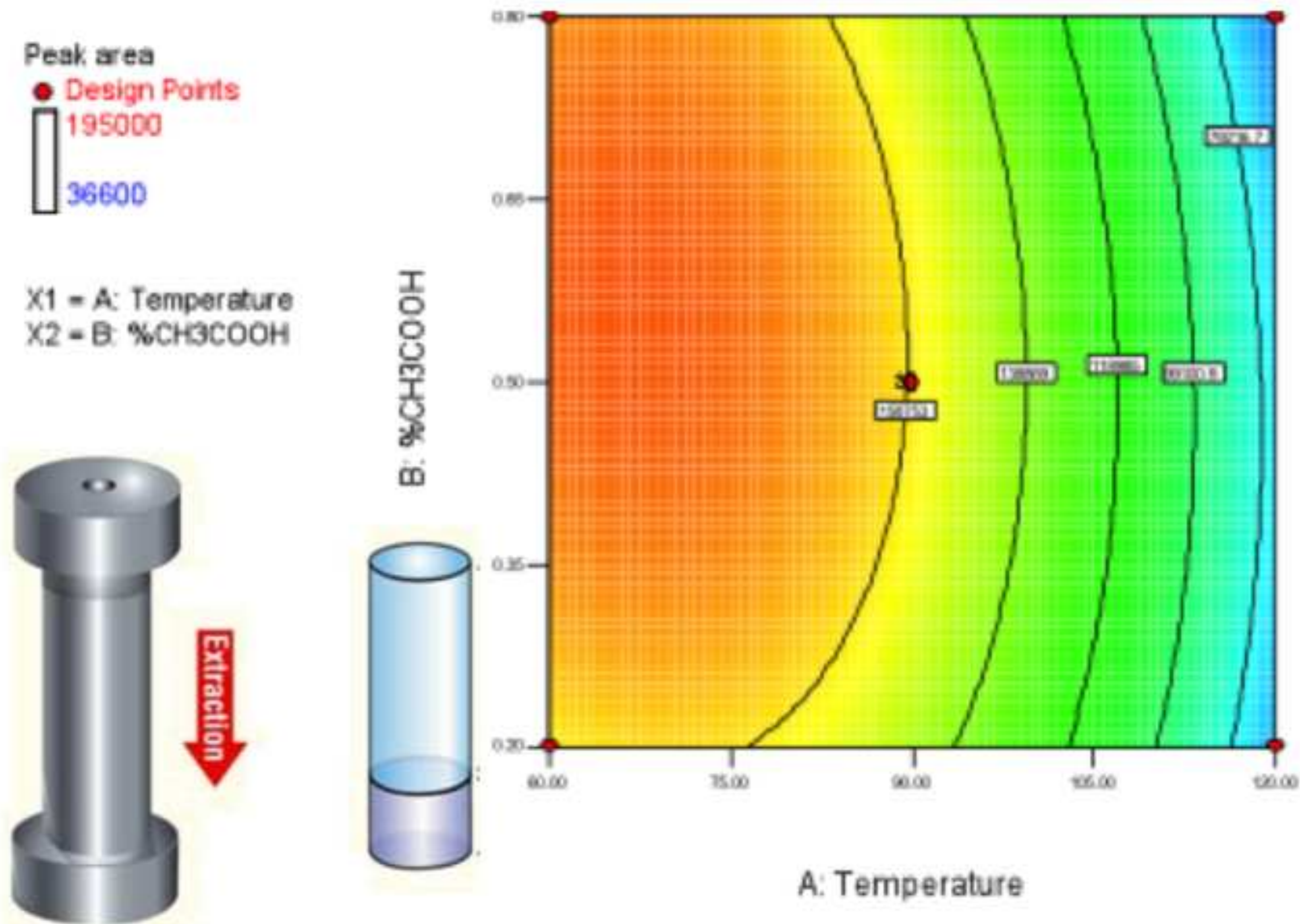

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Determination of sulfonamide antibiotics and metabolites in liver, muscle and kidney samples by pressurized liquid extraction or ultrasound-assisted extraction followed by liquid chromatography–quadrupole linear ion trap-tandem mass spectrometry (HPLC-QqLIT-MS/MS)

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Abstract

Sulfonamides are widely used in human and veterinary medicine. The presence of sulfonamides residues in food is an issue of great concern. In the present work, we developed and validated a method for 16 sulfonamides and metabolites residue analysis in several matrices i.e. liver, muscle and kidney samples of poultry, ovine, equine, swine, and fish. Extraction and clean-up was statistically optimized using central composite design experiments. Two extraction methods were developed, validated and compared: i) pressurized liquid extraction, in which samples were defatted with hexane and subsequently extracted with acetonitrile and ii) ultrasound-assisted extraction with acetonitrile and further liquid-liquid extraction with hexane. Extracts were analyzed by liquid chromatography – quadrupole linear ion trap-tandem mass spectrometry. Validation procedure was based on the Commission Decision 2002/657/EC and included the assessment of parameters such as decision limit (CCα), detection capability (CCβ), sensitivity, selectivity, accuracy and precision. Method

performance was satisfactory, with CCα values in the range 111.2 -161.4 μg kg^{-1}, limits of detection of 10 μg kg^{-1} and accuracy for all compounds at three concentrations levels around 100%.

Keywords

Sulfonamides, veterinary drugs, metabolites, food analysis, ultrasound-assisted extraction, pressurized liquid extraction, central composite design.

Introduction

Sulfonamides were the first class of antimicrobial agents introduced in medicine [1]. These compounds are still widely used in human and veterinary medicine. In animal production, sulfonamides are used not only to treat infections but also for prophylactic purposes [2]. The potential presence of sulfonamide residues in animal tissues or products derived from animals (e.g. milk, egg, honey) is a public health concern, since these residues could provoke several effects in humans and in the environment [3]. In order to provide food safety, maximum residue limits (MRL) were established for several drugs in distinct food matrices. For sulfonamides, Brazil adopts a MRL of 100 mg kg^{-1}[4]. That value comprehends the sum of sulfonamides and their metabolism products. In order to ensure the MRL compliance, analytical methods able to detect and quantify drug residues in food matrices are a constant purpose to researchers.

Generally, residues of sulfonamides can be analyzed in food matrices using several techniques, as liquid chromatography, bioactivity-based assays, and capillary electrophoresis among others [5–10]. Currently, due to high specificity and selectivity, hyphenated methods based in mass spectrometry detection are the most applied approach to determine sulfonamides residues at low concentrations (mg kg^{-1} or μg kg^{-1}). Within hyphenated methods, the use of liquid chromatography–electrospray-quadrupole linear ion trap mass spectrometry (HPLC-(ESI)-QqLIT-MS/MS) permits analysis with high specificity and adequate detection limits [11–13].

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Extraction and clean-up techniques must be applied to food matrices, since they are generally complex samples, e.g. liver, muscle, kidney, milk and honey. Several methods are used to this purpose, from classical approaches as solid-liquid extraction to recent methods as single drop microextraction [14].

Pressurized liquid extraction (PLE) is a relatively recent extraction technique. PLE takes advantage of the increased analyte solubility and extraction kinetics at higher temperature to speed the extraction process and reduce solvent consumption versus traditional methods [15]. Despite their advantages, PLE has not become a popular technique in analytical chemistry. In a review, Runnqvist et al discuss some lacks of information about PLE settings optimization [16].

PLE has been mostly applied to environmental samples as plants, sediments, soil, sludge and manure [17–20]. Just few reports using PLE to extract polar and moderate polar drugs from animal tissues were published in the last years [21–25]. Recently, two methods using PLE to sulfonamide residues analysis in biological and environmental samples were reported [11,26]. García-Galán et al developed and validated a method able to analyze 22 sulfonamide residues in soil and sewage sludge, using PLE followed by hydrophilic-lipophilic balance solid phase extraction (SPE) cartridges [11]. Yu et al used the same approach (PLE-SPE) to determine 18 sulfonamides in muscle, kidney and liver of bovine, swine and poultry [26].

Generally, PLE produces semi-purified extracts. Thus, these extracts must be submitted to a further purification procedure, commonly by using SPE. Several reports show the use of PLE followed by SPE, being. Oasis HLB SPE cartridges the most frequently used [11,27–30].

Other suitable technique is ultrasound-assisted extraction (USE). The use of this technique in the analysis of food and environmental samples was recently reviewed [31,32]. The overall advantage of this technique is the feasibility of extracting several samples simultaneously. Moreover, the extraction process can be performed using an ultrasound bath, which is a simple apparatus

present in virtually all analytical laboratories. Despite that, only one report using USE for sulfonamides analysis in food was published in recent years [33].

In the present work, we developed and validated two extraction methods for sulfonamide residue analysis in several matrices of animal origin: a fully-automated PLE and an ultrasound-assisted method, both without the need of further SPE purification. After extraction, samples were analyzed by HPLC-(ESI)-QqLIT-MS/MS. The methods were validated according to the Commission Decision 2002/657/EC in terms of precision, sensitivity, decision limit (CCα) and detection capability (CCβ), among other performance parameters [34].

Material and Methods

Chemicals

Analytical standards with high purity (≥99%) were obtained from Sigma-Aldrich (St Louis, MO, USA) namely sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxazole (SMA), sulfamethoxypyridazine (SMPZ), sulfadiazine (SDZ), sulfapyridine (SPY), sulfadimethoxine (SDMX), succinyl-sulfathiazole(S-STZ), sulfaguanidine (SGA), sulfacetamide (SCA), sulfabenzamide (SBZ), sulfanitran (SNT), sulfisomidin (SIM), sulfamethizole (SMTZ), sulfaquinoxaline (SQX), sulfathiazole (STZ), sulfaisoxazole (SIX) and sulfadoxin (SDX).

The metabolite N_4-acetyl-sulfamerazine (AcSMR) and the isotope labelled compounds d4-sulfamethoxazole (d^4-SMA), d^4-sulfamethazine (d^4-SMZ) and d^4-sulfadiazine (d^4-SDZ), used as surrogate and/or internal standards, were purchased from Toronto Chemical Research (North York, Ontario, Canada).

Hydroxyl-sulfaquinoxaline (SQX-OH), N_4-acetyl-sulfaquinoxaline (AcSQX) and N_4-acetyl-hydroxyl-sulfaquinoxaline (AcSQX-OH) were obtained from equine liver extract further purified using HPLC-DAD analysis, based on peak purity evaluation and also by high resolution mass spectrometry, as described elsewhere [35].

Water, acetonitrile (ACN), methanol (MeOH), hexane and acetone of HPLC-grade were supplied by J. T. Baker (Deventer, The Netherlands). Ethyl acetate was from Merck (Darmstadt, Germany). Formic and acetic acid and sodium chloride (NaCl) were obtained from Sigma-Aldrich. Diatomaceous earth (Hydromatrix®) was supplied by Agilent Technologies.

Individual stock standard solutions were prepared in MeOH: acetone (50:50) at 1 mg mL^{-1} and stored at -4°C until use. Standard solutions of the mixtures of all compounds at appropriate concentrations were prepared by dilution of the individual stock standard solutions in MeOH or acetone.

Samples

Samples of ovine (muscle, liver and kidney), poultry (liver), equine (liver) and fish (muscle) were obtained from the Federal Inspection Service (SIF) of the Ministry of Agriculture, Livestock and Food Supply of Brazil (MAPA). Samples were frozen (-20C) until arrival at the laboratory. Following, a representative portion of each sample was freeze dried (-40°C and -0.044 mbar vacuum).

Extraction and clean-up – PLE method

Samples were extracted by PLE using an ASE 350 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA). Samples (0.5 g) were grinded and homogenized in order to decrease particle size and promote better interaction with solvents. Prior to extraction, d^4-SMA, d^4-SMZ and d^4-SDZ were added to the sample as surrogate standards at a concentration of 100 ng g^{-1}. Samples were mixed with diatomaceous earth dispersing agent in order to avoid particle clumping and to reduce the interstitial volume of the PLE cells. Prior to extraction, samples were submitted to a clean-up method in order to remove the lipids by using hexane as solvent. PLE conditions were as follows: temperature 60ºC, 2 cycles of 5 min each one, 5 min static time, pressure 1500 psi. Total flush volume of 80% and 300 s of purge time with nitrogen flow were applied.

After that, the same PLE cells with the samples were submitted to a second PLE run (extraction method). To optimize the extracting solvent composition and the extraction temperature, a central composite design experiment was performed (see Results and Discussion). Optimal extraction solvent was the following: ACN with 0.2% acetic acid. The optimized extraction temperature was 90ºC. A preheating period of 8 min was chosen and 3 cycles of 7 min each were carried out. A total flush volume of 80% and 60 s of purge with nitrogen flow were applied. Pressure was set at 1,500 psi as it has been demonstrated that this parameter is not decisive in PLE.

The obtained PLE extracts were maintained in a freezer by one hour (at approximately -18ºC). Following, samples were centrifuged at 3500 rpm for 10 min in a 5810 R centrifuge (Eppendorf). The supernatant was evaporated at 40°C under nitrogen flow using a Turbo-Vap system (Zymark) until dryness. Extracts were redissolved in 1.0 mL of the HPLC mobile phase (water-ACN, 85:15) and transferred to an HPLC vial.

Extraction and clean-up – USE method

Samples (0.5 g) were weighted in polypropylene centrifuge tubes of 15 mL and spiked as previously described for the PLE extraction method. Following, 10 mL of ACN was added and tubes were mixed in a mechanical vortex by approximately 10 s. After that, all samples were placed into an ultrasonic bath by 60 min. After the extraction time, samples were stored in the freezer (-18°C) by 1 h to promote protein precipitation. Then, samples were centrifuged at 3500 rpm for 10 min, the supernatant was brought to dryness at 40°C under nitrogen flow. The extracts were redissolved in 2.0 mL of the HPLC mobile phase (water-ACN, 85:15). An aliquot of 2 mL of hexane was added to remove the fat content. Tubes were mixed in a vortex by approximately 5 s followed by centrifugation (3500 rpm for 10 min). The lower layer was carefully transferred to an HPLC vial.

Instrumental analysis

Sulfonamide separation was performed in a Symbiosis™ Pico System (Spark Holland, Emmen, The Netherlands), equipped with a HPLC system consisting of an Alias™ autosampler, a loop injector and two binary pumps with a four-channel solvent selector for each one. Chromatographic separation was performed using a HPLC column Purospher® STAR (C18, ec, 150 x 4.6 mm, 5 µm) preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL min^{-1}, being eluent (A) HPLC grade water acidified with 10 mM of formic acid, and eluent (B) ACN with 10 mM of formic acid. The elution gradient started with 25% of eluent (B), increasing to 80% in 10 min and to 100% in 11 min. During the next 2 min the column was kept at 100% (B), readjusted to the initial conditions in 3 min and equilibrated for 7 min. MS/MS analyses were carried out in a 4000 QTRAP hybrid triple quadrupole-linear ion trap-mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray ionization source working in the positive mode (ESI+). The optimization of the MS/MS experimental conditions was carried out in previous studies [11,36,37]. For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring (SRM) mode, selecting the two most abundant transitions precursor ion/product ions. The optimal MS/MS parameters are listed in Table 1.

Table 1. Optimized MS/MS detection parameters.

Results and discussion

PLE optimization

Despite PLE has been considered an useful extraction technique in analytical chemistry, some drawbacks have reduced its applicability. Main limitations are the expensive cost of the equipment and the usual need for complementary clean up and/or concentration steps. Moreover, the optimization of a PLE method is time consuming. In order to improve the development and enhance the yield of extraction, a central composite design experiment to statistically evaluate the major parameters in PLE was performed.

As all samples included in this study showed high lipids content in a first attempt the PLE extracts obtained were refrigerated, centrifuged, evaporated and then were submitted to a liquid-liquid extraction (LLE) with hexane to obtain defatted extracts.

In order to avoid additional steps and to provide a higher degree of automation to the method, two PLE methods were tested successively in which the same cell was submitted to clean-up, extraction and elution, according to the solvent used in fully automated mode. Hexane was selected as lipids extracting solvent since sulfonamides are virtually insoluble in hexane. The hexane PLE deffating method was evaluated using 2, 3 and 4 cycles. The results (see Figure 1) shown that the number of cycles gave practically equivalent results, although 4 cycles removed approximately 16% of sample dry weight in fat content. However, extracts obtained with only 2 cycles did not present apparent fat and were clear enough to be directly injected after the evaporation step. The hexane phase was evaporated until dryness and redissolved in the HPLC mobile phase to evaluate potential losses of analytes. None detectable signal was observed in the corresponding chromatograms.

Figure 1. Fat removal yield using PLE with hexane as extracting solvent. Results are expressed in percentage of sample weight.

After lipids removal, the same PLE cells were submitted to an extraction method. Two solvent mixtures were initially evaluated: MeOH and ACN. Both solvents were separately tested, mixed in some degree with water. The obtained PLE extracts were further purified using a salting-out assisted liquid-liquid extraction (SALLE). . To each extract (around 20-25 mL), a certain amount of NaCl was added in order to obtain approximately 1.0 moL L^{-1}. An aliquot of 5 mL of ethyl acetate was added and the tubes were vortexed and centrifuged at 3500 rpm for 10 min. The upper phase (organic layer) was collected using a Pasteur pipette. This organic extract was evaporated at 40°C under nitrogen until dryness. Extracts were redisolved in 1.0 mL of the HPLC mobile phase and transferred to an HPLC vial.

For samples extracted with pure MeOH or ACN, the final LLE was unnecessary. In this case, samples were evaporated to dryness and then reconstituted in 1.0 mL of the HPLC mobile phase and transferred to an HPLC vial.

As expected, MeOH extracts were not able to be submitted to the SALLE procedure, as reported by other authors [38,39]. Moreover, pure MeOH extracts were much more turbid than pure ACN extracts. Thus, ACN was chosen as the solvent to perform the extraction step.

Optimization using a central composite design

Univariate optimization procedure is based in varying “one variable at-a-time”. This approach does not guarantee a real approximation from optimal conditions. For PLE extraction optimization, a central composite design (CCD) was applied. Control variables were ACN percentage in water (%) and temperature of extraction (°C). The response variable was sulfonamide peak area. Table 2 shows the experimental design, including 4 axial points and 3 replicates for the center point. Center point conditions were established as the initial extraction levels obtained in the solvent selection stage. Data analysis and mathematical models construction were processed using Minitab 16 statistical software (Minitab, State College, PA, USA) and Design-Expert 7.0.0 (Stat-Ease, Minneapolis, MN, USA). Raw data were tabled and regression analysis was performed. Mathematical models were validated using ANOVA. Thus, contour plots for surface responses data were plotted. The results were very similar for all analytes, as can be observed in Figure 2. Data show that most intense peak areas were obtained using high percentages of ACN and lower temperatures. As the optimal conditions were not achieved, a second CCD was performed, now using a lower range of temperatures (48 to 132°C) and pure ACN with acid additive as extraction solvent (acetic acid from 0.08 to 0.92%). Table 3 shows this second experimental design. The use of acid as additive was based on our previous report published elsewhere [40]. A simple experiment was included to compare the extraction efficiency with and without the acid additive in the ACN, using 3 samples spiked at MRL level. Figure 3 shows the differences observed in the extraction yield. The results obtained in the second CCD were much more

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

variable, showing that SFAs with a group attached to the aniline moiety, succinyl-STZ and N_4-acetyl-SMR, has an increasing signal at lower temperatures and higher amounts of acid additive. Some analytes were more affected by changes in control variables (e.g. SQX and STZ), whereas the major fraction of analytes shown low signal variability. In order to obtain a compromise between higher extraction yield and analytes response, we chosen to use 90°C and 0.2% of acetic acid in the PLE extraction method. Figure 4 presents some examples of the analytes which showed heterogeneous response in the second CCD experiment.

Table 2. First experimental design for PLE optimization.

Table 3. Second experimental design for PLE optimization.

Figure 2. Surface plot example for first CCD corresponding to sulfathiazole.

Figure 3. Peak area comparison for extraction with pure ACN (black bars) and ACN with acetic acid (grey bars).

Figure 4. Surface plot examples for second CCD corresponding to sulfaquinoxaline.

Method validation

Method validation is absolutely necessary in residue analysis, because of the important role in statutory programs involved in international trade of commodities. European Union (EU) has issued a specific regulation (Commission Decision 2002/657/EC) concerning the performance of analytical methods and the interpretation of results in the official control of residues in products of animal origin. According to this, several parameters must be calculated as for instance limit of decision (CCα) and detection capability (CCβ). In the present study, the HPLC-MS/MS methods were validated in accordance with Commission Decision 2002/657/EC: method performance parameters were determined and evaluated using samples of liver spiked with the appropriate

volume of the standard solution of sulfonamides at various concentration levels. The parameters studied included linearity, accuracy, precision, specificity, matrix effects, besides the parameters CCα and CCβ. The linear response was assessed using standard solutions injected three times, covering the range 25-400 ng mL^{-1}. The calibration curves were constructed using the ratio [peak area of analyte/area of internal standard peak] versus the concentration of analyte. Precision and accuracy were determined by the analysis of samples spiked at three concentration levels (50, 100 and 150 ng mL^{-1}). The intra-precision test was carried out through seven measurements in replicate for the three concentration levels, whereas the inter-precision test was performed during the execution of three batches into three consecutive days. Although the method was applied to several tissues (muscle, kidney, fish), liver was chosen as the matrix for validation studies because of it was the most complex matrix among all the analyzed samples.

Decision limit (CCα) and detection capability (CCβ)

The decision limit, CCα, and the detection capability, CCβ, were calculated plotting all data obtained from the precision determination and applying the calibration curves approach as described in the Commission Directive 2002/657/EC and also in accordance with the ISO 11843. Briefly, the signal was plotted against the added concentration and the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility gives the CCα values. CCβ were calculated taking the concentration at the CCα plus 1.64 times the standard deviation of the within-reproducibility of the mean measured content at the lowest concentration level. Table 4 reports the CCα and CCβ values for both PLE and USE methods. Although these parameters do not present criteria for upper limits, some sulfonamides present values considered unacceptably high and were removed from the method. That was the case for STZ in the PLE method and SIM in the USE method.

Table 4. Validation data for sulfonamides in liver by PLE and USE: decision limits (CCα) and detection capability (CCβ). Bold numbers means the lower values for each sulfonamide.

Determination of limit of detection (LOD), limit of quantification (LOQ) and linearity

Considering that the mathematical approach for LOD and LOQ determination using the deviation of blank samples resulted in improbably low values, these parameters were established using data from spiked samples. To carry out the experimental determination of the lowest concentration detectable as required by guidelines for implementation of the Commission Decision (LOD and LOQ), calibration curves with lower concentrations than those used in previous tests (0.10 and 0.25 × MRL) were analyzed. The lowest spiked points were correctly identified and quantified. Based on these experimental data, LOD and LOQ were defined as 5% and 10%, respectively, of the MRL for each compound for both PLE and USE extraction methods. Tables 5 and 6 show correlation coefficients and linearity data that match the internal criteria of our laboratory ($r > 0.95$ for matrix-matched calibration curves) for PLE and USE methods, respectively. To define the relationship between concentration and analytical response, a calibration curve with five levels of concentration, discounting the zeros, was prepared for quantification of each matrix studied. For linearity, a matrix-matched curve with nine levels of concentration was analysed, being linear into the studied range (10 to 400 ng g^{-1}).

Table 5. Validation data for sulfonamides in liver by PLE: linearity, LOD and LOQ.

Table 6. Table 2. Validation data for sulfonamides in liver by USE: linearity, LOD and LOQ.

Specificity

Blank samples (n=20) were tested for verification of interference, using both PLE and USE extraction procedures. No significant difference in retention times of analytes and internal standard were observed. Typical results for blank samples (for both extraction methods) are shown in Figure 5.

Figure 5. Total ion chromatogram (TIC) for blank extracts of the PLE (A) and USE (B) extraction methods.

Recovery and matrix effect estimation

Relative recoveries were determined using the approach proposed by Matuszewski to quantitative estimation of matrix effects, as described elsewhere [41,42]. Results shown in Table 7 demonstrate that PLE method and USE method provide similar recoveries and matrix effect values. Matrix effects are highly intense in both PLE and USE method. Moreover, both methods have considerable losses in extraction process, which results in low recovery values. This fact lead us to the use of isotope labelled internal standards associated with matrix-matched calibration curves, in which standard solutions were added in the beginning of the analysis and suffer all the extraction and concentration process. This approach takes into account the many variables present in these matrices and is adequate for both extraction procedures. Since this method is based on internal standardization, recovery values are not considered for calculations. A detailed matrix effects report comparing several approaches using the data from PLE and USE methods is recently submitted to publication.

Table 7. Matrix effect estimation and relative recovery values for PLE and USE methods.

Precision, accuracy and reproducibility

Precision and reproducibility data are summarized in Tables 8 (PLE) and 9 (USE). The accuracy for each concentration is also included. Accuracy was determined using a comparison between the calculated concentration and the analyte amount added to the sample in the spiking procedure.

Table 8. Validation data for sulfonamides in liver by PLE: precision and accuracy results (n=21 for each level).

Table 9. Validation data for sulfonamides in liver by USE: precision and accuracy results (n=21 for each level).

Application to real samples

Both validated methods were used to analyze real incurred samples, which contain SQX and some metabolites. Results were show in Table 10. Sulfaquinoxaline was correctly detected using both techniques, although a significant difference between calculated concentrations was observed. In the case of ovine liver samples, which were previously analyzed in a sulfonamide residues method with ISO 17025 accreditation and used for routine analysis in our laboratory since 2009 [37], USE method provided closest results than PLE method. However, the use of both methods in a proficiency test to sulfonamides residues analysis in liver is still necessary to perform a more precise comparison. Figure 6 shows the extracted ion chromatogram for the presence of SQX and some metabolites in ovine kidney using USE extraction. Albeit SQX metabolites could not be determined because standards were not available, the SQX metabolites can be qualitatively detected using the current method. The optimization of the MS/MS determination parameters for SQX metabolites was performed using a semi-purified equine liver extract.

Table 10. Calculated SQX amount in naturally incurred samples using PLE, USE and a reference method.

Figure 6. Extracted ion chromatogram for the presence of SQX (A), SQX-OH (B), AcSQX (C) and AcSQX-OH (D) in ovine kidney using USE extraction method.

Methods comparison

In general terms, both methods were able to correctly extract and determine more than 15 sulfonamides residues in tissues. PLE and USE extraction procedures give similar performance to several parameters.

Some more hydrophilic sulfonamides as SGD, SNT and S-STZ were recovered in low yields and with an unacceptable precision. Thus, those compounds were removed from the method scope. The majority of the analytes show similar responses in terms of linearity, precision and accuracy for both extraction methods. Interestingly, STZ could not be satisfactorily determined using PLE method and was removed from this method scope. A similar behaviour was demonstrated for SIM responses when the USE method was used. As regards the detection and quantification limits, these parameters were firstly estimated using the methods of noise standard deviation of blank samples and the mathematical approach based on calibration curves. As experienced previously in other methods, the first method produced values unrealistically lower and the calibration curve also resulted in levels unrealistically higher. Thus, LOD and LOQ were determined using real spiked samples as described before. Although the established LOD and LOQ (10 and 25 ng g^{-1}, respectively) can be seen as relatively high, these values were considered as satisfactory, taking into account that the methods do not include an SPE procedure as additional purification step. In other words, a compromise between sensitivity and feasibility of the methods was chosen.

Naturally incurred samples were analyzed using both the PLE and the USE developed methods. Previously, those samples were analyzed using a reference method. The USE method showed results closest to those achieved by the reference method, which use conventional extraction with ACN followed by clean-up with sodium sulphate and concentration of organic extract [37]. Thereby, and also considering the fastness, simplicity and low cost of USE, this approach was considered as the better method of choice for sulfonamide residue analysis in animal tissues.

Conclusions

Two new extraction methods for sulfonamides residues determination in biological samples were developed, optimized and validated. All figures of merit were established, as decision limits, detection capability, accuracy, precision and linearity. Both PLE and USE methods are suitable as routine methods, although USE method appears to be more efficient and easier to perform. Results lead us to appoint PLE and USE as useful extraction techniques for the trace analysis of sulfonamides and metabolites. However, still remain the need to explore more deeply the potentialities of both techniques for drug residue determination in other in biological samples.

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Table

Table 1. Optimized mass spectrometry detection parameters. Bold SEM transtitions are used for quantitative analysis.

Sulfonamide	$[M+H]^+$	SRM	Declustering potential (V)	Collsion energy (V)	Collision cell exit potential (V)	Internal standard	Rentention time (min)
SCA	215	**215>156**	46	21	10	SDZ-d4	9.6
		215>92	46	35	6		
SIM	279	**279>124**	76	33	8	SDZ-d4	10.3
		279>186	76	23	14		
STZ	256	**256>156**	40	25	14	SDZ-d4	14.8
		256>92	40	25	10		
SDZ-d4	255	**255>160**	46	27	10		14.9
		255>96	46	30	8		
S-STZ	356	**356>256**	71	25	16	SDZ-d4	15.0
		356>192	71	33	16		
N^4-SMR	307	**307>134**	60	35	8	SDZ-d4	15.1
		307>110	60	35	8		
SGD	215	**215>156**	56	13	10	SDZ-d4	15.1
		215>108	56	31	4		
SDZ	251	**251>156**	46	27	10	SMZ-d4	15.1
		251>92	46	30	8		
SPY	250	**250>156**	51	28	12	SMZ-d4	15.2
		250>92	51	31	6		
SMR	265	**265>92**	61	47	6	SMZ-d4	15.6
		265>156	61	27	8		
SMTZ	271	**271>156**	36	23	12	SMZ-d4	15.6
		271>108	36	23	8		
SMZ-d4	283	**283>160**	26	30	8		15.7
		283>96	26	35	4		

SMPZ	281	**281>156**	66	27	14	SMZ-d4	15.8
		281>126	66	27	12		
SMZ	279	**279>156**	26	30	8	SMZ-d4	15.9
		279>124	26	35	4		
SQX-OH	317	**317>156**	76	25	10	SMZ-d4	16.3
		317>108	76	47	12		
SMA-d4	258	**258>160**	56	25	10		16.8
		258>96	56	27	10		
SDX	311	**311>156**	46	29	12	SMA-d4	16.9
		311>92	46	45	4		
SMA	254	**254>156**	56	25	10	SMA-d4	17.0
		254>108	56	27	10		
SIZ	268	**268>156**	71	21	10	SMA-d4	17.1
		268>113	71	21	8		
SQX	301	**301>156**	76	25	10	SMA-d4	17.3
		301>108	76	47	12		
SDMX	311	**311>156**	76	31	8	SMA-d4	17.5
		311>92	76	31	6		
SBZ	277	**277>156**	56	17	10	SMA-d4	17.6
		277>92	56	41	6		
SNT	336	**336>156**	66	17	12	SMA-d4	18.2
		336>158	66	29	14		

Table

Table 2. First experimental design for PLE optimization.

Sample	Categorization		Real values	
	T˚C	%ACN	T˚C	%ACN
1	-1	-1	100	20
2	+1	-1	140	20
3	-1	+1	100	80
4	+1	+1	140	80
5 (central point)	0	0	120	50
6 (central point)	0	0	120	50
7 (central point)	0	0	120	50
8 (axial point)	-1.41	0	91.7	50
9 (axial point)	+1.41	0	148.3	50
10 (axial point)	0	-1.41	120	7.6
11 (axial point)	0	+1.41	120	92.4

T˚C = temperaure; %ACN = percentage of acetonitrile in water.

Table

Table 3. Second experimental design for PLE optimization.

Sample	Categorization		Real values	
	T (˚C)	% acetic acid	T (˚C)	% acetic acid
1	-1	-1	60	0.2
2	+1	-1	120	0.2
3	-1	+1	60	0.8
4	+1	+1	120	0.8
5 (central point)	0	0	90	0.5
6 (central point)	0	0	90	0.5
7 (central point)	0	0	90	0.5
8 (axial point)	-1.41	0	47.58	0.5
9 (axial point)	+1.41	0	132.42	0.5
10 (axial point)	0	-1.41	90	0.08
11 (axial point)	0	+1.41	90	0.92

T :temperaure; % acetic acid: percentage of acetic acid in pure ACN.

Table

Table 4. Validation data for sulfonamides in liver by PLE and USE: decision limits (CCα) and detection capability (CCβ). Bold numbers means the lower values for each sulfonamide.

Compound	USE		PLE	
	CCα (μg kg^{-1})	CCβ (μg kg^{-1})	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
SMR	**119.3**	**138.6**	119.9	139.6
SMZ	122.5	144.9	**111.2**	**122.4**
SMA	125.1	150.2	**122.5**	**145.0**
SMPZ	124.9	149.7	**118.0**	**136.0**
SDZ	125.4	150.9	**120.5**	**141.0**
SPY	121.4	142.8	**114.2**	**128.3**
SDMX	133.6	167.1	**127.2**	**154.4**
SCA	**139.5**	**179.0**	161.4	222.8
SBZ	140.3	180.7	**134.7**	**169.4**
STZ	**132.1**	**164.3**	ND	ND
SMTZ	**142.7**	**185.5**	154.1	208.2
SQX	130.5	161.1	**129.6**	**159.3**
SIZ	128.5	157.1	**121.7**	**143.3**
SDX	**124.1**	**148.3**	124.4	148.9
N4-SMR	**138.4**	**176.7**	160.6	221.2
SIM	ND	ND	**152.9**	**205.8**

Table

Table 5. Validation data for sulfonamides in liver by PLE: linearity, LOD and LOQ.

Compound	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Linearity		
			Equation	*r*	RSD_r (%)
SMR	10	25	y=1.2478x – 0.105	0.99329	4.9
SMZ	10	25	y=0.8381x – 0.059	0.97533	5.8
SMA	10	25	y=1.0683x – 0.082	0.98920	11.3
SMPZ	10	25	y=1.7707x – 0.229	0.98371	2.4
SDZ	10	25	y=1.4485x – 0.0253	0.98560	3.6
SPY	10	25	y=1.0822x – 0.0662	0.99206	11.6
SDMX	10	25	y=3.6084x – 0.2738	0.98432	11.0
SCA	10	25	y=0.4932x – 0.0095	0.98287	17.3
SBZ	10	25	y=0.5028x – 0.0731	0.98036	18.2
SIM	10	25	y=2.3348x – 0.1152	0.98174	38.5
SMTZ	10	25	y=0.66398x – 0.0953	0.96309	6.4
SQX	10	25	y=1.3288x – 0.1037	0.98455	5.9
SIZ	10	25	y=0.8747x – 0.1281	0.97131	0.7
SDX	10	25	y=3.2228x – 0.2638	0.95470	5.7
N4-SMR	10	25	y=1.3565x + 0.3449	0.97670	16.4

Table

Table 6. Validation data for sulfonamides in liver by USE: linearity, LOD and LOQ.

Compound	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Linearity		
			Equation	*r*	RSD (%)
SMR	10	25	y=1.0298x + 0.0336	0.99512	1.2
SMZ	10	25	y=0.8356x + 0.0044	0.99582	1.1
SMA	10	25	y=1.1480x + 0.0447	0.99421	2.0
SMPZ	10	25	y=1.7707x – 0.229	0.98371	2.4
SDZ	10	25	y=1.6161x + 0.0802	0.99505	1.9
SPY	10	25	y=1.0830x + 0.0345	0.99325	1.1
SDMX	10	25	y=3.9274x + 0.2113	0.99165	2.0
SCA	10	25	y=0.4868x – 0.0314	0.97769	1.0
SBZ	10	25	y=0.5554x – 0.0233	0.93560	1.8
STZ	10	25	y=1.2153x + 0.6426	0.94848	2.3
SMTZ	10	25	y=0.4931x – 0.0737	0.91017	1.6
SQX	10	25	y=1.5243x + 0.0265	0.98953	2.5
SIZ	10	25	y=0.7934x + 0.0179	0.99430	0.8
SDX	10	25	y=3.6125x + 0.1117	0.99343	1.2
N4-SMR	10	25	y=0.9441x + 0.2720	0.97553	2.3

RSD: relative standard deviation for slope (n=3).

Table 7. Matrix effect estimation and relative recovery values for PLE and USE methods.

Analyte	PLE		USE	
	R_R (%)	ME (%)	R_R (%)	ME (%)
SMR	38	-80	42	-80
SMZ	33	-79	40	-78
SMA	32	-90	45	-89
SMPZ	24	-77	35	-77
SDZ	40	-87	45	-81
SPY	42	-83	41	-74
SMDX	28	-79	42	-79
S-STZ	39	-78	13	-73
SGA	18	-88	53	-86
SCA	28	-27	16	-6
SBZ	21	-96	45	-97
SNT	78	-90	49	-97
SIM	41	-76	22	-75
SMTZ	9	-85	8	-80
SQX	29	-86	35	-84
STZ	41	-63	29	-75
SIZ	23	-93	35	-93
SDX	29	-75	45	-74
N4-SMR	61	-65	57	-73

R_R = relative recovery; ME = matrix effects, as signal supression in percentage.

Table

Table 8. Validation data for sulfonamides in liver by PLE: precision and accuracy results (n=21 for each level).

Compound	Accuracy (%)	RSD_r (%)	RSD_R (%)	Accuracy (%)	RSD_r (%)	RSD_R (%)	Accuracy (%)	RSD_r (%)	RSD_R (%)
	50 µg kg^{-1}			100 µg kg^{-1}			150 µg kg^{-1}		
SMR	112	9.2	10.7	105	8.6	8.8	104	6.9	10.7
	110	13.5		106	9.0		101	10.3	
	101	5.4		115	7.0		110	13.2	
SMZ	112	6.4	9.7	98	7.5	6.5	98	4.3	4.7
	102	3.8		100	7.4		101	3.8	
	93	6.2		95	3.5		102	5.3	
SMA	110	12.9	15.1	109	7.8	11.6	97	4.4	9.6
	103	14.1		107	8.1		97	12.4	
	88	8.6		89	6.0		87	6.9	
SMPZ	97	3.8	4.5	104	6.5	7.6	97	8.4	9.9
	97	4.9		104	9.5		96	10.5	
	92	2.8		104	7.8		100	11.5	
SDZ	108	13.4	11.2	99	9.1	7.9	95	7.0	10.5
	113	8.2		100	8.4		102	13.6	
	109	12.7		99	7.5		90	4.0	
SPY	119	9.2	11.0	107	7.2	8.1	108	5.9	6.6
	113	8.0		100	5.7		105	7.9	
	99	6.1		116	3.8		111	5.6	
SDMX	119	11.3	12.0	119	14.7	18.0	104	5.4	11.1
	111	11.3		108	9.8		107	14.1	
	101	6.6		86	11.2		94	7.1	
SCA	120	13.6	22.6	92	18.1	28.6	86	11.5	33.5
	136	16.5		143	27.8		141	34.9	
	93	21.8		111	14.0		107	15.7	

SBZ	109	20.3	16.7	109	25.2	20.7	100	18.3	16.2
	95	9.5		88	9.4		88	13.5	
	96	14.9		89	13.3		95	15.4	
SIM	96	16.2	17.9	84	9.9	27.4	87	7.1	27.3
	115	16.0		96	14.6		96	30.0	
	126	12.2		143	13.3		128	21.6	
SMTZ	55	29.5	22.8	84	10.9	15.1	57	13.8	17.4
	76	10.6		88	14.5		88	14.4	
	61	16.0		89	19.9		63	22.7	
SQX	113	9.3	12.3	108	16.9	17.3	92	7.9	12.2
	111	11.4		100	10.3		100	15.7	
	95	8.1		82	9.6		87	11.5	
SIZ	103	8.4	8.4	98	17.9	12.3	85	6.7	9.0
	99	8.1		92	6.5		92	10.5	
	100	9.5		92	9.2		93	7.2	
SDX	113	7.1	12.1	109	15.0	13.9	91	6.4	10.3
	98	16.3		108	6.7		108	14.0	
	99	7.1		88	5.9		92	5.0	
N4-SMR	141	20.7	19.6	74	13.0	13.2	77	16.2	20.4
	139	16.1		79	7.2		79	27.4	
	126	22.6		82	17.3		82	14.6	

RSD_r : relative standard deviation for intra-day precision; RSD_R: relative standard deviation for inter-day precision.

Table

Table 9. Validation data for sulfonamides in liver by US: precision and accuracy results (n=21 for each level).

Compound	Accuracy (%)	RSD_r (%)	RSD_R (%)	Accuracy (%)	RSD_r (%)	RSD_R (%)	Accuracy (%)	RSD_r (%)	RSD_R (%)
	50 μg kg^{-1}			100 μg kg^{-1}			150 μg kg^{-1}		
SMR	120	13.6	22.6	92	18.1	28.6	86	11.5	33.5
	96	14.1		103	6.1		103	12.6	
	104	16.0		103	8.0		111	10.8	
SMZ	94	8.2	11.5	101	7.8	6.5	99	12.2	14.5
	96	11.6		100	6.6		109	16.1	
	98	15.0		100	5.9		107	15.0	
SMA	94	13.3	14.0	107	7.2	9.4	108	6.5	12.2
	96	14.0		106	9.2		113	13.8	
	90	16.1		104	12.5		109	16.2	
SMPZ	100	6.6	7.5	102	7.8	7.7	100	13.8	13.4
	105	7.3		104	7.4		108	13.8	
	105	8.7		105	9.0		103	13.7	
SDZ	93	9.1	12.9	103	6.5	6.5	105	10.9	13.3
	93	13.3		101	7.9		106	13.8	
	97	16.5		105	5.2		105	17.4	
SPY	91	8.6	11.7	105	9.3	8.1	101	10.8	11.1
	95	13.5		109	7.6		111	11.4	
	100	12.0		104	7.7		107	11.3	
SDMX	95	9.2	13.9	107	10.8	10.9	108	10.2	14.5
	97	15.3		109	7.9		115	15.5	
	90	17.1		105	14.7		111	18.9	
SCA	85	15.1	18.8	83	10.5	13.5	90	16.0	13.6
	91	20.7		89	12.7		91	14.0	

	93	21.2		85	17.9		96	12.5	
SBZ	107	18.4	22.8	102	25.3	18.9	108	15.2	19.7
	89	22.0		100	15.4		100	21.8	
	78	18.3		94	15.3		111	23.6	
STZ	54	40.0	41.7	91	11.0	12.7	102	14.2	24.0
	62	39.9		91	12.4		91	22.2	
	58	49.9		94	15.7		113	32.8	
SMTZ	96	22.7	17.7	87	23.8	21.1	81	19.3	19.3
	108	11.2		90	21.3		90	18.0	
	106	18.9		91	21.2		83	20.6	
SQX	97	11.2	12.3	103	7.5	9.6	106	11.6	20.4
	103	11.2		106	7.9		106	19.1	
	93	14.2		103	13.5		120	26.5	
SIZ	99	12.3	12.6	107	8.8	10.2	101	9.2	18.2
	94	11.8		106	8.5		106	16.4	
	94	14.8		104	14.0		110	25.9	
SDX	94	12.2	12.7	102	7.8	9.6	106	5.4	15.7
	94	13.7		103	10.0		103	13.1	
	90	13.7		103	11.8		115	23.2	
N4-SMR	97	16.5	24.6	98	10.9	13.8	107	13.0	15.9
	84	40.7		89	11.7		89	14.8	
	83	9.7		109	11.0		119	19.4	

RSD_r : relative standard deviation for intra-day precision; RSD_R: relative standard deviation for inter-day precision.

Table

Table 10. Calculated SQX amount in naturally incurred samples using PLE, USE and a reference method[a].

Sample	PLE (ng g^{-1})	USE (ng g^{-1})	Reference method (ng g^{-1})
Fish (*Astyanax* sp.).	25	15	19
Ovine kidney	325	284	295
Ovine muscle	17	7.5	12

[a].- Hoff et al., J. Chromatogr. A. 1216 (2009) 8254–8261.

Figure
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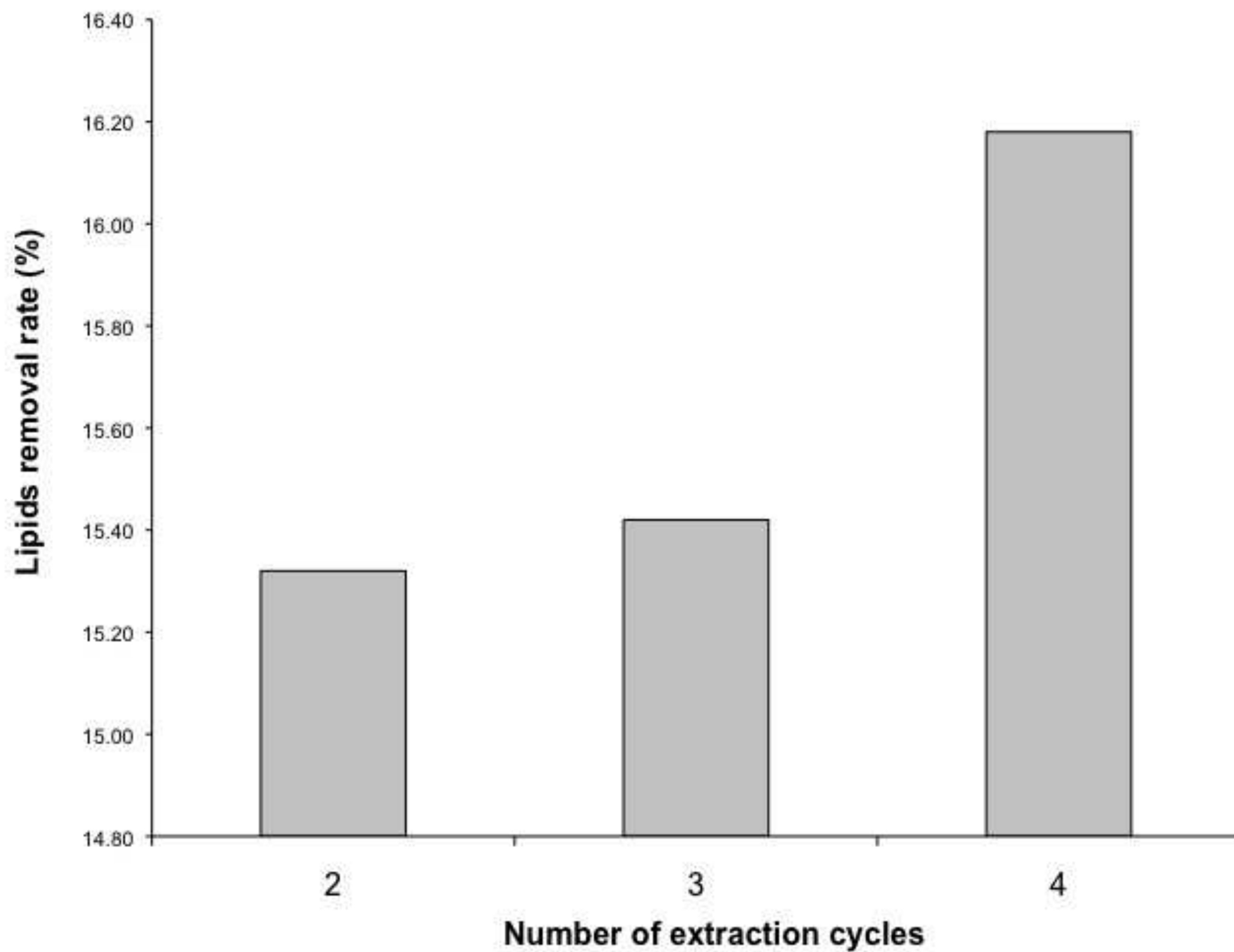

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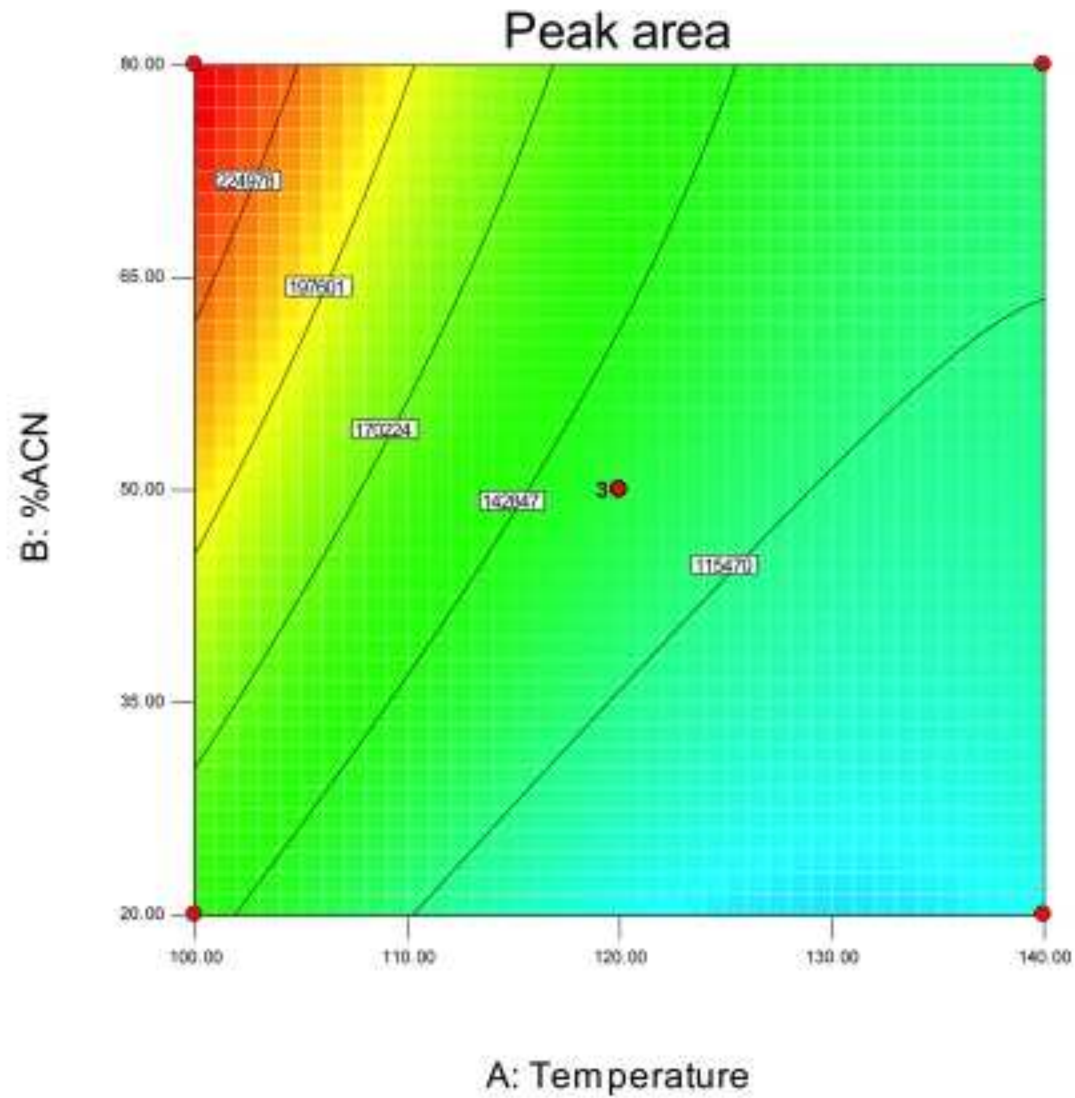
X1 = A: Temperature
X2 = B: %ACN



Figure
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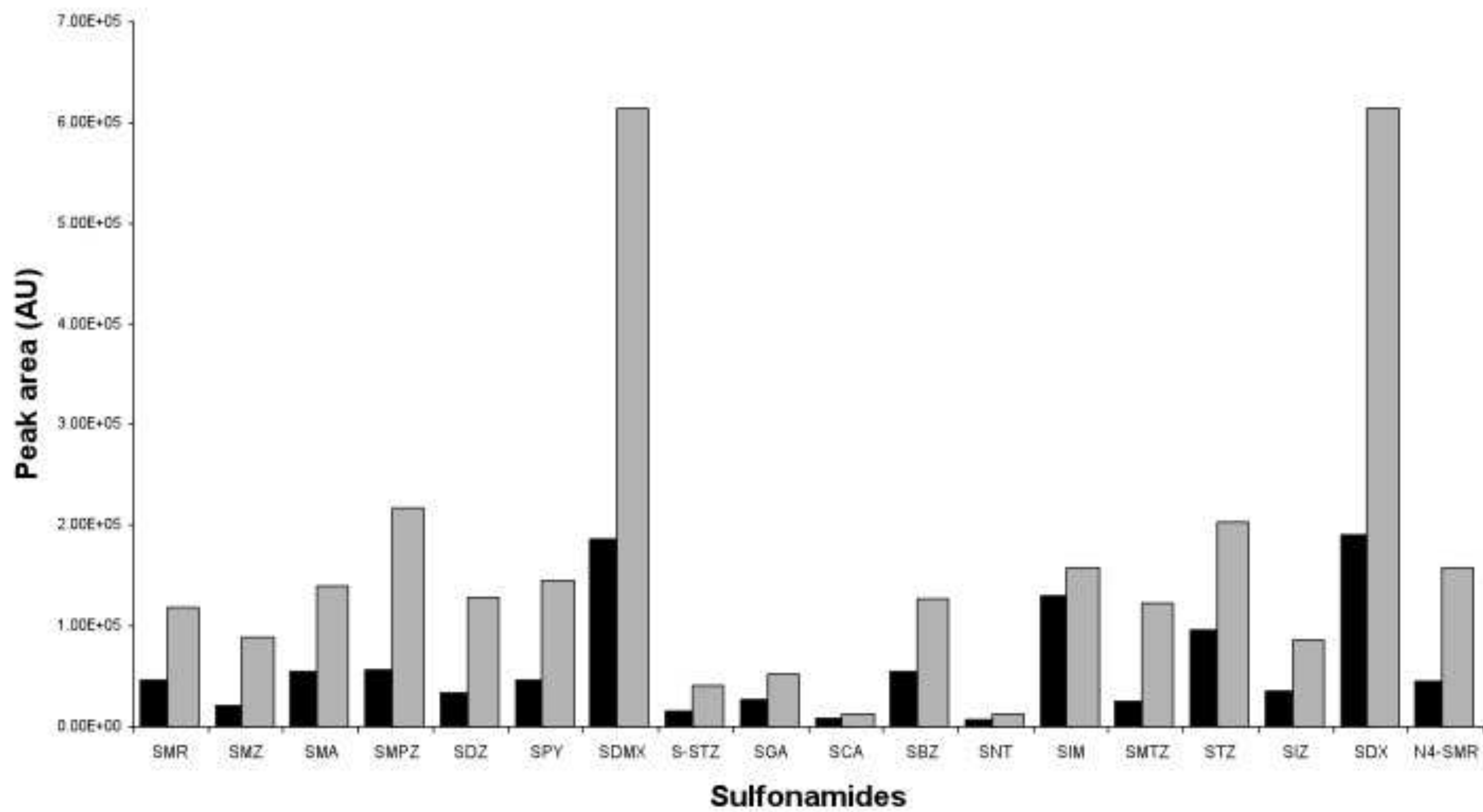

Figure

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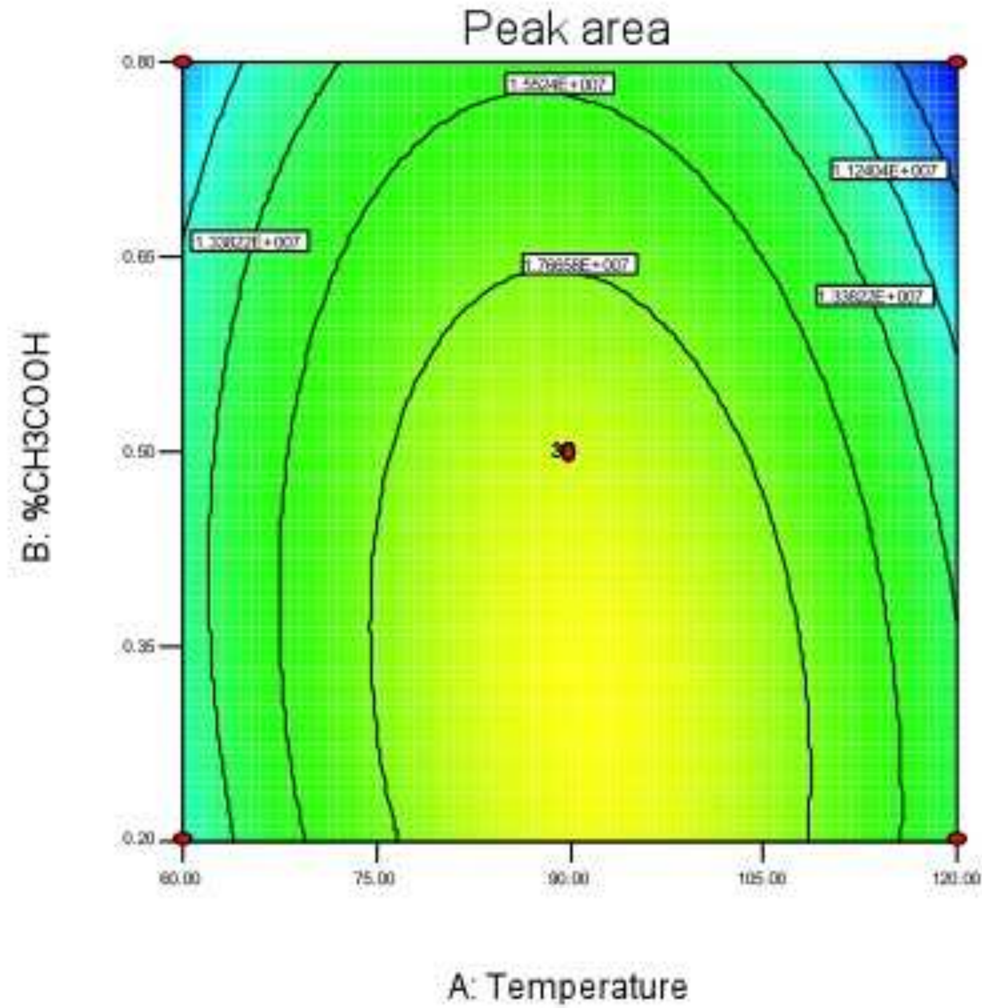
SQX



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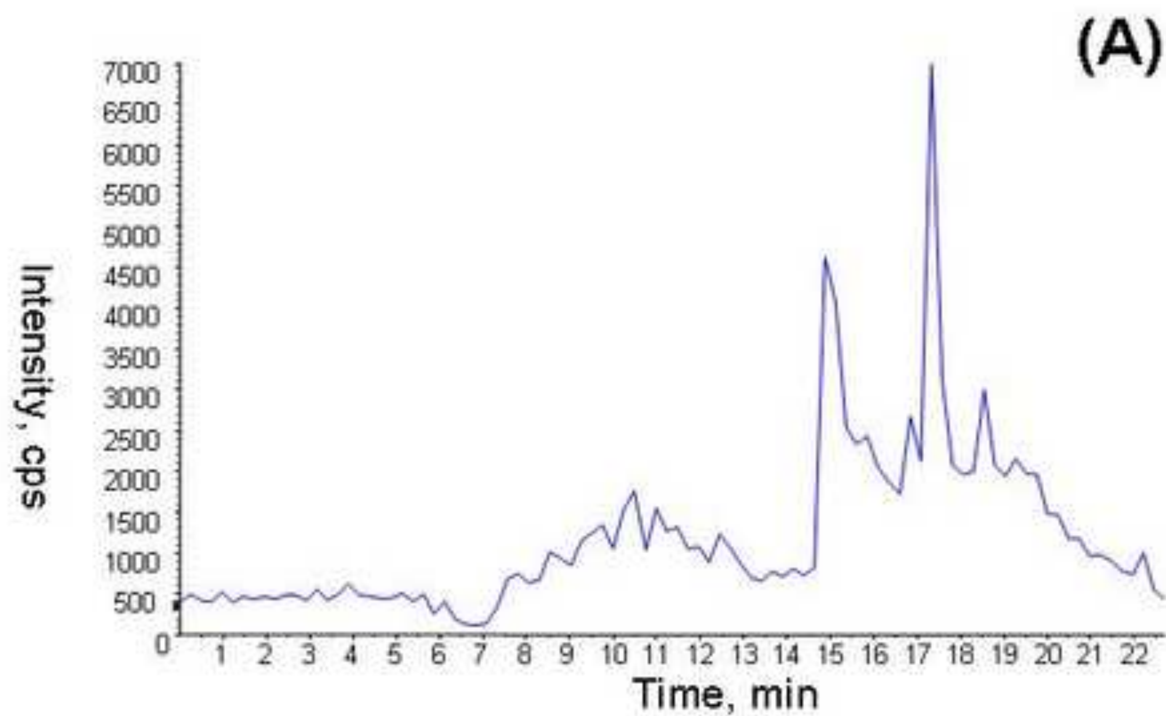

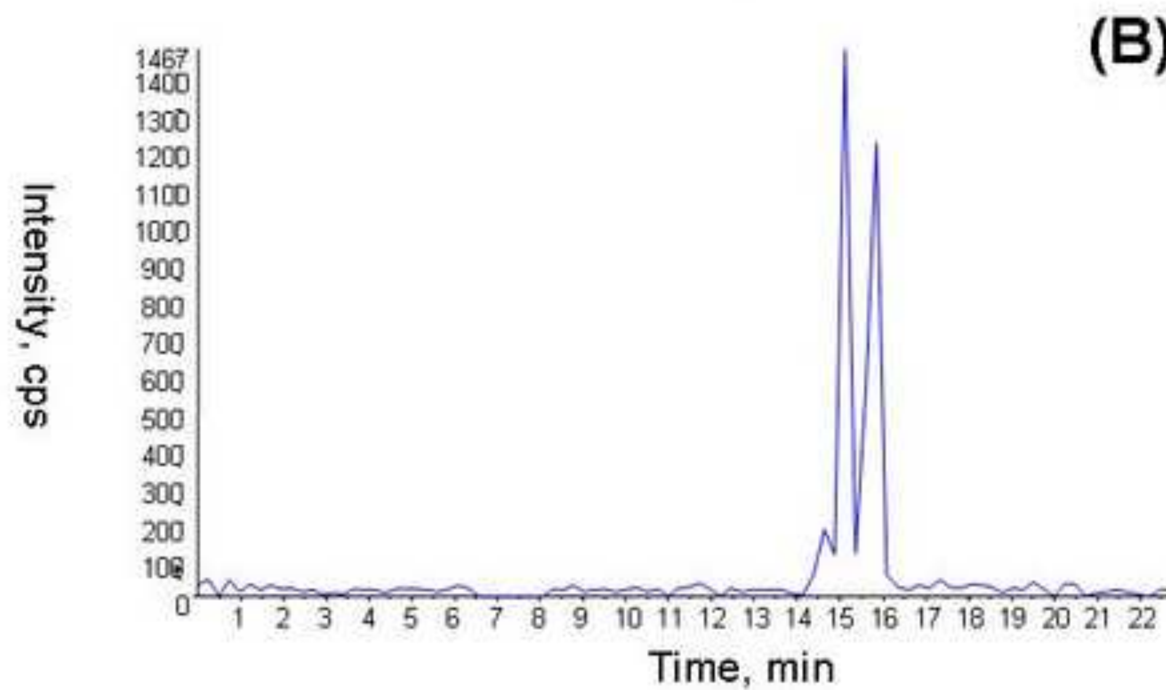

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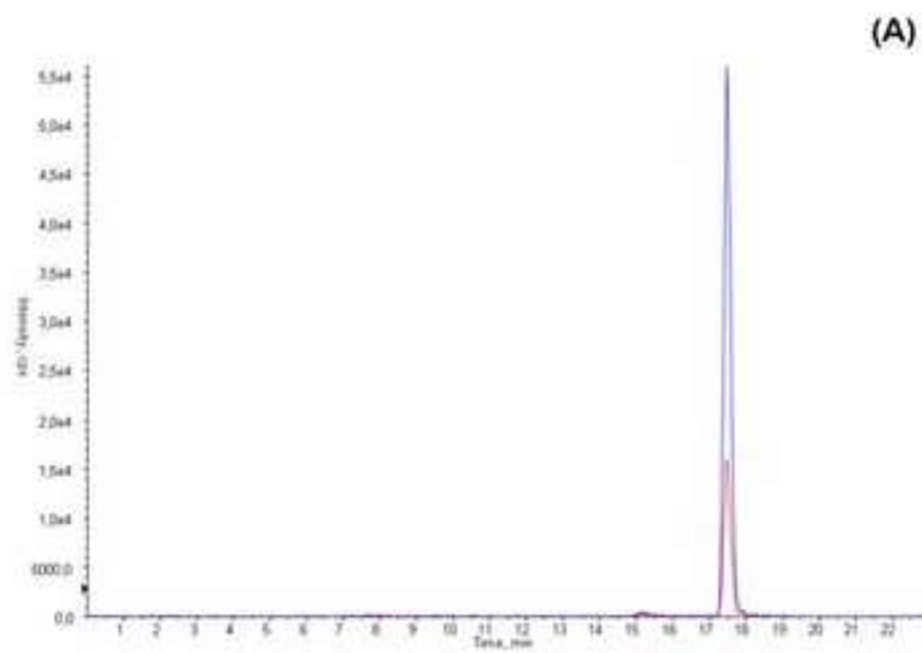

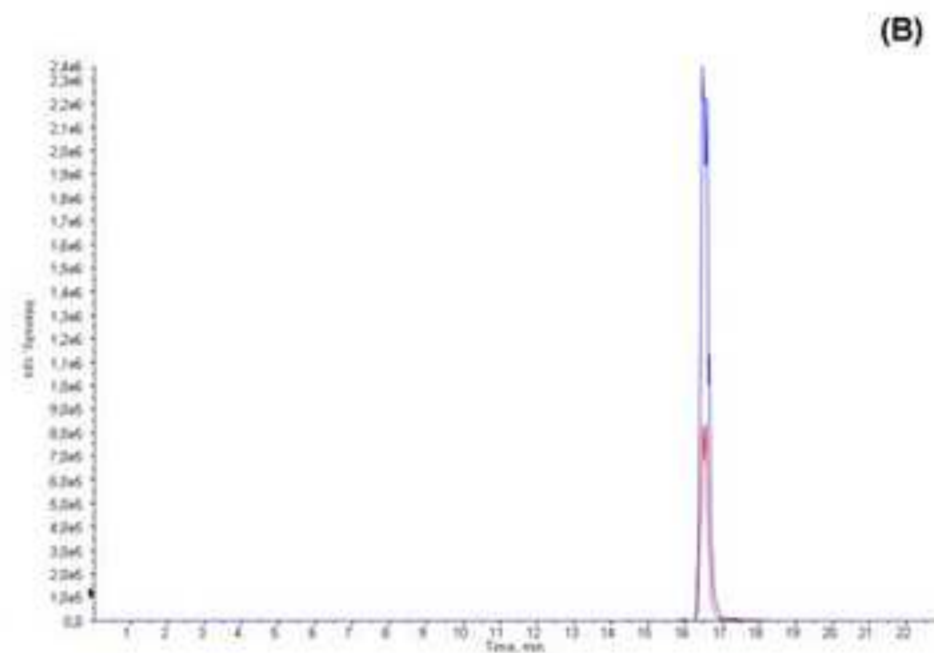

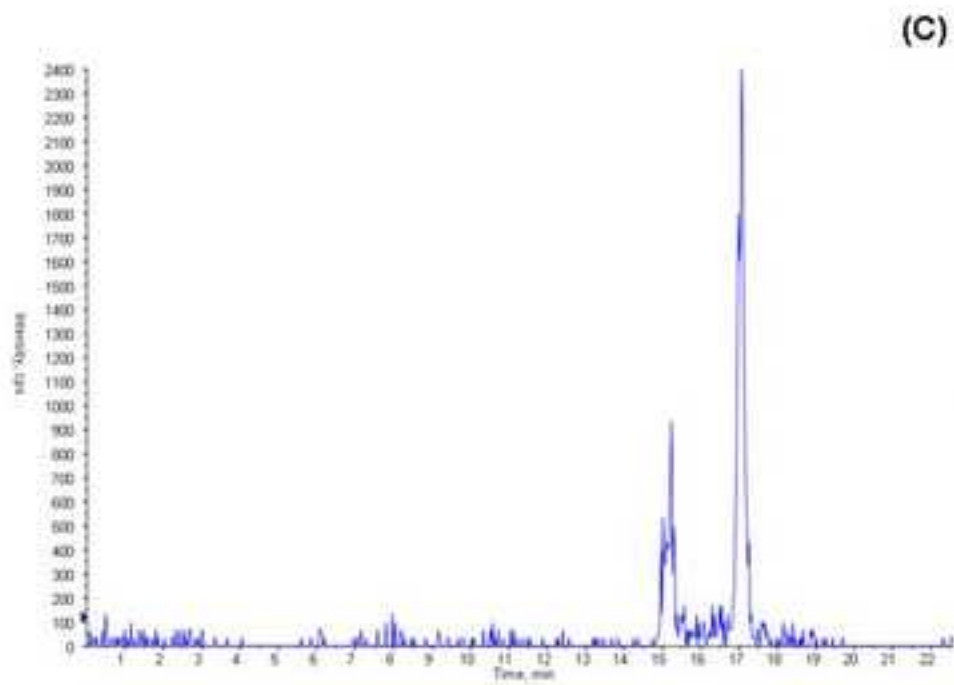

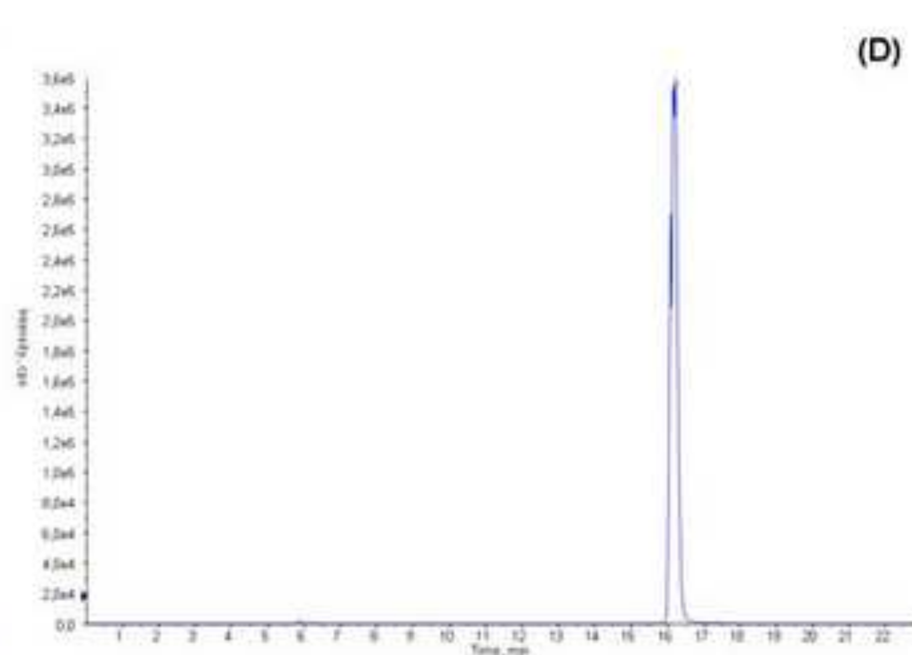

Conclusões gerais e perspectivas

Os dados do presente trabalho permitem as seguintes conclusões:

- A confirmação da presença de resíduos de medicamentos veterinários em alimentos e no meio ambiente reitera a grande preocupação, tanto no âmbito científico como no aspecto regulatório, visto que a presença dos mesmos gera implicações na saúde pública, no comércio internacional e no desequilíbrio do ecossistema;
- que existem vantagens e desvantagens nos diversos métodos para determinação de efeitos de matriz em espectroscopia de massas, implicando na proposição de um fluxo de trabalho de estimativa dos efeitos de matriz antes, durante e depois da validação de métodos de análise de resíduos de fármacos em alimentos através da associação das técnicas de cromatografia líquida e espectrometria de massas;
- que foi possível o desenvolvimento, a otimização e a validação de dois métodos de análise de resíduos de sulfonamidas em tecidos biológicos, usando extração por ultrassom ou por líquido pressurizado, os quais apresentaram eficiências similares, embora o método de ultrassom tenha demonstrado ser mais rápido e mais econômico em termos de execução;
- que as técnicas de cromatografia líquida de alta eficiência e de espectrometria de massas foram extremamente eficientes na identificação dos metabólitos de sulfaquinoxalina, uma das sulfonamidas mais utilizadas na avicultura e suinocultura brasileira, permitindo, pela primeira vez, a identificação dos mesmos em amostras de diversas espécies de tecido animal;.
- propor metodologia analítica, de aumento do número de compostos avaliados em um único procedimento analítico, no caso das sulfonamidas, em amostras ambientais e de produtos alimentícios;
- que as técnicas de espectrometria de massa de alta resolução são os métodos adequados para assinalar com a exatidão requerida as estruturas de metabólitos e produtos de degradação;

Com este trabalho foi igualmente possível apresentar uma revisão do estado-da-arte na análise de resíduos de sulfonamidas em amostras ambientais, traçando um panorama dos níveis de resíduos destas substâncias encontrados em diversos países. Igualmente, um modelo de priorização de substâncias a serem investigadas e monitoradas em alimentos e no meio ambiente baseadas em análise de risco e potencial de exposição foi elaborado.

Os resultados finais obtidos deixam como perspectivas de curto prazo a síntese dos metabólitos identificados de modo a realizar a avaliação completa das características físico-químicas, toxicológicas e farmacológicas destes compostos.