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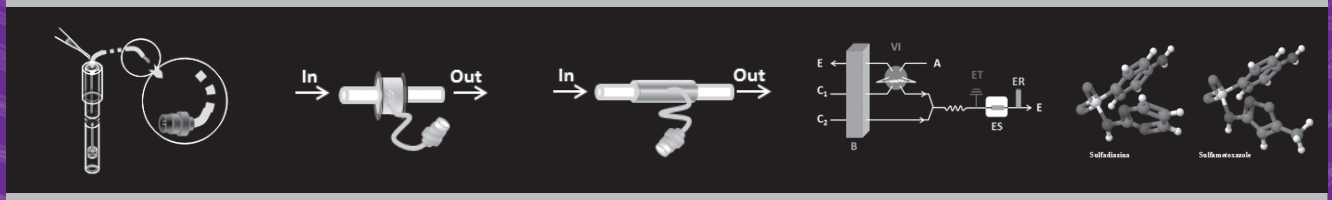
FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

(BIO)SENSORES PARA A DETECÇÃO/QUANTIFICAÇÃO DE ANTIMICROBIANOS EM ÁGUAS DE AQUACULTURA



Sofia Alexandra Alves Almeida

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(BIO)SENSORES PARA A DETECÇÃO/QUANTIFICAÇÃO DE ANTIMICROBIANOS EM ÁGUAS DE AQUACULTURA

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Trabalho realizado, sob orientação da Professora Doutora Maria da Conceição Branco da Silva de Mendonça Montenegro (REQUIMTE/Departamento de Ciências Químicas, Faculdade de Farmácia da Universidade do Porto) e co-orientação da Professora Doutora Maria Goreti Ferreira Sales (BioMark, Sensor Research/Instituto Superior de Engenharia do Instituto Politécnico do Porto), em parceria com o Departamento de Saúde Ambiental, Unidade de Água e Solo, Laboratório de Química e Toxicologia das Águas, do Instituto Nacional de Saúde Dr. Ricardo Jorge – Centro de Saúde Pública Dr. Gonçalves Ferreira.

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Aos meus pais, ao meu marido,

por caminharem comigo…

por estarem sempre presentes.

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“Vencer não é nada, se não se teve muito trabalho. Fracassar não é nada, se se fez o melhor possível”.

Nadia Boulanger

RESUMO

Os sistemas de aquacultura são fundamentais na nossa sociedade porque garantem um fornecimento regular de peixe às populações, preservando os recursos naturais no sector das pescas, muitas vezes já escassos, e garantindo a sobrevivência de espécies em risco. O aumento da produtividade está, contudo, associado à introdução de drogas que garantem o crescimento e a preservação das espécies, mas que acabam por se difundir para o meio aquático envolvente, promovendo alterações na biodiversidade e entrando, directa ou indirectamente, na cadeia alimentar. Quando estas drogas são agentes antimicrobianos de uso humano, como é o caso das sulfonamidas, existe um risco elevado de aparecimento de espécies bacterianas resistentes, algo que constitui uma grave ameaça à saúde pública.

A introdução de agentes antimicrobianos no meio aquático pelo sector da aquacultura pode ser minimizada através da monitorização contínua/regular dos níveis de antibiótico nas águas do sistema, tanto durante a sua aplicação, como antes da sua descarga para o meio aquático envolvente. Para este efeito, tornam-se necessários métodos analíticos de baixo custo que permitam uma frequência analítica elevada e contínua, nos tanques de cultivo dos peixes. Neste contexto, os eléctrodos selectivos de ião (ESIs) podem constituir uma preciosa ferramenta já que podem ser aplicados na análise de matrizes complexas, apresentando baixo custo e portabilidade e podendo também ser associados a metodologias automáticas, tal como a análise por injecção em fluxo (FIA, do inglês *Flow Injection Analysis*).

Neste trabalho, estudam-se os efeitos de diversos materiais sensores, comerciais ou sintetizados por técnicas de impressão molecular, na construção de (bio)sensores potenciométricos, apropriados para a monitorização de algumas das sulfonamidas mais utilizadas no sector da aquacultura. Estudam-se ainda diversos modos da sua adequação a sistemas de fluxo, incluindo configurações tubulares em sistemas FIA e planares em suportes cerâmicos de microfluídica. A possibilidade de redução significativa dos limites de detecção dos eléctrodos foi também objecto de estudo nesta dissertação. Todas as vertentes metodológicas desenvolvidas foram aplicadas, com sucesso, à análise de amostras de água de aquacultura.

Face aos resultados obtidos, considera-se que os vários métodos propostos constituem alternativas vantajosas aos métodos habitualmente utilizados para a determinação de sulfonamidas, nomeadamente aqueles que envolvem técnicas separativas, nas quais os reagentes utilizados são, só por si, geradores de contaminação ambiental. Os métodos potenciométricos desenvolvidos usam, de uma forma geral, materiais de baixo custo e sistemas simples, fornecem uma resposta rápida e requerem uma reduzida intervenção do operador. Do ponto de vista do seu impacto no meio ambiente, são também muito menos poluentes do que a grande parte dos métodos usados para avaliação deste tipo de compostos.

Palavras-Chave: materiais (bio)sensores, potenciometria, análise por injecção em fluxo, aquacultura.

ABSTRACT

Aquaculture ensures a constant fish supply to the population, preserving the finite and scarce resources of fish and ensuring the survival of endangered species. The rising profits of this sector have been achieved by the introduction of several drugs that ensure fish growth and preservation. However, these drugs may spread to the aquatic environment, promoting changes in biodiversity and entering, directly or indirectly, in the food chain. When these drugs are antimicrobials of human use, as is the case of sulfonamides, resistant microbial species may occur and pose a serious threat to public health.

The introduction of antimicrobial agents in the aquatic environment by the aquaculture sector can be minimized through continuous monitoring / regulating the levels of antibiotics in the water system, both during their application and before discharge to the aquatic environment. For this purpose, suitable analytical methods allowing low cost and high sampling rate in a continuous fashion for on-site application in fish ponds are required. In this context, ion-selective electrodes (ISEs, *ion selective electrode*) can be a powerful analytical tool. These detectors can be applied in the potentiometric analysis of complex matrices, with low cost and portability features. They can also be advantageously associated with automatic methods, such as flow injection analysis (FIA).

Thus, this work describes the production of suitable ISEs for sulphonamide determination in aquaculture environment. Different sensory materials of commercial origin or synthesized by molecular imprinting techniques have been employed to produce a wide range of new potentiometric (bio)sensors. Different ways of fitting these into flow systems have also been investigated, including tubular configuration for FIA systems and planar arrangement over ceramic substrates for microfluidic conditions. Finally, the conductive supports of the selective membrane and the composition of the inner reference solution have been explored to achieve a significant reduction in the detection limit. All methodological approaches developed have been applied successfully to the analysis of water samples from aquaculture.

The methods described herein represent an advantageous alternative to those most widely used for the determination of sulphonamides in aquaculture. In general, the proposed potentiometric methods apply low-cost materials and simple systems, providing quick responses and reduced operator intervention. They are also a green approach, discarding very low amounts of low toxic effluents and allowing on-site determinations.

Keywords: (bio)sensory materials, potentiometry, flow injection analysis, aquaculture.

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ABREVIATURAS E ACRÓNIMOS

APBA – Ácido 3-aminofenilborónico

APCI – Ionização química à pressão atmosférica

APTMS – 3-Aminopropiltrimetoxisilano

APTES – 3-Aminopropiltrietoxisilano

EC – Electroforese capilar

EDAC – *N*-Etil-*N'*-(3-dimetilaminopropil)carbodiimida

EMI – Emissão

ESpI – Ionização por *electrospray*

EXC – Excitação

DPTS – Difenildimetoxisilano

FA – Frequência de amostragem

FE – Fase estacionária

FIA – Análise por injecção em fluxo

FI-MS – *Flow injection-mass spectrometry*

FM – Fase móvel

FTIR – Espectroscopia de infravermelho com transformada de Fourrier

HPLC - Cromatografia líquida de alta eficiência

ESI – Eléctrodo selectivo de ião

ISG – Sol-gel com impressão molecular

K^{POT} – Coeficiente de selectividade potenciométrica

LC – Cromatografia líquida

LLLR – Limite inferior de resposta linear

LOD – Limite de detecção

LTCC – *Low Temperature Co-fired Ceramic*

NHS – *N*-hidroxisuccinimida

MIP – Polímero de impressão molecular

MRM – *Multiple reaction monitoring*

MS – Espectrometria de massa

MS/MS – Espectrometria de massa em *tandem*

MSPE – Extracção em fase sólida com partículas magnéticas

NIP– Polímero sem impressão molecular

NISG – Sol-gel sem impressão molecular

SAs – Sulfonamidas

SBZ – Sulfabenzamida

SC – Sulfacarbamida

SCM – Sulfacetamida

SCP – Sulfacloropiridazina

SDD – Sulfadimidina

SDM – Sulfadimetoxine

SDX – Sulfadoxina

SDZ – Sulfadiazina

SFN – Sulfanitran

SFX – Sulfisoxazole

SGD – Sulfaguanidina

SI – Suporte imobilizador

SM – Solvente mediador

SMD – Sulfametoxidiazina

SME – *Sulfameter*

SMM – Sulfamonometoxina

SMP – Sulfametoxipiradizina

SMR – Sulfamerazina

SMT – Sulfametizole

SMX – Sulfametoxazole

SMXo – Sulfamoxole

SMZ – Sulfametazina

SNM - Sulfanilamida

SPA - Sulfafenazole

SPD – Dispersão em fase sólida

SPE – Extracção em fase sólida

SPH – Sulfonamida

SPI – Sulfapiridina

SPME – Microextracção em fase sólida

SPS – Persulfato de sódio

SQX – Sulfaquinoxalina

SSD – Sulfisomidina

SSZ – Sulfasalazina

STiZ – Sulfatiazina

STP – Sulfaetoxipiridazine

STX – Sulfatroxazole

STZ – Sulfatiazole

TEOS – Tetraetoxisilano

TMP – Trimetoprim

TRIS – Tris(hidroxilmetil)aminometano

TSA – *p*-Toluenosulfonamida

UPLC – Cromatografia líquida de ultra eficiência

UV/Vis – Ultravioleta/visível

VA – Voltagem aplicada

VI – Volume de injecção

λ – Comprimento de onda

ORGANIZAÇÃO GERAL DA TESE

A presente dissertação está organizada em nove capítulos.

O Capítulo I apresenta uma reflexão acerca da necessidade do controlo de antimicrobianos em águas de aquacultura, centrando-se no grupo das sulfonamidas e nos métodos analíticos envolvidos na sua monitorização. Apresenta também uma abordagem geral aos sensores de natureza potenciométrica.

Segue-se um breve apontamento dos procedimentos gerais usados quando da realização de cada trabalho, no Capítulo II, onde se refere o material, o equipamento e as metodologias de avaliação envolvidas nos trabalhos descritos nos capítulos que se sucedem.

Os Capítulos III, IV, V, VI, VII e VIII apresentam a construção e a caracterização de sensores potenciométricos selectivos a diferentes sulfonamidas, sobretudo às que são mais utilizadas em aquacultura. Alguns destes sensores foram integrados em sistemas FIA.

No capítulo III, refere-se a construção de detectores potenciométricos preparados com membranas selectivas a sulfadiazina com um permutador iónico comercial, como ingrediente activo, incorporado em membranas de PVC plastificadas. Aqui, investiga-se a melhor composição qualitativa e quantitativa para a membrana selectiva e a possibilidade da sua incorporação em suportes de configuração tubular, construídos com agulhas de veterinária, para integração em sistemas FIA. As unidades com melhores características de funcionamento são aplicadas à análise de amostras ambientais, tanto em condições de estado estacionário como em condições de fluxo.

Nos capítulos IV e V, preparam-se detectores potenciométricos cujas membranas são selectivas a sulfadiazina e a sulfametoxazole, respectivamente, com base em ionóforos carregados e neutros. Apresenta-se a construção dos eléctrodos correspondentes, baseada na aplicação da membrana num suporte condutor sólido, e o mecanismo de funcionamento destes eléctrodos, através da vasta caracterização analítica de unidades contendo membranas com ou sem aditivos catiónicos ou aniónicos. Por fim, descreve-se a aplicação das unidades com melhores características à análise de águas de aquacultura.

No capítulo VI, desenvolvem-se e constroem-se eléctrodos selectivos a sulfadiazina e sulfametoxazole, incorporando como ingrediente activo novos materiais sensores, sintetizados por impressão molecular em sol-gel. Comparam-se as características daqueles que são baseados em membranas de sol-gel impressas, com a daqueles que incorporam este mesmo material em membranas de PVC, terminando-se com a aplicação destas unidades em amostras reais.

No capítulo VII, desenvolvem-se novos materiais sensores por impressão molecular na superfície de esferas de sílica e a sua incorporação em membranas de PVC, para serem utilizadas na preparação de membranas sensoras de detectores potenciométricos sensíveis a sulfametoxazole. Avaliação destes

detectores em sistemas de estado estacionário, em FIA e em sistemas de microfluídica construídos em suportes cerâmicos, é também referida. Apresenta-se, ainda, o acoplamento de detectores selectivos a trimetoprim nos suportes cerâmicos de microfluídica para uma análise biparamétrica simultânea. A utilidade analítica dos sistemas de fluxo implementados é testada pela análise das amostras de água de aquacultura.

No capítulo VIII desenvolvem-se novos materiais sensores por impressão molecular na superfície de nanoestruturas de grafite que foram utilizados na preparação de membranas sensoras, cuja aplicação foi efectuada em eléctrodos de suporte condutor sólido. Posteriormente, descreve-se a construção de eléctrodos com solução de referência interna, recorrendo ao uso das mesmas membranas. Neste caso efectua-se um estudo detalhado da composição das mesmas, com vista a reduzir os limites de detecção das unidades potenciométricas. Mais uma vez, detectores potenciométricos são aplicados à análise de amostras de águas de aquacultura.

O capítulo IX apresenta as conclusões gerais da dissertação, salientando-se a aplicabilidade dos sistemas implementados em análises de rotina.

De acordo com a legislação em vigor, reserva-se ao autor a opção de não aplicar o novo acordo ortográfico.

I. INTRODUÇÃO

I.1 AQUACULTURA

A aquacultura consiste no cultivo de organismos aquáticos como a criação de peixes, mariscos e plantas, envolvendo a intervenção do homem neste processo para aumentar a produção daí resultante (FAO, 2008). Este sector tem vindo a assumir uma importância crescente na economia nacional e mundial, pois garante o fornecimento de alimentos às populações e é, simultaneamente, responsável pela manutenção de algumas espécies, ao evitar a sua pesca intensiva (Cabello 2006, FAO 2006; Appleford et al., 2003).

Embora o aparecimento da aquacultura em Portugal remonte ao império romano (Mestre, 2008), os primeiros sistemas de aquacultura surgiram apenas no final do século XIX, com a estação de produção de truta arco-íris em Vila do Conde e a intensificação de apanha de ostras, no estuário do Tejo. Nos anos 30, a ostra Portuguesa alcança um volume de exportações de cerca de 13000 toneladas/ano. Depois de 1968, a aquacultura é promovida a actividade comercial, fazendo com que a truta arco-íris passe a ser produzida regularmente. Nos anos 80 surge o estímulo financeiro ao cultivo de bivalves nos estuários e na produção de truta.

Esta dinâmica evolutiva do sector da aquacultura é contrariada após a entrada de Portugal na União Europeia (1986), momento a partir do qual a produção em aquacultura de água doce decresce significativamente, cerca de 60% (Mestre, 2008). Este recuo no sector tradicional abriu portas a novos sistemas de cultivo, tecnologicamente mais avançados e baseados em espécies de elevado valor comercial, como o robalo, a dourada e o rodovalho. Esta realidade é transversal a nível mundial, dado o aumento da população mundial e a premente necessidade de lhe garantir uma alimentação saudável à base de proteína de origem animal.

A aquacultura é actualmente um dos sectores de produção de alimentos com maior expansão ao nível mundial, com um crescimento três vezes superior ao da produção de carne animal (FAO, 2009). As taxas de crescimento do produto cultivado podem ser superiores a 30 % por ano, beneficiando ainda da pronta disponibilidade de recursos naturais e da sua fácil interacção com o meio ambiente.

A intensificação do cultivo aquático tem levantado, porém, muitas reservas no que diz respeito à conservação dos recursos naturais envolventes. Com a protecção ambiental em pano de fundo, tem sido promovida a avaliação dos possíveis impactos ambientais resultantes das práticas de aquacultura e a monitorização desses mesmos impactos no meio ambiente. Estas avaliações não são porém uniformes, variando de país para país, e até dentro do mesmo país. As dificuldades de implementação de uma legislação transversal prendem-se com a necessidade de respeitar as diversidades verificadas no sector, como os recursos ou espécies utilizadas, o sistema de produção e o ambiente em que a produção é realizada. Em Portugal, as autoridades principais para o desenvolvimento da aquacultura são os ministérios de agricultura e desenvolvimento rural e das pescas. Estas entidades promoveram duas directivas, dirigidas a águas doces superficiais para a aquacultura (Directiva 78/659/CEE) e a águas costeiras (Directiva 79/923/CEE). Os padrões de qualidade aplicáveis a este tipo de água são determinados com base em parâmetros físico-químicos e microbiológicos (Decreto-Lei 236/98).

Independentemente das questões legais, a harmonia do sector da aquacultura com o meio ambiente passa sempre pelo seu desenvolvimento sustentável (Cabello, 2006). A utilização de recursos hídricos no sector deve estar associada a modelos de gestão ambiental, devendo ser acompanhado de medidas de prevenção e/ou controlo de contaminação aquática. Esta prática reveste-se de especial importância quando os compostos disseminados desencadeiam, ou podem desencadear, efeitos severos na biodiversidade e na saúde humana (Jones et al., 2004). Entre estes compostos encontram-se diversos fármacos, usadas tanto para fins terapêuticos como profiláticos, tendo em vista a intensificação da produção e o subsequente aumento de rendimento económico no sector (Les Burridge et al., 2010). Entre os compostos mais citados na literatura encontram-se os pesticidas, os antibióticos, os anestésicos e os desinfectantes. O uso destas drogas não se encontra regulado e coloca em risco a saúde pública, tanto pela sua ingestão directa no peixe, como através do consumo de água contaminada. Para além disso, a disseminação destas drogas no meio aquático coloca em risco a normal sobrevivência das espécies envolventes e daquelas que dependem directa ou indirectamente destes recursos.

Os antibióticos são aqueles que geram maior preocupação à sociedade em geral, reflectida pelo elevado número de trabalhos na literatura que focam estas drogas em particular (Figura I 1). Estes compostos têm sido usados, desde há várias décadas, na medicina humana e animal e entram no ambiente pelas fezes e/ou alimentação. O seu uso indiscriminado tem levado a uma crescente preocupação ambiental, devido às resistências microbianas a que podem conduzir, com impacto directo na saúde pública.

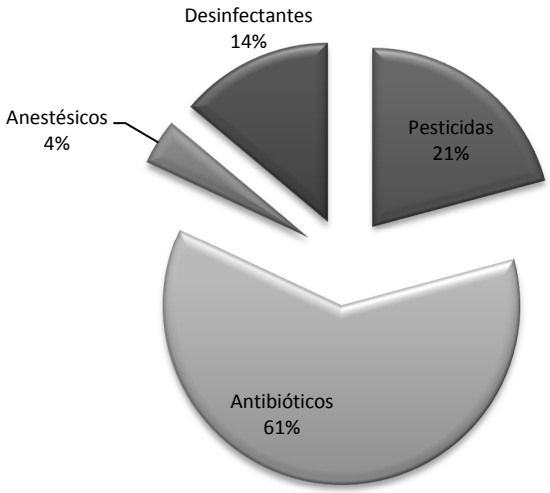


Figura I 1 Distribuição percentual dos grupos de compostos usados no sector da aquacultura (segundo dados da *ISI Web of knowledge*, para artigos publicados entre 1996 e 2009).

I.2 ANTIBIÓTICOS

Os antibióticos são compostos naturais ou sintéticos com propriedade de antibiose (Sousa, 2005), suprimindo o crescimento de alguns microrganismos (Brunton et al, 2006). Convencionalmente, eles foram produzidos por várias espécies de microrganismos (bactérias, fungos e actinomicetes) mas, actualmente, são obtidos por diversos

processos puramente sintéticos ou mistos. Do ponto de vista químico, os antibióticos podem ser agrupados, de acordo com a sua estrutura química ou o mecanismo de acção, em cefalosporinas, fluoroquinolonas, macrólidos, nitroimidazol, penicilinas, sulfonamidas e as tetraciclinas (Clarke e Smith, 2011).

Os antibióticos são convencionalmente incluídos nas práticas actuais de aquacultura para combater e prevenir as doenças infecciosas, um dos problemas mais frequentes e devastadores em vários compartimentos do ambiente aquático (Lupin et al. 2003). A expansão da aquacultura tem induzido uma tendência crescente no consumo de antibióticos em muitos países da União Europeia (Díaz-Cruz et al., 2003), rondando as 5000 toneladas/ano para a produção intensiva de animais.

O crescente consumo de antibióticos no sector da aquacultura origina, por consequência, a sua crescente disseminação no meio aquático. Esta contaminação do meio aquático tem vindo a ser associada a episódios de resistência a bactérias patogénicas (Zou et al., 2011), algo que constitui um gravíssimo problema de saúde pública (Jones et al., 2004; Rooklidge, 2004; Hernando et al., 2006; Sorum, 1999; 2006; Smith, 2008). Estes fenómenos de resistência a antibióticos têm assumido uma importância crescente nos países europeus, onde a consciência sobre a má utilização destes compostos tem estimulado a implementação de planos de monitorização e intervenção das autoridades sanitárias no sentido de vigiar o seu uso e fazer estudos de farmacovigilância. Algumas directivas Europeias, como a Directiva 2003/99/CE e a Directiva 2006/88/CE, denotam já alguma sensibilização das autoridades para esta área.

Para além dos próprios antibióticos ministrados, é possível encontrar no meio aquático resíduos dos mesmos, quer em amostras de esgoto, quer em águas superficiais e em água potável, muitos destes com efeitos mais nocivos do que o próprio composto original. A sua entrada no meio aquático faz-se sobretudo pela aplicação directa de antibióticos na alimentação dos peixes e através dos excrementos produzidos pelas espécies cultivadas (Hamscher et al. 2004). Outra forma possível é a lexiviação a partir de outros meios contaminados, nomeadamente o solo. Dos diferentes grupos de antibióticos conhecidos, segundo a literatura, os mais utilizados no sector da aquacultura são as sulfonamidas (Figura I 2).

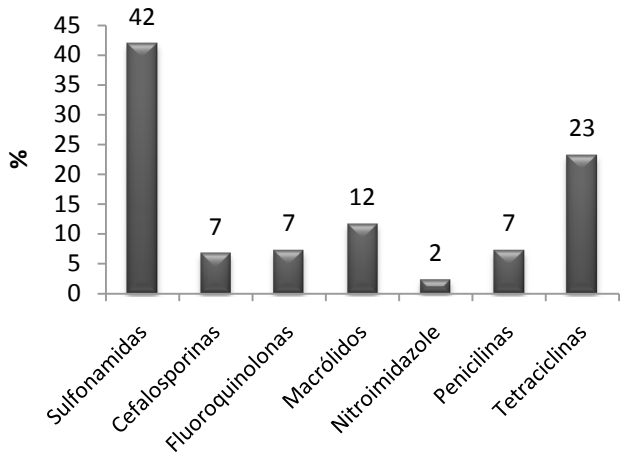


Figura I 2 Expressão percentual dos antibióticos referenciados em águas de aquacultura (segundo dados da *ISI Web of knowledge*, para artigos publicados entre 1995 e 2011)

I.3 SULFONAMIDAS

As sulfonamidas (SAs) representam um grupo de fármacos que contêm um núcleo comum de sulfanilamida. Este núcleo é constituído por um grupo arilo com uma amina e um grupo sulfonamida em posição *para* (Niessen, 1998, Alaburda e tal., 2007). O que distingue as diversas sulfonamidas é a substituição neste último grupo, podendo descrever-se cerca de 30 estruturas diferentes (Cai et al., 2008). As sulfonamidas são usadas como agentes antimicrobianos em aquacultura (Lupin et al. 2003), incluindo-se aqui a Sulfadiazina (SDZ) e o Sulfametoxazole (SMX) cujas estruturas são indicadas na tabela 1.

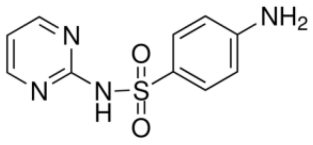
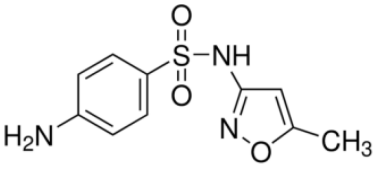
Nas bactérias, as SAs competem com o ácido *p*-aminobenzóico (Pérez-Trallero e Iglesias, 2003) para a síntese do ácido fólico, por questões de semelhança estrutural, impedindo deste modo a síntese de DNA e a subsequente divisão celular (Alaburda et al., 2007). As SAs apresentam, por isso, uma acção bacteriostática, exercida sobretudo em bactérias *gram*-positivas e negativas e em alguns protozoários (Pastor-Navarro et al., 2007). A sua eficácia terapêutica é aumentada sempre que são associadas a trimetoprim (TMP), um antibiótico do grupo das pirimidinas.

As SAs são ácidos fracos, apresentando tipicamente dois valores de pKa, relativos à protonação do grupo $-NH_2$ (pKa_1 2-2,5) e à desprotonação do grupo $-SO_2NH_2$ (pKa_2 5-8) (Seifrtová et al., 2009). Por consequência, elas podem apresentar-se carregadas positivamente em condições de pH inferior a 2, neutras a valores de pH entre $\approx$ 2 e 5, e carregadas negativamente a valores de pH superiores a cerca de 5 (Seifrtová et al., 2009).

As SAs são aplicadas tanto em terapêutica humana como animal (Wegener et al., 1999). A terapêutica humana não acarreta riscos para a saúde humana, sendo considerada segura a ingestão de SAs pelo ser humano desde que em doses controlados. O mesmo não se pode esperar quando são usadas na alimentação animal. Este procedimento pode levar o ser humano a ingerir, de modo não controlado e em níveis desconhecidos, quantidades vestigiárias das SAs acumuladas no animal ao longo do seu percurso metabólico. Este risco, associado à introdução de SAs na alimentação animal, levou à imposição de limites máximos de resíduo (LMR de 0,1 mg/mL) nos produtos de origem animal (Zheng et al, 2004).

A introdução de SAs no sector da aquacultura conduz, também, à sua disseminação no meio aquático, com consequentes riscos adicionais para a saúde pública, através da ingestão de produtos contaminados. As SAs podem atingir outras espécies no meio envolvente, afectando a biodiversidade do meio, podendo ainda difundir-se através do solo, da absorção das plantas e da alimentação das espécies vivas (Braschi et al., 2010 e Zuccato et al., 2010). Além de possíveis efeitos adversos sobre os microrganismos, e como foi referido anteriormente, o maior risco de introdução de SAs no meio ambiente é o desenvolvimento e a disseminação de agentes patogénicos resistentes (Zuccato et al., 2010).

Tabela I 1 Propriedades gerais das Sulfonamidas estudadas

Sulfadiazina	*Estrutura Química*	
	Nomenclatura	4-Amino-*N*-(2-pirimidinil)benzenosulfonamida
	Fórmula	$C_{10}H_{10}N_4O_2S$
	Peso molecular	250,3 g/mol
	pKa	6,50 ± 0,30 1,57 ± 0,10
Sulfametoxazole	*Estrutura Química*	
	Nomenclatura	4-Amino-*N*-(5-metil-3-isoxazolil) benzenosulfonamida
	Fórmula	$C_{10}H_{11}N_3O_3S$
	Peso molecular	253,3 g/mol
	pKa	8,81 ± 0,50 1,39 ± 0,10

A descarga de SAs no meio aquático deve ser por isso controlada. A primeira linha de intervenção neste contexto passa pela prevenção, através da monitorização apertada dos seus níveis, nas águas dos peixes cultivados. A segunda linha de intervenção será o controlo, através da verificação dos níveis de antibiótico nas águas adjacentes, garantindo que a sua libertação para o meio aquático, a jusante, não contribuirá com níveis excessivos de antibiótico.

A monitorização de SAs no meio aquático deve ser por isso agilizada, através da implementação de métodos analíticos de baixo custo e de resposta rápida, capazes de um reconhecimento selectivo de SAs em baixas quantidades.

I.4 MÉTODOS ANALÍTICOS PARA A DETECÇÃO DE SULFONAMIDAS

A escassez de informação disponível na literatura relativamente a métodos para a determinação de SAs no meio aquático levou a que, neste ponto, se incluíssem todos os métodos dedicados a determinação de SAs em amostras alimentares, bebidas e produtos farmacêuticos, publicados ao longo dos últimos 10 anos. Conforme se refere na Figura 1.3, são diversos os métodos descritos (Rodriguez *et al.*, 2009, Samanidou *et al.*, 2008, Wang *et al.*, 2006),, destacando-se em particular aqueles que envolvem processos separativos.

Nas secções seguintes apresentam-se, com mais detalhe, as vantagens/desvantagens destes métodos, fazendo-se uma análise crítica sobre a possibilidade de os adaptar à análise de antibióticos no meio aquático. Conforme foi referido, maioritariamente os métodos usados são de natureza separativa, muito embora os de natureza óptica e electroanalítica também tenham algum relevo (Figura 1.3). Muitos deles operam de um modo convencional, em estado estacionário, mas também são referidos alguns que operam em modo dinâmico, recorrendo a metodologias em fluxo, essencialmente a Análise por Injecção em Fluxo (FIA, do inglês *Flow Injection Analysis*).

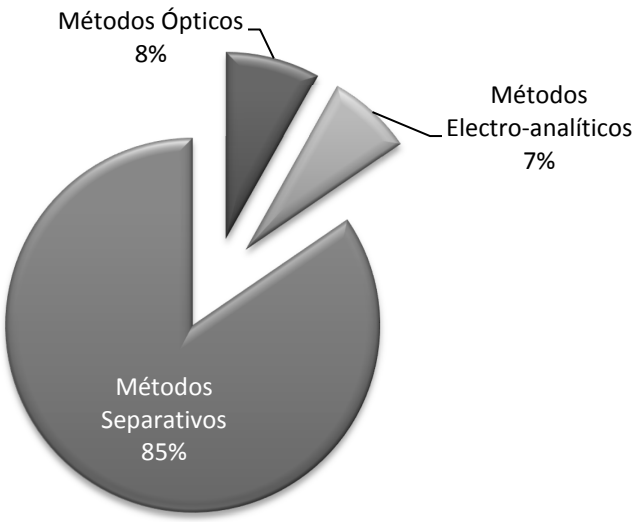


Figura I 3 Distribuição percentual da aplicação dos vários métodos instrumentais na determinação de sulfonamidas (segundo dados da *ISI Web of knowledge*, para artigos publicados entre 1995 e 2011).

I.4.1 MÉTODOS ÓPTICOS

Os métodos ópticos recorrem à interacção da radiação electromagnética com a matéria, em toda a gama do espectro, desde os raios X até aos microondas (Gonçalves, 1996). Os métodos ópticos são, por isso, baseados em fenómenos de óptica clássica. De um modo geral, classificam-se de acordo com a natureza da interacção em curso, nomeadamente absorção, emissão, difracção, refracção, dispersão, reflexão e polarização.

Os métodos ópticos descritos para a quantificação de SAs baseiam-se em medidas por espectrofotometria de absorção atómica, por espectrofotometria de absorção UV/VIS ou por quimioluminescência (tabela I 2). Estes métodos são aplicados sobretudo a produtos farmacêuticos, para além de fluidos biológicos, leite e cerveja. Alguns

dos métodos apresentados são efectuados em condições de fluxo, associados a sistemas FIA (Dadfarnia *et al.*, 2011, Córdova *et al.*, 2003, Gálvez *et al.*, 2002, Lattanzio *et al.*, 2008, Icardo *et al.*, 2003, Liu *et al.*, 2007, Aaron *et al.*, 1995). Os analitos considerados são SDZ, SMX, Sulfamerazina (SMR) Sulfametoxipiradizina (SMP), *Sulfameter* (SME), Sulfacetamida (SCM), Sulfadimidina (SDD), Sulfanilamida (SNM), Sulfatiazole (STZ) e Sulfaguanidina (SGD), por vezes associados a TMP.

Os métodos centrados em espectrofotometria de UV/Vis são maioritariamente aplicados à análise de produtos farmacêuticos e recorrem ao traçado de espectros na região UV. Devido à falta de selectividade oferecida por este processo de detecção, recorrem simultaneamente ao tratamento matemático dos sinais analíticos obtidos para os vários componentes analisados. A informação quantitativa subsequente decorre da monitorização da absorvância a(os) comprimento(s) de onda indicado(s) na tabela I 2. Tipicamente, as medidas são efectuadas em meio alcoólico (Hassouna, 1997, Ni *et al*., 2006) e/ou em meio alcalino, obtido por intermédio de tampão Britton-Robinson (Givianrad *et al.*, 2010), tampão fosfato (Goodarzi *et al*. 2009) ou tampão amónio (Martínez *et al*., 2002). No sentido de aumentar a solubilidade em meio alcoólico e consequentemente procurar um aumento de selectividade que se traduz numa melhor definição das bandas de absorção, Granero et al., 2002 usam a hidroxipropil-β-ciclodextrina como agente complexante.

Na maioria dos casos usam-se processos convencionais de análise, embora haja também referências ao controlo analítico das espécies, recorrendo a técnicas FIA, especialmente quando a determinação é dirigida a mais do que um parâmetro analítico. Neste contexto, Gálvez *et al.*, 2002 regista em contínuo medidas na região UV para obter, simultaneamente, dois perfis de dissolução de princípios activos presentes na mesma formulação farmacêutica, no presente caso SDZ e TMP. Em alternativa, e para tornar a determinação dos dois compostos referidos mais sensível e selectiva, Córdova *et al.*, 2003, propõe um sistema em que, na célula de fluxo, ocorre uma reacção de troca iónica que promove a separação on-line desses analitos. A chegada sequencial dos mesmos à zona de detecção leva a que seja possível a determinação simultânea dos dois compostos, apenas com uma injecção de amostra, e sem qualquer etapa de derivatização anterior.

No caso de amostras de cerveja, Nagaraja *et al*., 2002, propõe a determinação por espectrofotometria de UV/Vis mas precedida da formação de um composto corado, resultante da diazotação das SAs e subsequente reacção de acoplamento com iminodibenzili em meio aquoso. O produto resultante apresenta valores de máxima absorção entre 570-580 nm, com coeficientes de absorção molar entre $2{,}14\times10^4$ mol/L e $2{,}14\times10^4$ mol/L. Esta metodologia permitiu obter respostas lineares para concentrações inferiores às obtidas por leitura directa na região UV, requerendo, no entanto, a utilização de reagentes tóxicos para derivatizar o composto.

Tabela I 2 Determinação de Sulfonamidas por métodos ópticos.

Amostra	Analito	Detector	Modo de operação	Descrição	Características do método						REF
					λ	Linearidade	LD	Fluxo	VI	FA	
					nm	µg/mL	µg/mL	mL/min	µL	Amostras/h	
Produtos farmacêuticos, leite e urina	SDZ	Absorção atómica	FIA	Determinação indirecta de SDZ, por precipitação de sulfadiazinato de prata	328,1	0,005-0,5	0,0008	2,5	100	—	Dadfarnia *et al.*, 2011
Produtos farmacêuticos e fluidos biológicos	SMX, TMP	UV/Vis	*Batch*	Tampão Britton–Robinson a pH 10,0	256-304,7 224-272,4	—	16 11	n.a.	n.a.	n.a.	Givianrad *et al.*, 2010
Produtos farmacêuticos	SMX, TMP	UV/Vis	*Batch*	Tampão fosfato e etanol a pH 10	269 236	2-15 0,4-6	0,03 0,07	n.a.	n.a.	n.a.	Goodarzi *et al*. 2009
Produtos farmacêuticos	SDZ, SDD, SMX, SNM, TMP	UV/Vis	*Batch*	Etanol a pH 9,9	240-286	1-24	Entre 0,2 e 1,0	n.a.	n.a.	n.a.	Ni *et al.*, 2006
Produtos farmacêuticos	SMX, TMP	UV/Vis	*Batch*	Uso de Hidroxipropil - β-ciclodextrina para aumentar a selectividade em meio alcoólico	260	1,6-16,5 1,92-19,2	—	n.a.	n.a.	n.a.	Granero *et al.*, 2002
Cerveja	STZ, SDZ, SCM, SMX, SMR, SGD, SDD	UV/Vis	*Batch*	Composto corado (violeta) pela diazotação das SAs, seguida de uma reacção com iminodibenzilo em meio alcoólico	570-580	0,05-6	0,0330-0,0872	n.a.	n.a.	n.a.	Nagaraja *et al.*, 2002

λ: comprimento de onda; EXC: excitação; EMI: emissão; LD: limite de detecção; VI: volume de injecção; REF: referências; n.a.: não aplicável.

Tabela I 2 Determinação de Sulfonamidas por métodos ópticos (continuação).

Amostra	Analito	Detector	Modo de operação	Descrição	Características do método						REF
					λ	Linearidade	LD	Fluxo	VI	FA	
					nm	µg/mL	µg/mL	mL/min	µL	Amostras/h	
Produtos farmacêuticos	SMX, TMP	UV/Vis	*Batch*	Metanol a pH 6,9	288 240	4-20	—	n.a.	n.a.	n.a.	Hassouna, 1997
Produtos farmacêuticos	SMX, TMP	UV/Vis	FIA	Uso de $1x10^{-4}$ M de ácido clorídrico e 0,20 M acetato de sódio/ácido acético	269	50-250 10-70	9,5 0,6	1,4	500	14	Córdova *et al.*, 2003
Produtos farmacêuticos	SDZ, TMP	UV/Vis	FIA	Tampão amónia e etanol a pH 10	288 248,5	1-35 1-30	—	2,0	—	—	Gálvez *et al.*, 2002
Produtos farmacêuticos	SMX, SMP, TMP	*diode array*	*Batch*	Tampão amónio, a pH 10	257 289 251	0,3-40	0,34 0,56 0,19	n.a.	n.a.	n.a.	Martínez *et al*., 2002
Produtos farmacêuticos e fluidos biológicos	SDZ, SM, SDM	Quimioluminescência	*Batch*	Reacção de quimioluminescência entre o complexo $[Ag(HIO_6)_2]^{5-}$ e luminol, em solução alcalina.	—	—	Entre 0,0072 e 0,017	n.a.	n.a.	n.a.	Sun *et al.*, 2011
Produtos farmacêuticos	SDZ	Quimioluminescência	FIA	Reacção indirecta de quimioluminescência com peroxioxalato como precursor, peróxido de hidrogénio como oxidante, imidazol como catalisador e fluoresceína como agente fluorescente.	—	0,38-2	0,126	3,5	500	n.a.	Lattanzio *et al.*, 2008

λ: comprimento de onda; EXC: excitação; EMI: emissão; LD: limite de detecção; VI: volume de injecção; REF: referências; n.a.: não aplicável.

Tabela I 2 Determinação de Sulfonamidas por métodos ópticos (continuação).

Amostra	Analito	Detector	Modo de operação	Descrição	Características do método						REF
					λ	Linearidade	LD	Fluxo	VI	FA	
					nm	µg/mL	µg/mL	mL/min	µL	Amostras/h	
Produtos farmacêuticos	SDZ	Quimioluminescência	FIA	Reacção de quimioluminescência com formaldeído e permanganato de potássio -$H_6P_4O_{13}$ - SDZ	—	0,2-54	0,05	2,5	100	—	Liu *et al.*, 2007
Produtos farmacêuticos	SMX, SDZ, SMR, SMP, SCM, SDD, SNM, STZ, SGD	Quimioluminescência	FIA	Reacção de permanganato de potássio em ácido sulfúrico	n.a.	0,06-100	3-8	2,0	524	60	Icardo *et al.*, 2003
Leite	SDZ	Quimioluminescência	*Batch*	SAs derivatizadas com fluoresceína	EXC: 395 EMI: 485	n.a.	0,0043	n.a.	n.a.	n.a.	Díez *et al.*, 2007A
Leite	SDZ, SMZ, SMR	Quimioluminescência	*Batch*	SAs derivatizadas com fluoresceína	EXC: 395 EMI: 485	n.a.	n.a	n.a.	n.a.	n.a.	Díez *et al.*, 2007B
Leite	SAs	Quimioluminescência	*Batch*	Inibição de fluorescência do complexo de anidrase carbónica com densilamida	EXC: 280 EMI: 460	0,0200-0,344	0,0195	n.a.	n.a.	n.a.	Huaina e Yunxiang, 1998
Produtos farmacêuticos	SDZ	Quimioluminescência	*Batch*	Inibição de fluorescência de *Quantum Dots* de CdS	EXC: 360 EMI: 489	3,3-572	2,2	n.a.	n.a.	n.a.	Liu *et al.*, 2008
Produtos farmacêuticos	SDZ	Quimioluminescência	FIA	Metanol e água (50:50, v/v) irradiado por uma lâmpada de mercúrio de alta pressão	EXC: 295 EMI: 352	0,2-6,5	0,09	4,2	300	—	Aaron *et al.*, 1995

λ: comprimento de onda; EXC: excitação; EMI: emissão; LD: limite de detecção; VI: volume de injecção; REF: referências; n.a.: não aplicável.

Dado que as SAs não apresentam fluorescência intrínseca, os métodos que normalmente são descritos para a sua determinação baseiam-se na transformação das SAs num composto com propriedades quimiluminimétricas ou no efeito inibitório das SAs num sistema luminescente (tabela I 2). As reacções que são descritas envolvem fluoresceína (Lattanzio *et al.*, 2008, Díez *et al.*, 2007A, Díez *et al.*, 2007B), luminol (Sun *et al.*, 2011), permanganato (Liu *et al.*, 2007, Icardo *et al.*, 2003), estruturas luminescentes de anidrase carbónica/densilamida (Huaina e Yunxiang, 1998) ou *Quantum Dots* de CdS (Liu *et al.*, 2008). De uma forma geral, as gamas de linearidade conseguidas são mais alargadas e incluem concentrações inferiores às que são obtidas através das metodologias desenvolvidas para a região do UV. As concentrações mínimas a partir das quais a resposta é linear estão entre 0,02 µg/mL (Huaina e Yunxiang, 1998) e 3,3 µg/mL (Liu *et al.*, 2008). Alguns destes métodos operam em fluxo contínuo (Lattanzio *et al.*, 2008, Liu *et al.*, 2007, Icardo *et al.*, 2003 e Aaron *et al.*, 1995), minimizando as intervenções do operador e permitindo uma maior frequência analítica.

Dadfarnia et al., 2011 detém o único trabalho baseado em espectrofotometria de absorção atómica e reporta a monitorização indirecta de uma SA através da sua precipitação com prata. Este método analítico é usado na separação/pré-concentração e determinação indirecta de SDZ em diferentes matrizes. O método é simples e não recorre ao uso de solventes orgânicos tóxicos. Além disso, este apresenta o menor limite de detecção (0,0008 µg/mL) face aos demais métodos ópticos. O procedimento envolvido é, no entanto, muito mais caro no que diz respeito aos custos de equipamento/manutenção e aos consumíveis requeridos.

De uma forma geral, a natureza óptica dos métodos referidos anteriormente determina que os intervalos de linearidade sejam tipicamente estreitos. As concentrações a partir das quais a resposta é linear estão compreendidas entre 0,005 e 7200 µg/mL. Os valores de limite de detecção dependem da técnica propriamente dita, estando compreendidos entre 0,0008 e 9,5 µg/mL. De uma forma geral, os métodos baseados em fluorescência conseguem níveis de detecção inferiores aos obtidos pelos baseados em espectrofotometria UV e o método que detecta quantidades inferiores de SAs utiliza a espectrofotometria de absorção atómica (Dadfarnia *et al.*, 2011). A automatização de alguns dos métodos apresentados permite consumos reduzidos de amostra, entre 20 e 524 µL, e determinações expeditas, entre 14 a 60 amostras por hora, mas a diluição do volume injectado no interior do sistema FIA condiciona os diversos parâmetros analíticos do método, com grande incidência nos limites de detecção e nos intervalos de linearidade.

Independentemente das boas características analíticas que alguns dos métodos propostos apresentam, a sua grande limitação prende-se com a coloração e a turvação das amostras (Gonçalves, 1996), que se traduz numa interferência de matriz. Estes aspectos podem ter um impacto muito significativo em águas de sistemas de aquacultura. Alguns dos compostos aí utilizados, como desinfectantes, podem apresentar coloração e é comum a presença de partículas em suspensão no meio.

Neste sentido, os métodos ópticos não constituem uma boa alternativa para o desenvolvimento de métodos rápidos e contínuos de monitorização de SAs em sistemas de aquacultura. Na literatura, e como veremos na secção seguinte, encontram-se descritos, como alternativa, métodos baseados em técnicas separativas, que visam separar o analito da matriz da amostra antes de efectuar a sua quantificação, minimizando assim o efeito de interferência de espécies que coexistem com o analito na amostra.

1.4.2 MÉTODOS SEPARATIVOS

Os métodos instrumentais separativos são os que pressupõem uma separação dos constituintes de interesse na amostra, antes de se efectuar a medida analítica propriamente dita. São descritos na literatura diversos trabalhos que recorrem ao uso de técnicas separativas para a determinação de SAs, de natureza cromatográfica (tabela I 3) ou electroforética (tabela U 4), na maioria dos casos em amostras alimentares e fluidos biológicos. Contudo, aqui já se começa a assistir a uma aplicação destas técnicas a amostras de ambientais, sejam elas águas, solos efluentes ou mesmo produtos resultantes do tratamento das águas como é o caso das lamas (ver tabela I 3).

Nestes trabalhos, e dada a especificidade da técnica, é possível fazer-se uma determinação de vários analitos simultaneamente, referindo-se neste contexto um grande número de SAs, como por exemplo a Sulfabenzamida (SBZ), Sulfacarbamida (SC), Sulfacloropiridazina (SCP), SCM, SDD, Sulfadimetoxine (SDM), Sulfadoxina (SDX), SDZ, Sulfanitran (SFN), Sulfisoxazole (SFX), SGD, SME, Sulfamonometoxina (SMM), SMP, SMR, Sulfametizole (SMT), SMX, Sulfamoxole (SMXo), Sulfametazina (SMZ), SNM, Sulfafenazole (SPA), Sulfapiridina (SPI), Sulfaquinoxalina (SQX), Sulfisomidina (SSD), Sulfasalazina (SSZ), Sulfatiazina (STiZ), Sulfatroxazole (STX), STZ e Toluenosulfonamida (TSA), associadas ou não a TMP.

Os métodos cromatográficos descritos na literatura para a determinação de SAs são baseados em cromatografia líquida (LC). Esta é essencialmente um método físico de separação, em que os componentes a separar são distribuídos entre duas fases: uma fase estacionária, que se encontra fixa, ancorada numa coluna, e uma fase móvel líquida, que se desloca através da coluna. A cromatografia líquida de alta eficiência (HPLC) é a técnica analítica separativa mais popular neste contexto. A associação de HPLC com diferentes técnicas de detecção possibilita a análise qualitativa e quantitativa de um grande número de substâncias. As técnicas de detecção mais utilizadas são a espectrofotometria na região UV/Vis e a fluorescência (tabela I 3) e a espectrometria de massa (MS) (tabela I 4).

Analisando-se as tabelas I 3 e I 4 é possível notar que a utilização de colunas de fase reversa é predominante na determinação de SAs nas diferentes matrizes. Estas colunas são empacotadas com materiais inertes e resistem às pressões típicas dos sistemas de HPLC. As mais utilizadas nos trabalhos descritos são as colunas de octadecil (C_{18}, RP_{18}, ODS). De uma forma geral estas colunas representam um elevado custo nos sistemas cromatográficos e implicam a utilização de fases móveis com elevada pureza e um reduzido número de partículas com pequena dimensão.

Tabela I 3 Determinação de Sulfonamidas por comatografia líquida com detecção fotométrica.

Amostra	Analito	Detecção	Características do método								REF
			λ	Pré-tratamento	FE	FM	Linearidade	LOD	VI	Caudal	t_r	
			nm				µg/L	µg/L	µL	mL/min	min	
Águas do mar	SDZ, STZ, SMR, SMZ, SCP, SMX, SFX, SDM, SPD, SSD	UV/Vis	270	SPE, extracção acetonitrilo/ metanol (50:50, v/v)	Coluna C_{18} (150 × 4,6 mm e partículas de 5 µm)	Água com ácido trifluoroacético (pH 3,5) com acetonitrilo	5-500 500-20000	0,167	50	0,7	< 30	Bialk-Bielinska *et al.*, 2011
Água	SDZ, SMR, SMT, SMX	UV/Vis	262 270	LPME, com 1-octanol	Coluna C_{18} (150 × 3,0 mm e partículas de 3,5 µm)	Ácido fórmico (pH 2,6) e acetonitrilo	n.a.	0,0003 0,033	20	0,4	8,69-29,94 9,21-30,41	Payán *et al.*, 2011
Água	SDZ, SMR, SMT, SDM, SMX	UV/Vis	265	LPME, com 1-Octyl-3-methylimidazolium hexafluorophosphate e Tri-*n*-octylphosphine oxide	Coluna C_{18} (150 × 4,6 mm e partículas de 5 µm)	Acetonitrilo e tampão fosfato (pH 5,5)	1-2000	0,1-0,4	n.a.	1,0	~ 9	Tao *et al*. 2009
Águas Urbanas	SDZ, SMX, SMZ, SMR, TMP	UV/Vis	280	SPE, extracção com 95% de metanol	Coluna C_{18} (150 × 3,0 mm e partículas de 3,5 µm)	Ácido fórmico em água e acetonitrilo	0,1-50	—	5	0,3	< 22	Peng *et al*., 2008
Água de rio e esgoto	SDZ, STZ, SPD, SMZ, SMX	UV/Vis	260	SPE, extracção com metanol	Coluna C_{18} (250 × 4,6 mm e partículas de 4 µm)	Ácido fosfórico em água (pH 3) e acetonitrilo (25:75, v/v)	0-30	0,15 a 0,35	20	1,0	< 20	Li *et al*., 2007
Águas de suinicul-tura	SNM, SDZ, STZ, SMR, SMZ, SMP, SDM, SQX	UV/Vis	272	SPE, extracção com amónia e metanol (1:19, v/v)	Coluna C_{18} (250 × 4,6 mm e partículas de 4 µm)	Acetonitrilo e ácido acético em água	0,005-0,100	—	50	1,0	< 35	Malintan e Mohd, 2006
Leite	SMZ, SMX, SGD, SCP, SMM, SDM, SFX, SMR, SDX	UV/Vis	268	Extracção em pré-coluna de partículas de sílica (10 µm e poros de 1000 Å) modificadas por impressão molecular e de acesso restrito.	Coluna C_{18} (150 × 4,6 mm e partículas de 5 µm)	Tampão fosfato (pH 7,0), 2-propanol e tetrahidrofurano (84:10:6, v/v/v)	2-400	< 0,8	20	1,0	< 18	Xu *et al.*, 2010

* valores expressos em µg/Kg

Tabela I 3 Determinação de Sulfonamidas por comatografia líquida com detecção fotométrica (continuação).

Amostra	Analito	Detecção	Características do método								REF
			λ nm	Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Leite	SMX, TMP	UV/Vis	265	—	Coluna C_{18} (100 × 4,6 mm e partículas de10 µm)	Tampão fosfato (pH 6) e acetonitrilo (95:5, v/v)	25-800 50-400	—	150	1,0	< 28	Pereira e Cass, 2005
Leite	SDZ, SMR, SMZ, SMM, SDM, SMX	UV/Vis	260	—	Coluna C_{18} (150 × 4,6 mm e partículas de 5 µm)	Acetonitrilo e tampão de fosfato (pH 4) (25:75. v/v)	5-100000	0,08 a 0,86	10	0,6	< 20	Yang *et al.*, 2004
Ovos e carne	SDZ, SMR, SDM, STZ, SMX, SMT, SMP, SCP, SDX, SFX	UV/Vis	270	SPE, extracção com acetonitrilo	Coluna C_{18} (250 × 4,6 mm e partículas de 5 µm)	Metanol e água (22:78, v/v)	0,2-100	0,0041 a 0,010	20	1,0	23	Fang *et al.*, 2006
Mel	STZ, SMZ, SDM	UV/Vis	280	SPE, extracção com metanol e acetato de sódio	Coluna XBD-C8 (150x3,9 mm e partículas de 5 µm)	Acetato de sódio, metanol e acetonitrilo (pH 4,5)	25-200*	3 a 5*	—	0,75	< 30	Granja *et al*. 2008
Alimento para animais	SDZ, SCP, TMP	UV/Vis	270 240	SPE, extracção: com ácido acético em água e metanol	Coluna C_{18} (100 × 3,0 mm e partículas de 5 µm)	Ácido acético, trietilamina e metanol em água e metanol em acetonitrilo	250000-1000000* 50000-200000*	10400 a 4400000	100	0,5	< 10	Croubels *et al*., 2002
Amostra biológica	SDX, SMX	UV/Vis	256	—	Coluna RP_{18} (150 × 4,6 mm)	Acetonitrilo e acetato de sódio (pH 5,2) (1:2, v/v)	1260-20000	—	—	1,0	—	Lindkvist *et al*. 2009
Amostra biológica	SDZ, TMP	UV/Vis	270 240	Extracção com diclorometano ou acetato de etilo e remoção de proteínas com acetonitrilo e hexano	Coluna ODS-2 (250 × 4,0 mm com 5 µm de tamanho de partícula)	Acetonitrilo e tampão de fosfato (pH 4,5) com hidrogenosulfato de tetrabutilamónio (12:88, v/v)	50-604800 50-46200	5	20	1,0	< 10	Batzias *et al*., 2002

* valores expressos em µg/Kg

Tabela I 3 Determinação de Sulfonamidas por comatografia líquida com detecção fotométrica (continuação).

Amostra	Analito	Detecção	Características do método								REF
			λ nm	Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Produtos farmacêuticos	SMX	UV/Vis	269	Extracção com acetonitrilo e água (2:8, v/v)	Coluna C_{18} (50 × 4,6 mm)	Acetonitrilo e água (2:8, v/v)	0,25-2,5	—	10	1,0	—	Zheng *et al.*, 2004
Soro	SDM	UV/Vis	270	—	Coluna C_{18} (250 × 4,6 mm com 5 µm de tamanho de partícula)	Água, hidrogenofosfato de potássio e acetonitrilo (60:40, v/v)	100-9930	40	25	1,0	< 7	Primus *et al.*, 2007
Culturas microbiológicas	SDZ, TMP	UV/Vis	271	Extracção com diclorometano	Coluna C_{18} (250 × 4,6 mm com 5 µm de tamanho de partícula)	Acetonitrilo e ácido fosfórico (16:84, v/v)	5-150 25-750	3,1 18,2	100	1,0	—	Papapanagiotou *et al.*, 2002
Água	SCM, SDZ, STZ, SMR, SDD, SQX, SMX, SDZ, SMM	*Diode array*	265	LPME, extracção com 4-nitro-m-xylene	Coluna RP 18 (250 × 4,6 mm e partículas de 5 µm)	Acetonitrilo e 0,1M dihidrogenofosfato de potássio	1-500	—	20	0,6-1	< 35	Lin e Huang, 2008
Carne	SDZ, SDD, SMM, SMX, SDM, SQX	*Diode array*	267	SPD, extracção com etanol	Coluna RP-4 GP (250 × 4,6 mm e partículas de 5 µm)	Ácido acético em água (pH 2,7) e etanol (75:25, v/v)	50-500	—	20	0,8	< 30	Kishida e Furusawa, 2003
Leite	SDZ, STZ, SMT, SMZ, SMP, SMM, SMX, SFX, SDM, SQX	*Diode array*	265	SPE, extracção com uma mistura de acetato de etilo, *n*-hexano, e isopropanol	Coluna C_{18} (250 × 4,0 mm e partículas de 5 µm)	Ácido fórmico, acetonitrilo e metanol	—	2,3 a 9,7*	20	1,0	< 36	Tolika *et al.*, 2011
Leite	SCM, SMT, SGD, SMR, STZ, SMX	*Diode array*	*490*	—	Coluna C_{18} (250 × 4,6 mm e partículas de 5 µm)	Dodecilsulfato de sódio e propanol (pH 7)	200 a 10000	0,72-0,94	20	1,0	< 15	Raviolo *et al.*, 2007
Leite	SNM, SDZ, SMR, SMZ	*Diode array*	270	SPE, extracção com etanol e ácido acético (97:3, v/v)	Coluna C_8 (150 × 4,6 mm e partículas de 5 µm)	Acetonitrilo e água (5:95, v/v)	50-10000	30 a 60	20	1,5	< 15	*Huang et al.*, 2007

* valores expressos em µg/Kg

Tabela I 3 Determinação de Sulfonamidas por comatografia líquida com detecção fotométrica (continuação).

Amostra	Analito	Detecção	Características do método								REF
			λ nm	Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Leite	SDZ, STZ, SMZ, SMP, SMM, SMX, SDM, SQX	*Diode array*	270	SPE, extracção com metanol, acetonitrilo e ácido acético (1:1:8, v/v/v)	Coluna C_{18} (250 × 4,6 mm e partículas de 5 µm)	Ácido acético em metanol e acetonitrilo (1:1, v/v)	25-5000	0,8 a 15	40	1,0	< 31	Wu *et al.*, 2007
Leite	SMM, SDM	*Diode array*	267	Extracção com etanol e ácido acético (97:3, v/v)	Coluna SHP (250 × 4,6 mm e partículas de 5 µm)	Ácido acético em água (pH 3,1) e etanol (75:25, v/v)	—	—	20	1,2	—	Kishida e Furusawa, 2004
Ovos	SCM, SDZ, STZ, SMT, SMP, SMX	*Diode array*	*269*	Micro-extracção com tampão fosfato, pH 3	Coluna C_{18} (250 × 3,0 mm)	Acetonitrilo e metanol em ácido trifluroacético (pH 4) (60:40, v/v)	30-300*	≈ 9	—	1,0	< 15	Salami e Queiroz, 2011
Ovos	SMM, SDM	*Diode array*	267	—	Coluna com fase hidrofóbica (250 × 4,6 mm e partículas de 5 µm)	Ácido acético em água (pH 2,9) e etanol (75:25, v/v)	—	20 a 80	20	1,2	< 25	Kishida, 2007
Camarão	STZ, SMZ, SDM, SQX, SMX	*Diode array*	227	SPE, acetonitrilo e hidróxido de amónio (95:5)	Coluna C_{18} (150 × 3,0 mm e partículas de 5 µm)	Acetonitrilo e ácido acético	20-200*	1,5 a 6,3*	50	1,0	< 23	Santos e Maciel, 2011
Mel	SCM, SDZ, SDM, SGD, SMR, SMM, SNM, SPD, SQX, STZ, SFX	*Diode array*	275	Dissolução em SDS, ultra sons e filtração com filtro de Nylon 0,45 µm	Coluna C_{18} (100 × 4,6 mm e partículas de 5 µm)	Dodecilsulfato de sódio em propanol (pH 3,0)	100-3000	10 a 300	20	1,0	< 41	Caballero *et al.*, 2001
Amostra biológica	SDZ, STZ, SPD, SMR, SMT	*Diode array*	280	—	Coluna C_{18} (250 × 4,6 mm e partículas de 5 µm)	Ácido acético glacial e acetonitrilo (90:10, v/v)	2-30	0,2 a 1,0*	50	1,0	< 15	Tsai *et al.*, 2010A

* valores expressos em µg/Kg.

Tabela I 3 Determinação de Sulfonamidas por comatografia líquida com detecção fotométrica (continuação).

Amostra	Analito	Detecção	Características do método									REF
			λ nm	Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Amostra biológica	SMX, TMP	*Diode array*	270	SPE, extracção: na fase móvel	Coluna C_{18} (125 × 4,0 mm com 5 µm de tamanho de partícula)	Tampão fosfato (pH 3) e acetonitrilo (89:11, v/v)	300-180000 300-300000	—	15	2,0	—	Bedor *et al.*, 2008
Amostra biológica e ovos	SMM, SDM	diode array	270	—	Coluna RP-4 GP (150 × 4,6 mm com 5 µm de tamanho de partícula)	Etanol e ácido acético em água	—	—	20	1,0	—	Kishida e Furusawa, 2005
Amostra biológica	SMM, SDM	*diode array*	267	Extracção com Sulfato de amónio	Coluna C_{18} (150 × 4,6 mm com 5 µm de tamanho de partícula)	Acetato de sódio	100-2000	10 a 30	20	1,0	< 40	Kishida *et al.*, 2005
Amostra biológica	SDZ	*diode array*	269	—	Coluna C_{18} (100 × 4,6 mm com 3 µm de tamanho de partícula)	Metanol, água e ácido fórmico concentrado (50:950:1, v/v/v) e Metanol, água e ácido fórmico concentrado (500:500:1, v/v/v)	200-200000	—	—	0,5	< 9	Johannessen *et al.*, 2005
Amostra biológica	SGD, SMX, SMR, SMT, SMZ, SDD, SCM, SPD, SBZ, SMP, SMM, SDM, SSZ, SQX, SDZ, STZ	*diode array*	260	—	Coluna C_{18} (150 × 3,0 mm com 5 µm de tamanho de partícula)	Ácido acético em água e Ácido acético em metanol (85:15, v/v)	1,8-10	1,8 a 24,3	—	0,15	< 76	Msagati e Nindi, 2004
Amostra biológica	SDZ, TMP	*diode array*	270 240	—	Coluna C_{18} (100 × 3,0 mm com 3,5 µm de tamanho de partícula)	Ácido acético, trietilamina e metanol em água e metanol em acetonitrilo	100-1000 100-2000	19 a 24	100	—	—	Croubels *et al.*, 2003

Tabela I 3 Determinação de Sulfonamidas por comatografia líquida com detecção fotométrica (continuação).

Amostra	Analito	Detecção	Características do método									REF
			λ nm	**Pré-tratamento**	**FE**	**FM**	**Linearidade** µg/L	**LOD** µg/L	**VI** µL	**Caudal** mL/min	**t_r** min	
Amostra biológica	SDZ, STZ, SPD, SMR, SMT, SDD, SFX, SMX, STX, SCP	*diode array*	260	SPE, extracção: amónia e acetonitrilo (1:19, v/v)	Coluna C_{18} (250 × 2,0 mm com 5 µm de tamanho de partícula)	Acetato de amónio (pH 4,6) e acetonitrilo	10-100	—	25	0,35	< 18	Hela *et al*., 2003
Amostra biológica	SDZ, SDD, SMM, SMX, SDM, SQX	*diode array*	267	SPE, extracção com etanol a 70%	Coluna RP 18 (250 × 4,6 mm com 5 µm de tamanho de partícula)	Ácido acético em água (pH 3), acetonitrilo e *N,N*-dimetilformamida (78:22:5, v/v/v)	50-1000	3 a 12	10	0,3	< 15	Kishida e Furusawa, 2001
Amostra biológica	SDZ, TMP	*diode array*	270 240	SPE, extracção com amónio em metanol	Coluna C_{18} (250 × 4,6 mm com 5 µm de tamanho de partícula)	Ácido acético em água e acetonitrilo (85:15, v/v)	50-1000* 25-500*	11,4 a 36,7	50 100	1,0 1,5	30	Baere *et al*., 2000
Produtos farmacêuticos	SMX, TMP	*diode array*	254	—	Coluna C_{18} (250 × 4,6 mm com 5 µm de tamanho de partícula)	Água, acetonitrilo e trietilamina (77,7:22,2:0,1, v/v) com pH 5,9	2000-14000	6 a 9	20	2,5	< 6	Coutinho e Barbosa, 2009
Comida animal	SDZ, SDD	Fluorescência	EXC: 405 EMI: 485	extracção assistida por microondas	Coluna C_{18} (150 × 4,6 mm e partículas de 5 µm)	Ácido fórmico (pH 3,4) e fluoresceína em acetonitrilo	—	3,5-6,5	50	1,2	< 15	Jiménez *et al*. 2009
		Diode array	268			Acetonitrilo com ácido acético e tampão acetato (13:87, v/v)	50-1000	1,4-4,4	20	1,0		
Amostra biológica	SDZ, SPD, STZ, SDD	Fluorescência	EXC: 405 EMI:485	SPE, extracção com fosfatos de potássio (pH 6)	Coluna RP_{18} (150 × 3,9 mm com 5 µm de tamanho de partícula)	Ácido fórmico em água e acetonitrilo	50-800000*	60 a 1000	100	0,7	7,4-22,5	*Li et al*., 2008
		diode array	270				5000-1000000*	4000*	—	—	9,4-19,8	

* valores expressos em µg/Kg

Tabela I 3 Determinação de Sulfonamidas por comatografia líquida com detecção fotométrica (continuação).

Amostra	Analito	Detecção	Características do método									REF
			λ nm	Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Leite	SPD, SDZ, SMZ, SCP, SDX, SMX, SDM	Fluores-cência	265	SPE, extracção com clorofórmio e acetona (2:1, v/v)	Coluna C_{18} (150 × 4,6 mm e partículas de 5 µm)	Tampão citrato (pH 5,2), acetonitrilo e tetrahidrofurano (65:22:13, v/v/v)	5-120	6,2 a 3,6	100	1,0	< 14	Soto-Chinchilla *et al.*, 2005
Mel	SPD, SGD, SCP, SMM, SDM, SFX, SMR, SDX	Fluores-cência	EXC: 405 EMI: 495	Centrifugação	Coluna C_{18}-AR-II (250 × 4,6 mm e partículas de 5 µm)	Acetonitrilo e ácido acético	2-200*	0,6 a 0,9*	10	1,0	< 20	Tsai *et al.*, 2010B
Amostra biológica	SGD, SNM, SSD, STZ, SMR, SMZ, SMP, SMX	Fluores-cência	EXC: 406 EMI: 496	SPE, acetonitrilo	Coluna ODS 3 (250 × 4,0 mm e partículas de 5 µm)	Metanol e tampão acetato (pH 3,4)	1,0-300	0,10 a 0,30	20	1,0	< 4	Zotou e Vasiliadou, 2009
Amostra biológica	SNM, SDZ, STZ, SPD, SMR, SMZ, SMT, SMP, SCP, SMM, SDX, SMX, SDM, SQX	Fluores-cência	EXC: 400 EMI:495	SPE, extracção com acetona e acetato de amónio (1:1, v/v)	Coluna C_{18} (150 × 4,6 mm com 3,5 µm de tamanho de partícula)	Ácido acético, metanol e acetonitrilo	—	—	50	1,0	< 25	Gehring *et al.*, 2006
Amostra biológica	SDZ, SMR, SMZ, SMP, SMX, SDM	Fluores-cência	EXC: 405 EMI:485	SPE, extracção com acetonitrilo	Coluna C_{18} (150 × 4,6 mm com 3 µm de tamanho de partícula)	Metanol e ácido acético	0-500*	1-5*	10	0,5	< 22	Posyniak et al., 2005
Amostra biológica	SNM, SDZ, SMR, SDD, SMP, SCP, SDX, SMX, SDM, SQX	Fluores-cência	EXC: 405 EMI: 490	Extracção com acetato de etilo e acetona	Coluna RP_{18} (250 × 4,0 mm com 5 µm de tamanho de partícula)	Acetonitrilo e hidrogenofosfato de potássio (pH 3)	1-100*	—	100	1,5	< 30	Stoev e Michailova, 2000

* valores expressos em µg/Kg

Tabela I 4 Determinação de Sulfonamidas por cromatografia líquida com detecção por espectrometria de massa.

Amostra	Analito	Detecção	Características do método							REF
			Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Água de suinicultura	STZ, SMX, SMT, SDM, SMZ	LC-MS (ESI)	SPE, extracção diclorometano e acetona (3:2,v/v)	Coluna C_{18} (150 × 2,1 mm com 5 µm de tamanho de partícula)	Ácido fórmico em água, metanol e acetonitrilo	0,5-25	0,005 a 0,091	20	0,2	< 30	Ben *et al*., 2008
Água	SMX, SDZ, SMZ, SPD, STZ, TMP	LC-MS (ESI)	SPE, extracção com metanol	Coluna C_{18} (250 × 4,6 mm com 5 µm de tamanho de partícula)	Ácido acético (pH 3) e acetonitrilo	5-500	1-5	50	—	< 22	Pedrouzo *et al*., 2008
Águas residuais	SMR, SMZ, SMX, TMP	LC-MS (ESI)	SPE, extracção com metanol e ácido fosfórico (95:5, v/v)	Coluna C_{18} (150 × 2,1 mm com 5 µm de tamanho de partícula)	Ácido acético glacial e acetonitrilo	—	0,002 a 0,004	200	0,25	< 21	Renew e Huang, 2004
Carne	SMZ, SDM, STZ, SDZ, SMT, SPD	LC-MS (ESI)	SPE, extracção com acetonitrilo	Coluna ODS 2 (250 × 2,5 mm com 5 µm de tamanho de partícula)	Acetonitrilo, água e acetato de amónio	—	—	2	0,2	< 25	Kim e Lee, 2003
Carne	SDZ, SPD, SMR, SMZ, SMM, SFX, SDM, SQX, SPA	LC-MS (ESI)	SPE, extracção com *n*-hexano e acetonitrilo	Coluna C_{18} (125 × 2,0 mm com 5 µm de tamanho de partícula)	Ácido acético (pH 3,1) e acetonitrilo com ácido acético	50-500*	10*	10	0,20	< 25	Fuh e Chan, 2001
Leite	SDZ, STZ, SMP, SMX, SDM, SMZ	LC-MS (ESI)	SPE, extracção com metanol	Coluna RP_{18} (150 × 2,1 mm com 3,5 µm de tamanho de partícula)	Água e acetonitrilo, ambos com ácido oxálico	—	0,48 a 2,64	5	0,3	2-12	Koesukwiwat *et al*., 2007
Mel	SMX, SMR, SDM, SCP, SDZ, SMP, STZ, SGD, SMZ, SSZ, SMT	LC-MS (ESI)	SPE, extracção com acetonitrilo	Coluna C_{18} (150 × 2,1 mm com 3,5 µm de tamanho de partícula)	Acetonitrilo e ácido fórmico em água	—	25	5	0,2	< 30	Krivohlavek *et al*., 2005
Lamas	SMX, SDM	LC-MS (ESI)	SPE, extracção com metanol e água	Coluna C_{18} (250 × 4,6 mm com 4 µm de tamanho de partícula)	Metanol e acetato de amónio + ácido fórmico em água (pH 2,8)	0-500	—	5	0,4	< 20	Lillenberg *et al*., 2009

* valores expressos em µg/Kg

Tabela I 4 Determinação de Sulfonamidas por cromatografia líquida com detecção por espectrometria de massa (Continuação).

Amostra	Analito	Detecção	Características do método							REF
			Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Sedimentos marinhos	SDZ, TMP	LC-MS (APCI)	SPE, extracção com acetonitrilo	Coluna C_{18} (150 × 4,6 mm com 3,5 µm de tamanho de partícula)	Acetonitrilo e tampão acetato (pH 4,2)	10-100* 25-250*	4 0,9*	100	0,8	< 15	Sørensen e Hansen, 2002
Água subterrânea	SDZ, SDM, SMZ, SMT, SMX, SMP, SPD, SFX, STZ, SSD, SFN, SMR, SQX, SDX, STZ, SBZ	LC-MS/MS (ESI)	SPE, extracção com acetonitrilo e metanol	Coluna C_{18} (150 × 2,1 mm com 3 µm de tamanho de partícula)	Água e acetonitrilo, ambos com ácido fórmico	0,00001-0,5	0,00003 a 0,0033	—	0,2	< 15	García-Galán *et al.*, 2011
Água de rio	SMT, SFX, STZ, SDZ, SPD, SMZ, SMP, SDM, SMX	LC-MS/MS (ESI)	SPE, extracção com acetonitrilo e água (25:75, v/v)	Coluna C_{18} (150 × 3,1, mm com 3,5 µm de tamanho de partícula)	Ácido fórmico em água e acetonitrilo (pH 2,2)	0,00027-0,168	0,00005 a 0,0002	20	0,2	< 16	García-Galán *et al.*, 2010A
Água	SCM, SSD, STZ, SGD, SDZ, SPD, SMR, SMZ, SMT, SMP, SDX, SMX, SFX, SQX, SBZ, SDM, SFN	LC-MS/MS (ESI)	SPE, extracção com metanol e acetona (1:1, v/v)	Coluna C_{18} (150 × 2,1, mm com 3,0 µm de tamanho de partícula)	Água e acetonitrilo, ambos com ácido fórmico	—	0,00001 a 0,00784	—	0,2	< 12	García-Galán *et al.*, 2010B
Águas de rio e residuais	SDZ, STZ, SMR, SPD, SMT, SMX, TMP	LC-MS/MS (ESI)	SPE, extracção com 1 % amónia	Coluna C_{18} (150 × 2,1 mm com 3,0 µm de tamanho de partícula)	Água e metanol, ambos com ácido fórmico	1-500	0,01-0,25	20	0,2	< 33	Senta *et al.*, 2008
Amostras ambientais	SMT, SFX, STZ, SDZ, SPD, SMP SMX	LC-MS/MS (ESI)	SPE, extracção com amónio e acetonitrilo (1:19, v/v)	Coluna C_{18} (150 × 2,1 mm com 3 µm de tamanho de partícula)	Água e acetonitrilo, ambos com ácido fórmico (pH 2,2)	0,055-1000	0,00001 a 0,00113	20	—	< 15	Díaz-Cruz *et al.*, 2008

* valores expressos em µg/Kg

Tabela I 4 Determinação de Sulfonamidas por cromatografia líquida com detecção por espectrometria de massa (continuação).

Amostra	Analito	Detecção	Características do método								REF
			Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Amostras ambien-tais	bSFN, o-TSA, p-TSA	LC-MS/MS (ESI)	SPE, extracção com metanol	Coluna C_{18} (150 × 2,1 mm com 3,5 µm de tamanho de partícula)	Acetonitrilo e água (10:90, v/v)	0,02-5	—	10	0,2	< 39	Richter *et al*., 2007
Águas residuais	SCM, SDZ, SMX, STZ, SMR, SFX, SMT, SMZ, SM, SMM, SMP, SCP, SDM, SDX	LC-MS/MS (ESI)	SPE, extracção com água e metanol (80:20, v/v)	Coluna C_{18} (150 × 2,1 mm com 3,5 µm de tamanho de partícula)	Metanol e ácido fórmico em água	0,0012-0,0317	0,001 a 0,003	10	0,25	< 12	Ye *et al*., 2007
Águas residuais	SGD, SCM, SDZ, STiZ, SPD, SMR, SMT, SMX, SDM, SSZ	LC-MS/MS (ESI)	SPE, extracção com acetato de amónio; SPME, extracção com acetato de amónio em metanol	Coluna C_{18} (250 × 2,1 mm com 5 µm de tamanho de partícula)	Acetato de amónio em ácido fórmico (pH3) - ou acetato de amónio em acetonitrilo - e metanol: (2:1, v/v)	0,020-1,0	—	10	0,2	—	Balakrishnan *et al*., 2007
Águas residuais	SMZ, SMX, SDZ, SMR, STZ, SPD, TMP	LC-MS/MS (ESI)	SPE, extracção com metanol e acetato de etilo (1:1, v/v)	Coluna C_{18} (125 × 2,0 mm com 3 µm de tamanho de partícula)	Água e acetonitrilo, ambos com ácido fórmico (pH 2,1)	0,010-0,423	—	20	0,15	< 34	Gobel *et al*., 2004
Efluentes hospita-lares	SMX, TMP	LC-MS/MS (ESI)	Filtração por celulose (14 e 0,45 µm)	Coluna C_{18} (150 × 4,6 mm com 5 µm de tamanho de partícula)	Metanol, acetato de amónio e ácido acético (49,5:49,5:1, v/v/v)	0,8-100 0,5-100	0,25 0,80	5	0,6	< 4	Brenner *et al.*, 2011
Carne	STZ, SMP, SNM, SDZ, SMXo, SQX, SCP, SMR, SMX, SM, SSD, SFN, SFX, SDM, SDD, SMT, SPD	LC-MS/MS (ESI)	SPE, extracção com acetona e metanol (1:1, v/v)	Coluna C_8 (150 × 2,1 mm com 3,5 µm de tamanho de partícula)	Água e metanol, ambos com ácido fórmico	10-200	0,01-1*	10	0,2	< 33	Shao *et al*., 2005

* valores expressos em µg/Kg

Tabela I 4 Determinação de Sulfonamidas por cromatografia líquida com detecção por espectrometria de massa (continuação).

Amostra	Analito	Detecção	Características do método							REF
			Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Leite	SCP, STZ, SMD, SMR, SMZ, SMD, SMR, SMZ, SMP, SDM, SMT, SCM, SMM	LC-MS/MS (ESI)	MSPE, separação por acção magnética	Coluna VP-ODS (250 × 2,0 mm com 5,0 µm de tamanho de partícula)	Ácido fórmico em água e metanol	0,05-20	0,0005 a 0,0495	10	0,2	< 17	Gao *et al.*, 2010
Leite e ovos	SCM, SDZ, STZ, SPD, SMR, SMT, SMZ, SMX, SBZ, SDM	LC-MS/MS (ESI)	SPE, extracção com *n*-hexano	Coluna C_{18} (150 × 2,0 mm com 3 µm de tamanho de partícula)	Ácido fórmico em água e acetonitrilo em metanol	7,9-19*	—	20	0,4	—	Tamošiūnas e Padarauskas, 2008
		UPLC-MS/MS (ESI)		Coluna C_{18} (100 × 2,1 mm com 1,7 µm de tamanho de partícula)		7,1-26*	—	10	0,5	—	
Ovos	STZ, SPD, SDZ, SCM, SMR, SMX, SMT, SMZ, SBZ, SDM	LC-MS/MS (ESI)	SPE, extracção com metanol e acetato de amónio	Coluna C_{18} (150 × 2,0 mm com 3 µm de tamanho de partícula)	Ácido fórmico em água e acetonitrilo em metanol	1-45*	n.a.	20	0,4	< 20	Tamošiūnas e Padarauskas, 2007
Mel	SDZ, SMX, STZ, SMR, SMP	LC-MS/MS (ESI)	Membrana líquida de octanol e pentanol (55:45, v/v)	Coluna C_{18} (150 × 3,9 mm com 4 µm de tamanho de partícula)	Metanol e ácido fórmico + acetato de amónio em água	16–500*	5,1 a 27,4*	20	0,4	< 15	Bedendo *et al.*, 2010
Mel	STZ, SMZ, SMR, SPD, SDZ, SDX, SDM, SCP	LC-MS/MS (ESI)	SPE, extracção com ácido trifluoro acético em acetonitrilo	Coluna HLB (20 × 2,1 mm com 5 µm de tamanho de partícula)	Água e acetonitrilo, ambos com ácido fórmico	5-100	0,5-2*	50	0,9	< 9	Thompson e Noot, 2005

* valores expressos em µg/Kg

Tabela I 4 Determinação de Sulfonamidas por cromatografia líquida com espectrometria de massa (continuação).

Amostra	Analito	Detecção	Características do método							REF
			Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Comida animal	SDZ, TMP	LC-MS/MS (ESI)	SPE, extracção com ácido acético em água e metanol	Coluna C_{18} (100 × 2,0 mm com 5 µm de tamanho de partícula)	Ácido acético em água e metanol	—	52 22	10	0,3	n.a.	Croubels *et al.*, 2002
Amostra biológica	SDZ, STZ, SMR, SMT, SM, SMP, SCP, SMM, SMX, SDX, SFX, SDM, SQX	LC-MS/MS (ESI)	SPS, extracção com acetonitrilo e água (50:50, v/v)	Coluna C_{18} (50 × 2,1 mm com 2,7 µm de tamanho de partícula)	Ácido fórmico em água e acetonitrilo	0,1-100	0,75-3,0*	—	1,0	< 7	Lu *et al.*, 2011
Amostra biológica	SDM	LC-MS/MS (ESI)	SPE, extracção com acetonitrilo	Coluna C_{18} (150 × 2,1 mm com 5,0 µm de tamanho de partícula)	Ácido fórmico em água e acetonitrilo	0,5-2000	0,002-0,1	40	0,2	< 7	Li *et al.*, 2009
Amostra biológica	SMX, TMP	LC-MS/MS (ESI)	SPE, extracção com a fase móvel	Coluna de C_{18} (50 × 4,6 mm com 5,0 µm de tamanho de partícula)	Acetato de amónio + ácido fórmico em água (pH 3) e acetonitrilo (40:60, v/v)	880-80000 30-30000	—	2	0,8	< 3	Mistri *et al.*, 2010
Amostras biológicas	SMX, TMP	LC-MS/MS (ESI)	SPE, extracção com a fase móvel	Coluna C_{18} (150 × 4,6 mm com 5 µm de tamanho de partícula)	Acetonitrilo e água (50:50, v/v)	500-60000 50-50000 µg/mL	—	5	2,5	< 12	Bedor *et al.*, 2008
Amostras biológicas	SDZ, TMP	LC-MS/MS (ESI)	—	Coluna C_{18} (100 × 3,0 mm com 3,5 µm de tamanho de partícula)	Ácido acético em água e metanol	100-1000 100-2000	20 62	45	0,3	< 12	Croubels *et al.*, 2003
Solo	SDZ, SM, SMM, SQX	LC-MS/MS (ESI)	Extracção em microondas e SPE, extracção com acetonitrilo	Coluna C_{18} (250 × 4,6 mm com 5 µm de tamanho de partícula)	Ácido acético em água e acetonitrilo	—	1,4–4,8*	10	1,0	< 8	Chen *et al.*, 2009

* valores expressos em µg/Kg

Tabela I 4 Determinação de Sulfonamidas por cromatografia líquida com espectrometria de massa (continuação).

Amostra	Analito	Detecção	Características do método								REF
			Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VI µL	Caudal mL/min	t_r min	
Lamas e sedimentos	SDZ, SMR, SMX, SMT, TMP	LC-MS/MS (ESI)	SPE, extracção com metanol e água (1:1, v/v)	Coluna C_{18} (150 × 3,0 mm com 3,5 µm de tamanho de partícula)	Acetato de amónio e ácido fórmico em água e acetonitrilo	0,1-1000	2,2a 66,9*	5	0,25	< 19	Cai-Ming *et al.*, 2009
Lamas	SMZ, SMX, SDZ, STZ, SPD, TMP	LC-MS/MS (ESI)	SPE, extracção com metanol e acetato de etilo	Coluna C_{18} (150 × 2,0 mm com 3 µm de tamanho de partícula)	Metanol e ácido fórmico em água	—	3-41*	—	0,15	< 22	Gobel *et al*., 2005
Água de Aquacultura	SDZ, TMP	LC-MS/MS (APCI)	SPE, extracção com a fase móvel	Coluna C_{18} (150 × 4,6 mm com 5 µm de tamanho de partícula)	Metanol com ácido fórmico	—	0,001 a 0,002	50	0,60	< 20	Sørensen e Elbæk, 2004
Carne	SDZ, STZ, SMR, SMT, SMM, SMX, SQX, SDM	LC-MS/MS (APCI)	SPME, extracção com acetonitrilo e água (25:75, v/v)	Coluna C_{18} (250 × 4,6 mm com 5 µm de tamanho de partícula)	Água e acetonitrilo (70:30, v/v)	50-2000*	16 a 39*	—	0,5	< 40	Lu *et al.*, 2007
Carne	SMZ, SDM, STZ, SDZ, SMT	LC-MS/MS (APCI)	SPE, extracção com a fase móvel	Coluna ODS 2 (250 × 2,5 mm com 5 µm de tamanho de partícula)	Acetonitrilo, água: e acetato de amónio	—	—	2	0,2	< 25	Kim *et al*., 2002
Produtos farmacêuticos	SDZ, SMR, SDM, SMX, TMP	LC-MS/MS (APCI)	SPE, extracção com acetonitrilo e acetato de amónio com ácido acético (pH 3,5)	Coluna RP_{18} (150 × 2,0 mm com 3 µm de tamanho de partícula)	Acetonitrilo, água e ácido fórmico com ou sem acetato de amónio	5-5000*	0,4-1,3*	10	0,3	< 8	Pfeifer *et al*., 2002

* valores expressos em µg/Kg

Tabela I 4 Determinação de Sulfonamidas por cromatografia líquida com espectrometria de massa (continuação).

Amostra	**Analito**	**Detecção**	**Características do método**							**REF**
			Pré-tratamento	**FE**	**FM**	**Linearidade** µg/L	**LOD** µg/L	**VI** µL	**Caudal** mL/min	**t_r** min	
Carne	SGD, SNM, SCM, SSD, SDZ, STZ, SPD, SMR, SMXo, SMT, SM, SMP, SCP, SMX, SMM, SDM, SFX, SBZ, SDX, SQX, SFN, SPA	UPLC-MS/MS (ESI)	SPE, extracção com *n*-hexano	Coluna C_{18} (100 × 2,1 mm com 1,7 µm de tamanho de partícula)	Água e metanol, ambos com ácido fórmico	0,2-50*	—	10	0,2	15	Cai *et al*., 2008
Carne e Leite	SDZ, SDM, SMX, SCP, SMT, SQX, SFX, SMD, SPD, STZ, SMR, SMM	UPLC-MS/MS (ESI)	—	Coluna C_{18} (100 × 2,1 mm com 1,7 µm de tamanho de partícula)	Ácido fórmico em água e metanol	10-200	0,4-2,0	10	0,25	< 15	Zhao *et al.*, 2011
Leite	SGD, SNM, SSD, SCM, SDZ, STZ, SPD, SMR, SMXo, SMZ, SM, SMT	UPLC-MS/MS (ESI)	SPE, extracção com acetonitrilo	Coluna C_{18} (100 × 2,1 mm com 1,7 µm de tamanho de partícula)	Ácido acético em água e acetonitrilo	0,2-100	0,04 a 1,35*	5	0,25	< 10	She *et al.*, 2010

* valores expressos em µg/Kg

Tabela I 5 Determinação de Sulfonamidas por electroforese capilar.

Amostra	Analito	Detecção	Características do método								REF
			λ nm	Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VA kV	t_r min	
Água	SDX, SMX, SCP, SMR, SDM	UV/Vis	264	SPE, extracção com amoníaco e metanol a 60%	Sílica fundida (150 µm d.i. × 64,5 cm comp.)	Tampão de fosfato (pH 7,3) e metanol	75-100	0,23 a 0,48	25		Lara *et al.*, 2009
Carne e água	SPD, SMT, SMR, SM, SDZ, SDM, SMX, SCP, SMT	UV/Vis	265	n.a.	Sílica fundida (75 µm d.i. × 64,5 cm comp.)	Fosfato de sódio e metanol (pH 7,3)	5-250	2,6 a 23	25	—	Soto-Chinchilla *et al.*, 2006
Carne	SMX, SMZ, SMR, SDM	UV/Vis	254	SPE, extracção com metanol e água (50:50, v/v)	Sílica fundida (75 µm d.i. × 50 cm comp.)	Tampão de fosfato (pH 6,3)	500-50000*	4 a 6*	20	15	Farooq *et al.*, 2009
Carne	SMZ, SMR, SDZ, SDM, SMM, SPA, SQF, SFX	UV/Vis	205-210	SPE, extracção com acetonitrilo e água (50:50, v/v)	Sílica fundida (50 µm d.i. × 72 cm comp.)	Tampão de fosfato (pH 6,5)	500-50000	—	25	n.a.	Fuh e Chu, 2003
Amostra biológica	SMZ, SMR, STZ, SMX, SCP, SC, SGD	UV/Vis	200	SPE, extracção com fosfato e borato	Sílica fundida (75µm d.i. × 51 cm comp.)	Tetraborato de sódio (pH 9,3) e dodecilsulfato de sódio (20:80, v/v)	250-1000*	1,3-7,8	22	—	Kowalski *et al.*, 2011
Produtos farma-cêuticos	SMX, TMP	UV/Vis	205	Extracção com etanol e água (1:1, v/v)	Sílica fundida (50 µm d.i. × 56 cm comp.)	Tampão de borato (pH 9,0), dodecilsulfato de sódio e etanol	6,5-100	1,8	30	13	Injac *et al.*, 2008
Produtos farma-cêuticos	SDZ, SMX, TMP	UV/Vis	250	—	Sílica fundida (75 µm d.i. × 50 cm comp.)	Tampão de fosfato (pH 6,2)	1200-25000	100 a 300	30	—	Nevado *et al.*, 2001

λ: comprimento de onda; d.i.: diâmetro interno; l: comprimento; FM: fase móvel; LOD: limite de detecção; VA: voltagem aplicada; REF: referência; n.a.: não aplicável; * valores expressos em µg/Kg.

Tabela I 5 Determinação de Sulfonamidas por electroforese capilar (continuação).

Amostra	Analito	Detecção	Características do método								REF
			λ nm	Pré-tratamento	FE	FM	Linearidade µg/L	LOD µg/L	VA kV	t_r min	
Água	SPD, SDD, SDX, SDZ, SMR	*Diode array*	254	Microextracção líquido-líquido	Sílica fundida (75 µm d.i. × 50,2 cm comp.)	Dihidrogenofosfato de sódio e acetonitrilo com ácido fosfórico (pH 8,5)	500-50000	20 a 570	25	—	Wen *et al.*, 2011
Carne e água	SPD, SDX, SMZ, SDM, SM, SMR, SCP, SDZ, SMX, SMT, TMP	*Diode array*	264	SPE, extracção com acetonitrilo	Sílica fundida (50 µm d.i. × 96 cm comp.)	Acetato de amónio e hidróxido de amónio (pH 9,5)	5,5-10000	5,5 a 65,4	28	—	Soto-Chinchilla *et al.*, 2007
Amostra biológica	SMX, TMP	*Diode array*	220	SPE, extracção com acetato de etilo	Sílica fundida (50 µm d.i. × 56 cm comp.)	Tampão de fosfato (pH 6,2)	12500-80000 2500-12000	—	20	8	Teshima *et al.*, 2004
Soro	SMX, TMP	*Diode array*	260 206	—	Sílica fundida (75 µm d.i. × 60,2 cm comp.)	Tampão de borato, dodecilsulfato de sódio e acetonitrilo	—	40-60	30	—	Nevado *et al.*, 2005
Leite	SNM, STZ, SMX, SGD, SDZ	Fluorescência	EMI: 495	—	Sílica fundida (75 µm d.i. × 53,2 cm comp.)	Tampão de fosfato (pH 7,5) e dodecilsulfato de sódio	10-280	0,40-2,1	21	7	Lamba *et al.*, 2005
Produtos farma-cêuticos	SMX, SDZ, TMP	Ampero-metria	—	—	Sílica fundida (25 µm d.i. × 70 cm comp.)	Bórax e dihidrogenofosfato de potássio (pH 5,8)	0,05-100000	0,8 a 0,5	18	—	Cui *et al.*, 2008
Carne	STZ, SDZ, SMP, SGD, SNM, SDM, SPD, SCP, SFX, SSZ, SBZ	MS	—	SPE, extracção com metanol	Sílica fundida (75 µm d.i. × 50 cm comp.)	Acetato de amónio (pH 4,2)	50-500*	12,5*	23	—	Font *et al.*, 2007

λ: comprimento de onda; d.i.: diâmetro interno; l: comprimento; FM: fase móvel; LOD: limite de detecção; VA: voltagem aplicada; REF: referência; n.a.: não aplicável; * valores expressos em µg/Kg.

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São vários os solventes envolvidos na preparação de fases móveis para a determinação cromatográfica de SAs. Usados puros ou em misturas, quase todos os trabalhos referem acetonitrilo e/ou água, podendo incluir ainda trietilamina (Croubels *et al*., 2002, Croubels et al., 2003, Coutinho e Barbosa, 2009), metanol (Fang *et al*., 2006, Granja *et al*. 2008, Croubels *et al*., 2002, Tolika et al., 2011, Wu *et al*., 2007, Salami e Queiroz, 2011, Johannessen et al., 2005, Msagati e Nindi, 2004, Croubels et al., 2003, Zotou e Vasiliadou, 2009, Gehring et al., 2006, Posyniak et al., 2005, Lillenberg et al., 2009, Senta et al., 2008, Ye et al., 2007, Balakrishnan et al., 2007, Brenner et al., 2011, Shao et al., 2005, Gao et al., 2010, Tamošiūnas e Padarauskas, 2008, Tamošiūnas e Padarauskas, 2007, Bedendo et al., 2010, Croubels et al., 2002, Croubels et al., 2003, Gobel et al., 2005, Sørensen e Elbæk, 2004, Cai et al., 2008, Zhao et al., 2011), etanol (Kishida e Furusawa, 2003, Kishida e Furusawa, 2004, Kishida, 2007, Ben et al., 2008), propanol (Xu *et al.*, 2010, Raviolo *et al*., 2007, Caballero et al., 2001, Kishida e Furusawa, 2005), tetrahidrofurano (Soto-Chinchilla et al., 2005), *N,N*-dimetilformamida (Kishida e Furusawa, 2001). As soluções utilizadas incluem também, na maioria dos casos, ácido fosfórico/fosfatos de potássio (Tao *et al*. 2009, Xu *et al.*, 2010, Pereira e Cass, 2005, Yang *et al*., 2004, Batzias *et al*., 2002, Primus *et al*., 2007, Li *et al*., 2007, Papapanagiotou *et al*., 2002, Lin e Huang, 2008, Bedor et al., 2008, Stoev e Michailova, 2000), ácido acético/acetato de sódio (Lindkvist *et al*. 2009, Malintan e Mohd, 2006, Kishida e Furusawa, 2003, Wu *et al*., 2007, Kishida e Furusawa, 2004, Santos e Maciel, 2011, Tsai et al., 2010A, Kishida e Furusawa, 2005, Kishida et al., 2005, Msagati e Nindi, 2004, Croubels et al., 2003, Kishida e Furusawa, 2001, Baere et al., 2000, Jiménez et al. 2009, Tsai et al., 2010B, Zotou e Vasiliadou, 2009, Gehring et al., 2006, Posyniak et al., 2005, Pedrouzo et al., 2008, Renew e Huang, 2004, Kim e Lee, 2003, Fuh e Chan, 2001, Sørensen e Hansen, 2002, Brenner et al., 2011, Croubels et al., 2002, Croubels et al., 2003, Chen et al., 2009, Pfeifer et al., 2002, She et al., 2010), ácido trifluoroacético (Bialk-Bielinska *et al.*, 2011, Salami e Queiroz, 2011), ácido fórmico (Payán *et al.*, 2011, Peng *et al*., 2008, Tolika *et al.*, 2011, Johannessen et al., 2005, Jiménez et al. 2009, Li et al., 2008, Ben et al., 2008, Krivohlavek *et al*., 2005, Lillenberg et al., 2009, García-Galán et al., 2011, García-Galán *et al.*, 2010A, García-Galán *et al.*, 2010B, Senta et al., 2008, Díaz-Cruz et al., 2008, Ye et al., 2007, Balakrishnan et al., 2007, Gobel et al., 2004, Shao et al., 2005, Gao et al., 2010, Tamošiūnas e Padarauskas, 2008, Tamošiūnas e Padarauskas, 2007, Bedendo et al., 2010, Thompson e Noot, 2005, Lu et al., 2011, Li et al., 2009, Mistri et al., 2010, Cai-Ming et al., 2009, Gobel et al., 2005, Sørensen e Elbæk, 2004, Cai et al., 2008, Zhao et al., 2011), ácido cítrico/citrato de sódio (Soto-Chinchilla *et al*., 2005), ácido oxálico (Koesukwiwat *et al*., 2007) e/ou acetato de amónio (Hela et al., 2003, Lillenberg et al., 2009, Balakrishnan et al., 2007, Bedendo et al., 2010, Mistri et al., 2010, Cai-Ming et al., 2009, Kim et al., 2002, Pfeifer et al., 2002), para ajuste de pH. Algumas das fases móveis contêm pequenas quantidades de outros compostos iónicos como dodecilsulfato de sódio (Raviolo *et al*., 2007, Caballero et al., 2001) ou hidrogenosulfato de tetrabutilamónio (Batzias *et al*., 2002), tendo em vista melhorar a separação dos vários compostos presentes nas amostras através de um mecanismo de par-iónico. De uma forma geral, os requisitos específicos destas fases móveis fazem com que a análise cromatográfica tenha um elevado custo para o consumidor. Além disso, estes compostos iónicos não são desprovidos de toxicidade pelo que o seu débito contínuo pelo sistema, gera uma elevada

quantidade resíduos. Estes resíduos carecem de um acompanhamento adequado, acarretando custos adicionais e o seu uso levanta algumas reservas do ponto de vista ambiental, sobretudo se os métodos forem usados para análises de *Screening* numa perspectiva de rotina laboratorial.

Os detectores mais correntes associados aos métodos de LC para SAs são os fotométricos, como UV/Vis, *Diode Array* e fluorescência (tabela I-3). Este último pressupõe muitas vezes a utilização de compostos de derivatização, enquanto os primeiros recorrem a leituras de absorvância directa das SAs, não requerendo a sua modificação química. Independentemente da complexidade metodológica associada a cada uma, estas três abordagens de detecção não fornecem, contudo, uma informação qualitativa de cada composto eluido pela coluna, um aspecto que é essencial na análise de amostras complexas. Para colmatar essa lacuna surge a associação da espectrometria de massa (MS, do inglês *Mass Spectrometry*) aos métodos de LC para SAs (tabela I-4). Esta componente permite a identificação dos compostos eluidos na coluna, sobretudo quando o sistema está apetrechado com MS em *tandem* ou MS-MS (Barker, 1998). Este sistema é capaz de fragmentar o ião precursor com um determinado padrão de fragmentação e separar os iões filhos resultantes, para a identificação e quantificação (McMaster, 2005). A informação qualitativa e quantitativa fornecida é muitas vezes importante já que permite a identificação do composto de uma forma inequívoca. Também por isso, as concentrações que é possível determinar são muito inferiores às conseguidas através da utilização da LC com detecção fotométrica. No entanto, equipamentos com este tipo de detecção são extremamente dispendiosos, quer na aquisição quer na manutenção, não estando acessíveis aos laboratórios de análise de rotina, em que muitas vezes o número reduzido de amostras que possuem para analisar não justifica a sua aquisição.

Não pondo em causa a capacidade de separação característica dos métodos cromatográficos ou os avanços nos processos de detecção dos analitos, a obtenção de bons resultados analíticos implica, na maioria dos casos, um processo prévio de extracção/purificação dos compostos, que promova a remoção de interferências e/ou concentração do analito. A extracção em fase sólida (SPE, do inglês *Solid Phase Extraction*) é a técnica mais frequentemente usada como processo de preparação de amostras contendo SAs (Tabela I 3 e 4). Esta etapa extractiva, para além de acarretar um dispêndio de tempo de análise e não facilitar uma monitorização em tempo real, nem sempre permite uma recuperação total do analito, pelo que os resultados obtidos nem sempre correspondem à realidade.

De uma forma geral, os métodos LC apresentam as desvantagens de serem morosos e dispendiosos. Independentemente do tipo de detecção, os cromatogramas associados a misturas de SAs apresentam valores máximos de tempos de retenção que rondam os 30 minutos. Contudo, alguns trabalhos referem tempos superiores a estes (Malintan e Mohd, 2006, Lin e Huang, 2008, Tolika *et al.*, 2011, Wu *et al*., 2007, Caballero *et al*., 2001, Kishida *et al*., 2005, Msagati e Nindi, 2004, Senta *et al*., 2008, Richter *et al*., 2007, Gobel *et al*., 2004, Shao *et al*., 2005, Lu *et al*., 2007), não excedendo os 80 minutos. A este valor, que corresponde apenas à detecção da amostra, devem acrescentar-se o tempo de tratamento e preparação da mesma e do procedimento longo de calibração, o que limita a sua utilização para a

monitorização em tempo real. Os equipamentos requeridos são caros, especialmente aqueles com detecção de MS ou MS/MS e os custos de manutenção associados são também elevados. A detecção MS ou MS/MS adequa-se particularmente à análise de vestígios, mas é demasiado cara para implementar em regime de rotina laboratorial. Todos os solventes envolvidos são também mais caros para LC do que para os demais procedimentos gerais no laboratório e apresentam elevada toxicidade para os seres vivos e para o meio ambiente.

A electroforese capilar (EC) é a técnica electroforética escolhida para as SAs (tabela I 5). As separações são conseguidas por aplicação de voltagens entre 18 kV e 30 kV, as quais geram fluxos electro-osmóticos e electroforéticos de soluções tampão e de espécies iónicas, respectivamente, ao longo de um tubo capilar, em sílica fundida. As extremidades deste capilar são colocadas em reservatórios de tampão, que podem conter também outros solventes diferentes de água (Tabela I 5). Genericamente, a EC apresenta limites de detecção para valores de concentração bem mais elevados do que aqueles que são típicos dos sistemas LC, algo que constitui uma limitação à aplicação desta técnica na monitorização SAs em quantidades vestigiais.

Embora com todas as vantagens que lhe são inerentes, as metodologias cromatográficas ou electroforéticas poderão não ser as mais adequadas ao controlo de rotina, pela impossibilidade de realização de análises expeditas, a baixo custo e com baixo impacto ambiental. Em alternativa a estas, encontram-se também descritos na literatura, os métodos electroanalíticos.

1.4.3 MÉTODOS ELECTROANALÍTICOS

Os métodos electroanalíticos fornecem informação química sobre a solução em estudo, através da medida de uma propriedade de natureza eléctrica numa célula electroquímica. Estes métodos oferecem algumas vantagens relativamente aos métodos instrumentais anteriores: permitem obter informação rápida de natureza quantitativa para quantidades vestigiais de analito; utilizam equipamento de baixo custo e de fácil miniaturização o que aumenta a portabilidade, com possibilidade de efectuar análises de campo; podem fornecer informação sobre a estequiometria e constantes de equilíbrio dos compostos em estudo, e recorrem a processos de preparação de amostra pouco sofisticados e mais amigos do ambiente.

Os métodos electroanalíticos mais utilizados para a determinação de SAs aplicam-se a produtos farmacêuticos e leite, recorrendo a técnicas voltamétricas e amperométricas (Tabela I 6). A voltametria mede a corrente eléctrica produzida num sistema de três eléctrodos, através de uma variação contínua de potencial, enquanto a amperometria mede a corrente eléctrica produzida, a um potencial constante. Tipicamente, as medidas voltamétricas são mais lentas do que as amperométricas por efectuarem um varrimento de potenciais, mas estas últimas são menos selectivas do que as primeiras precisamente

pela aplicação de único potencial, não permitindo descriminar espécies com potenciais de oxidação/redução semelhantes. A selectividade destes sistemas depende, assim, do potencial ou da gama de potenciais aplicados.

Um aspecto de grande importância nos métodos voltamétricos e potenciométricos é o eléctrodo de trabalho utilizado. Incluem-se nestes trabalhos para SAs eléctrodos de carbono vítreo (Braga *et al.*, 2010) ou de diamante ou, ainda, versões modificadas destes através da inclusão de filmes de bismuto (Campestrini *et al.*, 2011), boro (Andrade *et al.,* 2010, 2009A e 2009B, Souza *et al.,* 2008) ou Nafion e nanotubos de carbono (Issac and Girish Kumar, 2009). De um modo geral, são os eléctrodos de diamante modificados com boro que apresentam as melhores características de detecção, detectando concentrações da ordem dos 10^{-8} mol/L. Os seus intervalos de linearidade são porém muito estreitos, estando localizados entre 4,0 e 32-40 μmol/L. Esta característica dificulta a sua aplicação *on-site*, uma vez que as concentrações reais nos sistemas de aquacultura podem variar para além deste intervalo de concentrações.

A potenciometria é, entre as técnicas electroanalíticas, aquela que permite obter gamas de resposta mais alargadas. Baseia-se na medida da diferença de potencial de dois eléctrodos mergulhados numa solução e a sua relação com a actividade de espécies iónicas presentes nessa mesma solução, em condições de corrente quase-nula. Tanto quanto se julga saber, o único método potenciométrico descrito na literatura para SAs (Kamel et al., 2009), para além daqueles desenvolvidos nesta dissertação, descreve detectores potenciométricos preparados com membrana de PVC constituídas com vários ionóforos ou permutadores iónicos, nomeadamente cloreto de *Bis*(triphenylphosphoranilideno)amónio, brometo de tetraoctilamónio e ftalocianina de Fe (II). Os eléctrodos apresentavam um contacto sólido e foram construídos com configuração convencional e tubular, tendo os estudos sido feitos com medidas em estado estacionário e em fluxo. Os limites de detecção conseguidos eram da ordem dos 3,4 μmol/L e 8,7 μmol/L, respectivamente quando avaliados em condições estacionárias ou em fluxo. Estes valores não se adequavam, contudo, a uma implementação local para a determinação de quantidades muito reduzidas de antibiótico. Por outro lado, as características de selectividade indicadas deixavam transparecer a necessidade de recorrer a outros materiais sensores.

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Tabela I 6 Métodos electroanalíticos para sulfonamidas.

Amostra	**Analito**	**Eléctrodo de trabalho ou indicador**	**Técnica**	**Características do método**						**REF**
				Modo de operação	**LLLR** µmol/L (mg/L)	**LOD** µmol/L (mg/L)	**VI** µL	**Caudal** mL/min	**FA** Amostras /h	
Produtos farmacêuticos	SDZ	Eléctrodo de carbono vítreo modificado com filme de bismuto	Voltametria	*Batch*	3,2-20,0 (0,8-5) 20,0-97,0 (5,0-25)	2,1 (0,5) 12,2 (3,0)	n.a.	n.a.	n.a.	Campestrini *et al.*, 2011
Produtos farmacêuticos	SMX, TMP	Eléctrodo de diamante dopado com boro	Voltametria	Multico-mutação	4,0-32 (1,0-8,0) 0,69-5,5 (0,20-1,6)	0,065 (0,0165) 0,063 (0,0183)	500	1,0	30	Andrade *et al.*, 2010
Produtos farmacêuticos	SDZ	Eléctrodo de carbono vítreo	Voltametria	*Batch*	62,7-340 (15,7-85)	10,9 (2,7)	n.a.	n.a.	n.a.	Braga *et al.*, 2010
Produtos farmacêuticos	SDZ	Eléctrodo tubular de contacto sólido de grafite e membrana de PVC	Potencio-metria	*Batch*	3,2-1000 (0,8-250)	3,4 (0,87)	n.a.	n.a.	n.a.	Kamel *et al.*, 2009
				FIA	10-1000 (2,5-250)	8,7 (2,18)	300	2,2	35	
Produtos farmacêuticos	SMX, TMP	Eléctrodo de diamante dopado com boro	Voltametria	*Batch*	4,0-40 (1–10) 6,9-69 (2–20)	0,015 (0,0037) 0,013 (0,0039)	n.a.	n.a.	n.a.	Andrade *et al.*, 2009A
Produtos farmacêuticos	SMX	Eléctrodo de carbono vítreo modificado com Nafion e nanotubos de carbono	Voltametria	*Batch*	50-1000 (12,7-2530)	10 (2,5)	n.a.	n.a.	n.a.	Issac and Girish Kumar, 2009
Produtos farmacêuticos	SDZ, SMX	Eléctrodo de diamante dopado com boro	Voltametria	*Batch*	8-1200 (2,0-300) 8-1200 (2,0-304)	2,2 (0,6) 1,2 (0,3)	n.a.	n.a.	n.a.	Souza *et al.*, 2008
Leite	SMX, TMP	Eléctrodo de diamante dopado com boro	Ampero-metria	*Batch*	0,2-3,1 (0,050–0,8) 0,086-1,4 (0,025–0,4)	0,099 (0,025) 0,059 (0,015)	n.a.	n.a.	n.a.	Andrade *et al.*, 2009B
Leite	SDZ, SMZ, SMR	Eléctrodo coulimétrico	Ampero-metria	FIA	(20,0-154,7) (39,2-169,1) (39,6-171,0)	—	20	2.5	—	Reguera *et al.*, 2008

De uma forma geral, a metodologia que combina as características de rapidez, economia e simplicidade desejadas para a monitorização *on-site* de SAs é a potenciometria com ESIs. Os ESIs oferecem ainda um sinal de elevada estabilidade ao longo do tempo e respondem numa ampla gama de concentrações. Esta é uma grande vantagem em comparação com todas as técnicas de detecção indicadas anteriormente, onde o intervalo de resposta analítica é muito menor. Além disso, os ESIs não são afectados pela cor ou turvação das amostras, como é o caso dos métodos ópticos. Na maioria dos casos são suficientemente selectivos e fáceis de operar, podendo ser facilmente construídos no laboratório. É ainda de salientar o baixo custo dos detectores e do equipamento envolvido. Neste sentido, o tempo e o custo decorrentes da determinação analítica com ESIs são reduzidos. Estes aspectos podem ainda ser melhorados quando se opta pela incorporação destes detectores em sistemas FIA.

Os ESIs são também facilmente acopláveis a FIA, uma associação muita vantajosa para estas unidades (Calatayud, 1996). Entre outras, incluem-se aqui vantagens como o aumento de precisão da resposta analítica, dado que o eléctrodo é exposto a todas as amostras por um período fixo de tempo; o aumento de selectividade, resultante da discriminação cinética da membrana selectiva para os iões interferentes (aos quais responde mais lentamente); e o aumento de tempo de vida, dado que a membrana selectiva não é sujeita a um contacto prolongado com substâncias presentes nas amostras que a possam "danificar".

A potenciometria baseada em ESIs poderia, por isso, constituir uma alternativa vantajosa às demais descritas na literatura. Hoje em dia, é possível encontrar ESIs baseados num pequeno filme ou numa membrana selectiva como elemento de reconhecimento e construídos com as mais diversas configurações, variando desde um formato equivalente a um eléctrodo de vidro até um arranjo tubular, particularmente interessante para a passagem de soluções em condições de fluxo. A superfície sensora, tipicamente constituída por incorporação de uma espécie de reconhecimento numa matriz de PVC plastificado, pode ainda ser aplicada sobre um contacto condutor sólido ou líquido, um aspecto determinante para as características de resposta analíticas obtidas neste contexto. Por último, um dos aspectos de maior importância no desenvolvimento de um ESI prende-se com o material electroactivo incorporado na membrana, e que garante a interacção selectiva com o analito. Neste domínio, será interessante o aparecimento de novos materiais, desenhados especificamente para um composto alvo.

Por não existir mais nenhuma referência neste domínio e pelas vantagens já anteriormente apontadas à utilização da potenciometria com eléctrodos selectivos de iões, justifica-se por isso um estudo mais profundo que resulte na possibilidade de estabelecer procedimentos analíticos simples, versáteis, selectivos e rápidos que possam ser usados no controlo de rotina de águas de aquacultura. O sucesso deste estudo pressupõe alguma inovação no desenvolvimento de sensores potenciométricos habitualmente descritos na literatura, tais como a utilização de materiais sensores mais selectivos e variações na sua configuração e forma de contacto eléctrico com a membrana.

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II. PARTE EXPERIMENTAL

II.1 CONSIDERAÇÕES GERAIS

Genericamente, o trabalho desenvolvido centrou-se no desenvolvimento de eléctrodos selectivos a SAs. Foram preparados vários sistemas sensores para SDZ e SMX (as SAs escolhidas e as mais utilizadas em aquacultura), baseados em permutadores iónicos, condutores neutros (ciclodextrinas), condutores carregados (porfirinas), ou polímeros com impressão molecular em sol-gel, partículas de sílica ou partículas de grafite, contendo aditivos aniónicos ou catiónicos. Estas membranas foram aplicadas em corpos de eléctrodos de configurações distintas, visando, em muitos casos, o desenvolvimento de sistemas de fluxo, tanto para macro, como para micro fluídica. Os corpos dos eléctrodos de diferentes configurações passavam pela construção de eléctrodos convencionais, de contacto sólido (grafite/ resina epoxídica) e de contacto líquido (solução de referência interna em pontas de pipeta), eléctrodos tubulares de contacto sólido (prata metálica) ou planares, também de contacto sólido (prata/resina epoxídica). O desempenho dos sensores foi avaliado em função das características gerais de funcionamento das curvas de calibração, nomeadamente intervalo de resposta linear, sensibilidade, limite de detecção, repetibilidade, reprodutibilidade, exactidão e precisão. O efeito de pH e a selectividade de cada uma das unidades potenciométricas foram igualmente avaliados. Os eléctrodos com características adequadas foram aplicados à análise de amostras reais, águas de aquacultura.

Ao longo deste capítulo são referidas as metodologias envolvidas na construção dos corpos dos eléctrodos, na síntese de novos sistemas sensores, na avaliação e na aplicação dos sensores utilizados. Outros aspectos mais específicos são descritos nos capítulos correspondentes.

II.2 MATERIAL E EQUIPAMENTOS UTILIZADOS

Todas as soluções foram preparadas em balões volumétricos de classe A, com capacidades de 50,00 a 1000,0 mL. Para as medições de volumes rigorosos iguais ou superiores a 5,00 mL foram usadas pipetas volumétricas de vidro, classe A. Para volumes inferiores recorreu-se a pipetas automáticas Gilson, modelos P100, P1000 e P5000, de volume regulável. As pesagens foram realizadas numa balança Kern 770, com precisão igual a 0,00001 g.

Sempre que era necessário promover a dissolução de substâncias sólidas, numa tentativa de reduzir o tempo envolvido nesse processo, foi usado um banho de ultra-sons, com temperatura estabilizada da marca Fungilab, S.A, *ultrasonic cleaner*; para mistura mais eficiente dos compostos químicos utilizados, usou-se um vortex IKA.

As diferenças de potencial entre o eléctrodo indicador e o eléctrodo de referência foram medidas com um milivoltímetro de sensibilidade ± 0,001 V da marca Crison. A comutação entre os eléctrodos, quando a sua avaliação era feita em estado estacionário, foi feita através de um comutador "*home-made*" que

permitia a leitura consecutiva de seis eléctrodos. Para se medir a resistência eléctrica dos eléctrodos construídos utilizou-se um multímetro de marca Fluke 179.

As montagens FIA eram constituídas por um sistema de propulsão, uma unidade de introdução da amostra, um sistema de transporte e/ou mistura de soluções e, ainda, uma unidade de detecção e registo dos sinais analíticos. Como sistema de propulsão utilizou-se uma bomba peristáltica, Gilson, Minipuls 3, de quatro canais. Os tubos de propulsão das soluções eram da marca Tygon. As amostras eram introduzidas no transportador através de uma válvula de injecção rotatória manual, com quatro vias, da marca Rheodyne, 5020, ou através de uma válvula de injecção rotatória automática, Hamilton MVP. Estes sistemas de injecção permitiam a introdução de um volume de amostra fixo, cujo valor era definido pelo comprimento do tubo de Teflon, com 0,64 ou 0,80 mm de diâmetro interno (d.i). Os tubos usados para o transporte da amostra ou outras soluções, ligações entre os vários elementos da montagem de fluxo, bem como as unidades de reacção utilizadas para promover a mistura da amostra com outra solução, eram também em Teflon com 0,8 mm de d.i.. O sistema de detecção era constituído pelos eléctrodos indicador, selectivo às SAs estudadas e cuja construção se refere mais adiante, e de referência, Crison ou Orion, de acordo com a montagem em causa, de dupla junção, electricamente ligados ao decimilivoltímetro. As diferenças de potencial entre os eléctrodos eram registadas por um registador (Kipp & Zonen, BD 117 ou software digital).

Aquando do estudo da influência de pH das soluções nas características de resposta dos eléctrodos preparados, utilizou-se um eléctrodo de vidro combinado WTW, CWL/S7, ligado a um decimilivoltímetro Metrohm 654, pH Meter. A homogeneização da solução ensaiada foi garantida por um agitador magnético da marca IKA®.

A modificação das nanopartículas de carbono e das esferas de silica foi acompanhada através de ensaios espectrofotométricos usando um FTIR da marca Nicolet iS10 com um acessório de ATR que permitiu a leitura directa dos materiais sólidos. O espectrofotómetro UV/Vis, Shimadzu Pharmaspec UV-1700, foi usado sempre que se estudou a estequiometria das reacções de complexação.

O sistema cromatográfico LC-MS/MS, usado para validar alguns dos resultados potenciométricos, era da marca Agilent Technologies e continha um amostrador automático HP1100, um desgaseificador, uma bomba, uma coluna termostatizada C-18 Zorbax Eclipse XDB da Agilent, acoplados a um detector de massa System Sciex API 2000, da Applied Biosystems. Para aquisição de dados usou-se o software Sciex Analyst, versão 1.4.1 .

Para o desenho dos dispositivos de *Low Temperature Co-fired Ceramic* (LTCC), usou-se o programa AutoCAD 2009; para a sua fabricação usaram-se capas Dupont 951 PX, cortadas a laser (Protolaser 200) LPKF, Laser & Electronics. A termo-compressão das capas foi feita usando uma prensa Tallers Francisco Camps.

II.3 REAGENTES E SOLUÇÕES

Todos os procedimentos analíticos referidos ao longo da dissertação foram desenvolvidos recorrendo a reagentes de qualidade analítica pro-análise, sem terem sido submetidos a tratamento ou purificação prévia.

A água utilizada para a preparação das soluções aquosas era ultra-pura, com uma condutividade igual ou inferior a 0,054 µS/cm, a 25ºC. As soluções aquosas mais concentradas foram preparadas por pesagem rigorosa dos sólidos correspondentes e posterior diluição, em água ultra-pura ou em tampão HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*). As menos concentradas foram preparadas por diluição rigorosa das anteriores, também em água ou no referido tampão.

Para o estudo do efeito do pH na resposta dos eléctrodos utilizou-se ácido sulfúrico concentrado e uma solução saturada de hidróxido de sódio, sempre que se pretendia alterar o pH das soluções ensaiadas. Todas as soluções de possíveis espécies interferentes foram preparadas por pesagem rigorosa dos sólidos correspondentes, seguida da sua dissolução e diluição rigorosa em água.

II.4. CONSTRUÇÃO DE DETECTORES POTENCIOMÉTRICOS

Para a construção dos detectores potenciométricos seguiram-se metodologias similares àquelas propostas por Lima *et al.*, 1986, para as unidades de configuração convencional, e por Alegret *et al.*, 1984, para as unidades de configuração tubular. Outras metodologias foram aqui apresentadas pela primeira vez. De seguida, descrevem-se as várias etapas envolvidas na construção de todos os eléctrodos utilizados.

II.4.1 ELÉCTRODOS CONVENCIONAIS

II.4.1.1 DE CONTACTO SÓLIDO

Para a preparação de eléctrodos convencionais de contacto sólido utilizaram-se cilindros ocos de Perspex com 8 mm de d.i. (diâmetro interno) e 10 mm de d.e. (diâmetro externo), cortados em troços de cerca de 12 cm de comprimento (Figura II 1).

Uma das extremidades foi preenchida com pasta de grafite, que resulta de uma mistura homogénea entre grafite em pó, Araldite M, e endurecedor HY, na proporção de 55, 30 e 15 % (p/p), respectivamente. Na outra extremidade, introduziu-se uma placa de cobre onde se soldou no centro um cabo eléctrico co-axial com cerca de 20 cm de comprimento. Procedeu-se à sua fixação na parte superior do corpo do eléctrodo, de forma a garantir a solidez da montagem e evitar a entrada de líquidos. Para tal adaptou-se um terminal tipo BNC (Bayonet Nut Coupling) para estabelecer o contacto eléctrico entre estes e o decimilivoltímetro. Após a construção, e para que houvesse secagem completa da resina epoxídica, os eléctrodos foram deixados à temperatura ambiente, durante pelo menos 48

horas, ao fim das quais era medida a resistência eléctrica de cada uma das unidades. Recorreu-se ao uso de uma lixa para reduzir a rugosidade existente na superfície da grafite e para criar uma ligeira cavidade onde era aplicada a membrana sensora. Estes corpos de eléctrodos foram utilizados nos trabalhos descritos nos capítulos IV, V e VI.

Figura II 1 Representação esquemática de um eléctrodo de contacto sólido

II.4.1.2 DE SOLUÇÃO DE REFERÊNCIA INTERNA

Na preparação de detectores com solução de referência interna utilizaram-se pontas de pipetas em polipropileno de 1000 µL, que funcionaram como corpo do eléctrodo e onde a membrana sensora era adaptada, ficando também em contacto com a solução de referência interna. A parte inferior da ponta de pipeta era introduzida no recipiente que continha a membrana sensora, solubilizada em tetrahidrofurano, e esta, por tensão superficial, ficava fixada na extremidade da mesma, após secagem do solvente orgânico. Na parte superior da ponta da pipeta introduziu-se um fio de prata, recoberto com cloreto de prata e mergulhado numa solução de referência interna (Figura II 2). Estes corpos de eléctrodos foram utilizados nos trabalhos descritos no capítulo VIII.

Figura II 2 Representação esquemática de um eléctrodo com solução de referência interna

II.4.2 DETECTORES PARA FLUXO

II.4.2.1 TUBULARES DE GRAFITE

Os detectores tubulares de grafite foram construídos com base no procedimento descrito no ponto II.4.1.1, com ligeiras modificações. O cilindro de Perspex tinha 7 mm de comprimento e um pequeno orifício lateral para introdução de um cabo eléctrico coaxial blindado. De seguida, este foi preenchido com uma mistura condutora à base de grafite de acordo com Alegret *et al.*, 1987, de igual composição à descrita anteriormente (II.4.1.1). As unidades construídas foram igualmente colocadas numa estufa, para secagem completa da resina não condutora (Figura II 3). Com recurso a uma lixa, procedeu-se ao alisamento das superfícies de modo a ficarem o mais uniforme possível, garantindo-se a sua adequação aos módulos de suporte correspondentes. De seguida, procedeu-se ao isolamento da grafite através da aplicação de uma resina epoxídica não condutora, evitando-se, deste modo, o contacto entre a solução transportadora e a grafite condutora. Tal como para as unidades de configuração convencional, a secagem desta resina foi efectuada em estufa. Estes corpos de eléctrodos foram utilizados nos trabalhos descritos nos capítulos III e VII.

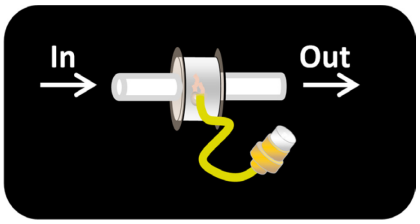


Figura II 3 Representação esquemática de um eléctrodo tubular de grafite

II.4.2.2 TUBULARES DE PRATA

Os eléctrodos foram construídos por corte transversal de um tubo de prata de 0,8 mm de d.i e com 0,5, 1,0 e 1,5 cm de comprimento. Soldou-se posteriormente um fio eléctrico ao suporte, como mostra a Figura II 4, isolando-se toda a superfície condutora. Na outra extremidade do fio adaptou-se, também, um terminal de BNC. Estes eléctrodos deram origem ao trabalho descrito no capítulo III.

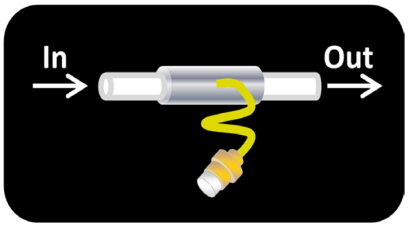


Figura II 4 Representação esquemática de um eléctrodo tubular de prata

II.4.2.3 PLANARES DE PRATA

Os dispositivos fluídicos miniaturizados em LTCC foram construídos com base na tecnologia de corte de LTCC através de um laser de infra-vermelhos, que corta os motivos desenhados previamente no autocad, e de acordo com o que se pretende experimentar. Neste caso, usaram-se 9 capas sobrepostas (Figura II 5), correspondendo cada capa a um corte do dispositivo a determinada cota.

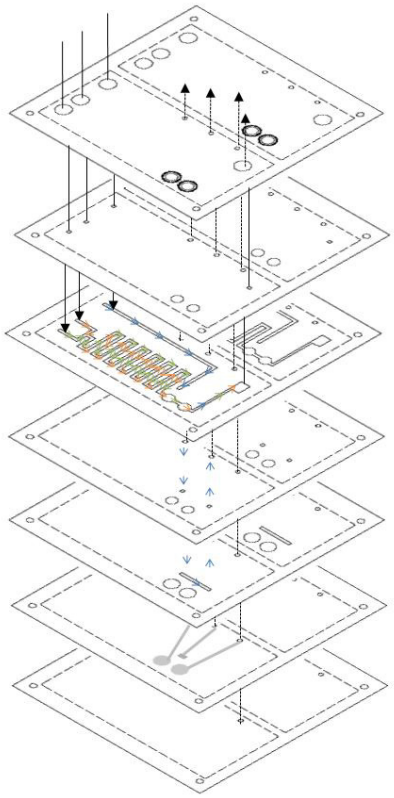


Figura II 5 Representação esquemática das várias capas que constituem o dispositivo cerâmico

Para este efeito, usaram-se capas Dupont 951, a variações de cota de 254µm. As capas foram cortadas a laser. De seguida, foi necessário aplicar pastas cerâmicas em algumas das capas cortadas para serigrafar os circuitos eléctricos ou os suportes condutores dos eléctrodos. Procedeu-se depois à laminação por termo-compressão, usando uma prensa constituída por dois pratos de aço 250x250 mm com controlo de temperatura e pressão. Finalmente, e para obtenção da estrutura final, foi necessário proceder-se à sinterização, primeiro entre os 200-400ºC, para volatilização dos compostos orgânicos, e depois entre os 600-850ºC, para a interpenetração de alumina entra as capas, para se obter o dispositivo final (Figura II 6). Estes eléctrodos foram desenvolvidos no capítulo VII.

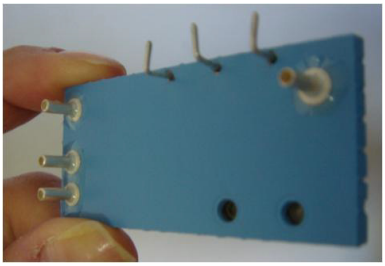


Figura II 6 Representação esquemática do dispositivo cerâmico

Uma vez construídos, os corpos dos eléctrodos encontravam-se prontos para receber a membrana sensora.

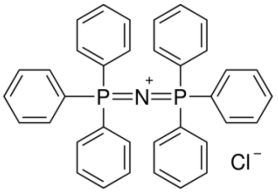
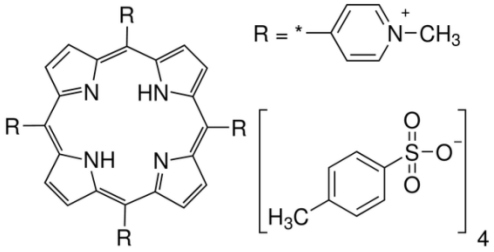
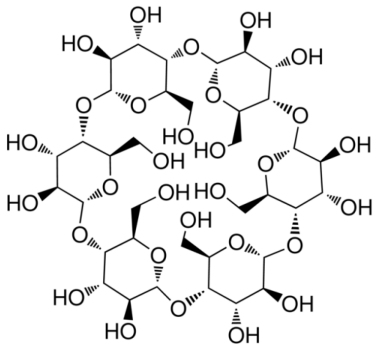
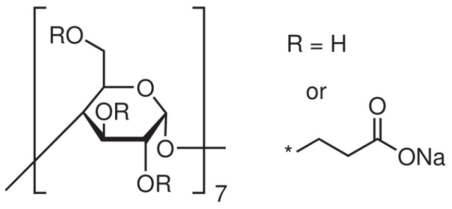
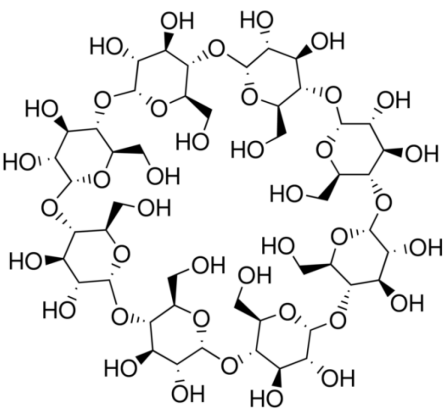
II.4.3 PREPARAÇÃO DE MEMBRANAS SENSORAS

Na preparação das membranas sensoras foram incluídos os seguintes componentes: ionóforo, aditivo, solvente mediador e PVC (matriz polimérica), dissolvidas num solvente orgânico que após volatilização dava origem a uma membrana plastificada. Os ionóforos usados foram comerciais ou sintetizados, como se descreve a seguir.

II.4.3.1 IONÓFOROS COMERCIAIS

Relativamente aos sensores iónicos, foram utilizados compostos comerciais, de naturezas diferentes, tais como sais de amónio quaternário, porfirinas e ciclodextrinas, cujas estruturas se indicam na tabela 1.

Tabela II 1 Moléculas orgânicas usadas como ionóforos no desenvolvimento dos (bio)sensores.

Ionóforo	Estrutura Química	ESIs para determinação de:
Cloreto de *bis*-trifenilfosforanilideno amónio		SDZ (Capítulo III)
Porfirina de Manganês		SDZ (Capítulo IV) SMX (Capítulo V)
α - Ciclodextrina		
β - Ciclodextrina		
γ - Ciclodextrina		

II.4.3.2 POLÍMEROS DE IMPRESSÃO MOLECULAR EM SÍLICA

A impressão da molécula SMX (capítulo VI) foi realizada na superfície de esferas de sílica, conjugando o conceito de "*self-assembled monolayers"* e de sol-gel (Lin et al., 1998).

Para este efeito, modificou-se a superfície das esferas de sílica com uma solução de 3-aminopropiltrimetoxisilano (APTMS) preparada em metanol. De seguida, procedeu-se à incubação das mesmas com gluteraldeído, por 12h, à temperatura ambiente. Adicionou-se primeiro uma solução da molécula que se pretendeu estudar, preparada em tampão, que esteve em agitação durante 1h. Seguidamente, adicionou-se uma solução tampão de tris(hidroxilmetil)aminometano (TRIS) e manteve-se a agitação por mais 30 minutos. Lavou-se a mistura anterior com água destilada, filtrou-se e deixou-se secar o filtro, durante pelos menos 12h.

Após este processo, adicionou-se, sob constante agitação, ácido 3-aminofenilborónico (ABPA, 50 nM) e deixou-se incubar, durante 45 minutos. De seguida, foi adicionado o iniciador persulfato de sódio (25nM) que incubou durante 1h. Removeu-se o analito ligado com ácido forte (ácido oxálico). A mistura foi posteriormente lavada, abundantemente, com água destilada, até que no filtrado não apresentasse pH inferior a 7. Por fim, a mistura sensora foi colocada a secar, durante cerca de 12h.

A solução sensora foi preparada posteriormente por solubilização do material sintetizado no solvente mediador. A esta mistura, e quando necessário, foi adicionado um aditivo iónico, como sais lipofílicos carregados positiva ou negativamente. Para a preparação das membranas, misturaram-se as soluções sensoras em PVC, previamente dissolvido em THF. Depois de preparadas, as membranas eram armazenadas num recipiente de vidro âmbar.

De uma forma geral, os (bio)sensores foram obtidos por aplicação, gota a gota, das membranas sobre o suporte condutor sólido, ou permitindo a sua subida, por capilaridade, pelo corpo do eléctrodo. Para cada aplicação efectuada, deixava-se o tetrahidrofurano evaporar totalmente, para se evitar a formação de bolhas de ar na superfície da membrana. Para a sua completa secagem, os eléctrodos foram mantidos numa posição vertical, ao ar, durante um período de aproximadamente 16 horas. Após este intervalo de tempo, eram colocados a condicionar numa solução aquosa do ião principal, com uma concentração igual a $1x10^{-6}$ mol/L, para que a membrana ficasse hidratada. Era também nesta solução que os eléctrodos eram mantidos enquanto estavam fora de uso.

II.4.3.3 NANOPARTÍCULAS DE CARBONO

Para a realização do trabalho descrito no capítulo VIII usaram-se nanopartículas de carbono como base de um material sensor. A impressão molecular deu-se à superfície destas partículas com dimensões muito reduzidas (<500 nm).

Para que fossem criadas as cavidades de reconhecimento molecular, procedeu-se inicialmente a um processo de acidificação das nanopartÍculas, com uma mistura de H_2SO_4:HNO_3 (3:1), em banho de ultra sons, durante 2h. De seguida, e após remoção do ácido com água, até pH neutro, misturaram-se, sob agitação constante, as nanoparticulas com o tampão HEPES 0,005 M, com *N*-hidroxisuccinimida (NHS, 50 mg/mL), durante 1h e com cloridrato de *N*-etil-*N'*-(3-dimetilaminopropil)carbodiimida (EDAC) fresco (10mg/mL), durante 30 min. Filtrou-se a suspensão e deixou-se secar durante 12h. Após este processo, adicionou-se ABPA (50 nM) e deixou-se incubar, sob agitação constante, durante 45 minutos. Juntou-se de seguida o iniciador persulfato de sódio (25nM), tendo esta mistura incubado durante 1h. Removeu-se posteriormente a SA ligada com ácido forte (ácido oxálico) e lavou-se abundantemente com água destilada, até que o filtrado apresentasse pH inferior a 7. O sólido recolhido foi deixado em estufa a secar, durante 12h.

A preparação das soluções sensoras a partir dos materiais sintetizados e a construção dos (bio)sensores seguiu um procedimento análogo ao indicado no ponto II.4.3.2.

II.4.3.4 SOL-GEL

O desenvolvimento de sensores baseados na técnica sol-gel envolveu um estudo sistemático de variáveis que podem influenciar esta tecnologia, nomeadamente o tipo de precursor, o pH, a natureza e a concentração dos catalisadores, a razão molar de H_2O:Si, o tipo de co-solvente, a temperatura e a natureza química do recipiente onde se dá a reacção.

No capítulo VI é apresentado um sensor para determinação de SDZ e SMX, recorrendo à técnica de sol-gel para a sua preparação. Esta envolve a hidrólise e a policondensação de compostos organometálicos. Para isso, misturaram-se cada uma das SAs (12.5 mg SDZ ou SMX) a imobilizar com precursor (1.5 mL 3-aminopropiltrietoxisilano, APTES) e um monómero (1.5 mL difenildimetoxisilano, DPTS), ambos dissolvidos num co-solvente (4.0 mL metanol), e agitando esta mistura durante 30 minutos. De seguida, adicionou-se o agente reticulante (0.5 mL tetraetoxisilano, TEOS), o catalisador (HCl 0,1M) e água destilada (2.5 mL). Deixou-se em agitação durante 7h, até que a mistura envelhecesse e adquirisse a viscosidade adequada. A deposição da membrana nos eléctrodos convencionais foi feita pelo método de "*dip-coating"* durante cerca de 7s. As unidades foram colocadas a secar, durante 2 dias à temperatura ambiente. Em alternativa, estes materiais sol-gel foram pulverizados e incluídos como ionóforos em membranas de PVC, de modo análogo ao descrito para os materiais obtidos nos pontos II.4.3.2 e II.4.3.3.

II.5 SISTEMAS DE FLUXO IMPLEMENTADOS

As montagens FIA utilizadas nas determinações potenciométricas foram constituídas por um sistema propulsor (que assegurou o movimento das soluções no seu interior), um sistema de injecção (que permitiu intercalar volumes de amostra, rigorosos e reprodutíveis, no fluxo transportador), e um sistema de detecção, constituído pelos eléctrodos selectivo (configuração tubular) e de referência, acoplados a um milivoltímetro e com um eléctrodo de "terra", constituído por um metal, que permitiu eliminar a variação de corrente produzida pelo movimento do fluxo. Este eléctrodo foi colocado imediatamente antes do sistema de detecção (Figura II 7) (Silva *et al.*, 2005). Os eléctrodos selectivos acoplados foram de configuração tubular em sistemas de macrofluídica ou planar em sistemas de microfluídica.

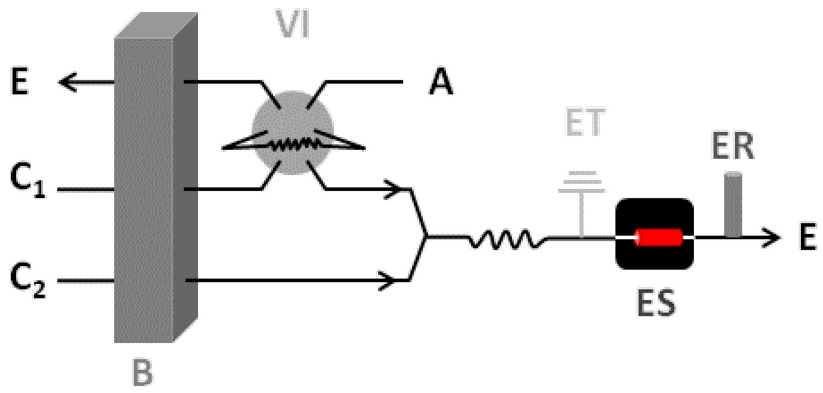


Figura II 7 Montagem FIA de baixa dispersão adaptada à avaliação da influência do pH no comportamento dos detectores tubulares. B: bomba peristáltica; VI: válvula de injecção; A: amostra; C_1 e C_2: canal de transporte de soluções; ET: eléctrodo de terra; ES: eléctrodo selectivo a sulfonamidas; ER: eléctrodo de referência; E: esgoto; mV: decimilivoltímetro; R: registador; EV: eléctrodo de vidro.

As montagens de fluxo apresentadas nos capítulos III e VII foram identificadas após estudos de optimização das condições hidrodinâmicas, nomeadamente o volume de injecção e o caudal transportador. Utilizou-se, para este efeito, o método univariado, fazendo variar-se uma das condições e mantendo a outra fixa.

II.6 CARACTERIZAÇÃO DOS ELÉCTRODOS

Para a avaliação das características gerais de funcionamento dos eléctrodos, realizaram-se traçados de curvas de calibração em soluções com diferentes concentrações de ião principal. Através destas, determinou-se o limite inferior de resposta linear, o limite de detecção e o declive. Além destes parâmetros, avaliou-se a estabilidade dos potenciais dos eléctrodos ao longo de um dia de trabalho e ao

longo do tempo. A zona de operacionalidade em que a resposta do eléctrodo se mantinha praticamente inalterada perante variações do valor de pH da solução foi identificada através do traçado de diagramas de Reilley. Foi também avaliada a extensão da interferência de alguns compostos no sinal analítico, através da determinação dos respectivos coeficientes de selectividade potenciométricos.

II.6.1 CURVAS DE CALIBRAÇÃO

A transdução potenciométrica baseia-se nas medidas entre a diferença de potencial do eléctrodo indicador e do eléctrodo de referência quando mergulhados numa solução e a actividade de espécies presentes na referida solução. As diferenças de potencial medidas (potencial do eléctrodo selectivo de ião *versus* potencial de eléctrodo de referência) variam linearmente com o logaritmo da actividade de um dado ião em solução (equação II 1), traduzindo-se esta resposta matematicamente pela seguinte equação II 1.

$$Emf = "Constante" + \frac{2{,}303RT}{Z_A F} x \log a_A$$

Equação II 1 Equação de Nernst-Nicolski. Emf: valor experimental de potencial de um eléctrodo selectivo de ião; R: constante dos gases perfeitos, 8,3144 J/K mol; T: temperatura termodinâmica, K; F: constante de Faraday, 9,64846x10^4 C/mol; z_A: carga do ião principal; a_A: actividade do ião principal, mol/L ou mol/kg; "constante": inclui o potencial normal do eléctrodo selectivo, o potencial do eléctrodo de referência e o potencial de junção líquida, mV.

Na presença de outros iões para além do ião principal, a resposta do eléctrodo pode ser representada pela equação II 2.

$$E = constante + \frac{2{,}303RT}{z_A F} log\left[a_A + \sum_{i=1}^{n} K_{A,B_i}^{pot} \left(a_{Bi}\right)^{z_A / z_{Bi}} \right]$$

Equação II 2 Equação de Nicolsky-Eisenman. E: valor experimental de potencial de um ESI; R: constante dos gases perfeitos, 8,3144 J K-1 mol-1; T: temperatura termodinâmica, K; F: constante de Faraday, 9,64846x104 C mol-1; aA: actividade do ião principal, mol l^{-1} ou mol kg^{-1}; aB: actividade de espécie iónica interferente; zA: número inteiro de sinal e magnitude da carga do ião principal; zB: número inteiro de sinal e magnitude da carga de iões interferentes; "constante": inclui o potencial normal do eléctrodo selectivo, o potencial do eléctrodo de referência e o potencial de junção líquida, mV; K: coeficiente de selectividade potenciométrica relativamente ao ião interferente.

II.6.1.1 CARACTERÍSTICAS GERAIS DE RESPOSTA

A representação gráfica de actividade em função da diferença de potencial é designada de curva de calibração. A partir desta, podem extrair-se os parâmetros gerais de resposta dos eléctrodos, sendo os mais relevantes: o limite inferior de resposta linear (LLLR, do inglês, *lower limit of linear range*), o limite de detecção (LOD, do inglês, *limit of detection*) e o declive (Figura II 8) (Castro *et al.*, 1989).

O LOD, definido como a concentração resultante da menor medição que pode ser detectada, com razoável certeza, para um certo procedimento analítico, foi calculado de acordo com as especificações da IUPAC (IUPAC, 1976). Também neste caso, o LLLR é o valor de concentração a partir do qual a resposta potenciométrica típica apresenta um comportamento linear. O declive traduz uma maior ou menor reversibilidade da reacção, do ponto de vista termodinâmico, devendo assumir, em condições normais de pressão e temperatura, um valor de cerca de $59/z_A$ mV/década a dividir pelo valor da carga do ião.

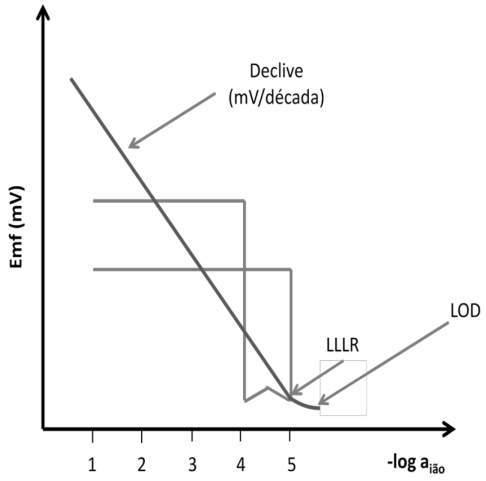


Figura II 8 Características gerais da curva de calibração

A precisão é um termo geral que pretende avaliar a dispersão de resultados entre ensaios independentes, repetidos sobre uma mesma amostra, amostras semelhantes ou padrões, em condições definidas. Para a avaliação deste parâmetro efectuaram-se 4 calibrações sucessivas calculando-se o declive médio e o respectivo desvio padrão, pelo cálculo do coeficiente de variação.

II.6.1.2 PROCEDIMENTO DE CALIBRAÇÃO

II.6.1.2.1 ESTADO ESTACIONÁRIO E FLUXO

Para as calibrações em modo estacionário, mergulharam-se os eléctrodos de referência e indicadores num copo, ao qual se adicionou um volume rigoroso de tampão, que também funcionava como ajustador da força iónica. A solução foi agitada continuamente, pela imersão de um magneto e colocada sobre uma placa de agitação magnética. Adicionaram-se sucessivamente, volumes crescentes de uma solução concentrada da espécie a determinar, registando-se, para cada uma das adições, o valor da diferença de potencial observada. A leitura do sinal analítico foi efectuada quando os potenciais variavam entre ±0,2 mV.

Sempre que os eléctrodos eram inseridos nos sistemas de fluxo, a calibração procedia-se inserindo repetidamente na solução transportadora, volumes definidos de solução padrão de sulfonamida com concentração crescente. A solução transportadora era simultaneamente ajustadora do pH e da força iónica.

II.6.2 ESTUDO DA INFLUÊNCIA DO PH

Este estudo foi realizado efectuando diagramas de Reilley e/ou realizando calibrações dos eléctrodos em soluções com diferentes valores de pH. O diagrama de Reilley representa a variação dos valores de ΔE (mV), fornecidos pela célula potenciométrica, em função dos valores de pH de uma solução de ião principal. Foi considerado como intervalo operacional do eléctrodo aquele cujos valores de pH davam origem a variações de sinal analítico que não ultrapassava os ± 5 mV.

Neste estudo foram adicionados volumes pequenos de uma base forte (NaOH) ou de um ácido forte (H_2SO_4) a uma solução de ião principal com uma concentração fixa e com um volume de cerca de 200 mL, de forma a promover uma variação de pH das soluções originais sem alterar significativamente a concentração do analito. A homogeneização da solução, cujo pH foi modificado, foi garantida com o auxílio de um agitador magnético. As variações de pH foram monitorizadas por um eléctrodo de vidro combinado, previamente calibrado por intermédio de soluções tampão padronizadas com 10, 7 e 4 unidades de pH. Em sistemas de fluxo o procedimento foi análogo, injectando-se aliquotas da solução de analito aos seus diferentes valores de pH.

As curvas de calibração a diferentes valores de pH foram efectuadas de acordo com os procedimentos descritos em II.6.1, realizando-se ensaios em soluções tampão com diferentes valores de pH.

II.6.3 AVALIAÇÃO DA SELECTIVIDADE DOS ELÉCTRODOS

A selectividade de uma unidade potenciométrica traduz a sua capacidade de discriminar, em meios complexos, o ião para o qual o ESI é selectivo. Neste sentido, o potencial de um eléctrodo numa solução que contenha o ião principal, A, e um outro ião, B, é expresso pela equação de Nicolsky-Eisenmam (equação II 2). A influência do ião coexistente é traduzida pela sua concentração e pelo coeficiente de selectividade potenciométrica, $K^{pot}_{A,B}$.

Os coeficientes de selectividade potenciométricos podem ser calculados por vários métodos: (i) o das soluções separadas; (ii) o das soluções mistas; (iii) e o *matched potential* (IUPAC, 1995). Embora os valores não coincidam quando as medidas são feitas por diferentes métodos, a ordem de grandeza dá-nos indicação do grau de interferência das espécies interferentes. Nos diferentes trabalhos realizados

optou-se por usar, para esta avaliação, o método das soluções separadas. Nos casos em que a espécie interferente estava presente nas amostras a dosear ou apresentava carga nula recorreu-se ao método *matched potential.* No primeiro caso, preparou-se uma solução que continha apenas o ião principal com uma actividade a_A e mediu-se a diferença de potencial (E_A). Seguiu-se o mesmo procedimento para o ião interferente (a_B), em que a_A=a_B, anotando-se a diferença de potencial (E_B). O coeficiente de selectividade foi calculado pela equação II 3.

$$K_{A,B}^{POT} = a_A\left(1 - \frac{Z_A}{Z_B}\right)e^{\frac{(E_B - E_A)Z_A F}{RT}}$$

Equação II 3 Expressão matemática utilizada no cálculo do coeficiente de selectividade pelo método das soluções separadas.

O método *matched potential* expressa a razão entre as actividades do ião primário e do ião interferente que produzem a mesma variação de potencial em condições semelhantes (Equação II 4). Para realizar os ensaios por este método, meteram-se os eléctrodos num copo de vidro no qual se introduziu um volume rigoroso de solução contendo apenas o ião primário (a_A), sob agitação constante. Adicionou-se um volume rigoroso de uma outra solução do ião primário com actividade a'_A, anotando-se o valor da diferença de potencial ocorrida. O procedimento foi repetido colocando os eléctrodos numa solução contendo o ião primário (a_A) e à qual se adicionou um volume rigoroso de uma solução contendo o interferente, com actividade a_B, de modo a obter a mesma diferença de potencial anteriormente registada. O valor do coeficiente de selectividade foi então determinado de acordo com a equação II 4 (Umezawa *et al*., 2000).

$$K_{A,B}^{POT} = \frac{(a'_A - a_A)}{a_B}$$

Equação II 4 Expressão matemática utilizada no cálculo do coeficiente de selectividade pelo *matched potential*.

Tal como no estudo do efeito do pH, o procedimento de avaliação da selectividade em sistemas FIA foi análogo ao referido anteriormente, injectando-se aliquotas da solução de analito e/ou interferente, contendo as concentrações desejadas.

II.7 DOSEAMENTO DAS AMOSTRAS

As amostras de água de aquacultura analisadas foram obtidas na região Norte do país. A colheita foi feita a montante e a jusante, a uma profundidade de 1 m. As amostras foram recolhidas, em duplicado, em frascos de vidro âmbar e colocadas sob refrigeração até à chegada ao laboratório.

A análise das amostras foi efectuada sempre por potenciometria directa, isto é, as concentrações de analito nas amostras foram calculadas por interpolação nas respectivas curvas de calibração. O número de ensaios realizados para cada amostra foi, no mínimo, igual a três.

A validação dos resultados foi efectuada, sempre que possível, recorrendo a métodos oficialmente descritos, nomeadamente ao LC-MS/MS do tipo triplo quadrupolo. O ião precursor foi obtido através da ionização do composto por "*electrospray*", sendo utilizado o azoto como gás de cortina, colisão e nebulização. Para estes ensaios, as soluções padrão de SDZ e SMX foram preparadas por dissolução de 10,0±0,01 mg em metanol, sendo a concentração final da solução stock de 1 mg/mL. Esta foi guardada a -80 ºC. As concentrações usadas para o traçado da curva de calibração foram preparadas a partir de uma solução de 250 µg/mL em metanol/água (1:4) de forma a enquadrar as concentrações das amostras. As soluções de amostra foram preparadas em paralelo e de modo análogo às soluções padrão. As condições cromatográficas usadas foram as seguintes:

Coluna Cromatográfica	Zorbax Eclipse XDB-C18, 5 µm, 150x2.1 mm
pré-coluna	Zorbax SB
Fase móvel	A: ácido fórmico:H_20 (1:9) B: Acetonitrilo:H_20 (9:1)
Fluxo do eluente	0,35 mL/min
Temperatura da coluna	450ºC
Volume de injecção	5 µL

II.7.1 TRATAMENTO ESTATÍSTICO DOS RESULTADOS DAS AMOSTRAS

A avaliação estatística dos resultados foi realizada com o software Origin 7 e com Microsoft Excel. Sempre que possível, recorreu-se ao teste *t*-student, para comprovar a exactidão dos resultados obtidos pela metodologia desenvolvida relativamente aos apresentados na literatura, e ao teste F de Fisher, que se baseia na comparação das variâncias entre o procedimento experimental e o procedimento aceite como verdadeiro. A aceitação do método pressupõe valores de *t* e F calculados, que sejam inferiores aos tabelados, para um nível de significância de 95% para *n-1* graus de liberdade.

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Desenvolvimento de um método automático para a monitorização de sulfadiazina em estações de aquacultura: montagem de análise por injecção em fluxo com um detector construído com uma agulha de veterinária revestida por uma membrana selectiva PVC

Com este trabalho pretendeu-se avaliar a possibilidade de utilizar um sistema de análise por injecção em fluxo, com detecção potenciométrica, para monitorizar a presença de SAs em águas de aquacultura. Como modelo, escolheu-se a SDZ, um dos antibióticos do grupo das SAs mais utilizado neste sector.

Começou-se por construir eléctrodos selectivos a SDZ preparados por deposição de uma membrana de PVC num suporte sólido à base de grafite. Foram preparadas diferentes membranas que incorporavam, como material electroactivo, um permutador iónico (sal de amónio quaternário) conjuntamente com um solvente mediador. Em algumas destas membranas foi também incluído um aditivo. Fez-se uma avaliação comparativa para as diferentes membranas e seleccionou-se aquela que fornecia melhores características de resposta para a determinação em vista.

Essa membrana, depois de optimizada, foi utilizada posteriormente para a construção de unidades potenciométricas tubulares, que eram adaptadas a um sistema de injecção em fluxo contínuo. Construíram-se eléctrodos tubulares com suporte condutor de grafite e eléctrodos de suporte tubular metálico feitos a partir de agulhas de veterinária. O sistema de fluxo era constituído por dois canais em que, num deles, circulava uma solução para ajuste de pH e força iónica e no outro circulava água. O sistema de fluxo estabelecido foi optimizado em relação às suas características hidrodinâmicas de fluxo, nomeadamente o volume de injecção e o caudal. Verificou-se que, após avaliação dos eléctrodos em fluxo, os que eram construídos com suporte metálico eram os que forneciam melhores características de funcionamento.

O sistema automático foi aplicado à determinação de SDZ em amostras de água de aquacultura fortificadas e forneceram resultados estatisticamente comparáveis com os obtidos por um método de LC-MS/MS utilizado para a análise das mesmas amostras.

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ORIGINAL PAPER

Rapid automated method for on-site determination of sulfadiazine in fish farming: a stainless steel veterinary syringe coated with a selective membrane of PVC serving as a potentiometric detector in a flow-injection-analysis system

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Abstract Sulfadiazine is an antibiotic of the sulfonamide group and is used as a veterinary drug in fish farming. Monitoring it in the tanks is fundamental to control the applied doses and avoid environmental dissemination. Pursuing this goal, we included a novel potentiometric design in a flow-injection assembly. The electrode body was a stainless steel needle veterinary syringe of 0.8-mm inner diameter. A selective membrane of PVC acted as a sensory surface. Its composition, the length of the electrode, and other flow variables were optimized. The best performance was obtained for sensors of 1.5-cm length and a membrane composition of 33% PVC, 66% *o*-nitrophenyloctyl ether, 1% ion exchanger, and a small amount of a cationic additive. It exhibited Nernstian slopes of 61.0 mV decade^{-1} down to 1.0×10^{-5} mol L^{-1}, with a limit of detection of 3.1×10^{-6} mol L^{-1} in flowing media. All necessary pH/ionic strength adjustments were performed online by merging the sample plug with a buffer carrier of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 4.9. The sensor exhibited the advantages of a fast response time (less than 15 s), long operational lifetime (60 days), and good selectivity for chloride, nitrite, acetate, tartrate, citrate, and ascorbate. The flow setup was successfully applied to the analysis of aquaculture waters. The analytical results were validated against those obtained with liquid chromatography–tandem mass spectrometry procedures. The sampling rate was about 84 samples per hour and recoveries ranged from 95.9 to 106.9%.

Keywords Sulfadiazine · Chemical sensor · Aquaculture · Flow-injection analysis · Potentiometry

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Introduction

Sulfadiazine (SDZ) is one of the sulfonamides most used [1] to treat veterinary and human infections. Its wide use in animal feed makes it a serious contaminant in food of

animal origin, posing serious problems to human health, such as allergic or toxic reactions [2] and the appearance of resistant bacteria [3–5]. This may be prevented by avoiding, first, its environmental dissemination and, later, its entry into the food chain.

Aquaculture activities are an important vehicle for dissemination of SDZ throughout the environment. The first measure to prevent spread of this drug is to have strict control of the applied doses by monitoring the waters from the tanks where the fish are being farmed. As the SDZ concentration may change with time (reaching several consecutive days of administration), monitoring these concentrations in real time is fundamental. This requires specific instrumental setups that allow monitoring of the drug online and in a continuous fashion. This must be achieved with a low-cost method, providing quick and selective responses.

Many papers may be found in the literature concerning SDZ determination in fish and feed [6–8]. Liquid chromatography with tandem mass spectrometry (LC–MS/MS) is currently regarded as the tool of choice for analysis of antibiotic residues in animal-derived food [6]. The use of LC–MS/MS also has the advantage of providing structural information. By contrast, only a few methods have been reported for monitoring SDZ in surface waters [2] or wastewaters [9]. These also rely on high-performance liquid chromatography, which is unsuitable for establishing continuous monitoring of SDZ in local fish farming units. For this purpose, rapid and inexpensive screening methods relying on more or less selective sensory surfaces are desired. The practical implementation of this idea requires low-cost equipment that is easy to operate.

Sensors relying on immunoassays [10, 11] are quite effective but are quite expensive for routine and continuous monitoring. Chemical sensors relying on charged and neutral carriers are also effective and have been proposed [12, 13]. Both sensors offer suitable analytical features for conducting continuous analysis of the administered drug, ion exchangers being much less expensive sensory materials than neutral/charged carriers.

Chemical sensors may also be a suitable methodological approach for monitoring SDZ on-site by means of flow-injection analysis (FIA). This association combines and enhances the advantages of both and avoids some of the limitations of potentiometric sensors [14]. The transient nature of the FIA signal may help overcome the effect of interfering ions on the sensors (if the response to these ions is slower than that of the target analyte) and the lifetime of the electrodes may be extended as the sensor surface is predominantly exposed to carrier solution [15, 16].

The performance of flow-injection potentiometric techniques depends largely on the design of flow-through potentiometric cells. Traditionally, potentiometric sensors are conceived with an internal solution, which is a source of sample contamination. Even when this effect is minimized [17], the internal reference hinders the development of miniaturized devices. Solid-contact electrodes should be employed instead [18], where the membrane materials are cast over conductive solid surfaces. When flowing conditions are used, the solid-contact electrodes should have a tubular shape. This configuration avoids the disturbance of the laminar flow and reduces the response time.

This work describes the construction of SDZ-selective electrodes made from stainless steel syringes (with tubular shape) of different lengths. The selective membrane coated the internal surface of the needle and the stainless steel acted as a solid contact. The analytical performance was evaluated in a FIA system and compared with the analytical performances of graphite-based solid-contact electrodes of conventional and tubular shapes. The flow assembly was optimized and applied to the analysis of aquaculture waters.

Experimental

Apparatus

Potentiometric measurements were made in an $Ag|Ag^{+}, KCl||$test solution$|$PVC membrane$|$solid-contact conductive material electrochemical cell. The emf was measured with a Crison μpH 2002 potentiometer(±0.1-mV sensitivity) coupled to a Kipp & Zonen BD 111 recorder. The reference electrode was an Orion 90-00-29 double-junction electrode.

The FIA system (Fig. 1) consisted of a Gilson Minipuls 3 peristaltic pump with PVC tubing (1.60 and/or 2.00 mm inner diameter) and a four-way Rheodyne 5020 injection valve. All components were joined by polytetrafluoroethylene tubing (Omnifit, Teflon, 0.8-mm inner diameter) and Gilson end fittings and connectors. Support devices for tubular and reference electrodes and the confluence point were constructed from Perspex as reported in [12].

The potentiometric data were validated against data obtained using the LC-MS/MS procedure. Measurements were made with an Agilent Technologies (Palo Alto, CA, USA) 1100 series liquid chromatography setup, equipped with a mass-selective detector, quaternary pump with vacuum degasser, autosampler, and thermostatic column compartment coupled to a Sciex API 2000 (Applied Biosystems, Foster City, USA) triple-quadruple system. The liquid chromatography system was equipped with a Zorbax Eclipse XDB C_{18}, 5 μm, 150 mm×2.1 mm column (Agilent Technologies, Palo Alto, USA).

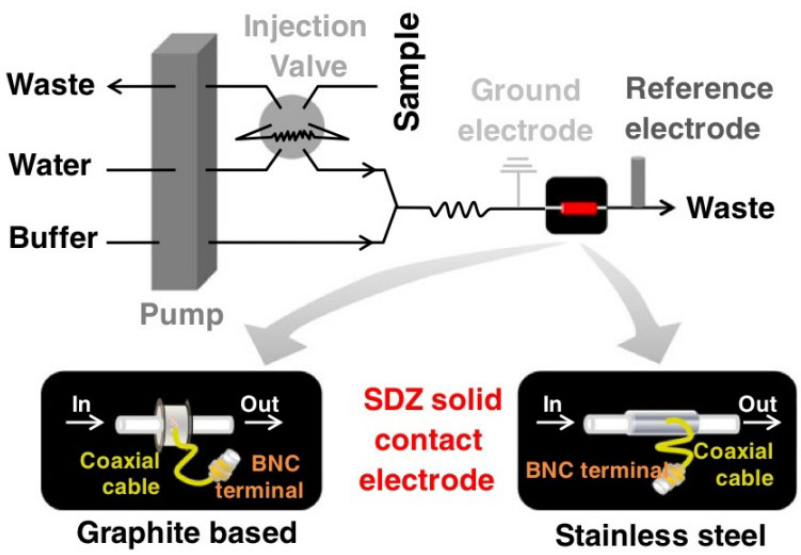

Fig. 1 Flow-injection-analysis system with tubular electrodes. *SDZ* sulfadiazine

Reagents and solutions

All chemicals were of analytical grade and deionized water (conductivity less than 0.1 μS cm^{-1}) was used throughout. Sodium SDZ (NaSDZ; Sigma), bis(triphenylphosphoranylidene)ammonium chloride (BTPPIACl; Aldrich), *o*-nitrophenyl octyl ether (oNPOE; Fluka), dibutylphthalate (DBP; Fluka), tetraoctylammonium bromide (TOABr; Sigma), 4-*tert*-octylphenol (TOP; Fluka), PVC (Fluka) of high molecular weight, tetrahydrofuran (Riedel-de Haën), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma) were used in this work.

The most concentrated SDZ standard solution had a concentration of 1.0×10^{-2} mol L^{-1}. It was prepared by dissolving about 1.2 g of NaSDZ in 500.0 mL of 0.01 mol L^{-1} HEPES (pH ~ 4.9) or in water. These standards were used in batch or flow assays, respectively. Less concentrated SDZ standards were prepared by suitable dilution of the previous solution in the same solvent.

The evaluation of the effect of pH and other interfering species required sodium hydroxide (Merck), concentrated sulfuric acid (Merck), sodium chloride (Merck), sodium nitrite (Merck), sodium acetate (Merck), sodium sulfate (Merck), potassium iodide (Aldrich), potassium bromide (Aldrich), sodium citrate (Riedel-de Haën), sodium tartrate (Riedel-de Haën), sodium ascorbate (Aldrich), and chlorpromazine hydrochloride (Riedel-de Haën).

Construction of the electrode body

Graphite-based electrodes were constructed with a conventional shape (similar to pH electrodes) or a tubular configuration for batch and flow evaluations, respectively [12]. The electrode body was made from Perspex® and had a conductive solid contact prepared with graphite/epoxy resin exposed on one side, and polished prior to membrane coating.

Stainless steel electrodes had a tubular configuration and were made from veterinary syringes (0.8-mm inner diameter) obtained from local drug stores. The needles were cut transversally to obtain a hollow cylinder of 5-, 10-, or 15-mm length. A shielded electrical wire was connected to the external surface and the remaining conducting external layer of the cylinder was isolated. The membrane solution was applied dropwise in the inner wall of the cylinder in order to coat it. The resulting detection unit was inserted in the flow assembly by placing small pieces of pumping tubes at both ends of the needle.

Preparation of the SDZ-selective electrodes

The anionic exchanger [bis(triphenylphosphoranylidene) ammonium sulfadiazine, BTPPIASDZ] was synthesized by mixing 50.0 mL of a 1.0×10^{-2} mol L^{-1} NaSDZ aqueous solution with 100.0 mL of a 1×10^{-2} mol L^{-1} BTPPIACl aqueous solution. The resulting solid was separated by filtration and thoroughly washed with water. It was dried after under a nitrogen atmosphere and kept in a dessicator.

Sensor solutions were prepared by dissolving about 0.0130 g of ion exchanger in about 1.0200 g of oNPOE (type A, B, and C sensors). The selective membranes were obtained by adding 0.0750 g of PVC (previously dissolved in about 2.0 mL of tetrahydrofuran) to 0.2 mL of each sensor. For membranes B and C, a small amount of TOABr or TOP, respectively, was added. An additional membrane equivalent to membrane B, but having DBP instead of oNPOE, was also prepared (type D sensor). Each of the previous membranes was applied on the solid conductive

support, made of graphite/epoxy resin (conventional and tubular) or stainless steel (tubular shape).

All electrodes were conditioned by soaking them overnight in a 1×10^{-6} mol L^{-1} SDZ solution before use and were stored in deionized water between daily measurements. For this purpose, the flow devices were inserted in a closed flow circuit filled with conditioning solution.

Flow potentiometric procedures

The flow assembly is shown in Fig. 1. All potentiometric measurements were conducted at room temperature. The main analytical features of the SDZ-selective electrodes were obtained from calibration curves following the IUPAC recommendations [19, 20]. The emf was measured after stabilization to ±0.2 mV and was plotted as a function of the logarithm of the SDZ concentration. All analytical signals were measured for increasing concentrations of SDZ, both in batch and in flow assays.

FIA assays were conducted similarly to batch evaluations. The selectivity was assessed by the separated solutions method [21], injecting separately into the FIA system solutions of SDZ or other possible interfering species. The effect of pH was evaluated by injecting a 1.0×10^{-3} mol L^{-1} SDZ solution prepared in water. A small adaptation of the manifold, consisting of a closed loop with continuous circulation of 200 mL of standard solution [22], was required for this purpose. Small amounts of a saturated sodium hydroxide solution were added to provide a pH increase up to pH 12. This was followed by the addition of small volumes of concentrated sulfuric acid until pH 2 was reached. This pH was monitored by a second potentiometric cell consisting of a combined glass electrode connected to another pH meter. Operational pH ranges were defined for maximum potential variations of ±15 mV.

Analysis of aquaculture waters

Potentiometric analysis was conducted on aquaculture water samples. These were collected from several sweet water aquaculture units in the north of Portugal. The samples were spiked with SDZ up to 140 μg mL^{-1}, because by the time of collection there was no previous antibiotic application. The analysis was performed after calibration of the potentiometric units.

LC-MS/MS analysis used a binary gradient phase of 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient started with 90:10 solvent A to solvent B, changed linearly to 10:90 solvent A to solvent B in 15 min, and changed back to 90:10 solvent A to solvent B in 1 min, with a total run time of 16 min. The injection volume was set to 5 μL, the temperature oven was 40 °C and the flow rate was 0.35 mL min^{-1}. The mass spectrometer operated in positive electrospray ionization mode using the TurboIonSpray source. Nitrogen was used as a curtain gas, collision gas and nebulizer gas with flow rates of 7, 4, and 9 L min^{-1}. The ion source block temperature was set to 450 °C, and the electrospray capillary voltage was set to 5.0 kV. Data acquisition was performed in the multiple reaction monitoring mode.

Results and discussion

Selection of membrane materials

Selective membranes include a plasticizer and active ingredients, such as ionophores and ionic additives, in an inert matrix. The inert matrix must be hydrophobic, flexible, nonporous, and crack-resistant and grant the membrane physical-mechanical stability and elasticity. PVC is widely used for this purpose, because it is inexpensive, allows plasticization, and grants good mechanical properties.

The binding between the ionophore and the target ion is the molecular-level event sensed by the potentiometric sensor. Thus, the selected ionophore must have high affinity for SDZ and low ability to leave the membrane for the measuring solution. In this work, BTPPIASDZ was selected as the ionophore. It exchanges negatively charged species (such as SDZ) because it bears a positive charge on the nitrogen atom of a quaternary amine group, but it also bears apolar groups that ensure its long residence in the membrane. Although BTPPIACl has not been widely used for potentiometric sensors, it has been proven to be a suitable material to obtain low limits of linear range, wide dynamic concentration ranges, and long lifetimes [23].

The plasticizer should display high lipophilicity to prevent its exudation from the polymer matrix, and high capacity to dissolve the substrate and other additives [24]. It affects the selectivity of the ion-selective sensor by influencing the extraction of ions into the organic phase and controlling their ion exchange with the ionophore. The nature of the plasticizer influences both the dielectric constant of the membrane and the mobility of the ionophore and its complex [25]. Therefore, the effect of plasticizer type on the response characteristics of the SDZ-selective electrodes was investigated by testing two plasticizers of different polarities: oNPOE and DBP. Their dielectric constants are 23.6 and 6.4, respectively [26].

Ionic additives ensure that ion-selective-electrode (ISE) membranes are permselective, reducing in the present case the cationic interference and lowering the electrical resistance [27]. They may also catalyze the exchange kinetics at the sample/membrane interface. In the present study, TOABr and TOP were used. They are surfactants with

polar or cationic heads and C_8 apolar chains, being sufficiently lipophilic to remain in the organic membrane phase when in contact with aqueous solution. In terms of analytical performance, the addition of lipophilic additives is expected to enhance the ISE performance, widening the linear range with theoretical slope [27].

Optimization of membrane composition

Several membrane compositions were tested, with about 1%, ion exchanger , 66% plasticizer, and 33% PVC. The influence of the nature of the plasticizer and the additive (when present) on the emf was investigated, and the results are shown in Table 1.

Calibration parameters

All calibrations indicated that BTPPIASDZ was a suitable ion exchanger for SDZ readings and that the additives employed contributed to a major decrease in the lower limit of the linear range, especially when TOP was present. It was also clear that a plasticizer of higher dielectric constant was essential to obtain near-Nernstian behavior.

In general, the best performance was displayed by SDZ-selective membranes having oNPOE as a plasticizer and TOP as an additive. The corresponding average slope and limit of detection in buffer were −59.2 mV decade^{-1} and 4.0×10^{-6} mol L^{-1}, with enhanced reproducibility and repeatability compared with other SDZ sensors.

Response time and lifetime

The time required to achieve a steady potential response (± 2 mV) for a tenfold increase of SDZ concentration starting at 5×10^{-5} mol L^{-1} was less than 15 s. This indicated that the complexation between SDZ and bis(triphenylphosphoranylidene)ammonium was kinetically fast, which in turn pointed out that the free energy barrier from free to complexed states was small. Replicate calibrations for each sensor indicated low potential drift and long-term stability, especially for type C sensors.

The proposed electrodes could be used for at least 2 months without any considerable change in the typical response, this being the maximum period observed. Detection limits, response times, linear ranges, and calibration slopes were reproducible within ±3% of their original values over this period.

Effect of pH

Reilley diagrams for a 1.0×10^{-3} mol L^{-1} SDZ solution prepared in water are presented in Fig. 2. The Useful pH ranges always corresponded to acidic media. Increasing the pH (towards the alkaline range) led to decreasing emfs.

Table 1 Analytical features of conventional sulfadiazine (SDZ) graphite solid-contact electrodes

Characteristics	ISE A	ISE B	ISE C	ISE D
Ionophore	BTPPIASDZ	BTPPIASDZ	BTPPIASDZ	BTPPIASDZ
Plasticizer	oNPOE	oNPOE	oNPOE	DBP
Additive	–	TOABr	TOP	TOP
Ionic strength adjuster (1×10^{-2} mol L^{-1})				
LOD (mol L^{-1})	1.6×10^{-4}	1.1×10^{-5}	7.8×10^{-6}	2.0×10^{-5}
LIRL (mol L^{-1})	5.2×10^{-4}	3.0×10^{-4}	1.2×10^{-5}	4.4×10^{-5}
Slope (mV decade^{-1})[a]	−20 (±10.0%)	−51 (±4.8%)	−53 (±3.4%)	−49 (±4.5%)
Intercept (mV)	−162	−158	−162	−110
R^2	> 0.9985	> 0.9983	> 0.9990	> 0.9970
Repeatability (mV)[b]	±1.45	±1.74	±0.23	±1.77
Reproducibility (mV)[c]	±1.53	±1.80	±0.25	±1.75
Operational pH range[d]	3.5-7.0	3.5-5.9	4.0-5.5	4.0-5.8
Buffer (pH 4.5 and 5.0×10^{-3} mol L^{-1} ionic strength)				
LOD (mol L^{-1})	2.0×10^{-4}	1.0×10^{-5}	4.0×10^{-6}	8.0×10^{-6}
LIRL (mol L^{-1})	5.2×10^{-4}	6.3×10^{-5}	1.0×10^{-5}	1.5×10^{-5}
Slope (mV decade^{-1})[a]	−40 (±10.3%)	−56 (±2.6%)	−59 (±2.5%)	−44 (±2.7%)
Intercept (mV)	−64	−149	−156	−124
R^2	> 0.9942	> 0.9950	> 0.9989	> 0.9935
Repeatability (mV)[b]	±1.45	±1.74	±0.23	±1.77
Reproducibility (mV)[c]	±1.53	±1.80	±0.25	±1.75

ISE ion-selective electrode, *BTPPIASDZ* bis(triphenylphosphoranylidene)ammonium sulfadiazine, *oNPOE* *o*-nitrophenyl octyl ether, *DBP* dibutylphthalate, *TOABr* tetraoctylammonium bromide, *TOP* 4-*tert*-octylphenol, *LOD* limit of detection, *LLLR* lower limit of linear range

[a]Relative standard deviation for 18 determinations with two different units

[b]Tested with 5.2×10^{-4} mol L^{-1}

[c]Tested with 4.5×10^{-4} mol L^{-1}

[d]For a potential change of ±10 mV

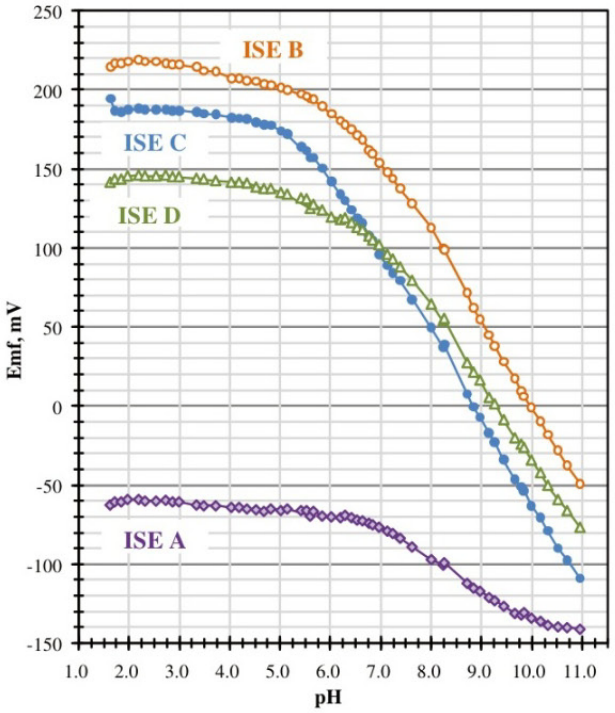

Fig. 2 Reilley diagram plotting the emf of a 1.0×10^{-3} mol L^{-1} SDZ solution against pH. *ISE* ion-selective electrode

This behavior was attributed to hydroxyl interference. A slight precipitation of SDZ in solution was observed at extremely acidic pH values.

As indicated in Table 1, the widest operational pH range was recorded for the type A detector and the narrowest was recorded for the type C detector. Compared with the type A device, the type B device presented a narrower operational pH range as a result of a decrease of about 1 pH unit in the upper limit. Compared with the type B device, the type C device presented an even narrower operational pH interval, resulting from a decrease of about 0.5 pH units in both upper and lower limits.

In general, the operational pH ranges were strongly dependent on the additive, becoming particularly narrow when TOP was used. The presence of TOP enhanced the general operating features but increased the pH interference at the same time. This corroborated with the fact that TOP improved the potentiometric analytical performance by promoting hydrogen bonding with the analyte. This way, it also acted as a proton exchanger when dissociated as phenolate anion [28]. This feature pointed out the need to buffer the measuring solutions with a pH between 4 and 5.5.

Selectivity

The selectivity was assessed by the separate solutions method [29]. The emf was measured for two separate solutions: one with only SDZ (of fixed activity) and the other with only interfering ion (and equal activity). Less interference was observed for more negative log K^{POT}.

The selectivity was examined for several organic and inorganic species of different charges, lipophilicity, and relative molecular mass (Fig. 3). In general, the sensors obeyed the Hofmeister lyotropic anion selectivity sequence, $I^->NO_3^->Br^->Cl^->CH_3COO^->SO_4^{2-}$, just as expected because the potentiometric response relied on an ion-exchange process. This sequence represented the order of extractability of these anions into organic solvents [30], which is mainly governed by differences in hydration energy.

Excluding the effect of iodide and nitrate in ISE types A and B, all species displayed a negligible interference. Membranes with TOP displayed better selectivity than those with no additive or with TOABr. This was attributed to hydrogen bonding between the hydroxyl of the phenol group of TOP and SDZ [31]. Lower interferences were found for ions having small proton affinity. Acetate, citrate, and tartrate showed a similar interfering effect in all electrodes. Despite their high proton affinities, these particular species contributed to significant pH changes, meaning that the observed interference was a combined effect of their ionized chemical structure and the H^+/HO^- ratio in solution. Furthermore, they have carboxylate groups within the molecular structure that may establish intramolecular hydrogen bonding, which reduces their affinity for intermolecular hydrogen bonding and consequently their degree of interference.

Flow assembly

A flow-injection manifold operating with a potentiometric detector is typically set up with minimum complexity, requiring only online ionic strength/pH adjustment. That was achieved by a double-channel manifold that alters online the matrix of the injected solutions before they reach the sensor. Under this condition, all solutions were prepared in water and merged online with a supporting electrolyte solution that worked as an ionic strength adjuster (Fig. 1). These solutions were mixed by means of a reactor, assembled in a knitted coil as this configuration minimizes dispersion and ensures adequate mixing of the injected sample and the carrier before they reach the potentiometric cell.

The final FIA assembly was obtained after checking/setting the several chemical, hydrodynamic, and physical variables indicated in Table 2. A small amount of SDZ was added to the buffer carrier to improve baseline stabilization and increase the lifetime of the detector. The exact SDZ concentration varied and was typically 10 times less than that of the first standard used for calibrating the system.

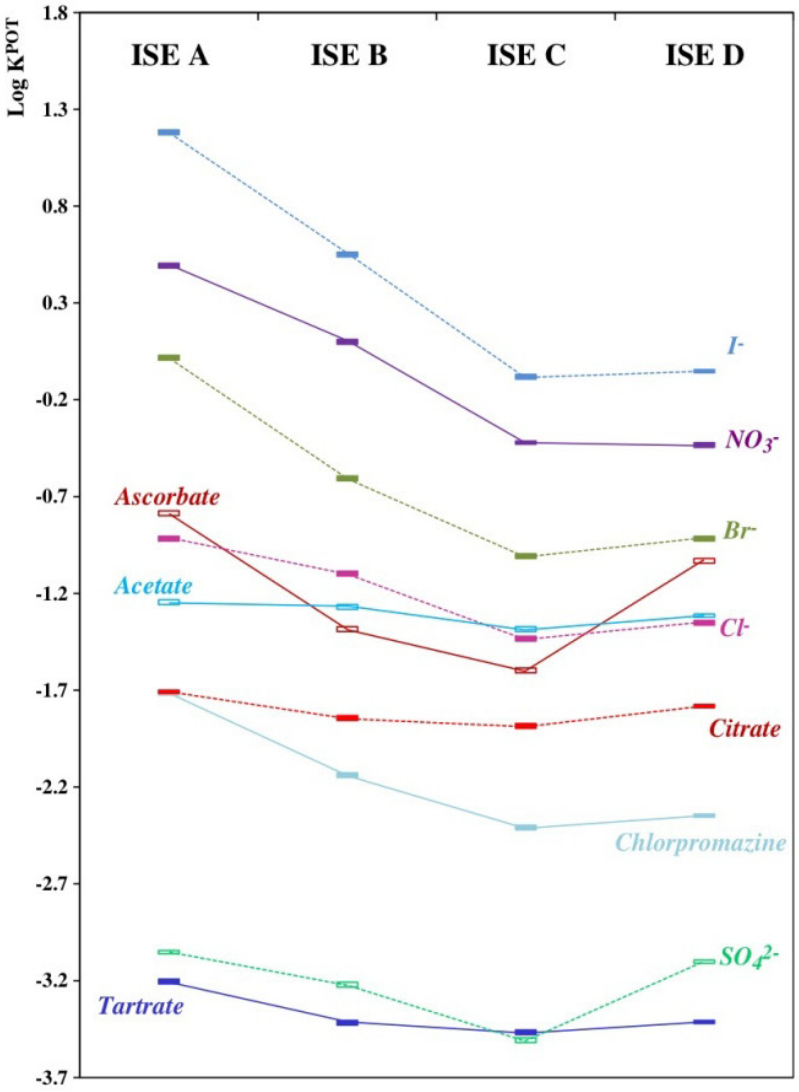

Fig. 3 Potentiometric selectivity coefficients (log K^{POT}) obtained in steady-state conditions

Selection of the sensor length

The effect of the electrode contact area was investigated for tubular SDZ sensors of different lengths. Table 3 shows the general operating characteristics of 1.0- and 1.5-cm sensors. Their slopes varied within 50 and 62 mV decade^{-1} and the dynamic concentration range was similar to that observed in batch conditions. The longer electrodes were the only ones displaying Nernstian behavior, and these were selected for further studies.

Table 2 Selected conditions for the flow-injection potentiometric system

Parameter	Condition	Range studied	Selected conditions
Chemical variables	Ionic strength/pH adjuster	–	HEPES, pH 4.5
	Concentration (mol L^{-1})	–	1.0×10^{-5}–1.0×10^{-3}
Hydrodynamic variables	Flow rate (mL min^{-1})	2.0–8.0	3.0
	Injection volume (μL)	200–1,000	300
Sensing unit	Length (mm)	10 and 15	15
	Conditioning	–	1.0×10^{-6} mol L^{-1} SDZ
	Measurement mode	–	Time base

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Table 3 Main analytical features of the SDZ tubular electrodes with different solid contacts

Characteristics	Graphite	Metal	
		1.0 cm	1.5 cm
LLLR (mol L^{-1})	2.5×10^{-5}	1.0×10^{-5}	1.0×10^{-5}
LOD (mol L^{-1})	7.6×10^{-6}	3.0×10^{-6}	3.1×10^{-6}
Slope (mV $decade^{-1}$)[a]	50.4±0.3	49.5±0.9	61.6±1.2
R^2	0.994±0.004	0.996±0.003	0.996±0.001
Repeatability (mV)[b]	±0.8	±0.8	±0.2
Reproducibility (mV)[c]	±3.5	±2.4	±1.6
Precision (%)	3.5	2.5	2.5
Accuracy (%)	2.2	3.0	1.3
Baseline drift (mV)	< 5.0	< 5.0	< 5.0
Baseline stability (mV)[d]	< 0.7	< 0.7	< 0.7
Sampling rate (sample h^{-1})	~80	~82	~84

[a]Relative standard deviation of 18 determinations with three units
[b]At 4.0×10^{-4} mol L^{-1}
[c]At 4.0×10^{-4} mol L^{-1}
[d]Maximum observed potential deviation in a single calibration

Because of the transient signal, the response time is lower for detectors of shorter length because the time of contact between the selective membrane and the analyte is limited to the size of the electrode. However, the sampling rates were about 80 samples per hour for all lengths tested. This was most probably a result of the quick response of the sensors, taking only a few seconds to reach a stable potential.

Selection of the main flow variables

The most relevant flow variables were the sampling volume and the flow rate. This optimization process was performed by investigating the effect of these variables on the slope and sampling frequency of six SDZ standard solutions, from 1.0×10^{-4} to 1.0×10^{-2} mol L^{-1}.

Injection volume

In general, the FIA peaks were higher for increasing injection volumes, meaning the detector had higher sensitivity. However, the residence time of the sample in the electrode surface was also longer, leading to a longer time to return to the baseline (and hence smaller sampling rates) and greater consumption of sample/buffer. Thus, a compromise between these two opposite effects was sought for 200-, 500-, or 1,000-μL injection volumes. For the maximum injected volume (corresponding to about 2 m of FIA tube), the steady state was obtained regardless of the flow rate used. Theoretical slopes were obtained after 500 μL (Table 4). Above this, the peak heights increased slightly but had no significant effect on the slope of the calibration run and provided dispersion values close to 1. This condition ensured a small dispersion, corresponding always to peaks higher than 90% of the maximum peak height obtained, less time to reach the baseline, and less reagent consumption. The average sampling rate was 84 samples per hour. For higher injected volumes, the average sampling rate decreased by 8% and the average sample consumption increased by 35%.

Flow rate

For a constant injected volume, the residence time of the sample is inversely proportional to the flow rate and the recovery time increases with the residence time of the sample. Thus, higher sampling rates are expected for higher flow rates, but this condition may hinder sensitivity. A compromise between these two features was checked from 2 to 8 mL min^{-1}. The flow rates of each single channel were

Table 4 Analytical response of the SDZ tubular sensors of 1.5-cm length (dispersion calculated for 5×10^{-4} mol L^{-1})

Loop (μL)	Flow rate (mL min^{-1})	Slope (mV $decade^{-1}$)	Sampling rate (samples h^{-1})	Dispersion coefficient
200	2	44.1±0.8	48±0	1.4
	4	61.2±3.5	79±15	1.4
	6	55.1±0.8	111±0	2.4
	8	49.7±0.7	152±11	2.8
500	2	52.2±3.7	32±3	1.2
	4	65.4±0.3	82±4	1.2
	6	65.7±2.2	112±6	1.5
	8	61.8±2.1	144±0	2.4
1,000	2	59.9±1.2	27±1	1.1
	4	59.6±0.8	49±8	1.2
	6	52.6±3.2	67±2	1.3
	8	52.7±2.2	90±0	1.4

set equal by keeping the same pumping tubes on the peristaltic pump and the total flow rate was measured after the detector. SDZ sensors displayed near-Nernstian slopes for flow rates of 3 mL min^{-1} and above and these remained more or less constant after that (Table 4). Under this flow-rate range, the peaks became sharper and had higher resolution. Thus, the flow rate was set to 3.0 mL min^{-1}. About 90% of the maximum peak height was found to reach the baseline within a short time and less carrier was consumed. The sampling rate was about 84 samples per hour. For higher injected volumes, the sampling rate decreased by 9% and the sample consumption increased by 40%.

Main analytical features

The main analytical features of the 1.5-cm metal electrodes were similar to those obtained in batch conditions. This observation indicated that the selective membrane governed the analytical response of the devices, regardless of the solid contact used. For comparison purposes, a tubular graphite-based solid-contact electrode of 1.0-cm length (Fig. 1) was also constructed and compared with the novel metal configuration, also of 1.0-cm length (Table 3). No significant differences were obtained in terms of calibration parameters, selectivity, and pH effect, thus confirming that the observed behavior was attributed to the selective membrane. These electrodes were, however, less sensitive than those of 1.5-cm length, which could be straightly correlated to the effective contact time between the sample plug and the membrane, which is greater for long electrodes.

The metal electrodes of 1.5-cm length were selected for further studies. These were the only ones displaying Nernstian behavior. An extended calibration is shown in Fig. 4.

Lifetime and precision

The electrode lifetime was investigated by testing periodically SDZ solutions ranging from 1.0×10^{-4} to 1.0×10^{-2} mol L^{-1}. The electrodes exhibited good stability in terms of slope, with negligible changes after 2 months of continuous use. The repeatability of the emf was 1.1% for a 4.0×10^{-4} mol L^{-1} SDZ standard solution (n=15).

The precision of the method was investigated by intraday and interday determination of SDZ with three different concentrations and was determined by calculating the relative standard deviation (Table 5). For intraday studies, each concentration was evaluated by five repeated measurements for five times in a working day. For interday measurements, studies were conducted for 1 week. No significant differences were found between intraday and interday assays. The variation coefficients ranged from 0.7 to 8.7%, thus confirming the high precision of the potentiometric readings.

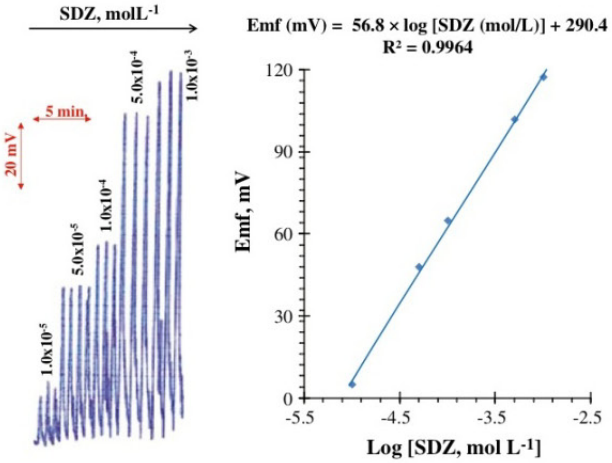

Fig. 4 Typical diagram obtained with 1.5-cm stainless steel electrodes and the corresponding calibration curve (1.0×10^{-6} mol L^{-1} SDZ flowing with the carrier buffer)

The accuracy was assessed by estimating the percentage relative error between measured and added concentrations. Relative errors were always less than 10% and the recoveries ranged from 93.1 to 104.6% (Table 5), confirming the accuracy of the method.

Analysis of aquaculture waters

The applicability of the SDZ electrodes was tested in aquaculture waters collected from commercial fish farms in the north region of Portugal. Estimating the levels of SDZ in polluted waters from aquaculture facilities was not possible for two main reasons: (1) there is no legal limit for SDZ in aquaculture use and (2) the effective concentration depends on the time of sampling after drug administration, which in turn depends on the purpose of its use (different doses for different fish conditions). Indeed, it is quite probable that on many occasions the system will be unable to perform a direct reading of the drug. In this case, the use of solid-phase microextraction techniques is advised. They allow a significant preconcentration of SDZ and, at the same time, contribute to sample cleanup.

All assays were conducted in (1) batch conditions with the conventional-shape graphite-based solid-contact electrodes and in (2) flow conditions with tubular metal electrodes of 1.5-cm length. Type C membranes were always used for this purpose. Table 6 reports the mean results of at least five determinations. In batch experiments, average recoveries and the corresponding relative standard deviations were 101.4% and 3.7%, respectively. For FIA mode, these were 102.7% and 2.0%, respectively.

Table 5 Analytical precision and accuracy of the assay of SDZ with flow-injection analysis (FIA)

Concentration (mmol L^{-1})	Intraday[a]				Interday[b]			
	Found (mmol L^{-1})	Recovery (%)	δ (%)	VC (%)	Found (mmol L^{-1})	Recovery (%)	δ (%)	VC (%)
0.16	0.17	104.6	4.4	8.7	0.16	100.7	0.0	5.5
1.00	0.94	93.7	−6.3	2.7	0.93	93.1	−7.0	0.7

Recovery (%)=$[SDZ]_{found}/[SDZ]_{added}\times 100$; δ (%)=$([SDZ]_{found} - [SDZ]_{added})/[SDZ]_{added}\times 100$; variation coefficient (%)=standard deviation/$[SDZ]_{mean\ found}\times 100$

VC variation coefficient

[a] Average of five measurements in 1 day

[b] Average of five measurements in 1 week

The potentiometric set of results was validated by LC-MS/MS procedures. Good agreement was found between added and found amounts of SDZ. The results showed recoveries ranging from 96 to 107%, which corresponded to relative errors within −4.1 and +6.7 %. The Student *t* test confirmed no significant differences between the means of added amounts of potentiometric and chromatographic procedures. The *p* value was 0.033 and 0.72 for batch and FIA analysis, always below the critical value (1.8).

Each calibration procedure with seven standards required approximately 30 mL of HEPES buffer and less than 1 mL of each standard solution (considering three injections per standard). At least five diluted samples may be analyzed without recalibration.

The environmental effects of the discharged effluents were considered to be negligible. They contain only buffer and SDZ (at 7.8 μg mL^{-1}). Still, for safety purposes, it is advisable to remove SDZ or make it inactive against microbes. The first suggestion may be achieved by its adsorption in activated carbon or its removal by extraction into solid-phase extraction cartridges. The second choice could be complete oxidation with a strong oxidant, such as permanganate. This latter case should be avoided because of the introduction of additional toxicants. It should also be coupled with ecotoxicity assays to ensure low biological toxicity of the oxidation products. The total volume of effluent is also quite low, producing an average of 180 mL per hour.

Conclusions

The sensor performance was favored by a high dielectric constant mediator solvent, improving the slope, lower limit of the linear range, and reproducibility. TOP was found to be essential to improve the detector sensitivity, but

Table 6 Batch and FIA potentiometric determination of SDZ in aquaculture water samples (n=5) and comparison with the liquid chromatography–tandem mass spectrometry (*LC-MS/MS*) method

Sample	Added (μg mL^{-1})	Found (μg mL^{-1})			Recovery(%)		
		Batch	FIA	LC-MS/MS	Batch	FIA	LC-MS/MS
1	30.0	31.8	30.8	31.5	106.0	102.7	102.7
	140.2	141.4	141.4	145.0	100.9	100.9	100.9
2	30.0	31.3	30.3	29.9	104.3	101.0	101.0
	140.2	134.9	143.4	142.0	96.2	102.3	102.3
3	30.0	31.8	31.3	32.3	106.0	104.3	104.3
	140.2	144.2	147.4	145.6	102.9	105.1	105.1
4	30.0	32.0	31.0	32.1	106.7	103.3	103.3
	140.2	140.7	144.7	143.9	100.4	103.2	103.2
5	30.0	29.8	31.3	30.8	99.3	104.3	104.3
	140.2	138.2	141.9	140.7	98.6	101.2	101.2
6	30.0	30.3	29.5	30.5	101.0	98.3	98.3
	140.2	134.4	148.2	144.6	95.9	105.7	105.7

enhanced the pH interference. In general, SDZ sensors using oNFOE as a mediator solvent and TOP provided a rapid, sensitive, inexpensive, and reliable method for SDZ determination in aquaculture waters with minimal sample pretreatment.

The proposed FIA method displayed excellent reproducibility, a high sampling rate, a wide linear range, a low detection limit, high sensitivity, and good selectivity. Moreover, the overall method required a low level of operator intervention and produced low volumes of effluents. The proposed system is accurate, precise, inexpensive regarding reagent consumption and the equipment involved, and simple, and is adequate for routine procedures. Furthermore, it can be easily applied to the automation of routine determination of SDZ in real samples.

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Determinação selectiva de sulfadiazina em amostras de aquacultura: transdução potenciométrica com base em ionóforos neutros e carregados

Neste trabalho optou-se por manter o analito, SDZ, e procurar encontrar unidades potenciométricas que fossem simultaneamente mais sensíveis e mais selectivas. Isto tinha como objectivo poder usar os sistemas potenciométricos no controlo em linha sem haver necessidade de pré preparação das amostras.

O trabalho baseou-se na preparação de membranas de PVC selectivas a SDZ, nas quais se incluíram materiais electroactivos cujo funcionamento era diferente daquele que é característico dos permutadores iónicos, estudados anteriormente. Assim, optou-se por utilizar compostos organometálicos do tipo das porfirinas e também oligossacarídios naturais do tipo das ciclodextrinas.

Foram preparadas diferentes membranas contendo porfirina de manganês e também α-, β-, e γ-ciclodextrinas. As membranas incorporavam solvente mediador e, algumas delas, aditivos aniónicos e catiónicos. A utilização de aditivos visava a modelação das características de resposta das membranas potenciométricas e compreender o mecanismo de funcionamento dos materiais sensores usados.

As membranas preparadas foram aplicadas directamente em suportes condutores à base de grafite e comparadas relativamente às características analíticas fornecidas pelos eléctrodos correspondentes. Neste estudo usaram-se também as unidades que ofereceram melhores características de funcionamento para testar a utilidade analítica do método desenvolvido na análise de amostras de águas de aquacultura.

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Sulfadiazine-selective determination in aquaculture environment: Selective potentiometric transduction by neutral or charged ionophores

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ABSTRACT

Solid-contact sensors for the selective screening of sulfadiazine (SDZ) in aquaculture waters are reported. Sensor surfaces were made from PVC membranes doped with tetraphenylporphyrin-manganese(III) chloride, α-cyclodextrin, β-cyclodextrin, or γ-cyclodextrin ionophores that were dispersed in plasticizer. Some membranes also presented a positive or a negatively charged additive. Phorphyrin-based sensors relied on a charged carrier mechanism. They exhibited a near-*Nernstian* response with slopes of 52 mV decade^{-1} and detection limits of 3.91×10^{-5} mol L^{-1}. The addition of cationic lipophilic compounds to the membrane originated Nernstian behaviours, with slopes ranging 59.7–62.0 mV decade^{-1} and wider linear ranges. Cyclodextrin-based sensors acted as neutral carriers. In general, sensors with positively charged additives showed an improved potentiometric performance when compared to those without additive. Some SDZ selective membranes displayed higher slopes and extended linear concentration ranges with an increasing amount of additive (always <100% ionophore). The sensors were independent from the pH of test solutions within 2–7. The sensors displayed fast response, always <15 s. In general, a good discriminating ability was found in real sample environment. The sensors were successfully applied to the fast screening of SDZ in real waters samples from aquaculture fish farms. The method offered the advantages of simplicity, accuracy, and automation feasibility. The sensing membrane may contribute to the development of small devices allowing in locus measurements of sulfadiazine or parent-drugs.

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1. Introduction

Aquaculture is the farming of aquatic organisms, practiced for centuries. This practice is today accompanied by the introduction of many chemical substances for therapeutic or prophylactic purposes. Particular attention is given to the use of antibiotics since they enter the environment once leached from faeces and/or uneaten antibiotic feed. Most of the antibiotics given in feed are exported to the surrounding environment and accumulate in the sediment. Thus, the routine analytical control of aquaculture waters should contribute to decrease this environmental contamination.

Sulfonamides are among the most used antibiotics in European countries with contributions between 11 and 24% [1]. They have been used in human medicine against a wide variety of microbes, being their current use primarily in the treatment of urinary tract infections and in farm animal feedstuff and fish cultures as veterinary drugs [2]. Consequently, sulfonamides may reach the foodchain by means of meat or water contamination by transport through soil or surface runoff and uptake by plants [3,4]. Besides possible adverse effects on microorganisms, the major risk of introducing antibiotics into the environment is the development and spreading of resistant pathogens [4].

Sulfonamides are *N*-derivatives of 4-aminobenzenesulphonamide, a large group of synthetic antibacterial compounds. They inhibit the conversion of *p*-aminobenzoic acid, interrupting bacterial use of this compound in the synthesis of folic acid and ultimately of purine and DNA. Due to resistance records in formerly susceptible microorganisms, only a few sulpha drugs are used today, among which sulfadiazine (SDZ, Fig. 1).

The analytical control of SDZ is required in several kinds of samples, such as commercial drugs, and biological and food samples. Ideally, this could be achieved by non-destructive and highly selective/sensitive measurements, such as those employing potentiometric sensors. These have found vast applications in many fields of analysis [5–7]. They offer high precision and rapidity, low cost of analysis, enhanced selectivity and sensitivity over a wide range of concentrations [8,9]. In addition, they are easy to construct and manipulate and no sample pretreatment is needed before the

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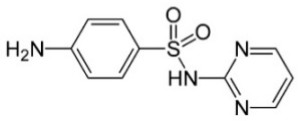

Fig. 1. Chemical structure of SDZ.

analysis itself. Short response times, in the order of seconds, make them appropriate devices for process control and screening analysis [8,9].

Ion-selective membrane sensors, as their name implies, are based on membranes that enable the selective recognition of a specific ion by transferring it (selectively) across the interface between the sample and membrane phase and generating a potential difference [10]. This electrochemical signal is a measure of the activity of that ion. Many mechanisms have been suggested for the selective recognition of different ions [11], most of which mention the selective complexation between the target ion (guest) and a specific carrier (host). This carrier is incorporated in the membrane of the sensor, in order to create the desired selectivity. In general, the overall selectivity of the host–guest interaction depends on several factors such as: (i) the size of the cavity of the host that should be large enough to accommodate the guest species (as the complexation happens, the hydration shell of the target species is removed and substituted by the donor atoms of the host or ligand); (ii) the number of donor atoms in the ligand should be sufficient, to match the coordination number of the target species ; (iii) the flexibility of holding of donor atoms by the host backbone must be limited, so that their positions are suitable to match the shape of the coordination sphere of the target species [12].

An obvious binding strategy for anionic species such as SDZ is to use a positively charged host molecule. However, electrostatic interactions are non-directional and all anions may bind to cations and form either a solvent-separated or contact-ion pair. Anions also bind neutral receptors when there is a difference in electrostatic charge [13]. Thus, neutral or charged carriers, such as cyclodextrin (CD) or porphyrin (PPHR) derivatives may be employed for the potentiometric transduction of SDZ.

CDs are the most widely used receptors in host–guest inclusion chemistry [13,14]. They are a family of cyclic oligosaccharides with a variable number of D-glucopyranoside units linked by 1,4-glycosidic bonds. CDs possess a cage-like supramolecular structure that is generally represented as a cylindrical funnel with an upper (wide) and lower (narrow) rim. The upper rim consists of the secondary hydroxyl groups and the lower of primary hydroxyl groups [13]. It allows carrying out chemical reactions involving "host–guest" electrostatic interactions of intramolecular nature. No covalent bonds are formed or broken in this context. The main driving force of complex formation is the release of enthalpy-rich water molecules from the cavity; water molecules are displaced by more hydrophobic guest molecules present in the solution to attain an apolar–apolar association, resulting in a more stable lower energy state [14].

The PPHR molecule contains four pyrrole rings linked via methine (=CH–) bridges and exhibits aromatic character. The PPHR nucleus is a tetradentate ligand in which there is space available for a coordinated metal. When coordination occurs, two protons are removed from the pyrrole nitrogen atoms, leaving two negative charges [15]. PPHR complexes with transition metal ions are very stable and are often used to construct structures in supramolecular chemistry, taking advantage of the Lewis acidity of the coordinated metal [16]. Several metalloporphyrins have been employed as electroactive components in the membrane of potentiometric sensors, and their response to anions has been interpreted by a dissociation ion-exchange mechanism or metal–ligand interaction mechanism [16–20].

In the presented work, new potentiometric sensors are proposed for SDZ by doping PVC membranes with neutral (CD) or charged ionophores (PPHR) that selectively and reversibly form complexes with the analyte. In addition, it is well known that the nature/amount of ionophore and additives in the selective membrane affect the analytical performance of a potentiometric sensor. A literature survey showed that the usual range of composition in the preparation of PVC matrix membrane sensor was 1–7% ionophore, 28–33% PVC (internal matrix), 60–69% plasticizer (solvent) and 0.03–2% lipophylic additive [21]. Hence, in this work, SDZ sensors were prepared in a similar way, having CD or PPHR based different electroactive materials and different additives in different amounts. Only *o*-nitrophenyl octyl ether (*o*-NPOE) was used as plasticizer, according to a previous study on porphyrin-based sensors [22]. The response behavior of the corresponding sensors is reported herein.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade without further purification. SDZ, tetraoctylammonium bromide (TOABr), dimethyldioctadecylammonium bromide (DDABr), tetrakis (4-chlorophenyl) borate (KTpClPB), tetrahydrofuran (THF), alpha, beta or gamma-CDs (CD), *meso*-tetraphenylporphyrin manganese (III) chloride complex (Mn^{III}TPPCl) and high relative molecular weight PVC were purchased from Sigma–Aldrich. De-ionized water (conductivity <0.1 $\mu S\,cm^{-1}$) was employed in all experiments.

Stock solutions of SDZ 0.01 mol L^{-1} were prepared in water. Less concentrated SDZ standards were prepared by suitable dilution in ultra-pure water. Buffer solutions were 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES, pH ~ 5.4).

The effect of pH was studied by imputing pH variations on 200 mL of a SDZ solution 1.0×10^{-3} mol L^{-1}. The pH of this solution was altered by little additions of either concentrated sulphuric acid or saturated sodium hydroxide solution, freshly prepared.

Interference of other chemicals was evaluated for 1.2×10^{-4}, 5.0×10^{-4}, and 1.0×10^{-3} mol L^{-1} solutions of sodium carbonate, sodium chloride, sodium fluoride, sodium nitrate, bicarbonate and sodium nitrite. All these solutions were prepared in buffer.

2.2. Apparatus

Measurements were carried out with the electrochemical cell AgCl(s)/Ag double junction reference electrode/test solution/SDZ selective membrane, graphite-epoxy. An Orion, 90-00-29, double-junction electrode was used as reference. Potential differences between indicator and reference electrodes were measured by means of a Crison® mpH 2002 decimilivoltammeter. The analytical output signal was transferred to a commutation point with six ways out, enabling the reading of six sensors dipped in the same solution. Each way presented an electrical antenna connector that provided suitable adaptation to each sensor.

The pH was measured by a Crison CWL/S7 combined glass electrode connected to a Crison decimilivoltammeter, pH meter, GLP 22. Spectrophotometric assays were carried out on a Shimadzu Pharmaspec UV-1700.

2.3. Preparation of the SDZ sensor

The sensors used an epoxy-graphite matrix as solid contact [9]. This matrix was prepared by mixing Araldit/Hardener with graphite powder in a 1:1 (w/w) ratio and placed before dry on

Table 1
Membrane composition of SDZ sensors.

ISE	Membrane composition				Slope (mV decade^{-1})	R^2 (n = 4)	LOD (mol L^{-1})	LLLR (mol L^{-1})	Response time (s)
	Active ingredient	Plasticizer	Additive	Weight (mg)					
1	Mn^{III}TPPCl	*o*-NPOE	–	5.5:370	51.70 ± 2.10	0.9946	3.88×10^{-5}	1.28×10^{-4}	<15
2	Mn^{III}TPPCl	*o*-NPOE	TOABr	5.5:370:1.1	58.66 ± 0.95	0.9933	3.43×10^{-5}	1.13×10^{-4}	<15
3	Mn^{III}TPPCl	*o*-NPOE	TOABr	5.5:370:2.1	60.23 ± 1.20	0.9956	2.55×10^{-5}	8.41×10^{-5}	<15
4	Mn^{III}TPPCl	*o*-NPOE	TOABr	5.5:370:4.3	61.42 ± 0.81	0.9956	2.99×10^{-5}	9.87×10^{-5}	<15
5	Mn^{III}TPPCl	*o*-NPOE	DDABr	5.5:370:1.2	59.11 ± 2.26	0.9938	2.10×10^{-5}	6.94×10^{-5}	<15
6	Mn^{III}TPPCl	*o*-NPOE	DDABr	5.5:370:2.4	62.02 ± 2.66	0.9940	2.10×10^{-5}	6.94×10^{-5}	<15
7	Mn^{III}TPPCl	*o*-NPOE	DDABr	5.5:370:4.9	61.89 ± 2.85	0.9953	2.10×10^{-5}	6.94×10^{-5}	<15
8	Mn^{III}TPPCl	*o*-NPOE	KTpClPB	5.5:370:1.0	26.57 ± 0.68	0.9948	2.10×10^{-5}	6.94×10^{-5}	<15
9	Mn^{III}TPPCl	*o*-NPOE	KTpClPB	5.5:370:1.9	36.30 ± 2.92	0.9932	2.55×10^{-5}	8.41×10^{-5}	<15
10	Mn^{III}TPPCl	*o*-NPOE	KTpClPB	5.5:370:3.9	43.40 ± 0.92	0.9966	2.55×10^{-5}	8.41×10^{-5}	<15
11	α-CD	*o*-NPOE	–	5.5:370	–				
12	α-CD	*o*-NPOE	TOABr	5.5:370:0.8	47.56 ± 0.66	0.9934	3.91×10^{-5}	1.29×10^{-4}	<15
13	α-CD	*o*-NPOE	TOABr	5.5:370:1.5	54.85 ± 0.51	0.9979	2.56×10^{-5}	8.46×10^{-5}	<15
14	α-CD	*o*-NPOE	TOABr	5.5:370:3.1	53.27 ± 1.93	0.9946	1.88×10^{-5}	6.21×10^{-5}	<15
15	α-CD	*o*-NPOE	DDABr	5.5:370:0.9	52.71 ± 0.82	0.9948	4.95×10^{-5}	1.63×10^{-4}	<15
16	α-CD	*o*-NPOE	DDABr	5.5:370:1.8	59.30 ± 3.36	0.9946	1.20×10^{-5}	3.97×10^{-5}	<15
17	α-CD	*o*-NPOE	DDABr	5.5:370:3.5	64.66 ± 1.03	0.9940	1.80×10^{-5}	5.94×10^{-5}	<15
18	β-CD	*o*-NPOE	–	5.5:370	–				
19	β-CD	*o*-NPOE	TOABr	5.5:370:0.6	49.79 ± 1.61	0.9935	3.43×10^{-5}	1.13×10^{-4}	<15
20	β-CD	*o*-NPOE	TOABr	5.5:370:1.3	57.17 ± 1.49	0.9960	1.87×10^{-5}	6.19×10^{-5}	<15
21	β-CD	*o*-NPOE	TOABr	5.5:370:2.6	65.31 ± 1.07	0.9957	1.21×10^{-5}	3.98×10^{-5}	<15
22	β-CD	*o*-NPOE	DDABr	5.5:370:0.7	59.13 ± 0.27	0.9937	3.91×10^{-5}	1.29×10^{-4}	<15
23	β-CD	*o*-NPOE	DDABr	5.5:370:1.5	60.47 ± 1.56	0.9942	4.71×10^{-5}	1.56×10^{-4}	<15
24	β-CD	*o*-NPOE	DDABr	5.5:370:3.1	59.08 ± 0.90	0.9949	2.71×10^{-5}	8.94×10^{-5}	<15
25	γ-CD	*o*-NPOE	–	5.5:370	–				
26	γ-CD	*o*-NPOE	TOABr	5.5:370:0.6	52.10 ± 1.91	0.9963	3.31×10^{-5}	1.09×10^{-4}	<15
27	γ-CD	*o*-NPOE	TOABr	5.5:370:1.2	64.96 ± 1.35	0.9960	1.21×10^{-5}	3.99×10^{-5}	<15
28	γ-CD	*o*-NPOE	TOABr	5.5:370:2.3	65.16 ± 1.28	0.9952	1.21×10^{-5}	3.99×10^{-5}	<15
29	γ-CD	*o*-NPOE	DDABr	5.5:370:0.7	51.92 ± 1.14	0.9926	4.68×10^{-5}	1.55×10^{-4}	<15
30	γ-CD	*o*-NPOE	DDABr	5.5:370:1.3	56.07 ± 0.91	0.9973	4.95×10^{-5}	1.63×10^{-4}	<15
31	γ-CD	*o*-NPOE	DDABr	5.5:370:2.7	63.70 ± 3.07	0.9975	2.54×10^{-5}	8.40×10^{-5}	<15

top of a cylindrical body. Each PVC membrane was prepared by mixing 5.5 mg of active ingredient with 370 mg of *o*-NPOE acting as plasticizer and variable amount/kind of additive (Table 1). The resulting solution was homogenized in a 180 mg of PVC previously dissolved in about 5 mL THF and casted over the previously indicated a graphite-based conductive supports. Membranes were let dry and conditioned in a 1.0×10^{-3} mol L^{-1} SDZ aqueous solution before use. The sensors were also kept in this solution when not in use.

2.4. Potentiometric procedures

All potentiometric measurements were carried out at room temperature and in solutions of fixed pH and ionic strength. Calibration curves followed the Litre beaker method [21]. All sensors were placed in a convenient support over a magnetic stirrer and immersed in 50.00 mL of HEPES Calibration procedures were made by transferring 0.0200–10.0 mL aliquots of 1.0×10^{-3} mol L^{-1} SDZ aqueous solution into this electrolyte. The potential readings of the stirred SDZ solutions were measured at room temperature and recorded after stabilization to ±0.2 mV. The calibration graph plotted logarithm concentration (mol L^{-1}) against electromotive force (mV).

2.5. Binding studies

The binding between Mn^{III}TPPCl or γ-CD and SDZ was monitored at 475 or 300 nm, respectively. These wavelengths were selected by plotting the spectra of a solution with ionophore and analyte with 1.0×10^{-6} mol L^{-1}. The spectra of single solutions with 1.0×10^{-6} mol L^{-1} of ionophore or SDZ prepared in HEPES/THF (50:50) was also recorded, serving as blank.

The molar ratio between the analyte and the ligand was calculated by adding 300 μL aliquots of a more concentrated SDZ solution to a suitable volume of 1.0×10^{-6} mol L^{-1} ionophore solution. Spectra were recorded for each concentration level of SDZ.

Binding constants were calculated by the Sandwich method. The conductive support of the sensor was first coated with membranes without ionophore and after with membranes carrying the ionophore. The sensors were let stand for 12 h in 1.0×10^{-2} mol L^{-1} SDZ.

2.6. Sample preparation

Samples were collected from several aquaculture units, placed in sweet waters by the north region of Portugal. The waters were collected from the tank itself and from the surroundings, in order to estimate antibiotic dissemination. By the time of collection, there was no previous antibiotic application and these were blank samples. Typically, a dose of 30 mg of active ingredient is administered *per* kg bodyweight of fish daily for a period of 7–10 days. Therefore, the waters were spiked taking into consideration the concentrations expected in each tank after application of a commercial formulation with 333.3 g sulfadiazine *per* kg. The concentration on samples lied within 25 and 250 μg mL^{-1}. The direct potential method was applied to determine SDZ in spiked waters. The analysis was conducted after calibration.

3. Results and discussion

Several parameters, such as binding features, calibration slopes, reproducibility, dynamic linear range, limit of detection, response time, effect of pH and selectivity, were investigated to evaluate the effect of Mn(III) PPHR and CD ionophores on the analytical

performance of SDZ sensors and the need for a charged additive (membrane composition was indicated in Table 1).

3.1. Porphyrin as charged ionophore

Metalloporphyrins are electrically charged carriers in the membrane when uncomplexed and neutral when bounded to anions by axial ligation of the metal center [23]. This ionophore is expected to respond selectively to anionic species such as SDZ if there is a selective coordination of the anion by axial bound to the positively charged metal center.

Thus, the selective interaction between SDZ and the metal center of the PPHR (Mn^{III}) was confirmed by spectrophotometric assays. PPHRs are Naturally Occurring Planar Microcycles with highly sensitive chromogenic properties and their metal chelates generally exhibit characteristic absorption bands in the visible region. The region from 400 to 500 nm, which is called the Soret band, shows the most intensive absorption [16]. Mn^{III}TPPCl provides a Lewis acid binding site for electron donors such as SDZ and the extended *p*-system points out the binding events by means of optical spectrophotometry. Free Mn^{III}TPPCl displayed a sharp absorption band with 475 nm maximum in HEPES/THF (50:50) medium. The increasing addition of SDZ decreased this absorbance, with maximum absorbance at 473 nm, until all PPHR was complexed with the analyte. This was observed when a 1:1 molar ratio of each compound was present (Fig. 2), suggesting that one mole of Mn^{III}TPPCl bound to one mol of SDZ. The average binding constant between SDZ and Mn^{III}TPPCl was 6.40. This value was calculated potentiometrically by means of the Sandwich method.

Mn^{III}TPPCl membrane sensors without additive (Fig. 3, left) exhibited a linear emf response against logarithmic SDZ concentration. They showed near-Nernstian behavior, with average slopes of 52 mV decade^{-1} from 1.3×10^{-4} to 1.0×10^{-2} mol L^{-1}. Although some concerns have been reported in literature about the spontaneous hydroxy-bridged dimer formation of metalloporphyrins in ion-selective membranes originating super-Nernstian response slopes [24–26], this was not observed in the present studies.

3.2. Cyclodextrin as neutral ionophore

CDs are widely known by their ability to form an inclusion complex with hydrophobic guest molecules, because their cavity is hydrophilic outside and hydrophobic inside [13]. Although hydrophilic hydroxyl groups occupy both rims of the cone and the inside of the cavity, they are hydrophobic in character for being covered by C_3–H, C_5–H, and C_6–H and by the ether link oxygen between each glucopyranosyl units [27].

The more or less extent of each inclusion complex is a function of space or thermodynamic aspects. The former one points out that the size of the CD cavity should fit to the size of the guest molecule. The CDs used in this work were α-CD, β-CD and γ-CD. They have six, seven and eight α-(1,4) glycosyl units, respectively, and different cavity sizes. The height of the cavity is equal for all three types, but their diameters vary within 4.7–5.3, 6.0–6.5, and 7.5–8.3 Å, corresponding to estimated volumes of 174, 262, and 427 Å [14]. Based on these dimensions, α-CD can typically accommodate the smaller molecules and γ-CD the larger molecules. To know in anticipate which CD carries the most suitable cavity size to accommodate SDZ is quite difficult because SDZ presents many different conformations and different protonation states varying with the pH. Besides, recent studies have revised the known structure of SDZ, showing that SDZ contains two S–O single bonds instead the typical S=O double bond, bears negative charges over the pyrimidine nitrogen atom and is a very flexible structure [28].

SDZ was studied first as an inclusion guest on the larger CD host cavity. Binding experiments were carried out by analysing the maximum absorbance of SDZ, β-CD and SDZ plus β-CD UV spectra. The addition of SDZ to β-CD increased the 300 nm absorbance. This increase was perceptible after comparing the individual spectra of the two components with that of their mixture. The experimental results suggested that two molecules of SDZ bound to one molecule of CD. Although CDs usually form 1:1 host–guest complexes, 1:2 complexes are also possible [13]. In aqueous solution, the *hydrophobic* cavity is filled with water and the weak nature of these apolar–polar interactions replace it by another less polar guest molecule. The average binding constants between SDZ and α-Cd, β-Cd, and γ-CD were 5.27, 5.46, and 5.22, respectively. As previously indicated, these were calculated by the Sandwich method.

Considering CD membranes without additive (Table 1), none of the CDs in this study were able to provide a suitable emf variation with SDZ concentration (Fig. 3, left). Since the uncharged carriers are neutral when uncomplexed in the membrane and the complexes have the same charge as the analyte ion, the respective membranes require the additional incorporation of lipophilic ions of opposite charge to ensure permselectivity [29]. Thus, CD SDZ membrane sensors found essential the inclusion of an ionic additive.

3.3. Effect of additive

The role of lipophilic anionic and cationic additives on the potentiometric anion selectivity of the membranes prepared with the PPHR or CD ionophores as anion selective ionophores was examined. The additives were employed to produce ionic sites among the sensing membranes. Generally, this procedure improves the general analytical response of the potentiometric sensor by ensuring that membranes are perm-selective, reducing the ionic interference and lowering the electrical resistance of the membranes [30]. It may also catalyze the exchange kinetics at the sample–membrane interface and enhance the sensitivity of the membrane [22].

In general, with positively charged receptors, lipophilic anionic sites should be added to the membrane in order to optimize the sensor selectivity, whereas in the case of neutral receptors cationic sites should be added [31,32]. In addition, depending on the organic ligand and the metal center, PPHR receptors applied in potentiometric sensors may contain both charged and neutral receptors. The type of ligand and metal center influence the sensor selectivity due to differences in the electron-accepting character of the complex.

Thus, the additives employed for PPHR-based sensors were either positively (TOABr, DDABr) or negatively charged (KTpClPB) while CD-based sensors had always positively charged additives. All these were sufficiently lipophilic to remain solely in the organic membrane phase when in contact with aqueous solution. The effect of carrier:SDZ molar ratios on the sensor response was studied by doping membranes with 25, 50 to 100% additive relative to the ionophore; the corresponding mass ratios are indicated in Table 1, and vary according to the molar mass of each additive.

As a general rule, the positively charged additives enhanced the sensitivity of the PPHR-based sensors from 52 to 60 mV decade^{-1} (Fig. 3B). A slight increase in slope was also observed for an increasing amount of additive. Both limit of detection (LOD) and lower limit of linear range (LLLR) remained unaffected by the additive amount. In turn, it is believed that the anionic additive acted as an excluder of anionic-species (including SDZ), leading to a decreased sensitivity.

The positive additive played a fundamental role in the CD sensors. TOABr increased more significantly the slopes of the CDs with larger cavities while DDABr increased with more relevance the slopes of the smaller carriers. Comparing CD-based sensors with additive, only those with β-CD displayed Nernstian behavior, independently of the amount of additive. The other sensors showed increasing sensitivities for an increased amount of additive.

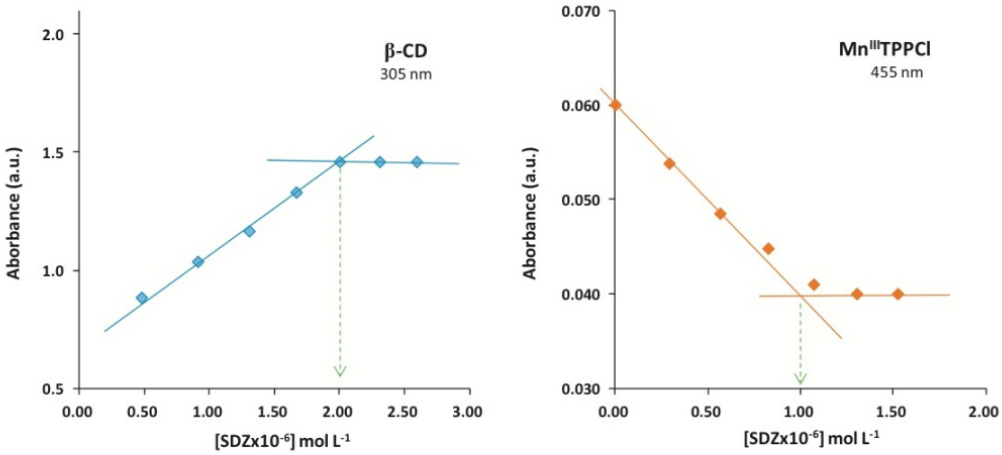

Fig. 2. Molar ratio between SDZ and CD (left) or PPHR (right), for 1.0×10^{-6} mol L^{-1} of ionophore.

Generally, potentiometric sensors with higher amount of additive presented extended working ranges, with lower LLLR and LOD. These results suggest that the cavity size of β-CD was the most suitable one for interacting with the SDZ hydrophobic portion.

3.4. Effect of pH

The effect of pH on the sensor potential was studied for a SDZ solution of 1×10^{-3} mol L^{-1}. The pH was adjusted by small additions of concentrated sulfuric acid or sodium hydroxide solution and recorded by a combined glass-pH electrode. When pH values were plotted against emf (mV), it was observed that the potential was not steady but almost independent from pH in the range of 2–11.0, with potential variations within ±15 mV. Consequently, this interval was considered a plausible pH working range. α-CD sensors with DDABr displayed however a potential drop after pH 7, suggesting an HO^- interference effect. Sensors with Mn^{III}TPPCl/KTpClPB behaved like a pH electrode (Fig. 4), probably in response to its negatively charged additive, present in the same amount as PPHR.

3.5. Response time, lifetime and validation

The time required to achieve a steady potential response (±1 mV) for a 10-fold concentration increase from 5×10^{-5} and 1×10^{-4} mol L^{-1} SDZ was <15 s (Table 1). These results indicated that the complexation process between SDZ and the different ionophores was kinetically fast, pointing out that the overall free energy barrier for the free to complexed states was small enough for complexation to occur rapidly. Replicate calibrations for each sensor indicated low potential drift, long-term stability and negligible change in the response of the sensors.

Having sensors stored and conditioned in 1×10^{-3} mol L^{-1} SDZ solution, the detection limits, response times, linear ranges and calibration slopes were reproducible within ±3% of their original values, and over a period of at least 2 months. This was the maximum period observed, meaning that the sensors could last longer.

During this period, leaching of electroactive materials from the membranes was not perceptible, and ionophore and additive remained preferentially in the hydrophobic phase. PPHR deriva-

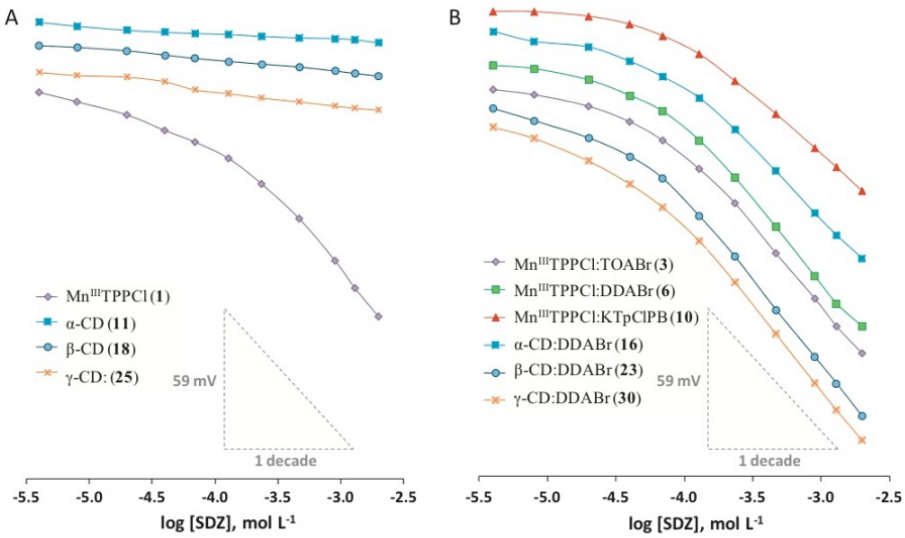

Fig. 3. Typical calibration curves of SDZ sensors without additive (A) or with different additives (B) in HEPES buffer.

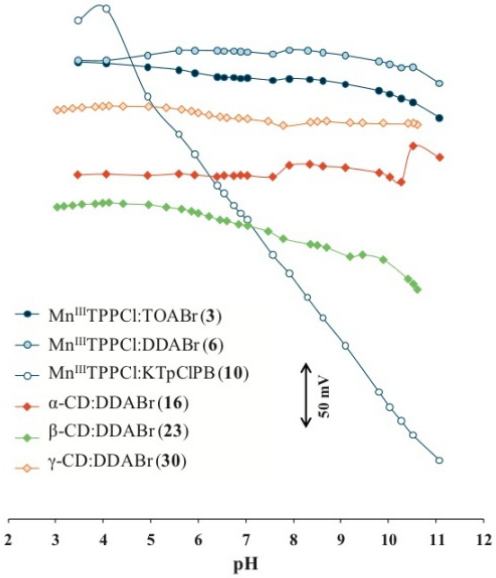

Fig. 4. Influence of pH on the potentiometric response of SDZ sensor ([SDZ] = 1×10^{-3} mol L^{-1}).

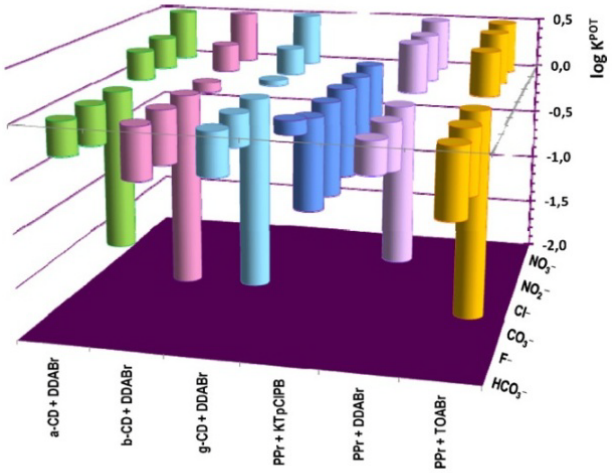

Fig. 5. Potentiometric selectivity coefficients ([SDZ] = 1×10^{-3} mol L^{-1}).

tives are typically hydro-soluble, while CD showed less water solubility (α-Cd, β-Cd and γ-CD are 14.5, 1.85 and 23.2 g, 100 mL^{-1} at 20 °C). Their aromatic rings or uncharged groups are however responsible for allowing them to stand inside a hydrophobic membrane. The water solubility of the ionic additives is much lower than that of the ionophores, because they bear a strong hydrophobic structure.

Validation of the potentiometric data was assessed with regard to precision, accuracy, within-day and between-day variability, and recovery (Table 2). These parameters were selected due to the intended analytical application. In general, all sensors displayed similar and good analytical behavior.

In general, an exceptional potential stability was observed for a solid-contact sensor. This may be attributed to the membrane good adhesion, its impossible lamination by water and its high thickness. The membrane is applied over a cavity where graphite occupies only its deep side [9] and the walls are made of Perspex. Since Perspex dissolves with tetrahydrofuran, the liquid membrane solution reacts with the wall, leading to a dry membrane that constitutes a solid block resistant to water lamination. Hydrated layers are formed therefore by water molecules moving through the plasticized membrane only. Still, the water reaches the graphite because it is responsible for establishing the internal potential, along with O_2 [33,34]. Since the membrane is quite thick (ca. 2 mm), it seems that the water gradient remains constant for a long period of time and the film of water reaching the graphite is unable to drift the potential with significance.

3.6. Sensors selectivity

The selectivity behavior of sensors is defined by the ion exchange constants which depend on the selectivity of complexation as well as on the standard free energies of the respective ions in the aqueous and organic phases [35]. The former requisite suggests the use of ligands that strongly bind the preferred ion and only weakly all the others [35], as the mechanism of selectivity is mainly governed by stereospecific and electrostatic aspects, being the lipophilic environment dictated by the plasticizer. For metalo-porphyrins, the kind and oxidation state of the coordinated central are also relevant.

The selectivity of a potentiometric sensor for the main ion (analyte) over other ions present in the solution is usually expressed in terms of the potentiometric selectivity coefficient, $K^{pot}_{A,B}$. The selectivity coefficients were determined by the separated solutions method [36]. The emf values of SDZ or interfering species solutions were measured separately and the corresponding selectivity coefficients were calculated by using the following equation:

$$K^{POT}_{SDZ^-,J^{z-}} = \frac{E_2 - E_1}{S} + \left(1 - \frac{-1}{Z}\right) \times \log C_{SDZ}$$

where E_1 and E_2 are the sensor potentials in 1.0×10^{-3} mol L^{-1} solutions of SDZ and interfering ion J^{Z-}, respectively, C_{SDZ} the concentration of SDZ and S the practical slope of the calibration plot calculated in mV decade^{-1}.

The potentiometric selectivity coefficients of the proposed sensor are shown in Fig. 5. Carbonate, chloride, nitrate, nitrite, fluoride and hydrogencarbonate (bicarbonate) were selected as possible interfering species because they are usually present in drinking water, as well as in wastewaters, industrial effluents, soils, etc.

The presence of cationic sites from DDABr or TOABr into the membrane lead to the following general relative order of interfering effect: nitrate > nitrite > chloride > fluoride ~ hydrogen hydrogencarbonate > carbonate (Table 3). This seems a near-Hofmeister behavior [37]. It is believed that the presence of ionic sites of opposite charge as the analyte ion (as required for neutral carriers), forces uncomplexed ions to be extracted for electroneutrality reasons, which gives a selectivity sequence that reflects the relative lipophilicity of the sample ions and is not influenced by the complexation with the charged ligand [38]. The experimental results also point out that the selectivity behavior did not depend on the ionophore in use, neither Mn^{III}TPPCl nor α-CD, β-CD or γ-CD. The fact of having α, β or γ-CD provided no significant differences between these with regard to selectivity.

Generally, the ionic sites of the same charge sign as the primary ion improved the selectivity. Membranes having PPHR as ionophore and negatively charged additives displayed a completely different selectivity behavior. All possible interfering compounds showed similar potentiometric selectivity coefficients, lying within -0.92 ± 0.06 log K^{POT}. Hydrogen carbonate was the only exception to this observation.

Table 2
Validation data of several SDZ sensors.

Validation	ISEs					
	Mn^{III}TPPCl TOABr (**3**)	Mn^{III}TPPCl DDABr (**6**)	Mn^{III}TPPCl KTpClPB (**10**)	α-CD DDABr (**16**)	β-CD DDABr (**23**)	γ-CD DDABr (**30**)
Cv_w, %	1.99	4.29	2.12	5.67	2.58	1.62
Accuracy, %	1.81	4.83	26.64	0.24	2.21	5.22
Within-day variability, %	1.70	1.91	2.31	1.99	1.32	1.89
Between-day variability, %	2.05	2.13	3.69	2.50	1.64	3.69
Recovery, %	99.1	97.9	98.9	104.3	101.1	98.6

Table 3
Selectivity coefficients for SDZ selective sensors for various interfering ions using separate solution method.

ISEs	log $K^{POT}_{SDZ,J^{z-}}$					
	CO_3^{2-}	Cl^-	F^-	HCO_3^-	NO_3^-	NO_2^-
Mn^{III}TPPCl + TOABr (3)	−2.04	0.43	−0.63	−0.66	1.39	0.65
Mn^{III}TPPCl + DDABr (6)	−1.49	0.48	−0.45	−0.31	0.99	0.45
Mn^{III}TPPCl + KTpClPB (10)	−0.90	−0.88	−0.86	0.12	−1.02	−0.95
α-CD + DDABr (16)	−1.56	0.28	−0.37	−0.32	1.37	0.31
β-CD + DDABr (23)	−1.86	−0.10	−0.52	−0.50	1.34	0.27
γ-CD + DDABr (30)	−1.84	0.06	−0.30	−0.42	1.48	0.26

3.7. Response mechanism

For PPHRs, the nature of the ionophore anion interaction mechanism correlates with the charge of the metal ion chelated by the PPHR [39,40]. Trivalent metal ion complexes with PPHR were found to serve as either neutral or charged carriers depending on the existence and number of bound axial ligands. In the case of sensors based on neutral carriers, ionic sites with an opposing charge to that of the primary ions are necessary to decrease the membrane resistance, which in turn reduces the co-ion interferences, and achieves a Nernstian response with an acceptable selectivity, and improves the detection limit. In the present study, ion-selective membranes formulated with Mn(III)PPHR required a cationic additive to enhance the potentiometric response. Thus, these results pointed out that the PPHR ionophore acted as neutral carrier in the membrane.

As expected, CDs also acted as neutral carrier. These neutral anion receptors incorporate strong, multiple, hydrogen bond donor groups (–OH) on its surface. Typically, once inside the CD cavity, the guest molecule makes conformational adjustments to take maximum advantage of the weak van der Waals forces that exist [14].

The effect of plasticizer and additive upon the observed potentiometric response was tested by evaluating two news sensors, carrying plasticized membranes (i) with no ionophore and no additive or (ii) with no ionophore and with negatively charged additive. The first sensor was quite unstable and the potential was unable to decrease with increasing SDZ concentrations. Its potential change ranged ±3 mV along each calibration. The second sensor showed sub-Nernstian behavior, with slopes of −25 mV decade^{-1} after a concentration of 1×10^{-4} mol L^{-1} SDZ. Especially for CD-based sensors, this indicated that the ionophore played a significant role on the observed response.

3.8. Complementary sample analysis

The previous selectivity study is mainly theoretical and suggested high interfering effects from nitrate, nitrite and chloride upon the potentiometric response. Thus, before proceeding with samples analysis, the most contaminated waters samples were analyzed in terms of their main chemical composition. The parameters selected for this purpose were those indicated on the Portuguese law for waters. The average levels found were indicated in Table 4. The obtained results are always below the legal limits and suggested that the proposed potentiometric method could be applied to the analysis of waters in aquaculture environment.

3.9. Application

The determination of SDZ in aquaculture waters was carried out on the previously prepared samples by direct potentiometry. The obtained results are summarized in Table 5. The conventionally shaped sensors selected for this purpose were sensors 3 and 23 (see Table 1); they presented increased sensitivity of response (mV decade^{-1}), good potential reproducibility and stability.

The samples were spiked by adding small increments of 1.0×10^{-2} mol L^{-1} standard SDZ solution to 20.0 mL aliquot samples of various concentrations. The change in potential reading was recorded for each increment and used to calculate the concentration of SDZ sample in the sample solutions using the following equation:

$$C_{SDZ} = \frac{C_s \times V_s}{(V_{SDZ} + V_s) \times 10^{\frac{\Delta E}{S}} - V_{SDZ}}$$

Here C_{SDZ} is the SDZ concentration of testing sample, C_s is the concentration of the standard, V_x and V_s are the corresponding volumes, S is the slope of the potentiometric response, and ΔE is the change in potential [41].

Table 4
Chemical parameters of the water samples.

	Sample A	Sample B	Sample C
Turbidity (UNT)	<0.2	<0.2	0.7
pH (Sorensen scale)	7	6.9	6.7
Conductivity (μS cm^{-1})	41	41	135
Nitrate (mg L^{-1})	1.7	1.3	12.1
Nitrite (mg L^{-1})	<0.03	<0.03	<0.03
Ammonium (mg L^{-1})	<0.05	0.07	<0.05
TOC (mg L^{-1})	<1	<1	<1
Cu (mg L^{-1})	<0.01	<0.01	<0.01
BOD (mg L^{-1})	<3	<3	<3
COD (mg L^{-1})	<30	<30	<30

TOC: total organic content; BOD: biochemical oxygen demand; COD: chemical oxygen demand.

Table 5
Determination of SDZ in aquaculture by applying the standard addition method and the corresponding statistical data.

Sample	Added, mol L^{-1}	Found, mol L^{-1} ($n=3$)	RD (%) ($n=3$)	Recovery(%) ($n=3$)
1	2.55×10^{-4}	$2.45\times10^{-4}\pm1.2\times10^{-5}$	4.0	96.0
	5.39×10^{-4}	$5.22\times10^{-4}\pm2.6\times10^{-5}$	3.0	97.0
	1.06×10^{-3}	$1.03\times10^{-3}\pm4.6\times10^{-5}$	3.0	97.0
2	2.55×10^{-4}	$2.62\times10^{-4}\pm3.2\times10^{-5}$	−2.9	102.9
	5.39×10^{-4}	$5.51\times10^{-4}\pm5.6\times10^{-5}$	−2.3	102.3
	1.06×10^{-3}	$1.01\times10^{-3}\pm7.2\times10^{-5}$	4.4	95.6
3	2.55×10^{-4}	$2.39\times10^{-4}\pm1.1\times10^{-5}$	6.4	93.6
	5.39×10^{-4}	$5.30\times10^{-4}\pm3.0\times10^{-5}$	1.6	98.4
	1.06×10^{-3}	$1.07\times10^{-3}\pm2.1\times10^{-5}$	−1.1	101.1
4	2.55×10^{-4}	$2.54\times10^{-4}\pm2.2\times10^{-5}$	0.5	99.5
	5.39×10^{-4}	$5.54\times10^{-4}\pm5.0\times10^{-5}$	−2.9	102.9
	1.06×10^{-3}	$1.03\times10^{-4}\pm6.9\times10^{-5}$	3.0	97.0
5	2.55×10^{-4}	$2.49\times10^{-4}\pm1.1\times10^{-5}$	2.2	97.8
	5.39×10^{-4}	$5.07\times10^{-4}\pm1.5\times10^{-5}$	5.8	94.2
	1.06×10^{-3}	$1.05\times10^{-3}\pm1.7\times10^{-5}$	1.2	98.8
6	1.07×10^{-4}	$1.01\times10^{-4}\pm1.7\times10^{-5}$	5.5	94.5
	5.39×10^{-4}	$5.19\times10^{-4}\pm2.4\times10^{-5}$	3.6	96.4
	1.06×10^{-3}	$1.00\times10^{-3}\pm3.7\times10^{-5}$	5.4	94.6

Found: mean ± standard error; RD: relative deviation.

The values reported in Table 5 were calculated from three determinations in aquaculture water samples collected from different farming places. The obtained results confirmed the accuracy and precision of the present work. Recoveries ranged 93.6 and 102.9%, thus confirming the accuracy of the analytical results. Relative standard deviations were also low, and confirmed the precision of the proposed method. Student t test (at 95% confidence level) confirmed the accuracy of the analytical data because

Table 6
The main features of the proposed method and previously reported sensors for SDZ determination.

Method	Small description	Samples	Detection limit	Response time	Reference
Fluorimetry	Competitive immunocomplex capture format making use of an immobilized protein A/G sorbent or a restricted access support in a novel homogeneous–heterogeneous (HH) assay mode.	Water Honey	0.11 $\mu g\,L^{-1}$0.85 $\mu g\,L^{-1}$	18 min2 min	[42]
SPR	Immunoassays based on the plasmon of gold diffraction grating surface for simultaneous detection of antibiotics from different groups, including sulfapyridine as a sulphonamide compound.	Milk	0.29 $\mu g\,L^{-1}$	≈30 min	[43]
SPR	Inhibition assay format in an optical biosensor chip.	Chicken serum	7–1000 $\mu g\,L^{-1}$	≈10 min	[47]
WIOS	Lab-on-a-chip for multi-antibiotic competitive immunoassay based on competitive immunoassay based on wavelength interrogated optical sensor technology and a polymer-based self-contained microfluidic cartridge.	Milk	100 $\mu g\,L^{-1}$	10 min	[44]
WIOS	Competitive immunoassay format using immunoreagents previously developed for the generic detection of sulfapyridine and evaluated by enzyme-linked immunosorbent assay. The immunoreagents were immobilized onto the surface of the waveguide chip, and a fluidic cell allowed flowing analyte and detection solutions above the surface.	Milk	0.5 $\mu g\,L^{-1}$	≈30 min	[45]
Electrochemistry	The immunological reaction for the detection of sulfonamide antibiotics performed on the magnetic bead is based on a direct Competitive assay using a tracer with horserodish peroxidase for the enzymatic labeling and modified magnetic beads captured by a magneto sensor made of graphite–epoxy composite acting as the transducer.	Cream milk	1.44 $\mu g\,L^{-1}$	–	[46]
Potentiometry	PVC membrane selective electrodes for SDZ for flow and batch measurements with iron phthalocyanine as ionophores	Drugs Biol. fluids	0.87–7.0 mg L^{-1}	<30 s	[9]
Potentiometry	PVC membranes with Mn^{III}TPPCl, α-CD, β-CD, γ-CD molecules as ionophores.	Aquaculture Water	3 mg L^{-1}	<30 s	This work

UV/Vis: Ultraviolet Visible; SPR: Surface Plasmon Resonance; WIOS: sensitive wavelength interrogated optical sensor.

the calculated t (0.28) did not exceed the theoretical value (2.07).

In order to know if the proposed method exhibited any fixed or proportional bias, a simple linear regression of the taken amounts against found was calculated. A small displacement of the zero origin (equal to 1×10^{-5}) and of the unit slope (0.9736) was found, confirming the absence of the above bias.

3.10. Comparison to previous sensors

The proposed work is compared in terms of analytical figures of merit with other sensors previously reported in the literature for the determination of different sulphonamides (Table 6). In general terms, it is possible to conclude that the main advantage of the presented work is the low response time and the low cost the proposed sensors. They display very quick responses and are inexpensive in terms of regular laboratory materials. Their main disadvantage compared to others is the high limit of detection. This last feature is mostly correlated to the use of conventional materials instead of nanostructured ones, which are not included in elegant platforms. Thus, further work is conducted to incorporate the membranes upon microfluific devices. Of course, this will turn out more expensive, but much cheaper than all other reported methods. These rely only on immunoassays and therefore require highly expensive consumable reagents. Furthermore, some of these use very expensive transducers that are quite far from the regular potentiometer in everyday laboratory.

The proposed sensors offer similar analytical features to those in [9]. The here presented sensor offers however higher stability in terms of analytical signal. The use of regular CD compounds as sensors is also an advantage in terms of routine laboratory.

4. Conclusions

Mn(III) PPHR and CD were suitable ionophores for the preparation of SDZ sensors. They acted both as neutral carriers. The presence of positively charged additives in the selective membrane enhanced the potentiometric performance. Selectivity profiles followed a near-Hofmeister pattern with the exception of PPHR-based sensors with negatively charged additives. Despite their good selectivity behavior, they suffered from a great pH interference effect.

The proposed sensors were found useful for the control of SDZ in waters from aquaculture origin. The corresponding detectors were constructed in a simple and inexpensive way. The overall procedure was considered precise, accurate, and inexpensive regarding reagent consumption and equipment involved. Considering its routine application, the main advantages arise from the composition and quantity of emitted effluents, with small concern in terms of environmental issues. The proposed method also enabled high sampling frequencies with low operator intervention, meaning that it was suitable for the routine procedures carried out in analytical laboratories; it is particularly suitable for screening assays.

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CAPÍTULO V

Desenvolvimento de eléctrodos de contacto sólido selectivos a Sulfametoxazole com base em ionóforos neutros e carregados: aplicação a sistemas de aquacultura

Neste trabalho procurou-se estudar a aplicabilidade dos eléctrodos desenvolvidos no capítulo anterior à determinação de outras espécies químicas do grupo das SAs, nomeadamente a SMX. Para este efeito, foi realizado um estudo comparativo entre dispositivos selectivos a estas SAs, usando como sistemas sensores os mesmos apresentados no capítulo anterior.

Com a modificação do analito verificou-se que as membranas que continham ciclodextrina não apresentavam resposta potenciométrica na ausência de aditivo. Foi necessária a adição de um aditivo de natureza catiónica para obter resposta Nernstiana. Ao contrário do que acontecia para a SDZ, neste caso, a ciclodextrina que mais se adequava à resposta potenciométrica era a γ-ciclodextrina cuja cavidade era superior à β ciclodextrina, anteriormente usada. Estas diferenças advêm de questões energéticas associadas à interacção "host-guest"que se verifica entre o analito e a ciclodextrina devido à dimensão do analito.

Para as membranas baseadas em metaloporfirina verificou-se, que a resposta dos eléctrodos era sub-Nernstiana quando não se usava aditivo na membrana. A sensibilidade da resposta aumentava após a adição de aditivo.

Em conclusão, os eléctrodos com porfirina de manganês apresentaram um melhor desempenho analítico no que se refere ao limite de detecção, limite inferior de resposta linear, declive, tempo de resposta, coeficientes de selectividade potenciométricos e efeito do pH, relativamente aos de γ-ciclodextrina, sendo, por isso, aplicados ao doseamento de SMX em águas de aquacultura.

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Solid contact PVC membrane electrodes based on neutral or charged carriers for the selective reading of anionic sulfamethoxazole and their application to the analysis of aquaculture water

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Sulfamethoxazole (SMX) is among the antibiotics employed in aquaculture for prophylactic and therapeutic reasons. Environmental and food spread may be prevented by controlling its levels in several stages of fish farming. The present work proposes for this purpose new SMX selective electrodes for the potentiometric determination of this sulphonamide in water. The selective membranes were made of polyvinyl chloride (PVC) with tetraphenylporphyrin manganese (III) chloride or cyclodextrin-based acting as ionophores. 2-nitrophenyl octyl ether was employed as plasticizer and tetraoctylammonium, dimethyldioctadecylammonium bromide or potassium tetrakis (4-chlorophenyl) borate was used as anionic or cationic additive. The best analytical performance was reported for ISEs of tetraphenylporphyrin manganese (III) chloride with 50% mol of potassium tetrakis (4-chlorophenyl) borate compared to ionophore. Nersntian behaviour was observed from 4.0×10^{-5} to 1.0×10^{-2} mol/L (10.0 to 2500 μg/mL), and the limit of detection was 1.2×10^{-5} mol/L (3.0 μg/mL). In general, the electrodes displayed steady potentials in the pH range of 6 to 9. Emf equilibrium was reached before 15 s in all concentration levels. The electrodes revealed good discriminating ability in environmental samples. The analytical application to contaminated waters showed recoveries from 96 to 106%.

Keywords: Sulfamethoxazole; aquaculture; ion selective electrode; cyclodextrin; porphyrin

1. Introduction

Aquaculture is a food source of great impact on the economy, especially facing the current global economic crisis [1]. However, public health concerns on residues and resistance of antimicrobial agents [1–5] have emerged as aquaculture intensifies and diversifies. The antimicrobials used to treat fish diseases are the same compounds given to humans [6–8]. They contaminate the aquatic environment with excrements and uneaten antibiotic feed [9,10], being able to reach water treatment facilities [11] and increasing the risk of

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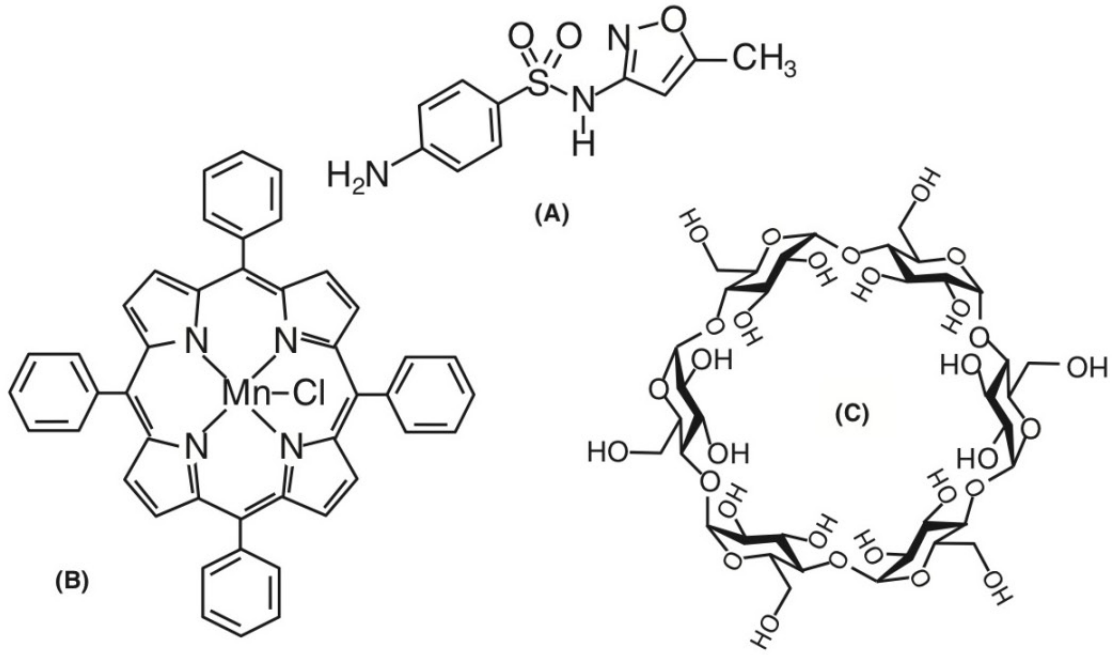

Figure 1. Chemical structures of SMX (A), Mn^{III}TPPCl (B) and CD (α structure presented).

contamination in drinking water supplies. In addition, the escalating spread of resistance in pathogens [12,13] is today advising restrictions on the veterinary use of antibiotics.

Sulfamethoxazole (SMX) is one of the several antibiotics used in fish farming practices (Figure 1). It is a member of the sulfonamide family of antibiotics that have a high potential to resist degradation [5,14], and acts by inhibiting the utilization of *p*-aminobenzoic acid by bacteria in the synthesis of folic acid [15–17].

To contain the environmental spread of SMX, its levels must be rigorously and continuously controlled in all stages of fish farming. Microbiological and chromatographic methods are employed for this purpose [18], but they are time consuming and too expensive to be used in a routine basis. They are also unable to carry out on-site screening analysis. New simple methods that offer quick and selective readings are thus required. Ideally, this could be achieved by non-destructive and highly selective/sensitive measurements, such as those employing ion-selective electrodes (ISEs). They offer high precision, rapidity, enhanced selectivity and sensitivity over a wide range of concentrations [19].

Ion-selective membrane sensors are able to discriminate selectively the analyte to which they are designed by means of a selective complexation between the target ion (guest) and a specific carrier (host) that is incorporated in the membrane of the electrodes [20]. In general, the overall selectivity of the host guest interaction depends on several factors such as: (i) the cavity of the host should be large enough to accommodate the guest species; (ii) the number of donor atoms in the ligand should be sufficient, to match the coordination number of the target species; (iii) the flexibility of the donor atoms in the host backbone must be limited, so that their positions are suitable to match the shape of the coordination sphere of the target species [21].

SMX binds to positively charged host molecules because it is negatively charged in aqueous media. Thus, a positively charged carrier could be tested as potentiometric ionophore. Electrostatic interactions are however non-directional and other anions my

bind to form either a solvent-separated or contact-ion pair. As an alternative, neutral host molecules may also be tried out as ionophore. Although the forces between anions and cations are larger, anions also bind to neutral receptors if there is a difference in electrostatic charge [22]. It has been argued that neutral receptors offer the possibility of enhanced selectivity when they turn out *invisible* for the competing counter-anion. These assumptions justify the application of neutral or charged carrier compounds, such as cyclodextrin (CD) or porphyrin (PPHR) derivatives, respectively, for the potentiometric transduction of anionic species.

In the present work, new potentiometric sensors are proposed for SMX by doping polyvinyl chloride (PVC) membranes with neutral CD or charged PPHR. The effect of positively or negatively charged additives was also studied as the nature/amount of ionophore and additive in the selective membrane affect the analytical performance of ISEs. The response behaviour of all ISEs is reported and compared herein.

2. Experimental

2.1 *Apparatus*

The potential build up across the membrane electrode was measured using the galvanic cell as AgCl(s)/Ag double junction reference electrode || test solution | PVC membrane | Graphite-based conductive support. Emf was measured in a Crison μpH 2002 decimilivoltammeter, using an Orion, 90-00-29, double-junction electrode as reference.

The analytical output signal was transferred to a commutation point reconnecting to one among six ways out. Each way presented an electrical antenna connector compatible to each electrode. The selective electrodes had no internal reference solution and used graphite/epoxy resin as conductive solid contact [23].

The pH of the solutions was measured by means of a Crison CWL/S7 combined glass electrode connected to a decimilivoltammeter Crison, pH meter, GLP 22. Spectrophotometric assays were carried out on a Shimadzu Pharmaspec UV-1700.

The LC system (Agilent Technologies, Palo Alto, CA, USA) consisted of an HP1100 thermostatted autosampler, degasser, gradient pump, and thermostatted column, coupled to a Triple Quadrupole System Sciex API 2000 (Applied Biosystem, Foster City, USA) mass detector was used for sample analysis. It was equipped with a Zorbax Eclipse XDB – C18, 5 μm, 150 × 2.1 mm column (Agilent Technology, Palo Alto, USA). Data acquisition was controlled by Sciex Analyst® software, version 1.4.1.

2.2 *Reagents and solutions*

All chemicals were of analytical grade and deionised water (conductivity < 0.054 μS/cm at 25°C) was used. SMX, tetraoctylammonium bromide (TOABr), dimethyldioctadecyl-lam-monium bromide (DDABr), 2-nitrophenyl octyl ether (2-NPOE), potassium tetrakis (4-chlorophenyl) borate (KTpClPB), tetrahydrofuran (THF), α-, β- or γ-CDs, *meso*-tetraphenylporphyrin manganese (III) chloride complex (Mn^{III}TPPCl), and PVC of high relative molecular weight were purchased from Sigma-Aldrich (Barcelona, Spain). Formic acid, methanol, acetonitrile were high-performance liquid chromatography (HPLC) grade and purchased from Merck (Darmstadt, Germany).

Stock solutions of SMX were 0.01 M and prepared in water. More diluted standards were prepared by rigorous dilution with buffer. Buffered solutions were prepared in 0.01 M

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma, Barcelona, Spain), pH 5.4

The effect of pH was studied for a 1.0×10^{-3} M SMX solution. Its pH was altered by adding either sulphuric acid or sodium hydroxide diluted solutions.

Interference studies relying on the separated solutions method used 1.19×10^{-4}, 4.94×10^{-4} and 1.01×10^{-3} M solutions in SMX or sodium carbonate, sodium chloride, potassium nitrate, sodium nitrite, sodium fluoride, sodium hydrogen carbonate (bicarbonate), sodium citrate, sodium tartrate, sodium acetate, sodium phosphate, sodium borate, sodium cyanide, sodium sulphate and glucose. For the matched potential method, a SMX concentration of 5.9×10^{-5} M or variable concentrations of each interfering species were added to a 7.9×10^{-5} M SMX solution. All solutions were prepared by suitable dissolution/dilution of the corresponding reagents (Merck, Darmstatd, Germany) in buffer.

2.3 *Construction of the SMX selective electrodes*

The solid conductive support was made by mixing Araldite M and hardener in a 1:0.4 (w/w) ratio. This epoxy resin was then mixed with graphite powder in a 2:1 (w/w) ratio and applied at 0.2 cm of the end of the electrode body made of Perspex tube (external ø 12 mm, internal ø 10 mm, length 15 cm).

The selective membrane was prepared by mixing 5.5 mg of active ingredient (CD or PPHR) with 370 mg of plasticizer (2-NPOE) and different amounts of different additives (Table 1). The resulting solutions were added of 180 mg of PVC previously dissolved in about 5 ml THF. After successive applications of the membranes onto the solid conductive support, the different units were left to dry for 2 days at room temperature, guaranteeing the complete evaporation of THF.

2.4 *Potentiometric procedures*

The response characteristics of the coated graphite electrodes were assessed by measuring the emf against increasing concentrations of SMX. This was made at room temperature, according to [24] and following IUPAC recommendations [25]. Aliquots of a 1.0×10^{-3} M SMX aqueous solution ranging from 0.0200 to 10.0 mL were transferred into 50.0 mL of electrolyte solution. HEPES solutions of 0.01 M ionic strength were used for this purpose. The solutions were stirred and potential readings recorded after stabilization to ± 0.2 mV. A calibration graph plotted logarithm concentration against emf.

The effect of pH was evaluated for 1.0×10^{-3} M SMX solution prepared in water. Volumes from 5 to 2000 µL of saturated NaOH solution were added to increase the pH up to 11. This was followed by the addition of diluted H_2SO_4 until the pH 3 was reached. The operational pH ranges were found for maximum potential variations of ± 5 mV. The selectivity was assessed by the separated solutions or matched potential methods [26], reading single or mixed analyte compositions of SMX standard or interfering species.

2.5 *Sample preparation*

Various kinds of aquaculture water were sampled from the north region of Portugal. The waters were collected from the tank itself and from the surroundings, in order to estimate

Table 1. Membrane composition and main analytical features the SMX sensors.

	Membrane composition				Slope	r	LLLR	LOD	
ISE	Active ingredient	Plasticizer	Additive	Weight (mg)	(mV/decade)	($n=4$)	(M)	(M)	Response time (s)
I	$Mn^{III}TPPCl$	2-NPOE	____	5.5:370	48.79 ± 1.94	0.9992	4.97 E-05	1.51 E-05	<15
II	$Mn^{III}TPPCl$	2-NPOE	TOABr	5.5:370:1.1	70.25 ± 2.91	0.9977	3.98 E-05	1.21 E-05	<15
III	$Mn^{III}TPPCl$	2-NPOE	TOABr	5.5:370:2.1	64.46 ± 2.09	0.9981	2.00 E-05	6.06 E-06	<15
IV	$Mn^{III}TPPCl$	2-NPOE	TOABr	5.5:370:4.3	69.76 ± 1.73	0.9972	2.50 E-05	7.56 E-06	<15
V	$Mn^{III}TPPCl$	2- NPOE	DDABr	5.5:370:1.2	68.35 ± 1.65	0.9979	4.31 E-05	1.31 E-05	<15
VI	$Mn^{III}TPPCl$	2-NPOE	DDABr	5.5:370:2.4	65.26 ± 2.09	0.9989	3.98 E-05	1.21 E-05	<15
VII	$Mn^{III}TPPCl$	2-NPOE	DDABr	5.5:370:4.9	64.64 ± 2.31	0.9988	4.97 E-05	1.51 E-05	<15
VIII	$Mn^{III}TPPCl$	2-NPOE	KTpClPB	5.5:370:1	46.72 ± 2.51	0.9969	4.97 E-05	1.51 E-05	<15
IX	$Mn^{III}TPPCl$	2-NPOE	KTpClPB	5.5:370:1.9	58.98 ± 1.61	0.9952	3.98 E-05	1.21 E-05	<15
X	$Mn^{III}TPPCl$	2-NPOE	KTpClPB	5.5:370:3.9	70.11 ± 0.97	0.9976	2.00 E-05	6.06 E-06	<15
XI	α-CD	2-NPOE	____	5.5:370	24.54 ± 1.05	0.9974	3.98 E-04	1.21 E-04	<15
XII	α-CD	2-NPOE	TOABr	5.5:370:0.8	28.61 ± 1.06	0.9993	3.98 E-05	1.21 E-05	<15
XIII	α-CD	2-NPOE	TOABr	5.5:370:1.5	56.22 ± 1.07	0.9979	3.98 E-05	1.21 E-05	<15
XIV	α-CD	2-NPOE	TOABr	5.5:370:3.1	59.40 ± 0.44	0.9983	3.98 E-05	1.21 E-05	<15
XV	α-CD	2-NPOE	DDABr	5.5:370:0.9	57.78 ± 3.24	0.9967	6.95 E-05	2.11 E-05	<15
XVI	α-CD	2-NPOE	DDABr	5.5:370:1.8	62.35 ± 1.21	0.9970	3.49 E-05	1.06 E-05	<15
XVII	α-CD	2-NPOE	DDABr	5.5:370:3.5	66.33 ± 2.81	0.9972	3.32 E-05	1.01 E-05	<15
XVIII	α-CD	2-NPOE	KTpClPB	5.5:370:0.7	20.09 ± 1.93	0.9965	3.98 E-04	1.21 E-04	<15
XIX	α-CD	2-NPOE	KTpClPB	5.5:370:1.4	31.50 ± 2.06	0.9979	3.98 E-05	1.21 E-05	<15
XX	α-CD	2-NPOE	KTpClPB	5.5:370:2.8	29.87 ± 1.20	0.9979	3.98 E-05	1.21 E-05	<15
XXI	β-CD	2-NPOE	____	5.5:370	24.36 ± 2.52	0.9967	3.97 E-04	1.20 E-04	<15
XXII	β-CD	2-NPOE	TOABr	5.5:370:0.6	57.72 ± 2.05	0.9975	3.97 E-05	1.20 E-05	<15
XXIII	β CD	2-NPOE	TOABr	5.5:370:1.3	58.71 ± 0.77	0.9971	3.97 E-05	1.20 E-05	<15
XXIV	β-CD	2-NPOE	TOABr	5.5:370:2.6	59.16 ± 1.20	0.9967	2.98 E-05	9.03 E-06	<15
XXV	β-CD	2-NPOE	DDABr	5.5:370:0.7	52.54 ± 2.26	0.9974	3.91 E-05	1.18 E-05	<15
XXVI	β-CD	2-NPOE	DDABr	5.5:370:1.5	55.58 ± 2.31	0.9979	3.91 E-05	1.18 E-05	<15
XXVII	β-CD	2-NPOE	DDABr	5.5:370:3.1	49.66 ± 2.23	0.9990	3.42 E-05	1.04 E-05	<15
XXVIII	β-CD	2-NPOE	KTpClPB	5.5:370:0.6	20.80 ± 1.18	0.9984	1.26 E-04	3.82 E-05	<15
XXIX	β-CD	2-NPOE	KTpClPB	5.5:370:1.2	23.16 ± 0.97	0.9979	2.34 E-04	7.09 E-05	<15

(*continued*)

Table 1. Continued.

ISE	Membrane composition				Slope	r	LLLR	LOD	Response time (s)
	Active ingredient	Plasticizer	Additive	Weight (mg)	(mV/decade)	($n=4$)	(M)	(M)	
XXX	β-CD	2-NPOE	KTpClPB	5.5:370:2.4	29.36 ± 0.26	0.9980	2.34 E-05	7.09 E-06	<15
XXXI	γ-CD	2-NPOE	———	5.5:370	24.97 ± 1.03	0.9960	3.98 E-04	1.21 E-04	<15
XXXII	γ-CD	2-NPOE	TOABr	5.5:370:0.6	58.71 ± 0.77	0.9968	3.42 E-05	1.04 E-05	<15
XXXIII	γ-CD	2-NPOE	TOABr	5.5:370:1.2	57.78 ± 0.40	0.9983	3.42 E-05	1.04 E-05	<15
XXXIV	γ-CD	2-NPOE	TOABr	5.5:370:2.3	61.23 ± 1.26	0.9981	3.42 E-05	1.04 E-05	<15
XXXV	γ-CD	2-NPOE	DDABr	5.5:370:0.7	56.03 ± 2.25	0.9974	3.91 E-05	1.18 E-05	<15
XXXVI	γ-CD	2-NPOE	DDABr	5.5:370:1.3	61.08 ± 1.72	0.9976	3.91 E-05	1.18 E-05	<15
XXXVII	γ-CD	2-NPOE	DDABr	5.5:370:2.7	61.21 ± 0.37	0.9967	2.94 E-05	8.89 E-06	<15
XXXVIII	γ-CD	2-NPOE	KTpClPB	5.5:370:0.5	21.93 ± 1.21	0.9962	1.26 E-03	3.82 E-04	<15
XXXIX	γ-CD	2-NPOE	KTpClPB	5.5:370:1.0	25.52 ± 1.01	0.9971	1.26 E-04	3.82 E-05	<15
XL	γ-CD	2-NPOE	KTpClPB	5.5:370:2.1	26.86 ± 0.56	0.9973	1.26 E-04	3.82 E-05	<15

LOD: Limit Of Detection; LLLR: Lower Limit of Linear Range.

antibiotic dissemination. All samples were collected in duplicate in amber glass bottles with polypropylene open-top screw caps and Teflon-silicon septa. All sampling personnel were latex gloves when collecting samples, and changed gloves between sites. After collection, the samples were labelled, wrapped with Parafilm®, and immediately chilled and sent to the laboratory.

2.6 *Sample analysis*

The direct potential method was applied to determine SMX in the previous water samples. The electrodes were calibrated prior to potentiometric analysis. The analytical results were compared with those obtained by LC-MS-MS procedures.

A binary gradient phase was used for LC-MS/MS analysis, at a flow rate of 0.35 mL/min, with 0,1% formic acid (A) and acetonitrile (B). The gradient starts with 90% of (A) and goes linearly to 10% in 15 min, finishing at 90% of (A) during 1 min with a total run time of 16 min. Sample injection volume was 5 µl and the temperature oven set to 40°C.

The mass spectrometer was operated in positive electrospray ionisation mode using the TurboIonSpray source. Nitrogen was used for curtain gas, collision gas and nebulizer gas at flow rates of 7, 4 and 9 L/min. The ion source block temperature was set to 450 °C, and the electrospray capillary voltage to 5.0 kV. Data acquisition was performed in the multiple reaction monitoring (MRM) mode. The precursor was 254 m/z and the product ions were 156 and 92 m/z. Collision energy were 23.35 eV. The dwell time was set to 100 msec for all the analytes.

SMX stock solutions were prepared by dissolving 10.00 mg of standard in 10 mL methanol resulting in 1.0 mg/mL stock solution. This solution was kept at − 80°C. A working standard solution of SMX was prepared at the concentration of 250 µg/mL in MeOH:H_2O (1:4). This working standard solution was used to prepare the calibration curve. These were evenly distributed into four concentrations between 25 and 250 µg/mL. Sampling volumes of 5 µL were analyzed in two replicates.

3. Results and discussion

SMX sensors were casted over solid contact devices. This configuration helps further miniaturization in subsequent studies and avoids sample contamination by traditional internal reference solutions [27]. Graphite/epoxy resin was selected for solid contact, as coated-wire versions present random drift, attributed to the formation of a thin aqueous layer between the polymeric ion-selective membrane and the inner metal electrode [28].

In general, the sensitivity, selectivity, working range, and stability of an ion-selective electrode depend not only on the nature of the ionophore, but also on the nature and amount of the plasticizer and lipophilic additives. 2-NPOE was selected for plasticizer because previous works showed enhanced sensitivity and selectivity when this solvent was employed [29,30]. The effect of the additive and kind of ionophore is reported here by preparing electrodes with different compositions (Table 1). The electroactive material employed was either of PPHR (Mn^{III}TPPCl) or of CD (α-CD, β-CD, or γ- CD) nature, doped or not with different lipophilic additives of positive or negative charge.

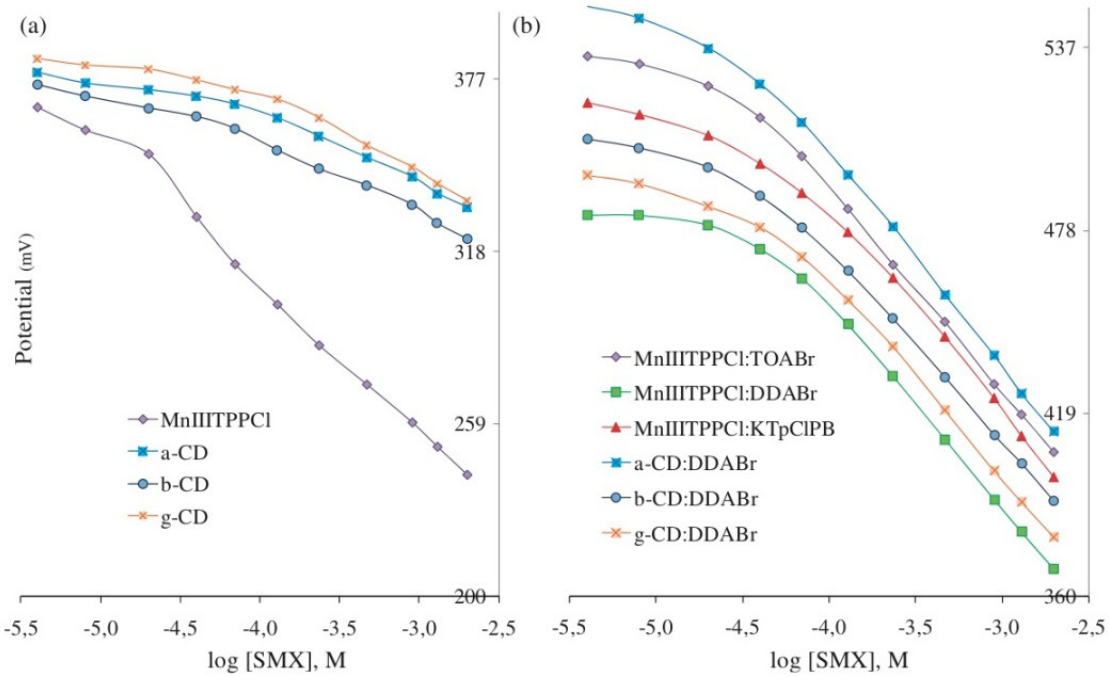

Figure 2. Typical calibration curves of SMX membranes prepared without (left) or with (right) ionic additives.

3.1 *Porphyrin-based sensors*

The selective response of the PPHR-based sensor for SMX was evaluated in plasticized PVC membranes with 1 wt.% ionophore, 66 wt.% 2-NPOE, and 33 wt.% PVC. The PPHR-based ionophore acted as anion carrier because it had a Lewis acidic metal as coordinating site. According to the literature, the nature of the ionophore-anion interaction is strongly influenced by the charge of the central metal ion from the porphyrin structure. The phorphyrin used was coordinated by Mn (III) meaning that it could act either as charged or as neutral carrier. The results suggested that a charged carrier mechanism governed the electrode performance because no charged additive was required to obtain a near-Nernstian behavior. The sensors exhibited a linear correlation of emf against log[SMX, M] (Figure 2, Left) from 5.0×10^{-5} to 1.0×10^{-2} M, with average slopes of 49 mV/decade. The response of the electrodes was also further enhanced by the addition of positively charged derivatives, which supported the charged carrier mechanism of the potentiometric transduction.

The binding between SMX and Mn^{III}TPPCl was confirmed by spectrophotometric studies. A slight deviation was recorded in the maximum wavelength of Mn^{III}TPPCl absorption spectra when SMX was present. Furthermore, the absorbance values of a blank PPHR solution decreased in the presence of SMX, in agreement with the linear correlation: Absorbance $= -15127 \times$ [SMX, M] $+ 0.066$ ($r = 0.9971$). This correlation was observed between 2.9×10^{-7} and 1.3×10^{-6} M. The binding constant was calculated by the sandwich method. The average value obtained was 4.5 for Mn^{III}TPPCl acting as guest molecule.

Although some concerns have been reported previously about the spontaneous hydroxyl-bridged dimmer formation of metalloporphyrins in ion-selective membranes originating super-Nernstian response slopes [31–33], this was not observed in the present studies.

3.2 *Cyclodextrin-based sensors*

CDs contain a hydrophilic outer surface and a hydrophobic cavity that may entrap hydrophobic molecules by means of electrostatic interactions. The inclusion complexes between ligand and CD are a result of hydrophobic and polar interactions established between these [34]. The size of the CD cavity also affects the formation of these inclusion complexes. Three major types of CDs are known, α-, β- and γ-CD; the α-CD contains six, β-CD seven, and γ-CD eight glucose units. A general trend is that hydrophobic organic compounds with a relatively small molecular volume form the strongest complexes with α-CD and the weakest with γ-CD [35]. The binding constant between SMX and β-CDs obtained by the sandwich method was 5.8, a similar value to that obtained when PPHR was used.

When SMX was used as ligand, none of the CD sensors prepared with either α-, β- and γ-CD and plasticized PVC (Table 1) was able to produce a suitable emf variation with SMX concentration (Figure 2, left). The uncharged carriers were neutral when uncomplexed in the membrane and the complexes had the same charge as the analyte ion. Thus, the respective membranes required lipophilic ions of opposite charge to ensure permselectivity [36].

3.3 *Effect of additive*

The effect of positive or negative charged sites in the selective membranes was investigated by doping them with DDABr, TOABr or KTpClPB. The ionophore:additive ratio tested was 1:1, 1:0.5 and 1:0.25 (mol:mol), with the additive relative amount changing from 25 to 100% mol (relative to the ionophore).

For PPHR materials, the cationic additives produced super-Nernstian behaviour, with slopes ranging from 66 to 70 mV/decade (Table 1). In general, the other operating features were unaffected. The electrodes prepared with an ionic additive displayed different behaviour according to the amount of additive. Sub-Nernstian behaviour was observed for a 25% mol of KTpClPB while super-Nernstian behaviour was obtained for the 100% mol additive (equal parts of additive and ionophore). The membranes with 50% mol amount of additive presented the best performance, with theoretical slopes and linear concentration ranges starting in 4×10^{-5} M. The use of an anionic additive may also provide advantages in terms of selectivity, because it acts as an anionic excluder in the membrane, reducing the interference from concomitant anionic species in real samples.

For CD based sensors, the addition of anionic additives gave slopes <32 mV/decade, which supported the fact that the CD ionophores acted as neutral carriers of SMX in the membrane. The cationic additives affected the anionic response to SMX depending on the size of the cavity of the carrier. Nernstian slopes were achieved with DDABr or TOABr when α-CD or β-CD was used as ionophore, respectively. For γ-CD, presenting the largest cavity for SMX inclusion, both cationic additives could be used, enabling suitable and similar analytical features in the potentiometric devices.

In general, ISEs IX and XXXVI exhibited the best analytical features. The slopes were 60.2 and 61.1 mV per decade concentration with the usable concentration range from 2.0×10^{-5} to 1.0×10^{-2} and 3.9×10^{-5} to 1.0×10^{-2}, respectively. The detection limit was 6.0×10^{-6} and 1.2×10^{-5}, respectively and the response time always ≤ 15 s (Figure 2). These electrodes were thus selected in most of the following experiments. To enable a systematic study of the additive effect upon the other analytical features to be studied, CD

electrodes with 50% mol of DDABr and PPHR-based electrodes with each additive in 50% mol were included along the following studies.

3.4 *Effect of pH*

The effect of pH upon the potentiometric response was tested by measuring the emf changes of a 1.0×10^{-3} M SMX solution over the pH range of 3 to 11 (Figure 3). The pH was altered by adding diluted H_2SO_4 or saturated NaOH hydroxide solutions and measuring continuously the resulting pH by a combined glass electrode dipped in the same solution.

In general, the small potential drop for higher pHs was evident in electrodes with cationic additives, which could suggest a hydroxyl interference. On the contrary, the increasing emf in the acidic range could indicate the lower concentration of anionic SMX in the solution.

The results showed that the operational pH ranges depended on the ionophore and the additive. PPHR ionophores showed a more intense dependence on the pH of the solution than CDs. For PPHR sensors with DDABr and TOABr cationic additives, steady emf was obtained within 6–9 and 5.5–9.5, respectively. For anionic additives the sensors behaved like a pH electrode, turning out selective to H^+. For CD sensors the increase in the cavity

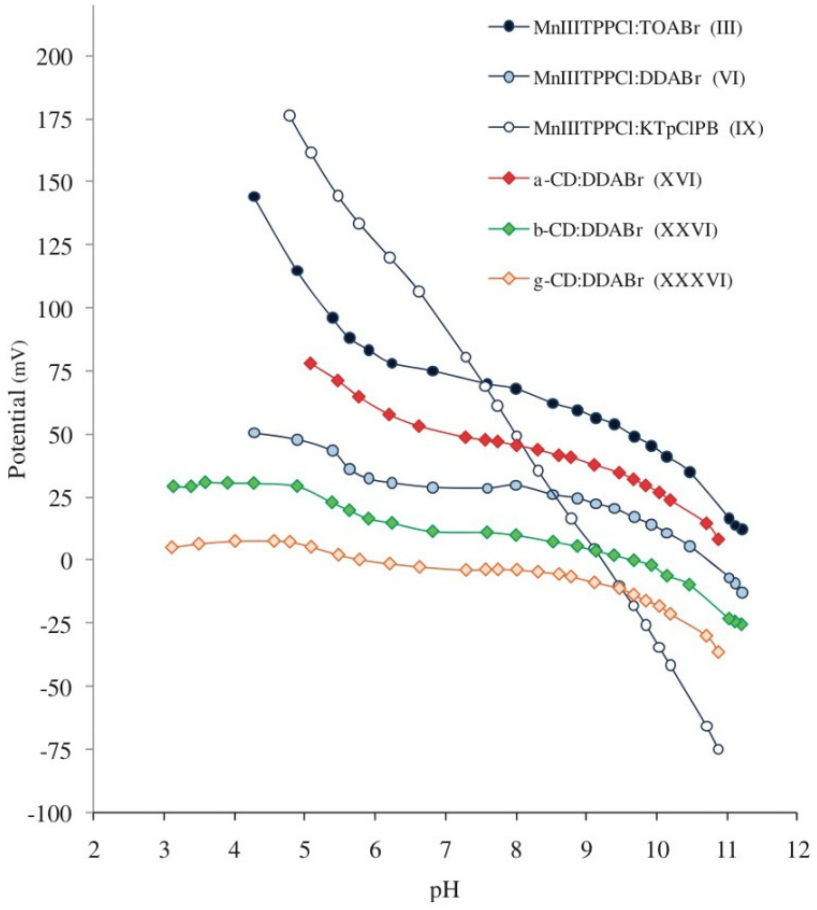

Figure 3. Emf variations of SMX selective electrodes against the pH of a 1×10^{-3} M SMX solution.

size originated wider operational pH range (Figure 3). Thus, γ-CD was the less affected by pH, with an operational pH range lying within 3-9.

3.5 *Selectivity of the electrodes*

The selectivity was initially assessed by the separate solutions method (SSM) [37]. The potential was measured with two separate solutions, one containing the SMX ion of activity a_{SMX} (but no interfering species) and the other containing the interfering ion J of the same activity $a_{SMX}=a_J$ (but no SMX anion). The equation applied was:

$$K^{POT}_{SMX,J} = a^{(1-1/Z_J)}_{SMX} e^{(E_J - E_{SMX})/S} \quad (1)$$

where the potential values were expressed as E_{SMX} and E_J, respectively; $K^{POT}_{SMX,J}$ was the corresponding potentiometric selectivity coefficient; and Z_J the ionic charge of the interfering ion.

All $K^{POT}_{SMX,J}$ data obtained were expressed in log $K^{POT}_{SMX,J}$ (Ttable 2). Lower interference was observed for more negative values. In general, the electrode was capable to discriminate SMX against other inorganic anions. The results indicated the following relative order of interference: carbonate $\ll$ chloride, nitrite; hydrogenocarbonate and fluoride interference depended on the electrode in use. The observed series deviated from the Hofmeister pattern, suggesting that the electrode selectivity for anions was not governed by anion lipophilicity. In the present case it seemed to depend on specific chemical interactions between the ionophores and the anions in the sample solution.

The matched potential method (MPM) was also employed to test the feasibility of using the electrodes on the analysis of real samples. In this method, $K^{POT}_{SMX,J}$ is the activity ratio of primary and interfering ions that give the same potential change under identical conditions. A known activity of SMX (a'_{SMX}) is added into a solution containing a fixed activity of SMX (a_{SMX}), and the corresponding potential change (ΔE) is recorded. A solution of interfering ion is added after to another reference solution of a_{SMX}, until the same potential change ΔE is obtained. The selectivity coefficient is calculated by:

$$K^{POT}_{SMX,J} = (a'_{SMX} - a_{SMX})/a_J \quad (2)$$

Table 2. Selectivity coefficients for SMX selective electrodes for various interfering ions using separate solution method.

	log $K^{POT}_{SMX,j^{z-}}$					
ISE	CO_3^{2-}	Cl^-	F^-	HCO_3^-	NO_3^-	NO_2^-
III	−2.68	−0.963	−1.59	−1.29	0.33	−0.56
VI	−2.86	−0.953	−1.54	−1.19	−0.41	−0.92
IX	−1.77	−0.51	−0.83	−0.39	−1.32	−0.46
XIII	−2.85	−1.14	−1.87	−1.32	0.35	−0.75
XVI	−1.59	−1.30	−1.44	−0.39	−0.64	−1.31
XIX	−2.97	−1.81	0.39	−1.76	0.21	−1.44
XXIII	−2.79	−1.20	−1.73	−1.40	0.45	−0.72
XXVI	−2.79	−1.07	−1.48	−1.34	0.28	−0.81
XXXIII	−2.93	−1.18	−1.84	−1.61	0.44	−0.79
XXXVI	−2.28	−0.80	−1.33	−1.13	0.34	−0.67

Table 3. Selectivity data for SMX selective electrodes for various interfering ions using both Separate solution (SSM) and Matched Solution (MSM) methods.

	SSM		*MPM*	
Interfering	ISE IX	ISE XXXVI	ISE IX	ISE XXXVI
Acetate	−1.31	−1.69	−0.77	−0.76
Borate	−3.08	0.2.89	−0.62	−0.65
Cyanide	−1.25	−1.75	−0.57	−0.57
Citrate	−2.86	−3.14	−0.50	−0.51
Phosphate	−3.74	−3.65	−0.70	−0.72
Glucose	−1.59	−1.79	−0.60	−0.69
Sulphate	−3.43	−2.91	−0.48	−0.54
Tartrate	−2.90	−3.10	−0.53	−0.47

The electrodes used for this purpose were ISEs IX and XXXVI for combining the best analytical features for a practical application: high sensitivity, extended linear range and good selectivity. The anionic species selected for this study were acetate, borate, cyanide, citrate, glucose, phosphate, sulphate and tartrate. In general, the obtained $\log K^{POT}$ confirmed the preference of the electrodes for SMX over other interfering species (Table 3). All $\log K^{POT}$ values were negative and confined to a narrow range: −0.47 to −0.77. The relative order of tolerance levels for the proposed sensor was: Sulphate, Citrate, Tartrate > Cyanide > Glucose, Borate > Phosphate > Acetate. The SSM gave lower $\log K^{POT}$ values than the MPM, which is logical because primary and interfering ion are competing for the same phenomenon in this last method.

3.6 *Response time and lifetime*

The response time was measured by placing the electrodes in a SMX standard solution and increasing the concentration in about tenfold, from 4.0×10^{-5} to 1.0×10^{-3} M. The static response time thus obtained was 7 s for 1.0×10^{-3} M SMX. At lower concentrations, the response time was longer and reached 15 s for a SMX concentration of 1.0×10^{-4} M. The actual potential *vs.* time plot is shown in Figure 4.

In general, the potentials remained constant for approximately 3 min, after which a small deviation was detected. The behaviour of the membrane did not depend on whether the potentials were recorded from low to high concentration or *vice versa.*

Every kind of electrodes was tested over a period of about two months. During this period each electrode was used daily and stored in 1.0×10^{-6} M SMX solution when not in use. No significant changes in the performance of the electrodes (slopes and linear range) were observed during this period.

3.7 *Analysis aquaculture waters*

The proposed method was applied to determine SMX in water samples from aquaculture environment. The collected samples were confirmed as blank and spiked with 35–340 μg/mL of SMX. The electrodes used for this purpose were ISEs IX and XXXVI for displaying

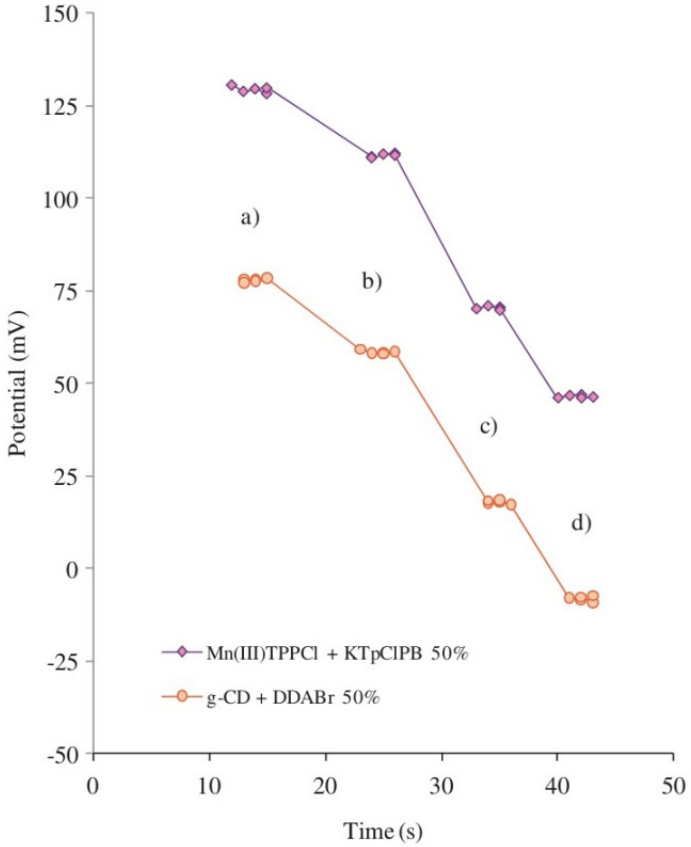

Figure 4. Response time for step changes in concentration of SMX: (a) 4×10^{-5} M, (b) 1×10^{-4} M, (c) 5×10^{-3} M, (d) 1×10^{-3} M.

the better analytical features in terms of sensitivity, linear range and selectivity (see Table 1).

The obtained results are summarized in Table 4 and correspond to the mean of at least 3 independent determinations. A good agreement was found between added and found amounts of SMX. The potentiometric set of results showed recoveries ranging from 96 to 106%, which corresponded to relative errors within −3.9 and +5.9%. They were also precise, with relative standard deviations always below 6%. The *t*-student test confirmed that there were no significant differences between the means of added amounts and potentiometric sets of results (Tables 3 and 4). The *p* value was 0.03 and 0.77 for Mn(III) PPHR or γ-CD sensors, always below the critical value (2.07).

The potentiometric data were validated against an LC-MS/MS method. For this purpose, some of the samples were tested again by both methods. A typical chromatogram for SMX is presented in Figure 5, with a retention time of 13.72 min. The results obtained are indicated in Table 5 and suggest the accuracy and precision of both methods. Using the LC-MS/MS procedure as reference, the *t* student ($p = 0.33$) was below the critical one (2.20), thus confirming the accuracy of all data.

The above results pointed out that the composition of the water samples collected from aquaculture environment did not interfere in the potentiometric determination of SMX. So, the samples were further analyzed with regard to their main composition; the legal parametric data applied to environmental waters were selected for this purpose [38]. The obtained values are indicated in Table 6, where the minimum as maximum values have

Table 4. Determination of SMX in aquaculture by potentiometric method, using PPHR and γ-CD electrodes.

		PPHR ISE ($n=3$)		γ-CD	
Sample	Added, μg/mL	SMX, μg/mL	RD (%)	SMX, μg/mL	RD (%)
1	35.0	32.8 ± 0.8	5.9	34.9 ± 0.8	−0.3
	83.3	83.1 ± 3.3	0.2	82.3 ± 0.7	−1.2
	174.6	177.0 ± 5.2	−1.4	180.8 ± 3.0	3.5
	338.1	339.5 ± 12.3	−0.4	329.6 ± 5.4	−2.5
2	35.0	33.6 ± 2.0	3.9	35.9 ± 0.5	2.5
	83.3	81.6 ± 2.4	2.1	82.7 ± 1.7	−0.7
	174.6	172.6 ± 5.8	1.2	174.2 ± 6.0	−0.2
	338.1	329.3 ± 4.1	2.6	330.9 ± 2.3	−2.2
3	35.0	33.7 ± 0.3	3.7	36.8 ± 0.8	5.2
	83.3	84.4 ± 2.9	−1.3	82.0 ± 2.3	−1.5
	174.6	173.6 ± 7.9	0.6	180.3 ± 2.7	3.3
	338.1	330.1 ± 2.7	2.4	333.6 ± 3.3	−1.4
4	35.0	34.4 ± 0.9	1.6	36.5 ± 1.6	4.3
	83.3	83.0 ± 1.4	0.4	82.4 ± 2.8	−1.1
	174.6	172.1 ± 0.8	1.4	182.1 ± 2.3	4.3
	338.1	330.6 ± 7.8	2.2	344.2 ± 17.3	1.8
5	35.0	35.8 ± 0.9	−2.4	34.2 ± 1.0	−2.3
	83.3	81.9 ± 0.5	1.7	81.4 ± 1.7	−2.2
	174.6	175.5 ± 6.3	−0.5	178.9 ± 4.1	2.5
	338.1	334.8 ± 6.7	1.0	329.9 ± 1.6	−2.4
6	35.0	34.8 ± 0.3	0.5	35.3 ± 1.2	1.0
	83.3	84.0 ± 1.4	−0.8	82.1 ± 1.4	−1.5
	174.6	176.1 ± 4.2	−0.9	177.7 ± 2.5	1.8
	338.1	331.1 ± 10.0	2.1	331.1 ± 7.5	−2.1

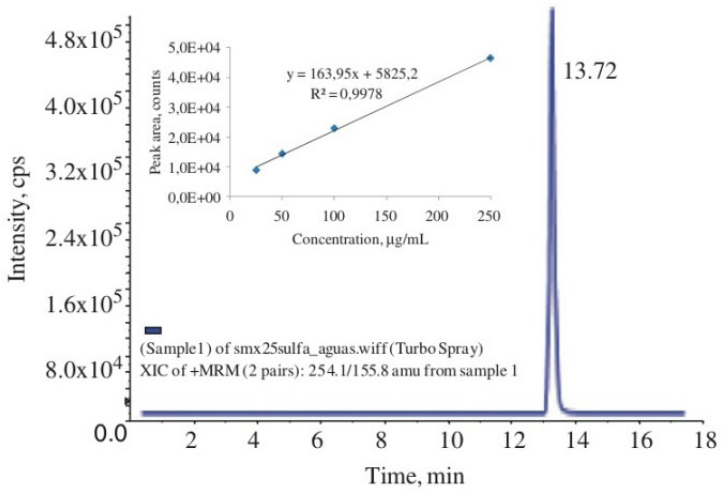

Figure 5. Typical MS chromatogram record for SMX (inset: calibration curve).

Table 5. Determination of SMX in aquaculture by potentiometric (POT) and the comparison methods (LC–MS/MS).

Sample	Added, μg/mL	POT ($n=3$) SMX, μg/mL	POT ($n=3$) RE (%)	LC–MS/MS SMX, μg/mL	LC–MS/MS RE (%)	RD (%)
1	40	39.6 ± 0.8	−2.8	44.2	10.5	−10.5
	100	101.8 ± 0.8	2.3	98.5	−1.5	3.3
	150	150.9 ± 0.2	0.5	158.0	5.3	−4.5
	250	245.7 ± 0.9	−1,.6	241.0	−3.6	2.0
2	40	43.0 ± 0.5	−1.3	49.4	23.5	−13.0
	100	102.1 ± 0.3	2.4	97.1	−2.9	5.1
	150	150.3 ± 1.2	−0.7	144.0	−4.0	4.4
	250	246.5 ± 1.6	−2.0	212.0	−15.2	16.3
3	40	38.6 ± 0.6	−3.2	37.2	−7.0	3.7
	100	101.2 ± 0.7	0.5	103.0	3.0	−1.7
	150	149.3 ± 0.6	−0.9	143.0	−4.7	4.4
	250	249.2 ± 0.4	−0.3	247.0	−1.2	0.9

Table 6. Main chemical parameters of the water samples.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Minimum	Maximum
Turbidity (UNT)	<0.2	<0.2	<0.2	<0.2	0.7	0.7	<0.2	0.7
pH (Sorensen scale)	7.0	7.0	6.9	6.8	6.7	6.6	6.6	7.0
Conductivity (μS/cm)	41.0	40.0	41.0	39.0	135.0	137.0	39.0	137.0
Nitrate (mg/L)	1.4	1.3	12.4	12.7	3.9	3.9	1.3	12.7
Nitrite (mg/L)	<0.03	<0.03	<0.03	0.41	<0.03	0.22	–	0.41
Ammonium (mg/L)	<0.05	<0.05	<0.05	<0.05	<0.05	0.34	<0.05	0.34
Sulphate (mg/L)	1.9	2.0	8.7	8.7	1.8	1.9	1.9	8.7
Chloride (mg/L)	6.3	6.4	21.2	20.5	5.9	5.9	5.9	21.2
Fluoride (mg/L)	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–
Phosphate (mg/L)	<0.03	<0.03	0.12	0.11	<0.03	<0.03	<0.03	0.12
Iron (μg/L)	<50	<50	<50	<50	<50	<50	<50	–
Potassium (mg/L)	<1.0	<1.0	2.0	2.0	<1.0	<1.0	<1.0	2.0
Sodium (mg/L)	5.6	5.7	14.9	15.4	4.7	4.7	5.6	15.4
Calcium (mg/L)	1.6	1.6	4.8	5.1	1.5	1.5	1.5	4.8
Magnesium (mg/L)	<1.0	<1.0	3.9	3.9	<1.0	<1.0	<1.0	3.9
TOC (mg/L)	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	–
Cu (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–
Zn (mg/L)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	–
BOD (mg/L)	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	–
COD (mg/L)	<30	<30	<30	<30	<30	<30	<30	–

TOC: total organic content; BOD: biochemical oxygen demand; COD: chemical oxygen demand.

been pointed out. These levels may be established as known (practical) tolerance limits for future potentiometric application.

4. Conclusions

In Mn(III) PPHR sensors an anionic sensor improved the potentiometric response but was not fundamental to obtain a significant emf variation against log SMX concentration, while in CD SMX membrane sensors required the inclusion of an ionic additive. Thus, the corresponding sensors acted, respectively, by a charged or neutral carrier mechanism to produce the potentiometric response. It is possible to exploit a practical application with both of the ionophores and even to attach the corresponding electrodes to the end of a chromatographic column, in case it is found necessary to discriminate several sulphonamides or confirm the existence of a specific sulphonamide.

The proposed electrodes displayed good reproducibility, high sampling rate, wide linear range, low detection limit, high sensitivity and good selectivity. The method is simple, accurate, precise, and inexpensive regarding reagent consumption and equipment involved, for which it is adequate for local screening procedures. It displayed comparable analytical data to a much more sophisticated procure, with much lower cost and shorter time, producing less volume of wastewaters. Furthermore it can be easily applied to the automation of routine determination of SMX in environmental contaminated samples.

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Materiais de sol-gel impressos com sulfonamidas utilizados na transdução potenciométrica

Uma alternativa mais recente para outros materiais sensores é a inclusão de cavidades receptoras numa matriz de sol-gel. Esta matriz, *per si*, apresenta as características eléctricas, térmicas e de estabilidade necessárias à preparação detectores potenciométricos de sucesso, tal como se deduz da sua utilização ao longo destas duas últimas décadas. A formação polimérica pode permitir o desenho de cavidades internas adequadas à recepção de um composto alvo, estabelecendo também interacções "host-guest" com esse mesmo composto. As estruturas impressas são também reconhecidas como anticorpos plásticos, pela sua semelhança conceptual para com os naturais. Tendo em conta estes aspectos, neste trabalho optou-se por preparar materiais de sol-gel impressos com diferentes composições, de modo a optimizar as características potenciométricas de eléctrodos para SDZ ou SMX. A melhor composição encontrada foi entretanto aplicada em eléctrodos de suporte sólido e testada com e sem aditivo catiónico. A sua capacidade de reconhecimento molecular foi também avaliada através da preparação de membranas de sol-gel "brancas", que não apresentavam impressão da molécula modelo. A transdução potenciométrica directa da membrana de sol-gel revelou resposta sub-Nernstiana, correspondendo isso a uma incapacidade de reconhecimento molecular.

Na sequência destes resultados, optou-se pela pulverização dos materiais de sol-gel e pela sua incorporação como ionóforos em membranas de PVC plastificado. Neste contexto, e perante a ausência total de informação na literatura sobre este tipo de eléctrodos, estudaram-se todos os parâmetros de resposta para os eléctrodos construídos. Relativamente ao material sensor propriamente dito foram preparados eléctrodos com materiais impressos ou não impressos, em quantidade diferentes. Para o aditivo foram avaliados os efeitos da sua presença ou ausência e da sua natureza química. Finalmente, foram ainda estudados diferentes solventes mediadores plastificantes, com permitividades muito diferentes. Todas as membranas preparadas foram avaliadas potenciometricamente e as melhores unidades foram aplicadas, com sucesso, à análise de águas de aquacultura. Incluíram-se também neste trabalho a análise de soro e de produtos farmacêuticos.

Comparando com os ionóforos testados nos capítulos anteriores, conclui-se que as estruturas de sol-gel podiam constituir excelentes receptores para a SDZ e a SMX, desde que incorporadas numa matriz de PVC plastificado. Verificou-se igualmente que o uso deste tipo de ionóforo permitia diminuir o limite de detecção dos eléctrodos.

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Sulphonamide-imprinted sol–gel materials as ionophores in potentiometric transduction

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ABSTRACT

This work proposes different kind of solid-contact graphite-based electrodes for the selective determination of sulphonamides (SPHs) in pharmaceuticals, biological fluids and aquaculture waters. Sulfadiazine (SDZ) and sulfamethoxazole (SMX) were selected for this purpose for being the most representative compounds of this group. The template molecules were imprinted in sol–gel (ISG) and the resulting material was used as detecting element. This was made by employing it as either a sensing layer or an ionophore of PVC-based membranes and subsequent potentiometric transduction, a strategy never reported before. The corresponding non-imprinted sol–gel (NISG) membranes were used as blank. The effect of plasticizer and kind/charge of ionic lipophilic additive was also studied.

The best performance in terms of slope, linearity ranges and signal reproducibility and repeatability was achieved by PVC membranes including a high dielectric constant plasticizer and 15 mg of ISG particles. The corresponding average slope was −51.4 and −52.4 mV/decade, linear responses were 9.0×10^{-6} and 1.7×10^{-5} M, and limits of detection were 0.74 and 1.3 μg/mL for SDZ and for SMX, respectively. Good selectivity with log Kpot<−0.3 was observed for carbonate, chloride, fluoride, hydrogenocarbonate, nitrate, nitrite, phosphate, cyanide, sulfate, borate, persulphate, citrate, tartrate, salicylate, tetracycline, ciprofloxacin, sulphamerazine, sulphatiazole, dopamine, glucose, galactose, cysteine and creatinine. The best sensors were successfully applied to the analysis of real samples with relative errors ranging from −6.8 to +3.7%.

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1. Introduction

Sulphonamide (SPH) drugs were the first effective chemotherapeutic agents employed for preventing bacteria from synthesizing folic acid, an essential chemical to their growth and are among the most used antibiotics in aquaculture activities [1]. Their similarity with the structure of *p*-aminobenzoic acid (PABA, a key ingredient in bacterial synthesis of folic acid), makes bacteria to mistakenly convert the drug instead of PABA into folic acid, thus inhibiting the normal growth of the microorganism.

Due to the development of resistance in formerly susceptible microorganisms, only a few sulpha drugs are used today, among which sulfadiazine (SDZ) and sulfamethoxazole (SMX) (Fig. 1). SDZ is used to treat toxoplasmosis as well as to prevent certain types of meningococcal meningitis [2]. SMX has been used successfully for the treatment of bacterial infections, including those of the respiratory and urinary tract, as well as in the treatment of opportunistic infections in transplantation and for AIDS related complications [3]. They have been employed also as an antimicrobial in aquaculture environment, posing serious risks of food and environmental contamination [2], and subsequent drug resistance episodes [4].

Most methods for SPH determination in pharmaceutical samples require capillary electrophoresis with UV [5] or electrochemical detection [6], and optical [7–9] and electrochemical methods [10–12]. Other methods for SPH determination are meant for the analysis of food or feeding stuffs [13–15], and biological fluids [16]. Few methods have been devoted to environmental samples [17–20], and these concern complex and high cost pre-sampling procedures, unsuitable for in situ or for routine control of waters such as those in the fish tanks.

Ion-selective electrodes (ISEs) could be an alternative to the previous methods, because they offer high precision and rapidity, low cost of analysis, and enhanced selectivity and sensitivity over a wide range of concentrations [21,22]. The binding between the ionophore and the target ion is the molecular-level phenomenon, sensed by an ISE [23]. Therefore, the ISE selectivity towards several ions is regarded to derive from the difference in the binding strengths between the selected ionophore, to be used in the sensor, and the different ions.

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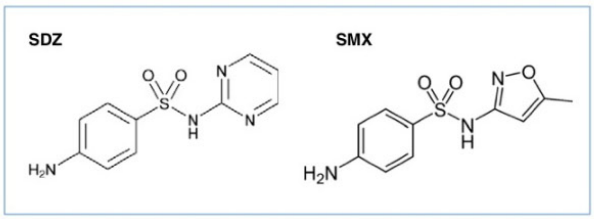

Fig. 1. Binding isotherm (A) and Scatchard plot (B) for ISG particles. Q is the amount of ISG bound to 15.0 mg of polymer; t = 25 °C; V = 10.00 mL; binding time: 20 h.

The ionophore or the ion carrier is thus a vital component in the selective membrane [23]. Ion exchangers and neutral macrocyclic compounds have been employed with success for this purpose over the past decades. However, the design of new sensing materials that are complementary to the size and charge of a particular ion may lead to very selective interactions [24–26]. This could be done by imprinting the target analyte (SDZ and SMX) on a solid surface [27,28].

Molecular imprinting (MI) allows the preparation of synthetic polymers with specific binding sites for a target molecule. This can be achieved if the target is present during the polymerization process, thus acting as a molecular template [29]. The template molecule is removed without disturbing the geometry of the solid matrix and the molecularly-imprinted polymer (MIP) keeps the ability to rebind it. Template rebind produces electrical, optical or mass changes in the nanostructured sensor, thus enabling its detection [30]. Essentially, there are two main routes to produce MIP structures: polymerization of vinyl monomers carrying different chemical functions or sol–gel technology, a useful method to prepare glass-like or ceramic materials through the hydrolysis and condensation of suitable metal alkoxides [31].

The sol–gel process can be described as the construction of an oxide network by progressive polycondensation reactions of molecular precursors in a liquid medium. This technique is currently gaining importance [32] because it is a rather convenient and simple way to incorporate heat sensitive compounds in porous ceramic materials [33]. It also permits designing new materials with tailor-made pore sizes and shapes for specific analyte recognition. Inorganic materials prepared by a sol–gel process have been implemented into a variety of technologies, including nonlinear optical devices, luminescent solar concentrators, and chemical sensors. Still, the application of imprinted sol–gel materials to the production of potentiometryic sensors is in its infancy [34,35].

Thus, this work proposes the construction of SDZ and SMX selective electrodes based on ISG material. The ISG is also ground and used as electroactive material on PVC SDZ and SMX selective membranes. Non-imprinted sol–gel (NISG) materials were used as a blank control of the imprinting process. The sensors were evaluated in steady-state conditions and applied to the analysis of drugs, water and biological samples.

2. Experimental

2.1. Apparatus and electrodes

All potential measurements were made by a Crison μpH 2002 decimilivoltammeter, at room temperature, and under constant stirring, by means of a Crison, micro ST 2038. The output signal in steady state evaluations was transferred to a commutation unit and reconnected to one of six ways out, enabling the simultaneous reading of six ISEs. The assembly of the potentiometric cell was as follows: conductive graphite | SDZ selective membrane | buffered sample solution (HEPES, 1×10^{-3} M, pH 5.0) || electrolyte solution, KCl | AgCl(s) | Ag. The reference electrode was an Orion Ag/AgCl double-junction (Orion 90-02-00). The selective electrodes were prepared in conventional configuration, with no internal reference solution and an epoxy–graphite solid contact [36].

2.2. Reagents and solutions

All chemicals were of analytical grade and de-ionized water (conductivity <0.1 μS cm^{-1}) was employed. SDZ, SMX, p-*tert*-octylphenol (TOP), tetraoctylammonium bromide (TOABr), tetraphenylborate (TPB), o-nitrophenyloctyl ether (oNPOE), bis(2-ethylhexyl)sebacate (bEHS), dibutyl phthalate (DBP), poly (vinyl chloride) (PVC) of high molecular weight, 3-aminopropyltriethoxysilane (APTES), diphenyldimethoxysilan (DPTS), tetraethyl orthosilicate (TEOS) were purchased from Fluka. Methanol (MeOH) and tetrahydrofuran (THF) were obtained from Riedel-deHäen. Buffer solutions were 0.001 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Interference of other chemicals was evaluated for 1.2×10^{-4}, 5.0×10^{-4}, and 1.0×10^{-3} M solutions prepared in buffer.

2.3. Synthesis of host-tailored sol–gel material and preparation of ISG membrane electrode

The water/alkoxide ratio was set to 3, ensuring a transparent smooth surface without cracks and with suitable porosity for the intended purposes (higher ratios decrease the thickness, shrinkage, and pore volume [37] of the matrix). ISG polymers were obtained by dissolving 12.5 mg of each SPH in 1.5 mL of APTES and 1.5 mL of DPTS, acting as functional monomers, in 4.0 mL of methanol. This mixture was stirred for 30 min at room temperature. Then TEOS (0.5 mL), the reticulating agent, was added, followed by hydrochloric acid (0.1 M, 0.3 mL) and desionized water (2.5 mL) to produce the alkoxysilan hydrolysis. The sol was stirred for a further 7 h until a viscous solution was achieved. The conductive graphite surface of the electrodes was immersed into this solution for 7 s. The membranes obtained were dried at room temperature for 2 days. NISG sensor was prepared in a similar way, by excluding the template from the procedure.

2.4. Preparation of the membrane electrodes

Sol–gel membranes were obtained by coating a clean graphite support [25] with 200 μL of the viscous ISG solution. This solution was allowed to dry at room temperature. NISG electrodes were prepared similarly and acted as control of the imprinting effect.

PVC membranes were prepared by including ISG particles inside the PVC matrix. For this purpose, the solid ISG material was grounded and sieved to particle sizes ranging 50−150 μm. SPH was removed from the ISG particles by washing them several times in water (the absence of SPH was confirmed by controlling the absorbance of the washout solution at 260 nm; the particles were repeatedly washed until SPH was no longer detected). The polymer was dried after at 60 °C under vacuum until constant weight. Variable amounts of ISG particles for SDZ were then mixed with 350 mg of plasticizer (*o*NPOE, bEHS or DPB) (Table 1). Some membranes were also added of TOABr, TOP and TPB, acting as additive. The sensor solution was added to 210 mg of PVC previously dissolved in 5.0 mL THF. These membranes were applied over graphite conductive supports. Membranes were let dry for 24 h. NISG PVC membranes were prepared similarly as control. SMX selective membranes were prepared in parallel for some compositions, following similar procedures.

2.5. Potentiometric procedures

All potentiometric measurements were carried out at room temperature. Emf values of each electrode were measured in solutions of fixed pH and ionic strength. Increasing concentration levels of SPH were obtained by transferring 0.02–10.0 mL aliquots of

Table 1
Composition SDZ selective electrodes of imprinted sol–gel and membrane sensors, main analytical features in 1×10^{-3} mol L^{-1} HEPES buffer, pH 5.0.

Characteristic	ISE I	ISE II	ISE III	ISE IV	ISE V	ISE VI	ISE VII	ISE VIII	ISE IX	ISE X	ISE XI	ISE XII	ISE XIII
Sensing material (SM)	MIP/ISG	MIP/ISG	NIP/ISG	MIP/PVC	MIP/PVC	NIP/PVC	MIP/PVC	MIP/PVC	MIP/PVC	MIP/PVC	MIP/PVC	MIP/PVC	MIP/PVC
Amount of SM, mg	–	–	–	15.0	15.0	15.0	15.0	15.0	15.0	15.0	5.0	10.0	30.0
Additive	–	TOP	–	–	TOP	–	–	–	TOABr	TPB	–	–	–
Plasticizer	oNPOE	oNPOE	oNPOE	oNPOE	oNPOE	oNPOE	bEHS	DPB	oNPOE	oNPOE	oNPOE	oNPOE	oNPOE
Slope, mV/decade	−46.3 ±1.5	−51.8 ±1.5	−44.2 ±3.2	−51.4 ±1.8	−43.5 ±1.1	−32.3 ±2.0	−50.9 ±1.6	−43.3 ±0.0	−60.2 ±0.3	−12.0 ±1.9	−41.6 ±1.3	−39.6 ±0.1	−42.6 ±3.9
R^2 (n = 3)	0.993	0.992	0.996	0.992	0.992	0.995	0.994	0.992	0.992	0.995	0.998	0.995	0.991
Cv_w (%)	6.1	7.3	3.2	8.0	0.8	1.0	5.3	0.0	1.8	9.1	4.4	4.4	6.4
LOD, μg/mL	5.6	6.5	14	0.74	4.0	6.5	17	11	0.87	4.2	2.4	1.2	3.1
LOD, mol/L	2.0×10^{-5}	2.4×10^{-5}	4.9×10^{-5}	2.7×10^{-6}	1.5×10^{-5}	2.4×10^{-5}	6.3×10^{-5}	4.0×10^{-5}	3.2×10^{-6}	1.5×10^{-5}	8.9×10^{-6}	4.5×10^{-6}	1.1×10^{-5}
LLLR, μg/mL	19	9.0	14	2.5	9.0	11	4.4	25	4.1	21	3.3	4.4	6.4
LLLR, mol/L	7.0×10^{-5}	3.3×10^{-5}	5.0×10^{-5}	9.0×10^{-6}	3.3×10^{-5}	4.0×10^{-5}	1.6×10^{-5}	9.0×10^{-5}	1.5×10^{-5}	7.6×10^{-5}	1.2×10^{-5}	1.6×10^{-5}	2.4×10^{-5}

LOD: Limit of Detection, LLLR: Lower Limit of Linear range; R^2: Squared correlation coefficient; SD: Standard deviation; Cv_w: Reproducibility.

1.0×10^{-3} M SPH aqueous solutions to a 100 mL beaker containing 50.0 mL of 1.0×10^{-3} M of buffer. Potential readings were recorded after stabilization to ±0.2 mV and emf was plotted as a function of logarithm SPH concentration. Calibration graphs were used for subsequent determination of unknown SPH concentrations.

2.6. Binding affinity assays between template/host molecules

Binding experiments were carried out by placing 15.0 mg of ISG washed particles in contact with 8.0 mL of SPH solution with varying concentrations ranging 0.4−4 mM. The mixtures were shacked for 12 h at room temperature and the solid phase separated by centrifugation (3000 rpm, 10 min). The concentration of free SPH in the supernatant was also detected by UV spectrophotometry at 260 nm. The amount of SPH bound to the polymer was calculated by subtracting the concentration of free SPH from the initial SPH concentration. The data obtained was used for Scatchard analysis [38].

2.7. Determination of SDZ and SMX in serum, aquaculture water and pharmaceuticals

Potentiometric analysis was carried out on oral dosage forms of pharmaceutical preparations, commercially available as Broncodiazina® (Vitoria, Portugal), with a labeled amount of 3.6 g SDZ per 100 mL. This suspension was dispersed in water by sonication and diluted in buffer to a concentration within the linear range.

For the analysis of aquaculture water or biological fluids, an amount of 250 μl of water or serum were placed in a 25 mL volumetric flask and diluted to final volume with 1.0×10^{-3} M HEPES (pH 5.0). The direct potential method was applied to determine SDZ and/or SMX in all samples.

3. Results and discussions

In the sol–gel process, a sol is first formed by mixing a liquid alkoxide precursor, water, a co-solvent (usually ethanol or methanol) and a catalyst (acid or base) at room temperature [39]. Through continuous monomer hydrolysis and condensation reactions, a porous gel network is thus obtained. Gel aging and drying can be conducted under controlled conditions in order to obtain dense solid matrices. The sol–gel route provides a useful method to prepare glass-like or ceramic materials through the hydrolysis and condensation of suitable organically modified precursors and permits the creation of an oxide network by progressive polycondensation reactions of the molecular precursors in alcoholic medium.

APTES was selected in this study to copolymerize with TEOS because an amine function would ensure a positive electrostatic environment in the sol–gel layer under sufficiently acidic media. This feature may enhance the sensitivity to negatively charged compounds, such as SDZ or SMZ in aqueous media. The selectivity of this material to a specific compound was attributed by MI technology. This was made by preparing the sol–gel in the presence of the template and removing it afterwards by suitable washout procedures.

3.1. Rebinding properties of ISG particles

Binding experiments were carried out by incubating fixed amounts of ISG particles with different concentrations of template until equilibrium was reached. The resulting binding capacity of ISG particles was calculated according to the following equation:

$$Q = \frac{\mu mol(SPH\ bound)}{g(ISG)} = \frac{(C_i - C_f)V_s \times 1000}{M_{ISG}} \quad (1)$$

where Q is binding capacity of ISG (μmol/g), C_i the initial SPH concentration (μmol/mL), C_f the final SPH concentration (μmol/mL), V_s the volume of solution tested (mL), M_{ISG} the mass of dried ISG particles (mg).

The binding capacities were plotted against the initial SPH concentration. The adsorption data showed that the binding capacity of particles increased with the increasing of the initial concentration of SPH, reaching saturation for higher concentrations.

The binding data were further processed with Scatchard analysis, using the equation

$$Q/C_{free} = (Q_{max} - Q)/K_d \quad (2)$$

where Q was the binding capacity; C_{free} the free analytical concentration in equilibrium (μM); Q_{max} the maximum apparent binding capacity; and K_D the dissociation constant at binding site. The equilibrium dissociation constant was calculated from the slopes and the apparent maximum number of binding sites from the *y*-intercepts in the linear plot of Q/C_{free} versus Q. The Scatchard plots showed a tendency to a linear behavior, suggesting the existence of a single kind of binding site in ISG particles. The corresponding equilibrium dissociation constants K_d for dry polymers were 1319 and 1410 μM for SDZ and SMX, respectively.

3.2. Physical features of the polymer

SEM analysis evidenced the formation of thick films without cracks, and with porous and rough structure. Images suggested that the thickness of the film was 846.1 nm and the diameter of the pore ranged approximately 27 and 52 nm (Fig. 2). The swelling of the membrane was about 3% in mass gain when in contact with water

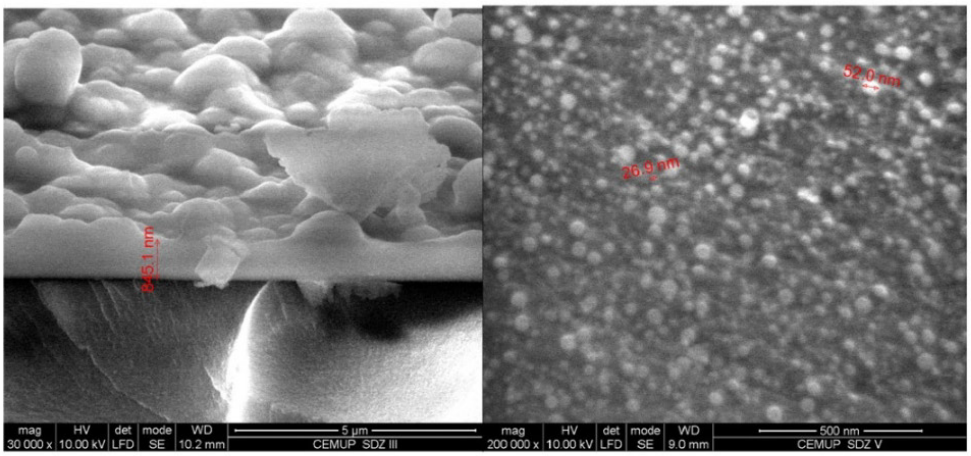

Fig. 2. SEM images of ISG polymer.

before calibration. FTIR spectra were made for grounded sol–gel matrix by an ATR accessory. The spectra displayed intense absorption band of ether bonds at about 1023 cm^{-1} (Fig. 3).

3.3. Sensors performance

The analytical data obtained for all ISEs is indicated in Tables 1 and 2. The reported limits of detection and linear behavior displayed relative standard deviations below 10%.

First, sol–gel membranes were prepared with imprinted (ISE I) or non-imprinted (ISE III) matrices. The ISG sensors displayed linear responses after 7.0×10^{-5} and 4.0×10^{-5} M, average anionic slopes of −46.3 and −49.3 mV/decade and detection limit of 5.6 and 3.1 µg/mL, respectively. The NISG sensors displayed a similar behavior, as may be seen in Fig. 4. This pointed out that the analyte was recognized by general electrostatic interactions with the silica groups of the sol–gel matrix. The possibility of improving the behavior by adding an additive was tested also by preparing sol–gel membranes including TOP (ISE II). This additive has an acid–base character, being mostly anionic or neutral. Under acidic conditions the molecule is essentially neutral with strong positive polarization on the hydrogen atom. This additive improved the analytical performance of the sensors in terms of slope and detection limit (Fig. 4A). Thus, it seems that the analyte was attracted to the membrane by hydrogen bonds [40], favoring the interaction of the anionic SPH with the hydrophobic membrane interface.

Similar electrodes to ISEs I to III were prepared by including the same materials in a PVC matrix (ISEs IV to VI), in order to check the influence of the membrane material on the performance of the sensors. This was done by doping PVC membranes with about 5% of the same ISG material that was finely pulverized. MIP/PVC ISEs showed some improvements in terms of limit of detection, decreasing from 5.6 to 0.74 µg/mL for SDZ (Table 1) and 3.1 to 1.3 µg/mL for SMX (Table 2). The imprinting effect was only evident here, with PVC based membranes. NISG PVC membranes displayed lower slopes than the corresponding ISG membranes; when a sol–gel layer was

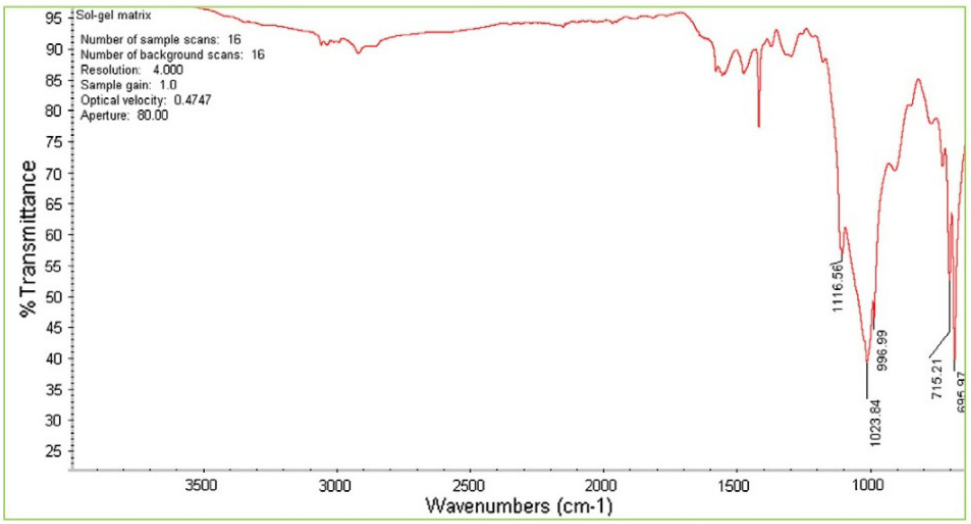

Fig. 3. FTIR spectra of the sol–gel matrix.

Table 2
Composition SMX selective electrodes of imprinted sol–gel and membrane sensors, main analytical features in 1×10^{-3} mol/L HEPES buffer, pH 5.0.

Characteristic	ISE I	ISE II	ISE III	ISE IV	ISE VI	ISE VII	ISE IX
Sensing material (SM)	MIP/ISG	MIP/ISG	NIP/ISG	MIP/PVC	NIP/PVC	MIP/PVC	MIP/PVC
Amount of SM, mg	–	–	–	15.0	15.0	15.0	15.0
Additive	–	TOP	–	–		–	TOABr
Plasticizer	oNPOE	oNPOE	oNPOE	oNPOE	oNPOE	bEHS	oNPOE
Slope, mV/decade	-49.3 ± 0.9	-51.1 ± 0.2	-41.9 ± 0.4	-52.4 ± 0.6	-36.1 ± 1.1	-51.9 ± 0.3	-59.5 ± 1.4
R^2 (n = 3)	0.993	0.996	0.999	0.996	0,995	0.996	0.997
Cv_w (%)	1.8	0.32	0.91	1.2	3.1	0.61	2.4
LOD, μg/mL	3.1	2.6	3.9	1.3	3.8	1.7	1.5
LOD, mol/L	1.2×10^{-5}	1.0×10^{-5}	1.6×10^{-5}	5.1×10^{-6}	1.5×10^{-5}	6.7×10^{-6}	6.1×10^{-6}
LLLR, μg/mL	10	8.4	13	4.3	13	5.1	5.1
LLLR, mol/L	4.0×10^{-5}	3.3×10^{-5}	5.0×10^{-5}	1.7×10^{-5}	5.0×10^{-5}	2.0×10^{-5}	2.0×10^{-5}

used no significant differences were observed between imprinted and non-imprinted versions. The TOP additive was not found relevant in the present case.

The use of PVC implicated the presence of a plasticizer material, for which different kinds of plasticizers were tested: oNFOE, bEHS and DBP, corresponding to ISEs IV, VII and VIII. This test was only conducted for SDZ and suggested that higher dielectric constant mediating solvents favored the potentiometric response produced by the selective membrane. oNFOE was found the best plasticizer (Fig. 4B), with the corresponding electrodes displaying the lower limits of detection.

The effect of additive in PVC-based membranes was also tested (Fig. 4C) and, as in the previous studies, this test was only conducted for SDZ membranes. TOP, TOABr and TPB were used for this purpose, corresponding to ISEs V, IX and X. Typically, the addition of cationic compounds of lipophilic nature to potentiometric sensors reduces the anionic interference and lowers the electrical resistance of the membranes [41]. TOABr was selected for this purpose (Table 1). The corresponding sensors showed Nernstian responses but the limit of detection was higher than that obtained for the equivalent membrane without additive. When TOP was used as additive instead, no improvements were observed. This compound is not ionized within the membrane but is able to establish hydrogen-bonds to the target analyte. The benefits provided by a positively charged additive were confirmed by using an additive of opposite charge in the membrane. The observed analytical response in this case was hindered, which was most probably a result of the anionic exclusion role played by the membrane.

The amount of ISG particles used as ionophore in the PVC membranes was also tested from 5 to 30 mg. The results pointed out that 15 mg was required to achieve higher slopes. Lower and higher values displayed narrower linear ranges and higher limits of detection.

3.4. Response time and lifetime

The time required to achieve a steady potential response (±3 mV) using the proposed sensors in 10^{-6} to 10^{-4} M SDZ or SMX solutions with a rapid 10-fold increase in concentration was <15 s. Replicate calibrations for each sensor indicated low potential drift, long-term stability and negligible change in the response of the sensors. The sensors were stored and conditioned in 10^{-6} M SDZ or SMX solution of pH 5.0. With all sensors examined, the detection limits, response times, linear ranges and calibration slopes were reproducible within ±3% of their original values over a period of at least 1 week for ISG sensing surface or at least 2 months for PVC membranes.

3.5. Sensor selectivity

Selectivity tests were conducted for ISEs I, III and IV, with the purpose of observing the effect of the sol–gel matrix and the imprint effect. ISE IX was also included as it was the best electrode found in this work. Selectivity was assessed by means of potentiometric selectivity coefficients (K^{POT}), calculated by the mixed solution method (MSM). The logarithmic values of log K^{POT} were indicated in Fig. 4. These were calculated by means of Eq. (3),

$$K^{POT}_{SPH,J} = a_{SPH}/\left(a_J\right)^{z_{SPH}/z_J} \quad (3)$$

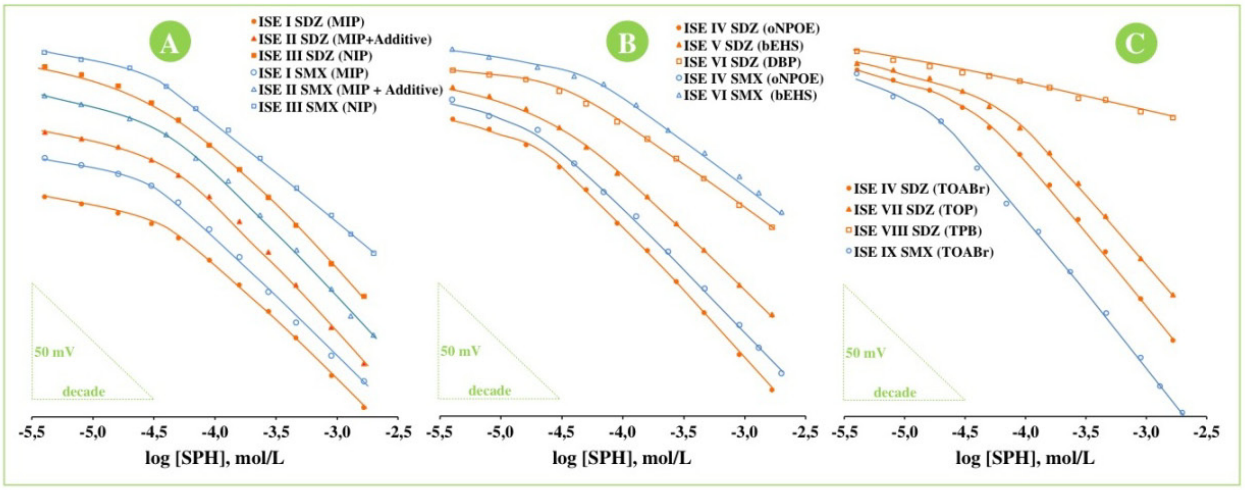

Fig. 4. Calibration plots of sol–gel and PVC membranes (A) with different plasticizers (B) and additives (C).

where a_J is 5.0×10^{-3} M of interfering species, Z the ionic charges of main and interfering ions and a_{SPH} the intersection of the extrapolated linear portions of the plot emf versus the logarithm of SPH concentration. In general, the values of log K^{POT} showed the degree of preferential interaction for SPH over different ionic species. Compounds that are commonly present in pharmaceuticals, biological or water samples were considered for this purpose.

A wide range of inorganic anions was tested, including carbonate, chloride, fluoride, hydrogencarbonate, nitrate, nitrite, phosphate, cyanide, sulfate, borate, persulphate, citrate, tartrate, and salicylate. These included species whose control in water is obliged and that may be present in biological fluids and pharmaceuticals. The obtained values were indicated in Table 3. In general, the results showed negligible interference in both SDZ and SMX selective electrodes, with log K^{POT} values lying within a narrow range: −0.20 to −3.9 and −0.29 to −3.8 for SDZ and SMX selective electrodes, respectively.

Several organic compounds were also selected, such as the antibiotics tetracycline, ciprofloxacin, others SPHs, namely sulphamerazine and sulphatiazole, dopamine, glucose, galactose, cysteine and creatinine. The selectivity of the ISEs against these species were evaluated only for SDZ because this was the only drug marketed alone (without trimethoprim) for human use. Negligible interference was also observed for all electrodes.

Overall, there was no evident difference between the observed behaviors of ISG or PVC based membrane electrodes.

3.6. Monitoring SDZ and SMX in serum, urine, drugs and aquaculture waters

To assess the applicability of the proposed method to real samples, commercial drug (Broncodiazine® syrup, Vitoria, Portugal), serum and aquaculture waters were analyzed for SDZ and SMX contents. Only SDZ was tested for pharmaceuticals and biological samples, given that the use of SMX is not that common in this context or is not done alone. The real samples were spiked when no SPH was found. The analysis was carried out after calibration procedure. To assess the reliability of the results, each sample was analyzed in triplicate.

The potentiometric results are given in Table 4. In general, the proposed sensors for SDZ and SMX operated suitably under laboratory conditions. The precision of the analysis of both SPHs were good and similar (Table 4). The relative standard deviations were always below 5%; for SDZ and SMX electrodes these results ranged from 0.6 to 1.4% and from 0.2 to 3.9%. A satisfactory agreement was obtained between found and claimed amounts. The mean recovery of the amounts taken in the proposed method ranged from 99.7 to 103.7, 93.2 to 94.8 and 97.4 to 105.6% for pharmaceuticals, serum samples and aquaculture waters, respectively. The corresponding relative errors varied from −0.3 to 3.7, −6.8 to −5.2 and 0.0 to 5.7%.

The *t*-student test confirmed that there were no significant differences between the means of claimed and potentiometric amounts of both SPHs (Table 4). The calculated t values (p) were 0.73 and 0.77 for SDZ and SMZ. In both cases, p values were below the tabulated critical t (2.36 and 2.20, respectively) for a 95% confidence level, demonstrating that there are no significant differences between the claimed and the potentiometric amounts.

4. Conclusions

ISG technique was employed to produce SDZ and SMX host-tailored sensors for potentiometric transduction. The sol–gel imprinted layer was used first as sensory surface and compared later to PVC-based sensors carrying the same imprinted material as ionophore. The best performance was obtained by ISG particles dispersed in PVC. A cationic additive offered some improvements in terms of analytical performance, although it was not essential to reach a near-Nernstian response. The sensors were found appropriate for a practical application to real samples.

Advantages of these sensors include the simplicity in designing, low cost, quick response, reproducible and repeatable data, low limit of detection and good selectivity. It also offers the possibility of high analytical trough output by adaptation to flow injection conditions. The proposed method is simple, cheap, precise, accurate and inexpensive regarding reagent consumption and equipment involved.

Table 3
Average potentiometric selectivity coefficients of SDZ and SMX membrane sensors.

Interfering species	K_{SPH-J}^{POT}							
	ISE I	ISE III	ISE IV	ISE IX	ISE I	ISE III	ISE IV	ISE IX
	SDZ	SDZ	SDZ	SDZ	SMX	SMX	SMX	SMX
CO_3^{2-}	−1.5 ± 0.16	−1.1 ± 0.065	−1.6 ± 0.067	−1.7 ± 0.044	−1.6 ± 0.059	−1.3 ± 0.032	−1.5 ± 0.071	−1.6 ± 0.034
Cl^-	−1.7 ± 0.078	−1.8 ± 0.024	−1.9 ± 0.045	−1.9 ± 0.077	−1.9 ± 0.022	−1.8 ± 0.054	−1.9 ± 0.063	−1.9 ± 0.046
F^-	−1.5 ± 0.11	−1.5 ± 0.045	−1.7 ± 0.034	−1.7 ± 0.075	−1.7 ± 0.027	−1.6 ± 0.075	−1.8 ± 0.034	−1.9 ± 0.057
HCO_3^-	−1.6 ± 0.026	−1.3 ± 0.092	−1.7 ± 0.096	−1.7 ± 0.062	−1.8 ± 0.024	−1.7 ± 0.056	−1.7 ± 0.042	−1.7 ± 0.043
NO_3^-	−0.95 ± 0.037	−0.85 ± 0.14	−1.3 ± 0.12	−1.4 ± 0.065	−1.3 ± 0.043	−1.2 ± 0.087	−1.4 ± 0.012	−1.6 ± 0.069
NO_2^-	−1.02 ± 0.10	−0.99 ± 0.064	−1.4 ± 0.076	−1.5 ± 0.041	−1.5 ± 0.037	−1.3 ± 0.053	−1.4 ± 0.026	−1.8 ± 0.071
PO_4^{3-}	−3.9 ± 0.025	−3.8 ± 0.083	−3.7 ± 0.046	−3.4 ± 0.091	−3.8 ± 0.012	−3.6 ± 0.013	−3.8 ± 0.069	−3.4 ± 0.039
CN^-	−1.2 ± 0.043	−1.3 ± 0.044	−1.2 ± 0.065	−1.2 ± 0.068	−1.3 ± 0.019	−1.2 ± 0.027	−1.0 ± 0.091	−1.2 ± 0.035
SO_4^{2-}	−0.51 ± 0.072	−3.3 ± 0.036	−3.2 ± 0.032	−3.5 ± 0.054	−3.2 ± 0.023	−3.4 ± 0.017	−3.5 ± 0.010	−3.4 ± 0.051
Borate	−2.9 ± 0.017	−3.0 ± 0.015	−2.9 ± 0.086	−3.0 ± 0.043	−3.0 ± 0.034	−3.1 ± 0.012	−2.9 ± 0.018	−3.0 ± 0.024
Persulphate	−0.41 ± 0.95	−0.37 ± 0.098	−0.45 ± 0.12	−0.32 ± 0.10	−0.42 ± 0.11	−0.38 ± 0.058	−0.37 ± 0.011	−0.29 ± 0.022
Citrate	−2.8 ± 0.063	−2.6 ± 0.033	−2.9 ± 0.034	−2.5 ± 0.067	−2.2 ± 0.032	−2.7 ± 0.026	−2.5 ± 0.11	−2.7 ± 0.014
Tartrate	−2.7 ± 0.10	−2.5 ± 0.053	−2.4 ± 0.039	−2.7 ± 0.073	−2.5 ± 0.028	−2.7 ± 0.031	−2.5 ± 0.048	−2.5 ± 0.043
Salicylate	−1.7 ± 0.071	−1.9 ± 0.032	−1.7 ± 0.052	−1.7 ± 0.031	−1.7 ± 0.014	−1.7 ± 0.039	−1.9 ± 0.026	−1.6 ± 0.087
Ciprofloxacin	−0.70 ± 0.025	−0.30 ± 0.023	−0.20 ± 0.026	−0.40 ± 0.013	–	–	–	–
Creatinine	−1.0 ± 0.063	−0.90 ± 0.041	−0.60 ± 0.057	−0.89 ± 0.022	–	–	–	–
Cysteine	−1.2 ± 0.032	−0.45 ± 0.049	−0.52 ± 0.085	−0.30 ± 0.037	–	–	–	–
Dopamine	−1.1 ± 0.044	−0.65 ± 0.078	−0.50 ± 0.091	−1.7 ± 0.093	–	–	–	–
Galactose	−1.0 ± 0.034	−0.90 ± 0.066	−0.30 ± 0.034	−1.5 ± 0.083	–	–	–	–
Glucose	−1.3 ± 0.012	−1.5 ± 0.071	−1.4 ± 0.043	−1.5 ± 0.042	–	–	–	–
Sulphamerazine	−1.1 ± 0.038	−1.5 ± 0.014	−0.70 ± 0.047	−0.50 ± 0.013	–	–	–	–
Sulfathiazole	−0.50 ± 0.027	−0.81 ± 0.013	−0.50 ± 0.039	−0.53 ± 0.045	–	–	–	–
Tetracycline	−0.90 ± 0.033	−0.90 ± 0.046	−0.58 ± 0.029	−0.30 ± 0.038	–	–	–	–

Table 4
Potentiometric determination of SDZ and SMX in commercial drugs, biological samples and aquaculture water.

Sample	Drug	Concentration (μg/mL)		Relative	Recovery
		Claimed	Found	Error (%)	(%)
Broncodiazine®	SDZ	25	25.9 ± 0.2	3.7	103.7
	SDZ	50	49.9 ± 1.8	−0.3	99.7
serum	SDZ	50	47.4 ± 2.1	−5.2	94.8
	SDZ	100	93.2 ± 3.3	−6.8	93.2
Aquaculture water	SDZ	35	34.7 ± 0.14	0.8	99.2
(Sample 1)	SDZ	100	105.9 ± 4.20	5.7	105.7
Aquaculture water	SDZ	35	35.2 ± 0.49	0.6	100.6
(Sample 2)	SDZ	100	101.5 ± 0.20	1.2	101.2
Aquaculture water	SDZ	35	35.4 ± 0.71	1.2	101.2
(Sample 3)	SDZ	100	100.2 ± 1.61	0	100
Aquaculture water	SDZ	35	34.1 ± 0.62	2.6	97.4
(Sample 4)	SDZ	100	98.5 ± 2.97	1.8	98.2
Aquaculture water	SMX	35	35.5 ± 0.21	1.5	101.5
(Sample 5)	SMX	100	99.2 ± 0.58	1.1	98.9
Aquaculture water	SMX	35	36.3 ± 0.28	3.7	103.7
(Sample 6)	SMX	100	98.0 ± 1.34	2.2	97.8
Aquaculture water	SMX	35	36.0 ± 0.21	3.0	103
(Sample 7)	SMX	100	100.6 ± 0.59	0.3	100.3
Aquaculture water	SMX	35	36.2 ± 0.35	3.4	103.4
(Sample 8)	SMX	100	99.4 ± 1.36	0.8	99.2

The wastewaters generated by this experimental work are of small concern to the environment regarding its volume and composition.

Acronyms

APTMS	3-aminopropyltrimethoxysilane
bEHS	bis(2-ethylhexyl)sebacate
C_{free}	free analytical concentration
DBP	dibutyl phthalate
DPTS	diphenyldimethoxysilan
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ISEs	ion-selective electrodes
ISG	imprinted sol–gel
K_d	dissociation constant
K^{POT}	potentiometric selectivity coefficients
MIP	moleculary imprinted polymer
MSM	mixed solution method
NISG	non-imprinted sol–gel
oNPOE	o-nitrophenyl octyl ether
PABA	*p*-aminobenzoic acid
Q	binding capacity
SDZ	sulfadiazine
SEM	scanning electron microscope
SMX	sulfamethoxazole
SPH	sulphonamide
TEOS	tetraethyl orthosilicate
THF	tetrahydrofuran
TOABr	tetraoctylammonium bromide (TOABr)
TOP	p-*tert*-octylphenol
TPB	tetraphenylborate

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Plataforma potenciométrica baseada em anticorpos plásticos e em LTCC para a determinação biparamétrica de compostos orgânicos: aplicação a Sulfamethoxazole e Trimethoprim

A impressão molecular numa estrutura polimérica pode ser realizada com recurso a várias técnicas, sendo a de impressão em superfície aquela que origina estruturas mais regulares, com cavidades de reconhecimento mais densas. Como suporte sólido para efectuar essa impressão controlada foram escolhidas esferas de sílica. Neste trabalho combinaram-se as técnicas de sol-gel e de "self-assembled monolayers". A molécula escolhida para este trabalho foi a SMX, uma das SAs anteriormente estudadas. As membranas foram preparadas com diferentes composições, tendo por objectivo avaliar o efeito do aditivo e da impressão na resposta potenciométrica.

Verificou-se, neste trabalho, que os eléctrodos apresentavam uma maior capacidade de reconhecimento molecular do que quando se utilizaram os materiais de sol-gel no capítulo anterior. Para além disso, os limites de detecção foram bastante inferiores aos encontrados nesse mesmo estudo.

Tendo em vista a aplicação deste trabalho em estações de aquacultura, os eléctrodos que apresentavam melhores características de resposta foram incorporados em sistemas de fluxo. Neste estudo, e uma vez que cada vez mais o tamanho dos dispositivos/sistemas é um factor determinante na escolha de um método analítico, recorreu-se a sistemas de microfluídica. Para este efeito, foram usados dispositivos LTCC em multicamada. Cada camada foi impressa com o desenho adequado para formação dos canais de passagem da solução e de suporte sólido das membranas dos eléctrodos após a sua junção e cozedura. Considerando que o SMX é comummente associado a TMP, optou-se pela inclusão de dois detectores no dispositivo de microfluídica permitindo assim uma determinação multiparamétrica. A membrana sensora a TMP era, também baseada em polímeros de impressão molecular.

A montagem de um dispositivo de microfluídica que permitisse a leitura simultânea de dois compostos orgânicos só foi possível depois de efectuar estudos preliminares, incluindo-se aqui a incorporação das membranas selectivas a SMX e a TMP em sistemas de análise por injecção em fluxo e a construção de dispositivos para a leitura individual de cada analíto. Todos estes sistemas foram amplamente testados relativamente aos vários parâmetros de operacionalidade e aplicados a amostras de água de estações de aquacultura.

Encontrados os sistemas sensores e as montagens em linha adequados à monitorização local de SAs nas estações de aquacultura, faltava apenas conseguir atingir os limites de detecção necessários à determinação directa de SAs no meio aquático dessas mesmas estações. Apesar que não haver qualquer legislação neste contexto, a concentração nas imediações das estações é previsivelmente muito inferior àquela que pode ser encontrada nos tanques dos viveiros.

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Novel LTCC-potentiometric microfluidic device for biparametric analysis of organic compounds carrying plastic antibodies as ionophores: Application to sulfamethoxazole and trimethoprim

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ABSTRACT

Monitoring organic environmental contaminants is of crucial importance to ensure public health. This requires simple, portable and robust devices to carry out on-site analysis. For this purpose, a low-temperature co-fired ceramics (LTCC) microfluidic potentiometric device (LTCC/μPOT) was developed for the first time for an organic compound: sulfamethoxazole (SMX).

Sensory materials relied on newly designed plastic antibodies. Sol–gel, self-assembling monolayer and molecular-imprinting techniques were merged for this purpose. Silica beads were amine-modified and linked to SMX via glutaraldehyde modification. Condensation polymerization was conducted around SMX to fill the vacant spaces. SMX was removed after, leaving behind imprinted sites of complementary shape. The obtained particles were used as ionophores in plasticized PVC membranes. The most suitable membrane composition was selected in steady-state assays. Its suitability to flow analysis was verified in flow-injection studies with regular tubular electrodes.

The LTCC/μPOT device integrated a bidimensional mixer, an embedded reference electrode based on Ag/AgCl and an Ag-based contact screen-printed under a micromachined cavity of 600 μm depth. The sensing membranes were deposited over this contact and acted as indicating electrodes. Under optimum conditions, the SMX sensor displayed slopes of about −58.7 mV/decade in a range from 12.7 to 250 μg/mL, providing a detection limit of 3.85 μg/mL and a sampling throughput of 36 samples/h with a reagent consumption of 3.3 mL per sample.

The system was adjusted later to multiple analyte detection by including a second potentiometric cell on the LTCC/μPOT device. No additional reference electrode was required. This concept was applied to Trimethoprim (TMP), always administered concomitantly with sulphonamide drugs, and tested in fish-farming waters. The biparametric microanalyzer displayed Nernstian behaviour, with average slopes −54.7 (SMX) and +57.8 (TMP) mV/decade. To demonstrate the microanalyzer capabilities for real applications, it was successfully applied to single and simultaneous determination of SMX and TMP in aquaculture waters.

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1. Introduction

The (bio)analytical chemistry field needs miniature and portable analytical devices for on-site control of several compounds. In this context, flow methods with potentiometric detection are an advantageous combination. Simple flow assemblies are capable of automatic sample collection and carry out most classical analytical procedures in-line, while potentiometric detectors are easily adapted to flowing media and small size sensory surfaces. This combination in microsize dimensions may produce a lab-on-a-chip device.

The introduction of micropotentiometric (μPOT) systems in analytical procedures relies mostly on solid conductive materials coated by ion-selective membranes. *Nernstian* responses are obtained when the analyte is the only major ion that is selectively transferred across the interface between sample and membrane

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phases. In general, the selectivity is achieved by doping the membranes with a hydrophobic ion (ionic site) and a hydrophobic ligand (ionophore or neutral/charged carrier) that selectively and reversibly forms complexes with the analyte (Amemiya, 2007).

A good ligand should selectively bind the analyte and remain in the membrane phase, i.e., it should have binding sites for SMX and low water solubility. Molecularly imprinted polymers (MIPs) are a route to obtain such materials and have been introduced successfully in polymeric membranes (Kamel et al., 2008; Moreira et al., 2010). They are specifically designed to display stereochemical interactions with the analyte and act similarly to natural antibodies. They are prepared by growing a reticulated polymer around the analyte and removing the entrapped molecules later. The vacant sites are complementary to the imprinted analyte and able to rebind it. Many imprinting techniques may be employed to create MIP materials. Surface-imprinting ensures a layer-by-layer control (Moreira et al., 2011), offering a higher number of binding sites *per* contact area.

There are not many ways to introduce a μPOT sensor in a microfluidic device. Basically, it should include a solid conductive contact to apply the selective membrane, a reference electrode and microchannels driving the fluids through the sensory surfaces. Ideally, it should also carry out in-line all necessary operations to turn out a successful candidate to a Lab-on-a-chip device.

The low temperature co-fired ceramic (LTCC) technology is a possible receptor of μPOT sensor. It is a well-established technique for microfabrication (Ibañez-Garcia et al., 2008; Budniewski et al., 2004; Chou et al., 2002; Gongora-Rubio et al., 2003; Golonka et al., 2003), producing three-dimensional (3D) structures using a multi-layer approach. The LTCC devices offer good electrical and mechanical properties, as well as high reliability and stability. They may integrate in a single unit all steps associated to an analytical procedure, being of special interest to micro-fluidic applications (Golonka et al., 2006; Lopes et al., 2007; Khanna et al., 2005; Patel et al., 2006; Bergstedt and Persson, 2002; Muller et al., 2005; Zawada, 2006) and lab-on-a-chip devices.

The integration of μPOT sensors on LTCC platforms has been successfully proven in the past, although only a few works are reported. These include nitrate and chloride determinations (Ibañez-Garcia et al., 2006, 2010). However, no organic compounds have been yet considered. One of the most critical steps regarding the membrane integration to the ceramic device resides on its adhesion to the solid contact screen-printed on the ceramic surface. Therefore, additional efforts should be performed to obtain an appropriate structure to place the membrane. Moreover, these devices may last for a long time if a deteriorated selective membrane may be replaced by a new one.

In this work, the construction and evaluation of a compact LTCC/μPOT system is proposed for the on-site determination of an organic environmental contaminant. Sulfamethoxazole (SMX) has been selected as target analyte because of its environmental spread (along with other antibiotics) is becoming of great danger to public health. It has been used for long as veterinary/human antibiotic and has been found in waters, coming from municipal wastewaters and fish farming practices (Hirsch et al., 1999). A new MIP-based ligand was designed to act as SMX ionophore, as no previous MIP sensor was reported earlier. Silica beads have been modified for this purpose by surface imprinting and included in polymeric selective membranes. The optimized composition was selected after steady-state assays and dropped on tubular electrodes (for testing the membrane suitability to flow-readings) and on LTCC devices (to set operational microfluidic conditions).

Since SMX is always used in conjunction with trimethoprim (TMP), the setup was adjusted to allow the simultaneous determination of SMX and TMP. A previously reported MIP-based sensor for TMP was used for this purpose.

2. Experimental

2.1. Apparatus

EMF in steady-state assays was measured by a Crison pH-meter GLP 21 (±0.1 mV sensitivity), at room temperature, and under constant stirring. The output signal was transferred to a home-made commutation unit with six ways out. The reference electrode was a Crison, 5240, of double-junction.

The flow setup consisted of a peristaltic pump (Minipuls 3, Gilson, WI) fitted with Tygon tubing (0.64 and 1.14 mm i.d.) purchased by Ismatec (Zurich, Switzerland). A six-port distribution valve (Hamilton, MVP, Reno, NV) of variable injection volumes was used. The several components were joined by PTFE tubing (Omnifit, Teflon, 0.8 mm i.d.). The potentiometer was coupled to acquisition/recording signal software purchased to TMI (Barcelona, Spain).

Green tapes for LTCC were machined by a laser machine (Protolaser 200, LPKF, Laser & Electronics, Germany). A thermo-compression press (Talleres Francisco Camps, Granollers, Spain) consisting of two 250 × 250 mm heating plates, whose temperature was controlled by means of a probe and a resistance was used for ceramic layers lamination. The devices were sintered in a programmable box furnace (Carbolite CBCWF11/23P16, Afora, Spain) following the thermal profile recommended by the ceramics manufacturer.

Infrared spectra were collected by a Nicolet iS10 FTIR spectrometer (Thermo Scientific), coupled to an ATR (Attenuated Total Reflectance) sampling accessory of diamond contact crystal from Nicolet.

2.2. Reagents/materials and solutions

MilliQ water was used for reagents preparation and as carrier solution. All reagents were of analytical grade. SMX, TMP, D,L-lactic acid 85%, 3-aminophenylboronic acid (APBA), tris(hydroxymethyl)aminomethane (TRIS), aminopropyl silica (particle size 15–40 μm, mean pore size 75 Å), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), tetraoctylammonium bromide (TOABr) and sodium sulphate were from Sigma. *o*-Nitrophenyloctyl ether (*o*NPOE), poly (vinyl chloride) (PVC) of high molecular weight, 3-aminopropyltrimethoxysilane (APTMS), sodium persulphate (SPS), glutaraldehyde (GLU) and oxalic acid were obtained by Fluka and tetrahydrofuran (THF) by Riedel-deHäen.

The LTCC device was fabricated with green tapes (951 PX, thickness before sintering: 254 μm) and silver based pastes (6142, 6141 and 6146), both from Du Pont.

Stock solutions of SMX and TMP were prepared in water. TMP was previously solubilised in D,L-lactic acid 85% (Sigma). Working standards were prepared by single dilution of the stock solutions in HEPES buffer (steady-state assays) or water (flow assays). SMX solutions ranged from 5×10^{-5} to 1×10^{-3} M and for TMP from 2×10^{-6} to 2×10^{-5} M. Solutions with both SMX and TMP (for biparametric readings) were prepared similarly.

All solutions were measured in 1×10^{-2} M HEPES buffer, of pH 5.4. LTCC assays required the addition of 1×10^{-4} M sodium sulphate to ensure a stable baseline signal.

2.3. LTCC device fabrication

The design of the device consisted on nine layers that once overlapped provided the three-dimensional structure required for this application. The fabrication process regarding LTCC-based

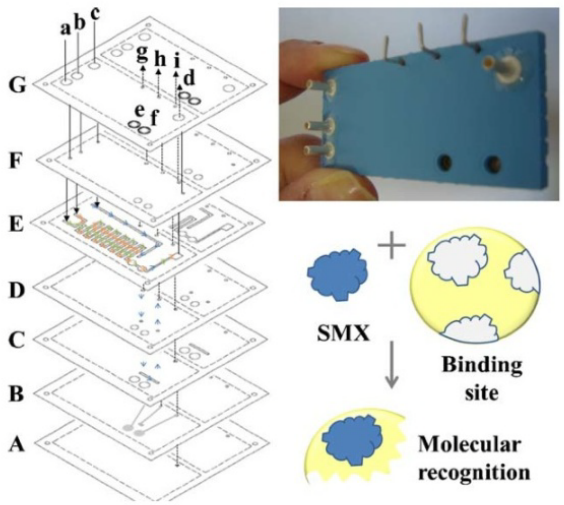

Fig. 1. Biparametric LTCC device layers (in the left; dotted lines represent the electrical contact to the external set-up and solid lines the hydraulic connections; where (a) conditioning solution inlet; (b) carrier solution; (c) KCl inlet; (d) outlet; (e) and (f) cavities for membrane deposition; (g, h) and (i) electrical connections to the external set up). Photograph of the corresponding device (top, right) and SMX recognition with the synthesized ligand (down, right).

devices is described in detail elsewhere (Ibañez-García et al., 2008). Fig. 1 presents the microanalyzer developed (2 mm height × 24 mm deep × 53 mm long), including the individual layers that integrates it. Circuit CAM software based on Windows was used to transfer the CAD layouts to the laser machine. Holes, channels, cavities and printing conductors were then mechanized on the green LTCC tapes as designed. Silver pastes were screen-printed on the correct places, to act as conductive support of all electrodes. Lamination and sintering was conducted as previously described.

The device included two liquid inlets that converged in a point downstream before getting into a bidimensional mixer. The conductive pads and their corresponding cavities for membrane deposition were defined after the mixer. The conductive pad regarding the reference electrode was defined downstream the working electrodes. The membranes were applied drop-by-drop inside the corresponding cavity, over the conductive material support, and left to dry at room temperature. Finally, hydraulic and electronic connections between the device and the external set-up were established.

2.4. Flow set-up

The conventional flow assembly consisted of a double-channel set-up, where a HEPES buffer carrier merged with a water channel transporting the injected sample. All details on electrode construction and set-up may be found in Kamel et al. (2009).

The LTCC was set-up for single (SMX or TMP) or biparametric readings (SMX and TMP together, as in Fig. 1). The carrier was always HEPES buffer with sodium sulphate and merged inside the microfluidic channels with a water channel receiving the injected samples. An auxiliary channel was used to flow 0.1 M KCl through the reference electrode to ensure its constant EMF. Single-analyte readings used a similar setup to that in Fig. 1, with only one solid contact for the selective membrane deposition.

2.5. Synthesis of the novel SMX ligand

Surface imprinting was selected to control each modification step and promote a higher number of effective binding sites. Sol–gel, self-assembled monolayer and molecular-imprint concepts were merged in the overall procedure (Fig. 2).

About 0.1 g of silica beads (15–40 μm diameter and 75 Å pore size) were amine-modified by dipping in 10% (w/v) APTMS prepared in methanol (Fig. 2) for 1 h and washing after that (ethanol first and water later). The beads were then incubated in 1% GLU prepared in 0.01 M HEPES for 12 h at room temperature and washed after with water. The amine groups on the silica beads underwent a nucleophilic addition reaction with the aldehyde groups in GLU, forming an imine bond (–C=N–).

Then, they were dipped in 1.0 mg/mL SMX in HEPES, transferred to 1 M Tris, and thoroughly washed with water. Free-aldehyde groups from GLU on the outer layer were bound to SMX by means of the same nucleophilic addition reaction with the amine group in SMX. Tris was added after to block unreacted aldehyde functions.

Finally, the beads were dipped for 1 h in 0.05 M APBA solution prepared in water, to arrange the monomers around SMX and the amine/hydroxyl groups on the outer layer. A volume of 1 mL of 0.03 M SPS was added to initiate and carry out the polymerization for 1 h. Finally, the beads were washed again with deionised water, incubated in 1 M oxalic acid, and washed again with water. The vacant sites enabled complementary interactions with the imprinted molecule.

Non-imprinted materials (NIPs) were obtained by a similar process, where no SMX was present.

2.6. SMX and TMP selective membranes

SMX selective membranes were prepared by mixing 210 mg of PVC, 350 mg of *o*NPOE and 15 mg of modified silica beads (MIP or NIP). An amount of 0.2 mg of additive (TOABr) was also added to some membranes (Table 1). All these were weighted and dissolved in THF. The mixture was stirred until the PVC was well moistened. A successful TMP MIP-based sensor had already been prepared before, and was used as described by Rebelo et al. (2011).

The liquid membranes were applied over solid conductive supports and let dry for 24 h. These conductive supports were included in electrodes of conventional or tubular shape (Kamel et al., 2009) or in the microfluidic device.

2.7. Potentiometric procedures

All potentiometric measurements were carried out at room temperature. The electrochemical operating characteristics of the SMX and TMP selective electrodes were assessed after calibration curves (Buck and Cosofret, 1993) following IUPAC recommendations (Buck and Lindner, 1994). These were conducted for increasing drug concentrations in HEPES buffer 1×10^{-3} M, reaching the detector in either steady-state or flow conditions. This buffer also had 1×10^{-4} sodium sulphate in assays with the LTCC device. Potential readings were recorded after stabilization to ±0.2 mV and plotted as a function of logarithm SMX or/and TMP concentration.

Selectivity studies followed the matched potential method for SMX assays in steady-state. The anions PO_4^{2-}, CH_3COO^-, BO_3^{3-}, CN^-, SO_4^{2-}, F^-, CO_3^{2-}, Cl^-, HCO_3^-, NO_3^-, NO_2^- and tartrate were tested for a concentration of 1×10^{-4} M. In flow conditions, selectivity was assessed by recovery assays. For this purpose, a preset drug concentration (2×10^{-4} M SMX and/or 5×10^{-6} M TMP) was spiked with different anions (NO_3^-, NO_2^-, Cl^-, HCO_3^- or SMX) or cations (K^+, $NH4^+$, Na^+ or TMP) until the maximum admitted level of each ion in water was found (excluding the drugs, to which no limit is established).

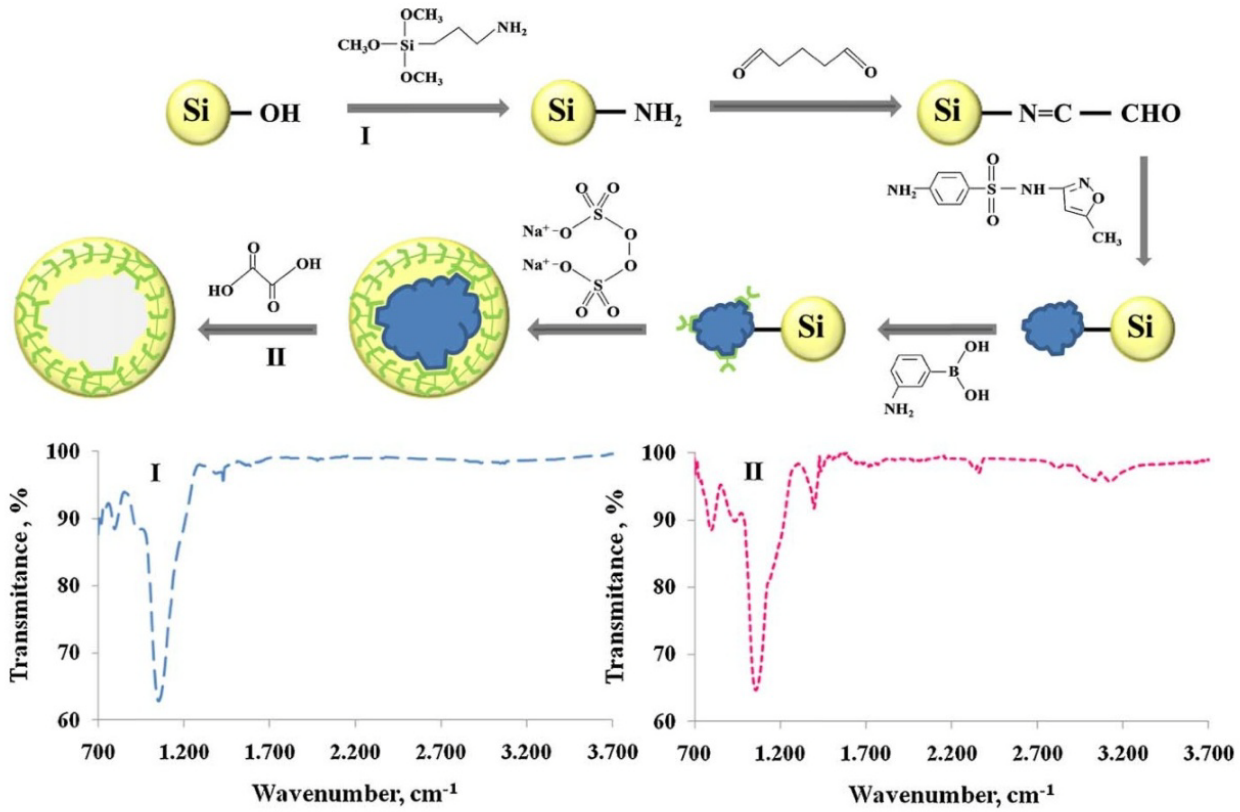

Fig. 2. Design of the plastic antibodies over silica beads and the FTIR spectra of starting and final products.

2.8. Sample analysis

Samples of aquaculture water were collected from different fish farming units in sweet waters from the north region of Portugal. The waters were not contaminated with the drugs and analysed with regard to their major organic and inorganic composition. No cation/ion was found in interfering concentrations, for which no special care was taken prior to potentiometric analysis. After, the water was spiked between 51–127 μg/mL SMX and 1.5–5.8 μg/mL TMP.

3. Results and discussions

3.1. Design control by FTIR

FTIR assays were made in all steps to control the chemical modification of the beads. The spectra of non-modified silica beads and imprinted ones are indicated in Fig. 2. Both showed significant bands at about 900 and 1100 cm^{-1}, revealing the absorption bands from the Si–OH and Si–O–Si vibrations, respectively.

Table 1
Main analytical features of all SMX and TMP sensors.

Analytical parameters	Steady-state					Flow injection			
	SMX					Tubular electrode		LTCC	
	ISE I	ISE II	ISE III	ISE IV	ISE V	ISE I	TMP	ISE I	TMP
Sensing material	MIP/SMX	MIP/SMX	NIP	NIP	–	MIP/SMX	–	MIP/SMX	–
Additive	*TOABr*	–	*TOABr*	–	*TOABr*	*TOABr*	–	*TOABr*	–
Plasticizer	*o*NPOE	*o*NPOE	*o*NPOE	*o*NPOE	*o*NPOE	*o*NPOE	–	*o*NPOE	–
Slope, mV/decade	−56.1 ± 0.2	−46.8 ± 2.0	−43.6 ± 0.7	−31.0 ± 1.7	−34.0 ± 0.8	−61.7 ± 1.4	58.7 ± 0.5	−58.7 ± 0.2	61.2 ± 0.2
R^2 (n = 3)	0.992	0.995	0.998	0.996	0.994	0.999	0.998	0.999	0.997
LLLR, μg/mL	5.49	16.2	18.9	34.9	35.4	12.7	0.290	12.7	0.580
LOD, μg/mL	1.66	4.92	5.73	10.6	10.7	3.85	0.0879	3.85	0.176
Cv_w (%)	0.41	4.26	1.50	5.67	2.26	2.28	0.815	0.358	0.370
Accuracy, %	5.11	20.9	26.3	47.6	42.5	4.29	0.778	3.31	3.45
Within-day variability, %	2.77	0.802	1.53	0.46	2.19	1.76	0.983	1.25	1.17
Between-day variability, %	2.15	1.17	2.10	0.766	1.73	1.86	1.13	0.979	1.04
Recovery, %	92.7	97.1	95.1	104	101	98.4	99.0	98.4	96.3
Repeatability RSD (%)[a]	–	–	–	–	–	1.21	1.38	1.42	1.29

LOD, limit of detection; LLLR, lower limit of linear range; R^2, squared correlation coefficient; RSD, relative standard deviation; Cvw, reproducibility.
[a] n = 10 for 5×10^{-6} to 5×10^{-4} M.

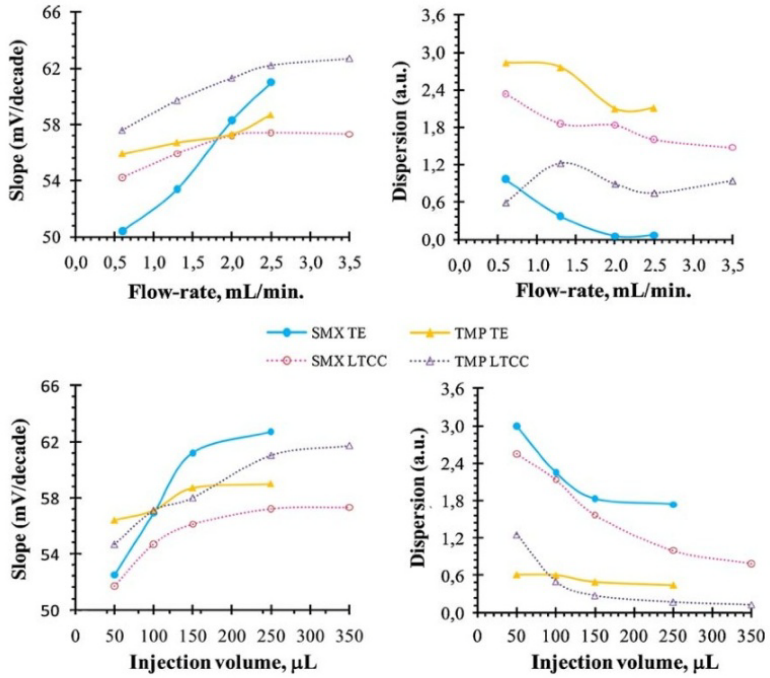

Fig. 3. Slope and dispersion values against injection volume and flow-rate, obtained with the tubular electrodes (TE) and the single LTCC/µPOT device.

The FTIR spectra of the imprinted beads showed specific peaks that confirmed the chemical modification of the Si–O core. The N–H bending of the secondary amines was evidenced by the small intensity peak at 1450 cm^{-1}. Two small peaks centered on 3000 cm^{-1} evidenced the C–H stretch from bonded GLU and APBA monomer molecules.

No chemical differences were observed between the spectra of NIP and MIP beads. This was already expected because FTIR is unable to detect stereochemical differences between equivalent materials. If any chemical difference was observed that would only be attributed to template molecules trapped inside the imprinted cavities.

3.2. Selection of SMX membrane composition

The effect of using MIP materials was tested by preparing electrodes with the imprinted/non-imprinted particles acting as ionophores (Table 1). MIP-based sensors (ISE II) displayed linear responses after 16.2 µg/mL, average anionic slopes of −46.8 mV/decade and detection limits of 4.92 µg/mL. The corresponding NIP sensors were unable to reach such performance, as may be seen in Table 1. The slope was much smaller and linearity was observed only for higher concentration ranges.

MIP/NIP sensors were also prepared with TOABr (ISEs I and III). It acted as cationic additive and improved the permselectivity of the membrane. In general, the additive was fundamental to obtain Nernstian responses with MIP-based sensors (−56.1 mV/decade). Improvements in limit of detection and linear range were also observed. Moreover, these ISEs displayed the best precision and accuracy features of all. The additive alone was not responsible for these improvements, considering the performance of ISEs III and V (Table 1).

The selectivity was assessed by calculating potentiometric selectivity coefficients against many (in)organic ions (Section 2.7). Log K^{POT} were <−1.0 and the relative order of interference was, $BO_3^{3-} < CO_3^{2-} \approx SO_4^{2-} < F^- < HCO_3^- < Cl^- < PO_4^{3-} < CH_3COO^- \approx CN^- < NO_2^- <$ tartrate $< NO_3^-$. As expected, a deviation from the Hofmeister pattern was observed, confirming that the selectivity was not governed by ion-extraction. In general, the MIP-based sensors were more selective than NIP ones for HCO_3^-, CO_3^{2-} and Cl^-. The other tested species did not provide significant differences on the behaviour of the electrodes. The additive generated a slight improvement in the observed selectivity.

The membrane in ISE I was selected for further testing. It was applied first on tubular electrodes to check its suitability for flow experiments and after in the LTCC devices to optimize the performance under microfluidic conditions. TMP based MIP membranes were always evaluated in parallel, aiming a biparametric analysis with the LTCC/µPOT.

3.3. SMX and TMP in tubular electrodes

3.3.1. Selection of flow variables

There are only two variables deserving attention in flow-injection potentiometric systems: injection volume and flow-rate. Injection volumes were varied within 50 and 250 µL while flow-rates ranged 0.6 and 3.5 mL/min. This study was carried out by univariant mode, starting by the injection volume selection. The slope and the signal dispersion were recorded for each condition tested (Fig. 3).

In general terms, increasing the injected volume up to 150 µL lead to higher peaks and higher slopes. The sensitivity increased about 2.5% and 0.5% by changing the injection volume from 50 µL to 150 µL, for SMX and TMP, respectively. Injecting more than 150 µL produced a slight slope increase but reduced sampling-rates and almost doubled reagent/sample costs and volume of generated wastes (Yang et al., 1998). The flow-rate displayed a similar effect because only low values were tested (above 3.5 mL/min flow-rates become unsuitable for microfluidic operations). A volume of 2.5 mL/min was selected as it gave the highest sensitivity.

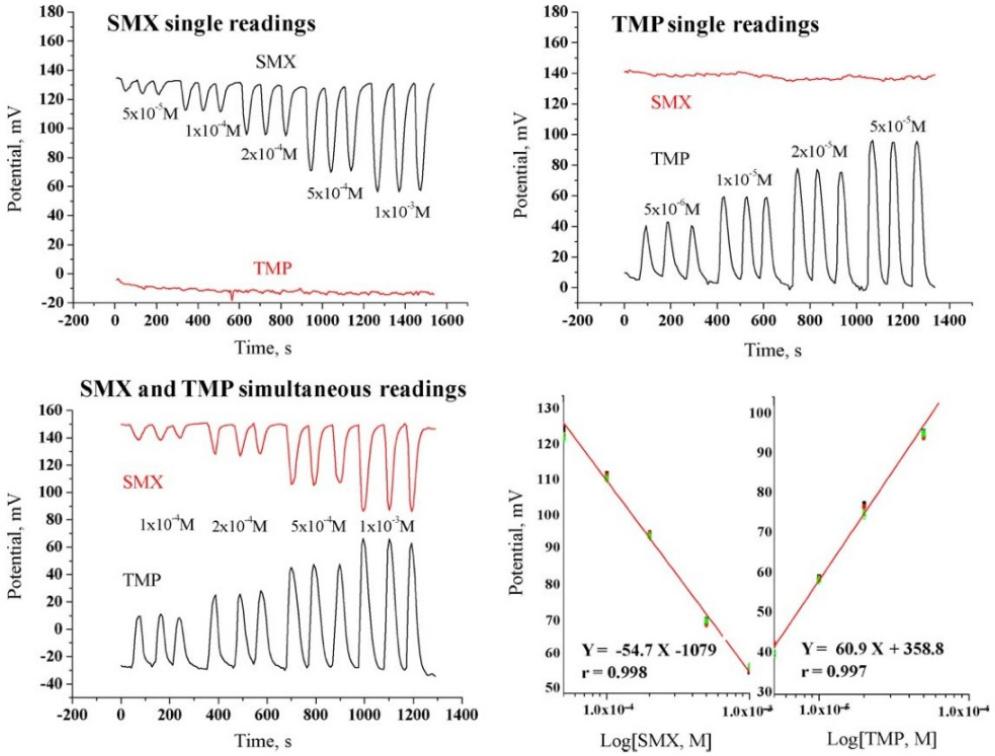

Fig. 4. Calibration of the biparametric LTCC/μPOT device with single and mixed solutions of SMX and TMP.

3.3.2. Analytical performance

SMX and TMP tubular electrodes were tested independently in conventional flow-injection setups operating with the previously established conditions. The results obtained are listed in Table 1. Linear dynamic responses ranged 5×10^{-5}–1×10^{-3} (12.7–253 μg/mL) and 1×10^{-6}–5×10^{-5} (0.290–14.5 μg/mL) M with average slopes of −61.7 and 58.7 mV/decade for SMX and TMP, respectively. The corresponding detection limits were 1.7×10^{-5} and 3.0×10^{-7} M. The repeatability of the analytical signal was assessed by injecting consecutively ten standard solutions. The relative standard deviation (RSD) was always below 1.5% (Table 1).

When SMX was added of other anionic interferences, the signal changed only slightly. NO_3^-, NO_2^- and Cl^- decrease the EMF while SO_4^{2-} increased it. A similar behaviour was observed for TMP. NH_4^+ and K^+ increased slightly the peak while Na^+ decreased it. The relative errors in terms of concentration were always below 5% for interfering concentrations up to 2×10^{-5} M.

3.4. SMX and TMP in single LTCC/μPOT

3.4.1. Set-up and LTCC design

LTCC platforms have 3D structures created by overlapping ceramic layers. Thus, the number of layers, the maximum dimensions of the device, the retraction of the materials in use and the resolution of the equipment involved were carefully considered before designing the device.

To define an optimum structure of the microanalyzer, different configurations of the detection chamber were tested. A wall-jet configuration was evaluated as a first approach. Nevertheless, in this case a brief lifetime was observed. To overcome this issue, a planar configuration, where the solutions were parallel pumped along the surface of the membrane was tried. This configuration was more suitable in terms of electrical noise and membrane adherence to the ceramic surface during longer periods of time.

The detection chamber in layer C (Fig. 1) was made larger than that in layer D. This way, when the membrane solution was applied it flew through the layer below, promoting its retention to the conductive support and avoiding liquid leakages to the latter.

The hydrodynamic parameters (flow-rate and injection volume) were optimized in the LTCC/μPOT device. The optimization was carried out in a multivariate mode, by checking the analytical signals produced after injecting 50, 100, 150, 250 and 350 μL sampling volumes, against carrier streams of 0.5, 1.3, 2.0, 2.5 and 3.5 mL/min flow rates. EMFs were evaluated in terms of dispersion and slope (Fig. 3). The best compromise was found by injecting 250 μL flowing at 2.0 mL/min.

3.4.2. Analytical performance

SMX and TMP LTCC/μPOT devices were tested independently, using the previously selected conditions. The results obtained are listed in Table 1 and indicated similar performance to that observed with the tubular electrodes. SMX μPOT showed a linear dynamic response range from 5×10^{-5} to 1×10^{-3} M (12.7–253 μg/mL) with a slope of −58.7 mV/decade and TMP μPOT from 2×10^{-6} to 5×10^{-5} M (0.580–14.5 μg/mL) with a slope of 61.2 mV/decade. The corresponding detection limits were 1.5×10^{-5} and 6.1×10^{-7} M. The selectivity behaviour was also equivalent to that observed with the corresponding tubular electrodes.

3.5. SMX and TMP in biparametric LTCC/μPOT

The calibration graphs obtained by injecting single solutions on the biparamatric system are shown in Fig. 4(top). Essentially, the analytical performance remained the same as that observed

Table 2
Results obtained by LTCC/μPOT and tubular electrodes (TE) in the determination of SMX and/or TMP in aquaculture waters.

Sample	SMX single readings					TMP single readings					SMX and TMP together[a]				
	Taken (μg/mL)	Found (μg/mL)		Recovery (%)		Taken (μg/mL)	Found (μg/mL)		Recovery (%)		Taken (μg/mL)	Found (μg/mL)		Recovery (%)	
		TE	LTCC	TE	LTCC		TE	LTCC	TE	LTCC		SMX	TMP	SMX	TMP
1	50.7	49.2	49.7	97.2	98.1	1.5	1.4	1.3	94.0	91.4	50.7	51.3	56.3	101.3	96.9
	126.7	131.7	130.2	104.0	102.8	5.8	6.1	5.9	105.0	101.3	126.7	125.8	144.8	99.3	99.7
2	50.7	48.9	50.9	96.6	100.5	1.5	1.4	1.3	94.0	91.5	50.7	47.9	57.9	94.6	99.7
	126.7	131.0	123.2	103.5	97.3	5.8	5.9	6.0	101.6	102.8	126.7	128.1	145.7	101.1	100.3
3	50.7	48.2	48.9	95.1	96.6	1.5	1.4	1.3	94.5	92.2	50.7	50.3	56.9	99.3	97.9
	126.7	126.4	127.0	99.8	100.3	5.8	5.9	5.7	101.3	98.8	126.7	127.1	146.1	100.4	100.6

[a] Biparametric LTCC/μPOT device.

for SMX and TMP solutions in the single LTCC/μPOT plaftorm. The sampling-rates were about 36 injections per hour and Nernstian slopes were observed (Fig. 3).

The signals obtained by injecting mixed solutions are indicated in Fig. 4. The average slope and the detection limits were −54.7 and 57.8 mV/decade, and 3.0×10^{-5} and 1.5×10^{-5} M for SMX and TMP, respectively. While SMX showed a similar behaviour, some changes were observed for TMP readings. This small cross-interference of SMX on TMP was found irrelevant because the accuracy of the EMF was ensured under this condition. Recovery values of single standard solutions lied within 95% and 101%, with relative standard deviations below 4%.

3.6. Analytical application

The flow set-ups were used to determine SMX and TMP in aquaculture waters. This was carried out with all flow set-ups established: tubular electrodes and single/biparametric LTCC/μPOT devices. The obtained results are summarized in Table 2 and correspond to the mean of at least 3 independent determinations. The standard deviations were always below 9%.

A good agreement was found between added and found amounts of the antibiotics. For single readings, the results showed recoveries ranging from 95% to 104% for SMX and from 93% to 104% to TMP. The *t*-student test indicated no statistical differences between the means of found amounts with tubular electrodes and LTCC devices. The *p* value was 0.68 for SMX and 0.38 for TMP, in both cases below the critical value (2.57).

The results obtained by the biparametric LTCC/μPOT devices were comparable to those provided by the tubular electrodes. The mean recoveries ranged from 94% to 105% and 91% to 103% for SMX and TMP, respectively. Considering as null hypothesis that the two configurations agree, a paired two-tail test for 5% level of significance gave a calculated $t(p = 0.56$ and $p = 0.15$, for SMX and TMP, respectively) below the tabulated one (2.57), therefore accepting the null hypothesis.

4. Conclusions

A new SMX ligand was successfully introduced as an ionophore in potentiometric transduction. The imprinting process was successful as the non-imprinted materials were not able to produce a Nernstian response, not even when a cationic lipophilic additive was included in the selective membrane.

A novel biparametric microfluidic potentiometric device based on the LTCC technology is developed and successfully tested to determine simultaneously SMX and TMP. SMX and TMP imprinted materials presented good behaviour in flow conditions, both in conventional tubular electrodes and in LTCC/μPOT. Biparamattic readings were enabled with little cross-interference playing no effect upon the analytical application. A sampling throughput of 36 samples per hour was achieved with the LTCC/μPOT device, producing about 4.2 mL of wastewaters *per* biparametric determination.

The biparametric LTCC/μPOT device is a good approach to a Lab-on-a-chip tool to carry out in-field analysis and simultaneous determination of SMX (or eventually other sulphonamide) and TMP.

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Desenho de anticorpos plásticos em nanopartículas de carbono e sua aplicação para a quantificação de vestígios de sulfamethoxazole no meio aquático com eléctrodos de solução de referência interna construídos numa ponta de pipeta: aplicação *on-site* em águas de aquacultura

Este trabalho teve como objectivos desenvolver biosensores baseados em materiais de baixo custo para uma impressão nanoestruturada e tentar baixar os limites de detecção dos eléctrodos pelo controlo de difusão das membranas sensoras. Assim, optou-se por desenvolver novos materiais sensores por impressão em nanoestruturas de grafite e por construir os eléctrodos com uma configuração convencional, incluindo solução de referência interna. Este estudo foi igualmente aplicado a SMX.

Para a síntese de materiais sensores, optou-se por substituir as esferas de sílica, utilizadas como suporte de impressão no trabalho anterior, por nanoestruturas de carbono presentes na grafite. A técnica de impressão implementada anteriormente foi ajustada a este material, optando-se pela oxidação do carbono nas nanoestruturas. O material resultante foi testado em membranas poliméricas de PVC plastificado e aplicado num suporte sólido condutor à base grafite. Concluiu-se que o comportamento obtido foi semelhante ao encontrado no trabalho anterior, indicando que as esferas de sílica podem ser substituídas por este material de baixo custo, sem qualquer prejuízo do ponto de vista analítico.

Encontrado o sistema sensor adequado, procurou-se baixar os limites de detecção dos eléctrodos construindo unidades convencionais por aplicação da membrana na parte inferior da ponta de uma micropipeta. No sentido de optimizar as condições de difusão na membrana, fizeram-se estudos da composição mais adequada para a solução de referência interna, no que respeita à concentração do analito e ao tipo de espécie interferente. Na presença de uma concentração do analito de $1x10^{-8}$ mol/L e de ião interferente $1x10^{-4}$ mol/L, foi possível baixar o limite de detecção dos eléctrodos para valores de cerca de 10^{-8} mol/L. Esta redução no limite de detecção foi muito significativa, comparativamente aos valores indicados na literatura para eléctrodos selectivos a espécies orgânicas. Concluiu-se assim que o trabalho desenvolvido constitui uma alternativa analítica vantajosa de baixo custo, muito sensível e selectiva para monitorização em campo.

Optimizing potentiometric ionophore and electrode design for environmental on-site control of antibiotic drugs: application to sulfamethoxazole

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Abstract

Potentiometric sensors are typically unable to carry out on-site monitoring of environmental drug contaminants because of their high limits of detection (LODs). Designing a novel ligand material for the target analyte and managing the composition of the internal reference solution have been the strategies employed here to produce for the first time a potentiometric-based direct reading method for an environmental drug contaminant. This concept has been applied to sulfamethoxazole (SMX), one of the many antibiotics used in aquaculture practices that may occur in environmental waters.

The novel ligand has been produced by imprinting SMX on the surface of graphitic carbon nanostructures (CN) < 500 nm. The imprinted carbon nanostructures (ICN) were dispersed in plasticizer and entrapped in a PVC matrix that carried (or not) a small amount of a lipophilic additive. The membrane composition was optimized on solid-contact electrodes, allowing near-Nernstian responses down to 5.2 μg/mL and detecting 1.6 μg/mL. The membranes offered good selectivity against most of the ionic compounds in environmental water.

The best membrane cocktail was applied on the smaller end of a 1000 μL micropipette tip made of polypropylene. The tip was then filled with inner reference solution containing SMX and chlorate (as interfering compound). The corresponding concentrations were studied for 1×10^{-5}-1×10^{-10} and 1×10^{-3}-1×10^{-8} mol/L. The best condition allowed the detection of 5.92 ng/L (or 2.3×10^{-8} mol/L) SMX for a sub-Nernstian slope of -40.3 mV/decade from 5.0×10^{-8} to 2.4×10^{-5} mol/L.

The described sensors were found promising devices for field applications. The good selectivity of the sensory materials together with a carefully selected composition for the inner reference solution allowed LODs near the nanomolar range. Both solid-contact and “pipette tip”-based sensors were successfully applied to the analysis of aquaculture waters.

Keywords: antibiotic contaminants; low detection limit; potentiometry; solid-contact electrodes; plastic antibodies.

1. Introduction

Analytical devices are becoming more and more sophisticated within time, being capable of reaching amazing low detection levels. However, this growing sophistication often increases the price and maintenance expenses around the analytical applications. Moreover, it obstructs portability, which is an emerging requirement among the analytical field. Most controls should be carried on-site and, ideally, via wireless connections.

Considering mainly portability and low-cost, ion-selective electrodes (ISEs) are by far one of the most attractive devices for field applications. The most disseminated way to prepare such detectors is to use a polymeric membrane as active layer consisting of PVC matrix carrying an appropriate ligand and eventually other additional ingredients. Trace level monitoring seems feasible after decreasing the relatively high LODs, typically lying within the micromolar range, and their associated limited selectivity.

The response of most membrane ISEs may be attributed to ion-transfer processes across the membrane/sample solution interfaces. However, the ion flux across the bulk membrane affects the analyte activity at this interface (Amemiya, 2007). The analyte ions leaching from the membrane may reach very high levels at the phase boundary when the concentration of primary ions in the sample solution is very low, which in turn hinders the LODs. Forcing a flux of analyte ions toward the inner solution has been given as a simple way to prevent this leaching and lowering LODs (Sokalski et al., 1997; Amemiya, 2007). Numerous parameters may be adjusted for this purpose, including the composition of the inner electrolyte and the selective membrane (Sokalski et al., 1999B).

The concentration of the primary ion in the inner compartment should be set to a low value, in order to generate a net flux of primary ions towards this side of the membrane. The exact concentration required for this purpose must be studied, because if a high flux is generated, super-Nernstian responses at intermediate concentrations and insensitivity at very low concentrations are expected (Michalska et al., 2004). Moreover, the ion-exchange with interfering ions at the inner membrane side becomes important under this limiting condition, supporting the need of an interfering compound inside the inner compartment (Bakker et al., 2004). This concept has been proven feasible to inorganic ionic species but never tested in organic trace levels.

Lowering LODs is only possible as long as the ISE shows good selectivity for the target ion (Sokalski et al., 1999B). In general, the selectivity of an ISE is reached by doping the membranes with an ionophore or neutral/charged carrier establishing selective and reversible complexes with the target analyte (Amemiya, 2007). In essence, a good ligand should selectively bind the analyte and remain in the membrane phase. Molecular imprinting polymers meet these criteria because they consist on synthetic robust network materials carrying specific binding sites for the imprinted molecules. These materials have been introduced successfully as potentiometric ionophores in PVC membranes for detecting pesticides (Kamel et al., 2008), antibiotics (Moreira et al., 2010) or proteins (Moreira et al., 2011). The

first and second works use bulk imprinting and confirmed that the conventional ionophores could be successfully replaced by these new man-tailored polymers, expected to carry out host-guest interactions. The last work employed surface-imprinting, ensuring a layer-by-layer control. This approach was used because protein imprinting has many difficulties associated to the many conformational changes of the target analyte (Moreira et al. 2011). This technique anchored the protein, diminishing the number of its possible orientations on the sensory surface and offering a higher number of binding sites *per* contact area.

The above concepts have been merged to develop a simple and low-cost device for on-site detection of water organic contaminants. In general, water matrices are regularly contaminated with antibiotics, pesticides, herbicides, hormones, pigments, minerals, and vitamins (Goldberg et al., 2001; JSA, 2007). Antibiotics are here a major concern because they have been associated to many emerging cases of resistant bacteria (Jones et al, 2001).

Thus, sulphamethoxazole (SMX) has been selected as test compound. It is a sulphonamide antibiotic drug for human or veterinary use (Bracket et al., 2004, Strom et al., 2003) that has been detected in environmental waters (García-Galán et al, 2011), coming from domestic or industrial sectors, including aquaculture activities. It shows potential toxicity to aquatic organisms and has also been related to resistant bacteria episodes (Jones et al., 2004). Its determination has been conducted by liquid-chromatography (LC) coupled with mass spectrometry (MS), UV, fluorescence or electrochemical detection, gas-chromatography (GC)/MS, capillary electrophoresis (CE), and immunochemical assays (Cai et al., 2008, He et al., 2008, Stoev and Michailova, 2000, Fuh et al., 2003, Vanesa et al., 2010). All these are too expensive or unable to carry out on-site analysis. The potentiometric sensors described so far do not have the necessary LOD for field applications (Almeida et al,, 2011 A and B).

In this work, a novel ligand compound for SMX was designed by carrying out for the first time surface imprinting on carbon nanostructures (CN). This material was tested first as a potentiometric ionophore in PVC membranes, displaying suitable selective character for the target ion. After that, the electrolyte composition of an electrode body consisting of a pipette tip was optimized and used to carry out steady state analyses of waters from different aquaculture facilities.

2. Experimental

2.1 Equipment

Emf in steady-state assays was measured by a Crison pH-meter GLP 21 (± 0.1mV sensitivity), at room temperature, and under constant stirring. The reference electrode was a Crison, 5240, of double-junction. When necessary, the pH was measured by a Crison GLP 21 combined glass electrode connected to a Crison pH meter. An SBS vortex, MVOR 03, was used to grant the good mixing of the reacting solutions. Insoluble materials were suspended in a Sonorex digitec sonicater. Infrared spectra were collected by a Nicolet iS10 FTIR spectrometer (Thermo Scientific), coupled to an ATR (Attenuated Total Reflectance) sampling accessory of diamond contact crystal, also from Nicolet. A scanning electron microscope JEOL JSM 35C/Noran Voyager, was used.

2.2 Reagents and solutions

All chemicals were of analytical grade and deionised water (conductivity < 0.054 μS/cm at 25ºC) was used. SMX, 3-aminophenylboronic acid (APBA), tris(hydroxymethyl)aminomethane (TRIS), graphite (nanopowder <500 nm and mean pore size of 137 Å), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), tetraoctylammonium bromide (TOABr) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich. *o*-Nitrophenyloctyl ether (*o*NPOE), poly (vinyl chloride) (PVC) of high molecular weight, sodium persulphate (SPS), and *N*-hydroxysuccinimide (NHS) were obtained from Fluka. Tetrahydrofuran (THF) was purchased to Riedel-deHäen and sulphuric acid and nitric acid to Merck.

Working SMX solutions ranged from 1×10^{-10} to 1×10^{-5} mol/L. They were prepared by accurate dilution of a stock solution of 1×10^{-4} mol/L in 1×10^{-4} mol/L HEPES buffer, pH 5.1.

2.3. Synthesis of Imprinted Carbon Nanostructures

About 200 mg of graphitic carbon nanostructures (CN) were suspended in a mixture of concentrated sulphuric acid and nitric acid (3:1 v/v), and sonicated for 2 h. The nanostructures were then filtrated by a 0.45 μm nylon membrane, washed with deionized water until the wash out solution had no acid character and dried at room temperature under a nitrogen atmosphere for 12 h.

About 100 mg of oxidized CNwere dispersed in 5.0 ml of deionized water by 1 min sonication. Then, 2.0 ml of a 0.05 mol/L HEPES buffer solution (pH ~ 5.1) and 5.0 ml of a 50 mg/ml NHS aqueous solution were added to the above suspension. 1.5 ml of fresh EDAC aqueous solution (10 mg/ml) was added quickly, and the mixture was continually stirred at room temperature for 30 min. The suspension was then filtered and rinsed thoroughly with a 0.005 mol/L HEPES buffer solution to remove excess EDAC and NHS.

The reactive CN were re-dispersed in 9.0 ml of 0.005 mol/L HEPES buffer and 1.0 ml of 1 mg/ml SMX solution. The nanostructures were then incubated with 1mol/L TRIS, washed with deionised water and finally added of 1.0 mL of 0.05 mol/L ABPA solution. After 45 min, 1.0 mL of 0.025 mol/L SPS solution was added. The subsequent polymerisation was carried out at room temperature for 1h, and the modified CN were washed again with deionised water. Finally, a diluted acid solution was added to break the amide bond binding the template to the modified material. This was made at room temperature for 12 h. The imprinted carbon nanostructures (ICN) were then filtered, thoroughly washed with water and dried.

Non-imprinted carbon nanostructures (NICN) materials were also prepared by following the same steps and excluding the template from the procedure.

2.4. Membrane composition and ISE constructions

SMX selective electrode membranes were prepared by dissolving 15 mg of ICN and 0.4 mg of TOABr, together in PVC and oNPOE (1:3, w/w) to give a total cocktail mass of 500 mg. This solution was then mixed with 2.0 mL of THF.

The ICN was tested as potentiometric ionophore in PVC membranes casted over a solid-state contact. This conductive support was prepared by mixing Araldite M and hardener in a 2.5:1.0 (w/w) ratio. This epoxy resin was then mixed with graphite powder in a 2:1 (w/w) ratio and applied at 0.2 cm of the end of the electrode body made of Perspex tube (external ø 12 mm, internal ø 10 mm, length 15 cm).

ISEs of internal reference solution were used to reach lower LOD. The electrode body consisted on pipette tips of 1000 µL. These were dipped into the membrane solution until the column of the liquid reached ~ 4 mm. Then, the pipettes were placed in a holder and the THF was left to evaporate overnight, yielding solvent polymeric membranes located at the end of the pipette tip. An Ag wire with a thin layer of AgCl was dipped in the internal electrolyte and used as internal reference material.

A shielded electrical wire was employed to connect the ISEs to the commutation unit. The remaining conducting surfaces were isolated with heat shrink sleeve. The free end of the cable was linked to a BNC (Bayonet Nut Coupling) terminal.

2.5 Potential measurements

All readings were conducted at room temperature (19-21 ºC) and in stirred solutions. The assembly of the potentiometric cell was Ag, AgCl (s) | KCl, 1 mol/L || buffered sample solution (1×10^{-4} mol/L HEPES) | SMX selective membrane | inner electrolyte | AgCl (s), Ag. The inner electrolyte had SMX and/or chlorate, of variable concentrations. The electrodes were conditioned in 1×10^{-8} mol/L SMX to minimize the effect of co-extraction from the sample side during conditioning.

The electrochemical operating characteristics of the ISEs were assessed by calibration curves (n=3) (Buck and Cosofret, 1993) following IUPAC recommendations (Buck and Lindner, 1994). These were conducted in HEPES buffer, 1×10^{-4} M, for increasing SMX concentrations. The emf was recorded after stabilization to ±0.2 mV and plotted as a function of logarithm SMX concentration.

Selectivity studies followed the matched potential method (MPM) for background SMX solutions of 8.0×10^{-5} mol/L that were increased up to 1.4×10^{-4} mol/L.

The coefficient of variation (Cv_w) was the ratio of the standard deviation to the mean. The within-day variability was assessed by measuring the closeness of agreement between 5 readings of a known

standard concentration during the same day. The between-day variability was assessed similarly but in 5 different days. The concentrations of SMX were 1.3×10^{-4} and 5.9×10^{-6} mol/L for electrodes of solid contact and with inner reference solution, respectively. The percentage recovery was calculated for these concentrations.

2.6 FTIR analysis

Every step of the chemical modification of the CN was followed by FTIR analysis. The infrared spectra were collected after background correction. The number of scans was 32 for both sample and background. X-axis was wavenumber, ranging 500 to 4000 cm^{-1}, and Y-axis was % transmittance. Resolution was 4000.

2.7 Analysis of Aquaculture waters

Aquaculture water samples were obtained in several points of sweet water aquaculture units in the Douro River. All samples were collected in duplicate to amber glass bottles, with polypropylene open-top screw caps and Teflon-silicon septa. The samples were labelled immediately after collection and kept chilled in the laboratory.

The samples had no antibiotic and were spiked with SMX up to 18100 or 181 ng/L when the analysis was conducted by ISEs of solid or liquid-contact, respectively. The analysis was carried out immediately after calibration of the potentiometric units.

3. Results and Discussion

3.1 Imprinting on Carbon Nanostructures

The CN play the very important role of acting as a physical support to the imprinted layer and providing extensive surface area for the surface imprinting, thus allowing a nanostructured controlled process. This material consisted of layers of sp2 hybridized carbon atoms mostly arranged in a hexagonal lattice with a distance between planes of 0.335 nm. Each of these layers was thus too small to be observed individually by scanning electron microscopy. Their random packing was however evident, as may be seen in Figure S1, presenting mostly a hexagonal form. These are relatively inexpensive materials, displaying suitable features for application in electrical devices. Surface imprinting was used for this purpose. The overall process may be found in Figure 1. The CN were first oxidized to ensure carboxylic acid groups on its surface. These groups were then activated by EDAC and added of NHS to form a stable ester. The resulting ester reacted by nucleophilic substitution with the amine group on SMX, linking the CN materials to the target analyte by an amide bond. APBA was then added to the system, in order to arrange the monomers around SMX. SPS was added later to initiate the polymerization. Finally, the template was removed by breaking the amide bond, leaving behind the imprinted cavities.

At a first glance, this may seem a similar process to that presented by Almeida et al., 2011A. However, the chemical path follows a completely different scheme, accounting for the great chemical differences between the silica spheres employed there and the CN used here. Overall, the silica spheres were linked to sol-gel materials and modified with a glutaraldehyde layer to enable introducing the protein on top of it, while the graphitic carbon nanostructures were oxidized and the carboxylic groups activated by EDAC/NHS prior to protein binding. Comparing these two imprinting schemes, this last one turns out more effective because the imprinting layer is constructed immediately over the oxidized carbon material and has less monolayers. Evidently, each additional monolayer is an additional critical step to the overall procedure.

All modification steps were followed by FTIR experiments. The spectra of oxidized and imprinted CN are presented in Figure 2. In general, the oxidation of the carbon material to carboxylic acid groups was confirmed by large absorption bands near 3500-3400 cm^{-1}. The chemical imprinting over this oxidized surface depleted this band; the acid groups being now aromatic secondary amines. The strong absorption of the carbon materials limited this observation, but the absence of the carboxylic band was an indirect way to confirm this chemical alteration. Moreover, the band around 2000 cm^{-1} was also decreased on the imprinted materials, as may be seen on the inset of Figure 2.

The imprinting efficiency was controlled by preparing NICN materials, where no SMX was added to the procedure. As expected, no differences could be observed on the FTIR spectra of ICN and NICN materials, because they differed only on the spatial arrangement of the molecular structure of the outer layer and presented the same chemical composition.

Electron microscopy observation was also tried out (Figure S1). The graphitic nanostructures and their chemically modified materials were however too small to be observed either in scanning or in electron transmission electron microscopes, as may be seen in Figure S1 (also presented below). The transmission images showed a slightly different packing for the CN, but this could not be attributed to a successful modification of the ICN/NICN forms, because the observed matrix was heterogeneous and this pattern was not regular. Their chemical modification seemed clear by the different behaviour of materials when they were suspended for observation under transmission: while CN dispersed readily in chloroform, ICN and NICN dispersed immediately in ethanol but deposited in the tube when chloroform was used.

3.2 Selective membrane composition

3.2.1 General components

The above materials were used as ionophores for the potentiometric transduction of SMX on PVC membrane ISEs. The selective sensors were prepared by dispersing the active ingredients (ICN or NICN) in a high dielectric constant plasticizer and PVC. A cationic additive was also added to some of the membranes (table 1), selected according to previous experiments (Almeida et al., 2011 B).

All the membranes were applied over a solid-contact conductive material made of graphite and epoxy resin. The best composition was set after varying the amount of cationic additive and electroactive material, and checking the analytical performance of the corresponding devices by means of consecutive and periodic calibrations. These studies were conducted for SMX concentrations ranging from 2×10^{-5} to 2×10^{-3} mol/L (table 1).

3.2.2 Cationic additive

The cationic additive was employed to improve the permselectivity of the membranes, thus favouring the electrode performance. However, it was important to check that the observed behaviour could be attributed mostly to the novel designed ligand and not to the additive itself. For this purpose, several ISEs were tested having only additive (VII-IX) in different minimal amounts. The results pointed out that the additive was unable to provide a Nernstian response. The lower amounts of additive decreased the sensitivity of the response, but widened the linear concentration range. Thus, an amount of 0.2 mg of additive was selected to test the behaviour of both ICN and NICN materials.

In general, the response of the electrodes pointed out that the additive was essential to reach an acceptable slope, especially for ISEs with ICN materials. The addition of this compound, even in a very

low amount, resulted in a significant slope increase, around 40 % (comparing ISE IV with II and VI with V). This behavior was attributed to the existence of neutral-ionophore material: the anionic analyte in the membrane formed negatively charged structures with the neutral ionophore SMX receptor sites (Figure 1), thus requiring the additional incorporation of lipophilic ions of opposite charge to ensure permselectivity. The anionic SMX was bound to the imprinted sites most probably by hydrogen bond formation with the amine or borate groups of the imprinted layer.

3.2.3 Amount of SMX ligand

Nernstian responses are obtained when the primary ion is the only major ion being transferred between samples and membrane phases (Amemiya 2007) and the free primary ion activity in the membrane is buffered by its receptor (Antonisse and Reinhoudt, 1999). For neutral anion receptors, this constant ion activity is achieved by ensuring a constant ratio between the concentration of the anion-receptor complex and the free receptor, which in turn depends on the amount of lipophilic positive sites added to the membrane to compensate the charge of the complexed anions (Antonisse and Reinhoudt, 1999). In the present study, the ratio of cationic additive/receptor site is impossible to calculate, because the chemically modified CN are polymeric materials of unknown molar mass per number of binding sites.

Thus, no molar ratio may be established and the best amount of ionophore can only be found by experimental testing. The amount of electroactive material acting as SMX ligand added to the membrane ranged from 5 to 30 mg (ISEs I to III). An amount of 15 mg was the one leading to the highest slopes. The other conditions tested gave lower sensitivities, displaying a slope decrease of about 13 and 8 %, respectively. The linear concentration ranges were narrower only for the minimum amount tested. So, the ISEs used in further experiments included 15 mg of ICN. Overall, the time taken by the electrodes to reach a steady potential (± 1 mV) was small, always less than 30 s, even for the highest concentrations tested.

3.3 Imprinting effect

The imprinting effect could be observed by comparing ISEs differing only on this membrane ingredient: ISEs II with V and ISEs IV with VI. The main differences displayed by each pair of ISEs regarded mostly slope, limit of detection and linear range. All membranes relying on imprinted materials showed much higher slopes and lower limits of detection than those with non-imprinted ones, as may be seen in table 1. The enhanced performance of the imprinted-based devices may be therefore attributed to the ability of carrying out stereochemical recognition of the analyte. The small response observed for NICN was attributed to non-specific electrostatic interactions between SMX and this material.

3.4 Effect of pH

The effect of pH on the response of the ISEs was studied by checking the emf of a 1×10^{-2} mol/L SMX solution over the pH range of 3 to 10. Each pH tested was obtained by adding small amounts of concentrated sulphuric acid or saturated sodium hydroxide solutions. In general, the potential remained within ± 5 mV from pH 5 to 8. Increasing pHs gave decreasing emfs that were attributed to hydroxyl interference. Decreasing pHs gave also rise to decreasing emfs, this time attributed to a chloride interference, occurring simultaneously with the deprotonation of SMX.

This operational pH range was confirmed later by calibrating ISEs II and V in pHs 4.0, 5.1, 6.5 or 9.0. As expected, the more extreme conditions were outside the above range. The analytical performance was completely opposite for pHs 4.0 and 9.0, with ISEs changing from super-Nernstian (104 mV/decade) to sub-Nernstian (44 mV/decade) behaviour and the detection limits decreasing from 63.8 to 3.29 µg/mL, respectively. The pH condition displaying a closest behaviour to that expected by the Nernst equation was pH 5.1. This was the one selected for further studies.

3.5 Sensor selectivity

Selectivity studies followed the matched potential method (MPM) and were conducted against PO_4^{2-}, CH_3COO^-, BO_3^{3-}, CN^-, SO_4^{2-}, F^-, CO_3^{2-}, Cl^-, HCO_3^-, NO_3^-, NO_2^-, ClO_3^- and tartrate. The initial concentration of primary ion was set to 8.0×10^{-5} mol/L and the activity of the primary ion solution added into this was 1.4×10^{-4} mol/L. The average corresponding potential change was about 13 mV. The interferences were assessed by adding small aliquots of the corresponding solutions into the primary ion solution of a_A until the same potential change was observed.

Overall, the log K^{POT} values ranged from -3.6 to 0.47, showing negligible interference for most of the observed species. Only chlorate showed a positive logK^{POT} and was taken as a severe interference. The relative order of interference was $ClO_3^- > NO_3^- > NO_2^-$ > tartrate ~ $CN^- > Cl^- > CH_3COO^- > HCO_3^- > F^- > SO_4^{2-} > PO_4^{2-} > CO_3^{2-} > BO_3^{3-}$. This sequence deviated for the Hofmeister series, indicating that the mechanism of action was not governed by ion-exchange.

In general terms, the more interfering ion was chlorate. Thus, this specific ion was used as interfering compound in the inner electrolyte of ISEs made from pipette tips.

3.6 Liquid contact ISEs

3.6.1 Inner electrolyte composition

The composition of the inner electrolyte should be set in such a way that the net flux of primary ions should be forced towards the membrane side contacting with this electrolyte. A low activity of primary ion and a sufficiently high activity of an interfering ion should favour this process. Thus, the inner electrolyte composition was tested for SMX concentrations ranging from 1×10^{-3} to 1×10^{-10} mol/L and chlorate from 1×10^{-4} to 1×10^{-8} mol/L. An electrolyte with just HEPES was also used and tested as control. The general analytical features of such electrodes were indicated in table 2 and the general set-up may be seen in Figure 3.

The concentration of SMX in the inner electrolyte was studied for a solution containing only HEPES with variable amounts of SMX (table 2). No interfering species was added at this stage. The obtained results pointed out that for concentrations of SMX lowers than 1×10^{-8} mol/L no significant improvement was observed. The lower limit of linear range (LLLR) followed a similar behaviour. The need to add SMX (in a low concentration) to the inner electrolyte was confirmed by checking the analytical performance of electrodes carrying only HEPES (ISEs M, table 2). These devices presented a similar behaviour to those carrying a higher amount of SMX (ISE A, table 2).

The conditioning solution was set by evaluating the effect of different concentrations of primary ion upon the LOD of ISE D. Concentrations of 1×10^{-6} mol/L and 1×10^{-8} mol/L were selected for this purpose. The electrodes were let stand in these solutions for at least 16 h. The subsequent calibration curves were carried by washing the electrodes with water, transferring them to HEPES buffer 1×10^{-4} mol/L and adding small aliquots of 1×10^{-4} mol/L SMX standard solution. The LODs were nearly identical in both conditioning conditions, equal to 39.2 and 39.1 ng/mL, respectively. The conditioning of all electrodes was therefore set to 1×10^{-8} mol/L, thus avoiding the "saturation" of the ligand sites on the membrane interface when the calibration is carried out at really low SMX concentrations.

The chlorate concentration on the inner electrolyte was tested for ISEs having a fixed amount of SMX (1×10^{-8} mol/L) and HEPES. The use of a 1×10^{-4} mol/L chlorate concentration favoured the electrode performance, leading to a LOD of 2.3×10^{-8} mol/L. All other conditions tested gave similar results. The exact effect of chlorate was identified by preparing additional electrodes with inner electrolyte having only chlorate (1×10^{-8} mol/L) in HEPES buffer. These electrodes displayed higher LOD than ISE H (125 ng/mL) and lowers slopes (-38.7 mV/decade), thus confirming the need to include an interfering species in the inner electrolyte.

Overall, the concentration of SMX was set to 1×10^{-8} mol/L and the interfering species to 1×10^{-4} mol/L. The observed behaviour seems to indicate that the LOD is mainly dependent on the interfering species,

as long as a sufficiently low concentration of primary ion is ensured. Moreover, a small amount of SMX is necessary for a slight slope increase.

3.7 Analysis of aquaculture waters

The applicability of the SMX electrodes was tested in aquaculture waters collected on commercial fish farms on the north region of Portugal. The obtained results are summarized in table 3 and correspond to the mean of at least 3 independent determinations. The standard deviations were always below 5.4 %.

A good agreement was found between added and found amounts of the antibiotics. For solid contact electrodes, the results showed recoveries ranging from 96.6 to 99.1%, and for pippette tips electrodes from 94.6 to 102 %. The *t*-Student and *F* tests indicated no significant statistical differences betweeen the means of claimed and potentiometric amounts of both types of electrodes. The calculated t values (*p*) were 1.2 and 1.5 and the *F* values were 5.3 and 3.9, for solid contact ISEs and electrodes with inner reference solution, respectively. In both cases, *p* values were below the tabulated critical value (*t* 1.8 and 1.9 and *F* 6.4 respectively) for 95% confidence level, demonstrating that there are no significant differences between claimed and found amounts.

4. Conclusions

A new SMX ligand was successfully introduced as an ionophore in potentiometric transduction. The imprinting process was effective and used inexpensive material. Improved LODs were enabled by managing the composition of the inner electrolyte with regard to primary and interfering ion concentrations. This was obtained for the first time for an organic anionic species. The devices were coupled with a simple construction, low power requirements and low manufacturing costs, relying on pipette tips. The obtained sensors showed suitable analytical performance to carry out on-site analysis.

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Captions for Tables

Table 1

Membrane composition of SMX sensors evaluated in solid contact electrodes and their potentiometric features in 1×10^{-4} mol L^{-1} HEPES buffer, pH 5.1.

Table 2

Comparison of SMX pipette tips electrodes with different inner solutions.

Table 3

Results obtained with solid contact and liquid-contact ISEs in the determination of SMX in aquaculture waters.

Captions for Figures

Figure 1

Design of the plastic antibodies over graphitic carbon nanostructures.

Figure 2

FTIR spectra of oxidised and imprinted carbon nanostructures.

Figure 3

Schematic diagram of the potentiometric cell. a) Ion-selective electrode; b) inner reference (Ag/AgCl); c) inner filling solution; d) ion-selective membrane; e) reference electrode; f) reference electrolyte; g) bridge electrolyte. Insert: photography of the pipette-tip electrode).

Table 1 – Membrane composition of SMX sensors evaluated in solid contact electrodes and their potentiometric features in 1×10^{-4} mol L^{-1} HEPES buffer, pH 5.1.

Characteristics	**ISE I**	**ISE II**	**ISE III**	**ISE IV**	**ISE V**	**ISE VI**	**ISE VII**	**ISE VIII**	**ISE IX**
SMX ligand (mg)	ICN (5)	ICN (15)	ICN (30)	ICN (15)	NICN (15)	NICN (15)	—	—	—
Additive (mg)	TOABr (0.2)	TOABr (0.2)	TOABr (0.2)	—	TOABr (0.2)	—	TOABr (0.2)	TOABr (0.5)	TOABr (1.0)
Slope, mV/decade	-45.8 (±1.3)	-52.5(±0.4)	-48.4(±1.1)	-39.3 (±0.6)	-38.7 (±0.5)	-27.1 (±2.9)	-30.5 (±1.6)	-32.2 (±1.0)	-35.4 (±0.5)
R^2 (n = 3)	0.993	0.997	0.994	0.995	0.994	0.997	0.996	0.995	0.997
LLLR, ng/mL	10.4×10^3	5.18×10^3	5.18×10^3	5.19×10^3	8.64×10^3	10.4×10^3	33.2×10^3	17.9×10^3	15.3×10^3
LOD, ng/mL	3.10×10^3	1.57×10^3	1.61×10^3	1.57×10^3	2.62×10^3	3.14×10^3	10.1×10^3	5.43×10^3	4.64×10^3
Cv_w (%)	2.83	0.796	2.27	1.44	1.42	10.7	5.15	3.12	1.55
within-day variability, %	1.35	1.01	1.76	2.98	2.08	0.639	2.47	0.283	1.13
between-day variability, %	1.58	1.22	2.21	2.67	2.71	1.11	1.79	0.231	0.954
Recovery, %	95.2	99.3	97.3	98.0	98.7	90.3	92.7	100.8	96.7

LOD: Limit of Detection, LLLR: Lower Limit of Linear range; R^2: Squared correlation coefficient; RSD: Relative Standard deviation; Cv_w: Reproducibility;

Table 2 - Comparison of SMX sensors with different inner electrolyte solutions.

Characteristics	SMX (mol/L)*						Chlorate (mol/L) **						Hepes
	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}	1×10^{-9}	1×10^{-10}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}	---
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
Slope, mV/decade	-47.5	-45.4	-44.8	-45.4	-43.8	-44.1	-44.3	-40.3	-45.0	-44.7	-44.0	-43.6	-46.9
	(±1.8)	(±0.9)	(±1.6)	(±0.6)	(±0.9)	(±1.5)	(±0.4)	(±0.4)	(±0.8)	(±1.6)	(±0.2)	(±1.2)	(±2.0)
R^2 *(n = 3)*	0.995	0.995	0.993	0.996	0.996	0.995	0.999	0.997	0.996	0.996	0.999	0.994	0.994
LLLR, ng/mL	1500	415	405	129	129	129	129	19.5	129	129	129	129	1500
LOD, ng/mL	454	126	123	39.2	41.8	40.4	40.1	5.92	39.2	38.8	41.8	40.4	457
Cv_w *(%)*	3.70	1.96	3.51	1.37	2.12	3.37	0.937	7.64	1.83	3.79	0.549	3.14	4.35
within-day variability, %	1.13	1.50	1.47	1.30	2.55	3.46	0.806	1.82	0.990	1.11	1.70	1.98	1.28
between-day variability, %	—	—	—	0.651	—	—	—	1.56	0.896	1.43	—	—	—
Recovery, %	105	105	101	102	97.2	96.6	98.6	101	107.6	94.7	105.2	97.0	102

Table 3 - Results obtained with solid contact and liquid-contact ISEs in the determination of SMX in aquaculture waters.

	Solid-contact ISEs			**ISEs with inner electrolyte solution**		
Sample	**Taken (ng/mL)**	**Found (ng/mL)**	**Recovery (%)**	**Taken (ng/mL)**	**Found (ng/mL)**	**Recovery (%)**
1	18.1×10^3	$17.6\times10^3 \pm 0.809\times10^3$	97.5	181	180 ± 4.55	99.6
	100×10^3	$97.8\times10^3 \pm 2.06\times10^3$	97.8	1000	1002 ± 15.5	101.6
2	18.1×10^3	$17.9\times10^3 \pm 0.404\times10^3$	99.1	181	177 ± 6.8	98.2
	100×10^3	$96.6\times10^3 \pm 1.31\times10^3$	96.6	1000	946 ± 20.7	94.6

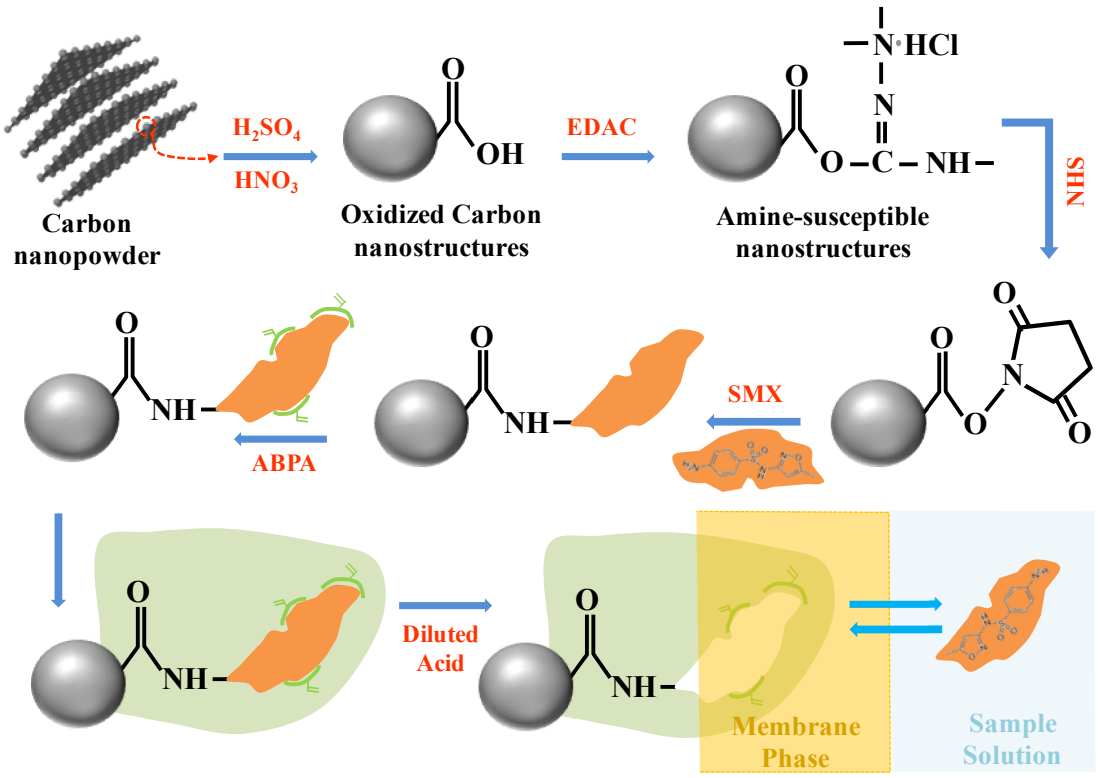
Carbon
nanopowder
H_2SO_4
HNO_3
Oxidized Carbon
nanostructures
EDAC
Amine-susceptible
nanostructures
NHS
ABPA
SMX
Diluted
Acid
Membrane
Phase
Sample
Solution

Figure 1

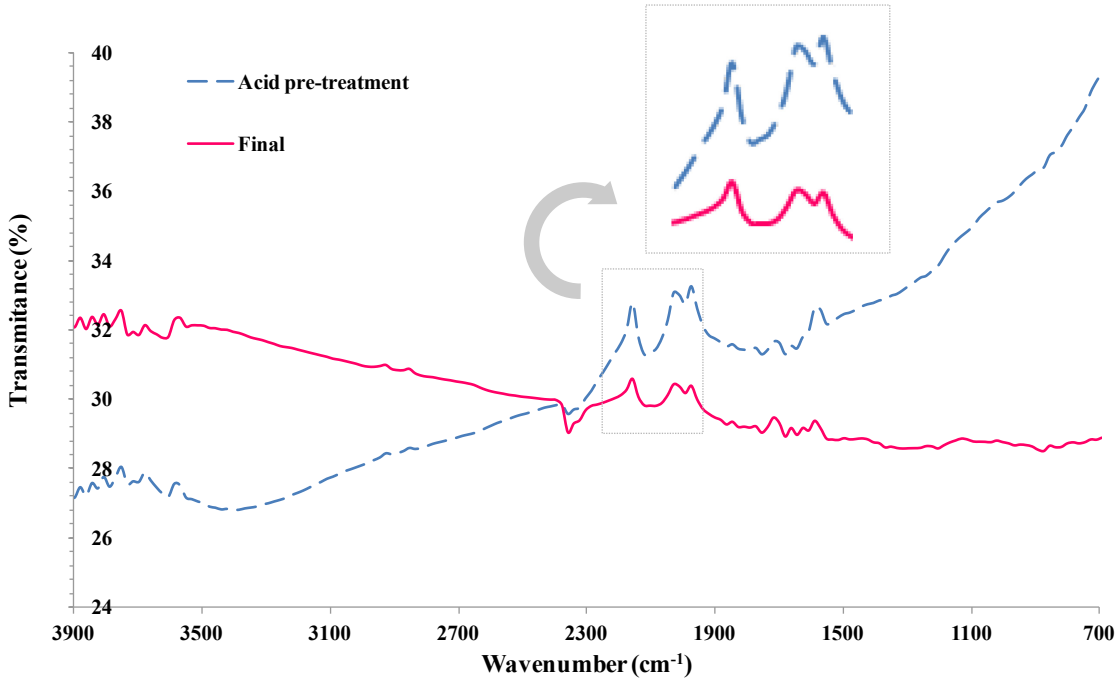
Acid pre-treatment
Final
Transmitance (%)
40
38
36
34
32
30
28
26
24
3900
3500
3100
2700
2300
1900
1500
1100
700
Wavenumber (cm-1)

Figure 2

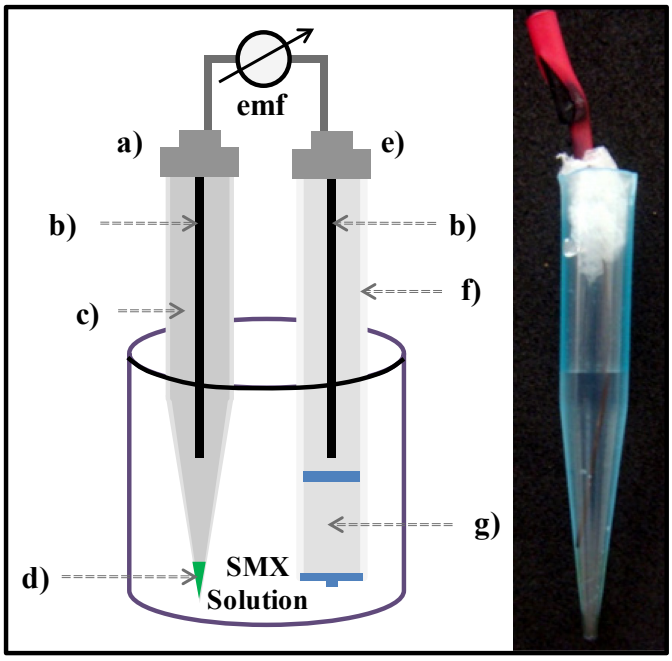

Figure 3

IX. CONCLUSÕES

O presente trabalho permitiu desenvolver novos (bio)sensores com aplicação no sector da aquacultura, para monitorização de SAs no meio aquático, ao longo das várias fases de produção. Estes dispositivos introduziram várias inovações técnicas no domínio do desenvolvimento de ESIs, tendo permitido construir eléctrodos selectivos a espécies aniónicas de carácter orgânico com características excepcionais de operação. Estas inovações prendem-se, sobretudo, com os sistemas sensores utilizados para a preparação das membranas selectivas, com a configuração dos (bio)sensores e com a ligação a sistemas de microfluídica.

Os sistemas ionóforos comerciais utilizados (baseados em porfirinas e ciclodextrinas) provaram ser capazes de originar membranas sensoras com respostas potenciométricas adequadas à monitorização dos analitos estudados, sobretudo no que se refere à selectividade das medidas, embora os níveis de detecção alcançados pelos eléctrodos, por si só, não fossem adequados ao controlo *in situ* em águas de aquacultura. A utilização de sensores baseados em polímeros de impressão molecular, tanto em matriz de sol-gel como em matriz polimérica, usando como suporte materiais de sílica ou de carbono, contribuiu para uma redução significativa dos limites de detecção dos eléctrodos. Desta forma, ficou patente que a potenciometria, associada a materiais sensores apropriados, pode constituir uma alternativa vantajosa aos métodos habitualmente usados para a análise destes compostos em matrizes reais.

No que diz respeito à configuração dos eléctrodos, foram testadas configurações convencionais em que a membrana era directamente aplicada a um suporte condutor (eléctrodos de contacto sólido), configurações convencionais baseadas na adaptação das membranas a pontas de pipeta e com solução de referência interna, ambas para determinações em estado estacionário e também configurações tubulares, de reduzido custo, baseadas na utilização de agulhas de veterinária que foram depois acopladas a sistemas FIA. Todos os tipos de eléctrodos mostraram boas características de funcionamento, embora sejam de destacar os de membranas selectivas em contacto uma solução de referência interna. O facto de se poder optimizar a composição desta solução pode contribuir para um decréscimo significativo dos limites de detecção dos eléctrodos, o que se torna vantajoso, sobretudo se os antibióticos em estudo se apresentam em baixas concentrações. A inserção das unidades em microfluídica abriu novas portas na direcção das análises de campo, já que permitiu a determinação simultânea de mais do que um analito sobre a mesma amostra, constituindo assim a primeira etapa na direcção da concepção de um *Lab-On-a-Chip* multiparamétrico.

De uma forma geral, as soluções analíticas propostas mostram ser um contributo valioso para, de uma forma expedita, económica e eficaz, controlar a presença de antibióticos, poluentes orgânicos muitas vezes utilizados de uma forma fraudulenta em indústrias de aquacultura.

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