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Emprego e automatização de técnicas miniaturizadas de preparo de amostra para análise de desreguladores endócrinos em águas residuais

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RESUMO

O preparo de amostra é uma etapa crítica do método analítico, afetando a eficiência analítica, custos, impacto ambiental e é a principal fonte de introdução de erros e contaminação da amostra. Uma das melhores abordagens para aumentar a confiabilidade e a eficiência desta etapa é miniaturizar e automatizar o processo. Atualmente, existem vários tipos de microextrações em fase líquida ou sólida disponíveis e diferentes estratégias de automação. Este trabalho foi realizado utilizando microextração por sorvente empacotado (MEPS) como abordagem em fase sólida e microextração por gota única (SDME) como abordagem em fase líquida; sendo a estratégia de automação uma plataforma robótica controlada por programação em Arduino anteriormente desenvolvida pelo grupo. Os parâmetros operacionais dos métodos foram otimizados por meio de experimentos univariados e multivariados. A separação e detecção dos analitos foi realizada por cromatografia líquida de alta eficiência (HPLC) e espectrometria de massas sequencial (MS/MS). Os analitos são contaminantes relatados na literatura como possíveis desreguladores endócrinos em matrizes ambientais, sendo que a aplicabilidade dos métodos foi feita em amostras de águas residuárias coletadas de um rio na cidade de São Carlos (SP, Brasil). O método (MEPS-LC-MS/MS) emprega um dispositivo MEPS reutilizável feito em laboratório que proporciona flexibilidade para testar pequenas quantidades (2 mg) de várias fases de extração. Em condições otimizadas, a extração foi realizada a partir de um pequeno volume de amostra de 1,5 mL e os limites de detecção variaram de 0,15 a 0,30 ng L−1. Os limites de quantificação variaram de 0,15 a 0,6 ng L−1 e as variações relativas padrão intra e interdias situaram-se entre 3 e 21%. O método (SDME-LC-MS/MS) foi automatizado empregando-se um robô cartesiano que controla o desempenho automático de todas as etapas da SDME, incluindo o preenchimento da seringa, exposição da gota, reciclagem do solvente e coleta do extrato. Em condições otimizadas, os limites de detecção foram determinados em 0,3 μg L-1 para todos os analitos, limites de quantificação e o método forneceu respostas lineares na faixa de 0,6 a 10 μg L-1 para todos os analitos, com repetibilidade adequada, com coeficiente de variação intra-dia (RSDs) entre 5,54% e 17,94% (n = 6), e inter-dia entre 8,97% e 16,49% (n = 9). Os métodos de MEPS e SDME automatizados mostraram-se viáveis, robustos e confiáveis, demonstrando ser uma estratégia competitiva e ambientalmente amigável para a determinação de contaminantes orgânicos em amostras ambientais.

Palavras-chave: MEPS; SDME; preparo de amostra; Química Verde; contaminantes ambientais

ABSTRACT

Sample preparation is a critical step in the analytical method, affecting analytical efficiency, costs, environmental impact, and is the main source of error introduction and sample contamination. One of the best approaches to enhance reliability and efficiency in this step is to miniaturize and automate the process. Currently, there are various types of liquid or solid phase microextractions available along with different automation strategies. This work was carried out using packed sorbent microextraction (MEPS) as the solid-phase approach and single drop microextraction (SDME) as the liquid-phase approach, with the automation strategy being a robotic platform controlled by Arduino programming previously developed by the group. The operational parameters of the methods were optimized through univariate and multivariate experiments. The separation and detection of analytes were performed by high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS). The analytes are contaminants reported in the literature as possible endocrine disruptors in environmental matrices, and the applicability of the methods was tested on wastewater samples collected from a river in the city of São Carlos (SP, Brazil). The MEPS-LC-MS/MS method employs a reusable homemade MEPS device that provides flexibility to test small amounts (2 mg) of various extraction phases. Under optimized conditions, the extraction was carried out from a small sample volume of 1.5 mL, and the detection limits ranged from 0.15 to 0.30 ng L^{-1}. The quantification limits ranged from 0.15 to 0.6 ng L^{-1}, and the intra-and inter-day relative standard deviations ranged from 3 to 21%. The SDME-LC-MS/MS method was automated using a cartesian robot that controls the automatic performance of all SDME steps, including syringe filling, drop exposure, solvent recycling, and extract collection. Under optimized conditions, the detection limits were determined to be 0.3 μg L−1 for all analytes, quantification limits, and the method provided linear responses in the range of 0.6 to 10 μg L−1 for all analytes, with adequate repeatability, with intra-day coefficient of variation (RSD) between 5.54% and 17.94% (n = 6), and inter-day between 8.97% and 16.49% (n = 9). The automated MEPS and SDME methods proved to be feasible, robust, and reliable, demonstrating to be a competitive and environmentally friendly strategy for the determination of organic contaminants in environmental samples.

Keywords: MEPS; SDME; sample preparation; Green Chemistry; environmental contaminants

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

DI-SDME – Microextração em gota única por imersão direta

DLLME – Microextração líquido-líquido dispersiva

GC – Cromatografia gasosa

HF-LPME – Microextração em fase líquida com fibra oca

HS-SDME – Microextração em gota única por *headspace*

IT-SPME – Microextração em fase sólida em tubo

LC – Cromatografia líquida

LLE – Extração líquido-líquido

LOD – Limite de detecção

LOQ – Limite de quantificação

LPME – Microextração em fase líquida

MEPS – Microextração por sorvente empacotado

MS/MS – Espectrometria de massas sequencial

PCP – Produtos de cuidado pessoal

SBSE – Extração sortiva em barras de agitação

SDME – Microextração em gota única

SPE – Extração em fase sólida

SPME – Microextração em fase sólida

UHPLC – Cromatografia líquida de ultra eficiência

UV – Ultravioleta

SUMÁRIO

1 INTRODUÇÃO

1.1 A importância do preparo de amostras

O preparo de amostra é crucial no desenvolvimento de um método analítico. Geralmente possui várias etapas, sendo uma parte tediosa que ocupa a maior parte do tempo do método, sendo ainda hoje o gargalo e a maior fonte de introdução de erros. O preparo de amostra permite eliminar interferentes da matriz e concentrar a amostra, o que se torna ainda mais imprescindível em análises em que o analito de interesse está presente em uma matriz complexa e em baixas concentrações. Portanto, um planejamento bem feito do preparo de amostra permite reduzir o tempo total de análise, equalizar custos e desenvolver um método sensível e robusto o suficiente para ser reprodutível. Ainda, é possível reduzir a necessidade de limpeza e manutenção de aparelhos como cromatógrafos e colunas cromatográficas, assim como seus vários tipos de detectores disponíveis (KATAOKA, 2023; KATAOKA et al., 2009).

Técnicas clássicas como Soxhlet e extração líquido-líquido (LLE) foram as primeiras a serem usadas com sucesso como preparo de amostra. Tais técnicas utilizam grande quantidade de solventes orgânicos que, além de caros, são nocivos à saúde do analista e ao meio ambiente. Para reduzir as limitações dessas técnicas, foram desenvolvidas extrações baseadas em materiais sorventes, destacando-se a extração em fase sólida (SPE). A SPE é um pouco mais simples de ser executada e utiliza menor quantidade de solventes orgânicos que a LLE. Atualmente, muitos dos métodos de preparo de amostras são desenvolvidos a partir da SPE e sua literatura é extensa e muito bem fundamentada. Diversos materiais sorventes estão disponíveis comercialmente permitindo que uma enorme gama de analitos sejam extraídos, quer seja de amostras biológicas, ambientais, de alimentos, entre outras (ALI et al., 2019).

1.2 Técnicas modernas de preparo de amostras

Apesar do sucesso e grande utilização das técnicas clássicas de preparo de amostra até hoje, elas possuem limitações e são reconhecidas como processos não amigáveis ao meio ambiente, devido à necessidade de grandes quantidades de amostra e de solventes, muitos deles tóxicos; resultando em riscos ao analista e gerando grandes quantidades de resíduos que precisam ser corretamente tratados e dispostos. As preocupações com os impactos ambientais

na química analítica a partir da década de 90 incentivaram o desenvolvimento de métodos analíticos que estivessem mais alinhados ao conceito da Química Verde e, desde então, a literatura sobre esse assunto aumentou exponencialmente (ARMENTA; GARRIGUES; DE LA GUARDIA, 2008). De maneira geral, os fundamentos do conceito de química verde estão intrinsecamente ligados à eliminação ou redução do uso de substâncias químicas, à minimização do consumo e desperdício de energia, à facilidade de automação e eficiência elevada, e ao aumento da segurança do analista. Todos esses elementos são requisitos para o desenvolvimento de métodos de preparo de amostras mais sustentáveis, mantendo, contudo, a premissa de assegurar um ótimo desempenho analítico. Dessa forma, a preferência recai sobre a análise direta das amostras. No entanto, o preparo de amostra é uma etapa que raramente pode ser contornada, seja pela complexidade da amostra ou pela necessidade de pré-concentração dos analitos (PERIS-PASTOR et al., 2023).

A miniaturização de técnicas de extração data da década de 80, quando, na tentativa de miniaturizar a SPE, os pesquisadores substituíram os cartuchos convencionais por discos e membranas extrativas. Porém o grande marco da miniaturização da SPE se deu com o desenvolvimento da microextração em fase sólida (SPME) por Pawliszyn na década de 90, em que uma fibra é recoberta com um material sorvente e a extração se dá pelo equilíbrio de partição ou adsorção dos analitos presentes na amostra com a fase estacionária que reveste a fibra. A extração pode ser totalmente livre de solventes e facilmente automatizada. A partir dela, houve o desenvolvimento de várias outras microtécnicas de extração, como a microextração em fase sólida em tubo (IT-SPME) e a extração sortiva em barras de agitação (SBSE). Já a microextração por sorvente empacotado (MEPS), foi desenvolvida como uma miniaturização da SPE clássica, em que um cartucho é preenchido com material sorvente e a amostra é percolada para que os analitos fiquem retidos na fase extratora (SARTORE et al., 2023).

Paralelamente aos avanços nas extrações em fase sólida, a atenção também centrou-se sobre a utilização de pequenos volumes de líquidos para extrações analíticas, com o ideal de realizar uma extração líquido-líquido de forma miniaturizada, nomeada microextração em fase líquida (LPME). Em particular, este campo foi introduzido em 1996 pelos trabalhos de Dasgupta et al. e Cantwell et al.. Posteriormente, Lee et al. e Andrews et al. também se envolveram no desenvolvimento da técnica. Nesse conceito, uma gota suspensa na ponta de uma fibra ou na agulha de uma microsseringa servia de interface de amostragem para extração do analito de interesse. A partir desses primeiros trabalhos realizados com microextração em

fase líquida foram desenvolvidas diferentes variantes para a LPME, cada uma delas apresentando características específicas como, por exemplo, outras interfaces para estabilização da gota e aumento da superfície de contato. Um exemplo de variação da técnica para aumento da superfície de contato é chamada de microextração em fase líquida com fibra oca (HF-LPME) introduzida em 1999 por Pedersen-Bjegaard et al., em que uma fibra oca serve de interface para a interação do solvente de extração com a amostra. Mais tarde, em 2006 foi introduzida a técnica chamada microextração líquido-líquido dispersiva (DLLME) por Rezaee et al., consistindo basicamente em um sistema ternário de solventes (amostra aquosa, solvente dispersor e solvente extrator). Uma mistura de poucos µL dos solventes extrator e dispersor é introduzida rapidamente na amostra, proporcionando a formação de uma enorme quantidade de microgotas que permitem a formação de uma área de contato grande entre o solvente extrator e a amostra. Decorrido um certo tempo, a fase extratora decanta para o fundo do frasco, permitindo ser recolhida com o auxílio de uma seringa e ser injetado no sistema cromatográfico. Para alguns sistemas, a decantação ou separação do solvente pode ser melhor atingida com centrifugação ou outros processos (REZAEE; YAMINI; FARAJI, 2010)(MOREIRA; YOKOYA; GAITANI, 2014).

1.3 Automatização do preparo de amostra

A automatização do preparo de amostras traz muitos benefícios para o método analítico, apesar do grande desafio que é desenvolver um aparato que funcione bem em menor escala e que seja confiável e robusto. É a maneira mais efetiva para desenvolver um método simples, rápido e eficiente, já que essa etapa é a que mais consome tempo no método. Também é uma maneira de diminuir a introdução de erros manuais, pois essa etapa é também a principal fonte de introdução de erros. Permite trabalhar com pequenas quantidades de amostras, o que em muitos casos é muito pertinente, em áreas como pesquisa clínica, biológica e farmacêutica, entre outras. É uma estratégia mais alinhada com a química verde por consumir menos recursos. Por fim, como trabalha com pequenos volumes, é mais adequada à injeção em sistemas cromatográficos (VARGAS MEDINA; MACIEL; LANÇAS, 2023).

Consequentemente, nas últimas décadas, esforços significativos foram concentrados no desenvolvimento de sistemas analíticos automatizados, e diversos tipos de sistemas foram aprimorados e estão disponíveis comercialmente, como autosamplers e pipetadores eletrônicos. Sistemas robóticos são uma das abordagens mais versáteis para automatizar o preparo de

amostras pois podem executar várias tarefas de manipulação de amostras, como pipetagem, mistura, diluição, derivatização e extração. Aumentam a capacidade de processamento, a reprodutibilidade e a precisão, podendo ser programados para executar fluxos de trabalho complexos sem intervenção humana. Além disso, uma plataforma robótica pode ser adaptada e programada para diferentes microtécnicas de extração (SARTORE et al., 2023).

1.4 Microextração por sorvente empacotado (MEPS)

A MEPS é uma técnica de preparo de amostra recente, desenvolvida em 2004 por Mohamed Abdel-Rehim (ABDEL-REHIM, 2004). Essa técnica é a miniaturização da SPE convencional, sendo que os dispositivos sortivos passaram da ordem de muitos miligramas para poucos miligramas e os volumes da ordem de mililitros para microlitros. Diferentemente da SPE, se o método for bem concebido e dependendo da natureza da amostra, o cartucho de MEPS pode ser reutilizado até 100 vezes. Dessa forma, além da facilidade de automatização e utilização mínima de solventes e materiais sortivos, nessa microtécnica o volume do solvente utilizado para a eluição dos analitos no processo de extração é compatível para a injeção direta em sistemas de cromatografia líquida, cromatografia gasosa, eletroforese capilar e espectrometria de massas (QUEIROZ, 2011).

A extração é realizada por meio da utilização de uma microsseringa que contém um cartucho preenchido com a fase sortiva entre o seu corpo e a agulha. Atualmente, há uma diversade de fases sortivas disponíveis em cartuchos para utilização em MEPS. Porém, essa diversidade não é tão grande como na SPE de modo que uma solução seria a utilização de dispositivos *lab-made* em que se possa preencher com as diversas fases sortivas disponíveis comercialmente para SPE.

Como desvantagens, pode-se citar a formação de bolhas e entupimento do cartucho no caso de amostras muito complexas. Outro fator ao qual se deve ter atenção é a velocidade do embolo nos ciclos de cada etapa, pois uma velocidade muito grande pode prejudicar a recuperação dos analitos. Além disso os ciclos de cada etapa devem ser feitos com a mesma velocidade para cada amostra, pois a velocidade de aspiração da amostra, por exemplo, impacta na recuperação dos analitos e isso pode prejudicar muito a repetibilidade do método. Portanto, com a automatização do método, pode-se eliminar ou mitigar todas estas desvantagens, permitindo a elaboração de um método confiável e robusto (NOVÁKOVÁ; VLČKOVÁ, 2009).

1.5 Microextração por gota única (SDME)

A microextração por gota única (SDME) é uma técnica de preparo de amostra resultante da miniaturização da LLE. O trabalho pioneiro foi de Jeannot et al. que usou uma gota de n-octano suspensa na ponta de um tubo de Teflon para extrair compostos orgânicos em uma amostra aquosa (JEANNOT; CANTWELL, 1996). Um ano depois, He e Lee modificaram a técnica usando uma microsseringa para cromatografia gasosa (GC) no lugar do tubo de Teflon. Dessa maneira, o lúmen da microsseringa foi preenchido com o solvente orgânico extrator e a extração foi realizada mergulhando a agulha na amostra. Empurrando-se o embolo da seringa a gota foi gerada e entrou em contato com a amostra, para que os analitos pudessem migrar para o solvente extrator. Após esse procedimento, a gota foi recolhida e introduzida no injetor de GC (HE; LEE, 1997).

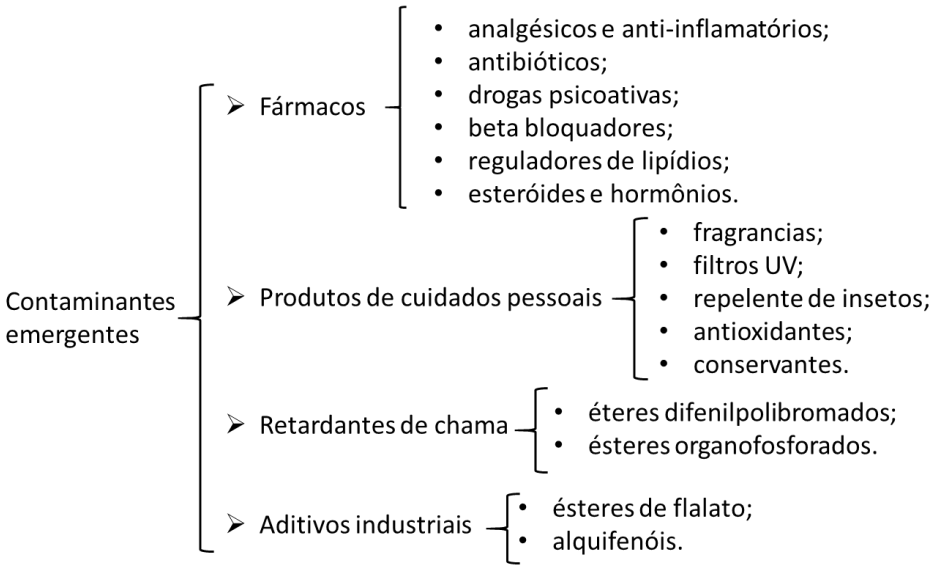
Ao longo dos anos, diversas variações da técnica foram desenvolvidas, sendo que hoje as principais são a microextração em gota única por *headspace* (HS-SDME) e a microextração em gota única por imersão direta (DI-SDME). Na primeira, a gota de solvente extrator é exposta ao headspace de um tubo contendo a amostra, para a extração de analitos voláteis e semi-voláteis. Na segunda, a gota é imersa na amostra e os analitos passam para o solvente extrator utilizado para a gota de extração. Geralmente emprega-se agitação mecânica, pois ela acelera o transporte dos analitos que estão presentes no meio da solução para a vizinhança da gota, promovendo uma migração de analitos mais rápida e eficiente.

A SDME apresenta muitas das vantagens que toda microtécnica apresenta em relação às tradicionais. Porém, toda miniaturização traz também alguns desafios. O primeiro deles é fazer com que a gota seja estável, principalmente quando se usa agitação. Um fluxo muito turbulento pode desestabilizar a gota e abortar toda a extração que está sendo feita. Para melhorar a estabilidade da gota, pode-se desenvolver aparatos como plugs que proporcionem maior base de contato à gota de solvente e consequentemente aumentem sua estabilidade. Outro grande desafio é a realização do procedimento exposição/retração da gota, pois é muito difícil para o analista controlar o volume da gota manualmente. Consequentemente, cada amostra sofrerá a introdução de erros manuais subsequentes que podem comprometer a repetibilidade do método. Isso pode ser solucionado automatizando-se o método e eliminando o procedimento manual, que consequentemente diminuirá a introdução de erros à cada etapa e resultará em um método mais robusto e confiável (PINTO; PEDROSO, 2015)(DIONÍSIO et al., 2010).

1.6 Produtos de Cuidados Pessoais

Compostos oriundos de Produtos de Cuidado Pessoal (PCP) são uma classe grande de compostos químicos usados em muitos produtos de uso diário, com o intuito de aumentar a qualidade de vida das pessoas (BOXALL et al., 2012). Esses compostos estão presentes em produtos como batons, filtros de proteção solar, tinturas para cabelo, xampus, pastas de dentes, alimentos, desodorantes, produtos farmacêuticos, alimentos, etc. Os estudos recentes enquadram esses compostos como contaminantes emergentes. São usados em grande quantidade pelas pessoas e incluem um grupo diversificado de substâncias (MAHUGO-SANTANA et al., 2011), como mostrado na Figura 1.

Figura 1 Compostos utilizados em PCP classificados como contaminantes emergentes.



FONTE: Adaptado de MAHUGO-SANTANA et al., 2011.

Uma parte desses compostos são absorvidos pelo organismo e excretados, tendo como destino o esgoto. Outra parte não é absorvida, mas geralmente acaba também tendo o esgoto como destino final seja pelo seu curso natural de uso ou por descarte impróprio. Como as estações de tratamento de efluentes não removem esses compostos completamente, as águas residuárias contaminadas entram em contato com os corpos hídricos adjacentes, contaminando posteriormente tanto o ecossistema aquático quanto o terrestre que entra em contato com essas

águas (KINNEY et al., 2006; TERNES; JOSS; SIEGRIST, 2004). Portanto, os seres humanos e a natureza como um todo são expostos à uma série de moléculas xenobióticas que nem sempre são biodegradáveis, ao contrário disso, potencialmente, algumas são bioacumuladas no organismo, prejudicando o bom funcionamento do metabolismo e consequentemente a saúde (ANAND et al., 2022).

Muitos dos compostos listados como PCP são classificados como desreguladores endócrinos, ou seja; são moléculas que possuem a capacidade de interferir no desempenho do sistema endócrino afetando negativamente seu desenvolvimento e funcionamento, afetando as funções gerais do corpo influenciadas por hormônios provenientes dessas glândulas (WITORSCH; THOMAS, 2010). Além disso, estudos relacionam o contato com alguns compostos presentes nos PCP's como responsáveis pelo aumento da incidência de câncer de pele e do câncer de mama em mulheres (DARBRE; HARVEY, 2008). Assim, com a crescente urbanização e reuso da água, a compreensão do destino e ocorrência desses compostos são fatores importantes de avaliação da qualidade da água disponível para seu uso diário (KOLPIN et al., 2002). Portanto, faz-se necessário métodos analíticos robustos, sensíveis e confiáveis para o estudo e ocorrência de moléculas oriundas dos PCP em matrizes ambientais, tanto para garantir a qualidade da água como para desenvolver tecnologias adequadas para remediação e remoção.

2 OBJETIVOS

2.1 Objetivo geral

O objetivo proposto dessa tese é desenvolver dois métodos analíticos miniaturizados e automatizados para análise de contaminantes orgânicos em amostras ambientais. Um deles proponto uma abordagem de extração em fase sólida, tendo-se escolhido a de MEPS. Para a MEPS, a maioria dos métodos na literatura utiliza dispositivos comerciais que além de caros, limitam o desenvolvimento pela menor possibilidade de parâmetros que podem ser testados. Assim, o propôs-se para MEPS o desenvolvimento utilizando um dispositivo *lab-made* para acomodação da fase extratora, permitindo que uma maior gama de opções de teste esteja disponível e um método mais otimizado possa ser desenvolvido.

O outro método foi desenvolvido propondo-se uma abordagem de extração líquido-líquido por meio da SDME. Para este método, propôs-se a concepção e testes de um plug de estabilização da gota formada, permitindo utilizar maiores volumes de gota e maior velocidade de agitação, permitindo explorar mais opções que possam resultar em extrações mais eficientes.

Para a automatização dos métodos, foram utilizadas e adaptadas as plataformas robóticas desenvolvidas pelo grupo CROMA do IQSC/USP.

Além disso, este trabalho se propôs visando atender ao tripé de sustentabilidade (*triple-bottom-line)*, desenvolvendo uma abordagem analítica economicamente viável, ambientalmente favorável e socialmente justa, tendo como principais focos o impacto a redução de custos, os impactos ambientais e análises de alto interesse social em se tratando de contaminantes.

2.2 Coletânea de artigos

Esta tese foi concebida na modalidade de coletânea de artigos, em que ao menos dois artigos científicos originais aceitos/publicados em revistas indexadas compõe a parte experimental do trabalho desenvolvido durante o doutoramento. Todos os 3 trabalhos aqui apresentados foram desenvolvidos sob a orientação e coautoria do Prof. Dr. Álvaro José dos Santos Neto. Os dois primeiros artigos são documentos originais de pesquisa e o candidato é o

primeiro autor. O terceiro artigo é um artigo de revisão em que o candidato também é o primeiro autor.

O primeiro artigo[(1)], apresentado no Capítulo 3 refere-se ao desenvolvimento de um método automatizado MEPS-LC/MS/MS para a determinação de contaminantes orgânicos em amostras ambientais. Esse trabalho também contou com a colaboração do Prof. Dr. Fernando Mauro Lanças e do Dr. Deyber Arley Vargas Medina.

[(1)]Automated microextraction by packed sorbent of endocrine disruptors in wastewater using a high-throughput robotic platform followed by liquid chromatography–tandem mass spectrometry

Marcio David Bocelli, Deyber Arley Vargas Medina, Fernando Mauro Lanças, Álvaro José dos Santos-Neto

Analytical and Bioanalytical Chemistry (2023)

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O segundo artigo[(2)], apresentado no Capítulo 4, refere-se ao desenvolvimento de um método automatizado DI-SDME-LC-MS/MS para a determinação de contaminantes orgânicos (parabenos) em amostras ambientais. Esse trabalho contou com a colaboração do Prof. Dr. Fernando Mauro Lanças, do Dr. Deyber Arley Vargas Medina e da Dra. Julie Paulin García Rodriguez.

[(2)]Determination of parabens in wastewater samples via robot-assisted dynamic single-drop microextraction and liquid chromatography–tandem mass spectrometry

Marcio David Bocelli, Julie Paulin García Rodriguez, Deyber Arley Vargas Medina, Fernando Mauro Lanças, Álvaro José dos Santos-Neto

Electrophoresis (2022)

DOI: 10.1002/elps.202100390

O terceiro artigo[(3)], apresentado no Capítulo 5, refere-se a um artigo de revisão que o doutorando produziu referente à miniaturização da cromatografia líquida de alta eficiência em escala capilar, abordando seus aspectos teóricos e práticos, bem como uma breve retrospectiva até seu desenvolvimento. Esse trabalho contou com a colaboração do Dr. João Victor Basolli Borsatto.

[(3)]UHPLC capilar: aspectos teóricos e práticos

Marcio David Bocelli, João Victor Basolli Borsatto

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CAPÍTULO 3 - Automated microextraction by packed sorbent of endocrine disruptors in wastewater using a high-throughput robotic platform followed by liquid chromatography–tandem mass spectrometry

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RESEARCH PAPER

Automated microextraction by packed sorbent of endocrine disruptors in wastewater using a high-throughput robotic platform followed by liquid chromatography–tandem mass spectrometry

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Abstract
An automated microextraction by packed sorbent followed by liquid chromatography–tandem mass spectrometry (MEPS-LC–MS/MS) method was developed for the determination of four endocrine disruptors—parabens, benzophenones, and synthetic phenolic antioxidants—in wastewater samples. The method utilizes a lab-made repackable MEPS device and a multi-syringe robotic platform that provides flexibility to test small quantities (2 mg) of multiple extraction phases and enables high-throughput capabilities for efficient method development. The overall performance of the MEPS procedure, including the investigation of influencing variables and the optimization of operational parameters for the robotic platform, was comprehensively studied through univariate and multivariate experiments. Under optimized conditions, the target analytes were effectively extracted from a small sample volume of 1.5 mL, with competitive detectability and analytical confidence. The limits of detection ranged from 0.15 to 0.30 ng L^{-1}, and the intra-day and inter-day relative standard deviations were between 3 and 21%. The method's applicability was successfully demonstrated by determining methylparaben, propylparaben, butylated hydroxyanisole, and oxybenzone in wastewater samples collected from the São Carlos (SP, Brazil) river. Overall, the developed method proved to be a fast, sensitive, reliable, and environmentally friendly analytical tool for water quality monitoring.

Keywords Automated sample preparation · Microextraction by packed sorbent · Liquid chromatography · Mass spectrometry · Organic pollutants · Wastewater analysis

Introduction

Endocrine disruptors are a group of chemical compounds that can disrupt the normal functioning of the endocrine system by mimicking or antagonizing hormone properties [1, 2]. These compounds include various organic substances, such as parabens, benzophenones, and which are commonly found in consumer products like food, personal care items, cosmetics, pharmaceuticals, and sunscreens [3]. Consequently, endocrine disruptors can contaminate water sources through processes such as wastewater discharge, runoff, and leaching, and their prevalence in the environment has given rise to significant public concerns regarding the potential risks they pose to human and animal health, as well as the ecological balance.

The determination of endocrine disruptors (EDs) is of utmost importance in monitoring the quality of water and facilitating the development of remediation processes [4]. However, EDs, like other pollutants, are typically found in trace concentrations within water samples. Consequently, their sensitive analysis necessitates the use of efficient analytical tools capable of high sample throughput while ensuring reliable analytical confidence [5]. In this regard, the continued development of rapid, sensitive, and efficient sample preparation strategies for the accurate determination of EDs in water samples remains both relevant and necessary [6].

Nowadays, accurate detection of trace pollutants in water samples is efficiently achieved thanks to modern advancements in liquid chromatography–tandem mass spectrometry (LC–MS/MS). LC–MS/MS is a highly influential technique capable of providing enhanced sensitivity and specificity. However, conducting water quality surveillance via LC–MS/

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MS still presents significant challenges due to the presence of numerous interfering compounds that can compromise sensitivity and selectivity, leading to inadequate limits of detection (LODs) and quantification (LOQs) [7]. Therefore, despite the remarkable instrumental advancements, it remains crucial to develop efficient sample preparation methods capable of selectively extracting and pre-concentrating the target analytes, to achieve the requisite detectability and uphold the robustness of LC–MS/MS instruments.

EDs encompass a diverse range of compound families, exhibiting significant variations in molecular structures and physicochemical properties. So, selecting a technique or developing a new sample preparation method for the determination of EDs can depend on the specific class of compounds under analysis. Solid-phase extraction (SPE) has been widely utilized as an efficient technique for the extraction and preconcentration of EDs and various organic pollutants from water samples [8]. While SPE has shown remarkable recovery and enrichment factors, conventional methods employing this technique require the use of substantial amounts of toxic solvents and involve drying and resuspension stages. These characteristics render the methods unsustainable, labor-intensive, costly, and time-consuming [9]. To overcome these drawbacks, modern miniaturized versions of SPE have emerged as a beneficial alternative. These advanced techniques deliver comparable SPE performance while reducing reagent and sample consumption, minimizing waste generation, lowering costs, increasing sample throughput, and simplifying implementation and automation [10]. Consequently, contemporary methods employing miniaturized sorbent-based techniques for microextraction of EDs have gained prominence. Notable examples of such techniques include solid-phase microextraction (SPME), stir sorptive bar extraction (SBSE), and microextraction by packed sorbent (MEPS) [11–16].

Among the various miniaturized formats of SPE, MEPS emerges as a straightforward and highly efficient technique [8]. MEPS has demonstrated success across diverse matrices and applications, encompassing environmental surveillance as well as pharmaceutical and clinical analysis [17], forensic analysis, drugs of abuse [18–21], food composition [22–24], and metabolomics [25]. Introduced nearly two decades ago, MEPS has continuously evolved and is now available in a wide range of formats and extraction devices [26]. When coupled with gas chromatography, MEPS can be easily automated at-line using suitable autosampler instruments [27]. On the other hand, when used in conjunction with liquid chromatography, MEPS is typically performed in an offline and non-automated manner, which can be tedious and prone to errors due to the multiple draw/eject cycles required in each stage [28].

While several commercial solutions for semi- or fully automated MEPS are currently available, their high cost and limited accessibility pose challenges for many laboratories [29]. Consequently, researchers have actively pursued strategies to automate MEPS and increase its affordability. For instance, Serenjeh et al. successfully developed a semi-automated setup for MEPS, specifically focusing on the extraction of volatile polycyclic aromatic hydrocarbons in soil [30]. Our research group has also made significant contributions in this field by developing a Cartesian robot capable of complete automation and online coupling to high-performance liquid chromatography (HPLC) for various microextraction techniques, including MEPS [31–34]. Moreover, our team has designed a dedicated multi-syringe autosampler specifically tailored to enable the development of high-throughput MEPS methods. This innovative autosampler has demonstrated excellent performance and versatility, for example, in the MEPS of cannabinoids from human urine [35].

On the other hand, the commercial versions of MEPS face limitations due to the non-repackable nature of barrel insert and needle (BIN) devices, as well as the limited availability of extraction phases [36, 37]. So, the development of alternative MEPS devices that allows the exploitation of alternative or lab-made sorbents at reduced cost and with a high degree of reusability also becomes pertinent to make the technique more affordable and accessible. For example, our research group has introduced repackable MEPS devices, adaptable to gastight syringes [38], and cheaper versions in propylene Luer Slip syringes [39], allowing the exploration of diverse lab-made innovative sorbents.

In this study, we demonstrate the association of an easily repackable lab-made MEPS device with the Arduino-controlled multi-syringe lab-made robot as an alternative method for the development of sensitive, reliable, and high-throughput MEPS methods for environmental surveillance. To illustrate its effectiveness, we selected methylparaben (MeP), propylparaben (PrP), butylated hydroxyanisole (BHA), and oxybenzone (Oxi) as model compounds representing endocrine disruptors from the families of parabens, benzophenones, and synthetic phenolic antioxidants. A MEPS-UHPLC-MS/MS method was developed to determine these compounds in wastewater samples, demonstrating to be a competitive, fast, and sustainable analytical tool for environmental analysis.

Material and methods

Standards and reagents

MeP, PrP, BHA, and Oxi were procured from Sigma-Aldrich (St. Louis, MO, USA). The standard stock solutions were prepared by dissolving the analytes (1 mg mL^{-1}) in methanol and stored in amber bottles at – 20 °C. Working solutions

were freshly prepared on a daily basis. For detailed information on the physicochemical characteristics and compound structures, please refer to Table S1, which is available in the supplementary material.

Methanol and acetonitrile of chromatographic analysis grade were obtained from Tedia (Fairfield, OH, USA). Ammonium hydroxide (MS grade, 98%) was obtained from Fluka (Buchs, Switzerland), and ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA), both used as a buffer in the aqueous mobile phase. Ultrapure water was generated using a Milli-Q purification system (Millipore, USA). Formic acid (98%) was obtained from Fluka (Darmstadt, Germany).

For the development of the MEPS method, several commercial extraction phases were evaluated, including Strata-X (33 µm particle size, from Phenomenex), Strata C18-E (55 µm particle size, from Phenomenex), Oasis MAX (30 µm particle size, from Waters), and BAKERBOND spe Amino (40 µm particle size, from Phenomenex).

Samples

Lab-made wastewater samples were prepared following the procedure described by Gomes et al. [40]. These samples were spiked with the compounds and used to create controlled samples for method development and subsequent analytical validation. As for the real sample, water was collected from Rio Monjolinho in São Carlos (SP, Brazil), a creek that receives both treated and untreated sewage discharges. The collected water was filtered through a 0.22-µm cellulose membrane and stored in amber flasks at − 20 °C.

Automated MEPS

The extraction procedure using MEPS was carried out with a Hamilton 500 µL gastight syringe (Nevada, USA) with a removable needle. A modified ferrule nut was inserted between the needle and the syringe, which housed a lab-made repackable MEPS device. This extraction microdevice (Fig. 1a) consists of three PTFE detachable parts (base, body, and lid) and two stainless steel screens of 10-µm porosity (patent BR1020130254517) [41]. This repackable microcartridge can accommodate approximately 2.0 mg of any desired sorbent phase, commercial or lab-synthesized. Once the microdevice is sealed with the sorbent phase inside, the syringe can be integrated in a programmable robotic apparatus.

The automated MEPS procedures were performed using a lab-made multi-syringe autosampler (patent pending BR1020180046080), which consists of a Cartesian robot built of aluminum parts (Fig. 1b). The prototype had a horizontal rack platform with six syringes trails, and each trail could hold eight different containers to use for solvents or

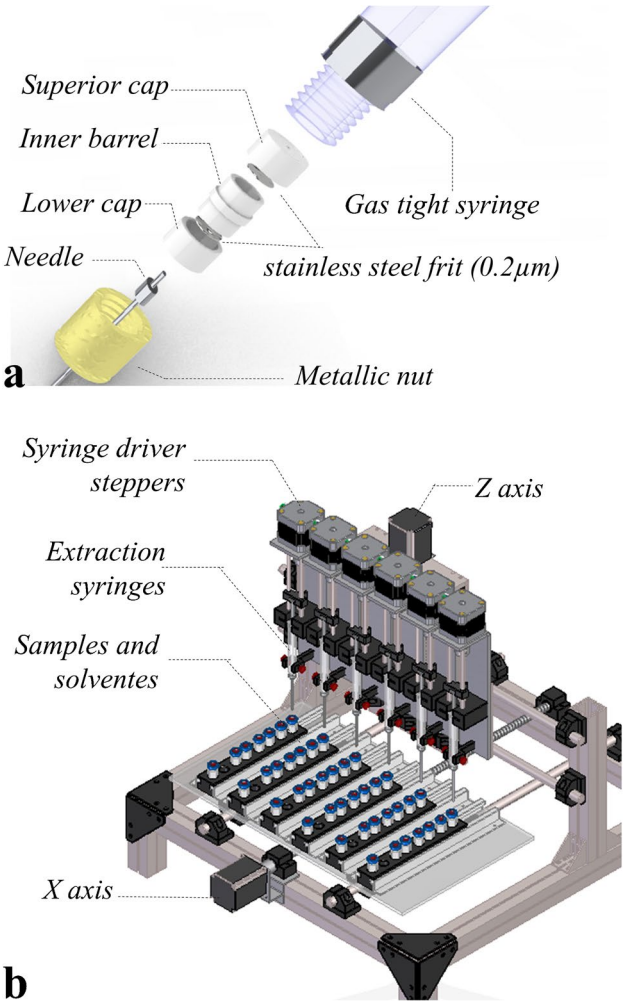

Fig. 1 Instrumental setup for the performance of robot-assisted MEPS. **a** Schematic representation of the lab-made replaceable MEPS device. **b** Schematic representation of the lab-made multi-syringe robot

samples. Linear actuators and stepper motors were employed for spatial positioning. The commanding electronic circuit contains an Arduino® Mega 2560 microcontroller board, which controls the eight stepper motors: one for each syringe, plus one motor for the horizontal platform, and another to move the array of syringe drivers. The prototype was controlled via USB from a personal computer, utilizing pre-programmed instructions written in the Arduino Integrated Development Environment (IDE) [31, 35].

Optimization of the automated MEPS procedure

Univariate and multivariate analyses were performed to thoroughly assess the influence of key parameters on the performance of MEPS.

The selection of the MEPS sorbent was carried out through a univariate comparison of extraction performance

using Strata-X, Strata C18-E, Oasis MAX, and Amino. Repackable MEPS devices were filled with approximately 2.0 mg of each sorbent, and extractions of lab-made wastewater samples fortified with 200 µg L^{-1} of each analyte were performed in triplicate to assess the relative extraction efficiency of the tested sorbents. The MEPS protocol used for evaluating the sorbent phases is provided in Table S2.

Subsequently, a 2^{6-2} fractional factorial experimental design was employed to identify the steps of the automated MEPS procedure that significantly influenced the extraction of EDs (Tables S3-S6). The volumes of each step were maintained at a constant 500 µL, while the number of draw/eject cycles was varied between 3 (−) and 20 (+). To determine the optimal conditions (volumes and number of cycles) for the most influential step (sampling), a central composite design was conducted over an experimental range of 100 ($-\sqrt{2}$) to 500 ($\sqrt{2}$) microliters for volume and 2 ($-\sqrt{2}$) to 20 ($\sqrt{2}$) for the number of cycles. The complete experimental design is presented in Table S7, and the data obtained from these analyses were processed using Statistica 13 software (StatSoft Inc., Tulsa, USA, 2013).

HPLC–UV-Vis method

The development of the MEPS method was conducted using HPLC–UV-Vis. This encompassed the selection of the sorbent phase, determination of the most influential step in MEPS, and subsequent optimization. The HPLC–UV-Vis consisted of a Shimadzu (Kyoto, Japan) LC system consisting of a CBM-20A communication bus module, SIL-20AC autosampler, DGU-20AS degasser, two LC 20-AD pumps, CTO-20A column oven, and SPD-20A UV–Vis detector set to monitor at wavelengths of 250 nm and 290 nm. The mobile phases selected were water and acetonitrile, both containing 0.1% formic acid. The flow rate was set to 0.250 mL min^{-1}. Initially, an isocratic elution with 12% acetonitrile was programmed for 5 min. From 5 to 15 min, gradient elution was performed by linearly increasing the acetonitrile content from 12 to 90%. Finally, in the subsequent 5 min, a linear change was made to return the organic modifier to 12%.

UHPLC-MS/MS method

The analytical validation of the method and the analysis of the real sample were carried out using a UPLC Acquity Waters coupled to a triple quadrupole XEVO TQ-MS system. Chromatographic separation was achieved using a Kinetex EVO C18 analytical column (150 mm × 2.1 mm, 5 µm) from Phenomenex (Torrance, CA, USA), which was maintained at a temperature of 40 °C. Water with acetate buffer (25 mM ammonium acetate + 25 mM ammonium hydroxide) and acetonitrile were used as mobile phases A and B, respectively, at a flow rate of 0.25 mL min^{-1}. Initially, an isocratic elution was programmed with 10% of acetonitrile for 2 min. Gradient elution was then performed from 2 to 3 min, with a linear increase in acetonitrile content from 10 to 90%. From 3 to 6 min, an isocratic elution was kept with 90% of acetonitrile, then changing linearly to 10% of organic modifier in 1 min, and finally kept in 10% of acetonitrile for 5 min (7 to 12 min) for conditioning of the analytical column.

For MS/MS detection, the operation and data acquisition were performed using Waters MassLynx 4.1 software (Milford. MA, USA). The optimal parameters for ED detection were selected by direct infusion of aqueous solutions. The analysis was carried out using the multiple reaction monitoring (MRM) acquisition mode, with negative electrospray ionization (ESI−) employed. Monitored transitions and selected operational parameters are shown in Table 1. Nitrogen was used as a desolvation gas at a temperature of 400 °C and a flow rate of 800 L h^{-1}. The source temperature was set to 150 °C.

Method validation

The developed method was validated using the matrix-matching calibration approach and lab-made wastewater samples. Method selectivity was ensured through MS/MS detection, where precursor ions and two transitions provided at least four identification points (IPs) for each of the investigated analytes [41]. The calibration curves were generated in triplicate by utilizing six levels of concentration. To determine the LODs and LOQs, a series of successively diluted

Table 1 UHPLC-MS/MS parameters for the detection of EDs

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Cone energy (eV)	Collision energy (eV)
MeP	3.48	151	92[a]/136	0.065	30/30	15/15
PrP	3.65	179	92[a]/136	0.065	30/30	20/15
BHA	3.82	179	164[a]/149	0.065	20/20	15/20
Oxi	3.97	227	211[a]/167	0.065	30/30	20/30

[a]quantification ion

samples were prepared and injected until reaching the lowest concentrations that yielded a signal three times and ten times higher than the noise, respectively. Precision was assessed under both inter- and intra-day conditions at two concentration levels, with a sample size of $n = 6$, employing the LC–MS/MS method.

The matrix effect (ME), total process efficiency (PE), and recovery (RE) were studied following the approach outlined by Matuszewski et al. [42]. In this case, the *ME* was calculated as $(B/A) \times 100$, *PE* as $(C/A) \times 100$, and *RE* as $(C/B) \times 100$. Here, *A* represents the analytical response for the direct injection of a standard solution of the analytes at a concentration equivalent to 100% MEPS efficiency. *B* represents the analytical response for the injection of extracts obtained after MEPS of spiked matrix samples (1.0 ng L^{-1}). *C* represents the analytical response for the injection of a solution prepared by spiking an extract obtained by MEPS of blank matrix samples at a concentration equivalent to 100% MEPS efficiency.

Results and discussion

Development of the MEPS method

MEPS has been widely utilized as a sample preparation technique for the determination of EDs. For instance, Silvera et al. recently presented a method for analyzing 16 EDs (such as parabens, benzophenones, bisphenols, and triclocarban) in human urine using commercial MEPS syringes with C18 as the extraction phase [42]. Similarly, Matin et al. investigated the effectiveness of a montmorillonite-reinforced polystyrene nanocomposite coated onto cellulose filter paper as an extraction sorbent in MEPS for the layered extraction of fluoxetine from environmental water and wastewater samples [43]. In both studies, the development of the MEPS method involved assessing the extraction phase, determining the necessary MEPS stages (conditioning, sampling, washing, drying, elution, and clean-up), and defining the volumes and number of draw/eject cycles to be employed in each stage. This rational workflow closely aligns with the approach we have adopted for the development of our MEPS method.

Selection of the extraction phase

As previously mentioned, EDs comprise a wide range of compound families with diverse molecular structures and properties. Therefore, the selection of an appropriate sorbent for extracting these compounds depends on the specific compound family under investigation. Currently, active research is focused on synthesizing and evaluating new extraction sorbents to develop advanced strategies for detecting EDs in water samples.

One notable trend in this field is the emergence of magnetic solid-phase extractions (MSPE), which employ magnetic sorbents like porous carbons [44, 45], graphene [46], covalent organic frameworks (MOFs) [47], chitosan [48], and histamine [49], among others. Additionally, other sorbent-based microextraction methods are being explored, including the use of coated devices with molecularly imprinted polymers [50] and packed bed-based techniques like sorptive stir bar microextraction with packed membranes. Some examples of these techniques include the use of cyclodextrin-based sorbents in stir bar sorptive extraction with packed fibers [51] and the use of some commercial phases such as divinylbenzene grafted polyvinylpyrrolidone (DVB@PVP) in SPE [52] and C18 in SPE [53] and MEPS [54].

In this study, to assess the performance of a proposed automated setup, utilizing a repackable MEPS device for the determination of EDs in wastewater samples was assessed. The commercial sorbents Strata-X, C18, Oasis Max, and aminopropyl silica were evaluated. 2.0 mg of each extraction phase was packed in the MEPS device, and extractions were performed as described in the "Optimization of the automated MEPS procedure" section. Subsequently, the obtained extracts underwent analysis using HPLC–UV-Vis, and the resulting chromatographic areas for each compound were plotted in Fig. 2a.

To investigate the statistical significance of the chromatographic areas obtained with each tested sorbent, a two-way analysis of variance (ANOVA) test was conducted (Table S8). In all cases, the calculated *F* values exceeded the critical *F* value, and the corresponding *p*-values were below the pre-determined significance level ($\alpha = 0.05$). These findings indicate the presence of statistically significant differences in the recovery of each analyte with each extraction phase and the extraction efficiency of the different analytes by each sorbent.

Further analysis of the ANOVA results was performed using a Tukey test (Table S9). The test revealed that, with an honestly significant difference observed in the chromatographic area of 1268.3, all the chromatographic areas exhibited statistically significant differences, except for the extractions of MeP with the amino and C18 phases, BHA with Strata-X and C18, and Oxi with amino and Oasis Max.

Among the tested sorbent phases, Strata-X consistently demonstrated higher chromatographic areas for all the tested analytes and exhibited suitable precision with a relative standard deviation (RSD) below 10%. Consequently, Strata-X was selected for the subsequent stages of the extraction method development.

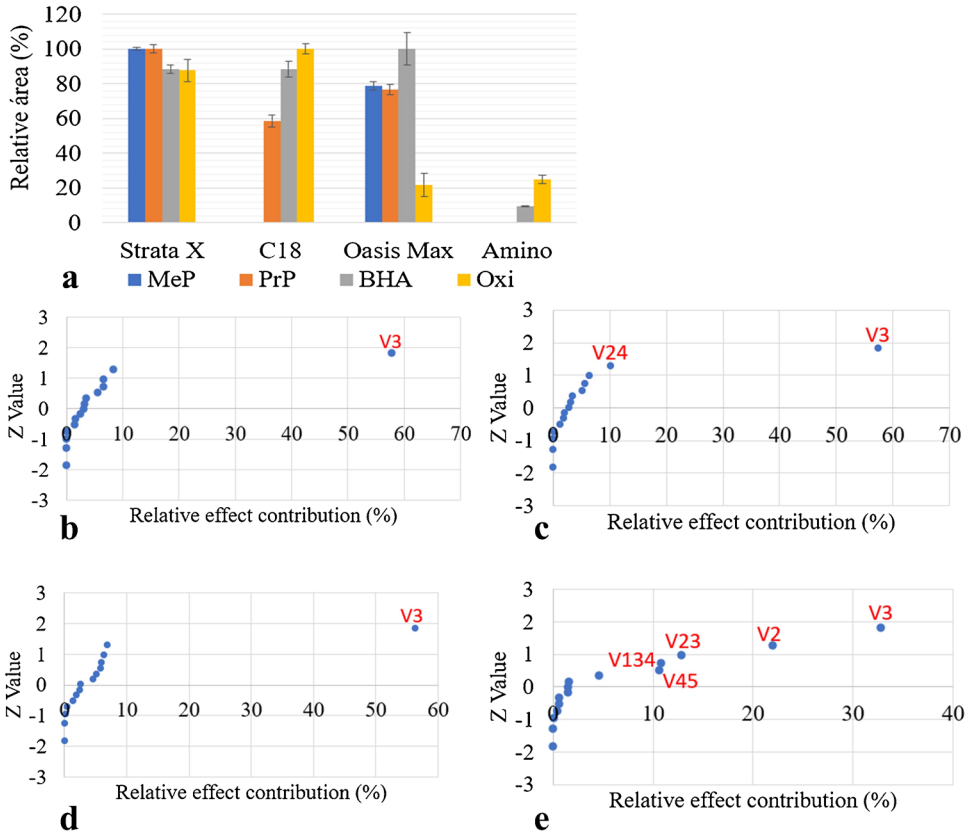

Fig. 2 Selection of the best MEPS conditions. **a** Selection of the extraction phase; normal probability plot of the effects for the 2^2 factorial experimental design for **b** methylparaben; **c** propylparaben; **d** butylated hydroxyanisole; and **e** response surface for oxybenzone

Determination of the most influential MEPS steps

The MEPS process involves several stages, including sorbent conditioning, sampling, washing, drying, desorption, and clean-up cartridge. To assess the impact of each stage on the process's efficiency, a 2^{6-2} fractional factorial experimental design was utilized (see Table S3-S6).

The influence of each MEPS stage was evaluated by constructing normal probability plots, which plotted the *Z* value against the standardized percentage effect. The effects were calculated by determining the difference between the responses obtained at the high and low levels for each variable, including their interactions. The percentage contribution of each variable's effect was calculated by summing the squared effects and their contribution to the total sum. These calculated effects are associated with equal areas of a Gaussian curve. In a normal probability plot (*Z* value vs. % standardized effect), smaller effects tend to cluster around zero, while statistically significant effects deviate from zero.

To determine the magnitude of each MEPS stage's impact on the chromatographic peak area, probability plots (Fig. 2b–e) were analyzed. Although other MEPS steps, such as water conditioning (v2), exhibited a relatively significant effect on the extraction performance of oxybenzone, the sampling (v3) stage emerged as the most influential. It accounted for approximately 60% of the increase in chromatographic area for MeP, PrP, and BHA and over 30% for Oxi. Consequently, we optimized the sampling stage using a multivariate experimental design.

Fine-tuning of the sampling step

To optimize the MEPS procedure and develop a fast and efficient method, the sampling step was meticulously refined using a surface response methodology. The conditioning, clean-up, elution, and washing steps were maintained at a lower level of 100 µL and three draw/eject cycles. A central composite design was employed, with the sample volume and the number of draw/eject cycles as the variables of interest. The experimental range for the sample volume was set between 100 and 500 µL, while the number of cycles ranged from 2 to 20. Figure 3 displays the surface responses obtained from the conducted experiments, and the ANOVA tables for the resulting models can be found in the supplementary information (Tables S10-S13).

The models derived from the experiments included six coefficients: an independent term, two linear coefficients for the number of cycles and volume, two square coefficients for these variables, and two coefficient products representing the interaction between the variables. However, not all coefficients were statistically significant ($p < 0.05$). Nevertheless, with a probability higher than 95%, none of the models

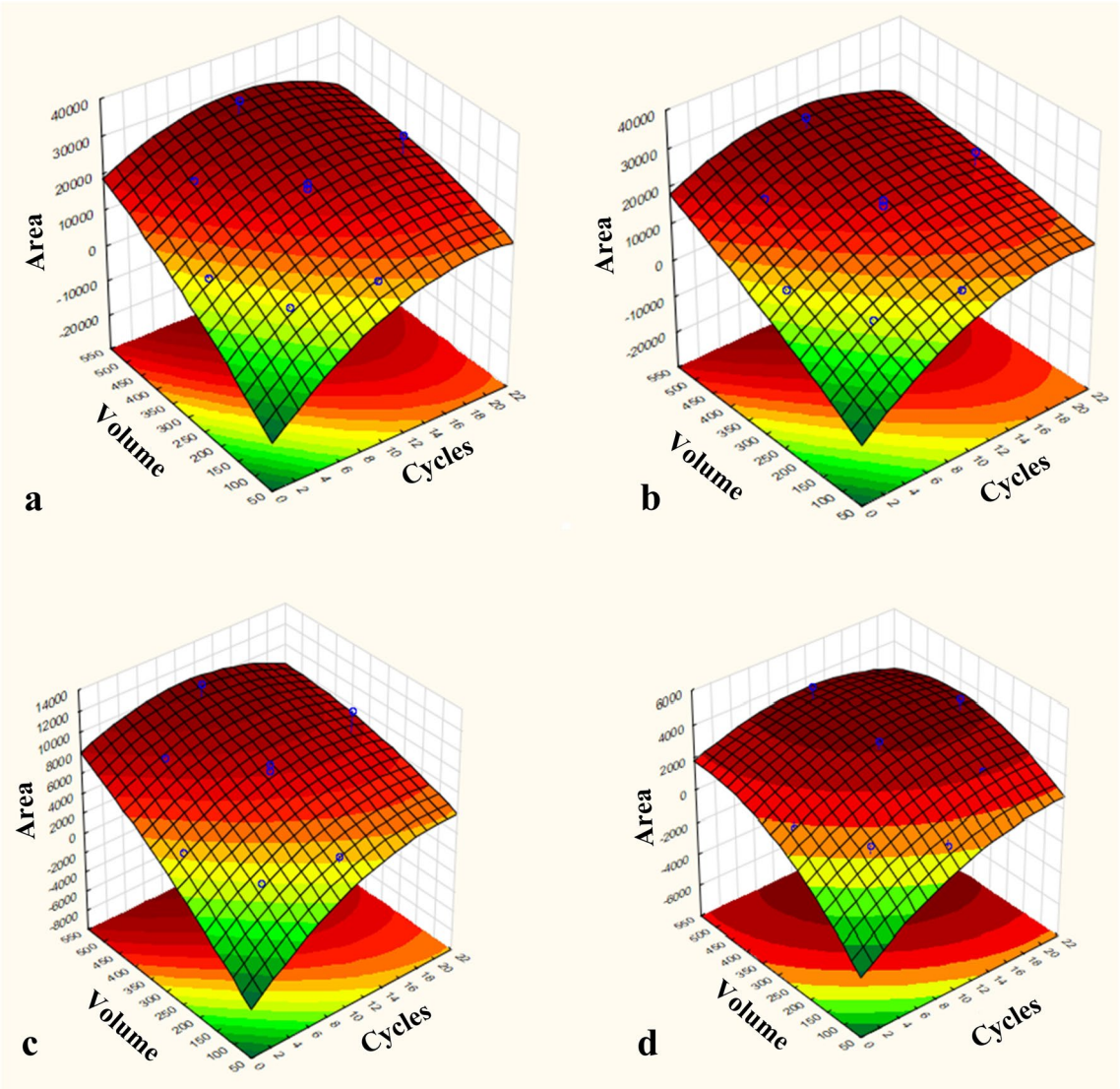

Fig. 3 Multivariate optimization of the sampling stage. **a** Response surface for methylparaben. **b** Response surface for propylparaben. **c)** Response surface for butylated hydroxyanisole. **d** Response surface for oxybenzone

exhibited a significant lack of fit. These results indicate that the obtained models effectively represent the experimental data and provide reliable predictability of the analytical responses, to obtain the experimental conditions capable of providing the maximum chromatographic area.

The highest analytical responses for all analytes were observed when passing 500-µL aliquots of the sample through the packed bed approximately 10 to 16 times. Based on these findings, the optimal conditions for the sampling stage were determined as a sample volume of 500 µL and 14 draw/eject cycles. Implementing these conditions in the MEPS procedure ensures a more effective and improved sampling step, resulting in better outcomes for all analytes.

Finally, the MEPS procedure was developed, which comprised seven steps: conditioning the extraction phase with organic solvent (methanol), conditioning the extraction phase with aqueous solvent (water), aspirating the sample (wastewater), washing the extraction phase (water), drying (air), eluting the analytes (acetonitrile), and cleaning the extraction phase (methanol). The specific volume and number of cycles for each step were determined and are presented in Table 2.

Analytical performance of the MEPS-LC–MS/MS method

Under the selected experimental conditions, the analytical performance of the proposed automated MEPS method was evaluated by considering some figures of merit. These

Table 2 Description of the developed MEPS procedure

Step	Cycles	Volume cycle (µL)	Volume vial (mL)
Conditioning sorbent (methanol)	3	100	1.5
Conditioning sorbent (water)	3	100	1.5
Sampling (sample)	14	500	1.5
Washing (water)	3	100	1.5
Drying (air)	3	100	-
Eluting (acetonitrile)	20	100	0.1
Sorbent cleaning (methanol)	10	100	1.5

include selectivity, LOD, LOQ, linearity, linearity range, precision, and enrichment factors (Table 3).

The selectivity of the developed method was successfully achieved through the utilization of MS/MS detection. Confident identification of the analytes was accomplished by obtaining a minimum of four identification points (IPs), encompassing known retention times from standard injections, precursor ions, and two transitions [41]. To ensure accurate quantification of the analytes with the necessary selectivity, black lab-made wastewater samples were subjected to extraction and analysis. No endogenous or exogenous interferences at the same retention time as the analytes were observed in any extracted ion chromatogram for every monitored MS/MS transition. This confirmation of selectivity for both quantification and confirmation ion transitions across all analytes guarantees the suitability of the chromatographic separation and the method's MS/MS detection selectivity for accurate quantification.

The LODs and LOQs were determined experimentally through a series of successive injections using consecutively diluted solutions. As a result, LODs and LOQs were established as the minimum concentrations capable of generating signal-to-noise ratios greater than 3 and 10, respectively, for the two monitored transitions of each analyte. The corresponding values can be found in Table 3.

This experimental determination ensures that each analyte can be detected with signal-to-noise ratios higher than 3 for both monitored transitions. Additionally, at the reported LOQ (which corresponds to the first point on the calibration curve), analytes not only can be identified with a signal-to-noise ratio higher than 10 for both transitions, but they could also be quantified with the appropriate level of precision, as indicated by the relative standard deviation (RSD). The obtained LOQs are in agreement with the criteria established for the regulatory agencies for determining EDs in wastewater samples, which depend on the ED compound range in the pg-ng L^{-1} order [55].

Matrix-matched calibration curves were established using MEPS of spiked lab-made synthetic wastewater samples, followed by UHPLC-MS/MS analysis (Table 3). The concentration range of the calibration curves spanned from the LOQ of each analyte to 5.0 ng/mL, and the experiments were performed in triplicate. The obtained linear correlations demonstrated acceptable relationships across the studied concentration range, with coefficients of determination (r^2) ranging from 0.9807 to 0.9863.

Although the obtained r^2 values explained only slightly over 98% of the data variability, residue analysis revealed that the proposed regression models could predict concentration values with an error below 20%. Supplementary information (Figure S1) presents the plots of relative residues (%). For MeP, PrP, and BHA, a $1/x^2$ weighted calibration was employed to minimize the absolute sum of residues ($\sum$%RE) and ensure the heteroscedasticity of the data. Conversely, no improvement in residue behavior was observed for Oxi with the application of weighting, rendering a weighted calibration unnecessary for this analyte. Notably, relative residues remained below 20% for all cases, indicating a highly acceptable level of accuracy in predicting sample concentrations within the investigated concentration range.

An unusual relationship between the intercept and slope values was observed in the calibration curve for oxybenzone. Various factors, including instrument-specific effects, matrix interferences, and experimental limitations, could contribute to this phenomenon. While there is limited literature explaining this fact, some of these factors can significantly impact the extraction and mass spectrometry detection of oxybenzone. For instance, during the development of an SPE method for determining household chemicals using GC and LC–MS/MS, Threnholm et al. reported that although oxybenzone was easily extracted from reagent water during

Table 3 Parameters obtained from the qualification of the developed analytical method

Analyte	Linear range (ng L^{-1})	Slope (ng L^{-1})	Intercept	R^2	LOD (ng L^{-1})	Intra-day RSD (%)		Inter-day RSD (%)	
MeP	0.3–5.0	3656.3	728.7	0.9889	0.15	6[a]	3[b]	11[a]	10[b]
PrP	0.15–5.0	7897.4	930.1	0.9869	0.05	7[a]	9[b]	10[a]	7[b]
BHA	0.6–5.0	3711.7	457.8	0.9827	0.3	20[b]	21[c]	17[b]	17[c]
Oxi	0.6–5.0	391.8	625.7	0.9807	0.3	17[b]	9[c]	15[b]	21[c]

$a = 0.5$ (ng L^{-1}), $b = 1.3$ (ng L^{-1}), and $c = 4$ (ng L^{-1})

method development, it exhibited poor recoveries in matrix spikes, which limited the analysis to qualitative observations [56]. The authors attributed the low recovery to the pH effect, noting that oxybenzone displayed improved recoveries at pH levels below 7 but poor recoveries at pH levels above 7. In their study, the pH of the reagent water ranged from 5.0 to 6.5, while the surface water and wastewater effluent had pH values ranging from 7.3 to 8.1, respectively. Similarly, although not providing a specific explanation, Chen et al., in their work on developing a method for screening chlorinated transformation products of aromatic pharmaceuticals and personal care products, reported the inability to detect oxybenzone using high-resolution mass spectrometry (HRMS) in spiked natural organic matter water samples [57]. These studies highlight the challenges associated with the detection and quantification of oxybenzone, particularly in complex matrices, and suggest that factors such as pH and the presence of organic matter can have a significant impact on its analysis.

Despite the atypical relationship between the intercept and slope values, the reliability and accuracy of the oxybenzone calibration model can be supported by considering other analytical parameters. Analysis of variance (ANOVA) confirmed the statistical significance of the regression for oxybenzone. The calculated F value of 815.13 for Oxi significantly exceeds the tabulated F value at a 0.05 probability level with 1 and 16 degrees of freedom (0.0041), indicating a highly significant regression. The ANOVA table for the oxybenzone linear regression can be found in the supplementary information (Table S14). Furthermore, as previously mentioned, the calibration curves were obtained in triplicate, resulting in relative standard deviation (RSD) values below 20% for all concentration levels and analytes. Additionally, the relative residues for the oxybenzone calibration curve were consistently below 20% across all concentration levels, further indicating the accuracy of the calibration model.

Precision was evaluated by calculating the intra- and inter-day relative standard deviations (% RSD) based on sextuplicate experiments at two concentrations for each analyte, as presented in Table 3. The intra-day RSD values ranged from 3.0 to 21.0%, while the inter-day RSD values ranged from 7.0 to 21.0%. These results demonstrate the method's capability to accurately quantify the target analytes within the linear range studied.

In the field of analytical methods employing mass spectrometry, matrix effects play a crucial role in influencing the performance of sample preparation and the efficiency of analyte ionization in the ESI source. To assess these effects accurately, we employed the methodology proposed by Matuszewski et al. to determine the matrix effect (ME), extraction recovery (ER), and process efficiency (PE), as described in the "Optimization of the automated MEPS procedure" section [58].

The RE provides an estimate of the influence of matrix constituents on MEPS performance which ranges from 60 to 76%. These values indicate that certain matrix constituents can be adsorbed during the extraction phase, impeding the uptake of analytes. Similarly, the PE values ranged from 47 to 75%, suggesting a significant decrease in analyte ionization due to the presence of matrix constituents. Furthermore, the ME, which represents the combined effects of matrix constituents on both extraction performance and ionization efficiency, varied from 79 to 112% (Table 4). These RE, PE, and ME values offer an estimation of the impact of matrix constituents on method performance and emphasize the importance of employing matrix-matched calibration when developing methods for the treatment and analysis of complex samples.

Although notable effects of matrix constituents were observed during both the MEPS and ESI processes, these effects do not hinder the applicability of the robot-assisted MEPS setup. This is evidenced by the determination of LODs, LOQs, and other performance parameters using the matrix-matched approach, which ensures that matrix effects are considered during their estimation.

Comparison with recently previously reported methods

Table 5 presents a comparison between the method proposed in this study and the recently reported sorbent-based methods for the determination of EDs in water samples. The automated MEPS-LC–MS/MS method demonstrated competitive performance in terms of detectability, feasibility, and sample throughput. Its automated nature, along with the efficient utilization of sorbents, samples, and organic solvents, contributes to its economic and green attributes. Likewise, the extended reusability of the extraction devices further enhances its sustainability.

In comparison to conventional manual [53] and automated SPE [52] methods, the setup proposed in this study achieves competitive limits of quantification (LOQs) while consuming significantly lower amounts of sorbent, samples, and elution solvents. When compared to other miniaturized techniques such as MEPS [43], SPME [50], and some dispersive techniques such as the magnetic effervescence-assisted

Table 4 Calculated matrix effect (ME), total process efficiency (PE), and recovery (RE)

Analyte	ME (%)	PE (%)	RE (%)
MeP	79 ± 3	47 ± 3	60 ± 6
PrP	67 ± 7	50 ± 4	76 ± 13
BHA	103 ± 1	70 ± 2	68 ± 2
Oxi	112 ± 6	75 ± 2	67 ± 4

Table 5 Comparisons of the performance of the method reported in this study with recent sorbent-based methods for determining endocrine disruptors in water sample

Technique	Analytes	Sorbent (amount)	Sample amount (mL)	Analysis	LOQs (ng/L)	Ref
MSPE	Triclosan, triclocarban, bisphenol A, and tetrabromobisphenol A bis(allyl)ether)	Fe3O4/N-HCSCs (5.0 mg)	10	HPLC–DAD	0.3–1.5	[45]
MNER-EM	Bisphenols, E3, EE2	NiFe2O4@COF	5	HPLC-FLD	86–316	[47]
SPME	Steroid hormones	Estrone-MIP	15	HPLC–UV	690–2600	[50]
SPE	Bisphenol A and nonylphenols	C18 (1000 mg)	1000	HPLC-FLD	10–21	[53]
Automated SPE	Steroid hormones and BPA	DVB@PVP (200 mg)	10	HPLC–MS/MS	0.5–3.0	[52]
MEPS	fluoxetine	Montmorillonite/OS	1.5	FL	7.0	[43]
Automated MEPS	MeP, PrP, BHA, Oxi	Strata-X (2.0 mg)	1.5	UHPLC-MS/MS	0.3–0.6	This study

sorbent-based extraction (MNER-ME) [47], the MEPS-LC–MS method reported in this study demonstrates LOQs up to ten times lower. Additionally, it offers the advantages of automation and high-throughput capabilities provided by the robot, allowing for the simultaneous preparation of up to six samples. Some other magnetic dispersive extraction, such as the one using a Fe_3O_4/N-HCSCs sorbent [45], are also highly efficient and fast techniques capable of providing comparable LOQs with small amounts of sorbent and samples. However, the reusability of the sorbents can be limited, and for example, in the case of the Fe_3O_4/N-HCSCs sorbent, it was limited to only 12 times. In contrast, our method utilizing a single packed device with 2.0 mg of extraction phase achieved more than 100 reuses without appreciable loss in its extraction capabilities.

Application of the developed method to the analysis of real wastewater sample

To demonstrate the applicability of the proposed setup in analyzing real samples, we collected a flask of running water from Rio Monjolinho in São Carlos, SP. Four extractions were performed, yielding the following concentrations: MeP: 0.58 ± 0.07 ng L^{-1}; PrP: 1.7 ± 0.06 ng L^{-1}; Oxi: 5.1 ± 0.6 ng L^{-1}; and BHA was not detected in the sample.

Conclusion

The utilization of an automated MEPS method for sample treatment has demonstrated its efficiency in determining some organic pollutants in environmental samples. Additionally, the application of factorial design proved to be practical in selecting the optimal extraction phase and parameters for sample treatment, resulting in enhanced extraction efficiency. Both the automated system and the MEPS device utilizing a commercial syringe offer excellent alternatives that can be implemented in the laboratory to meet the demands associated with the development of new analytical methods. The multi-syringe capability of the MEPS device offers the possibility of treating six samples simultaneously, enabling high-throughput characteristics to the developed method. Furthermore, the utilization of a microextraction technique makes it an excellent environmentally friendly option. Finally, the incorporation of MS/MS detection yielded exceptional LODs and LOQs even when using a reduced amount of sample. Consequently, the proposed approach represents an advantageous strategy for monitoring some organic pollutants as MeP, PrP, BHA, and Oxi in wastewater samples.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00216-023-04888-0.

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Declarations

Competing interests The authors declare no competing interests.

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CAPÍTULO 4 - Determination of parabens in wastewater samples via robot-assisted dynamic single-drop microextraction and liquid chromatography–tandem mass spectrometry

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RESEARCH ARTICLE

Determination of parabens in wastewater samples via robot-assisted dynamic single-drop microextraction and liquid chromatography–tandem mass spectrometry

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Abstract

Dynamic single-drop microextraction (SDME) was automatized employing an Arduino-based lab-made Cartesian robot and implemented to determine parabens in wastewater samples in combination with liquid chromatography–tandem mass spectrometry. A dedicated Arduino sketch controls the auto-performance of all the stages of the SDME process, including syringe filling, drop exposition, solvent recycling, and extract collection. Univariate and multivariate experiments investigated the main variables affecting the SDME performance, including robot-dependent and additional operational parameters. Under selected conditions, limit of detections were established at 0.3 μg/L for all the analytes, and the method provided linear responses in the range between 0.6 and 10 μg/L, with adequate reproducibility, measured as intraday relative standard deviations (RSDs) between 5.54% and 17.94%, ($n = 6$), and inter-days RSDs between 8.97% and 16.49% ($n = 9$). The robot-assisted technique eased the control of dynamic SDME, making the process more feasible, robust, and reliable so that the developed setup demonstrated to be a competitive strategy for the automated extraction of organic pollutants from water samples.

KEYWORDS
automated sample preparation, liquid chromatography, liquid-phase microextraction, mass spectrometry, single-drop microextraction

1 | INTRODUCTION

Parabens are organic compounds with an estrogenic activity, the presence of which in the environment can negatively affect the endocrine system of humans, aquatic organisms, and wildlife. However, due to their antibacterial and antifungal properties, parabens are still extensively used as preservatives in the food, personal care products, and pharmaceutical industries [1]. Hence, due to their massive consumption and irregular disposal, parabens are among modern life's most recurrent organic pollutants [2]. Therefore, their monitoring and quantitative determinations are primordial to grant the water quality and develop appropriate water remediation technologies.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is the paramount technique for analyzing

Abbreviations: BT, butylparaben; BzP, benzylparaben; CCD, central composite design; EP, ethylparaben; LLE, liquid–liquid extraction; ME, matrix effect; MP, methylparaben; PE, process efficiency; PrP, propylparaben; RE, recovery of the extraction procedure; SDME, single-drop microextraction; SPME, solid-phase microextraction.

organic pollutants from water samples [3, 4]. LC–MS/MS provides spread applicability scope and outstanding sensitivity and selectivity. However, surficial water is a complex sample rich in metals, biomolecules, humid acids, and other contaminants that impair the determination of trace organic molecules [1]. Therefore, an LC–MS/MS analysis of pollutants—such as parabens—from water samples should be preceded by an adequate sample preparation procedure to extract and concentrate the targeted analytes [5].

Organic pollutants have been mainly extracted from water samples via conventional solid-phase extraction and liquid–liquid extraction (LLE). Despite being very efficient, those techniques are laborious and consume large amounts of samples, sorbents, and toxic organic solvents, while generating considerable waste. Therefore, according to the precepts in green analytical chemistry, microextraction techniques have been replacing traditional procedures. Among these, single-drop microextraction (SDME) has emerged as an environment-friendly alternative to the traditional LLE and a cheaper technique than the consolidated fiber-based solid-phase microextraction [6–8].

SDME was the first reported solvent-based microextraction [9] and, nowadays, is a well-established technique with multiple applications in many analytical areas. SDME relies on using a needle-hanging drop of an organic solvent as extraction media, reducing solvent and sample consumption while maintaining the extraction efficiency [10–12]. The technique's performance depends on the physicochemical properties of the extraction solvent, the volume ratio between organic and aqueous phases, and the number of extraction batches, among many others [6].

In addition to its environment-friendly characteristics, SDME can provide good extraction capacity in an affordable, feasible, and inexpensive way. Although slower than other miniaturized LLE, SDME facilitates the uptake of the enriched extract without decantation, centrifugation, or solidification steps. However, the technique endures some drawbacks regarding the extractant drop's stability, especially under stirring conditions, elevated temperatures, and long extraction periods [10–12]. Besides, under the dynamic mode of extraction, the manual performance of the technique becomes more challenging. In this case, an amount of organic solvent larger than the drop volume is charged into the syringe barrel, so that a portion of the extraction solvent is recycled and renewed by performing a controlled sequence of drop exposition/retraction (draw/eject) cycles [13].

Although diverse examples of manual performance of SDME have been reported [14], the need to form stable and reproducible extractant drops with controlled volume and area surface makes SDME a technique highly dependent on automation—especially in the dynamic mode [15]. On-flow analysis techniques [16–18] and commercial Cartesian robots (autosamplers) have been versatile strategies employed in the automation of SDME [19]. Techniques, such as sequential injection analysis and flow injection analysis, have been preferred for coupling with spectrophotometric detectors [16, 20], whereas commercial autosamplers have been especially useful in coupling with gas chromatography [19]. In this case, the autosampler performs the stages of solvent loading, drop exposition, and extract injection, improving the technique's feasibility [21, 22]. Nevertheless, commercial autosamplers are costly, limited to a few preprogrammed functions, and the performance of SDME is not an available option in all cases.

Our research group has developed Arduino-based Cartesian robots dedicated explicitly to the automation of solid and liquid phase microextraction, which can be online hyphenated with liquid chromatography [23–26] and perform simultaneous multi-syringe extraction tasks [27]. This paper describes the robotic performance of dynamic SDME of parabens followed by their determination by LC–MS/MS. All the robot operational parameters involved in the SDME process were comprehensively studied. Under selected extraction conditions, the developed setup demonstrated high precision, reliability, and robustness in controlling the drop volume and performing the drop exposition/retraction cycles of the dynamic process.

2 | MATERIALS AND METHODS

2.1 | Analytical standards and reagents

Methylparaben (MP), ethylparaben (EP), propylparaben, butylparaben, and benzylparaben were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared by dissolving the analytes (1 mg/ml) in methanol and stocked in amber flasks at −20°C. Working solutions were prepared daily.

Dichloromethane, ethyl acetate, and acetonitrile (chromatographic analysis grade) were acquired from Tedia (Fairfield, OH, USA). Ultrapure water was produced in a Milli-Q purification system (Millipore, USA).

2.2 | Samples

Lab-made wastewater samples were prepared according to Lima Gomes et al. [28], spiked with the parabens, and used to prepare the controlled samples for method development solutions.

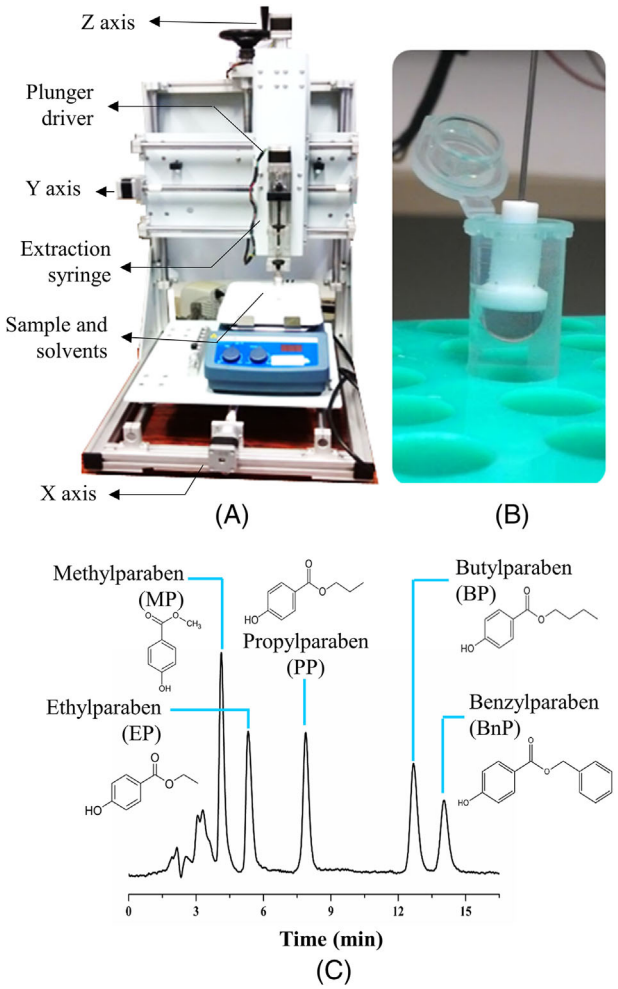

FIGURE 1 Instrumental setup employed in the automated SDME determination of parabens: (A) lab-made Cartesian robot; (B) needle-adapted large drop holder; (C) HPLC–UV–Vis Chromatogram of the tested parabens. HPLC, high-performance liquid chromatography; SDME, single-drop microextraction

2.3 | Automated dynamic SDME

For parabens extraction, a lab-made Arduino Cartesian robot (Figure 1A) was equipped with a 500-µl gastight syringe and a polytetrafluoroethylene (PTFE) adjustable needle endpoint drop-holder (Figure 1B), which facilitates the formation of large stable drops. The written Arduino sketch for automated dynamic SDME, containing the detailed description of the automated process, is available in the Supporting Information, and the procedure is briefly described as follows:

(i) The robot moves to the solvent position and loads 250 µl of extraction solvent into the syringe barrel.
(ii) The robot scrolls to the sample position, introduces the drop holder into 2 ml of lab-made wastewater, and slowly depresses the plunger to expose the extractant drop.
(iii) The robot performs the programmed sequence of drop draw/eject cycles to allow the continuous renovation/exposition of the drop solvent.
(iv) When the programmed number of draw/cycles is completed, the robot collects the drop solvent and dispenses it into a clean conic tube.

Finally, the extracts are dried under a nitrogen stream, reconstituted into 50 µl of ultrapure water for injection in the UV–Vis chromatographic system, and into 100 µl of acetonitrile for the analysis via ultra HPLC (UHPLC)–MS/MS.

2.4 | Optimization of the automated dynamic SDME procedure

Parameters independent of the Cartesian robot operation, such as extraction solvent, stirring, and salting-out effect, were investigated through univariate experiments. A 2^4 factorial experimental design was employed to determine the effect of drop volume (v1), drop exposure time (v2), drop draw/eject cycles (v3), and time between drops (v4). The complete 2^4 factorial experimental design is presented in Table S1. Finally, the extraction performance was studied by fine-tuning the drop exposure time and the number of drop draw/eject cycles through a central composite design (CCD), maintaining the drop volume and the time between drops at the lower tested levels (20 µl and 0 s, respectively). The experimental region ranged between 0 ($-\sqrt{2}$) and 26 ($\sqrt{2}$) draw/eject cycles and between 18 ($-\sqrt{2}$) and 103 ($\sqrt{2}$) s of drop exposition. The complete CCD, with different levels and exact values used, is shown in Table S2. Data obtained were processed by Statistica 13 (StatSoft, Inc., Tulsa, USA, 2013).

2.5 | HPLC–UV–Vis analysis

For SDME method development, chromatographic analyses were performed using a Shimadzu high-performance liquid chromatography (HPLC) equipment (20A Prominence), equipped with two LC-20AD pumps, a SIL-20A autosampler, a CTO-20A column oven, and a UV/Vis SPD detector 20A. A Poroshell 120 EC-C18 analytical column (100 mm x 3.0 mm; 2.7 µm) from Agilent Technologies was used. The analyses were performed in the isocratic mode, using a methanol/ethyl acetate (80/20):water (50:50) mixture as a mobile phase at a 0.23-ml/min flow rate. Figure 1C shows an example of the LC–UV–Vis separation.

TABLE 1 UHPLC–MS/MS parameters for the detection of the parabens

Analyte	Retention time (min)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Dwell time (s)	Cone potential (V)	Collision potential (V)
Methylparaben	2.52	151	92[a]/136	0.094	38/38	22/14
Ethylparaben	2.63	165	92[a]/136	0.094	38/38	24/16
Propylparaben	2.76	179	92[a]/136	0.094	42/42	24/16
Butylparaben	2.85	193	92[a]/136	0.094	42/36	24/15
Benzylparaben	2.81	227	92[a]/136	0.094	42/42	24/15

Abbreviations: MS/MS, tandem mass spectrometry; UHPLC, ultra high-performance liquid chromatography.
[a]Quantification ion.

2.6 | UHPLC–MS/MS analysis

The validation stage was performed in a more sensitive UPLC ACQUITY Waters system coupled with a triple quadrupole-type XEVO TQ–MS detector. Chromatographic separations were carried out in a Kinetex EVO C18 analytical column (150 mm x 2.1 mm, 5 µm) from Phenomenex (Torrance, CA, USA) with a temperature of 40°C. Water with a phosphate buffer (25-mM ammonium acetate + 25-mM ammonium hydroxide) and acetonitrile was used as mobile phases A and B, respectively, at a flow rate of 0.25 ml/min. Initially, an isocratic elution was programmed with 10% acetonitrile for 1 min. Then, gradient elution was performed from 1 to 2 min by linearly increasing the acetonitrile content from 10% to 80%. Next, from 2 to 4 min, an isocratic elution was kept with 80% acetonitrile, then changed linearly to 10% of organic modifier in 1 min, and finally kept in 10% acetonitrile for 3 min (5–8 min). For the MS/MS detection, the operation and data acquisition parameters were selected using Waters MassLynx 4.1 software (Milford. MA, USA). Parabens were detected in negative electrospray ionization(ESI−), and the optimal parameters were established by a direct infusion of standard solutions containing a mixture of tested parabens at a 10-µg/L level of concentration. MS/MS detection operational parameters and monitored transitions are shown in Table 1. Nitrogen was used as a desolvation gas at a temperature of 400°C and a flow rate of 800 l/h. The source temperature was set to 150°C.

3 | RESULTS AND DISCUSSION

An extraction procedure was designed and programmed in which, with the help of a lab-made PTFE drop holder, samples of 2 ml of wastewater, enriched with a mixture of parabens, were extracted with 250 µl of organic solvent upon sequential exposures by the direct immersion of different portions of the solvent in drops of between 20 and 60 µl. Once the extraction was completed, the organic solvent was dried, and the recovered analytes were redissolved in 50 µl and analyzed by HPLC–UV.

For the experiment described previously, an Arduino sketch was created. A 500-µl syringe is previously filled with the 250-µl organic solvent, which is operated by the prototype to (see the attached Video S1)

(i) fill the Teflon expander outside the sample (avoid the formation of bubbles air),
(ii) insert the device into the sample,
(iii) form the drop according to the size indicated by programming,
(iv) perform the exposure/retraction cycles according to the program, and
(v) collect the extract inside the syringe, transport it and deposit it in a conical Eppendorf tube for subsequent drying, resuspension, and analysis.

The written program for the execution of this experiment is available in the annexes.

3.1 | Study of the dynamic SDME influencing variables

3.1.1 | Univariate conditions selection

The extraction efficiency in SDME depends on a wide range of factors and their interactions. In the specific case of the proposed experiment, although the main determinant variables of the process can be easily controlled via the programming of the Cartesian robot and were studied using multivariate factorial planning, factors such as temperature, the nature of the solvent employed, the salting-out effect, the sample agitation, among others, should be preliminarily assessed in a univariate manner. Initially, the droplet volume was fixed at 60 µl, the droplet exposure time kept at 30 s, performing 11 exposure/retraction cycles and a time between droplets of 2.5 s.

Although a systematic investigation of the room temperature effect on the extraction efficiency was not conducted in this report, this variable proved to be a determinant of the reproducibility of the extractions and was strictly controlled at 25°C.

To the best of our knowledge, there are just two studies describing the extraction of parabens employing the SDME technique, and they report the use of hexyl acetate [29] and toluene [30] as extraction solvents. An extraction solvent suitable for SDME must be immiscible with the aqueous sample and provide a high analyte extraction capacity. In this case (direct immersion of big drops), an appropriate solvent additionally should be (i) dense enough to allow the formation of stable "big and heavy" drops under prolonged stirring but (ii) susceptible to fast evaporation for further resuspension of the analytes into a solvent compatible with the injection under reversed phase HPLC conditions. Big drops from solvents that are lighter than water are easily disclosed from the tip, and most of the solvents heavier than water are not volatile enough for short extract drying and resuspension. Based on these criteria, dichloromethane, ethyl acetate, and a mixture of dichloromethane/ethyl acetate (50:50, v/v) were evaluated as extraction solvents.

Unfortunately, ethyl acetate was not viable due to its partial and gradual miscibility in water, leading to unstable drops. On the other hand, as shown in Figure 2A, the mixture of dichloromethane/ethyl acetate (50:50, v/v) showed the best extraction efficiency for all parabens. Nevertheless, when that mixture is used, the extractant drop slowly fades by partial dissolution of the ethyl acetate, making the procedure unstable and susceptible to errors. That effect is more significant in prolonged extractions. So, dichloromethane was selected as the extraction solvent for further experiments.

Stirring is a simple parameter to modulate the extraction efficiency. High agitation rates favor the mass transference between the phases but can cause drop dislodgement. That parameter was evaluated at two levels, and, as shown in Figure 2B, the magnetic stirring of the sample (600 rpm) led to increments in the extraction efficiency of up to 40% without affecting the drop stability.

Finally, when an electrolyte is added to an aqueous solution, the solubility of the organic molecules decreases. This effect is known as the salting-out effect. However, in liquid-phase microextraction, this effect can be positive or negative as the increase in ionic strength can also increase the viscosity of the aqueous phase and reduce the transfer rate of the analytes between the phases. In general, this effect then depends on the characteristics of the analyte, the sample, and the type of salt used. Experiments with different additions of Na_2SO_4 (0 and 50, and 100 mg) were performed to assess the impact of ionic strength. The extraction efficiency decreased as the amount of sodium sulfate salt added increased (Figure 2C). Besides, the addition of small amounts of salt increased the density of the water sample and made extractant drop unstable. Therefore, further experiments were carried out without the addition of salt.

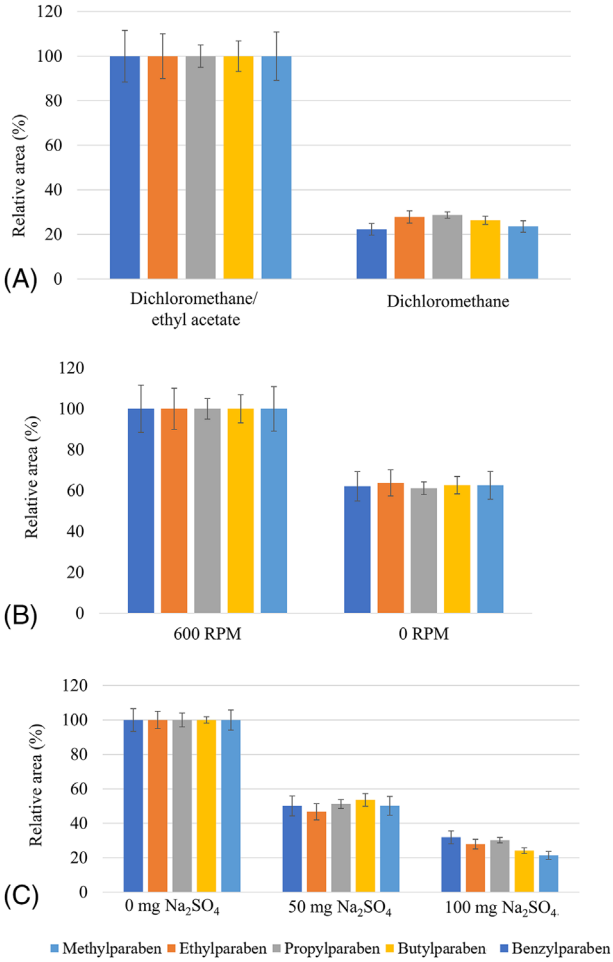

FIGURE 2 Univariate assessment of the relative extraction efficiency as a function of (A) the nature of the solvent; (B) shaking the sample; (C) adding salt

3.1.2 | 2^2 Factorial experimental design

Those variables influencing the extraction efficiency on SDME—such as the volume of the drops used, their exposure time, the number of extraction cycles performed, and the time of equilibrium between drops—can be easily controlled via the programming of the Cartesian robot. Therefore, these variables were studied through multivariate optimization in two stages: (i) the study of the effect of each variable on the chromatographic response via a factorial 2^4 experimental design and (ii) the determination of the optimal operating conditions via fine adjustment

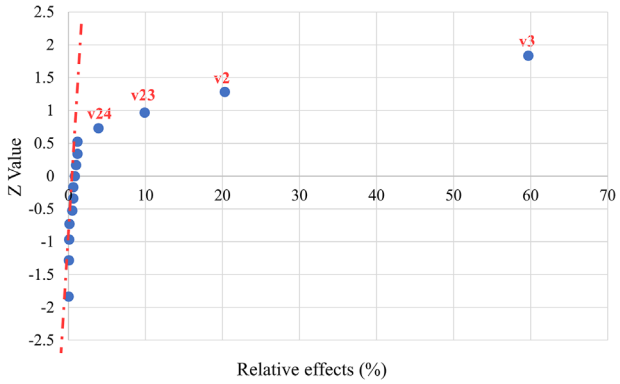

FIGURE 3 Normal probability plot of the effects for the 2^2 factorial experimental design. The negligible effects are close to zero on the *x*-axis (red line), and the significant effects (2 and 3) are far from zero. v1: drop volume; v2: drop exposition time; v3: number of draw/eject cycles; v4: time between drops

of the two more influencing variables by response surface methodology.

Initially, a 2^4 factorial design was performed (Table S1), and based on the chromatographic areas, the effects of each of the variables on the extraction efficiency were determined. Effects were calculated from the difference between responses at high and low levels for each variable and their interaction. Variables contributions were calculated as the percentage of each square effect concerning the summation square effect. The magnitude of the effect of each one of the variables on the total chromatographic response observed was determined from the probability graph shown in Figure 3. In this case, each of the calculated effects (as a percentage) is related to equal areas of a Gaussian curve. Each assigned area average is related to a *Z* value, and in a graph of *Z* value versus effects, minor effects are clustered around zero, whereas the more significant effects are far from zero.

The number of extraction cycles, the exposure time, and the interaction between these variables are responsible for more than 90% of the effects over the chromatographic response—the interactions between variables 2 and 4 and the second-, third-, and fourth-order interactions cause the 10% of the remained effects (Figure 3).

According to these results, variables 3 (number of extraction cycles) and 2 (drop exposure time) were selected to fine-tune the process using the response surface methodology, seeking to find extraction conditions that provide high analytical responses and suitable sample throughput robustly. As the effects due to the variables drop volume and time between drops (and their interactions) were responsible for less than 10% of the effects, they were kept at a low level in the execution of the subsequent experiments, trying to obtain the fastest method and with less solvent consumption.

3.1.3 | Central composite experimental design

Optimal conditions for carrying out a given experiment can be obtained by building mathematical models that describe the relationship between the response and the variables involved in the process. In analytical chemistry, these models are frequently built using the response surface methodology, and different types of experimental designs can be used for this purpose. For example, a CCD allows modeling the parabens extraction as a function of the number of extraction cycles and the exposure time of the drop in dynamic SDME. A surface response was obtained for each analyte.

For MP, a parabolic model was obtained, dependent on the linear coefficients for the number of cycles and the exposure time, and the quadratic coefficient of the exposure time (Figure 4A). The Pareto chart (Figure 4B) shows significant interactions between the studied variables. The model presented a good fit, demonstrating an excellent ability to predict the chromatographic responses of MP as a function of the number of extraction cycles and the drop exposure time (Figure 4C).

Similar models were obtained for ethyl, propyl, butyl, and benzyl paraben (Figures S1–S4). In all cases, the models did not lack adjustment and demonstrated the ability to predict responses as a function of the experimental values of the tested variables.

For all the analytes, the best analytical responses were obtained at the highest studied levels (30 extraction cycles and 103 s of drop exposure time). Despite the optimal point of the process will be beyond the studied experimental region, the highest studied levels already lead to time-consuming procedures (~1.0 h per sample). Looking for a more suitable sample throughput, we selected 20 extraction cycles and 90 s for the drop exposure as conditions for assessing the analytical performance of the method. As shown in the following section, although the analytical parameters were not established at the optimal conditions regarding the peak area/recovery response, the developed method demonstrated suitable detectability, and linearity, for the application, within a more reasonable time. Moreover, the robotic control provided suitable precision to work out of the optimal region.

3.2 | Analytical performance of the offline robot-assisted SDME–LC–MS/MS method

Under the selected experimental conditions, the analytical performance of the proposed automated dynamic SDME method was evaluated according to figures of merit such as selectivity, limit of detection (LOD), limit of quantitation

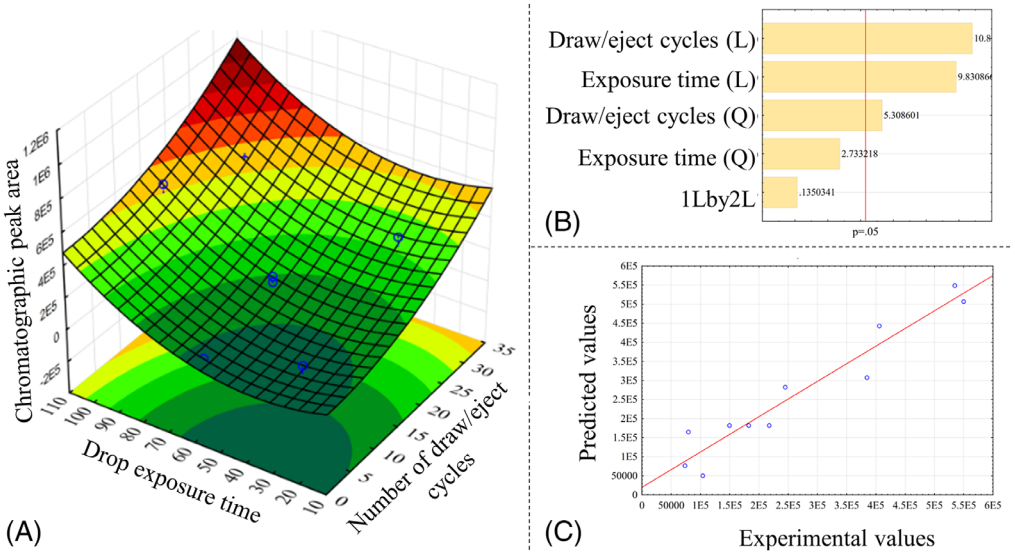

FIGURE 4 Droplet's exposure time versus the number of exposure cycles: (A) response surface for methylparaben; (B) Pareto graph for the generated model, showing the significance of the model's coefficients; (C) graph of correlation between predicted and observed values

(LOQ), linearity, precision, matrix effects (MEs), enrichment recovery, and the total efficiency of the process.

Method selectivity was ensured by MS/MS detection, monitoring one precursor ion and two fragment ions, and obtaining at least four identification points for each one of the tested analytes [31]. No endogenous or exogenous interferences were observed in the same retention time as the targeted analytes, confirming the MS/MS detection method's selectivity for appropriate quantification (Figure 5).

LODs and LOQs were determined experimentally through successive injections of consecutively diluted solutions until the minimal concentration was able to generate an analytical response with a signal 3 and 10 times higher than the noise for the 2 monitored transitions in the MS/MS chromatogram. Although lower and specific LOD and LOQ values for each one of the analytes could be obtained after fine-tuning, the reported values demonstrate the potential of the robot-assisted SDME–LC–MS/MS approach to provide sensitive methods.

Calibration curves were obtained in triplicate, with five concentration levels and in the range between 0.6 and 10 μg/L. Precision was assessed as inter- and intraday relative standard deviation (% RSD), at two concentration levels, with $n = 6$ and $n = 9$, respectively. Intraday RSD ranged between 5.54% and 17.94%, and interdays RSD between 8.97% and 16.49%, standing in good agreement with acceptable values for the measured concentrations (<20%) [32] (Table 2).

In this type of study, matrix constituents can interfere in the sample preparation step and cause suppression or enhancement of the ionization during the LC–MS/MS analysis. To assess those effects separately, we studied the ME, recovery (RE) of the extraction procedure, and overall "process efficiency" (PE), according to Matuszewski and collaborators [33]. That approach compares the analytical responses for

(i) *A*: Direct injection—without SDME—of mobile phase spiked at a concentration of the analytes equivalent to 100% SDME efficiency (40.0 μg/L).
(ii) *B*: Solution prepared by the reconstitution of a dried extract of blank matrix with mobile phase and containing the analytes at a concentration equivalent to 100% SDME efficiency (40.0 μg/L).
(iii) *C*: Solution prepared following the SDME of blank matrix spiked at 2.0 μg/L, meaning a theoretical 40.0-μg/L concentration in the reconstituted phase in case of 100% recovery.

The ME% was estimated as the ratio $(B/A) \times 100$ and, in this case, that value reflects the effect of the matrix interferents into the ionization process. The recovery (RE%) of the extraction procedure was calculated as the ratio $(C/B) \times 100$. The total PE was estimated as (C/A), and that value reflects the sum of the effects of the matrix interferents into the ionization and the SDME process. Table 3 summarizes the results.

The analytical responses for the reconstituted extract in the mobile phase after a blank matrix (*B*) extraction were higher than those obtained for the injection of the spiked mobile phase with no matrix interferences and at the same level of concentration (*A*). This result shows that matrix interferences remaining in solution caused enhancement

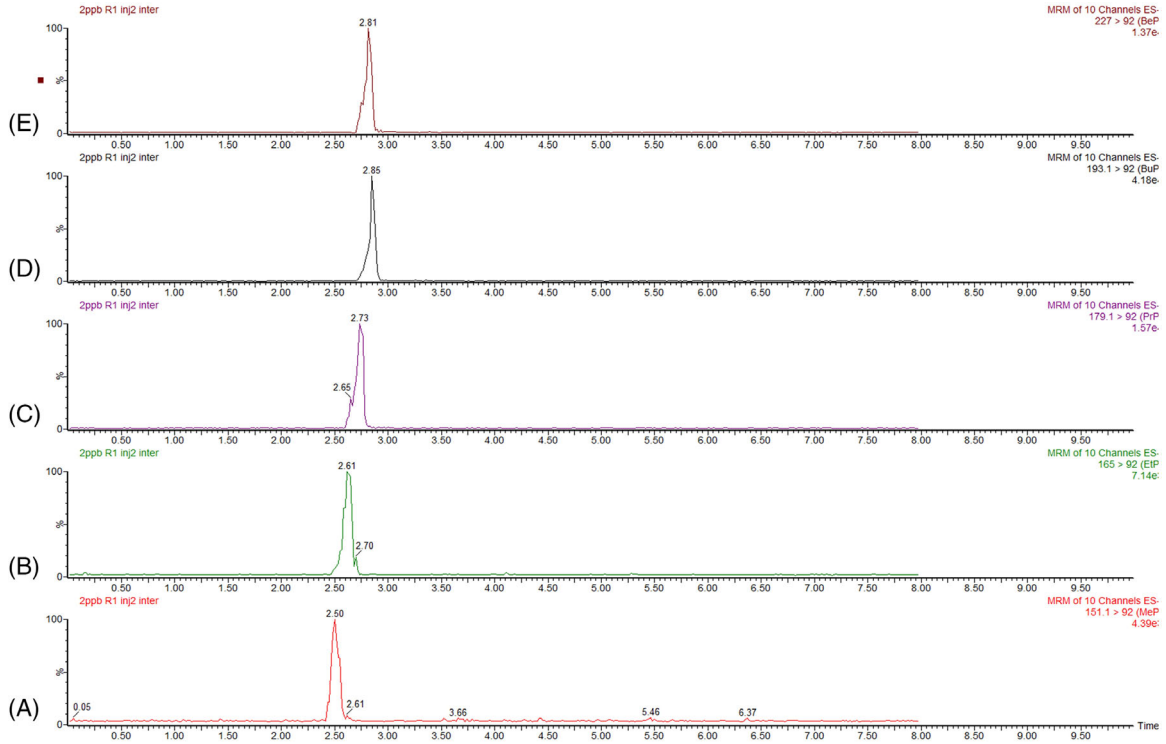

FIGURE 5 Extracted ion chromatogram of the five parabens analyzed after SDME of a wastewater sample (2 ml) spiked at 2 μg/L. SDME, single-drop microextraction

of the analyte's ionization. ME ranged between 119% and 131%, being suitable for method application in the analyses of wastewater samples. REs ranged between 22% and 72% and were directly proportional to the analytes' hydrophobic character (log *P*). The larger the log *P* of the analyte, its affinity for the organic phase, the higher the SDME performance.

Similarly, PE ranged between 27% and 94%, as a product of the previous two parameters. These results denote a significant influence of the SDME performance over the process's overall performance. Although RE was particularly low for MP and EP compounds (22% and 46%, respectively), that result does not hinder the method's applicability. Obtained LODs and LOQs demonstrated the dynamic SDME potential to provide sensitive methods. Furthermore, figures of merit were not assessed at the optimal condition in the studied analytical region. So, improvements in RE, PE, and detectability could still be obtained by performing more time-consuming SDMEs.

4 | CONCLUDING REMARKS

Although liquid phase microextraction is an intricate procedure—mainly due to the complications in handling small amounts of extraction solvent—the combination of miniaturization, automation, and experimental design offers a comprehensive range of possibilities to be explored in the development of modern sample preparation strategies. A multistage dynamic SDME procedure was success-

TABLE 2 Parameters obtained from the qualification of the automated dynamic SDME–LC–MS/MS method

Analyte	LOD (μg/L)	Linear range (μg/L)	Slope (L/μg)	Intercept	R^2	Intraday RSD (%)		Interday RSD (%)	
						0.6 μg/L	2.0 μg/L	0.6 μg/L	2.0 μg/L
MP	0.3	0.6–10	180.8 ± 0.004	4.710 ± 18.2	0.9898	15.73	6.03	12.72	8.97
EP	0.3	0.6–10	417.6 ± 0.418	19.43 ± 37.5	0.9919	14.85	6.49	14.14	11.78
PP	0.3	0.6–10	718.9 ± 0.012	102.65 ± 59.4	0.9931	8.45	4.56	6.89	13.85
BP	0.3	0.6–10	1337.2 ± 0.025	307.49 ± 109.4	0.9933	12.13	6.00	9.91	13.82
BnP	0.3	0.6–10	5367 ± 0.018	168.61 ± 79.28	0.9783	17.94	5.54	16.49	15.20

Abbreviations: EP, ethylparaben; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; MP, methylparaben; RSD, relative standard deviation; SDME, single-drop microextraction.

TABLE 3 Calculated ME, recovery (RE) of the extraction procedure, and, overall, PE

Analyte	Chromatographic peak areas			ME (%)	RE (%)	PE (%)
	C	*B*	*A*			
MP	436.5 ± 2 6.3	1620 ± 80.6	1979.2 ± 113.3	122.2	22.1	26.9
EP	970 ± 62.9	1796.3 ± 128.8	2130.1 ± 205.3	118.6	45.5	54.0
PP	1909.5 ± 87.1	2322.7 ± 86.5	2893.2 ± 307.7	124.6	66.0	82.2
BP	3862.5 ± 231.9	4339.7 ± 62.7	5619.4 ± 542.2	129.5	68.7	89.0
BnP	1685.2 ± 93.4	1685.2 ± 93.4	2351.4 ± 296.0	131.1	71.7	94.0

Abbreviations: EP, ethylparaben; ME, matrix effect; MP, methylparaben; PE, process efficiency.

fully automated in this work, demonstrating robustness, affordability, and reliability for routine applications. The flexibility of the Arduino sketch allowed the mechanical reproduction of all the dynamic SDME steps, making viable the exploration of experimental parameters hardly controllable in a manual way. The developed automated dynamic SDME demonstrated a competitive, straightforward, and environment-friendly approach for determining trace pollutants in surficial water samples by appropriately tuning the extraction variables. Future work will be directed toward the online integration of the robot-assisted sample preparation set up with chromatographic and mass spectrometry instrumentation.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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CAPÍTULO 5 - UHPLC capilar: aspectos teóricos e práticos

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UHPLC capilar: aspectos teóricos e práticos

Capillary UHPLC: theoretical and practical aspects

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Resumo

A UHPLC capilar é uma ferramenta bastante interessante. Ela une as principais características da UHPLC e da cromatografia capilar, como capacidade de trabalhar com pequenas quantidades de amostra e analises rápidas e eficientes. Embora esta técnica apresente características interessantes, não possui um grande número de publicações, estando restrita basicamente a separações cromatográficas na área de proteômica. Essa revisão apresenta os principais aspectos práticos e teóricos sobre UHPLC capilar. Fundamentos relevantes como efeito do diâmetro da partícula, efeito do diâmetro interno da coluna, efeito da pressão, aquecimento ficcional e efeito da parede são abordados de forma sucinta. São apresentadas também abordagens históricas sobre a evolução da UHPLC e da miniaturização de colunas. As principais características da instrumentação também são abordadas.

Palavras-chave: UHPLC capilar, colunas empacotadas, aquecimento friccional, efeito de parede, colunas capilares.

Abstract

Capillary UHPLC is a very interesting tool. It combines the main characteristics of UHPLC and capillary liquid chromatography, such as the capability to work with small amounts of sample as well as rapid and efficient analysis. Although this technique presents interesting characteristics, it is not present in a large number of publications, being restricted, basically, to chromatographic separations in the area of proteomics. This review presents the main theoretical and practical aspects about capillary UHPLC. Relevant fundamentals such as particle diameter effect, column inner diameter effect, pressure effect, fictional heating and wall effect are concisely described. A historical approach is presented on the evolution of UHPLC and the columns miniaturization. The main features of instrumentation are also addressed.

Keywords: Capillary UHPLC, packed columns, frictional heating, wall effect, capillary columns.

1. Introdução

A cromatografia líquida capilar é uma ferramenta extremamente interessante. Ela mantém as principais características da cromatografia líquida convencional (HPLC), porém utiliza vazões baixas (0,2 a 20 µl ml^{-1}) em colunas de diâmetros internos inferiores a 0,5 mm. A combinação desses dois fatores faz com que a escala capilar apresente vantagens em relação à cromatografia líquida convencional. O diâmetro reduzido facilita a aplicação de programação de temperatura em colunas capilares[1] e propicia o uso de vazões baixas, o que melhora o acoplamento com a espectrometria de massas com ionização por electrospray[2]. Adicionalmente, soma-se a esses fatores uma economia considerável no consumo de fase móvel, de amostras e também de fase estacionária.

Para um melhor entendimento sobre a evolução da UHPLC capilar, sugere-se a abordagem do assunto por duas perspectivas: a evolução da UHPLC convencional e a evolução da HPLC capilar.

1.1. Evolução da UHPLC convencional

Por volta de 1960, havia vários desafios para o desenvolvimento da cromatografia líquida. O principal deles era a otimização do empacotamento das colunas, por meio de melhorias em três fatores: (i) síntese de uma variedade de fases estacionárias que fossem mecanicamente e termicamente estáveis; (ii) redução do diâmetro médio das partículas (e sua faixa de distribuição de tamanhos) e (iii) produção de partículas esféricas de fases estacionárias[3].

Nesta mesma década (1960), Horváth e colaboradores utilizaram partículas peliculares de diâmetro entre 40-50 µm produzidas a partir de pequenas esferas maciças de vidro revestidas por uma fina camada porosa de fase estacionária. Essas partículas peliculares permitiam uma rápida transferência de massa e uma considerável melhoria nas separações, embora possuíssem baixa capacidade de carga de analito[4].

Em 1970 foram produzidas e utilizadas partículas totalmente porosas com diâmetros em torno de 10 µm. A redução do diâmetro das partículas permitiu um empacotamento mais denso, o que melhorou o leito cromatográfico[3]. Esse tipo de partícula (totalmente porosa) é um dos principais tipos de partículas utilizadas em HPLC atualmente, dividindo o espaço com partículas superficialmente porosas, estas mais recentes[5-7] (Figura 1).

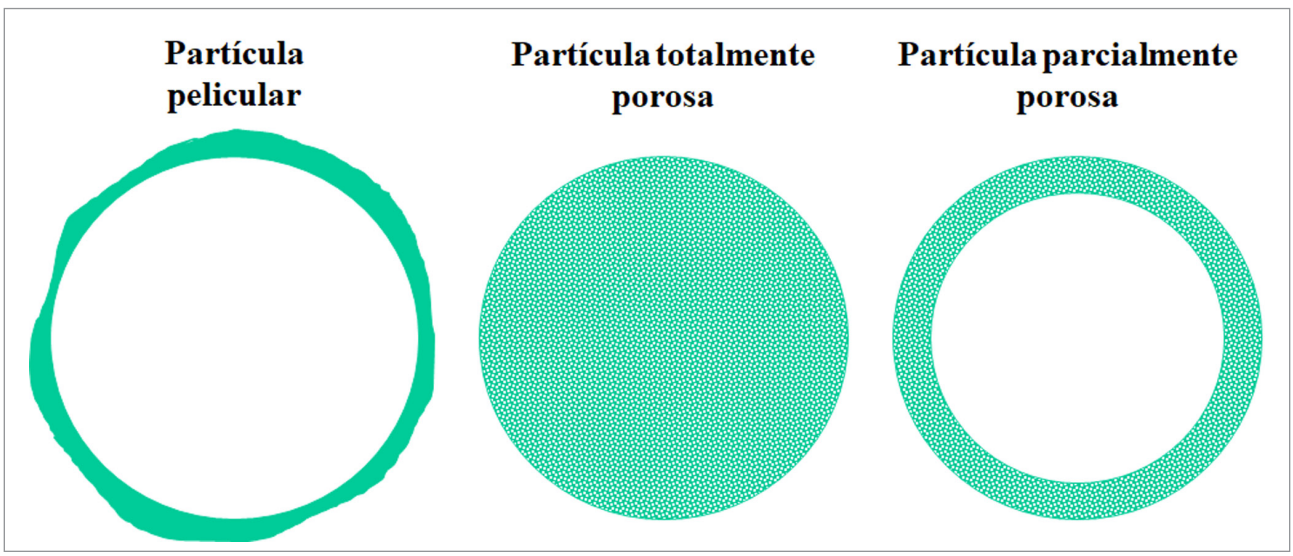

Figura 1. Representação de partículas peliculares, totalmente porosas e parcialmente porosas. A região em verde representa a camada na qual os analitos podem particionar e a região branca representa a conta de vidro utilizada em partículas peliculares e o núcleo sólido (sílica fundida) para as partículas superficialmente porosas.

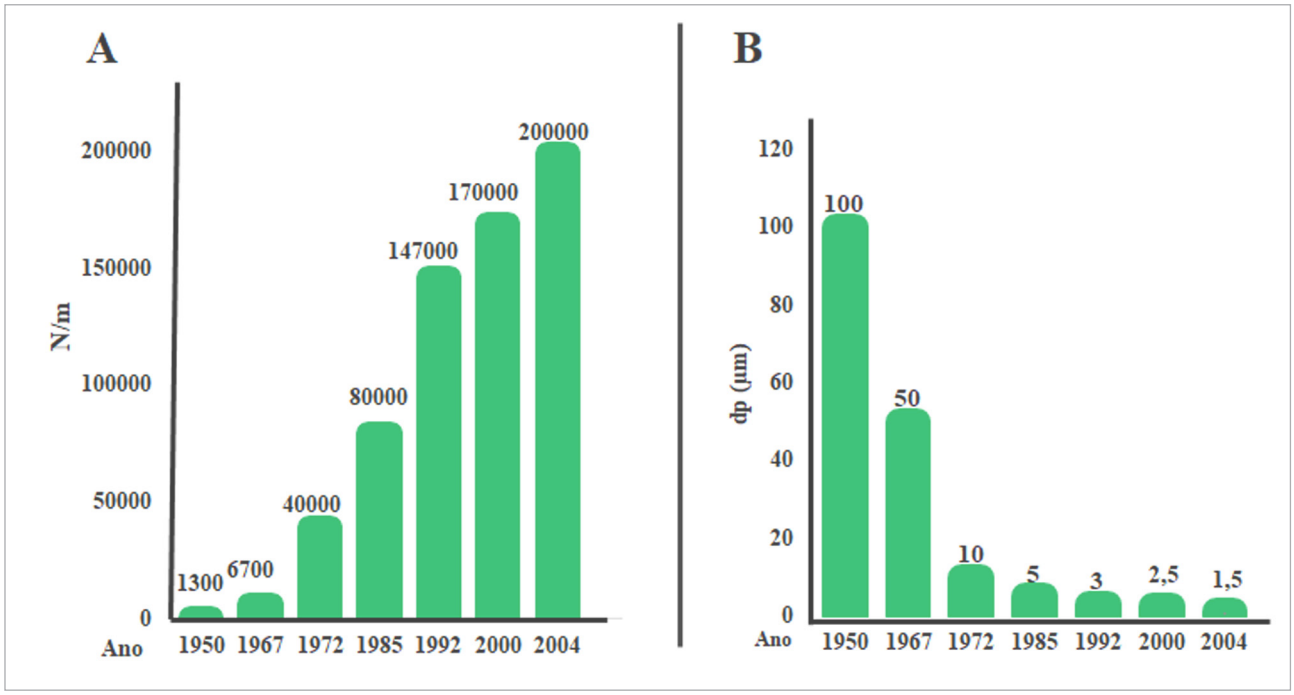

Figura 2. Evolução da eficiência (A) e diâmetro de partículas (B) com o passar dos anos. Fonte: Construída a partir de dados disponíveis em [9].

Na década de 1980, popularizou-se o uso de partículas com uma faixa de diâmetros em torno de 5 µm. Essas partículas apresentavam um estrutura esférica mais aprimorada e uma faixa de distribuição de tamanho mais estreita em comparação às partículas da década anterior[8]. Nos anos 1990, essas partículas evoluíram ainda mais e partículas de diâmetros de 3 a 3,5 µm começaram a ser utilizadas (Figura 2).

A evolução das partículas usadas como fase estacionária continuou[9]. Nos anos 2000 já havia colunas cromatográficas com partículas de diâmetro médio de 2,5 µm disponíveis comercialmente (Figura 3). Finalmente, em 2004, a *Waters Corporation*® produziu partículas de diâmetro médio de 1,7 µm, introduzindo assim uma nova classe de partículas: as partículas sub-2 µm[10]. Atualmente, fabricantes comercializam colunas com partículas de até 1,3 µm.

Embora reduzir o diâmetro das partículas possa implicar em ganho na eficiência, também resulta na elevação da pressão necessária para que a fase móvel percole a coluna. Segundo a Lei de Darcy (Equação 1), a queda de pressão (ΔP) na coluna é inversamente

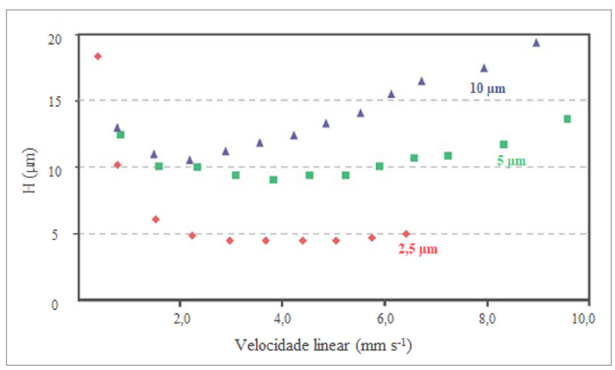

Figura 3. Curvas de van Deemter para partículas de 10, 5 e 2,5 µm. FONTE: Adaptado com permissão de Analytical Chemistry, 77 [23], 2005, Mazzeo JR, D.Neue U, Kele M, Plumb RS. Advancing LC performance with smaller particles and higher pressure.460A 467A.

proporcional ao quadrado do diâmetro da partícula porosa. μ é a velocidade linear da fase móvel, L é o comprimento da coluna, η é a viscosidade, ø é a resistência ao fluxo e dp é o diâmetro da partícula[9].

$$\Delta P = \frac{\mu L \eta \phi}{100 d_p^2} \qquad (1)$$

Para lidar com a exigência de pressões mais altas e permitir explorar todo o potencial que as partículas sub-2 µm propiciaram, foram desenvolvidos os cromatógrafos

líquidos a pressão ultra alta - UHPLCs (*Ultra High Pressure Liquid Chromatography*); também nomeados em português como cromatógrafos líquidos de ultra eficiência (CLUE). A empresa Waters® desenvolveu e lançou no mercado o primeiro cromatógrafo comercial (série *Acquity*) capaz de operar a pressões na ordem de 1000 bar, sob a sigla patenteada de UPLC (*Ultra Pressure Liquid Chromatography*). Atualmente, alguns fabricantes produzem equipamentos equivalentes à mesma finalidade, porém, poucos destes, com versões de instrumentação miniaturizadas para UHPLC capilar.

Como havia muitos cromatógrafos convencionais sendo utilizados no mundo todo e o custo de um aparelho de UHPLC era (e ainda é) elevado, as concorrentes da *Waters* bem como essa própria empresa desenvolveram partículas *core shell* de diâmetro sub-3 µm. Essas partículas superficialmente porosas apresentaram a propriedade de possuir menor resistência à transferência de massa, menor faixa de variação nos diâmetros e maior uniformidade. Portanto, apesar de não apresentarem eficiências superiores ou até mesmo idênticas àquelas das partículas sub-2 µm totalmente porosas; essas partículas *core shell* possuíam eficiência quase equiparável à elas, mesmo quando em uso em um cromatógrafo adequado a pressões convencionais (400 a 600 bar), desde que devidamente otimizado para baixa dispersão extracoluna da banda cromatográfica[11].

1.2. Evolução das colunas capilares

Apesar de colunas capilares para cromatografia líquida serem foco de estudos desde a década de 60. A miniaturização de sistemas cromatográficos começou a ganhar relevância a partir da década de 90, em especial pela necessidade de atender à demanda farmacêutico-bioquímica, que possuía quantidade limitada de amostras[12]. Além disso, um sistema de cromatografia capilar utiliza quantidades bem menores de fase móvel comparativamente ao sistema convencional. Do mesmo modo, para a confecção de uma coluna capilar a quantidade de fase estacionária utilizada é bem menor que para uma coluna convencional. Um cromatógrafo capilar usualmente opera com um volume de apenas 50 nL de amostras, enquanto um cromatógrafo líquido convencional opera com cerca de 10 a 400 vezes mais amostra (500 nL a 20 µL). A diferença no consumo de fase móvel também é muito grande, enquanto um cromatógrafo capilar trabalhando a 10 µL min^{-1} consome apenas cerca de 1,25 L ao ano (operando 8h/dia e 5 dias/semana), um cromatógrafo convencional operando com uma vazão de 0,5 ml min^{-1} consome mais de 62 L ao ano.

Apesar do crescente interesse pela área, o conceito de miniaturização teve seu início atribuído a Golay em 1958, o qual estudou os efeitos da diminuição do diâmetro das colunas utilizadas em GC (*Gas Chromatography*)[13]. Já para cromatografia líquida, os estudos da diminuição do diâmetro interno da coluna se iniciaram com Horváth e colaboradores em 1967, que estudaram e compararam a eficiência de colunas tubulares abertas e colunas empacotadas de 0,3 e 1,0 mm de diâmetro. Quase 10 anos mais tarde, em 1973, Ishii e colaboradores utilizaram colunas empacotadas com partículas de politetrafluoretileno de 30 µm e com diâmetro interno de 0,5 mm[14].

Na década de 80 em diante, diversos estudos utilizando colunas empacotadas, monolíticas e tubulares abertas foram realizados. O desenvolvimento de colunas miniaturizadas, assim como da instrumentação necessária para sua utilização é alvo de intenso estudo nos dias de hoje[12].

Diversos autores classificaram as colunas de cromatografia líquida de forma diferente[15]; uma das mais conhecidas é a classificação apresentada por Saito[16], representada pela Figura 4.

A tecnologia de produção de colunas capilares empacotadas evoluiu tanto para colunas produzidas em laboratório, quanto para comerciais[5,6,17]. Partículas de diâmetros equivalentes a UHPLC (<2 µm) passaram a ser utilizadas em colunas capilares, elevando a eficiência dos sistemas capilares e fazendo necessário o desenvolvimento de cromatógrafos líquidos capilares aptos a trabalharem na faixa de ultra pressão (UHPLC).

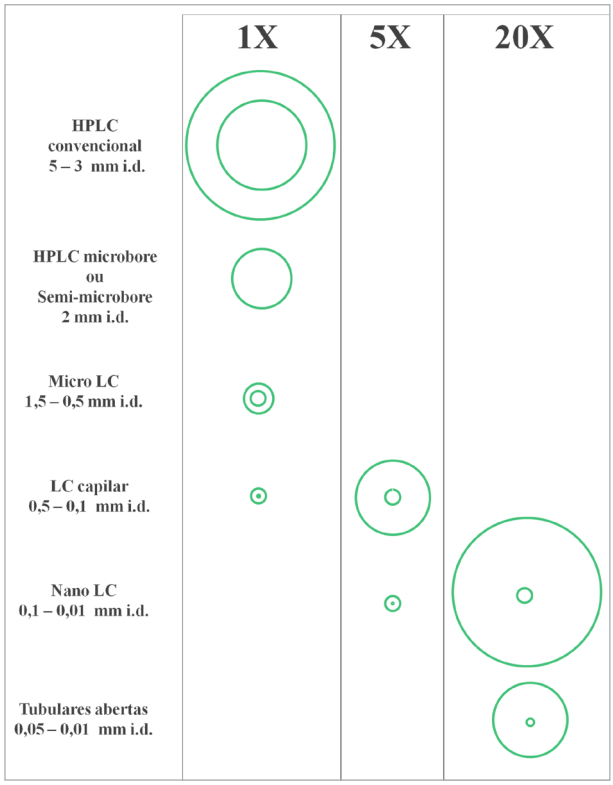

Figura 4. Ilustração em escala (ampliações de 1, 5 e 20 vezes) dos diferentes diâmetros de colunas disponíveis para LC. O círculo externo representa o maior diâmetro dessa categoria e o círculo interno as de menor diâmetro. Elaborada a partir de dados disponíveis em [16].

2. Instrumentação

É desejável que a instrumentação para cromatografia líquida capilar apresente alta robustez, porém, que seja capaz de trabalhar eficientemente com volumes muito baixos nos sistemas de bombeamento, injeção e, principalmente, nas tubulações e detectores, para evitar alargamento extracoluna da banda cromatográfica[18]. A instrumentação para cromatografia líquida capilar está descrita na literatura[19], sendo aqui apresentada de forma sucinta.

2.1. Sistemas de bombeamento

Um sistema de bombeamento para uso em UHPLC capilar deve possuir a capacidade de atingir altas pressões, bombear pequenos volumes, gerar o mínimo de pulso possível e, preferencialmente, ter capacidade de operar gradientes de eluição. Os dois principais tipos de bombas desenvolvidas para essa finalidade são as bombas de pistões e as bombas tipo seringa.

As bombas de pistões são similares às utilizadas em UHPLCs (e HPLCs) convencionais. Elas possuem, em geral, um par de pistões que trabalham alternadamente empurrando a fase móvel em direção à coluna. Estas bombas podem ser de pistões reciprocantes em série ou em paralelo. Este tipo de sistema permite a operação em modos gradiente e isocrático, sendo o tipo de bomba mais comum tanto em sistemas convencionais quanto em capilares[19].

As bombas do tipo seringa são menos comuns, porém também integram alguns modelos de equipamentos comerciais. Produzidas de forma semelhante a uma seringa, a fase móvel é empurrada por um êmbolo para a coluna. Este tipo de sistema não permite a utilização de modo gradiente, a menos que duas seringas sejam posicionadas em paralelo. Adicionalmente, outro limitante para este tipo de bomba está na necessidade de interrupção da sequência de análises para a recarga do dispositivo[1].

2.2. Injetores

Os sistemas de injeção de amostras são um ponto crucial em um sistema miniaturizado. Ele deve ser resistente a altas pressões e capaz de injetar o volume exato, fator ainda mais importante na escala capilar.

Válvulas associadas a alças de amostragem (*loop*) para injeção são os dispositivos padrões para a introdução de amostras em cromatografia líquida. Geralmente elas empregam um rotor que desliza dentro de um estator circundante. O rotor fornece caminhos que se alinham com as portas do estator. É necessária uma vedação eficiente, que não permita vazamentos entre o rotor e o estator. Para isso, geralmente são utilizados polímeros de alta resistência. O principal desafio para a injeção em UHPLC capilar é a obtenção de sistemas que injetem na ordem de nanolitros (<50 a 1000 nl) de forma exata e precisa, garantindo boas figuras de mérito para as análises.

2.3. Detectores

Os detectores usados em HPLC capilar são similares aos que são utilizados em escala convencional, porém, ajustados para vazões reduzidas, para que não haja perda na detectabilidade e alargamento de banda. Detectores UV-Vis necessitam de uma redução no volume da cela de detecção, uma vez que o volume de fase móvel é muito pequeno. Em escala nano, existem celas com menos de 10 nL de volume; em escala capilar, na ordem de 50 nL; enquanto, em escala micro existem celas na ordem de 200 nL de volume.

A espectrometria de massas (MS – *Mass Spectrometry*) é um dos principais sistemas de detecção da atualidade e funciona muito bem com a UHPLC capilar. A cromatografia em escala capilar utiliza pequenos volumes na saída da coluna, o que é ideal para o sistema de ESI (electrospray ionization) do MS. A eficiência da ionização aumenta consideravelmente quando a vazão é reduzida, de forma que esse tipo de ionização é extremamente atraente para o acoplamento de UHPLC capilar-ESI-MS[2]. Na atualidade, existem diversas interfaces comerciais e de diferentes fabricantes apropriadas para operar nas vazões típicas dessa escala, com o máximo de eficiência e robustez.

3. Fundamentos

O alargamento de banda em colunas empacotadas para cromatografia gasosa foi estudado na década de 50 principalmente por van Deemter. Suas considerações permanecem válidas para cromatografia líquida, seja na escala convencional ou capilar. Van Deemter correlacionou o alargamento de banda com a altura do prato, ou seja; quanto menor o alargamento de banda, menor será a altura do prato da coluna e, por consequência, quanto menor o prato, maior a eficiência de uma coluna.

A equação de van Deemter (Equação 2) é dividida em três termos, sendo estes: o efeito dos múltiplos caminhos (A), a difusão longitudinal (B) e a resistência à transferência de massa (C). O efeito de múltiplos caminhos, termo A, é subdividido em cinco fatores: a difusão intracoluna, (ocorre entre as paredes da coluna), a difusão intrapartícula, (ocorre dentro dos poros da partícula), a difusão intracanal (ocorre no interstício entre as partículas), e as difusões intercanais de curta e longa distância (ocorrem devido a heterogeneidade do empacotamento).

O termo B, a difusão longitudinal, representa o espalhamento longitudinal dos analitos na fase móvel. Esse termo é estritamente dependente da viscosidade da fase móvel e, em geral, é mínimo para a cromatografia líquida, tendo contribuição considerável apenas em vazões muito reduzidas. Já o termo C é relativo à transferência de massa, ou seja, à resistência à movimentação dos analitos entre a fase móvel e a fase estacionária.

$$H = A + \frac{B}{\mu} + C\mu \qquad (2)$$

3.1. Influência do diâmetro das partículas

A redução do diâmetro de partículas melhora significativamente a eficiência de uma coluna (Figura 5). A Equação 3, uma aproximação da equação de van Deemter, mostra que a altura do prato é diretamente proporcional ao diâmetro da partícula[20].

$$H = \frac{d_p}{2} + \frac{2D_m}{\mu} + \frac{d_p^2\mu}{5D_m} \qquad (3)$$

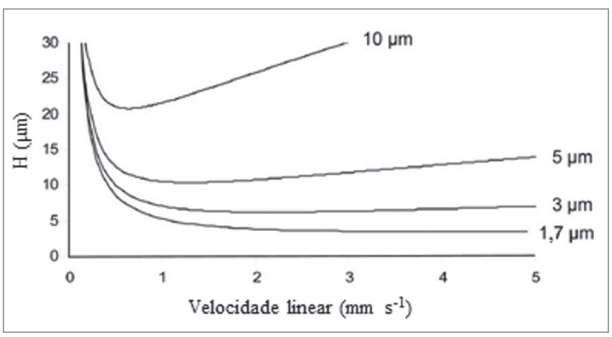

Figura 5. Altura do prato para partículas de vários diâmetros. FONTE: Adaptado com permissão de Journal of Separation Science 29 [12], 2006. Nguyen DT-T, Guillarme D, Rudaz S, Veuthey J-L. Fast analysis in liquid chromatography using small particle size and high pressure. J 1836–1848. Copyright (2008), com permissão de John Wiley and Sons.

3.2. Influência do diâmetro das colunas

Embora seja um assunto controverso, diversos trabalhos demonstraram a dependência da eficiência com o diâmetro interno da coluna[17,21,22]. Patel e colaboradores avaliaram a redução do diâmetro da coluna de 150 para 10 µm[23]. Em seus resultados é nítido que reduzir o diâmetro interno da coluna empacotada reduz diretamente a altura do prato (Figura 6).

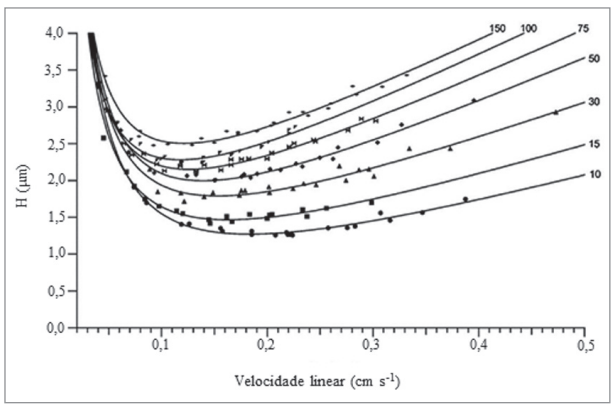

Figura 6. Diminuição da altura do prato de uma coluna obtida pela redução do diâmetro da coluna. FONTE: Adaptado com permissão de Analytical Chemistry, 76 (19), 2004, Patel KD, Jerkovich AD, Link JC, Jorgenson JW. In-depth characterization of slurry packed capillary columns with 1.0-µm nonporous particles using reversed-phase isocratic ultrahigh-pressure liquid chromatography 5777-5786 de [23].

3.3. Influência da pressão

Em condições ordinárias para a cromatografia líquida convencional, o efeito da pressão no fator de retenção é considerado desprezível ou inexistente. Por outro lado, Fallas e colaboradores demonstram que alterações (grandes) na pressão afetam o fator de retenção[24]. Em fase reversa, compostos ionizados apresentaram maior aumento no fator de retenção conforme a pressão é elevada, em comparação a compostos neutros (Figura 7). O oposto foi observado para HILIC, na qual bases ionizadas apresentaram uma redução no fator de retenção com o aumento da pressão. Isto foi atribuído à hipótese de que a variação na retenção se deve a uma redução no volume molar do soluto à medida que ele se transfere da fase móvel para a fase estacionária; e que isso poderia ser causado pela perda da camada de hidratação de soluto quando entra na rede hidrofóbica de cadeias da fase estacionária[25].

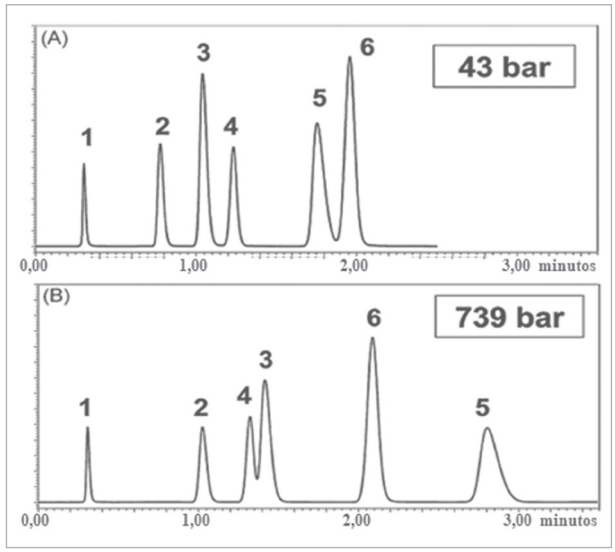

Figura 7. Cromatogramas de uma mistura de compostos neutros e básicos obtidos em diferentes pressões médias de coluna: 43 bar (A) e 739 bar (B) obtidas sem e com um capilar de restrição de 25 cm, respectivamente. Picos: 1, tioureia; 2, propranolol; 3, difenidramina; 4, acetofenona; 5, protriptilina; e 6, nitrobenzeno. FONTE: Republicado com permissão de Journal of Chromatography A, 1209(1–2), Fallas MM, Neue UD, Hadley MR, McCalley D V. Investigation of the effect of pressure on retention of small molecules using reversed-phase ultra-high-pressure liquid chromatography, 195–205. Copyright (2008), com permissão de Elsevier.

3.4. Aquecimento friccional e efeito de parede

Quando a fase móvel passa através de um leito empacotado, ocorre atrito e uma pequena quantidade de energia térmica é gerada. Esse aquecimento é pouco significativo quando em pressões convencionais (<400 bar). Entretanto, quando partículas sub-2 µm são usadas faz-se necessário o uso de pressões ultraelevadas. O atrito entre as fases móvel e estacionária libera energia térmica, aumentando a temperatura na coluna[26,27]. A energia térmica gerada na coluna causa um gradiente de temperatura (Figura 8). Esse gradiente de temperatura ocorre de duas maneiras diferentes. O aquecimento radial faz com que o centro da coluna fique mais quente que a região mais próxima das paredes da coluna, alterando a viscosidade da fase móvel e, por consequência, o fator de retenção dos analitos nessas diferentes regiões, causando alargamento de banda. Outra forma de aquecimento ocorre longitudinalmente, ou seja, a energia é acumulada ao longo da coluna, de forma que as porções finais da

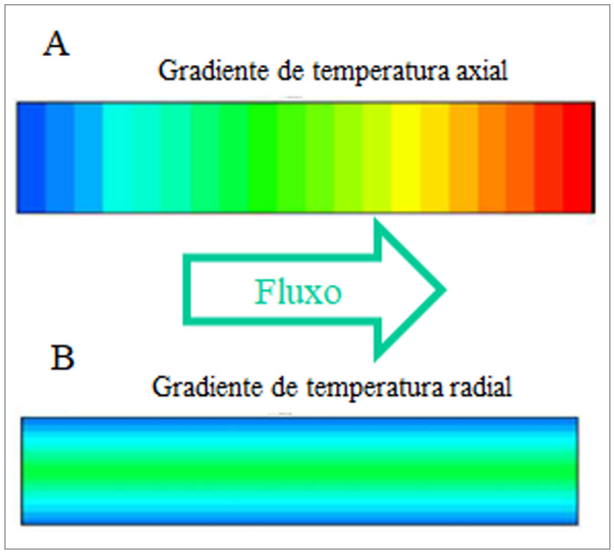

Figura 8. Formação de gradiente de temperatura axial (A) e radial (B) na coluna cromatográfica. FONTE: Adaptado com autorização de Journal of Chromatography A 1216(9), 2009, Gritti F, Guiochon G. Optimization of the thermal environment of columns packed with very fine particles, 1353–1362. Copyright (2009), com permissão de Elsevier.

coluna, mais próximas à sua saída, estejam mais quentes do que aquelas situadas próximas à sua entrada.

Esse calor gerado (Tabela 1) pelo atrito da fase móvel com o leito cromatográfico, também conhecido como *frictional heating*, é mais bem dissipado tanto quanto menor for o diâmetro da coluna. Consequentemente, o alargamento de banda devido a esse efeito é minimizado em colunas com diâmetros pequenos, como em capilares[8].

Colunas capilares também minimizam o alargamento de banda devido ao efeito de parede (*wall effect*). Apesar de todas as causas desse efeito não serem ainda inteiramente esclarecidas, observa-se que há uma tendência de as partículas menores se disporem mais perto da parede da coluna; fazendo com que o centro do leito cromatográfico abrigue partículas maiores. Dessa forma, a região próxima à parede da coluna terá uma velocidade de difusão do analito diferente da velocidade de difusão no centro do leito cromatográfico, causando assim um alargamento de banda. Diminuindo o diâmetro da coluna, essa discrepância entre as regiões é suavizada e consequentemente o alargamento é reduzido[28,29].

Tabela 1. Colunas de diferentes diâmetros, com L = 25 cm e empacotadas com partículas de 1 µm e P = 2000 bar. FONTE: Construído com dados de [8].

Classificação	Diâmetro interno da coluna	Vazão
HPLC convencional	4,6 mm	820 µL/min
Micro LC	1,0 mm	38 µL/min
LC Capilar	0,3 mm	3,5 µL/min
LC capilar/Nano LC	0,1 mm	0,380 µL/min
Nano LC	0,05 mm	0,096 µL/min

4. Conclusão

A UHPLC capilar é uma ferramenta poderosíssima para análises, pois mantém as principais vantagens da UHPLC convencional; como o tempo curto de análise e a elevada eficiência que pode ser obtida otimizando-se os parâmetros cromatográficos. Porém, tem como virtude adicional a capacidade de trabalhar com quantidades muito pequenas de amostra e volumes de fases móveis bem reduzidos. Embora apresente grande potencial, esta técnica ainda apresenta pouco uso em pesquisas aplicadas. Em várias situações ela também apresenta grande sensibilidade, além de ser bastante vantajosa para o acoplamento com a ESI-MS. Embora bastante vantajosa nos aspectos práticos, a cromatografia líquida capilar em pressões ultraelevadas tem como maior limitação o custo elevado de sua instrumentação; visto que é necessário o uso de equipamentos com elevada resistência mecânica e robustez, além de ser capaz de trabalhar com volumes pequenos; sendo este o principal fator limitante. Além disso, o usuário também deve estar mais familiarizado ao uso desse tipo de sistema, pois a escala miniaturizada pode ter alguns componentes mais delicados, além de exigir atenção para que se evitem situações de alargamento de banda extracoluna e vazamentos de fase móvel (este último mais difícil de ser detectado em situações de escala reduzida).

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7 CONCLUSÃO

A combinação entre miniaturização do preparo de amostras, design fatorial e automatização mostrou-se uma excelente abordagem no desenvolvimento de métodos simples, rápidos e de baixo custo para a determinação de contaminantes orgânicos em amostras ambientais, apresentando-se como uma alternativa efetiva e ambientalmente amigável. A combinação dos métodos de MEPS e SDME com detecção por espectrometria de massas proporcionou ótimos LOQ e LOD para métodos analíticos que utilizam uma quantidade muito pequena de amostra.

A plataforma robótica aliada à flexibilidade de ferramentas de código aberto como o Arduino permitiu avaliar todas as etapas dinâmicas da microextração, tornando viável a exploração de parâmetros experimentais dificilmente controláveis manualmente, obtendo-se um método mais bem otimizado.

Adicionalmente, o dispositivo para fase extratora que utiliza uma seringa comercial para MEPS é uma ferramenta importante para se trabalhar com as diferentes fases extratoras disponíveis comercialmente, sem depender da restrição a dispositivos comerciais.

Os trabalhos apresentados nesta tese mostram que a abordagem pode ser utilizada para fins de pesquisa em análises ambientais, mas pode ser estendida também para análises de rotina. Para isto, como perspectivas futuras pode-se deixar a plataforma automatizada ainda mais robusta e rápida, tornando o ciclo de extração ainda mais eficiente e isento de erros que comprometam a análise, como travamentos, entupimentos e queda de conexão com o computador. Além disso, como a plataforma robótica é ajustável e facilmente passível de mudanças em sua configuração; novas técnicas miniaturizadas de extração podem ser desenvolvidas, diminuindo a dificuldade que essa microtécnica teria se fosse realizada de forma manual.