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Sistemas automatizados integrando preparo de amostra à cromatografia líquida e espectrometria de massas como uma ferramenta analítica eficiente e ambientalmente amigável.

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Dedicatória

Aos meus pais Edvaldo e Cida que me mostraram o impacto imensurável que a educação e o amor podem exercer na vida de uma pessoa, independente de quaisquer circunstâncias. O amor nos levará para melhores dias, sempre!

A todos os professores que passaram pela minha vida desde o pré-ecola até este dia. Assim como a vida precisa de um meio ideal para se criar, nós precisamos de professores para nos ensinar!

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O que está embaixo é como o que está no alto
O que está no alto é como o que está embaixo

Hermes Trismegistus

RESUMO

Esta tese é construída na forma de coletânea de artigos científicos publicados durante o período de doutorado do autor. Seis publicações distintas são destacadas e discutidas, para evidenciar a linha de pesquisa, os objetivos e conclusões do projeto. Entre os principais assuntos abordados está o estudo da etapa de preparo de amostras, priorizando o desenvolvimento de métodos miniaturizados e automatizados para posterior acoplamento com sistemas de cromatografia líquida. Já é consendo entre os químicos analíticos que o preparo da amostra representa uma das etapas mais trabalhosas, demoradas e passível de erros analíticos durante o processo de análise. Além destas características contribuírem negativamente no resultado, prejudicando reprodutibilidade analítica, estas também influenciam no consumo de reagentens e amostra, e geração de resíduos químicos. Atualmente a consciência ambiental é um fator que todos devemos levar em conta para nossas vidas, e dentro do âmbito científico, algumas diretrizes neste sentido foram compiladas como conceitos da Química Verde. Visando equacionar tais conceitos, diversas técnicas miniaturizadas de amostra têm surgido desde o desenvolvimento da microextração em fase sólida no início de 1990. Todas estas têm como prioridade a redução de etapas analíticas, consumo e geração de resíduos tóxicos. Levando isso em consideração, nesta tese optou-se pelo desenvolvimento e utilização apenas de métodos considerados miniaturizados. Apesar do sucesso proveniente da miniaturização, a atual problemática relacionada a existência de resíduos e contaminantes em concentrações cada vez mais baixas e em diversas matrizes distintas, tem exigido dos químicos analíticos um aumento da produtividade e eficiência analítica. Neste sentido, aliado a miniaturização, a automatização do preparo de amostra representa um caminho promissor para diminuir o tempo total de análise e os erros analíticos. Desta forma, o presente trabalho focou no desenvolvimento de métodos miniaturizados e automatizados, baseados em cromatografia líquida para análise de resíduos e contaminantes em matrizes complexas. Três métodos analíticos foram desenvolvidos: parcialmente miniaturizado/automatizado, totalmente miniaturizado/automatizado – ambos empregados em matrizes líquidas –, e um especialmente desenvolvido para análise de matrizes sólidas. De forma complementar ao desenvolvimento dos métodos, foi realizada a síntese e emprego de materiais a base de grafeno como fase extratora, uma vez que estes apresentam características ambientalmente favoráveis e boa capacidade de extração. Neste trabalho, todas as colunas de extração utilizadas para o preparo da amostra automatizado foram produzidas no laboratório e utilizando os materiais a base de grafeno como fase extratora. Sendo assim, a tese aborda dois tópicos de estudo: o desenvolvimento dos métodos analiticos e o emprego de "novos" materiais sorventes. Todo o material é apresentado em detalhes nas publicações, as quais evidenciam a qualidade do trabalho e reforçam que os principais objetivos iniciais foram atingidos. Em resumo, os métodos aqui desenvolvidos foram aplicados para diversos analitos (pesticidas, fármacos, micotoxinas, etc) em matrizes alimentares, biológicas e ambientais, evidenciando a aplicabilidade do projeto. Esta tese apresenta métodos e conceitos importantes para a obtenção de métodos analíticos que priorizam um menor consumo de reagentes e amostra, e consequente geração de resíduos – estando em concordância com alguns dos conceitos da Química Verde.

ABSTRACT

This doctorate thesis is a compendium of scientific papers published by the author during this period as a PhD student. Six selected publications were discussed to highlight the research topics, main goals and obtained conclusions. The sample preparation step is a major topic in this work, especially focusing on the development of miniaturized and automated approaches coupled with liquid chromatography-mass spectrometry platforms. It is widely known that sample preparation is laborious, time-consuming and source of analytical errors. Despite these features contribute negatively over the analytical results, they also influence in the consume of reagents and samples as well as toxic waste production. Nowadays, environment awareness it is an essential point in our lives, and for analytical chemistry, some tenets are compiled as green chemistry concepts. In according to such concepts, several miniaturized sample preparation techniques started to sprung up since the creation of solid-phase microextraction in the 1990s. All of them, prioritizing reduction of reagents and sample consumption, and waste generation. Taking this into account, this thesis focused on the development of only miniaturized approaches. Although the success of miniaturization, the current challenges associated with the trace levels occurrence of pollutants in several matrices, has been demanded analytical high-throughput and efficiency. Therefore, in combination with miniaturization, automation emerged as a promising strategy to reduce time-per-analysis and analytical errors. This work focused on developing miniaturized and automated analytical approaches to cope with residues and contaminants in complex matrices. Basically, three analytical methods were proposed: partially miniaturized/automated, fully miniaturized/automated – both to be used for liquid matrices – and, one miniaturized/partially automated, specially designed for solids. Complementary to the development of these sample preparation approaches, the synthesis and use of graphene-based materials, as sorbents, was another point of study, as they present good extraction capacity and greener properties. It is worth mentioning that the extraction columns herein used were all in-lab made, using only graphene-based materials as sorbents. In short, this work coped with two main topics: the development of modern analytical methods, and the employment of graphene-based materials as sorbents. Detailed information is presented in the six following scientific publications, which reinforces the quality of this thesis and, that most of the main goals were accomplished. At last, all herein developed analytical methods were applied for several classes of analytes (e.g., pesticides, pharmaceutical drugs, mycotoxins) in a broad range of matrices such as food, biological, and environmental ones, underscoring the real applicability of this study. This doctorate thesis presents methodologies and important concepts for developing environmentally-friendly analytical methods in according to some concepts of the Green Chemistry.

SUMÁRIO

CAPÍTULO 1

Introdução

1 Contextualização do trabalho

A química analítica é, atualmente, parte indispensável para o desenvolvimento da ciência, contribuindo diretamente para o avanço da nossa sociedade através do fornecimento de métodos e técnicas de análise cada vez mais eficientes.(1) O químico analítico é responsável por criar e propor métodos e estratégias para a identificação, quantificação e monitoramento de uma infinidade de tipos de compostos (exógenos ou endógenos) em diversas matrizes. Desta forma esta área da ciência atua conjuntamente e em harmonia com diversos outros setores científicos e industriais, como a medicina, farmácia, biologia, toxicologia, forense, esportes, meio ambiente, segurança alimentar, etc.(2) Levando em consideração esta amplitude de atuação, fica mais fácil compreender o porque da existência de diversas técnicas de preparo de amostra, separação e detecção de compostos. Espera-se que, independentemente do tipo de analito ou da matriz de análise, a química analítica possa oferecer uma alternativa eficiente para a realização do estudo. Os atuais desafios enfrentados pela nossa sociedade, em suas mais diversas facetas, necessitam de raciocío complexo e integrador para a criação de soluções efetivas, realçando o papel colaborativo que o químico analítico deve ter, estando apto a dialogar com profissionais de diferentes áreas científicas. Por outro lado, é preciso entender que, apesar da química analítica oferecer suporte as áreas anteriormente mencionadas, ela é uma ciência a qual necessita de dedicação focada em seu próprio desenvolvimento teórico e prático.(3)

A análise de amostras complexas contendo uma infinidade de substâncias interferentes juntamente com o composto alvo – seja este uma proteína, ou contaminantes como pesticidas e fármacos – não é uma particulariedade, e nem uma demanda específica da química analítica.(2) Diversas áreas necessitam deste tipo de abordagem para progredir com suas pesquisas (e.g., o estudo de doenças neurodegenerativas dentro da medicina precisa do constante suporte da química analítica para fornecer melhores dados experimentais e, assim, auxiliar os médicos em seus diagnóticos e desenvolvimento de tratamentos).

É importante ter em mente essa capacidade de atuação do química analítico – o qual pode atuar na pesquisa e resolução de problemas de outras áreas científicas, aplicando os conceitos e técnicas provenientes da química analítica ou, então, focar no desenvolvimento teórico e prático da "caixa de ferramentas" de técnicas e estratégias para o avanço da química analítica, enquanto ciência independente. Dentro deste

contexto, o presente trabalho teve como objetivo propor experimentos e estratégias que visassem o avanço da própria química analítica focando em uma das principais e mais trabalhosas etapas de uma análise, o preparo da amostra, apesar de que outros desafios analíticos também foram abordados no trabalho.

O sucesso no desenvolvimento de um método análitico para identificar resíduos ou contaminantes em uma matriz complexa é dependente principalmente de três etapas distintas: o preparo da amostra, separação e detecção dos analitos, e o processamento e interpretação efetiva dos dados experimentais. Quando a concentração destes compostos é baixa – geralmente variando entre ng L^{-1} e µg L^{-1} – o preparo da amostra se torna ainda mais indispensável.(4) Basicamente, a etapa de preparo de amostra atua na: (i) limpeza da matriz através da remoção da maioria dos interferentes; (ii) extração dos analitos de interesse e transferência para um solvente apropriado; (iii) pré-concentração dos analitos antes da etapa de separação/detecção.(4) Todas essas sub-etapas juntas tem como principal objetivo um aumento na relação sinal analítico/ruído para, consequentemente, aumentar a sensibilidade do método, permitindo a diminuição dos limites de detecção e quantificação. Devido ao caracter multi-função do preparo da amostra, esta etapa é reconhecidamente laboriosa e demorada, podendo representar mais de 70% do tempo total de análise.(5) Além disso, o consideral número de etapas é um fator determinante para a ocorrência de erros análiticos devido a manipulação da amostra por parte do analista.

Apesar das técnicas de separação e detecção, como a cromatografia e a espectrometria de massas, terem atingido um alto nível de excelência e compatibilidade entre si, o preparo da amostra ainda é imprescindivel para obtenção de resultados confiáveis e reprodutíveis. O desenvolvimento e utilização de técnicas eficientes para o preparo da amostra auxília na injeção de amostras mais compatíveis com o instrumento analítico, o que certamente exerce influência positiva também na vida útil do equipamento. Sistemas de cromatografia líquida acoplados a espectrometria de massas vendidos atualmente exigem um investimento financeiro considerável para sua aquisição e manutenção. Portanto, fica evidente, mais uma vez, a importância em melhorar o preparo da amostra, o que torna compreensível o fato deste ser um assunto constantemente de interesse dos pesquisadores em todo o mundo.

Diversas técnicas de preparo de amostra têm sido desenvolvidas e aplicadas ao longo dos últimos 75 anos. Em resumo, podemos dividí-las em dois grandes grupos de acordo com o processo de extração dos analitos alvo: (i) técnicas baseadas em sorção, com emprego de sorventes sólidos, e (ii) técnicas baseadas em partição entre solventes líquidos.(6) Esse tipo de classificação está diretamente relacionado com o surgimento de duas técnicas que podem ser consideratas clássicas atualmente, a extração líquido-líquido (LLE, *liquid-liquid extraction*) e a extração em fase sólida (SPE, *solid-phase extraction*).(6) Apesar destas técnicas serem efetivas e terem desempenho satisfatório – ainda são recomendadas por orgãos governamentais ao redor do mundo – estas requerem volumes substanciais de solvente orgânico e amostras.(7) Isto implica na geração consideravel de resíduo químico e exposição do analista a um ambiente tóxico.(8) Impulsionada por estes fatores, a partir da década de 1990 houve o surgimento e o desenvolvimento de uma variedade de técnicas de preparo de amostra miniaturizadas, as quais visam equacionar os pontos ambientais e melhorar a performance analítica.(8) A principal contribuição inicial e técnica considerada pioneira entre as miniaturizadas é a microextração em fase sólida (SPME, *solid-phase microextraction*) desenvolvida por Arthur e Pawliszyn.(9)

De maneira análoga à divisão das técnicas clássicas, podemos dividir as miniaturizadas entre as baseadas em sorção e as baseadas em solventes. Pertencentes ao primeiro grupo podemos destacar a pioneira SPME, além da microextração sortiva em barra de agitação (*SBSE*), a microextração por sorvente empacotado (*MEPS*), e a microextração dispersiva em fase sólida (*DμSPE*).(10) Entre as principais técnicas baseadas em solvente podemos citar a microextração líquido-líquido (*LLME*) , microextração líquido-líquido dispersiva (*DLLME*), microextração em gota suspensa (*SDME*) e a microextração em fibra oca (*HFME*).(10) Basicamente todas estas técnicas oferecem uma considerável redução no volume de solvente e amostra utilizados e, consequente, menor geração de resíduo, além da capacidade de reutilização dos dispositivos de extração – um fator raro para as técnicas clássicas. Apesar destas reconhecidas melhorias, a crescente demanda por métodos mais sensíveis e de elevada produtividade (tempo por análise) tem representado um ponto ainda a ser melhorado na etapa de preparo da amostra.(11) Ainda que as técnicas miniaturizadas apresentem melhor performance quando comparadas às clássicas, alguns aspectos práticos ainda são similares. Neste sentido destaca-se a necessidade de manuseio pelo operador, o que

eleva a probabilidade de erros em reprodutibilidade e tempo total do método analítico, além de o expor ao ambiente tóxico por maiores períodos.(11)

Portanto, estudos focados no desenvolvimento de técnicas de preparo de amostra mais rápidas e produtívas com elevado poder de extração e seletividade dos analitos alvo têm representado um importante nicho de pesquisa dentro da química analítica.(12) Tal vertende de pesquisa tem dado origem, por exemplo, aos métodos automatizados ou parcialmente automatizados de preparo de amostra.(13) Encontram-se inseridos neste contexto os métodos multidimensionais capazes de executar a extração/pré-concentração on-line com posterior separação/detecção através de cromatografia líquida acoplada a espectrometria de massas – sistema analítico utilizado neste trabalho.(13)

Em resumo, os métodos automatizados baseados em cromatografia líquida caracterizam-se por realizar a transferência dos analitos alvo de uma primeira para uma segunda dimensão de separação, geralmente com auxílio de válvulas elétricas de comutação.(13) Este tipo de estratégia é aplicada para algumas finalidades como realização do preparo da amostra automatizado acoplado a técnicas de separação e detecção, ou, utilização de duas etapas complementares de separação cromatográfica para refinamento da separação dos picos analíticos antes da etapa de detecção. Independente do caso, ambas estratégias tem alguns objetivos em comum incluindo a melhora da seletividade e sensibilidade analítica.(12) Em nosso caso concentraremos na primeira abordagem, trabalhando com preparo da amostra automatizado e baseado em sorção (primeira dimensão), seguido de cromatografia líquida e espectrometria de massas (segunda dimensão).

Entre as técnicas de preparo de amostra automatizadas, aquelas que utilizam um dispositivo extrator semelhante a uma coluna cromatográfica oferecem uma boa compatiblidade com os sistemas baseados em cromatografia líquida. Neste caso, o sistema automatizado é composto por duas ou mais colunas responsáveis por extrair, pré-concentrar, purificar e separar os analitos dos interferences da matriz de análise.(14) As principais vantagens deste tipo de arranjo são a realização das etapas de análise (preparo, separação e detecção) sem intervenção do analista, além de, consequentemente, acarretar um aumento da produtividade devido a redução no número de etapas entre as fases de uma análise.(14) Ou seja, complementar às qualidades já atingidas pelas técnicas miniaturizadas, a automatização visa

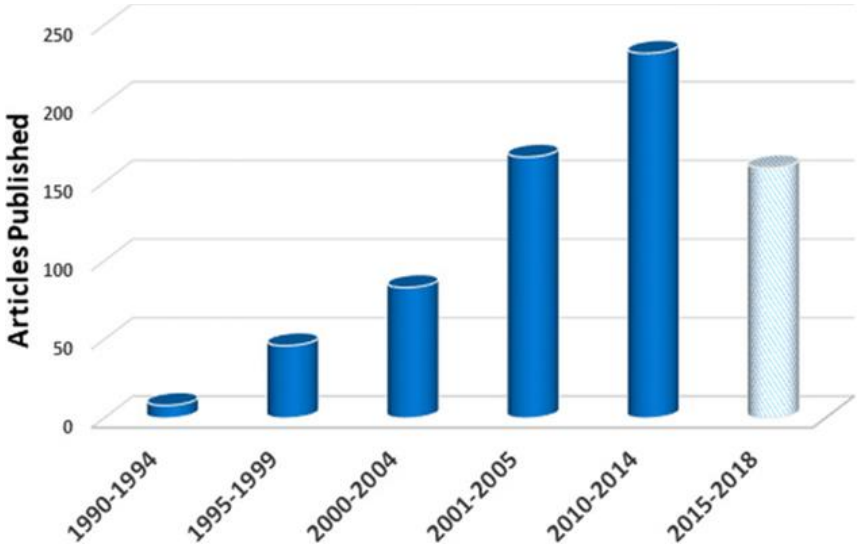
complementá-las com a diminuição do tempo total de análise e probabilidade de erros. Vale ressaltar que em nosso trabalho foi priorizado o desenvolvimento de metodologias de preparo de amostra automatizas e miniaturizadas, para não "perder" as boas características já evidenciadas pelas microtécnicas de preparo de amostra.

Por definição, as técnicas de preparo da amostra que permitem acoplamento com sistemas de cromatografia e utilizam colunas de extração para tal finalidade, são definidas como do tipo *"column switching"*.(12) Inseridas nesta classificação estão a on-line *SPE*, microextração em fase sólida no tubo (*In-tube- SPME*) e a extração por fluxo turbulento (*TFC*). Como em todas essas abordagens há o emprego de uma coluna de extração e outra analítica, iremos padronizar neste trabalho a denominação método analítico multidimensional para esses sistemas que empregam o preparo de amostra e cromatografia líquida acoplada a espectrometria de massas.(12) Para evidenciar o carácter atual deste trabalho, Maciel e colaboradores efetuaram uma revisão bibliográfica(13), ressaltando a tendência na utilização de métodos analíticos multidimensionais para preparo da amostra acoplado a cromatografia líquida desde o início da década de 1990 – surgimento dos primeiros trabalhos abordando o tema (**Figura 1**). Pode-ve observar o crescente uso destes sistemas para o preparo da amostra, o que é facilmente compreensível devido as diversas vantagens já mencionadas como elevada produtividade, diminuição de erros e consequente aumento de reprodutibilidade, e um melhor equacionamento dos fatores ambientais de acordo com os conceitos da Química Verde (redução no uso de reagentes e amostras, assim como, menor geração de resíduos).(15)

Outro importante parâmetro utilizado para avaliar a efetividade do método de preparo de amostra é a seletividade – capacidade de isolar determinado composto alvo ou classe de analitos com especificidade em relação a inúmeros interferentes presentes na matriz de análise.(16) Neste caso, o principal componente atuante é denominado de meio extrator ou fase extratora, podendo ser este um material sólido (sorvente), ou líquido (solvente). A escolha por uma fase extratora adequada relaciona-se diretamente com as características físico-químicas dos analitos alvo (e.g., polaridade, hidrofilicidade ou hidrofobicidade), e com a composição da matriz (e.g., tipos de interferentes identificados).(17) Em especial, quando trata-se de um método muldimensional para preparo da amostra acoplado a cromatografia líquida, também é

importante levar em consideração a compatibilidade desta fase extratora com a coluna analítica utilizada.

Figura 1 – Crescente tendência de publicações nos últimos 28 anos envolvendo sistemas multidimensionais empregando preparo da amostra na primeira dimensão. Adaptada a partir de (13), com permissão da Elsevier.



* Fonte: base de dados Scopus utilizando os termos: "on-line SPE, in-tube SPME, online sample preparation, liquid chromatography e TFC.

As principais fases extratoras comercialmente disponíveis incluem: (i) grupos específicos quimicamente ligados a sílica – C8, C18, -NH_2 (amino), cianopropil, fenil, cicloexil; (ii) fases poliméricas tradicionais – polidimetilsiloxano (PDMS) e poliestireno entrecruzado com divinilbenzeno (PD-DVB); e (iii) fases híbridas, desenvolvidas por empresas reconhecidas na área de química analítica como a Oasis HLB da Waters, ou a Strata-X da Phenomenex.(17) Apesar da variabilidade de opções e bons resultados obtidos com estas fases extratoras, a busca por materiais cada vez mais especificos e seletivos para determinados analitos ou classes de analitos tem impulsionado a síntese de novos materiais adaptados as necessidades do químico analítico.(18) Diversas classes de novos materiais têm sido reportadas na literatura incluindo polímeros molecularmente impressos (MIP), monolitos, líquidos iônicos (ILs), materiais magnéticos, meios de acesso restrito (RAM), imunosorventes, materiais a base de carbono, etc.(19) Dentre estes, os materiais baseados em carbono foram os escolhidos, sintetizados e modificados neste presente trabalho – mais precisamente os derivados de grafeno.

Desde o trabalho publicado em 2004 por Novoselov e colaboradores confirmando a sua existência(20), o grafeno e seus derivados vem sendo utilizados em

diversas areas científicas e técnológicas, incluindo a química. Os primeiros trabalhos empregando grafeno e seus derivados como fase extratora no preparo da amostra são de 2011.(21,22) Desde então, estes materiais tornaram-se uma das principais classes de sorventes de alta eficiência para o preparo de amostra. Isto deve-se as suas excelentes características físico-químicos, principalmente a elevada área superficial para interação (~2630 m^2 g^{-1}), capacidade de funcionalização química para customização e maior seletividade, síntese relativamente simples e de baixo custo, e boa interação com analitos contendo grupamentos aromáticos – propriedades existentes em diversas classes de resíduos e contaminantes frequentemente analisados.(23) Levando isso em consideração, e a experiência do grupo de cromatografia do IQSC no tema, optou-se por utilizar materiais a base de grafeno como outro tópico de estudo. O laboratório de cromatografia da USP – São Carlos (CROMA) exerce papel importante dentro do tema, sendo este documentado através de publicações utilizando sorventes a base de grafeno em diversas aplicações como a determinação de parabenos em amostras ambientais(24); fármacos em alimentos e fluidos biológicos(25,26); micotoxinas em bebidas alcólicas e não alcólicas(15); isoflavonas em fluídos biológicos(27); pesticidas em bebidas, solo e abelha(28).

De maneira geral, o foco deste trabalho foi no estudo e desenvolvimento de metodologias miniaturizadas e automatizadas para o preparo de amostra em matrizes complexas. Para que tal objetivo fosse abordado com eficiência, buscou-se trabalhar o desenvolvimento de mais de um tipo de método, destinado a matrizes líquidas e sólidas comumente utilizadas pelos químicos analíticos. Optou-se por também realizar a síntese e emprego de novos materiais a base de grafeno como complemento positivo visando uma contribuição dentro deste assunto atual. Por fim, como este trabalho foi pensado e executado visando equacionar as questões ambientais que estivessem ao meu alcance, juntamente com a miniaturização e utilização de técnicas automatizadas de preparo de amostra, o emprego da cromatografia líquida miniaturizada foi incluída em boa parte dos estudos. Desta forma podemos dizer que o foco final foi no desenvolvimento e aplicação de metodologias totalmente miniaturizadas para preparo de amostra, separação e detecção de diversas classes de analitos em mais de uma matriz complexa.

Como esta seção destina-se apenas a contextualizar o leitor dentro das motivações para a execução do trabalho, as informações e dados experimentais serão

apresentados e discutidos a seguir, principalmente dentro dos artigos publicados e adicionados como ponto-chave desta tese de doutorado.

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3 Objetivos

3.1 Objetivo geral

- Desenvolvimento de métodos analíticos multidimensionais totalmente automatizados integrando preparo de amostra à cromatografia líquida acoplada a espectrometria de massas. Avaliar a empregabilidade destes em matrizes complexas, focando na obtenção de resultados eficientes enquanto busca-se diminuir o uso de reagentes, quantidade de amostra, e consequentemente a geração de resíduos tóxicos ao meio-ambiente.

3.2 Objetivos específicos

- Síntese e caraterização de materiais baseados em grafeno para posterior uso como fase extratora na etapa de preparo da amostra. Neste caso foram abordados os seguintes materiais: óxido de grafeno (GO), óxido de grafeno ancorado em aminopropil sílica (SiGO), SiGO funcionalizado com octadecil silano e subsequente processo de *end-capping* ($SiGOC18_{edc}$).

- Desenvolvimento de métodos analíticos multidimensionais utilizando colunas de extração a base de óxido de grafeno, e seu posterior emprego na separação de analitos pertencentes a diversas classes químicas presentes em amostras líquidas. Tal abordagem foi empregada para analitos como antibióticos, canabinóides, micotoxinas, hormônios e pesticidas em matrizes como água, cachaça, cerveja, urina e vinho.

- Desenvolvimento de método analítico multidimensional focado em matrizes complexas sólidas. Tal abordagem foi utilizada para avaliar a presença de pesticidades em amostras de solo proveniente de região com cultivo de cana-de-açucar, e também para avaliar a presença destes pesticidas em amostras de abelhas da espécie africanizada *Appis melifera*.

CAPÍTULO 2

Coletânea de artigos científicos

Artigos científicos em destaque

Como essa tese de doutorado é apresentada em forma de coletânea de artigos, nesta serão discutidos os principais pontos que foram destaque em cada uma das publicações. Desta forma o autor mostrará ao leitor a linha de raciocínio para a realização dos experimentos e planejamento do doutorado como um trabalho coeso tendo um propósito final bem definido. Vale ressaltar que neste documento constarão e serão explicados na íntegra os artigos em destaque. Porém, em "outras publicações", o leitor poderá consultar outros trabalhos publicados pelo autor, que tiveram relevância significativa e positiva durante todo o doutorado e "estadía" como aluno do laboratório de cromatografia da USP São Carlos – CROMA.

Antes de iniciar uma breve "resenha" sobre cada um dos trabalhos, gostaria de salientar mais alguns pontos. O fato desta tese anexar grande parte dos dados em forma de publicações trás consigo algumas características:

(i) Uma considerável amplitude de divulgação alcançada por boa parte dos dados aqui discutidos, devido a estes já constarem na literatura disponíveis como publicações. Isso em minha opinião é algo positivo que força a qualidade e a capacidade deste trabalho em potencialmente contribuir para o avanço científico, mesmo que esse seja sútil, além de poder auxiliar na formação acadêmica de outros indíviduos no futuro.

(ii) Como o Brasil é um país de dimensões continentais, nem todos os alunos de pós graduação têm o mesmo tipo e profundidade de acesso a base de dados científicas como tem os pertencentes as grandes universidades nacionais – como a Universidade de São Paulo. Desta forma, a existência de um documento como este aqui, amplamente divulgado e acessível via "Biblioteca Digital da USP", em minha opinião, também é algo benéfico. Assim, alunos que provavelmente não teríam acesso a artigos científicos de qualidade, poderão consultar e ler os contidos neste trabalho bem como outras publicações pertencentes a demais teses de doutorado apresentadas e defendidas nos mesmos moldes.

Declaro que todos os trabalhos aqui apresentados foram idealizados, coordenados e supervisionados em parceria com meu orientador e co-autor, Professor Dr. Fernando Mauro Lanças.

Primeira publicação *"Towards a universal automated and miniaturized sample preparation approach"*: Trata-se de um artigo de revisão focado principalmente na discussão dos principais conceitos, vantagens, desvantagens e propriedades dos sistemas do tipo "*column switching*" totalmente automatizados para o preparo de amostra do tipo *"column switching"*. Desta forma, o mesmo trás uma introdução sobre o tema explicando os principais conceitos e definições, além de apresentar aplicações selecionadas. Um tópico importante que é abordado neste artigo é uma comparação entre sistemas multidimensionais para preparo de amostra em escala convencional, parcialmente miniaturizada e totalmente miniaturizada. Em resumo, este artigo trás boa parte do conteúdo teórico para explicar a escolha pelo tema geral com que esta tese se propoe a trabalhar.

DOI: https://doi.org/10.1016/j.scp.2021.100427

Segunda publicação *"New materials in sample preparation: Recent advances and future trends"*: Complementar ao primeiro artigo de revisão (apresentado no ítem anterior), este segundo foca na síntese e desenvolvimento de novos materiais para utilização como fase extratora no preparo da amostra. Esta revisão publicada em um dos periódicos de maior impacto dentro da área de Química Analítica representa um importante levantamento sobre as principais classes de compostos exploradas no decorrer dos últimos anos – com destaque para o tópico dedicado aos alótropos de carbono, principalmente os derivados de grafeno. Como essa tese também explora a síntese e caracterização de novos materiais derivados do grafeno, este artigo contextualizará o leitor sobre a importância, as principais características e seu emprego para o preparo da amostra. Vale ressaltar que a seção 6 do artigo em questão, é a que discuti especificamente sobre os materiais derivados de grafeno.

DOI: https://doi.org/10.1016/j.trac.2019.115633

Terceira publicação *"Evaluation of the tubing material and physical dimensions on the performance of extraction columns for on-line sample preparation-LC-MS/MS"*: Trata-se da primeira publicação considerando dados experimentais de laboratório obtidos durante o primeiro ano de doutorado. Este trabalho foi feito em colaboração com uma pós-doc do laboratório naquele momento (Dr. Ana Lúcia de Toffoli). Como ambos trabalhavamos com o desenvolvimento de colunas de extração para automatização do preparo da amostra,

achavamos que seria positivo realizar um estudo sistemático para otimização das condições de produção e característica dessas colunas. Vale ressaltar que a Dr. Ana Lúcia de Toffoli tinha como foco principal a síntese de outros tipos de fases extratoras, principalmente as baseadas em polímeros molecularmente impressos e líquidos iônicos. No caso deste trabalho, utilizamos as fases extratoras por mim sintetizadas, sendo todas baseadas em óxido de grafeno como reagente precursor. Em minha opinião este trabalho representa um importante passo inicial para a continuidade do meu doutorado, pois foi com ele que consegui chegar a condições padronizadas de produção de colunas de extração, as quais foram utilizadas em todos os trabalhos subsequentes até a finalização desta tese. Vale ressaltar que além da publicação no Journal of Chromatography A, dados parciais foram apresentados em 2018 no conceituado congresso "*XLII International symposium on capillary chromatography*" realizado em Riva del Garda – Itália.

DOI: https://doi.org/10.1016/j.chroma.2019.03.023

Quarta publicação *"Multidimensional liquid chromatography employing a graphene oxide capillary column as the first dimension: Determination of antidepressant and antiepileptic drugs in urine"*: Dando continuidade aos resultados experimentais obtidos na publicação anterior, este artigo é focado na aplicação da coluna de extração empacotada com SiGO para a extração automatizada de fármacos antidepressivos e anticonvulsivantes em urina humana. O ponto chave deste artigo está relacionado ao uso da coluna mais efetiva reportada no artigo anterior (considerando fase extratora, condição de empacotamento, e dimensões físicas do tubo) em uma aplicação atual e importante. Sabendo que as doenças mentais, principalmente ansiedade e depressão, são atualmente consideradas entre as 3 que mais acometem pessoas ao redor do mundo, os autores decidiram aplicar os conceitos de miniaturização e automatização do preparo da amostra em uma aplicação deste teor. Importante ressaltar que além do autor deste trabalho, outra aluna participou de iniciação científica colaborou com o intuito de absorver conceitos iniciais relacioados ao trabalho: empacotamente de colunas de extração e funcionamento de sistemas multidimensionais para o preparo de amostra. Desta forma, além de evidenciar o trabalho experimental, este artigo mostra outro importante aspecto em que o autor deste trabalho procurou contribuir durante seu doutorado: treinar e acompanhar alunos em seus primeiros contatos com a academia.

DOI: https://doi.org/10.3390/molecules25051092

Quinta publicação *"Evaluation of Two Fully Automated Setups for Mycotoxin Analysis Based on Online Extraction-Liquid Chromatography-Tandem Mass Spectrometry"*: Outro importante trabalho que merece citação dentre dessa tese foi realizado com o intuito de desenvolver e principalmente comparar quais eram os benefícios ambientas – redução no consumo e geração de resíduos – que poderiam ser atingidos se um sistema totalmente automatizado e miniaturizado fosse utilizado para a análise de micotoxinas em matrizes alimentares. Este trabalho foi realiazado em colaboração com uma aluna naquele momento de mestrado (M.Sc Karen Mejía-Carmona). A minha contribuição se deu exatamente no desenvolvimento de uma metodologia totalmente automatizada envolvendo coluna capilar de extração, cromatografia capilar e espectrometria de massas. Como coluna de extração, eu produzi uma similar a utilizada no artigo anterior (empacotada com SiGO), utilizando as condições encontradas da terceira publicação aqui discutida. Desta forma, fica evidente o caracter evolutivo e sequencial das publicações e da presente tese – parte dos resultados e conclusões foram sempre aproveitados para os trabalhos subsequentes. Por fim, outro ponto deste artigo que merece destaque, é que ele mostra a capacidade do autor desta tese em mais uma vez trabalhar em parceria com outros cientistas a fim de elevar a qualidade do trabalho e consequentemente das pesquisas por ele realizadas. Em minha opinião, a colaboração científica é parte chave para o sucesso de estudos significativos e contribuem de maneira mais efetiva para o avanço científico.

DOI: https://doi.org/10.3390/molecules25122756

Sexta publicação *"A cartridge-based device for automated analysis of solid matrices by online multidimensional sample-prep-capillaryLC-MS/MS"*: Como último trabalho de destaque e finalização do projeto de doutrado, o autor procurou utilizar os conceitos e a experiência adquirida e evidenciada nas publicações anteriores, para o desenvolvimento de um método multidimensional focado na extração de analitos presentes em amostras sólidas. Todas as metodologias descritas anteriormente foram destinadas ao preparo de amostras líquidas (bebidas, água, etc) e, em minha opinião, faltava uma atenção especial para as matrizes sólidas. Dessa forma, este trabalho concentrou-se na montagem de um sistema baseado em cartucho empacotado com amostras de solo de região de cultivo de cana-de-açucar para extrair, reter, separar e detectar pesticidas frequentemente utilizados nestes locais. A metodologia em questão pode ser considerada semi-automatizada, necessitando de

intervenção manual por parte do analista apenas na etapa inicial de empacotamento da amostra sólida dentro do cartucho. Após este momento, toda a análise é realizada em sequência – cartucho empacotado (extração) → coluna de extração (retenção) → coluna analítica e espectrometria de massas (separação e detecção). Como este trabalho trata-se da última, e mais recente contribuição deste autor, o mesmo ainda encontra-se em processo de publicação, tendo sido já submetido ao periódico *"Analytical and Bionalaytical Chemistry/Springer"*

Outras publicações

Além das publicações em destaque, o autor deste trabalho participou ativamente na execução de outros projetos científicos e publicações no decorrer do seu doutorado. Segue a lista de outros trabalhos publicados:

D.A. Vargas Medina, J.V. Bassolli Borsatto, **E.V.S. Maciel**, F.M. Lanças, Current role of modern chromatography and mass spectrometry in the analysis of mycotoxins in food, TrAC Trends Anal. Chem. 135 (2021) 116156. https://doi.org/10.1016/j.trac.2020.116156.

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D.C. Ferreira, A.L. de Toffoli, **E.V.S. Maciel**, F.M. Lanças, Online fully automated SPE-HPLC-MS/MS determination of ceftiofur in bovine milk samples employing a silica-anchored ionic liquid as sorbent, Electrophoresis. 39 (2018) 2210–2217. https://doi.org/10.1002/elps.201800053.

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CAPÍTULO 3

Towards a universal automated and miniaturized sample preparation approach.

E. V. S. Maciel, D. A. Vargas Medina, J. V. B. Borsatto and F. M. Lanças, *Sustain. Chem. Pharm.*, 2021, **21**, 100427
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Towards a universal automated and miniaturized sample preparation approach

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Green chemistry

ABSTRACT

Nowadays, analytical chemistry plays a fundamental role in developing strategies for monitoring residues and contaminants in complex matrices (e.g., biological, environmental, and food samples). Considering the significant number of interfering substances present, combined with the lower concentration levels in which the target analytes are commonly found, sample preparation is becoming a crucial step in the analytical workflow. For these reasons, automation and miniaturization of this process represent a current trend since by these means, several principles of Green Chemistry can be meet all together, including: (i) lower volumes of reagents and samples required; (ii) reduced waste generation; and (iii) high-throughput with lesser operators' handling. The employment of online sample preparation based on LC systems (e.g., online SPE and In-Tube SPME) can be considered promising tools to perform sample preparation in a more sustainable way than conventional approaches. For these reasons, herein, we present a review discussing the essential characteristics of miniaturized, automated systems, including column-switching modes and modern approaches based on chip-LC. These methodologies deserve attention mainly due to their greener character derived from automation and miniaturization of the process. Also, selected applications, mostly covering the last five years, are presented. A brief comparison between them and conventional LC-based approaches is carried out to bring an adequately discussed review to this journal audience.

1. Introduction

Analytical chemistry plays a fundamental role in many areas of technological science and industry by developing strategies to monitor the concentration of chemical species in several places. It covers analyzes of biological and environmental samples, food, pharmaceutical drugs, organic and inorganic pollutants, among several others (Fernández-Ramos et al., 2014). It is essential to understand that independently of the samples, most of them contain the target-compounds together with other thousand ones (e.g., carbohydrates, proteins, amino acids, salts, and others). These matrix interferents might alter the final analyses' results by affecting the method's ability to report the target analytes' exact concentration. For this reason, one of the most critical stages of amending this in the analytical workflow is the sample preparation step (Pérez-Fernández et al., 2017).

In short, the sample preparation procedure is crucial in a typical analysis once it is responsible for eliminating interferents by cleaning up the sample while pre-concentrating the target analytes (Nazario et al., 2017). Over approximately six decades, the dominant techniques were liquid-liquid extraction (LLE) and, posteriorly, solid-phase extraction (SPE) (Nazario et al., 2017). More precisely, SPE emerged as a sample preparation technique alternative to LLE, aiming to reduce the large amounts of organic solvents often used on it (Maciel et al., 2019). Although SPE brought new analytical possibilities by diversifying the extraction-media (e.g., several sorbents as C18, -NH_2, Al_2O_3), the relatively large amounts of reagents and samples, still required by this technique, continued to represent a challenging for the sample preparation step (de Toffoli et al., 2018a,b). Their frequent multiple steps-related methods made them laborious/time-consuming and a potential source of analytical errors (de Toffoli et al., 2018a,b).

Miniaturized sample preparation techniques emerged in the early '90s to meet these LLE and SPE shortcomings. In this way, the SPE-based solid-phase microextraction (SPME) developed by Pawliszyn et al. was the first one ushering to a new era of miniaturization on sample preparation techniques (Arthur and Pawliszyn, 1990). After, other

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Abbreviations

µ-TAS	micro-total analysis
BMA-EDMA	azobisisobutyronitrile
C18	octadecyl silane stationary phase
DLLME	dispersive liquid-liquid microextraction
FSPE	fabric-phase sorptive extraction
HPLC	high-performance liquid chromatography
I.D.	internal diameter
IL	ionic liquid
IT-SPME	In-Tube solid-phase microextraction
LLE	liquid-liquid extraction
LPS	large particle supports
MEPS	microextraction by packed sorbent
MIP	molecularly-imprinted polymer
MOF	metal-organic framework
OT	open-tubular column
PDMS	polydimethylsiloxane
PLOT	porous-layer open tubular
PS-DVB	poly(styrene-*co*-divinylbenzene)
PS-OD-DVB	poly(styrene-*co*-octadecene-*co*-divinylbenzene)
RAM	restrict access media
SBSE	stir bar sorptive extraction
SDME	single-drop microextraction
SPE	solid-phase extraction
SPME	solid-phase microextraction
TFC	turbulent flow chromatography
UHPLC	ultrahigh-performance liquid chromatography

miniaturized approaches began to continuously spring up, outstanding the stir bar sorptive extraction (SBSE) and microextraction by packed sorbent (MEPS) (de Toffoli et al., 2018a,b). Noteworthy, these microtechniques have their roots in SPE and are considered sorption-based approaches (Jalili et al., 2020). Conversely, LLE-based microtechniques also emerged at the same time, including dispersive liquid-liquid microextraction (DLLME), single-drop microextraction (SDME), among others (Płotka-Wasylka et al., 2016). The main goals of such miniaturized approaches are the drastic reduction in the use of organic solvents and samples according to the Green Chemistry principles, which was adequately achieved in the previously underscored ones (Jalili et al., 2020). On the other hand, they sometimes involve necessary additional procedures (e.g., centrifugation, drawn and drop cycles, vortexing, and others) and, by consequence, substantial handling by the analyst (Maciel et al., 2019). Like so, even considering all non-automated microtechniques' benefits, they are still related to multiple steps, increasing the analysis time, complexity, and propensity of analytical errors (Maciel et al., 2019).

For these reasons, automated approaches emerged basically to be faster, simpler, and more environmentally friendly than traditional and miniaturized ones (Medina et al., 2019). Within such a context, several strategies have been reported in the last decades, including the automation of SPE (online SPE), and by tailoring the microextraction techniques towards its automation (e.g., automated MEPS, automated SPME, in-tube SPME, automated SDME, automated SBSE, and others) (Pan et al., 2014). The sorption-based approaches, as our focus here, possess similarities relying upon its interaction mechanisms between sorbent/-solvent with the target analytes (Chen et al., 2019). As an example, the same extraction sorbents can be used in different approaches, which in some cases, opens up the possibility to standardize such approaches in one unique way. For example, a method based on SPME could be theoretically adapted to work with MEPS once the same sorbents (e.g., C18, or C8) can be packed inside the MEPS's syringe or used as a coating onto SPME's fibers. So, after preliminary tests to adequate the values of the main parameters (e.g. extraction cycles, solvent volumes, etc.), the methods could be transferred between these two techniques and to other sorbent-based approaches as well. (Chen et al., 2019).

Taken this into account, a universal automated sorption-based sample preparation approach can be proposed using multidimensional separations based on liquid chromatography systems (Franco et al., 2016). In this case, the first column would be constituted of the extractive phase, responsible for retaining the target analytes, instead of working as a chromatographic dispositive (Maciel et al., 2019). On the contrary, and after desorbing the analytes from the first (extraction) column and transferring them to the second one, the analyte's separation will be processed. Noteworthy, the first separation device, usually termed extraction column, might be constituted of a filled extractive phase (packed or monolithic) or an open tubular one (OT) (Maciel et al., 2019).

Considering that the first-dimension acts as an extraction column and the second as a chromatographic separation medium, in some cases, this approach can be also termed as a multidimensional separation or hybrid (extraction-chromatography) separation system. Independently of the terminology already employed, it seems that multidimensional separation will better fit in this case, once both extraction and chromatography are separation units. Theoretically, this strategy becomes, in several situations, feasible to achieve similar results to the off-line sorption-based methods in one unique automated platform (Franco et al., 2016). This combination can exhibit several positive features, including (i) excellent compatibility between the extraction and analytical columns, once they can possess similar characteristics favoring the automation as well as the selection of an appropriate solvent to enhance analyzes' results; (ii) more straightforward application and greater accuracy once when analysis started, there is no need for additional analyst's interference; (iii) faster analysis and high-throughput owing to the full automation of the analytical workflow; and (iv) demanding for lower amounts of solvent and samples, and consequently reduction of the toxic waste (de Toffoli et al., 2019). To demonstrate this, Maciel et al. recently published a work showing the advantages of miniaturization and automation of mycotoxin analysis using multidimensional separation based on an LC system (Maciel et al., 2020a,b). The excellent economies of mobile phases and samples were highlighted, obtained with a shorter analysis time. For instance, the authors estimated a mobile phase consumption of 100 times lower than other recently published similar works based on non-automated approaches.

Considering the current world situation in which many scientists are encouraging more sustainable and environmentally friendly practices, these multidimensional separation approaches, embracing sample preparation and analysis in a unique platform, emerge to help some analytical chemistry branches become more sustainable without losing their performance (Kurowska-Susdorf et al., 2019). Like so, this review is focused on presenting/discussing fundamental aspects and recent advancements in the field of multidimensional separations based on LC and employing an extraction column as the first dimension of such systems. Herein are discussed automated traditional column switching approaches and the modern ones, covering the last ten years and emphasizing the last five.

2. Multidimensional separation approaches

2.1. Column switching

Since the beginning of the HPLC development, the possibility to inject raw complex samples directly into the chromatographic system has encouraged the researchers to explore and develop different automated strategies that enable the efficient hyphenation of the sample

preparation procedures with the later separation and detection of the analytes. One of the most efficient strategies to meet this is implementing multidimensional systems based on the LC concepts. In this case, an extraction and an analytical column are online coupled via a switching valve.

In these systems, raw samples can be injected into the extraction column assisted by an additional high-pressure pump (loading pump), while the analytical column is conditioned with the mobile phase from the HPLC pump. Hence, the analytes are selectively retained, in the first dimension, while macromolecules and other sample interferents are excluded. After the sample clean-up step, the valve is switched, connecting the two columns online, being the analytes desorbed from the first one, and transferred by the mobile phase towards the analytical column for separation and further detection (Mao and Huang, 2019).

Although earlier multidimensional separation systems were developed by adapting approaches used for multidimensional GC (Deans, 1968) or low-pressure LC (Scott and Kucera, 1979a), the main difficulty in coupling LC columns was the lack of high-pressure valves able to withstand the operational conditions (Huber et al., 1973). Some early strategies were based on the "offline coupling," as in the system described by Ishii et al. (1976). To the best of our knowledge, the first multidimensional high-pressure separation system based on HPLC was reported in 1973 by Huber and Van Der Linden (Huber et al., 1973). They introduced a switching valve operating at high pressures with very low hold-up volume. A few years later, Scott and Kucera (1979b) developed an online multidimensional separation system to analyze raw urine and blood samples. Two six-port valves were employed, and the raw biological sample was injected into an open sample loop installed in the first valve.

Several similar approaches and strategies were developed and explored by many researchers in the following decades (Cai and Henion, 1997; Dolphin et al., 1976; Moore et al., 1991; Nielen et al., 1985a, 1985b; Pascual et al., 1996; Shen et al., 1997; Swart et al, 1998, 1999; Takeuchi et al., 1985; Van Der Heeft et al., 1998; Westerlund, 1987). With the emerging commercial HPLC instruments, the development and use of multidimensional separation systems skyrocketed during the '80s and '90s. A significant number of column switching approaches were reported, ranging from a simple two-column (bidimensional) system to multidimensional column networks. Various names were created to designate this new situation (e.g., coupled column chromatography, split chromatography, and column switching chromatography, among others) (Huber et al., 1980; Johnson et al., 1978; Kok et al., 1982; Lankelma and Poppe, 1978; Ramsteiner and Böhm, 1983; Snyder, 1970).

Nowadays, although multidimensional separation systems are well-consolidated, the nomenclature remains somewhat controversial and not standardized. For instance, the terms multidimensional LC and "column switching" gathers several modes of online coupling, including the comprehensive LCxLC and its different modes, as well as sample preparation procedures (e.g., online solid-phase extraction (online SPE), in-tube solid-phase microextraction (IT-SPME), turbulent flow chromatography (TFC), and others, which are emphasized in this review (Fumes et al., 2017).

In brief, comprehensive LCxLC gathered a series of analytical techniques to increase the resolution and peak capacity to separate very complex mixtures. This is accomplished by the coupling of analytical columns with orthogonal retention mechanisms. In LCxLC, all the sample parts are transferred from the first to the second dimension (Brandão et al., 2019; Cacciola et al., 2020; Knox and Gilbert, 1979; Montero and Herrero, 2019). On the other hand, online-SPE, IT-SPME, and TFC are multidimensional separation approaches focused on hyphenate the sample preparation step with chromatographic separation. In this manner, only the analytes of interest are selectively transferred from the first to the second dimension (Franco et al., 2016; Háková et al., 2020; Marlot and Faure, 2017).

Distinguishing among the various multidimensional separation modalities is not trivial, especially nowadays, due to the several recent advances in LC mainly focusing on its miniaturization achievements (Vargas Medina, Maciel, de Toffoli et al., 2020b). For instance, miniaturized LC includes sophisticated analytical devices such as modern chip-LC containing extraction and separation columns in a single small footprint platform (Maciel et al., 2020a,b). Nevertheless, all these techniques have their origin in the early efforts directed to injecting raw complex samples. They are based on coupling two or more columns and a switching mechanism, firstly for sample clean up and then for chromatographic separation (Queiroz and Acquaro, 2017). The most significant differences between online-SPE, IT-SPME, and TFC, are the particle size and some characteristics of the extraction column (physical dimensions and stationary phases, for example) (Fig. 1).

In this context, a comprehensive overview of the different extraction columns employed as the first dimension in automated multidimensional separation techniques was recently published by Maciel et al. (2019), highlighting their main features, performance, and fabrication material/extraction-phase and technologies.

Over recent decades, online SPE have had been one of the most used modes for multidimensional separations based on LC; this approach employs column-like support for the sorbent instead of the traditional cartridge style. The term "online SPE" appeared in the literature in the '80s. Since then, this technique has found great applicability in many analytical areas, covering from omic sciences to the extraction and determination of small organic molecules in clinical, forensic, environmental, and food analysis. Noteworthy, this term is being used for both conventional and miniaturized scales. In this case, the first dimension is typically a filled column (i.e., particle-packed, monolithic, or fiber-packed) with conventional dimensions (i.d. $>$ 1 mm). The generic procedure commonly involves four sequential steps: i) extraction and analytical column conditioning; ii) sample injection and clean-up; iii) analytes transference and chromatographic separation; and iv) washing of the extraction column. Like in other multidimensional separation techniques, the analytes' desorption can be performed either in the straight-flushing or backflushing column-switching modes (Fig. 2). The technique owes its name to the similarities with the off-line cartridge-based SPE technique. In this case, the main differences between off-line and online SPE formats are the reusability of the extraction column in the online mode and the importance of the washing step to avoid the carry-over effect in the sequential analysis of multiple samples. Thus, online SPE provides analytical frequencies several times superior to those obtained with the off-line format, while employs lower amounts of solvent and sorbent, reducing the sample handle and consequently, increasing the analytical accuracy (Rogeberg et al., 2014; Valsecchi et al., 2015; Yang et al., 2014).

In the last years, a substantial amount of research on the development of new online SPE procedures has been performed. One of the most active research areas in online SPE includes the synthesis and use of cutting-edge sorbents. Current online SPE extraction columns are packed with classical reverse-phase particles and innovative materials for raw sample direct extraction. These materials include modified silica particles with ionic liquids (da Silva and Lanças, 2018; Ferreira et al., 2018), molecularly imprinted polymers (S. Yang et al., 2019), aptamer-based magnetic materials (Gan and Xu, 2018), polymeric nanofibers (L. Q. Chen et al., 2020; Háková et al., 2018, 2020), carbon allotropes such as restricted access carbon nanotubes (Gan and Xu, 2018), and graphene oxide (S. Yang et al., 2019), among many others. Similarly, it can be currently found a great diversity of reports describing the use of online SPE for the analysis of diverse analytes by the direct extraction from raw biological samples such as plasma (S. Yang et al., 2019), oral fluids (Mulet et al., 2020), urine (L. Q. Chen et al., 2020), and feces (S. Yang et al., 2019). This approach has also covered food and beverage samples (K. Liu et al., 2020), as well as environmentally relevant matrices (S. Yang et al., 2019), cosmeceutical and personal care matrices(Wilcox et al., 2020), and phytotherapeutic products (Wilcox et al., 2020). Finally, recent reviews focused on online-SPE offer

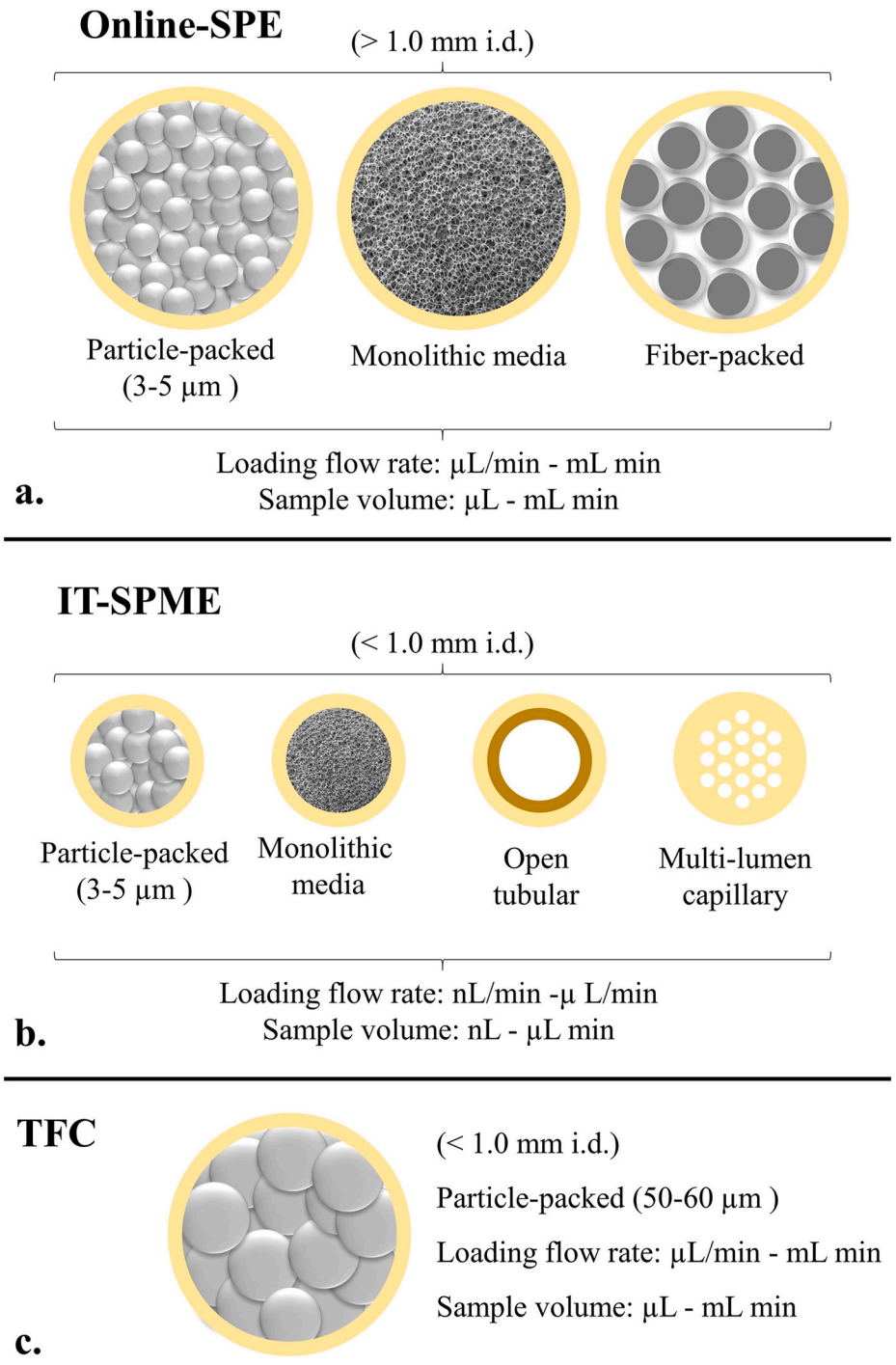

Fig. 1. Different types of extraction columns employed as the first dimension in multidimensional separation systems. **(a)** Extraction columns typically employed in online-SPE; **(b)** extraction columns typically employed in IT-SPME); (c) extraction columns typically employed in TFC.

comprehensive descriptions of the technique, their advantages, and additional relevant applications (Andrade-Eiroa et al., 2016; Mao and Huang, 2019; Wei et al., 2015; Zheng, 2019).

Following this line of reasoning, IT-SPME was introduced in 2000 by Pawliszyn and Gou to accomplish the automated hyphenation of SPME with liquid chromatography and mass spectrometry (LC-MS) (Gou and Pawliszyn, 2000). Under its original form, the extraction was performed employing an OT extraction column (Eisert and Pawliszyn, 1997). Currently, several publications are describing multidimensional separations as IT-SPME, even when miniaturized packed or monolithic extraction columns are used (Kataoka et al., 2016). Similarly to online-SPE, in the IT-SPME, the analytes' extraction and sample clean-up are carried out by loading the sample through the miniaturized column, low eluotropic strength mobile phase condition, and sequentially conducted to the analytical column. Although there is no official consensus about it, the IT-SPME concept has been applied to describe the majority of fully miniaturized multidimensional separations based on LC systems, which possess, in the first dimension, an extraction sorbent into a small diameter tube. However, in a more general definition of it, the IT-SPME systems are characterized by using a micro, capillary, or nano column in the first dimension, while the second one can be a miniaturized or conventional analytical column (Pascual Serra-Mora et al.,

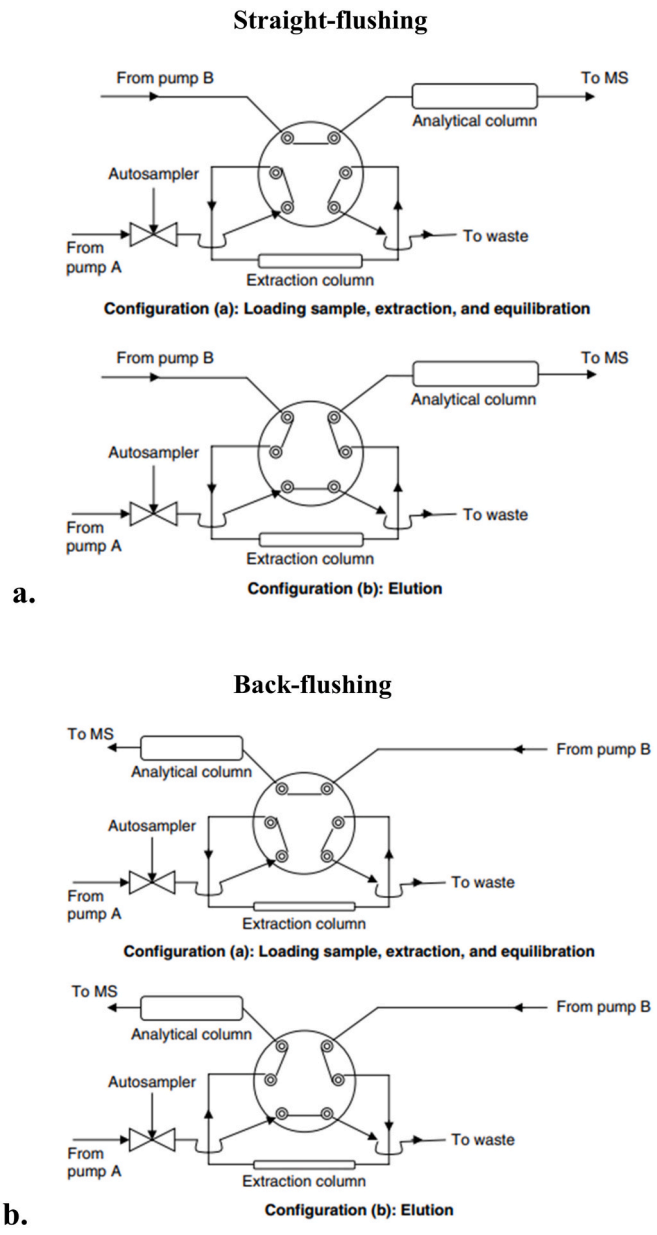

Fig. 2. Operation modes of multidimensional separation systems. a) Extraction-LC-MS/MS system with straight-flushing column switching; b) extraction-LC-MS/MS system with back-flushing column switching.

2017).

Another difference between IT-SPME and online-SPE is the sample loading mode, which in the first case, can be performed by i) pumping the sample through the miniaturized extraction column in a single step (flow-through extraction mode) or ii) performing several draw/eject cycles (draw/eject mode) thought the extraction column (Moliner-Martinez et al., 2015). Although the draw/eject mode can provide enhanced analyte enrichment and improved detectability, the flow-through mode is only employed with particle-packed and monolithic columns due to the chromatographic media's permeability. In contrast, the draw/eject mode is limited to extraction/preconcentration OT columns (Costa Queiroz et al., 2019). Also, the possibility of performing draw/eject cycles is available only in some of the modern LC instruments' automated injection systems, once it requires bidirectional pumping systems (Fernández-Amado et al., 2016). In this IT-SPME mode, the extraction column is placed between the injection needle and the sampling loop, so that, once the injection needle enters the sample vial, the sampler pass the sample through the extraction column in a sequence of draw/eject cycles until equilibrium is reached, while the analytical column is being conditioned with the mobile phase (Fernández-Amado et al., 2016).

IT-SPME is perhaps one of the most popular and spreading techniques that might be considered a multidimensional separation LC system. Due to the reduced amount of extraction phase required to prepare the miniaturized extraction column, IT-SPME is frequently the chosen technique for testing new and innovative columns' formats. Examples are the multi-lumen capillaries (da Silva et al., 2017), and cutting-edge sorbent materials, such as graphene (de Toffoli et al., 2018; Mejía-Carmona and Lanças, 2020), graphitized materials (Pang et al., 2019), polymeric ionic liquids (ILs) (Souza et al., 2019), immunity-affinity sorbents (Chaves and Queiroz, 2013), restrict access materials (RAM) (De Oliveira Isac Moraes et al., 2013; Santos Neto et al., 2006), imprinted polymers (MIP) based sorbents (Chaves and Costa Queiroz, 2013), Metal-organic frameworks (MOFs) (Pang et al., 2019), metallic nanoparticles (Pang et al., 2019), and natural materials as cotton (Pang et al., 2019). Hence, the literature describing IT-SPME systems and methods has been under continuous growth in the last two decades, and many current reviews on this topic can be found in the literature (Costa Queiroz et al., 2019; Moliner-Martinez et al., 2020; Ponce-Rodríguez et al., 2020).

Finally, TFC is a multidimensional separation technique for online sample clean-up of raw complex samples, particularly before LC-MS, which employs in the first dimension an extraction column packed with large particles (large particle supports, LPS) and high sample loading flow rates (Herman and Edge, 2012). In this case, the fluid's momentum exceeds the resistance to flow by a factor enough to promote turbulent flow. Higher mass transference is obtained under that condition. Large molecules — such as proteins or humic acids — are efficiently discriminated against from the analytes possessing lower molecular mass (Couchman, 2012; Lanças, 2014).

Several TFC operation modes have been explored, being the most used the one named as focus mode. In this case, the instrumental setup involves two switching valves, a loop for the desorption solvent storing, and an auxiliary high-pressure pump for sample loading (Fumes et al., 2017). The raw sample is loaded into the extraction column at flow rates in the order of 1.5 mL/min or higher, while the analytical column is conditioned with the mobile phase and then washed with a weak solvent (low eluotropic strength) for a limited time. After that, the valves switches, and the desorption solvent contained is transferred from the loop to the LPS media desorbing the analytes, which are online transferred to the analytical column contained in the second dimension. Finally, the loop is filled again with the desorption solvent, and the extraction column is washed and conditioned for the next analysis (Couchman, 2012; Lanças, 2014).

Considering the statements previously discussed and understanding the differences between each mode of multidimensional separations (e.g., online SPE, IT-SPME, and TFC), we consider that it becomes clear that distinguishing between them is still unclear in the literature. Noteworthy, this fact might represent a problem for researchers focused on literature revision. Hence, in the light of the current development towards miniaturized LC and online sample preparation approaches, we attempt to unify all these automated column-based techniques under the category of multidimensional separation techniques.

2.2. *Fully miniaturized multidimensional separation approaches*

Probably one of the most remarkable trends in modern liquid chromatography is the miniaturization of the separation media and the associated instrumentation (Mejía-Carmona et al., 2020; Vargas Medina et al., 2020b). Although the '70s were established that using miniaturized columns in liquid separations could provide superior chromatographic performance, the consolidation of capillary/nanoLC-MS required reengineering all related instrumentation. For this reason, its

consolidation should still take several decades. Nowadays, miniaturized LC-MS is an emerging technique, with spread and growing applications. Its main analytical advantage is the improved sensitivity, derived from its high compatibility with mass spectrometry detection (Vargas Medina et al., 2020b).

In practice, to access the remarkable sensitivity at capillary/nano-scale, sample volumes more considerable than those commonly supported by the miniaturized columns through direct injection should be analyzed (Blue et al., 2017). For this reason, at a miniaturized scale, the multidimensional separation approaches enable the online sample treatment and pre-concentration, becoming the best strategy to analyze much larger volumes of samples, usually greater than those supported by the miniaturized columns via direct injection, while improving detectability (Prinsen et al., 1998). A fully miniaturized multidimensional system employing extraction and analytical columns exhibit excellent compatibility with mass spectrometry detection. As an example, such a miniaturized and multidimensional system favors so much the hyphenation with electrospray ionization MS detectors, once this ionization mode shows improved performance at lower flow rates (Vargas Medina et al., 2020a). More recently, studies focusing on the hyphenation between nano-LC with electron ionization MS detectors have been reported (Rigano et al., 2019). The full miniaturization of multidimensional separation approaches seems an attractive idea within such a context since one major challenge on the nanoLC-EI-MS coupling is still the low sensitivity due to the limited injection volumes required (ca. nL). Therefore, implementing a first-dimension containing a miniaturized extraction column to concentrate the analytes will be useful in such cases.

The efforts to develop fully miniaturized multidimensional separation systems date back to the first attempts to develop miniaturized liquid chromatography systems themselves. Older miniaturized multidimensional systems were configured through the coupling of narrow bore packed extraction columns to analytical columns at the μ-scale (0.5–1.5 mm ID). Several examples of semi and fully automated configurations for the direct injection of raw samples in μ-LC were widely reported between the end of the '70s and the '90s. Over the years, the advances in column manufacturing and miniaturized LC instrumentation achievements made the migration to the downscaled capillary and nano-LC (150–500 μm ID; < 150 μm ID, respectively) feasible.

The first multidimensional separation system operating at the capillary scale emerged with the introduction of the IT-SPME. Gou and Pawliszyn demonstrated that the online coupling of an OT extraction column (ID 250 μm coated with PDMS) with a capillary analytical column could provide sensitivities more than 20 times greater than the observed by coupling the same extraction capillary with an analytical column of conventional scale (Gou and Pawliszyn, 2000). Nowadays, the capillary scale analytical advantages are well established, and it can be considered the most frequently used in the development of fully miniaturized multidimensional separation systems. In this way, capillary analytical columns have been employed in many configurations with several extraction media such as monolithic columns (Zhang et al., 2010), OT columns (Campíns-Falcó et al, 2015, 2018; 2015; González-Fuenzalida et al., 2016; Jornet-Martínez et al, 2015, 2018; Moliner-Martínez et al., 2015; Pla-Tolós et al., 2016; Prieto-Blanco et al., 2016), and packed columns including RAM-MIP particles (Santos-Neto et al, 2007, 2008), magnetic materials (Moliner-Martinez et al., 2014; Moliner-Martínez et al., 2012), and carbon allotropes derived sorbents (Campíns-Falcó et al., 2016; P. Serra-Mora et al., 2017).

Although the extra column band-broadening becomes a real challenge for these systems, the commercial capillary/nano-LC instruments' popularization has also boosted the development of multidimensional separation approaches at the nanoscale. Wilson et al. (2007) presented one of the first multidimensional separation systems used to quantify perfluorooctanoic acid and perfluorooctane sulfonate from water samples. The authors demonstrated increases in peak intensity up to 7-fold through a capillary to a nano-sample-prep-LC-MS approach.

Similarly, Falcó's research group has currently dedicated significant efforts to developing multidimensional separation systems at the nanoscale. On several occasions, they have demonstrated improvements in overall analytical performance by replacing the capillary analytical column with others of smaller inner diameters. For example, in a recent study, the authors compared the IT-SPME's analytical performance coupled to capillary- and nano-LC, in the determination of triazines and their degradation products from water samples (P. Serra-Mora et al., 2017). The IT-SPME-nLC showed higher performance than IT-SPME-cLC, with superior extraction efficiency. Although the processed sample volume was 4.0 mL for cLC and 0.5 mL for nLC, the sensitivity for IT-SPME-nLC was between 10 and 25 times higher than the observed for IT-SPME-cLC.

In another interesting recent example, Maciel and coworkers (Maciel et al., 2020a,b,c) compared the performance of two multidimensional approaches — employing extraction columns packed with graphene-based sorbents — for the analysis of mycotoxin in beverages. The use of a multidimensional capillary LC-MS/MS setup demonstrated to be a very environmental-friendly system with consumption of reagents and waste generation 100 times lower than conventional scale systems.

Nowadays, nano-LC-based multidimensional separation systems are turning out more common and widespread. Interesting examples reported the use of conventional C18 extraction columns (Wilson et al., 2007), but also innovative extraction media, such as the Hypercarb (Leonhardt et al., 2014) and OT capillaries coated with traditional porous coatings (Jornet-Martínez et al., 2018), as well as coatings modified with diverse nanoparticles (Serra-Mora et al., 2020).

2.3. Chip-based multidimensional separation approaches

One of the most critical aspects of the configuration of multidimensional separation systems at the nanoscale is the attenuation of extra-column effects over the separation performance. A remarkable strategy to meet these effects is developing and using chip-based LC-MS systems. Analytical chips are compact and robust devices, with complex microfluidic networks embedded, featuring multiple instrumental parts, such as injection systems, dilution mechanism, reactors, extraction/enrichment and/or separation columns, spectrophotometric detection cells, and even nano-electrospray emitters. All parts should be integrated into a compact block of reduced footprint, without the need for external union, tubing, or fittings (Vargas Medina et al., 2020a; Maciel et al., 2020a).

The origin of the multidimensional chip-based LC-MS systems date backs to the '90s, with the introduction of the "Lab-on-a-Chip" and "micro-total analysis (μ-TAS)" concepts (Janasek et al., 2006). This analysis mode has quickly evolved to robust and more sophisticated commercial platforms such as the first HPLC-chip introduced by Agilent in 2005 (Yin et al., 2005; Yin and Killeen, 2007). The Agilent HPLC-chip is a fully integrated multilayer polyimide small device, containing a nano extraction column, a 6-port switching valve, and a nano analytical packed column coupled to a nanoESI-MS emitter (Fig. 3a). Since its introduction, other instrument manufacturers, such as Waters and AB-Sciex, have also developed similar products. Nowadays, a broad portfolio of commercial chip-based multidimensional separation systems is available, integrating sample preparation and chromatographic separation in a single device (Maciel et al., 2020a). Chip-based approaches are becoming very popular. This system provided the users with the possibility of developing analytical methods involving miniaturized multidimensional separation systems in a straightforward way.

Commercial multidimensional nano-LC-MS chips can operate connected to the fluidic systems of a nano-LC benchtop instrument. In this case, a dedicated interface, easily coupled to the inlet of a mass spectrometer, establishes all fluidic and electric connections, just by "plugging and playing" the chip device. This characteristic has made the commercial chips especially promising and attractive for a wide

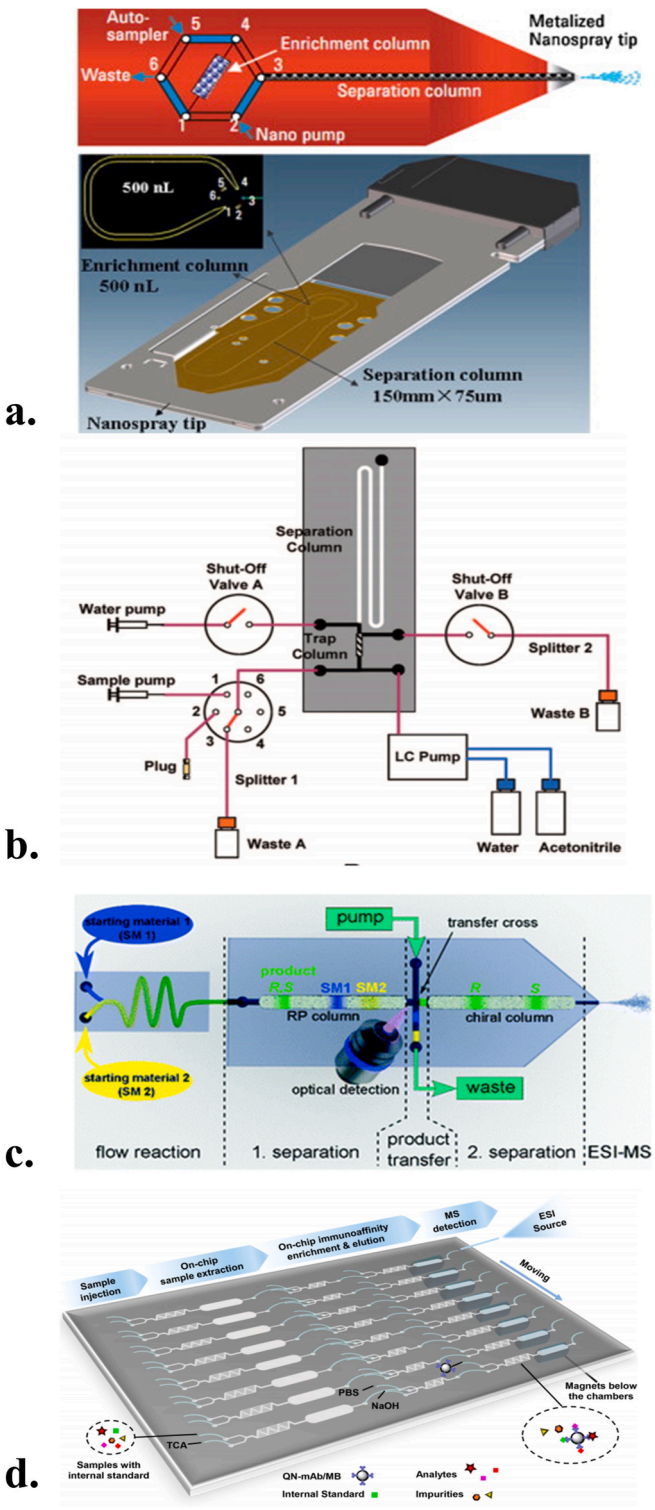

Fig. 3. Some examples of chip-based multidimensional separation approaches. **(a)** schematic design of the Agilent HPLC-chip (Zhao et al., 2011); **(b)** Design of the multidimensional chip developed by Liu et al. integrating online-SPE and chromatographic separation via a double-T passive injection mechanism (Liu et al., 2009). **(c)** Design of the on-chip two-dimensional heart-cut device developed by Lotter et al. (Lotter et al., 2016); **(d)** High-throughput chip-based multidimensional device developed by Zhao et al. (Zhao et al., 2019).

diversity of applications, such as the pre-concentration and reverse-phase separation of peptides (Yin et al., 2005), the determination of abused drugs and metabolites in human hair (Zhu et al., 2012), oligosaccharides in milk (Barile et al., 2010), 8-isoprostaglandin F2 in human urine (Bai et al., 2011), polyamines in human nails samples (Min et al., 2011), and aflatoxins in peanut products (Liu et al., 2013), among others.

Apart from the commercial approaches, chip-based analysis is considered one of the more remarkable trends in LC miniaturization (Desmet and Eeltink, 2013). In the last decades, a vast collection of lab-made fully integrated multidimensional LC-chips, containing sorption-based sample preparation and chromatographic separation have been developed and reported (Ai et al., 2019; Cui and Wang, 2019; Dubey, 2018; Haghighi et al., 2018). For example, Liu and coworkers (Liu et al., 2009) integrated solid-phase extraction and chromatographic separation in a polymethacrylate chip able to withstand pressures above 20 MPa (~2900 psi) for efficient pressure-driven HPLC analysis. The integrated chip design includes a passive double-T injection mechanism and a nano methacrylate monolith extraction/enrichment column. The proposed approach increased both sensitivity and the separation performance by coupling the extraction column to a nano reversed-phase polymethacrylate monolith analytical column. The chip design is also equipped with solvent splitting channels that allow the coupling with conventional scale LC pumps for on-chip gradient generation (Fig. 3b).

Another interesting on-chip multidimensional separation approach was reported by Lotter et al. (2016). They developed a heart-cut 2D LC-chip for interference-free determination of the enantiomeric excess via mass spectrometry. The device contains two different columns packed with reversed-phase and chiral stationary phase materials. The columns are linked through a transfer cross channel, allowing the heart-cut operation mode to transfer the chiral compound of interest from the first to the second chromatographic dimension in the microfluidic glass chip (Fig. 3c). The same research group developed a similar chip-based multiple heart-cutting 2D LC system to determine pesticides and peptides, integrating additional functionalities on the previous prototype (Piendl et al., 2020).

Another interesting prospect of the chip-based LC multidimensional separation systems is the development of parallel arrangements, containing multiple analytical systems into a single miniaturized device (Huft et al., 2013; Zhou et al., 2019). For example, recently, Zhao and coworkers developed a multi-parallel channeled chip to determine quinolones in milk samples via mass spectrometry (Zhao et al., 2019). The system includes various parallel channels, with sample extraction, immunoaffinity enrichment, and magnetic separation. Noteworthy, this system gathers all steps of automated sample loading and programmable gradient generation, without additional off-line clean-up procedures and in a high throughput configuration (Fig. 3d).

The chip technology paves its way in developing fully miniaturized multidimensional separation platforms, offering great flexibility in the system arrangement, and incorporating multiple analytical tasks in a single small footprint device.

3. Selected applications

Multidimensional separation has become a relevant field of analytical chemistry in the current century, receiving attention from various fields, such as food chemistry, proteomics, metabolomics, and health science (Franco et al., 2016). In this section, selected applications based on these systems and considered attractive from the author's point of view, are summarized and discussed. The subsection is focused on five different setups that are: (i) 1st and 2nd are packed columns; (ii) 1st dimension is an OT column and 2nd dimension is a packed or OT columns; (iii) 1st dimension is a monolithic column and the 2nd dimensions are OT or packed columns; (iv) chip-based separations; (v) key findings about the applications.

3.1. 1st and 2nd dimensions are packed columns

Packed columns are the most popular type in liquid chromatography for both extraction and analytical purposes. The packing material employed on it comprises various commercially available particles and allows modifications on the particle binders to produce more selective columns depending on the analysis. Besides its significant advantages, it presents the main limitation of the high-pressure drop provoked by the packing material. The particles' presence in the packed beds demands high pressure to overcome the resistance and makes the mobile phase flow through it.

The applications of this system differ expressively. The reversed-phase separation is more typical on this instrumental set up as well in any other LC configuration. In this way, Márta and coworkers (Márta et al., 2018) have evaluated a series of nonsteroidal anti-inflammatory drugs from drinking water, surface water, and wastewater by an on-line SPE-micro HPLC system. As those compounds are present in trace amounts in the water matrix, the detection method selected should be sensitive and selective. The authors found naproxen ketoprofen, ibuprofen, diclofenac in the range of 0.31–21.7 ng/mL in the wastewaters; no contaminants were found in other explored matrix. Another exciting work was performed by Acquaro and coworkers (Acquaro et al., 2017). Drugs that act in the central nervous system were monitored via multidimensional UHPLC-MS. Several drugs were determined in human plasma samples, which are used in the treatment of schizophrenia. Schizophrenia is usually treated by a combination of more than one compound. The concentrations of the drugs in the plasma should be controlled to keep the therapeutic range and reduce side effects. The extraction column used by Acquaro was a C8 - ADS (25 μm, 25 × 4 mm), and the analytical column was a superficially porous column C18 (1.7 μm, 100 × 2.1 mm).

Maciel and coworkers (Maciel et al., 2020a,b,c) have explored the use of graphene-based compounds anchored onto silica particles to produce extraction columns (50 μm, 0.254 × 200 mm) as the first dimension for the evaluation of mycotoxins in beverages. Two different equipment setups were explored for the 2nd dimension: one using a conventional scale and the other a capillary scale. In the conventional LC scale, the system configuration was Poroshell 120 SB-C8 (2.7 μm, 2.1 mm × 100 mm) and in the capillary LC scale, it was C18 THS (1.7 μm, 0.300 × 100 mm). Both performed adequately. The author concluded that the conventional LC scale performed better to analyze mycotoxins in matrices when a larger volume of samples is available and lower limits of quantification are required. However, in analysis requiring a higher throughput and a lower cost, the miniaturized LC system showed to be a better option.

Another good example of non-typical separations performed is presented by Hädener and coworkers (Hädener et al., 2017) that have quantified amphetamine enantiomers in human urine. The determination of chiral compounds in human samples is crucial to achieving the goal of using such compounds. Like so, the R-enantiomer results in cardiovascular and peripheral effects, but the S-enantiomer has stimulant proprieties; amphetamines produced illegally usually yields a 50:50 racemic mixture (George and Braithwaite, 2000). The Hädener instrument configuration was composed of a first dimension C18 extraction column (5 μm, 10 × 2 mm), and a second-dimension fitted with a chiral analytical column (AMP LC column, 3 μm, 150 × 3.0 mm). Noteworthy, the analytes were transferred from the extractive column to the analytical one via a backflush mode. Likewise, Hädener and coworkers (Hädener et al., 2016) have also investigated 11-nor-9-carboxy-delta9--tetrahydrocannabinol and 11-nor-9-carboxy-delta9-tetrahydrocannabinol glucuronide in blood samples to evaluate cannabis consumption frequency. The extraction column (10 × 2.0 mm) was packed with 4 μm RP particles, and the analytical column (30 × 2.1 mm) with C18, 2.6 μm core-shell particles. The elution was performed in a backflush mode.

Yan and coworkers (Yan et al., 2018) silylated oligosaccharides in mammalian milk using an SPE-LC-MS system operating in the HILIC separation mode. The system configuration was composed of a Click TE-GSH column (5 μm, 50 × 2.1 mm) as a trapping column and a XAmide (5 μm, 150 mm × 4.6 mm) as the analytical column. In the method explored, more than 30 compounds were detected in the human samples. The comparison with other no human samples was also evaluated. As the authors concluded, this online method presents advantages over the offline approaches later employed for those samples, especially the reduced analysis time due to the absence of sample preparation. Another example of HILIC applied to the multidimensional analysis is presented by Schriewe and coworkers. In that work, the authors have analyzed malonyl-coenzyme A in breast cancer cell cultures. The 1st dimension column was Oasis® weak anion-exchange column (20 × 2.1 mm, 30 μm) and the 2nd XBridge BEH Amide (100 × 2.1 mm, 2.5 μm). The amounts observed were 1 nmol/L (LOD) and 2.5 nmol/L (LOQ) for acetyl coenzyme A, 5 nmol/L (LOD), and 10 nmol/L (LOQ) for malonyl coenzyme A.

In a general view, the use of packed columns in both the 1st and 2nd dimensions is a robust and trustable approach to deal with large amounts of sample volume and different matrices. This kind of trapping column is the most popular used for several reasons stands out as its robustness and easy preparation procedure. The sensitivity of the analytical method allowed the determination of 0.10 ng/mL of arachidonoyl ethanolamine and 0.05 ng/mL of 2-Arachidonoyl glycerol.

3.2. 1st dimension is an OT column, and 2nd dimension is a packed or OT columns

Less popular than the packed extraction columns, the open-tubular columns also received attention and can be used as extraction columns, with good results being reported (Costa Queiroz et al., 2019; Kataoka, 2021). Souza and coworkers (Souza et al., 2019) explored using a polymeric ionic liquid in an open tubular capillary column as a 1st dimension column to determine endocannabinoids in plasma samples. A Phenomenex Kinetex C18, 1.74 μm column (100 mm × 2.1 mm) was used as the analytical column. Although reliability is a topic of concern when producing a lab-made column, the author found good repeatability for synthesizing the produced extraction column. The intra-batch and inter-batch relative standard deviation values were lower than 13.5%. Likewise, Chen and Xu have evaluated 8-hydroxy-2'-deoxyguanosine, 3-hydroxyphenanthreneand 1-hydroxypyrene by using a graphene oxide/poly(3,4-ethylene dioxythiophene)/polypyrrole composite film on an open tubular extraction column. The analytical column was a Waters symmetry C8 column (150 mm × 2.1 mm, 3.5 μm). The minimal LOQ found was 0.013 ng/mL for the 3-Hydroxyphenanthrene. The authors concluded that the method presented an excellent performance on the extraction of the analytes in human urine. Ma and coworkers (Ma et al., 2020) have used a coating of aptamer/gold nanoparticles as the 1st dimension for adenosine isolation in serum and urine. The analytical column was an Agilent C18 column (3.0 μm, 2.1 × 150 mm).

Open tubular columns can also be used for both extraction and analytical purposes. For example, Da Silva and coworkers (da Silva et al., 2017) have used an equipment setup combining open tubular columns in the first and second dimensions. An open tubular multi-hole crystal fiber solid-phase extraction column was used in the first dimension. This column is composed of 126 channels (8 μm of inner diameter each) of 12 cm coated with poly(styrene-*co*-octadecene-*co*-divinylbenzene) (PS-OD-DVB). Noteworthy, this strategy was used to form a porous-layer open tubular (PLOT) column in each channel. An analytical 10 μm, i.d. poly(styrene-*co*-divinylbenzene) (PS-DVB) PLOT column (2 m long and 0.4 μm film thickness), was used. The use of 126 channels allows a higher surface area than a conventional single-channel OT-SPE column and requires a lower column pressure. The extraction performance of these columns was also compared to packed and monolithic columns. It was observed that the multi-hole extraction column presented a higher extraction capacity (Fig. 4a).

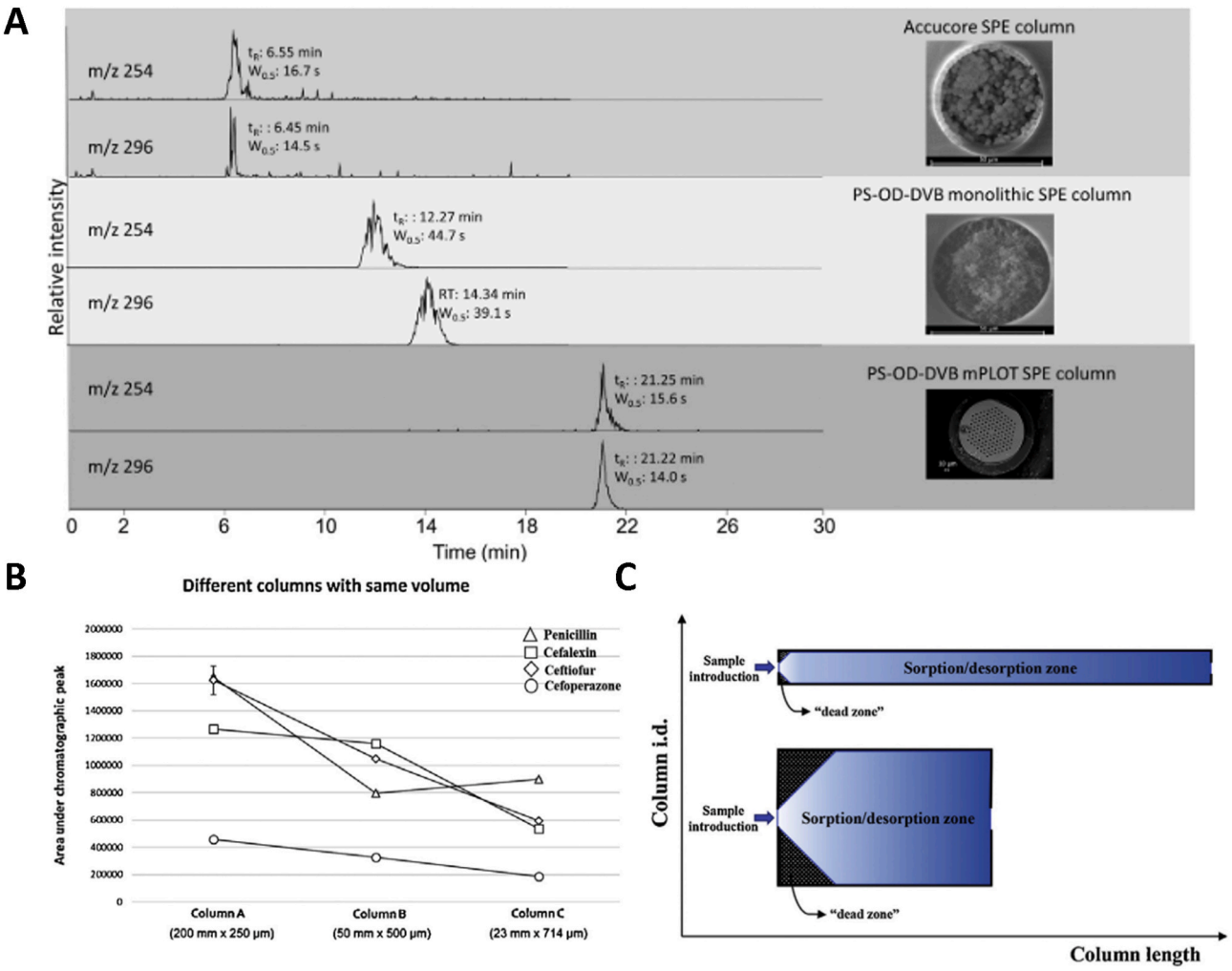

Fig. 4. **(A)** Comparison of the chromatographic performance of packed, monolithic, and multi-hole OT extraction column. Reprinted with permission of (da Silva et al., 2017). **(B)** Effect of the column dimensions but same volume on the extraction performance. Reprinted with permission of (de Toffoli et al., 2019). **(C)** Representation of the dead zones (region of low interaction of the analyte and extraction phase) in columns of different bore. Reprinted with permission of (de Toffoli et al., 2019).

Though OT column have presented great results as extraction and analytical column, they present as major limitation the absence of commercial columns, been in general needed to produce the column on the lab in other t use that kind of column. Another limitation is related to the small inner diameter and thin film of stationary phase that leads to a lower injection volume allowed.

3.3. 1st dimension is a monolithic column and the 2nd dimensions are OT or packed columns

The monolithic columns can be a good alternative to produce extraction columns. Generally, they are produced with materials similar to those used for OT columns. Rogeberg and coworkers (Rogeberg et al., 2013) have also developed an SPE-PLOT-LC-MS system using only parts available in commercial instruments (not including the column). A poly (butyl methacrylate-*co*-ethylene dimethacrylate monolithic extraction column (4.5 cm long and 50 µm inner diameter) was used in the first dimension, and a PLOT column was used as the analytical column in the second dimension. The work focused on using a monolithic SPE column combined with an OT analytical one to pre-concentrate the sample before the analysis avoided the saturation of the analytical column with the sample solution. Similar to the other types of extraction columns cited before, the monolithic also found application in different analysis types. For example, Domingues and coworkers (Domingues et al., 2015) have employed an organic-inorganic hybrid cyanopropyl monolithic column to extract a series of medicines in plasma samples of schizophrenic patients. The analytical column was a Waters XSelect CSH C18 XP 2.5 µm (100 × 2.1 mm). The authors concluded that the phase sorption capability is appropriate for analyzing drugs such as antidepressants, anticonvulsants, anxiolytics, and antipsychotics in plasma samples.

Additionally, the low backpressure and the high permeability of the monolithic capillary allowed the easy percolation of the sample through the extraction column that was reused over one hundred times. Likewise, a P(2-hydroxyethyl methacrylate-N-methacryloyl-L-phenylalanine) monolithic trapping column (200 × 4.6 mm) was used by Armutcu and coworkers (Armutcu et al., 2019) to evaluate polycyclic aromatic hydrocarbons in water samples. The 2nd dimension analytical column was a Pinnacle II PAH column (50 × 4.6 mm, 4 µm). The limit of quantification observed was 1.2 pg/mL for benzo(*a*)pyrene.

Analogous to the other two types of columns presented before, monolithic columns show advantages and limitations. As significant advantages, it presents different phases that can be produced and modified to match the analytes' characteristics, exhibit high mechanical strength, and be placed in any channel of different shapes. Additionally, the monolithic columns require less pressure to the mobile phase flow through the column than a similar packed column. Conversely, as it major limitation, monoliths can only be synthesized on narrow

channels; otherwise, the monoliths can form voids reducing the columns' performance (Namera and Saito, 2013).

3.4. Chip-based approaches

Chip-based analyses are state-of-the-art technology. Mainly, chip-based systems present dimensions compatible with the LC capillary and nanoscales. For example, Seo and coworkers (Seo et al., 2019) have applied a chip-based LC system to perform biotherapeutics' characterization by analyzing highly acidic glycans. Previously to the LC analyses, the analytes were selectively extracted by porous graphitized carbon SPE cartridges. The nano-LC chip contains a 9 × 0.075 mm extraction column and a 43 × 0.075 mm analytical column; both were packed with porous graphitized carbon material. The authors conclude that this study demonstrated a systematic analytical strategy for detecting various types of glycans such as neutral, phosphorylated, and silylated species within a single sample treatment.

Bai and coworkers (Bai et al., 2011) have used a microchip integrating a Zorbax300A SB-C18 (5 μm) enrichment channel of 500 nL and an analytical column packed with the same phase (150 mm × 75 μm) for the analysis of 8-isoprostaglandin F2α in human urine. The limit of quantification found was 0.01 ng/mL. According to the authors, the method showed good linearity and reproducibility, being considered a good alternative for automated analysis.

Liu and coworkers (Liu et al., 2013) developed methods for determining aflatoxins in peanut products. Two Agilent nanoLC-chips were explored; an SB-C18 and an SB-C8 Zorbax 300A containing a 500 nL enrichment channel (150 mm × 75 μm). The limit of detection found was 0.004 ng/g for Aflatoxin B1. The method demonstrated to be sensitive and appropriate for the analysis of aflatoxins in the selected samples.

Piendl and coworkers (Piendl et al., 2020) have designed a multiple heart-cutting two-dimensional chip-based LC to perform fluorescence detection in the 1st dimension and mass spectrometry detection in the 2nd dimension. Additionally, the multichannel chip was composed by a 1st channel packed with ProntoSIL 100-5-CYANO-2, 5 μm (50 mm × 90 μm × 40 μm depth) and a ProntoSIL 100-5-C18 SH-2, 5 μm (50 mm × 90 μm × 40 μm depth) in the 2nd channel. This system performed adequately for the separation of pesticides and tryptic digest. For all the reasons mentioned above, chip technology is an up-to-date technology that is receiving increasing attention due to advantages such as integrated extraction and analytical columns and ESI tips in the same platform. Consequently, low dead-volumes are reported due to the lack of connections between the modules, contributing to attenuating extra column band-broadening. Although those advantages, the system still found less application than the other ones due to the difficulties of producing lab-made chip-based approaches (Vargas Medina et al., 2020c).

3.5. Key findings of the applications

Considering the series of different extraction columns already described, it must be in mind that their dimensions combined with those of the analytical columns can affect the extraction and separation performances. A work performed by de Toffoli and coworkers (de Toffoli et al., 2019) brought a new light on this topic. In this work, different tubing diameters, lengths, and materials are investigated. It is observed that the extraction performance is directly related to the extraction phase's amount packed on the extraction column; in other words, larger and longer columns performed better. However, a surprising result was found when evaluating extraction columns of the same inner volume (approximately the same mass of extraction phase). A column of 200 mm length (L) and 0.25 mm inner diameter (i.d.) performed better than a 23 mm L and 714 mm i.d. (Fig. 4b). The explanation was that the thinner i.d. allows better interaction with the extraction phase; the thicker columns present zones where analytes present lower interaction with the extraction material (Fig. 4c).

Other advantages that are underscored by almost all cited authors in using automated sample preparation include (i) higher analytical-throughput and fast analysis and (ii) substantial economies on reagents and samples. These positive characteristics have been contributed to make some analytical methods environmentally friendly, according to Green Chemistry. In this way, the authors here believe that multidimensional separations systems represent a good-way-to achieve more environmentally friendly methods without losing the performance.

Table 1 gathers information about the selected applications herein discussed as well as their main characteristics.

4. Overall performance (multidimensional vs conventional approaches)

The gains in analysis efficiency by using miniaturized techniques become a focus of discussion as soon as the multidimensional separations get more popular in the '00s (Saito and Jinno, 2002; Santos Neto et al., 2006). Although it is not an emerging field anymore, very few works have directly compared miniaturized and conventional automated techniques for the extraction and analytical separation (Titato and Lanças, 2005), and MS detection (Abian et al., 1999). To the best of our knowledge, no work was recently published focusing on the direct comparison of multidimensional separation systems with conventional approaches used for sample clean-up, as one of the primary purposes. In the following paragraphs, the practical and theoretical aspects of conventional and multidimensional analysis are compared, and vantages and limitations are discussed.

Source of errors: A survey published in 2013 (Technical Analytical Methods Committee Briefs, 2013) summarized the most typical source of errors in analytical chemistry (Fig. 5). The sample preparation step is the primary cause of such errors, directly followed by human error and equipment problems. In this way, the automated multidimensional separation approach allows the reduction of the sample preparation steps, consequently reducing the error that comes from it. Additionally, the fully automated steps significantly reduce human error likelihood. Though the instrumental error has the same occurrence as the human error in Fig. 5, a well-calibrated instrument can eliminate it.

Sample: Conventional and non-automated sample preparation techniques can deal with many different samples. However, in most current analytical problems, the main issue is the trace-levels of contaminants. The matrix is also crucial for the extraction success since it can interfere in the analysis, reducing the performance. In some samples, the presence of proteins, fat, and other compounds as particulate materials can be a limitation for the use of direct online sampling preparation techniques, been required a pre-treatment step (Rogeberg et al., 2014). Filtration, dilution, centrifugation, and protein dilutions can be an alternative to deal with those samples.

A work conducted by Mortensen and coworkers (Mortensen et al., 2005) using an extraction method composed of liquid extraction with a mixture of ethyl acetate and cyclohexane (95:5) and a two-step solid--phase extraction (SPE) lead to a limit of detection of 0.01–0.5 μg L^{-1} of phthalate in milk. For the same matrix, Ferreira and coworkers used an online fully automated LC-based multidimensional system to evaluate ceftiofur presence in milk. The limit of detection and quantification were 0.1 and 0.7 μg L^{-1}, respectively. Even for a complex matrix such as milk, both the conventional and miniaturized multidimensional approaches performed similarly. Both conventional (Lindholm-Lehto et al., 2017) and multidimensional (Franco et al., 2016) approaches can reach very low detection and quantification levels if adequately performed. The detection limit is not related only to the extraction technique, but also to the separation and detection system (Petritis et al., 2002).

Phase availability: In both cases, conventional and miniaturized (including automated multidimensional separations) approaches, there is almost an infinity of possibilities of stationary phases (Burato et al.,

Table 1
Multidimensional separation-based applications discussed throughout the manuscript classified according to its main characteristics. Obs: Extraction (1st Dimension) and Analytical columns (2nd Dimension).

Column Type	Extraction column (L × i.d., d_p)[a]	Analytical column (L × i.d., d_p)[a]	Detection method	Analyte	Matrix	Amount detected	Ref
Packed	Agilent Eclipse XDB-C8 (12.5 mm × 2.1 mm; 5 μm)	Phenomenex Kinetex C18 100 Å (50 mm × 2.1 mm, 2.6 μm)	MS	Cocaine (and its metabolites)	Hair	LOD - 0.05 ng/mg (cocaine and benzoylecgonine) and 0.012 ng/mg (codethyline)	Alves et al. (2013)
	LiChroCART LiChrospher 100RP-18 (4 mm × 4 mm, 5 μm)	LiChroCART RP-18 endcapped Purospher STAR (55 mm × 2 mm, 3 μm)	MS	Testosterone	Human serum	LOQ - 0.01 μg L^{-1}	Borrey et al. (2007)
	Phenomenex Gemini C18 110 Å, (10 mm × 2 mm, 5 μm)	chiral Phenomenex Lux AMP LC column, 150 mm × 3.0 mm, 3 μm)	MS	Amphetamines enantiomer	human urine	0.20 mg/L (R amphetamine) 3.57 mg/L(S-amphetamine)	Hädener et al. (2017)
	Phenomenex Mercury SynergyTM Polar RP 80 Å (10 mm × 2.0 mm, 4 μm)	Phenomenex Kinetex PFP 100 Å (30 mm × 2.1 mm, 2.6 μm)	MS	THCCOOH and THCCOOH-glucuronide	Blood	LOQ – 5 μg L^{-1}(THCCOOH and THCCOOH-glucuronide)	Hädener et al. (2016)
	LiChrospher® C8 ADS (25 mm × 4 mm, 25 μm)	Phenomenex Kinetex® C18 (100 mm × 2.1 mm, 1.7 μm)	MS	CNS drugs	Plasma	LOQ – 0.075 (Olanzapine)	Acquaro et al. (2017)
Monolithic	Lab made organic–inorganic hybrid cyanopropyl monolithic column	Waters XSelect CSH C18 XP (100 mm × 2.1 mm, 2.5 μm)	MS	Antipsychotic drugs	Plasma	0.063 ng/mL (Carbamazepine)	Domingues et al. (2015)
	The C8-bonded monolithic silica column (450 mm0.2 mm, 3 μm)	GL Sciences Inertsil ODS-3 (150 mm × 0.3 mm, 3 μm)	UV–Vis	Pesticides	Clear sample	0.8 mg/L (Mecoprop)	Shintani et al. (2003)
	P(2-hydroxyethyl methacrylate-N-methacryloyl-L-phenylalanine) monolithic column (200 mm × 4.6 mm)	Pinnacle II PAH column (50 mm × 4.6 mm, 4 μm particle size)	Fluorescence detector	Polycyclic Aromatic Hydrocarbons	Water	1.2 pg/mL (benzo[*a*] pyrene)	Armutcu et al. (2019)
Open tubular	Polymer-ionic liquid OT column (100 mm × 0.53 mm)	Phenomenex KinetexC18 column 100 mm × 2.1 mm, 1.7 4 μm)	MS	Endocannabinoids	Plasma	0.10 ng/mL (Arachidonoyl ethanolamine) and 0.05 ng/mL (2-Arachidonoyl glycerol)	Souza et al. (2019)
	Poly(styrene-*co*-octadecene-*co*-divinylbenzene OT multi-hole crystal fiber solid phase extraction composed by a 126 channeled (8 μm) of 120 mm	Poly(styrene-*co*-divinylbenzene) PLOT column (2 m × 10 μm, 0.4 μm)	MS	Sulfamethoxazole	Clear sample	100 ng/mL	da Silva et al. (2017)

[a] Or pore size for monolithic columns, and film thickness for open tubulars.

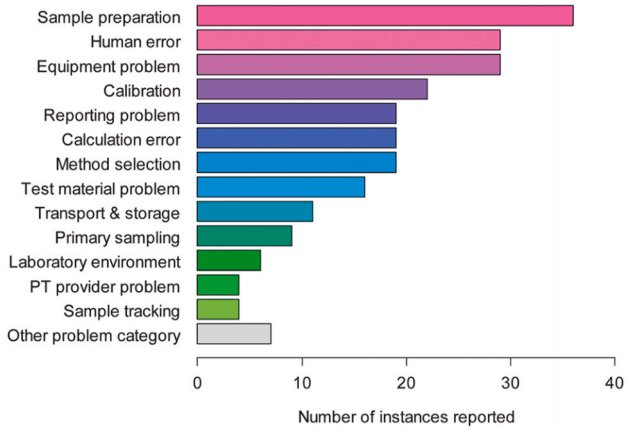

Fig. 5. Source of errors in analytical chemistry. Reprinted with permission of (Technical Analytical Methods Committee Briefs, 2013).

2020). Conventional LC phases (C8, C18, and others) present as its primary advantage the commercial availability. It can easily be purchased as bulk material, available at variable amounts, or obtained by unpacking an HPLC column. Lab-made phases are also extensively explored. Allotropes forms of carbon are of particular interest in sample preparation, especially graphene (Liu et al, 2011, 2012). A LOD of 0.15 ng μL^{-1} (150 μg L^{-1}) was obtained. Soares and coworkers (Maciel et al., 2020a,b) have applied a graphene oxide capillary extraction column to analyze antidepressant and antiepileptic drugs in urine (Fig. 6). A low LOD of 0.01 μg L^{-1} was achieved. Ionic liquids are also a focus of interest in sample preparation, employing conventional (Fontanals et al., 2012) and multidimensional systems (da Silva and Mauro Lanças, 2018). Molecularly-imprinted polymers are also present in conventional and online extraction methods (Turiel and Martín-Esteban, 2010).

Although both conventional and automated multidimensional methods present a significant similarity in packed extraction phases, only the multidimensional one offers the possibility of using an open tubular extraction column. To flow a sample through a packed material demands pressure; the thinner the packed powder and the longer the column (e.g., SPE cartridges or columns), the higher is the pressure required to perform the extraction. The absence of a packed material on OT columns drastically reduces the pressure demanded to flow the sampling, thus allowing performing the extraction using simpler pumping systems.

Environmental aspects: Miniaturized analytical methods implies a minimal amount of extraction solvents (Burato et al., 2020). Usually, a multidimensional separation approach permits capillary bore extraction columns, which demands low flow rates to carry the sample through the column. When combined with miniaturized LC, the economy of organic solvent improves extraordinarily. Fig. 7 compares conventional SPE followed by conventional LC, multidimensional separations based on conventional LC, and multidimensional separations based on nanoLC.

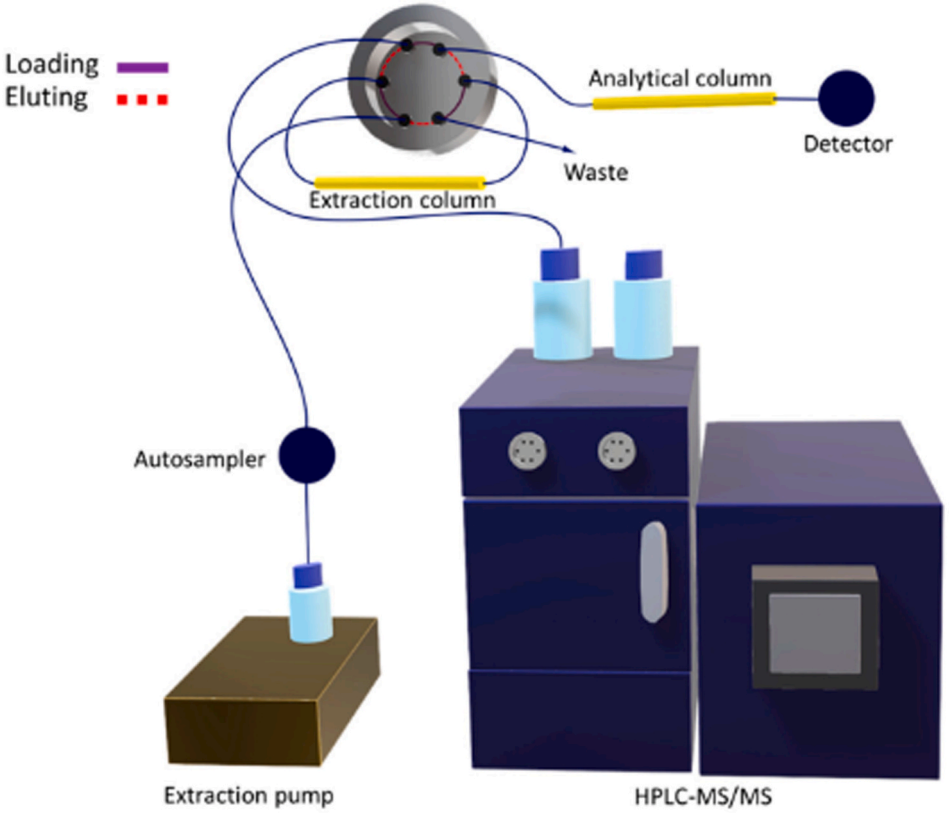

Fig. 6. (A) Representation of the fabric phase sorptive extraction procedure. Reprinted with permission of (Zilfidou et al., 2019) **(B)** Representing the multidimensional configuration. Reprinted with permission of (Maciel et al., 2020a,b).

The most critical economy here is over organic solvents consumption. When the automated multidimensional approach replaces the conventional SPE, the economy is 99% on organic solvent for the extraction procedure. When the automated approach is coupled to nano-LC, the total amount of organic solvent consumed is reduced from 16.5 mL on the conventional SPE and LC analysis to 0.0175 mL on online SPE coupled to nano-LC.

In this way, multidimensional and conventional sample preparation techniques can run similarly in selectivity (using the same extraction phase). However, the multidimensional separations are much easier to be automated, reduce human error, and provide an expressive solvent economy, which is excellent under an environmental viewpoint.

5. Conclusions

This review focused on presenting and discussing the most critical aspects and recent trends in multidimensional LC-based sample preparation approaches (i.e., multidimensional separations). From the authors' viewpoint, techniques based on it can meet the scientific community's current environmental aspects without losing or even enhancing the analysis performance. By examining the current literature, these approaches already proved their performance, possessing remarkable advantages including (i) great analytical-throughput and fast analysis, (ii) substantial economies on reagents and samples, (iii) and great accuracy over the analytical results, mostly owing to the reduced sample handling by the analysts. Additionally, considering the solvent savings and high-analytical throughput, such automated methods represent a significant economy in the long term run.

The essential characteristics of both conventional and modern methods using column-switching or chip-based approaches are described in detail. There are both commercially available instruments and laboratory-assembled systems, both designed to perform multidimensional separations. On the one hand, commercial instruments deliver the practice of being already assembled and ready-to-go through the most common analytical methods. On the other hand, lab-made multidimensional separation approaches often possess the ability to be tailored to a specific application resulting in a higher selectivity or better performance (e.g., using open-tubular columns, multi-channeled OT, chiral extractive phases, and others). Moreover, the recent popularization of analytical microchips combined with liquid chromatography miniaturization has paved the chip-based LC way in multidimensional separations over the last decade.

A series of selected applications was presented mostly to show the readers the versatility of multidimensional sample preparation approaches. In this way, applications dealing with food, environmental, and biological analysis achieved by conventional, miniaturized, and chip-based multidimensional systems were discussed. Within such a context, the good results achieved so far support such automated methods in several analytical chemistry fields. For the coming years, we can expect an increment of applications based on those approaches, mainly focusing on multidimensional separations based capillary- and nano-LC and the chip-based approaches. This trend towards fully miniaturized systems improves the hyphenation with mass spectrometry. In addition to including the use of the well-established electrospray ionization (ESI-MS), this miniaturization opens-up new horizons to include the emergent electron ionization (EI-MS) sources in multidimensional LC. Noteworthy, it is unarguably that obtaining better performances, faster analysis, and at the same time, environmentally friendly methods is a challenging task for analytical chemists. In this way, the herein discussed multidimensional approaches can represent an excellent way to achieve such results for chromatographic-based techniques while also contributing to a more sustainable analytical science.

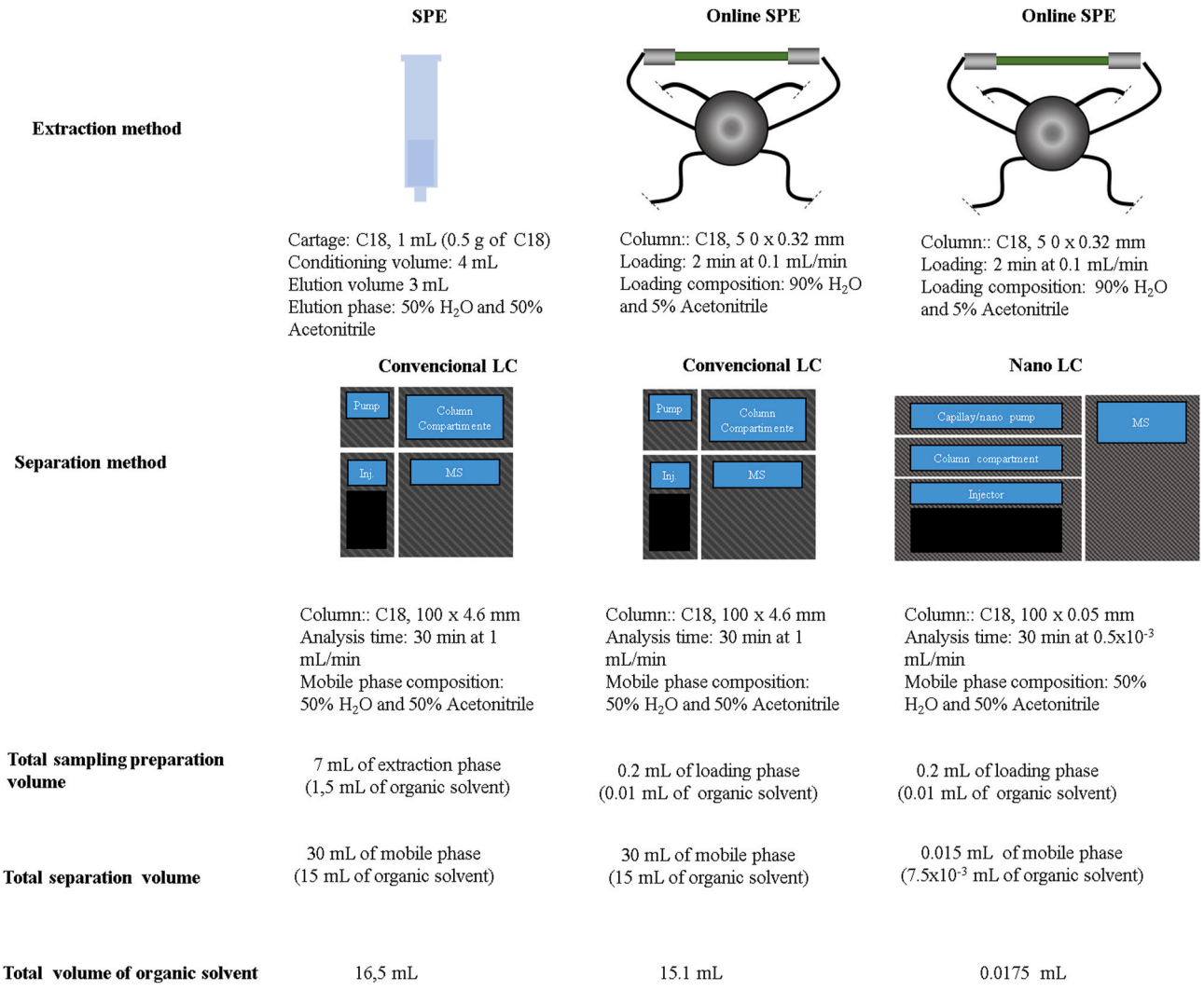

Fig. 7. Comparison of extractions solvent and mobile phase consumption for SPE, online SPE coupled to LC, and online SPE coupled to nano-LC.

Author statement

The authors inform that this manuscript has not been (and is not) submitted to any other place. All authors agree with its publication, if accepted. The authors have no conflict of interest regarding this work.

Declaration of competing interest

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

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CAPÍTULO 4

New materials in sample preparation: Recent advances and future trends.
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New materials in sample preparation: Recent advances and future trends

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ABSTRACT

The low concentration levels of different chemical compounds in complex matrices such as food, environmental, biological and pharmaceutical, requires adequate analytical methods to isolate, preconcentrate and accurately quantify the analytes obtaining satisfactory results. The sample preparation step is a critical part of the whole analytical process, once it is constantly prone to contamination and at the same time, it is more difficult to automate. Aiming to improve the extraction performance of sample preparation techniques, highlighting the microextraction techniques, several sorbent materials have been developed and used in different application areas. They are based upon sorption processes and usually are in accordance with the principles of green chemistry (greener analytical methods). This review summarizes the recent advances and the prospects for new materials to be utilized in sample preparation techniques, emphasizing microextraction techniques based upon a sorption process in line with the concept of green analytical chemistry. We also approached the synthesis and main properties of the sorbent materials as well as the main miniaturized techniques that use them, and their most relevant and representative applications.

1. Introduction

Nowadays analytical chemistry has reached an advanced level of technology in response to the increased demand for modern analytical methods aiming to monitor residues and contaminants in complex matrices [1]. The analysis of compounds at (very) low concentrations levels can be divided into several steps going from sample preparation to data processing in a way that any of them may affect the analytical performance [1]. In this context, regulatory agencies around the world are responsible to establish strict laws as well as create several procedures, including methods validation directives to assure the quality and reliability of these analytical methods [2]. Despite the recent advances in analytical chemistry instrumentation (mainly in LC and MS), a prior step before the chromatographic sample introduction, consisting in the processing of raw complex matrices, is still mandatory in most cases [3,4].

This sample preparation step is applied mainly to eliminate matrix interferents, to perform sample clean-up, and preconcentrate target compounds in order to minimize sample complexity before their injection into the chromatograph, which can enhance analyte's response signal [5]. In general, this procedure is frequently considered the main source of potential errors, as well as the most time-consuming step in a typical analytical workflow. Traditional extraction techniques including liquid-liquid extraction (LLE) and Soxhlet extraction, for instance, were developed decades ago and have been since applied to sample processing [6]. However, these traditional techniques show major drawbacks related to sample preparation including: (1) high solvent consumption and waste generation; (2) the laborious routine; (3) frequent source of sample contamination, and (4) analytical errors due to the operator's handling required to perform these techniques [6].

Solid phase extraction (SPE) was introduced to overcome the main disadvantages and limitations of LLE, especially the use of large amounts of organic solvent and low selectivity related. SPE can be considered as a simple technique presenting satisfactory extraction efficiency as well as a good pre-concentration capacity for most matrices. It involves a fairly low instrumentation cost, easy

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Abbreviations

([C4C12im]Br) 1-butyl-3-dodecylimidazolium bromide
[P(HEMAPA)] Poly(2-hydroxyethylmethacrylate-N-methacryloyl-(L)-phenylalanine)
μ-SPE Micro-solid phase extraction
15α-OHEs 15α-Hydroxyestrogens
2D-HPLC On-line two dimensional high performance liquid chromatography
amine-rGO Amino-functional reduced graphene oxide
BPA Bisphenol A
BSA Bovine serum albumin
C18 Octadecylsilane
C_{60} Fullerene
CD-IMS Corona discharge ion mobility spectrometry
CDs-DMIP Carbon quantum dots-coated dummy molecularly imprinted polymer
CEC Capillary electrochromatography
CF Cefotaxime
CFs Carbon fibers
CNTs Carbon nanotubes
CNTs/G Carbon nanotubes functionalized with graphene
CNTs-HF-SLPME Carbon nanotubes reinforced hollow fiber solid/liquid phase microextraction
COF Covalent organic frameworks
COF-COOH Covalent organic frameworks with carboxylic groups
COF-HBI Covalent organic frameworks with 2-(2,4-dihydroxyphenyl)-benzimidazole
−COOH Carboxyl
CPs Chlorophenols
d-μSPE Dispersive micro solid-phase extraction
DLLME Dispersive liquid-liquid microextraction
DON 4-deoxynivalenol
dSPE Dispersive solid phase extraction
EDCs Endocrine disrupting chemicals
EDOT 3,4-ethylenedioxythiophene
EGDMA Ethylene glycol dimethacrylate
EPD Electrophoretic deposition
ET-AAS Electrothermal atomic absorption spectrometry
F-AAS Flame atomic absorption spectrometry
Fe_3O_4 Magnetite
Fe_3O_4@G Graphene-magnetite composite
Fe_3O_4@mTiO_2@COFs Magnetic mesoporous titanium dioxide@covalent organic frameworks
Fe_3O_4@SiO_2@C18 MNPs Octadecylsilane-functionalized magnetic silica nanoparticles
Fe_3O_4@SiO_2@PIL Silica-coated iron oxide nanoparticles with imidazolium-based polymeric ionic liquid
Fe_3O_4@SWNTs Magnetic nanoparticles of ferroferric oxide/single-walled carbon nanotubes
Fe_3O_4@VTEO@IL-MIPs Magnetic ionic liquid-molecularly imprinted polymers
Fe_3O_4-CTS@DES-MIPs Molecular-imprinted polymers-based magnetic chitosan with facile deep eutectic solvent-functional monomers
Fe_3O_4PSS@ZIF-67 Magnetic beads embedded in poly (sodium-p-styrenesulfonate) and ZIF-67
Fe_3O_4-SP@GO Magnetite-sporopollenin/graphene oxide
G Graphene
G@PS-DVB Graphene-coated polystyrene-divinylbenzene
GCB Graphitized carbon black
GC-FID Gas chromatography-flame ionization detection
GDMA Glycerol dimethacrylate
GO Graphene oxide
GO-PET Polyethylene terephthalate nanofiber was doped with graphene oxide
HANPs Hydroxyapatite nanoparticles
HF- LPME Hollow fiber liquid phase microextraction
HFME Hollow fiber membrane extraction
HLB Hydrophilic-lipophilic balance
HPLC High performance liquid chromatography
HPLC-DAD High performance liquid chromatography-diode array detection
HPLC-UV High performance liquid chromatography-ultraviolet-visible
HSSE Headspace sorptive extraction
IACs Immunoaffinity columns
icELISA Indirect competitive enzyme-linked immunosorbent assay
ICP-MS Inductively coupled plasma mass spectrometry
IIPs Ionic imprinted polymers
IL [C4MIM][NCA] Ionic liquid naphthalene carboxylic acid-based
IL-IL-DLLME Ionic liquids in dispersive liquid-liquid microextraction
ILs Ionic liquids
IL-Si-LLE Ionic liquid-based salt induced liquid-liquid extraction
IOT-SPME In-out-tube SPME
LC Liquid chromatography
LC-HRMS Liquid chromatography - high resolution mass spectrometry
LC-MS/MS Liquid chromatography−tandem mass spectrometry
LE Liquid-liquid extraction
LOD Limit of detection
LPME Liquid-phase microextraction
mAb Monoclonal antibody
MEPS Microextraction by packed sorbent
MG@mSiO_2-C Carbon-functionalized magnetic graphene/mesoporous silica composites
MIMC-2D-LC Molecularly imprinted monolithic column in a two-dimensional liquid chromatographic
MIMs Molecularly imprinted monoliths
MIP-μ-SPE Micro-solid-phase extraction molecularly imprinted polymer
MIPs Molecularly imprinted polymers
MIPs-E1-IOT-SPME MIPs-E1-coated in-out-tube SPME
MISPE Molecularly imprinted solid phase extraction
MISPME Molecularly imprinted solid phase microextraction
MNPs Magnetic nanoparticles
MOFs Metal-organic structures
MS Mass spectrometry
MSPE Magnetic solid phase extraction
MTMOS−TEOS Methyl-trimethoxysilane-tetraethoxysilane
MWCNTs Multi-walled carbon nanotubes
MWCNTs/Fe_3O_4@PAPy Magnetic multiwalled carbon nanotubes/Fe_3O_4@poly (2-aminopyrimidine)
MWCNTs-rGO-IL Three-dimensional porous material
NP Nonyl phenol
NTD Needle trap devices
NTME Needle trap microextraction
−OH Hydroxyl
OTA Ochratoxin A
PAGF Phytic acid induced 3D graphene-based foam

PAHs	Polycyclic aromatic hydrocarbons
PANI	Polyaniline
PCBs	Polychlorinated biphenyls
PDMS	Polydimethylsiloxane
PEDOT	Poly(3,4- ethylenedioxythiophene)
PEDOT-PIL/MWCNTs	Poly(3,4-ethylenedioxythiophene)-ionic liquid polymer functionalized multiwalled carbon nanotubes
PEDs	Phenolic endocrine disruptors
PIL	Polymeric ionic liquid
PIL/MWCNTs	Multi-walled carbon nanotubes chemically bonded to polymeric ionic liquid
pNE	Polynorepinephrine
PPE	Pipette-tip extraction
PPY	Polypyrrole
PS-DVB	Polystyrene-divinylbenzene
PT-SPE	Pipette-tip solid-phase extraction
PY/GOx/C18/chitosan	Polypyrrole-coated graphene oxide/ octadecyl silica incorporated in chitosan cryogel
QuEChERS	Quick, easy, cheap, effective, rugged and safe
RACNTs	Restricted access carbon nanotubes
RAMs	Restricted access materials
RB	Rhodamine B
SBSE	Stir bar sorptive extraction
SP	Sporopollenin
SPE	Solid phase extraction
SPME	Solid phase microextraction
SWCNTs	Single-wallet carbon nanotubes
TC	Tetracycline
TCM	Traditional chinese medicine
TCS	Triclosan
TEOS	Tetraethoxysilane
TiO_2	Titania
TMOS	Tetramethoxysilane
TpBD	1,3,5-triformylphloroglucinol-benzine
UPLC-MS/MS	Ultra pressure liquid chromatography-tandem mass spectrometry
ZrO_2-PPO	Zirconia-polypropylene oxide.

automation, and good selectivity. Besides that, its basic theoretical and practical principles are based upon the miniaturization of classical liquid chromatography. For many years SPE was one of the most employed techniques for sample preparation due to its perceived advantages. At the beginning of the '90s, this technique was used as a theoretical base for the development of solid phase microextraction (SPME), now considered as the precursor of all miniaturized sample preparation techniques [7–9].

Solid phase microextraction techniques were introduced to overcome the SPE main drawbacks including (1) several extraction steps; (2) higher amounts of a solvent when compared to the miniaturized techniques; (3) non-reusable extraction cartridges, and so on [7,8]. In addition to solid phase microextraction (SPME), among the miniaturized techniques being considered as environmentally friendly, the following can be pointed out: in-tube solid phase microextraction (in-tube SPME), stir-bar sportive extraction (SBSE), magnetic SPE (MSPE), microextraction by packed syringe (MEPS), and dispersive solid-phase extraction (dSPE). Those miniaturized techniques based upon the use of sorbent materials are the most utilized for sample preparation, once they considerably decrease the organic solvent consumption during the extraction and preconcentration steps [7–9].

Aiming to improve the extraction efficiency during sample preparation several new materials have been developed. The selection of the most appropriate sorbent able to extract a wide range of analytes present in complex matrices is extremely important for the method success. Nowadays, a wide range of new materials is being developed including (1) molecularly imprinted polymers (MIPs); (2) ionic liquids (ILs); (3) immunosorbents (IS), (4) carbon-based materials, and (5) sol-gel-based compounds, among others. The synthesis of these novel compounds opens up new alternatives to combine the suitable miniaturized extraction technique with the most selective sorbents. Therefore, taking into account the analyte's chemical classes and the matrix main characteristics, the best combination will drive the analyst to obtain the best overall results [7–9].

This review updates a previous work reported by the same research group [10], now aiming to point out the recent advances on the issues discussed in that publication as well as to bring out new subjects emerged during the last few years. Moreover, since 2015 other works have been published discussing new sorbent materials advances on sample preparation [11–13]. As an example, de Faria et al. [12] recently published a review regarding restricted access-media advancements mainly over modified compounds based on silica, polymer, carbon, and solvents as well as discuss biological applications. Another important contribution by Masini and Svec [11] presents a well-discussed work about monolithic material's role in sample preparation technique. The review summarizes important topics on current advances in obtaining hybrid monolithic materials, including imprinted monoliths herein discussed, and their subsequent application based on automated sample preparation analysis. Furthermore, ionic liquids are reviewed in a recent report by Clark et al. [13] underscoring relevant aspects of IL-based sample preparation methods that included solid/polymer ILs and magnetic ILs, among others. In the same paper, the authors also described updates application of these materials in both liquid-phase extraction and solid-phase extraction techniques.

For these facts, the main trends in new materials used as sorbents emphasizing environment-friendly miniaturized techniques were herein highlighted. Therefore, information regarding synthesis procedures, the main properties, and intrinsic advantages are presented for several classes of materials including sol-gel techniques, ionic liquids, graphene and derived materials, restricted access materials (RAMs), immunosorbents, molecularly imprinted polymers and molecularly imprinted monoliths (MIMs). Furthermore, several different applications of them combined with sample preparation methods are discussed, resulting in updated information covering recent advances as well as future trends in sorbent developments applied to innovative sample preparation approaches.

Fig. 1 shows a representative scheme of the recent trends in analytical chemistry sample preparation, underscoring the main subjects covered in this paper.

2. Sol-gel synthesis route

Sol-gel chemistry is an interesting approach to design new sorbents for sample preparation techniques. The ability to use different precursors as building blocks and the possibility to control the sorbent morphology by varying the synthesis reaction are some of the main advantages related to sol-gel processes [14,15].

The most common synthetic route to develop sol-gel sorbents is based on the hydrolysis and condensation of metal alkoxides

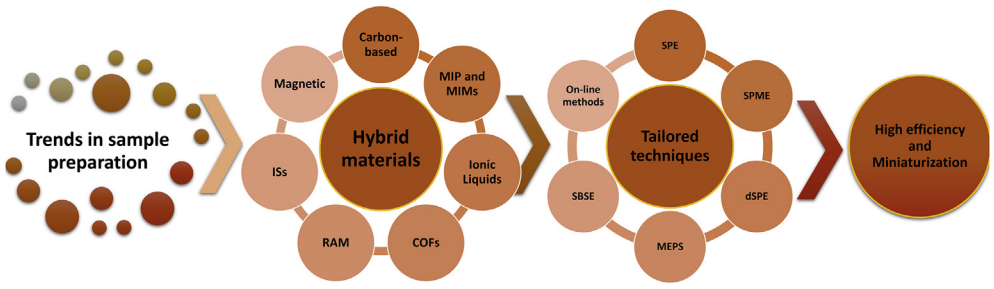

Fig. 1. Illustrative scheme of the recent trends in analytical chemistry underscoring the subjects covered in this paper.

$[M(OR)_x]$ driven by basic or acid catalysis [16]. Silicon is usually the main heteroatom employed, in its alkoxide form, for sol-gel chemistry processes aiming to produce selective sorbents for sample preparation. However, materials based on titanium or zirconium have been used since they demonstrate greater thermal/chemical stability than silica-based polymers [16].

The presence of functional groups on the polymer surface allows further reactions aiming to alter the sorbent selectivity. Additionally, several publications covering the synthesis of hybrid materials containing inorganic-organic structure have been demonstrated as being an interesting strategy to alter sorbents polarity and selectivity [15,17–19]. Table 1 summarizes some recent applications of sol-gel synthetic routes to obtain sorbent materials used in sample preparation techniques.

Sol-gel/nanoclays were recently prepared by Saraji et al. [20] for extraction of diazinon in water samples using MEPS and corona discharge ion mobility mass spectrometry (CD-IMS). In a comparison with a classical C18 sorbent, the sol-gel/nanoclay sorbent showed similar recovery. Further, the sorbent showed good extraction efficiency after 40 extraction cycles. Omar et al. [21] prepared a sol-gel hybrid methyl-trimethoxysilane-tetraethoxysilane (MTMOS–TEOS) sorbent for dispersive solid phase extraction (dSPE) step on QuEChERS. The sorbent was applied for quantification of acrylamide in Sudanese food. The analytical results are comparable to those obtained from studies using PSA as clean-up sorbent. Al Suhaimi et al. [22] prepared a novel porous silica monolith using potassium silicate precursors via an in situ sol-gel method. The monoliths were chemically functionalized with 5-amino-1,10-phenanthroline for selective solid phase extraction of Cu, Co, Ni, and Zn metal ions from groundwater samples. As a result, the material showed efficient performance and exhibited excellent chelating properties with fast adsorption capacities.

A sorbent based on functionalized multi-walled carbon nanotubes (MWCNTs) were produced to be held in the pores of a hollow

Table 1
Sol-gel based sorbents applied to sample preparation.

Precursors[a]	Sample	Analyte	Sample preparation	Analysis	Ref.
MTMOS TEOS	Sudanese food	Acrylamide	dSPE	GC-MS	[21]
TEOS MTMOS Nanoclay	Water	Organophosphorus pesticides	SPME	GC-MS	[175]
Ti TiO_2 Hydroxyapatite	River water Domestic sewage	Chlorophenols Triclosan Bisphenol A	SPME	HPLC-UV	[25]
Silicates IPTMS	Ground water	Metal ions	SPE (monolith)	ICP-MS	[22]
TEOS MWCNTs	Hospital drain water Tap water Wastewater	Piroxicam Diclofenac	HF-SLPME	HPLC-DAD	[23]
TEOS γ-MPTS EGDMA	Cucumber	Cyanazine Atrazine	PT-SPE	HPLC-UV	[24]
MTMOS CW-20 M	River water Wastewater	Personal care product	DFPSE	HPLC-MS/MS	[176]
TEOS PTMOS DPDMS MTMOS Nanoclay	River water Agricultural wastewater Well water	Diazinon	MEPS	CD-IMS	[20]
MTMOS PEG MWCNTs	Soil Wastewater Lake River Rain water	PAHs	SBSE	HPLC-UV	[177]
TEOS MTMOS	Tap water River water	Chlorobenzenes	NTME	GC/MS	[26]
ZBOT $ZrCl_4$ OH-PPO	Synthetic urine samples	Catecholamines Metabolites	In-tube SPME	HPLC-UV	[27]
MTMOS CW-20 M	River water Wastewater	Personal care product	CPME	HPLC-MS/MS	[178]
TEOS Silica (5 μm)	Biological sources	Nucleic acid	Microchannel on-chip	BAW	[179]

[a] The definitions about each precursor abbreviation herein presented are showed into the list of abbreviations.

fiber via sol-gel technology aiming the extraction of piroxicam and diclofenac in water matrices [23]. The carbon nanotubes reinforced hollow fiber solid/liquid phase microextraction (CNTs-HF-SLPME) using 1-octanol showed better extraction performance than HF-LPME and CNTs-HF-SLPME. Additionally, the CNTs π- π interaction and the presence of −COOH and −OH on the material surface probably enhance the interaction between the analytes and sorbents. Wang et al. [24] synthesized a hybrid sorbent with an extraction layer formed by ethylene glycol dimethacrylate (EGDMA). The PT-SPE followed by HPLC-UV was applied to the analysis of triazine herbicides in cucumber samples. The sorbent purification performance showed similar results in comparison with MCNTs, C18, and HLB phases. Among the evaluated materials, the hybrid sorbent exhibited the best recoveries (≥90.6%).

A new SPME fiber was synthesized via direct electrochemical anodization for in situ formation of titania (TiO_2) on a titanium wire. Then, hydroxyapatite nanoparticles (HANPs) were covered on the TiO_2 using sol-gel process [25]. According to the authors, the Ti@TiO_2@HANP fiber was used over 200 times showing excellent extraction efficiency and selectivity for polar chlorophenols (CPs), triclosan (TCS) and bisphenol A (BPA) compared to polyacrylate and polydimethylsiloxane coating.

Baktash and Bagheri [26] synthesized a superhydrophobic silica sorbent for needle trap microextraction (NTME) of chlorobenzene in water samples. The sorbent is highly temperature resistant showing a large surface area as well as high-water contact angle. A nonhydrolytic sol-gel approach was used to prepare zirconia-polypropylene oxide (ZrO_2-PPO) hybrid sorbent for in-tube SPME of catecholamines and metabolites [27]. The sorbent exhibits high pH stability (pH range: 0−14) and enrichment factors (1480−2650) and low LODs.

In addition, sol-gel chemistry has also been widely used in the synthesis of new materials based on MIPs, immunoaffinity, ILs, carbon derived materials and magnetic nanoparticles. A discussion on their sorption features, as well as selected applications, are covered in the following topics.

3. Ionic liquids (ILs)

Over the past years, ionic liquids have been used in many areas of science and engineering [13]. In the sample preparation area, ILs have been used as a sorbent for SPE, MEPS, SPME and as extractor solvent for miniaturized LPME, HF-LPME and DLLME [10]. As the main feature, the final properties of ILs may be tuned by binding different substituents in their structure. Thus, viscosity, density, melting point, solubility, thermal stability, hydrophobicity, electrical conductivity, acidity, and basicity are some physicochemical characteristics which can be modified according to the specific application purposes [13].

The most recently reported ILs applications are summarized in Table 2. It may be noted that several publications explore the advantages of ILs with MNPs since this combination provides unique features. These include the strong response to an external magnetic field which improves the phase separation while reducing clean-up steps (such as centrifugation or filtration) and the fact that this combination results in a greener magnetic sorbent material when compared to the common ferrofluids. Finally, the easy sorbent isolation from the matrix by a magnet can reduce the matrix effect [28]. For instance, Badragheh et al. [29] developed silica-coated iron oxide nanoparticles with imidazolium-based polymeric ionic liquid, (Fe_3O_4@SiO_2@PIL). The strong interaction between the analyte and this sorbent provided selective analysis enhancing the extraction performance.

Shah et al. [30] used an imidazolium-based ionic liquid composed by different chemical groups bonded on silica support according to general mechanism presents in Fig. 2. The π-π interactions between ILs and the analytes provided selectivity for naphthenic acid removal from dodecane/kerosene samples.

Sotolongo et al. [31] used an IL ([C4C12im]Br) to functionalize a graphene oxide (GO) for preconcentration and determination of mercury using μ-SPE followed by electrothermal atomic absorption spectrometry (ETAAS). The use of an IL-GO containing a long alkyl chain increased the interaction between GO and the analyte

Table 2
Ionic liquids employed for sample preparation and their recent applications.

Ionic liquid[a]	Compounds	Samples	Method	Ref.
Fe_3O_4@SiO_2@GO@β-CD, Fe_3O_4@SiO_2@GO	Plant growth regulators	Vegetables	MSPE UPLC-MS	[180]
[C_2MIM][BF_4], [C_4MIM][BF_4],[C_6MIM][BF_4] and [C_8MIM][BF_4]	Polydatin Resveratrol Emodin Physcion	Poly-gonum Cuspidatum	IL-SI-LLE HPLC-DAD	[32]
([A336][TS]) and 2-(methylthio) benzoate ([A336][MTBA])	Metal ions	Water	SBME FAAS	[181]
[C4MIM][PF6] (magnetic)	Paracetamol Ibuprofen Naproxen Diclofenac	Water	MSPE HPLC-UV-FL	[182]
3D-IL-Fe_3O_4-GO	PAHs	Human whole blood	PT-SPE GC-MS	[183]
Poly(3,4-ethylenedioxythiophene)-ionic liquid	Benzene derivatives	Water	SPME GC-FID	[184]
Poly(calixarene ionic liquid)	Flavonoids	Bottled fruit juice Green tea	MSPE HPLC-DAD	[185]
Imidazolium-based polymeric ionic liquid	Antidiabetic drugs	Human plasma	MSPE HPLC-UV	[29]
Ionic liquid-molecularly imprinted polymers	(Z)-3-(chloromethylene)-6-flourothiochroman-4-one	Urine	PT-SPE HPLC-UV	[186]
Magnetic ionic liquid [C4mim][FeCl4]	Triazine herbicides	Oilseeds	MSPD-MIL-DLLM UFLC-UV	[187]
Magnetic ionic liquid Fe@GO@AFDCIL	Proteins	Porcine and bovine blood	MSPE	[188]
SILs-SPE-ED	Bisphenol A	Water and plastic samples	SPE HPLC-UV	[189]
Ionic liquid-mediated imprinted monolith	Corilagin	Crude extract of *phyllanthus urinaria L.*	SPE HPLC-DAD	[190]
Eco-material based on montmorillonite clays ionic liquids	Organic compounds	Water	RDSE GC-ECD	[191]

[a] The definitions about each precursor abbreviation herein presented are showed into the list of abbreviations.

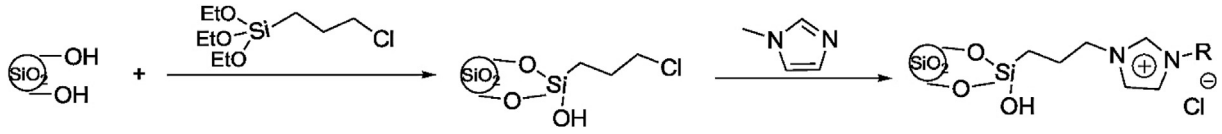

Fig. 2. General mechanism for synthesis of supported ionic liquid phases. {Reproduced by permission from Ref. [30]}.

resulting in enrichment factors up to 100 and a low detection limit of 14 ng L^{-1} in water samples.

Wang et al. [32] applied an ultrasound-assisted ionic liquid-based salt-induced liquid-liquid extraction (IL-Si-LLE) for analysis of polyphenols and anthraquinones in poly-gonum cuspidatum. In a comparative study with hot reflux extraction and ultrasound-assisted extraction, the sample preparation approach using alkylimidazole as ILs required less extraction solvent and time. Novel naphthalene carboxylic acid-based IL [C4MIM][NCA] was developed for IL-IL-DLLME of triclosan and methyltriclosan in bovine milk and chicken egg samples [33]. Due to the strong acidity and lower hydrophilicity of [C4MIM][NCA], the recovery achieved values from 92.6 to 94.2%.

Considering the several advantages of ionic liquids and the wide variety of commercially available anions and cations it will be possible to synthesize specific ionic liquids to be used in the sample preparation for preconcentration and extraction of target analytes. Besides that, some recent studies reported in the literature have successfully demonstrated the combination of ionic liquids with other materials such as graphene and derivatives, molecularly printed polymers, magnetic materials, among others, improving the overall extraction and efficient removal of matrix interferers.

4. Magnetic materials

Magnetic nanoparticles (MNPs) are materials that have a superparamagnetic characteristic. In general, the particles are attracted by an outside magnetic field. However, when the magnetic field is removed, the particles do not retain residual magnetism [9].

For sample preparation, the sorbent is composed of a rigid core (MNPs) covered by a layer of polymer that provides the necessary selectivity for the extraction of the target analytes. Among the MNPs, magnetite (Fe_3O_4) is the most used material for magnetic solid phase extraction (MSPE) [34]. By using the magnetic field appropriate to separate the sorbent from the aqueous extraction media, this sample preparation strategy avoids the usage of centrifugation or filtration systems. Other perceived advantages include its chemical stability, high surface area, reuse of magnetic nanoparticles and low consumption of organic solvents. Further, the sorbent may show high selectivity according to the covering strategy adopted [35]. With regard to the synthesis process, iron oxide MNPs have been developed by coprecipitation methods [36,37], hydrothermal synthesis [38,39], sol-gel [40], solvothermal [41], thermal decomposition [42], microemulsion [43] and sonochemical preparation [44].

Due to the instability and vulnerability to oxidations, as well as propensity to form a cluster, the magnetic core is covered by an external layer. Furthermore, the covering step helps to alter the sorbent selectivity. The MNPs external layer may be designed with inorganic, organics, or hybrid materials through chemical bonding or physical interactions [45,46].

As previously discussed, the control and optimization of the reaction conditions using the sol-gel procedure are important to control the silica layer thickness on MNPs [47]. In microemulsion synthesis, the MNPs are scattered in a solvent with water, oil, and surfactant to form the emulsion. This strategic advantage includes the uniformity of the silica layer avoiding clusters formation [48]. The presence of silanol groups on recovered MNPs (Fe3O4@SiO2) make possible the functionalization with other chemistry groups (C18, amino, thiol, graphene, MIP, ILs) aiming for high analysis selectivity and high analytes recovery.

Ma et al. [49] prepared Fe_3O_4@SiO_2@C18 MNPs that presented an enrichment factor of 500 times in the quantification of microcystin-LR in water reservoirs. External layer based on molecularly imprinted polymers (MIPs) and ionic imprinted polymers (IIPs) show high selectivity in MSPE. For instance, a Fe_3O_4-CTS@DES-MIPs sorbent was synthesized and applied to a selective recognition and separation of (+)-catechin, (−)-epicatechin, and (−)-epigallocatechin gallate in black tea [50]. Another interesting sorbent was designed by Xu et al. [51] using the characteristic of MNPs, MIP, and ILs. As a result, the Fe_3O_4@VTEO@IL-MIPs showed high selectivity for extraction of lysozyme.

A class of hybrid material that has been highlighted as a covering layer in MSPE are the metal-organic structures (MOFs), which have interactions through coordination bonding. These materials have a high surface area, uniform pore sizes, thermal stability, and exhibits great potential for further functionalization due to the structure of their internal walls [52]. Due to these properties, Yang et al. [53] synthesized a sorbent based on MOFs (Fe_3O_4-PSS@ZIF-67) with a large surface area (491 m^2 g^1) and good adsorption performance (Fig. 3).

Surfactants and organic acids with long alkyl chain are also used in MNPs owing to their amphiphilic properties [54]. The interaction between the magnetic core and the covering layer involves the adsorption of surfactant on the magnetic core. Based on this, SDS-coated Fe_3O_4 nanoparticles were developed for both preconcentration and the determination of Nystatin in urine and plasma samples [55].

MNPs covered with carbon-based materials show high surface area, low density, flexibility, and chemical stability [56]. Li et al. [57] synthesized a sorbent using single-walled carbon nanotubes (Fe_3O_4@SWNTs) for preconcentration of vanillin and ethyl vanillin from milk samples. Jalilian et al. [58] used a magnetic multiwalled carbon nanotubes/Fe_3O_4@poly (2-aminopyrimidine) (MWCNTs/Fe_3O_4@PAPy) as a sorbent to preconcentrate nortriptyline, cetirizine, naproxen, diclofenac, and ibuprofen using HPLC-DAD. As an advantage, the proposed sorbent was able to simultaneously extract acidic, basic and amphiprotic drugs. Graphene (Fe_3O_4@G) was synthesized and applied to the analysis of phenolic acids from stingless bee honey showing a high extraction efficiency [59]. Magnetite-sporopollenin/graphene oxide (Fe_3O_4-SP@GO) was engineered aiming to combine the high adsorption capability of sporopollenin (SP) with the chemical characteristic of graphene oxide (GO). As a result, the limits of detection (LOD) of a validated method using this material for analysis of polar organophosphorus pesticides in vegetables ranged from 0.02 to 0.05 ng mL^{-1} [60].

In short, magnetic materials (MM) have attracted wide interest due to several features such as excellent mechanical/thermal stability, delocalized π-electron system, and huge surface area. Considering the sample preparation step it has been applied to enrichment of biological macromolecules, contaminants in foods and pollutants in the environment, among others. Furthermore, magnetic materials can be fast and effectively separated from large

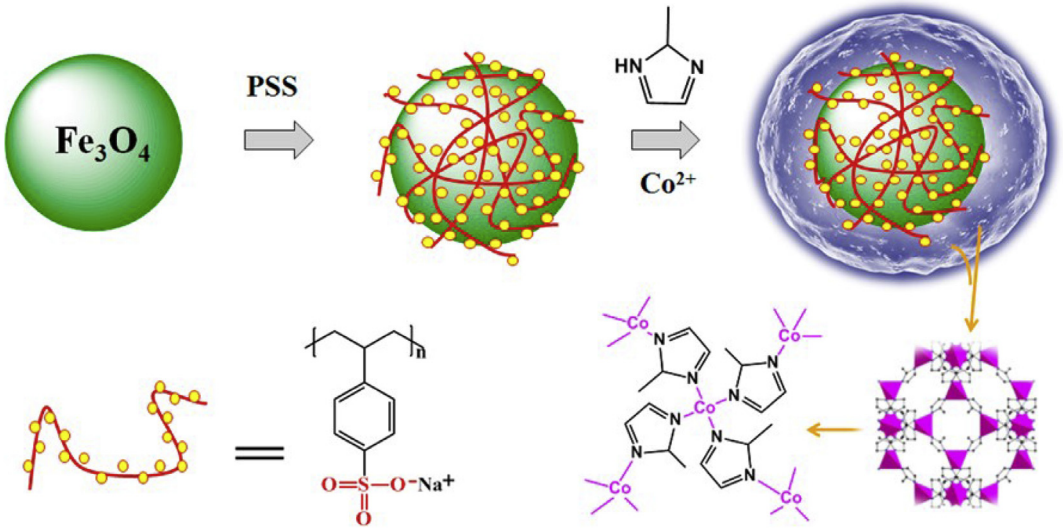

Fig. 3. Scheme for the preparation procedure of Fe_3O_4-PSS@ZIF-67 composites. {Reproduced by permission from Ref. [53]}.

sample volumes by using a magnet instead of high-speed centrifugation or filtration. Table 3 summarizes some recent applications of magnetic materials as an extractive phase in sample preparation.

5. Covalent organic frameworks (COFs)

Covalent organic frameworks (COFs) were synthesized for the first time [61] by condensation reactions of phenyl diboronic acid and hexahydroxytriphenylene. The final product is a crystalline porous polymer composed of building units with powerful covalent bonds [62]. COFs show excellent properties to be employed as sorbents, such as high surface area, tunable pore sizes, easy control of the structure and functional properties, high thermostability, good chemical stability and selectivity [62,63].

Although most reported papers about COFs cover mainly their synthesis and characterization, these materials have shown promising features for extraction of analytes with high enrichment factor [64]. Similar to a sol-gel process, different synthetic strategies may be used to alter the selectivity and extraction performance, including solvothermal [65–67], ionothermal [68,69], mechanochemical [70] and wave synthesis [71].

In 2017, for the first time, the COFs CTpBD were applied as a sorbent for analysis of ions (metals) using SPE online with inductively coupled plasma mass spectrometry (ICP-MS) [72]. Due to the CTpBD high surface area and porous structure, the new sorbent has contributed to achieving considerably low detection limits (2.1–21.6 ng L^{-1}). Liu et al. [73] prepared a spherical 1,3,5-triformylphloroglucinol-benzine (TpBD) COFs for preconcentration of phenolic endocrine disruptors (PEDs). The SPE-HPLC method exhibit a high enrichment factor with a limit of detection ranging from 56 to 123 ng L^{-1}.

Another approach was recently addressed on using a hydrothermal method to create a new magnetic mesoporous titanium dioxide@covalent organic framework (Fe_3O_4@mTiO_2@COFs) onto an Nd-Fe-B magnet for headspace sorptive extraction (HSSE) aiming trace analysis of polychlorinated biphenyls (PCBs) [74]. The PCBs can move freely inside the COF pore (exclusion of macromolecules) and selectively interact with the inner phase. In this

Table 3
Magnetic Materials employed for sample preparation and their recent applications.

Magnetic Material[a]	Compounds	Sample	Method	Ref.
3D MWNTs@ g-C_3N_4@Fe_3O_4	PAHs	Water	GC-FID	[192]
Fe_3O_4@$Cu_3(BTC)_2$	NSAIDs	Human urine Serum Plasma	HPLC	[193]
MWCNTs@ Fe_3O_4@PAPy	Nortriptyline Cetirizine Naproxen Diclofenac Ibuprofen	Water Plasma Urine	HPLC-DAD	[58]
(Fe_3O_4@mSiO_2-Ph-PTSA and Fe_3O_4@mSiO_2-Ph	PAHs	Soil	GC-MS	[194]
Co-Fe_2O_4@PEI	Tartrazine	Saffron spray Cotton candy Water samples	UV/Vis	[195]
Magnetic Graphene Oxide (MGO)	Pd (II)	Water	ETAAS	[196]
Fe_3O_4@CTAB-polyaniline	Pb	Effluent	FAAS	[197]
Fe_3O_4@SiO_2@ MMIPs	Chloramphenicol	Food	UV/Vis	[198]
Fe_3O_4@PEI-RGO	Non-steroidal anti-inflammatory drugs	Water	HPLC-DAD	[199]
Fe_3O_4@G	Acids namely 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid	Stingless bee honey	HPLC-UV	[59]
Fe_3O_4@SP@GO	Polar organophosphorus pesticides	Cucumbe Long beans Bell pepper Tomato	GC-μECD	[60]
Fe_3O_4@G	Non-steroidal anti-inflammatory drug	Human plasma Urine	UPLC-DAD	[200]
Fe_3O_4@VTEO@IL-MIPs	Lysozyme	Chicken egg	UV/Vis	[51]
Fe_3O_4@CTS@DES-MIPs	(+)-catechin, (−)-epicatechin and (−)-epigallocatechin	Black tea	HPLC-UV/Vis	[50]
Fe_3O_4@GC	Phthalate esters	Beverage Plastic bottles	HPLC-UV	[201]

[a] The definitions about each precursor abbreviation herein presented are showed into the list of abbreviations.

paper, the sorbent was reported to be used at least 80 times. Yang et al. [75] prepared a novel COF containing large amounts of carboxylic groups (COF—COOH) and 2-(2,4-dihydroxyphenyl)-benzimidazole (COF-HBI). As a result, both materials have great potential for enrichment of uranium from salt-lake water and seawater.

The diversity of COFs synthesis processes results in many different materials based on variable structures that ensure singular chemical functionalities to them. This feature is of great importance in sample preparation since this can improve selectivity and sorbent efficiency to extract a specific target compound. Due to this fact, COFs applications in analytical chemistry have been increasing in the last few years, revealing a promising class of compounds to act as sorbent as well as to be combined with the other materials discussed in this paper. Furthermore, Table 4 presents recent applications of covalent organic frameworks as a sorbent phase in sample preparation techniques.

6. Carbon-based materials

The utilization of carbon-based materials as a sorbent in analytical chemistry started with the discovery of fullerene (C_{60}) in 1985 by Smalley et al. [76]. This carbon derived molecule is characterized by a polyhedral structure formed by five and six-membered structure of sp^2 carbons bonded to other three carbon neighbors as an icosahedral polygon form, similar to a soccer ball. C_{60} has a spherical structure with a satisfactory surface area and low electron delocalization that ensures a good electron acceptance making fullerene a favorable compound to form change-transfer complexes. Despite these great mentioned features, their low solubility in aqueous and organic solvents represent an intrinsic negative property responsible to decrease their application as sorbent material in analytical chemistry [77]. Even so, the initial use of the fullerene boosted researchers to develop other carbon-based materials.

Carbon nanotubes (CNTs) have aroused in 1991 to become a promising alternative to fullerene as sorbent material. This class of carbon-based compounds can be classified into two main groups: single-wallet (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). In short, SWCNTs are composed by a single graphite sheet rolled around itself forming a cylinder structure, while MWCNTs are constituted by a rearrangement of multiple SWCNTs kept together by Van der Walls forces [78]. In general, CNTs have lengths ranging from several nanometers to micrometers with tube diameters between 0.2 and 2 nm for SWCNTs and 2–100 nm for each coaxial MWCNTs [79,80].

Due to the chemical structure composed by sp^2 carbons, CNTs have a great electrical property with high mechanical and thermal stability. However, the cylindrical shape represents a disadvantage since only its external surface area is able to interact with target compounds. This fact allied to poor solubility, as in the case of fullerenes, have boosted researches to improve carbon-based sorbents. For this purpose, modifications onto the external surface of CNTs aiming the support of organic groups to enhance sorption/desorption capacity were carried out [79].

Another interesting form of carbon allotrope is graphene, discovery in 2004 by Novoselov et al. [81]. This compound was first introduced by isolating a single nanosheet from a graphite pencil using an adhesive tape where the procedure was named mechanical exfoliation. Since its discovery graphene has been attracting large attention from the scientific community mainly due to its great physical and chemical properties including the good mechanical resistance, thermal and electrical stability; translucent form when fully isolated; fast mobility of charge carriers; and high Young's modulus [82]. These graphene-related characteristics made it one of the most applied new materials in several scientific fields (engineering, sports, and medicine, for instance). Furthermore, other significant characteristics are responsible for the increasing development of graphene-based sorbents in analytical chemistry. This includes its ultra-high surface area; a π-delocalized electron system, low cost, and easy synthesis process. In addition, the possibility to chemically modify its surface, as well as its planar structure granting both inner and external surface interactions, represents an advantage when compared with the other carbonaceous materials previously described [83]. The most applied synthesis process is the Hummers' method [84], in some cases with minor modification. This methodology starts with the oxidative conversion of graphite into graphite oxide. Afterward, by an exfoliation process graphene oxide (GO) is isolated from its precursor. The obtained GO might be reduced resulting in pure graphene (G) or used as sorbent material mainly to adsorb polar compounds due to the presence of oxygenated groups onto its surface (Hydroxyl: -OH; and Carboxyl: -COOH) [82–84]. This feature can also be used to insert another reactive chemical group onto its surface aiming to obtain another selective graphene-based sorbent. Fig. 4 illustrates the

Table 4
COFs employed for sample preparation.

Type of extraction	COFs[a]	Target analytes	Advantages	Ref.
SPME	TpPa-1	Polybrominated diphenyl ethers	Superior enrichment capacity	[202]
MSPE	Fe_3O_4@TpBD-DSS-Ab-MEG	Hsp90α	Enhanced ability to control the orientation of immobilized antibodies	[203]
_	SNW-1	Sulfonamides Cephalosporins Amino acids Parabens	The synthesized COF was used as stationary phase in a capillary column of the type open-tubular Showing good efficiency for analytical separations	[204]
MSPE	Fe_3O_4@mTiO2@ COFs	PCBs	The synthesized COF showed magnetic properties and has good extractions parameters	[74]
_	Imine-linked two-dimensional (2D) COFs	PFAS	Synthesis and application of COF as adsorbent to remove GenX and Alkyl compounds from water	[63]
_	3D-COOH-COF	Nd^{3+}, Sr^{2+} and Fe^{3+}	It presents an excellent capacity to extract lanthanides ions	[205]
SPE	TpBD COFs	PEDs	The prepared COFs were encapsulated in an SPE system to preconcentrate analysts, showing very low detection limits	[73]
On-line SPE	CTpBD	Cr (III), Mn (II), Co (II), Ni (II), Cd (II), V (V), Cu (II), As (III), Se (IV), and Mo (VI)	COF was used as an adsorbent in an SPE-ICP-MS online system. Making ultra-trace determinations	[72]
SPME	Cross-linked hydrazone COFs	Pesticide	Good selectivity due to the high number of phenyl and C double N groups	[64]
_	COF-COOH e COF-HBI	Uranio (VI)	The COFs were prepared for the first time in one single step	[75]

[a] The definitions about each precursor abbreviation herein presented are showed into the list of abbreviations.

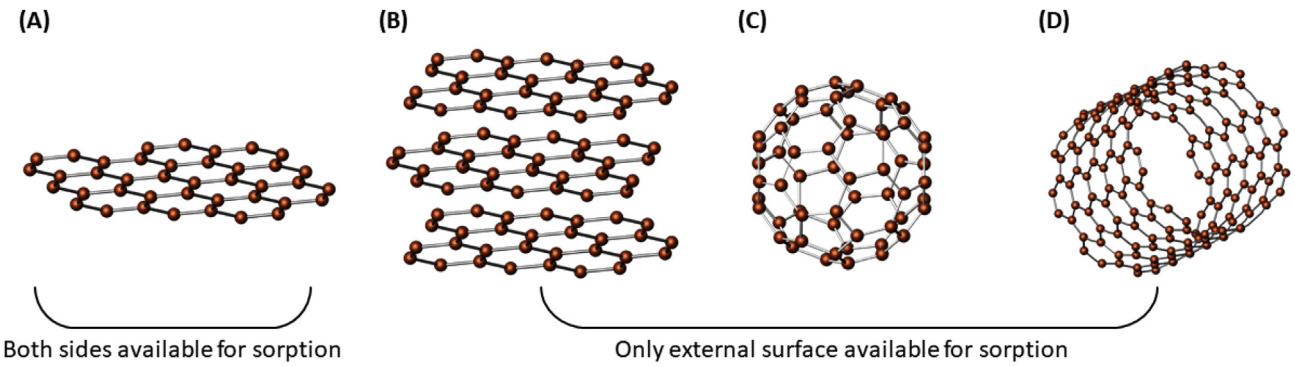

Fig. 4. Representative drawing of carbon allotropes considered as a precursor for main current carbon-based materials: (A) Graphene; (B) Graphite; (C) Fullerene; (D) Carbon nanotubes.

representative carbon allotropic forms considered as the precursors of the main carbon-based materials herein discussed.

The applications of carbon-based sorbents, especially of those derived from the compounds herein discussed, is a current trend in analytical chemistry. Nowadays, researches are faced off with many different sorbents to be used as extractive phases, including those "unmodified" (CNTs and G-based materials, for instance), as well as hybrid materials derived from combinations of them with other classes including: RAM, alkyl carbonic chains, polymeric skeletons, silica-based compounds, etc. The main use of these materials will be discussed in later topics, emphasizing the most applied according to the related extraction technique. This will bring a better overview of the actual sample preparation techniques that have been combined with carbon-based sorbents.

A large number of applications underscored below suggests carbon-based compounds as a versatile material once different allotropic forms (from unidimensional to tridimensional chemical structures) can be used for sample preparation. These structural differences between them may favor chemical modifications that enhance the extraction selectivity, being this fact one possible reason for a crescent demand for this compound class. Moreover, Table 5 summarizes other applications that complement this topic.

6.1. Solid-phase extraction (SPE)

SPE can be considered as one of the most widely applied and well-established extraction techniques, being still currently used in many laboratories. Its basic principles have supported the development of several other extraction techniques such as SPME, MEPS, dSPE, on-line SPE, etc. Besides the common commercially available phases utilized in packing SPE cartridges (C8, C18, -NH2, etc), several applications of carbon-based compounds have been reported in the literature for trace-analysis of different target analytes including pharmaceutical drugs, preservatives, organic pollutants, pesticides, metal ions, mycotoxins, and so on (Table 5). Considering the simplicity of the SPE procedure, this technique can be performed using a broad range of different carbonaceous materials. For this reason, both CNTs and graphene-based compounds have been increasingly applied as an extractive phase in SPE.

Reinholds et al. [85] evaluated four different carbon nanotubes (MWCNTs) aiming to extract 12 acidic non-steroidal anti-inflammatory drugs from environmental water samples (tap and wastewater) employing a solid phase-extraction method with subsequent determination by HPLC-MS/MS. These sorbents showed great adsorption performance reporting recovery values between 94 and 100% for all target compounds. Another interesting approach applying CNTs sponges as SPE sorbent was investigated by Wang et al. [86] aiming the simultaneous determination of metallic traces (copper, cobalt, and mercury) in water samples by HPLC-UV analysis. This paper applies for the first time this novel class of carbon nanotube called sponges to analyze residue of heavy metals in environmental water. It must be highlighted that this material might represent an emerging alternative to remove large-area oil, dyes, and other organic pollutants from aquatic ecosystems mainly due to its highly porous 3D framework.

Jiang et al. [87] synthesized a hybrid sorbent based on reduced graphene oxide coupled to gold nanoparticles to be used as SPE sorbent aiming the selective determination of nine mycotoxins in milk samples by UHPLC-MS/MS. This strategy of adding other nanoparticles in the reduced graphene oxide is carried out to enhance interlayer distances thus minimizing chances of G nanosheets agglomeration increasing the analyte's flow through the SPE cartridge.

A hierarchically porous structured sorbent composed by polypyrrole-coated graphene oxide/octadecyl silica incorporated in chitosan cryogel (PY/GOx/C18/chitosan) was synthesized by Klongklaew et al. [88] aiming to extract carbamate pesticides from fruit juices with subsequent determination by HPLC-UV. The main goal was the production of a highly porous composite to enhance analyte's diffusion allied to great sorption capabilities provided by the incorporated graphene oxide into its structure. Therefore, this paper reinforces the current trend to merge compounds with different properties to produce high performance/selective sorbent materials.

From the author's point of view, and considering the described well-known solid-phase extraction features in the cartridge format, it is clear that this approach will still be widely applied for the coming years in many different areas, mainly due to its instrumentation simplicity, easy to be performed, and well-established procedure. Several recently reported applications combining novel materials (as carbon-based sorbents) and classical SPE suggest that this combination is a potential sample preparation approach to be better explored in the coming years.

6.2. Solid-phase microextraction (SPME)

Considered as the pioneer of miniaturized sample preparation technique, SPME was originally proposed for the solvent-free analysis of volatile compounds. However, due to its success in the extraction procedure making feasible integrating sampling, purification and pre-concentration in a single step, a wide range of different applications were and still are being developed [89]. Included on this trend is the development of SPME fibers based upon carbon-based skeleton physically or chemically modified to enhance its sorption capacity as well as selectivity [19]. A list of new coating materials includes CNTs and graphene doped with

Table 5
Recent applications of carbon-based materials as sorbent in solid phase sample preparation techniques.

Carbon-based material[a]	Target compounds	Matrix	Sample preparation	Analytical technique	Ref.
Graphene oxide/polyvinyl chloride	Sulfonamides	Cosmetics	SPE	HPLC-UV	[206]
MWCNTs	Pesticides	Water	SPE	HPLC-MS	[207]
rGO-MIP	PAHs	Water	SPE	GC-MS	[208]
GO-CNTs-diethylenetriamine-functionalized	Metal ions	Water	SPE	ICP	[209]
Modified-MWCNTs	Metal ions	Food Water	SPE	AAS	[210]
Porous graphitic carbon	Adenine nucleotides	Cells	SPE	HPLC-MS	[211]
Banana-peel derived hierarchical porous carbon	Pesticides	Cucumber Watermelon	SPE	HPLC-DAD	[212]
Graphene	Food additives	Spaghetti Hard bouillon cube	SPE	HPLC-UV	[213]
GO-Sil	Aflatoxins	Cereal crops	SPE	HPLC-FLD	[214]
GO-IL	Phenolic acids	Food Biological	SPE	HPLC-UV	[215]
MWCNTs	Non-steroidal anti-inflammatory drugs	Water	SPE	HPLC-MS	[85]
CNTs sponges	Metal ions	Water	SPE	HPLC-UV	[86]
rGO-Au	Mycotoxins	Milk	SPE	HPLC-MS	[87]
PPY-C18-GO-chitosan cryogel	Pesticides	Beverages	SPE	HPLC-UV	[86]
MWCNTs-IL	Pesticides	Vegetable and Fruit	SPME	GC-FID	[90]
MWCNTs-rGO-IL	Alcohols	Water	SPME	GC-FID	[91]
NH_2-G	Synthetic musks	Water	SPME	GC-MS	[92]
N doped-G	Benzenes and xylenes	Water	SPME	GC-FID	[93]
3D phytic acid-induced graphene	Nerolidol	Tea	SPME	GC-FID	[94]
CNTs-polymeric chain	Pharmaceutical drugs	Fish	In vivo -SPME	HPLC-MS	[96]
GO	Furfural	Food	SPME	GC-MS	[98]
G	PAHs	Cigarette smoke	SPME	GC-MS	[99]
G-PS-DVB	Dyes	Water	d-SPE	SFC-UV	[100]
NH_2-rGO	Pesticides	Tea	d-SPE	UHPLC-MS	[101]
Oxidized MWCNTs	Lead ions	Water	d-SPE	F-AAS; ET- AAS	[110]
MWCNTs	Pesticides	Liquor Sorghum Rice hull	d-SPE	HPLC-MS GC-MS	[102]
Nano fibers-GO	Pharmaceutical drugs	Honey	d-SPE	HPLC-UV	[103]
Graphitized-MWCNTs	Pesticides	Tea	d-SPE	GPC-GC-MS	[104]
G-MWCNTs	β2-agonists	Pork tissues	d-SPE	HPLC-MS	[105]
MWCNTs	Pesticides	Vegetables	d-SPE	HPLC-MS	[106]
GO	Aflatoxins	Chine medicine products	d-SPE	HPLC-FLU	[107]
GO-framework	Herbicides	Water	d-SPE	HPLC-DAD	[108]
MWCNTs	Metal ions	Water	d-SPE	GF-AAS	[109]
RACNTs	Anticonvulsants	Human plasma	On-line SPE	HPLC-UV	[111]
RACNTs	Tetracyclines	Bovine milk	On-line SPE	HPLC-UV	[112]
RACNTs	Antihypertensive	Human serum	On-line SPE	UHPLC-MS	[113]
3D graphene-based foam	Bisphenol A	Disposable syringes	On-line SPE	HPLC-DAD	[114]
GO-monolith	Sulfonamides	Chicken Milk	On-line SPE	HPLC-MS	[115]
Polythiophene-GO	Pharmaceutical drugs	Water Urine Plasma	On-line IT- SPME	HPLC-UV	[116]
G-based monolith	Β-sitosterol	Food	On-line SPE	HPLC-DAD	[117]
Acrylamide-modified graphene	Heterocyclic amines	Food	On-line SPE	HPLC-UV	[118]
CFs-GO	PAHs Estrogens Phthalates	Water	On-line IT SPME	HPLC-UV	[120]
GO-Sil	Tetracyclines	Bovine milk	MEPS	HPLC-MS	[121]
Carbon foam	PAHs	Water	SB-μ-SPE	GC-MS	[122]
ZnO-MWCNTs-OH	Pesticides	Water Fruit Vegetables	Stir Brush micro extractor	GC-FID	[216]
IL-TGO	Auxins	Soybean sprouts	Pipette tip	HPLC-DAD	[124]
G-based gel	Pyrethroids	Water	In-syringe SPE	GC-MS	[125]
Cotton-GO-Sil	PAHs	Soil	Needle trap device	GC-FID	[126]

[a] The definitions about each precursor abbreviation herein presented are showed into the list of abbreviations.

other chemical classes as ionic liquids, polymers, nitrogen and acid compound [90–94]. Furthermore, the use of electrochemical strategies as electrophoretic deposition of GO onto custom fibers and electrochemical enhanced tools (sample preparation electrode assisted, for instance) are also being used for SPME procedures [95–97]. Besides that, there still are applications reporting the use of only GO-fibers to extract contaminants by SPME [98,99].

An example of MWCNTs chemically bonded to polymeric ionic liquid (PIL/MWCNTs) was reported by Wu et al. [90]. This hybrid sorbent was doped with electrochemical polymerization of 3,4-ethylene dioxythiophene (EDOT) to obtain an SPME fiber called PEDOT-PIL/MWCNTs. Furthermore, the authors pointed out that the fiber was dipped into a Nafion solution to obtain a protective outer layer improving durability, robustness as well as allowing direct-immersion of the fiber to SPME procedures. As another alternative, Li et al. [91] investigated a self-assembly synthesis to obtain a 3D porous material based on MWCNTs-rGO-IL that was deposited into a stainless steel wire by cyclic voltammetry. These two mentioned fibers (PEDOT-PIL/MWCNTs and MWCNTs-rGO-IL) were applied to an SPME-GC-FID analysis of pesticides in vegetables/fruits, and alcohols in tea samples, respectively. Another important field covered by SPME applications is the in vivo analysis as presented by Qiu et al. [96]. The authors developed an ultrafast SPME procedure based on electrosorption enhancement using a custom-made fiber composed by CTpBD). The short sampling time (~1 min), allied to better extraction capacity than commercially available SPME coatings, made the proposed approach as a new alternative to exploit ultrafast SPME procedures with high selective carbon-based fibers.

6.3. Dispersive solid-phase extraction (dSPE/d-μSPE)

dSPE has emerged as an alternative to SPE aiming simplification in the routine procedure, mainly reducing analysis time as well as solvent/reagent consumption [7]. In short, the procedure is carried out by dispersing a solid sorbent into a sample solution aiming the sorption of either the target analytes or the matrix interferents. Nowadays, dispersive solid-phase extraction has a miniaturized mode (d-μSPE) which consists of the same principles but applied to a reduced scale.

Regarding the applications of the synthesized sorbents described up to this point to dSPE, several methods have been reported, possibly due to the fact that dispersive procedures are not performed under high pressure. This fact might favor the testing of the sorption properties of new materials once problems related to obstruction are not too frequent as verified in other common sample preparation techniques.

The simple operation procedure and the requirement to be performed into a liquid media turns dSPE an interesting technique to exploit carbonaceous compounds as sorbent since those have high dispersibility in solution as well large specific surface area [7]. For these reasons, applications of dSPE or d-μSPE using carbon-based compounds became an important novel sample preparation approach with a large number of papers recently published. A list of publications includes methods applying amine-functional reduced graphene (amine-rGO); nanofibers doped with GO; G-coated/polystyrene-divinylbenzene (PS-DVB); MWCNTs; graphitized carbon black (GCB); carbon nanotubes functionalized with graphene (CNTs/G) [100–110].

The synthesis of amino-functional reduced graphene oxide (amine-rGO) compounds with several carbonic chains with different lengths was investigated by Ma et al. [101] These composites were evaluated for the dispersive extraction of catechins, pesticides, and caffeine in tea samples. The authors point out that insertions of amino-carbonic chains onto the GO are an alternative strategy to obtain useful sorbents aiming to eliminate matrix interferents and pre-concentrate target compounds. Besides the insertion of carbonic chains, another interesting approach was studied by Lou et al. [100] consisting in coating G sheets onto polymers as polystyrene-divinylbenzene (PS-DVB) to be used as a dispersive sorbent in the purification/pre-concentration of allergenic-disperse dyes in industrial wastewater samples. The modification was carried out to overcome the difficult separation of minuscule G sheets well dispersed into the solution during the dSPE procedure. Therefore, G sheets were supported onto PS-DVB microspheres to enhance particles size, thus facilitating their isolation while G adsorption properties were maintained. The G@PS-DVB sorbent showed satisfactory robustness being applied over 20 times with no loss of performance revealing to be a greener alternative material.

Another investigation towards a modification of carbon nanotubes was carried out by Feist [110]. In this work, a selective material was synthesized by the oxidation of MWCNTs to be used in d-μSPE for trace analysis of lead ions in fish samples with subsequent determination by flame and electrothermal spectrometry (F-AAS and ET-AAS). An interesting and unique form of a carbon-based compound called electrospun nanofiber was investigated by Arabsorkhi et al. [103] to be used as a sorbent in d-μSPE. This polyethylene terephthalate nanofiber was doped with graphene oxide (GO-PET) aiming the determination of tetracycline (TC) and cefotaxime (CF) in honey samples by HPLC-UV. The synthesized nanofiber exhibited the same extraction performance after at least 8 times without loss in analytical signal.

6.4. Multidimensional extraction systems

An important advance towards the development of more accurate, sensitive and fast methods is represented by an analytical approach generically termed as “online sample preparation techniques”. Generally, due to the reduced demand for operator's handling these approaches are more effective in terms of reproducibility as well as productivity than off-line techniques previously discussed. Considering these features, a crescent number of applications using carbon-based materials to produce extraction column are being developed, including restricted access carbon nanotubes (RACNTs), GO-based monoliths, acrylamide-modified graphene, polythiophene/graphene oxide, carbon nanofibers, and so on [111–119]. In these referred papers, applications of hybrid carbon-based compounds are the majority once these modified sorbents offer advantages in terms of permeability to overcome problems regarding high-pressure rates common in online sample preparation methods.

The use of a solid disk composed by modified graphene instead a packed extraction column is presented by an example of novelty based upon traditional on-line approaches in a work published by Yao et al. [114]. The extractive disc packed with a phytic acid induced 3D graphene-based foam (PAGF) embedded in the HPLC injection valve to measure traces of bisphenol A from disposable syringes. Toffoli et al. [119] packed an extraction column using graphene oxide supported on silica (Si-GO) as an extractive phase for the analysis of triazines in water by in-tube SPME-LC-MS/MS. The authors reported high sensitivity and good enrichment factor probably due to the affinity of graphene-derived materials for aromatic structure as present on triazines. Another interesting idea to obtain high-efficiency carbon-based sorbents consists in the use of electrophoretic deposition (EPD) coating of carbon fibers (CFs) with GO [120]. In this work, EPD was used to obtain GO-CF as in-tube SPME extraction device for the on-line extraction of PAHs in wastewater samples with subsequent HPLC-DAD analysis.

The application of an in-situ graphene-based monolith as an extraction column in an on-line SPE system to extract β-sitosterol from food samples was investigated by Cui et al. [117]. The synthesized material showed satisfactory extraction capacity (being compared to a C18 commercially available phase) as well as a simple and inexpensive production process. The main feature reported was the excellent permeability obtained by this sorbent when qualities of monolithic materials and carbon-based composites were maintained. The evaluation of a synthesized sorbent combining size exclusion mechanism with carbon-based properties by coating CNTs with a layer of bovine serum albumin was made by dos Santos et al. [113]. In this work, restricted access carbon nanotubes (RACNTs) were used as extraction column for the on-line determination of anticonvulsant drugs in human plasma by HPLC-UV. This method reported excellent analysis time being able to process 5 samples per hour including both sample preparation and chromatographic run. Moreover, its great robustness must be highlighted since the synthesized RACNTs was applied for at least 300 analytical cycles maintaining its analytical performance.

6.5. Other applications

The use of carbon-based materials is not limited to the techniques above-mentioned. Applications of graphene-based, CNTs, as well as hybrid materials as sorbent have also been reported utilizing other novel or well-established approaches such as microextraction by packed sorbent (MEPS), stir bar sorptive extraction (SBSE), micro solid-phase extraction (μSPE), pipette-tip extraction (PPE), in-syringe microextraction, needle trap devices (NTD),

hollow fiber membrane extraction (HFME), and so on [121–127]. Considering the wide application range some of them will be discussed next.

Developed for the fast and easy extraction of contaminants in environmental and biological samples, MEPS was combined with graphene-based compounds supported on amino silica particles (G-Sil, GO-Sil, and C18-GO-Sil) by Maciel et al. [121] to analyze four tetracyclines in milk samples. This approach for food analysis showed better extraction performance than commercially available phases (C8 and C18) as well as short analysis time (5 min) suggesting the MEPS applicability when combined with graphene-based materials to other matrices. An interesting study was conducted by Heidari et al. [126] aiming the isolation of PAHs from soil samples by a needle trap device (NTD). This approach (NTD) consists of packing the sorbent inside the needle utilizing the MEPS barrel for this purpose. The packed sorbent employed was GO-Sil covalently immobilized onto cotton which consists in cellulose derived sturdy material with a large number of oxygenated groups, ideal for graphene-based supporting.

Jilani et al. [122] developed a stir bar consisting of a low-cost carbon foam material for acting as a sorbent for the stir bar sorptive extraction of PAHs from wastewater samples. This novel carbonic compound is an alternative to the widely applied ones (MWCNTs, for instance) mainly due to its highly porous framework containing macropores and open cell structures which favors sorption interactions. Finally, another recent approach published by Zhang et al. [124] reports the development of an ionic liquid functionalized thiol graphene oxide (IL-TGO) to be used as sorbent. This material was designed to perform multiple adsorption mechanisms on a pipette tip extraction of growth hormones (auxins) in soybean sprouts. The main goal behind this strategy was obtaining a high-performance graphene-based composite merging with other sorbent class to avoid nanosheets aggregation.

All reports discussed in this topic support the combined application of carbon-based materials with sample preparation techniques as a future trend which can lead to improvements on the well-established extraction methods as well as boost the rise of novel sample preparation approaches.

7. Restricted access material (RAM) sorbents

The restricted access material involves two principles of exclusion: physical and chemical diffusion barriers. In the physical barrier, the material pores are small and do not allow the macromolecules entrance. Thus, only small analytes can be retained in the sorbent. On the other hand, in the chemical diffusion barrier, the functional groups that cover the surface of the particle are specific and exclude the macromolecules [125]. This material can be more widely used because they present mixed characteristics, since they combine the exclusion and adsorption principles to select the compounds of interest, thus becoming a more specific and selective material.

The use of RAM sorbents to the analysis of complex matrices containing macromolecules such as proteins helps in the sample clean-up resulting in better precision and accuracy. Although the RAM sorbents can be used in off-line sample preparation, it is in an online configuration that these sorbents have been highlighted [128]. Table 6 shows the most recent applications employing RAM sorbents. It may be noted that the application in biological matrices using SPE strategy is the most frequent case once the exclusion of proteins from the sorbent improves the method selectivity [128–131].

An online SPE-HPLC-DAD method was developed by He et al. [128] for rapid quantification of traces of amisulpride in human plasma. In the first step, a RAM column extracted the analyte of interest and eliminated the proteins. After turning the switching valve, utilizing a proper eluent the analyte is eluted to the analytical column (Fig. 5). As a function of the high enrichment factor, a low LOD value was found (3.5 ng mL^{-1}). Fig. 5 illustrate an automated online SPE-HPLC system (A) and summarize the principle behind the extraction and clean-up using RAM sorbents (B).

Huang et al. [129] developed an online SPE-HPLC-UV method to analyze enrofloxacin and gatifloxacin in milk bovine samples. A RAM sorbent was prepared through a combination of hydrophilic polymer poly(glycerol mono-methacrylate) and bovine serum albumin (BSA) as cross-linking to be used in an online system. Xiaodan et al. [130] prepared a carbon-functionalized magnetic

Table 6
Recent applications of RAM sorbents in sample preparation.

RAM sorbent[a]	Compounds	Samples	Methods	Ref.
Capcell Pak MF Ph-1	Amisulpride	Human plasma	On-line SPE-HPLC-DAD	[128]
Sil-g-p(St/DVB)-g-pGMMA	Enrofloxacin Gatifloxacin	Milk samples	On-line SPE-HPLC-UV	[129]
MG@mSiO_2-C	Miglitol Voglibose	Rat plasma	SPE LC-MS/MS	[130]
RAM-MIP	a-endosulfan Endosulfan Endosulfate Endosulfan-ether Endosulfan Lactone Heptachlor Heptachlor-exo-Epoxide Heptachlor-endo-epoxide	Pork	SPE GC-ECD	[131]
RAM trap column SUPELCOSIL LC-HISEP	Lipid mediators	Skeletal muscles	LC-MS/MS	[217]
C18-BSA	Antipsychotics Antidepressants Anticonvulsants Anxiolytics	Plasma from schizophrenic patients	DPX-LC-MS/MS	[218]
RAM-MINP	Hippuric acid	Human urine	RAM-MINP-SPE	[219]
graphene@mSiO_2-C_8	Glucocorticoids	Liver tissue	RAM-d-SPE	[132]
MG-mSiO_2-Ph	Parabens	Water-based skin toners	RAM-SPE-HPLC-DAD	[220]
RAM-MIP	Parabens	Breast milk	In-tube SPME-UPLC-MS/MS	[133]
RAM-BSA C18	Fluoxetine Norfluoxetine	Human milk	2D UPLC-MS/MS	[221]
RAM-MIMMs	Florfenicol	Milk	d-SPE-HPCL-UV	[222]
SUPRAS-RAM	Fusarium toxins	Cereals	SUPRAS-RAM-LC-IT-MS	[223]
RAM-MIP	Folic acid	Milk powder	SPE-HPLC-DAD	[224]
RAM-MIPs	Organophosphorus	Honey	SPE-GC-FID	[225]

[a] The definitions about each precursor abbreviation herein presented are showed into the list of abbreviations.

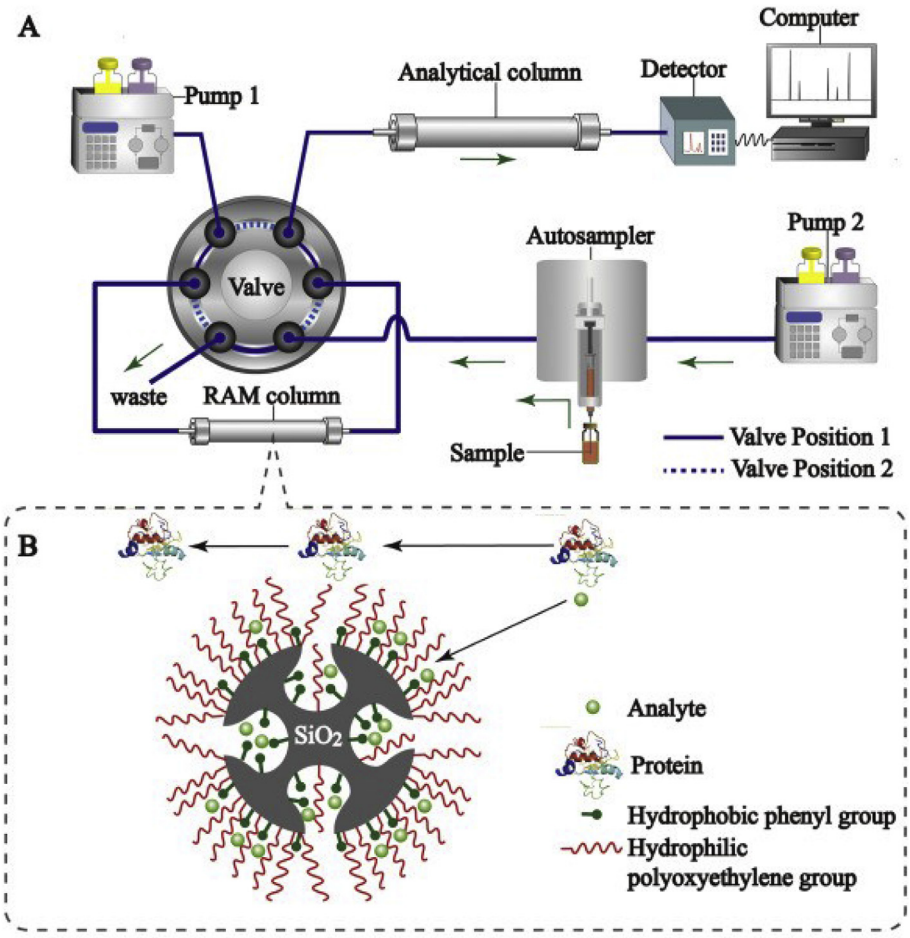

Fig. 5. Schematic of the automated online SPE-HPLC system (A), the extraction and clean-up principle of a RAM column (B). {Reproduced by permission from Ref. [128]}.

graphene/mesoporous silica composites MG@mSiO$_2$-C composites in RAM-SPE for the determination of miglitol and voglibose in rat plasma. The great advantage of this material is the hydrophilic interaction between carbon and glycans. Thus, miglitol and voglibose could be directly extracted from the matrix with no additional pre-treatment steps.

Feng et al. [132] synthesized a graphene@mSiO2-C8 sorbent (C8-RAM) for d-SPE aiming the analysis of glucocorticoids residues in liver tissue. In comparison with traditional methods that employ LLE and SPE, the use of microwave-assisted enzymatic hydrolysis followed by RAM-d-SPE reduced the analysis time from several hours to several minutes.

A new MIP-RAM was reported by Souza et al. [133] using benzylparaben as template and glycerol dimethacrylate (GDMA) as RAM phase. The synthesis was performed via in situ polymerization into open fused silica capillary for in-tube SPME. The sorbent exhibits high selectivity for the analysis of parabens in breast milk samples.

Initially, RAMs were most commonly used in biological sample preparations because of their unique feature related to the exclusion of macromolecules. However, nowadays RAMs extraction columns have been applied to extract different analytes in several other matrices, in special food. Due to their relevant advantages and ease of being synthesized, the use of this material has been encouraged in the sample preparation techniques involving the exclusion of macromolecules from complex matrices.

8. Immunosorbents (ISs)

Immunosorbents are prepared by immobilization of antibodies onto solid supports, either solid or gel, such as silica or cellulose, using the same principles as for affinity chromatography. This material is an alternative to the classical adsorbents to provide low limits of detection and high selectivity extraction of the target analytes mainly due to the specific interactions between antigen and antibody to afford the molecular recognition [134,135].

This adsorbent material is selective in retaining the analytes of interest whereas interfering matrix compounds are not sorbed on the column because the immobilized antibodies recognize a small group of the analytes (antigens), resulting in a highly specific enrichment process. The ISs have been used for several different applications such as environmental monitoring, medical diagnostics, food safety and biological applications [134,135].

A method recently described by Ok et al. [136] utilized an immunoaffinity column for sample cleanup followed by HPLC-UV detection and quantification. The immunoaffinity cleanup was efficient for the simultaneous determination of the analytes nivalenol and deoxynivalenol in rice and bran. After the validation, the method was employed to detect the analytes in 482 rice and 239 bran samples. Limits of detection and quantification ranged from 6.4 to 15.6 and from 21.2 to 52.0 μg kg^{-1}, respectively, demonstrating that the immunoaffinity cleaning step was important to obtain low limits.

Uhlig et al. [137] selected commercial immunoaffinity columns (IACs) with different commercial DON immunosorbents to provide a guide for choosing an appropriate IAC or combinations of columns for extraction of mycotoxin 4-deoxynivalenol (DON) derivatives. All columns were evaluated employing liquid chromatography-high-resolution mass spectrometry (LC-HRMS) and four out of the five tested IACs showed similar extraction profiles, while one has a distinctive extraction profile. The authors suggest in this work that the combination of two columns would enable extraction of all the evaluated compounds, except for DON-13-derivatives. In general, it is possible to notice that the immunoaffinity columns improve the methodologies since they promote greater selectivity between the immunosorbent and the analytes.

Yao et al. [138] developed a method for concentrating and purifying bisphenol A (BPA) in 14 different food samples using immunoaffinity cleanup material based on a monoclonal antibody (mAb). The authors compared the method developed with two conventional SPE methods [139,140] concluding that the immunoaffinity column demonstrated great ability to eliminate impurities, lower UPLC-MS/MS baseline noise, and have provided better selectivity. Important characteristics of the immunoaffinity column highlighted by the authors, included the column capacity and reusability of IAC because only a small amount of mAb was required to prepare the immunosorbent. The promising results obtained with this work, such as low limits of detection ranging from 0.001 μg L^{-1} to 0.01 μg kg^{-1} and the high recoveries from spiked samples that ranged from 82% to 104.9%, showed to be a promising sample pre-treatment tool for extraction and purification of BPA from complex matrices.

Nakagomi and Suzuki [141] established a method for quantifying 15α-Hydroxyestrogens (15α-OHEs) in rat urine using immunoaffinity extraction column and HPLC electrochemical detection. The immunosorbent demonstrated high selectivity for the three analytes of interest with detection limits obtained from the urine of male rats that range from 0.047 to 0.103 ng. The authors obtained satisfactory results with the immunosorbent employing this analytical method, with accuracy ranging from 97 to 109%.

Armutcu et al. [142] reported a comparison between an automated on-line two-dimensional high-performance liquid chromatography (2D-HPLC) and an off-line immunoaffinity-high-performance liquid chromatography system. The 2D-HPLC system is proposed to determine Ochratoxin A (OTA) in food samples (including beer, wine, corn, and Turkish coffee) as an alternative to OTA immunoaffinity column. The authors developed a poly(2-hydroxyethylmethacrylate-N-methacryloyl-(L)-phenylalanine) [P(HEMAPA)] monolithic column to act as the first dimension in a multidimensional setup while a reversed phase C18 column was employed in the second dimension. The on-line methodology minimized human error and eliminated the pretreatment processes for the analysis of OTA, which was required for Ochraprep IAC. The online system results demonstrated good extraction efficiency of the P(HEMAPA)-4 immunosorbent monolithic column developed for OTA with the recovery of the analyte ranging from 104.24% to 107.33%. The authors concluded that the monolithic column could be a potential alternative to disposable commercially available immunoaffinity columns due to the reusability, easy manipulation, and adaptability to the 2D-HPLC system.

Lan et al. [143] developed an immunoaffinity column for the improvement of selectivity and enrichment of spinosyn A in milk, fruits and vegetable samples. A new spinosad hapten immunosorbent was synthesized and a sensitive monoclonal antibody (mAb3E6) with high specificity for spinosad was produced. Then, the authors made a mAb3E6 based immunoaffinity column for enrichment extraction of spinosyn A and reduction of matrix interference. The results with indirect competitive enzyme-linked immunosorbent assay (icELISA) methods were validated by HPLC-UV and showed satisfactory results demonstrating that the method is potentially sensitive and a convenient tool for monitoring the analyte of interest in food matrices.

The immunosorbents, and consequently the affinity chromatography, have been showing great advances in recent years in different separation areas with satisfactory results. As reported in the articles, the antibodies can be immobilized onto supports and packed into columns. Although the immunoaffinity columns have become popular, especially for sample preparation, this type of column has as main drawback its high cost. In the face of this undesirable feature, researchers are evaluating the use of molecularly imprinted polymers that have the same principles of immunosorbent with some advantages such as lower price, easier and quicker preparation.

9. Molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers are simple and now well-established material that can be used for selective extraction of target molecules. They are considered synthetic polymers of predetermined selectivity toward a given analyte or a group of structurally related species. MIPs present high chemical, mechanical and thermal stabilities, in addition to their unique selectivity, which ensures their wide application in the extraction, pre-concentration, and separation of many different compounds in various analytical chemistry approaches [144–147].

The synthesis of a MIP requires the polymerization of functional monomers and a cross-linker around a template, then the recognition sites are imprinted in the polymer matrix by the presence of the template during their synthesis which is formed according to the size, shape and functional groups of the template molecule [144–147].

Several types of MIPs have been developed in the last years being used in various fields such as biological, environmental, catalysts, chiral separation, food, drugs, chromatographic stationary phases, pharmaceutical and highlighting the capacity of them specifically binding a target compound and mimicking natural receptor systems including enzymes, antibodies, hormones [144–147].

Due to their outstanding advantages such as high selectivity, easy preparation, good performance, reusability, being simple, high adsorption capacity and low cost, MIPs have attracted increasing attention and consequently, different combinations and procedures based on them have been developed. Some works show the use of MIPs as SPE sorbents (MISPE), MIPs as SPME sorbents (MISPME), MIPs in sensor technology, in magnetic separation, as stationary phases, in sample preparation techniques, conjugated with carbon-based materials and among other variations [148–150]. Besides that, MIPs have also been combined with liquid chromatography, capillary electrochromatography and capillary electrophoresis [144,145,151].

The MIPs synthesis allows the creation of selective and specific binding sites that are similar to a goal template in chemical and steric properties. In general, they are prepared by a process that includes the formation of a complex between the template (analyte of interest) and the functional monomers. After this, the process of polymerization in the presence of a crosslinking agent and a solvent occurs. Then, the template molecules are removed and the polymer with specific cavities is ready for use [144,145,151].

Depending upon the interaction between the self-assembled complex of target molecules and the functional monomer in the pre-polymerization and during rebinding, three different imprinting processes can be prepared for the synthesis of MIPs: covalent, non-covalent and semi-covalent [144,145,151]. The

covalent imprinting is formed by using the reversible covalent bonds between the template molecule and the functional monomer before the polymerization process. The covalent bond cleavage after synthesis eliminates the template from the polymer and can affect cavity functionality. One of the advantages of this synthetic route is a homogenous distribution of binding sites and as a disadvantage, there are not many types of functional monomers that can be used in this type of synthesis [144,145,151]. The non-covalent approach is the most widely used due to the ease of template removal without the need for formation and subsequent cleavage of chemical bonds. This synthesis depends on forming week binding interactions such as hydrophobic or hydrogen-bonds, dipole-dipole and ionic interactions between the functional monomer and templates. Besides that, the non-covalent character enables the imprinting of a wider range of compounds, being this approach more versatile and responsible for a more heterogeneous distribution of binding site affinities and structures [144,145,151]. Bulk polymerization [152], precipitation polymerization [153], emulsion polymerization [154], imprinted solid-phase [155] and core-shell imprinting [156] are some of the non-covalent strategies.

The semi-covalent or hybrid imprinting procedure is a mixture of the covalent and non-covalent imprinting ones. Initially, covalent bonds are formed during the molecular imprinting process and the target molecule binds with the monomer via non-covalent interactions [144,145,151]. Regardless of the MIP's synthetic route, this material has demonstrated promising applications in different areas since they have several advantages such as high specificity and selectivity for the molecule used in the imprinting process, low cost, simple synthesis process, reusability, robustness, allow pH variation and strong stability [144,145,151].

A new capillary extraction prepared by in-situ polymerization on inner and out surface namely MIPs-E1-coated in-out-tube SPME (MIPs-E1-IOT-SPME) combined the advantages of an extraction efficiency of IOT-SPME and the specific selectivity of MIPs developed by Wang et al. [157]. This approach demonstrated satisfactory extraction of six endocrine disrupting chemicals (EDCs), presenting large enrichment ability. The combination of MIPs-E1-IOT-SPME with HPLC-UV when compared with commercial fibers presented enhanced adsorption capacity being a potentially promising alternative to be applied in different areas such as environmental monitoring, food, drugs, emerging contaminants and biological.

Another novel on-line procedure using molecularly imprinted column was developed by Guo et al. [158]. In this work, the authors developed and validated a coupled molecular imprinted monolithic column with two-dimensional liquid chromatography. The imprinted columns were synthesized by in-situ polymerization and showed high selectivity adsorption capacity for the analysis of clenbuterol in pork liver and swine urine samples. When compared to other methods described in the literature, this online method has better specificity and can efficiently avoid the disturbance of endogenous impurities.

Da Silva et al. [159] synthesized a molecularly imprinted polymer for solid phase extraction of lumefantrine from human plasma to be used in drug monitoring. The molecularly imprinted solid phase extraction coupled to liquid chromatography (MISPE-HPLC-UV) was optimized using chemometric tools and validated according to international requirements showing advantages such as faster analysis, highly selective and sensitive when compared with conventional sorbents. The MIP sorbent exhibited high thermal stability, binding properties and affinity, adequate porosity and surface morphology. Another similar work was developed by Sánchez-González et al. [160]. The authors prepared a micro-solid-phase extraction molecularly imprinted polymer (MIP-μ-SPE) followed by HPLC-MS/MS for synthetic cannabinoids assessment in urine samples. The MIP-μ-SPE device consisted of a polypropylene porous membrane containing the adsorbent for operating in a batch mode.

MIPs have been demonstrated as promising sorbents for sample preparation in several fields because their specific recognition of the target analytes increasing the concentration power. The development and application of new MIP types in analytical chemistry have been growing significantly because this material shows high extraction capacity, simply production methodologies and fast extraction times.

10. Molecularly imprinted monoliths (MIMs)

Molecularly imprinting is a well-established technique which has several advantages such as low cost, robust materials, specific recognition properties, high stability, among others. Since the discovery of MIPs, this area has been growing significantly. Nowadays the molecularly imprinted monoliths have been highlighting in various areas, mostly as sorbents in microextraction techniques and as the stationary phase used in high performance liquid chromatography (HPLC) and capillary electrochromatography (CEC) because they have good permeability, easy preparation, rapid mass transfer, and versatile surface modification and high selectivity [11,161–164].

Molecularly imprinted monolithic columns are a good alternative for extraction columns, once they present a great potential to selectively extract the analytes of interest. One of the main advantages of this type of column is its high permeability, characteristic of the monoliths, that generates low pressures. Another advantage is the elimination of particles and frits in the preparation of the extraction column [11,161,162,164].

Molecularly imprinted monoliths can exist in several physical forms (e.g., disks, needles, columns, fibers, and others) highlighting the columns that are simple and can be produced by in situ polymerizations directly into a tubing resulting in a chromatographic or extraction column. The molecularly imprinted monoliths are easy to prepare, and their porosity and surface chemistry can be easily adjusted. Based on the material used for polymerization they can be classified as inorganic monoliths, organic polymer monoliths and organic-inorganic hybrid monoliths [11,161,162,164].

Inorganic imprinted monoliths can be prepared with silica-based or metal oxide-based and can have different pore morphologies. Organic imprinted monoliths are the most important because of the variety of monomers commercially available and their excellent stability at different pH.

Hybrid monoliths consist of two or more of them combined. One hybrid form can be done between an organic and inorganic monolith combining several advantages such as low density, flexibility, and excellent optical and mechanical properties. The organic-inorganic hybrid monoliths are a good selection for several imprinted monolith template preparation procedures [162–164].

Guo et al. [165] developed a molecularly imprinted monolithic column in a two-dimensional liquid chromatographic method (MIMC-2D-LC). The authors investigated in this work several characteristics of the polymer such as morphology, surface groups, adsorption performance, and polymerization of the monolith. The sorbent was further employed for the determination of estradiol in various cosmetic samples. Estradiol molecularly imprinted columns were prepared by in situ polymerization and the results showed that the imprinted monolithic extraction phase presented high selectivity towards estradiol, moderate towards some other steroid hormone and avoids the influence of possible impurities present in the matrix.

Another molecularly imprinted monolithic extraction column was reported by Shao et al. [166] for the selective extraction of hesperetin in the flesh of *Citrus reticulata cv. Chachiensis*, which is a

traditional Chinese medicine (TCM). The authors investigated the selectivity recognition properties demonstrating that the imprinted monolithic extraction column presented high adsorption capacity and selectivity of the interest analyte and can remove the interfering substances which promote cleaner extracts from the complex matrices.

A novel method was proposed by Liang et al. [167] to determine aflatoxin B_1 in peanut sample using a carbon quantum dots-coated dummy molecularly imprinted polymer (CDs-DMIP) monolithic column for sample pretreatment coupled with HPLC-FLD. The monolith extraction column was prepared by in situ polymerization and the results showed high extraction capacity and sensitivity with an enrichment factor over 71-fold.

Zhai et al. [168] reported a PDMS/glass chip integrated with RB-imprinted monolithic capillary array extraction columns coated with silanized graphene oxide (GO/SiO_2) as support matrix to determine low levels of rhodamine B (RB) in chili powder. The cheap-base array column device was used to effectively extract and enrich RB from chili powder indicating selectivity, a higher adsorption capacity, impurity removal, and affinity when compared with conventional MISPE extraction columns. Besides that, the authors related that the prepared columns and chips can be reused at least 30 times with reproducibility and enrichment factor over than 110-fold. Another interesting approach to chip-based systems was investigated by Huang et al. [169]. The authors developed an extraction method based on chip-based dual-imprinted array monolithic extraction columns synthesized by in situ polymerization to simultaneous extraction of BPA and nonylphenol (NP) from three kinds of fish samples. The extraction device showed satisfactory results with an enrichment factor of 113-fold for BPA and 92-fold for NP demonstrating that the material presents high binding capacity and selectivity.

MIMs is another material which has been attracted interest to be used for selective separation and determination of analytes from complex matrices. This material combines the high selectivity of MIP with the fast mass transfer of the monolithic approach, thus resulting in fast extractions and selective analysis. Nowadays, only a few reports referring to MIMs as extraction phase are described in the literature but considering the discussed features of this material within the next coming years, the potential of this material should be further exploited.

Apart from the monolithic sorbents obtained by molecularly printing procedures, other compounds can be underscored. As discussed by Masini and Svec [11] several types of monoliths to be utilized in sample preparation have been developed mainly since 2010. In this cited review the authors emphasized the differences between organic and silica-based monoliths as well as presents strategies to obtain hybrid monoliths containing other sorbent classes, as those herein discussed. In general, the idea over the hybrid compounds is the possibility to combine the known advantages of porous monoliths with other good intrinsic characteristics from other materials. For instance, the combination with nanoparticles provides a high surface area permeable material, which can result in a better extraction performance. The broad variety of nanoparticles (metal organic framework, carbon-based, magnetic, etc) allied to a large number of different monoliths represents a promising field to obtain tunable sorbent phases which can enhance the sample preparation selectivity [163]. Furthermore, the use of metal-organic frameworks containing a conglomerate of metallic ions as a template to enhance monolith porosity is another interesting approach currently reported [170]. This strategy provides hybrid materials with higher surface contact area when compared to those traditional representing then an excellent alternative in sample preparation [170].

Another clever strategy is carried out by insertion of ILs into the monolith preparation mixture. Due to the ILs chemical structure composed by an anion and a cation species, these materials when combined with monoliths can perform extraction of both polar and non-polar contaminants [171]. Monolithic materials also can be used as a sorbent phase in applications of macromolecules such as proteins, carbohydrates, nucleosides, and so on [172]. An interesting approach consists to produce boronate functionalized monoliths to extract molecules containing diol moieties being useful to extract glycoproteins, glycopeptides, carbohydrates, etc [172]. Following this trend, approaches based on molecular recognition as the immobilization of aptamers on monoliths and immunosorbents should be pointed out [173]. Aptamer-based monoliths are oligomeric compound supported onto a solid monolith, characterized by specific binding sites similar to those produced by antibodies when these are used as a coating in the immunosorbents [173]. The low molar mass of aptamers, generally ranging from 3 to 20 kDa, decreases steric hindrance favoring high coating rates, which can be useful for trace bioanalysis [173].

All these available options over monoliths combined to different chemical compounds are contributing to obtain high sorptive and permeable monolith-based materials to be used mainly in miniaturized extraction techniques.

11. Concluding remarks and future trends

There is a consensus that one of the current challenges in analytical chemistry includes the determination of contaminants at low concentration levels while, at the same time, more efficient and environmentally friendly methodologies are required. For this reason, miniaturized extraction techniques were developed allowing to reduce solvent/reagent consumption, analysis time and operator's handling (mainly for online approaches), resulting in more efficient and affordable analytical methods. However, considering the complexity of the common matrices of interest (biological fluids, foods, environmental samples, among others) more selective methods are still required. As a natural reaction, researches aiming the production of new materials to act as sorbent phases have emerged. In this review, different classes of them were discussed such as sol-gel process derived materials, ILs, magnetic-based, COFs, carbon-based, RAMs, ISs, MIPs, and MIMs. Moreover, several applications combined with more efficient sample preparation techniques are reviewed, given a broad idea of the current scenario to the readers.

The authors suggest that the merging of these different compound classes, to produce hybrid materials, is as a crescent trend in analytical chemistry as an efficient strategy to improve extraction selectivity, capacity as well as robustness, both for sample preparation as well as new materials. Beyond the development of sorbent materials, some modifications towards tailored modern sample preparation methods are currently being done as showed in topic 6.6. These novel approaches also include a trend for full automatization of the analytical workflow, as mentioned in Ref. [174], as well as punctual changes on common sample preparation rearrangements to obtain more singular and specific extraction techniques.

In this context, efforts have been made in the development of specifically modified extraction approaches based on the sample preparation techniques herein mentioned, as reinforced in Refs. [121–127]. Therefore, the current scenario suggests that the development of tunable hybrid materials (obtained by mixing up the chemical classes herein discussed) combined with miniaturized sample preparation techniques and approaches (both online and off-line) open up a huge research window for the years ahead.

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CAPÍTULO 5

Evaluation of the tubing material and physical dimensions on the performance of extraction columns for on-line sample preparation-LC-MS/MS.

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Evaluation of the tubing material and physical dimensions on the performance of extraction columns for on-line sample preparation-LC–MS/MS

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ABSTRACT

Nowadays, high analytical throughputs are required considering an increasing demand for faster, simple and improved methods to analyze contaminants in a considerable number of samples. Generally, these compounds are present in complex matrices in contact with a high number of interferents becoming their determination difficult at low concentration. In this context, on-line extraction techniques arose to improve the extraction as well as separation power, while minimizing errors related to human sample manipulation. This paper describes a study regarding the development and optimization of columns used as an extraction device in multidimensional liquid chromatography. The main goals were the evaluation of the material used as column body as well as the investigation of the tube dimensions (internal diameter and length) in the extraction performance. Firstly, several tube materials were tested (steel, fused silica, PEEK, among others) being steel whose reported the best performance and was consequently chose for further studies. The investigation about the effects of the columns physical dimensions revealed a linear relationship between performance and the amount of sorbent utilized as extractive phase. However, when different columns with same amount of sorbent were tested results suggests that both i.d. and lengths play an important role in extraction efficiency. The longest columns with lower internal diameter showed the best results favoring the radial as well as axial analytes diffusion into the extraction column. After evaluation of these column variables, applications were carried out employing several different analytes belonging to various chemical classes and practical utilization, in order to reinforce the versatility as well as the robustness of this proposed study.

1. Introduction

Sample preparation is a crucial step to obtain reliable results that require high precision and accuracy. This procedure usually consumes more than 70% of the total time of a typical analysis involving complex matrices (from the sampling to final result) being characterized by requiring extensive sample manipulation by the analyst which increases the probability of errors occurrence. However, this step is very important and frequently mandatory in order to isolate the analytes of interest from the matrix interferents. The determination of both target and untargeted compounds at very low concentrations requires precise analytical procedures that concentrate the analytes before the quantification step. Besides that, due to the number of steps required it represents a potential source of error [1–4].

Constantly, researchers have been seeking instrumental advances to become the analysis faster and, consequently, allowing an increase in the extraction and separation power of the target compounds whilst minimizing the human error by reducing the number of steps. Despite of some progress, in most cases the presence of a large number of interferers in complex matrices still makes difficult the achievement of analytically reliable results [1,5].

Systems that allow the on-line extraction and pre-concentration of the analytes followed by the chromatographic separation and detection, under full automation, have reported satisfactory results as already described in the literature [4,6–10]. These breakthrough in modern analytical instrumentation become very promising for the development of fully miniaturized techniques through the hyphenation of automated techniques resulting in reduced organic solvent consumption, samples, and analysis time [2,3,11].

On-line microextraction techniques including in-tube solid phase extraction (in-tube SPME), on-line solid phase extraction (on-line SPE), turbulent flow and others, are efficient techniques for

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sample preparation that employs either a filled (packed or monolithic) or coated ("porous layer open tubular" - PLOT and "wall coated open tubular" - WCOT) capillary tube with a proper sorbent to extract and pre-concentrate the analytes of interest [4,10,12].

On-line extraction systems can be directly coupled to liquid chromatography (HPLC) being this technique usually consisting of two steps (loading and elution) and two different columns (usually one for extraction and another for separation). The sample loading system can be operated in two main modes: draw/eject or in the flow through mode [2,13]. The draw/eject extraction mode requires a modification in the autosampler, being most adequate when coated capillary tubes are used [2,13]. Conversely, flow through extraction is more demanded for packed and monolithic capillary columns because draw/eject extraction exhibits higher backpressures in such types of capillaries [2,13]. For analytes elution there are two flow directions: straight flush and back flush. Elution in the straight-flush mode occurs in the same direction as the sample loading flow and in the back-flush mode in the opposite direction of the sample loading flow [4,6,9].

The selection of the type of extractive phase is of extreme importance to ensure the extraction of the analytes employing the on-line systems. Several types of materials are commercially available such as: C8, C18, Strata X, Oasis, and so on. However, in most cases there are limitations on the use of these materials due to their high cost as well as the disposable character of these extractive phases, mainly in the traditional SPE cartridge format. Thus, the development of new materials is of extreme importance to improve the efficiency and versatility of the extraction process since there are still few studies in this area, limiting the extraction selectivity when analytical methods are applied.

Considering the relevant limitations presented by the commercially available phases, recent studies have pointed out the need for new sorbent materials to be used for samples preparation [3]. Among these materials we can highlight graphene and its derivatives, which have been widely employed in several applications [14–17]. Graphene oxide (GO) is a graphene precursor material which contains epoxy, carboxyl and hydroxyl groups, differing from graphene in the number of oxygen containing functional groups. GO presents large specific surface area, good physical-chemical properties, easy synthesis and can be covalent bonding to aminopropyl silica (Sil). Due to its attractive properties, GO has been applied in a several fields such as: adsorption of organic and inorganic compounds, sensors, biosensors, component of nanocomposites, energy storage devices and so on [16–20].

Another important feature of the on-line systems is the type of miniaturized extraction column utilized. Different column dimensions have been used ranging from 250 to 530 μm with several combinations of lengths and types of column body materials. Therefore, it is important to evaluate and understand how the extraction column dimensional variables can influence the performance of the extraction, analytes pre-concentration and, consequently, the overall analytical results [4].

In the present work the main goal was the evaluation and optimization of miniaturized extraction column hardware materials and physical dimensions and their on-line utilization in fully automated multidimensional systems aiming the determination of analytes present in complex matrices at trace levels. The first step of this work was focused on the type of column body material for the on-line extraction directly coupled to a chromatography system. The second step was to evaluate other parameters such as length and internal diameter of the extraction columns. Finally, after selected the best column based upon optimization of the hardware conditions and the extraction performance the on-line fully automated system was applied to analyze different compound classes.

2. Materials and methods

2.1. Reagents

All the reagents used were of high purity. Twenty four analytical standards were utilized in this study being ceftiofur, cephalexin and ochratoxin A purchased from Fluka Analytical (Steinheim, NW, Germany); cefoperazone and penicillin G potassium from United States Pharmacopeia (Rockville, MD, USA); sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfachlorpyridazine, sulfamethoxazole, sulfadimethoxine, sulfaquinoxaline, simazine, atrazine, ametryn, β-zearalenol, methylparaben, ethylparaben, propylparaben, butylparaben, benzylparaben, Δ9-tetrahydrocannabinol and 11-Hydroxy-Δ9-tetrahydrocannabinol were all purchased from Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile (ACN), methanol, isopropanol and tetrahydrofuran (chromatographic analysis grade) were obtained from Tedia (Fairfield, OH, USA). Highly purified water was prepared employing a Milli-Q water purification system from Millipore (Concord Road, Billerica, MA, USA). Stock solutions of the individual analytes were prepared in methanol at a concentration of 1000 mg L^{-1} and stored at −32 °C. During the experiments, diluted solutions of the analytes in different concentrations were prepared by dilution of the stock solutions. For the graphene oxide (GO) synthesis and further support onto aminopropyl silica, graphite, potassium permanganate, sodium nitrate, dicyclohexylcarbodiimide (DCC) and dimethylformamide (DMF) were acquired from Sigma–Aldrich (Saint Louis, MO, USA); sulfuric acid from Tedia (Fairfield, OH, USA); hydrochloric acid from Qhemis (Jundiai, SP, Brazil); and hydrogen peroxide from Synth (Diadema, SP Brazil). The aminopropyl silica (for short "Sil") used to support GO was acquired from J.T. Baker (Hampton, NH, USA).

2.2. Instrumentation and analytical conditions

An Acquity Ultra Performance Liquid Chromatography system and a Xevo TQ Mass Spectrometer assisted by MassLynx Software, all from Waters (Milford, CT, USA), were utilized for the LC–MS/MS experiments. A Shimadzu Liquid Chromatography external pump LC-10A*i* coupled to a Shimadzu Degasser 14 A were used to load the samples into the extraction column employing water as the solvent. For the on-line system an electrically automated six-port valve from Supelco (Bellefont, PA, USA), maintained at room temperature, assisted in the complete automation system.

Chromatographic separation was performed in a Poroshell column 120 SB-C8 (100 mm × 2.1 mm, 2.7 μm) from Agilent (Santa Clara, CA, USA). The column was utilized under the following conditions: mobile phase consisting of ACN/water and loading phase consisting of water; column temperature, 40 °C; chromatographic flow rate of 0.2 mL min^{-1} and loading flow rate of 0.05 mL min^{-1}.

The mass spectrometer fitted with an Electrospray Ionization (ESI) source was optimized by direct infusion of the analytical standards (0.5 mg L^{-1}) diluted in the mobile phase. The optimized conditions for the MS/MS were: ESI in the positive mode; a capillary voltage of 3.0 kV; cone voltage of 20 V; source temperature at 150 °C; dessolvation gas temperature of 400 °C; dessolvation gas flow rate (N_2) at 800 L h^{-1} and collision gas flow (Ar) at 0.15 mL min^{-1}. For identification and detection of target compounds with maximum sensitivity, two Multiple Monitoring Reaction (MRM) transitions were optimized for each compound. Table 1 lists both the precursor and product ions as well as the optimized conditions for the associated collision energies, cone voltages and dwell-time.

Table 1
MS/MS parameters optimized for the analysis of the target β-lactams.

Analytes	Precursor ion (m/z) $[M+H]^+$	Product ion (m/z)	Cone (V)	Collision energy (V)	Dwell-time (s)
Ceftiofur	524	125	30	62	0.1
		209	30	22	0.1
Cephalexin	348	106	14	28	0.1
		174	14	16	0.1
Cefoperazone	646	143	18	34	0.1
		530	18	12	0.1
Penicillin G	335	128	34	26	0.1
		159	34	24	0.1

2.3. Synthesis of the extraction phase (GO-Sil)

The graphene oxide (GO) synthesis basically followed the method reported by Hummers [21] with minor modifications to bond it onto the silica surface. The synthesis product (graphite oxide) was dried for 72 h at 40 °C and subsequently re-dispersed in water at a concentration of 1 mg mL^{-1}. This solution of graphite oxide was sonicated for 1 h and lyophilized resulting in graphene oxide (GO). To supporting GO onto Sil surface was used the procedure described by Liu et al. [22], where 40 mg of the synthetized GO were dispersed in 100 mL of DMF and ultrasonicated for 1 h; then 1 g of Sil and 40 mg of DCC were added. The mixture was stirred at 50 °C for 30 h. The product obtained (GO-Sil) was washed with methanol to remove unbounded GO before the material lyophilization.

2.4. Column hardware and packing procedure

Different columns were made to be used in this study being all of them packed in the same methodology. Columns employing polyether ether ketone (PEEK), stainless steel, fused silica, silanized fused silica, polytetrafluoroethylene (Teflon) and a silica tube covered with stainless steel (steel silica) were packed with GO-Sil employing a slurry packing procedure using methanol as the packing phase. Different columns were packed once several parameters such as type of the hardware material; internal diameter and length were evaluated (as described in 2.5). Before starting, 10 mg of the extractive phase was weighed in a vial and suspended under 1 mL of isopropanol/tetrahydrofuran (1:1). The packing procedure was done by using a Haskell (Los Angeles, CA, EUA) pneumatic amplification pump to push the slurry inside the column under an average pressure of 500 bars and methanol as packing solvent. The column tube was fitted with a stainless steel frit (metallic screen - diameter, 1/16 in. porosity, 10 μm) at both ends to hold the extraction phase inside the extraction columns.

2.5. Extraction column optimization

A detailed study about the influence of different physical parameters on the quality of miniaturized extraction columns was performed. This study consisted in evaluating initially seven different column materials (PEEK, stainless steel, steel silica, fused silica, silanized fused-silica and Teflon) being for that purpose the size and internal diameter of the columns standardized (50 mm of length and 500 μm of i.d.). After defining the most efficient hardware material for the extraction columns, the next step was the evaluation of the influence of the column dimensions, such as the internal diameter (i.d.) and the length (L). For evaluating the role of the internal diameter parameter on the extraction performance, three different i.d. values were evaluated: 180 μm; 250 μm and 500 μm. The role of the length on the column's performance was evaluated by preparing three columns of different lengths: 50 mm; 100 mm and 200 mm. Finally, three different extraction columns containing the approximately same phase volume (0.01 cm^3) but different lengths and internal diameters (column A: L = 200 mm and i.d. = 250 μm; column B: L = 50 mm and i.d. = 500 μm and column C: L = 23 mm and i.d. = 714 μm) were evaluated. All columns were packaged using the procedure described in 2.4. The evaluation of the effect of all these variations on the extraction columns performance was done employing an on-line system [6,16,17] using four different analytes of the β-lactam class as probes. Photos containing all columns evaluated in this work are depicted in the supplementary material illustrating the relative proportion in their physical dimensions (Figs. S1–S4).

2.6. On-line extraction system

An on-line extraction system coupled to LC–MS/MS in a fully automated set up was used to evaluate all columns developed. A schematic diagram illustrating the on-line extraction and desorption steps utilized in this work is shown in the supplementary material (Fig. S5), including the electrically automated six-port switching valve that was used for the full system automation. The on-line system assembled and employed in this work was based on a flow through extraction configuration, where the sample passes only once through the extraction column and the analytes elution was carried out in the opposite direction of the sample loading, employing the back-flush mode [6,16,17,23,24].

The on-line extraction was carried out using a GO-Sil extraction column packed as described. An LC-10A*i* external pump from Shimadzu delivering water at a flow rate of 0.05 mL min^{-1} was used for loading the sample into the extraction column. During the extraction procedure, the on-line extraction step was carried out as following: initially the valve was set at the loading position and 50 μL (full loop) of the sample solution was pumped through the extraction column for one minute at a flow rate of 0.05 mL min^{-1} with a loading phase containing 100% of water. After finishing this step, the valve was switched to the elution position for the desorption step, where the analytes were eluted by the HPLC mobile phase from the extraction column being consequently shifted to the analytical column. The chromatographic separation was done in a C8 column employing as mobile phase acetonitrile:water (10:90 until 45:55 v/v) at a flow rate of 0.2 mL min^{-1}. The elution gradient started at 10:90 (acetonitrile:water v/v) at the time of the valve switch (2 min) and the concentration was changed to 45:55 (acetonitrile:water v/v) within 6 min. After the chromatographic separation the analytes are detected and quantified by tandem mass spectrometry (MS/MS). Thus, in the next step the extraction column is cleaned in line with the analytical column by flushing both with 45:55 (acetonitrile:water v/v) for 3 min. Then, the system returns to the original analytical and extraction column conditions where the analytical column is conditioned during 4 min with 10:90 (acetonitrile:water v/v) and the extraction column with 100% of water. The temperature of the analytical column oven was set at 40 °C and the extraction column was maintained at room tem-

Table 2
Time table schedule of the experimental on-line steps.

Steps	Event	Time (minutes)	HPLC flow rate (mL min^{-1})	Solvent composition	Switching valve position
1	Extraction	0:00–2:00	0.2	Water (100%)	Loading
2	Desorption	2:00–8:00	0.2	Acetonitrile/Water (10:90 until 45:55, v/v)	Elution
3	Cleaning of the column	8:00–11:00	0.2	Acetonitrile/Water (45:55, v/v)	Elution
4	Extraction column conditioning	11:00–15:00	0.2	Acetonitrile/Water (10:90, v/v)	Loading
5	Analytical column conditioning	11:00–15:00	0.2	Water (100%)	Loading

perature. The total run time including the extraction, desorption, separation, cleaning and conditioning steps was 15 min. All steps of the on-line system are displayed in Table 2.

2.7. On-line extraction optimization

Despite extraction column physical parameters being the main issue on this study other variables were also optimized in order to achieve an ideal condition for the β-lactams extraction. After the preliminary experiments it was verified that several parameters including sample loading time, sample loading flow rate and analytical column temperature should be investigated aiming further improvement of the method conditions. For this purpose, a factorial design 2^3 with the center point (4 times replicated) was applied taking into account the mentioned factors. A total of twenty experiments were carried out with the variables varying between 1 and 3 min for loading time; from 0.05 to 2 min for loading flow rate; and 30–50 °C for column temperature, respectively. The levels considered for each factor in the experiments performed during this step are shown in the supplementary material (Table S1).

2.8. Extraction column application

After all the optimization procedures involving the investigated columns it was planned a practical application involving the extraction column hardware that presented the best overall results during the optimization process, considering the type of tubing material, internal diameter and length. Firstly, this column was fitted into the on-line system and utilized to analyze standard solutions containing analytes belonging to different chemical classes, including β-lactams and sulfonamides (antibiotics), triazines (pesticides), cannabinoids (drugs), and parabens (preservatives). Afterwards, the same extraction column was employed to analyze four β-lactams in milk samples as well as two mycotoxins in wine samples. The analysis of these complex matrices required only a filtration step employing cellulose membrane prior to their direct introduction into the multidimensional system.

3. Results and discussion

3.1. Characterization of the synthesized and anchored extraction phase (GO-Sil)

In this work were utilized two complementary techniques to investigate the synthesized extraction phase main characteristics. Firstly, a scanning electron microscopy (SEM) was applied aiming to obtain information about the morphology of the GO-Sil material. The photomicrographs are shown in the supplementary material (Fig. S6).

Fig. S6-A depicts a raw particle of Sil utilized in this synthesis, while Fig. S6-B shows the nanosheets morphology characteristic of the GO material as well as its corrugated profile. Furthermore, it also can be seen (Fig. S6-C and -D) GO sheets clearly anchored onto the Sil surface, which confirms the formation of the GO-Sil extraction phase. The SEM images suggests that the supporting process of GO onto Sil surface still requires a further improvement aiming a higher coating efficiency, although the obtained material was successfully used recently to carry out other studies reporting similar results [25,26].

Another information to evaluate the effective coupling between graphene oxide and aminopropyl silica to produce GO-Sil material was obtained applying Fourier-transform infrared spectroscopy (FT-IR) being the IR spectra shown in Fig. S7. The adsorption bands appearing at 800 and 1080 cm^{-1} can be attributed to the Sil–OH bending and Sil-O-Sil stretching vibrations, respectively. An important band appeared at 1650 cm^{-1} correspond to a C=O bending vibration related to a peptide bond formed between oxygen groups belonging to GO and aminopropyl groups of Sil. This band suggests that the synthesized graphene oxide material was successfully supported onto the silica surface. The band shown at 3450 cm^{-1} can be assigned to a –OH bending vibration characteristic from both GO and Sil since these two compounds have hydroxyl groups into their chemical skeleton. However, an increase in the signal going from Sil to GO, which is in agreement with the photomicrographs previously discussed (Fig. S6-C and -D), corroborates with the hypothesis that GO-Sil particles were formed.

3.2. On-line extraction optimization

Initially, a series of preliminary experiments was carried out to determine which parameters most affected the extraction performance. Based upon the results of these experiments and by chromatographic separations already described in the literature, it was decided to use water and acetonitrile (both acidified with formic acid 0.1%) as the HPLC mobile phase A and B, respectively, as well as water as the sample loading solvent for the on-line extraction. Water and acetonitrile were chosen as solvents for the mobile phase, once they presented the best separation and resolution for the β-lactams.

In addition to the univariate optimization of the solvents, a multivariate experiment was performed involving the main factors chosen to be studied by the factorial design: loading time, loading flow rate, and analytical column temperature. The results showing the influence of these factors in the extraction performance of ceftiofur as model compound considered into the design of experiments (DoE 2^3) are depicted in a pareto chart (Fig. 1). From this data, the method seems to be significantly affected by all selected variables being the loading flow rate the one that most influenced the results. This trend can be related to the analyte concentration once by increasing the loading flow rate (from 0.05 to 2 mL min^{-1}) a more diluted sample is obtained, making the sorption process less favorable and decreasing the extraction efficiency. Therefore, a loading flow rate of 0.05 mL min^{-1} was set to continue this study. In the pareto chart (Fig. 1) it can be seen a negative result related to the loading time suggesting that lower values for this variable can improve the on-line extraction. This fact suggests that increasing the time during which the sample flows through the extraction column may favor the sorption of the target compounds, but also of those interferents present in the matrix. Considering these results, the lowest time evaluated (1 min) was selected as the best one. The temperature employed to the analytical column showed a positive effect into the extraction, although it was more significant

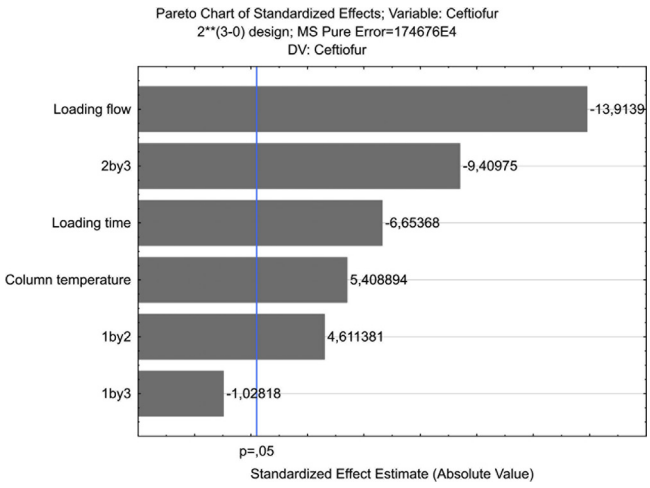

Fig. 1. Pareto chart from the design of experiments (DoE 2^3) showing the influences of selected experimental factors on the extraction performance. Model analyte: Ceftiofur.

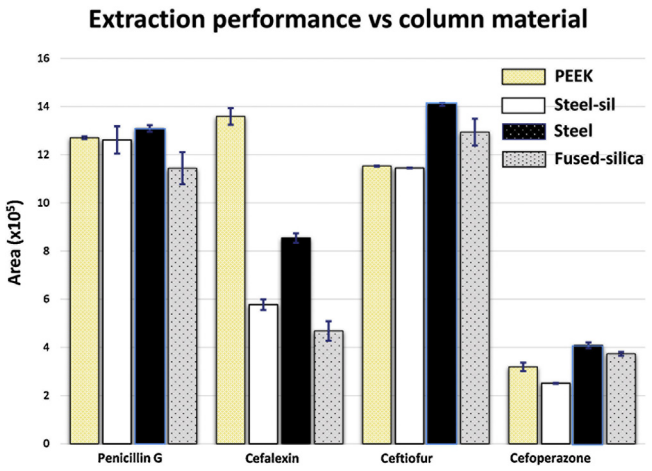

Fig. 2. Effect of the tubing material (four main columns) employed on the extraction performance of the investigated analytes using the on-line system.

for ceftiofur and penicillin. For this reason, the intermediary value considered in the central point was set. Therefore, the analytical column was maintained at 40 °C during the analysis. In spite of the fact that results here reported are related only to ceftiofur utilized as model compounds, for the other analytes a similar behavior was obtained.

A three-dimensional response surface study has also been investigated based upon the results previously discussed showing the relation between the variables (Fig. S8-A). The resulting graphics shows that optimum conditions can be achieved employing low values for the loading flow as well as for the loading time. When temperature is considered (Fig. S8-B and -C), a trend for higher extraction values is observed; however, as this variable was not significant in the pareto chart (Fig. S8-A), the central point mentioned before was maintained. Therefore, the conditions obtained by examining the pareto chart were confirmed by this three-dimensional response surface. To endorse these observations an analysis involving the observed vs predicted values (Fig. S9) for each experiment considered into the DoE 2^3 was carried out. The comparison between the actual points (blue dots) and the predicted values (red line) shows a satisfactory correlation suggesting that the generated model for this factorial design was predictive and the data can be considered. For this reason, the obtained results in this part of the work were used in the continuation of the on-line method development and on the studies related to the extraction column parameters.

3.3. *Influence of column body materials*

As the beginning of this study an investigation about the chemical composition of the tube material used to produce the extraction columns was carried out. A photo containing the columns investigated in this work is depicted in the supplementary material (Fig. S1), while Fig. 2 shows the results obtained in the extraction of each studied analyte using four different materials types. It is important to quote that the extraction columns made of teflon and silanized fused silica are not represented in Fig. 2 because they did not withstand the packing system pressure resulting in tube breaking. Although the evaluated columns presented similar overall results, those columns produced employing stainless steel and those with PEEK were chosen to be further explored as they provided the best results in the extraction of β-lactams antibiotics employed as a probe (Fig. 2). Another aspect to be considered is the good column reproducibility when extractions cycles were carried out applying two different extraction columns made of the same material and under same conditions. Three replicates of extraction were considered for each column. Those produced from stainless steel resulted in lower standard deviations than those produced employing other materials as can be seen examining the bars representing the deviation in Fig. 2. Comparing the area values by integrating each peak of the analytes of interest (Fig. 2) the column based on bare stainless steel was superior in the extraction of 3 (Penicillin G, Cefoperazone and Ceftiofur) out of 4 analytes, excluding only cephalexin that was better extracted on columns made of PEEK. The results obtained employing the PEEK columns, in special for Cephalexin, may suggests its inertness as a good property favoring extraction performance when compared to stainless steel columns. However, as our columns were designed to work at high-pressure conditions, stainless steel seems a more reasonable material since PEEK may suffer of swelling effects considering our working pressures close to 400 bar.

The previously discussed results can be attributed to some intrinsic properties of stainless steel tubes such as its excellent resistance to the higher pressures required to obtain a dense and homogeneous GO-Sil packed bed, which after all results in a more effective interaction between the target compounds and the extraction phase [27,28]. Moreover, its superior resistance to handling when compared to other materials such as fused silica that is more easily break than stainless steel should also be point out once it favors a routine use of these extraction columns in different instrumental setups, without stressing the packed bed [29,30].

It is widely known that column's dimensions (mainly length and i.d.) are relevant factors in liquid chromatographic analysis once they directly influences the efficiency of the analytical columns [31]. For this reason, a study to determine the role of these parameters in the case of extraction columns was carried out. Stainless steel was selected and fixed as the tube material to be used in the development of the new extraction columns. The aim of this step consisted in the evaluation on the influence of both length and inner diameter on the extraction efficiency as well as its singular and combined contribution to the automated extraction method performance.

3.4. *Influence of column dimensions (length and i.d.)*

These parameters were investigated using six different extraction columns developed as can be seen in the supplementary material (Figs. S2 and S3). The tubes used to perform the column's evaluation in this topic, were all made of bare stainless steel.

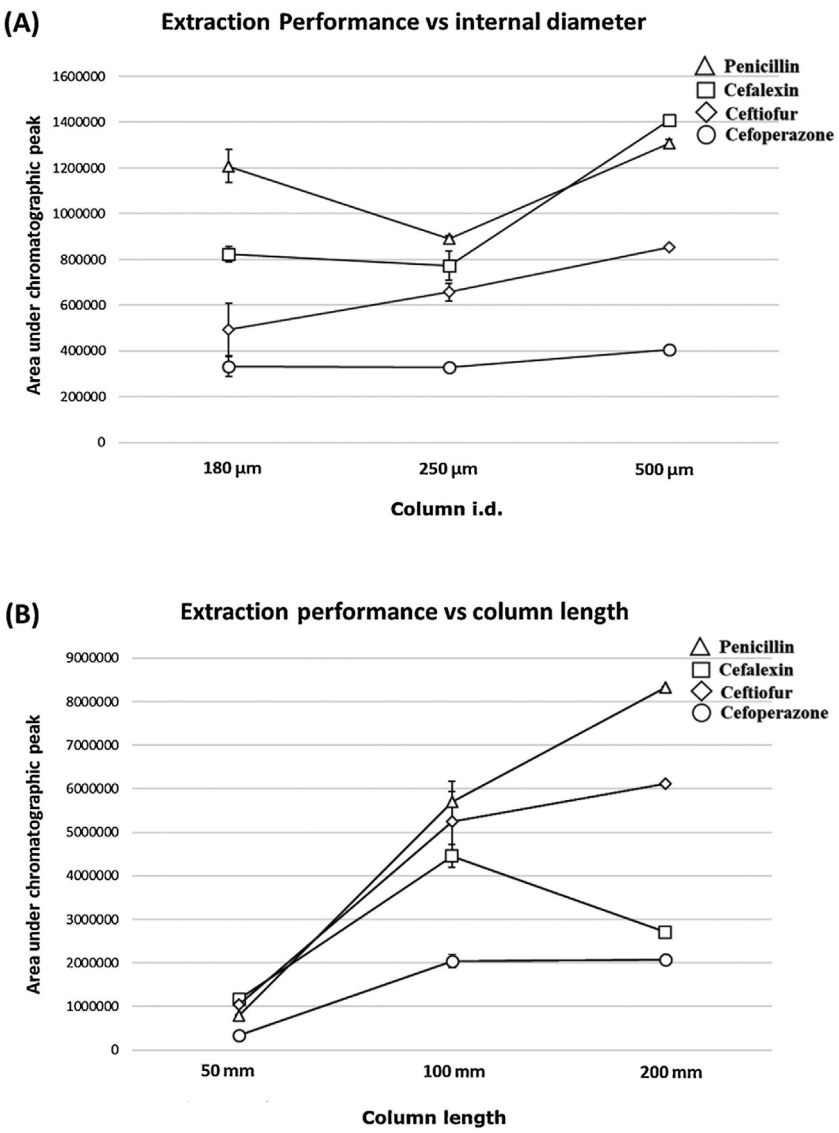

Fig. 3. Effect of selected column parameters on the extraction performance of the investigated analytes using the on-line system: **(A)** column internal diameter and **(B)** columns length.

A variation on the tube internal diameter while maintaining the column length constant may be an important factor in the extraction procedure, thus was selected to be investigated. In this case, three different columns (180 μm, 250 μm and 500 μm i.d.) were used being the length of them fixed in 50 mm. An increase in the extraction efficiency as a function of column i.d. was observed, being the best results obtained employing of extraction column of 500 μm (Fig. 3A), as expected, once larger column i.d. with the same length will contain more extraction phase. However, when compared columns having approximated same i.d. (180 μm and 250 μm; ca. 30% difference) the extraction efficiency was close. The 180 μm i.d. column showed better results than the 250 μm i.d. column for two analytes. Conversely, the results were higher in all cases for the column having the larger i.d. (500 μm).

Afterwards, an investigation regarding extraction performance as a function of tube length was carried out. The tubes used to procedure this part had same i.d. (500 μm - all made from the same piece tube cut as required) and three different lengths (50 mm, 100 mm and 200 mm). The longest extraction column showed better extraction efficiency as can be seen in Fig. 3B. This result was consistent since for columns with equal i.d., the amount of extraction phase responsible to retain the target compounds is directly proportional to the column length which corroborates with the prior study based on tube internal diameter.

For these reasons, a more detailed investigation regarding the role of tube physical dimensions (i.d. vs length) when simultaneously varied was performed and will be described in the next item (3.5).

3.5. Comparison among different columns exhibiting the approximated same phase volume

The mentioned results obtained by the investigation of column dimensions (length and i.d. – topic 3.4) boosted a third study regarding the combined relation between length vs i.d. in the extraction efficiency. The columns used in this step are shown in the supplementary material (Fig. S4).

In the previously experiments it was verified in most cases to exist a linear relationship between the quantity of packed phase utilized and the extraction performance. For this reason, three different columns were evaluated maintaining the approximated same tube internal volume as 0.01 cm^3, varying only its length as a function of the inner diameter, to result in equally conditions regarding the quantity of extracting sorbent. As were utilized the

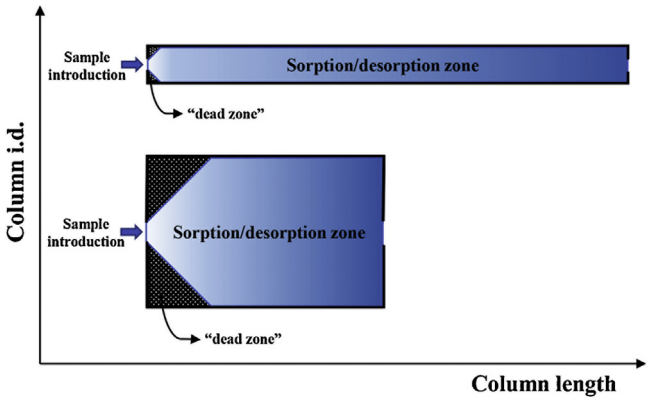

Fig. 4. Illustrative effect to remark the correlation between the relative main column dimensions (length and i.d.) and the formation of "dead zones".

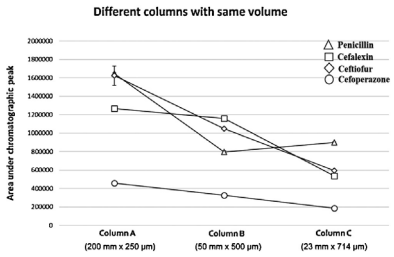

Fig. 5. Effect of different columns having the same packing volume in the extraction performance of the investigated analytes using the on-line system.

same packing material and technique, in principle the only variable will be the combination length – i.d. to generate the approximated same volume. As proposed by Knox et al. [32,33] when chromatographic column diameter is sufficiently large in relation to the tube length it is possible that unreactive zones are created being the analytes not able to interact with the extraction phase packed into these locals.

Fig. 4 shows a schematic representation of the mentioned effect. According to this model, analytes centrally injected onto the top of the column will travel a distance to reach the column walls that is a function of the internal diameter [32]. Columns having smaller i.d. will reach the walls before those having larger i.d. Therefore, once reaching the column walls the analytes can interact with the packed extraction phase while longitudinally flowing until the end of the tube [31–34]. Considering a column having a smaller length-to-internal diameter ratio (bottom portion of Fig. 4), it is expected that analytes might take more time to reach the column walls, thus decreasing the extraction efficiency when compared to the situation when a larger length-to-id column is available (top portion of Fig. 4). Consequently, this suggests that extraction efficiency is also proportional to the length of the tube once relatively lengthy tubes allow the analytes to spend more time interacting with the extraction phase, being another possible reason to improve analytes sorption.

To evaluate these effects together three different columns having the approximated same volume as well as extractive phase were evaluated: column A (200 mm × 250 μm i.d.), column B (50 mm × 500 μm i.d.) and column C (23 mm × 714 μm i.d.).

In agreement with the discussed model, higher extraction efficiencies were obtained utilizing column A (Fig. 5), which had the lower internal diameter and the larger length. Additionally, column A reported larger peak width and consequently lower resolution probably due the greater time spent by the analyte flows through column A than B and C which increased band broadening, although this effect was not so significant allowing to continue the study with column A. These discussed results suggests that the effect originally mentioned by Knox et al. [32] for LC columns is also observed for columns being used as extraction devices in the first dimension of a multidimensional separation system.

After additional experiments aiming to verify the accuracy of proposed model, column A (stainless steel; L = 200 mm; i.d. = 250 μm; packed with GO-Sil) was considered as having the required conditions to obtain good extraction performance for the automated instrumental setup used in this work. Thence, this extraction column was applied to the extraction of different classes of analytes under various analytical conditions.

3.6. Extraction column application

Once column A was defined as the best one among those evaluated, it was further employed as the extraction column (first dimension) in an automated multidimensional separation system aiming the analysis of several compounds from different chemical classes and in different matrices. The main goal was the qualitative evaluation of its robustness, precision and ability to extract different target compounds.

Fig. S10 depict the total ion chromatograms (TIC) obtained for the different analytes investigated belonging to the following distinct chemical classes: β-lactams, sulfonamides, triazines, parabens and cannabinoids. The main chemical characteristics of these compounds are displayed in Table S2. As it can be seen from the results, column A extracted with good performance molecules having different chemical structures and physical-chemical properties. As example, the analytes belonging to both β-lactams and sulfonamides (SFs) are polar compounds with amphoteric properties (logP ranging from -0.12 to 2.0) while triazines and parabens both have a less polar character than SFs [35–37]. In addition, cannabinoids analyzed in this work are compounds with pronounced nonpolar characteristics (logP > 5.7, in general) [38]. These facts confirm the versatility related to the packing phase employed (GO-Sil) as already reported by us [24,26,39], and the excellence of the optimized extraction column as well.

In relation to the robustness of column extraction C it is estimated that over 150 injections were made during the method development, optimization and applications, with the column still retaining its original performance. Only one column A was utilized for the entire work, suggesting its capability to work in routine analysis. This number of injections retaining the column original quality suggests a satisfactory result when compared with other reported works using automated analysis combined with a column in the first dimension as extraction device [23,40–42]. Another important aspect to underscore is the pressure rate for these extraction columns, which stood between 30–150 bar. This range represents a useful threshold for HPLC scale once the pressure values summing up that of the extraction column plus the analytical column do not surpass 400 bar.

We must point out that the extraction column A was already applied into the analysis of real samples, including wine and milk, to reinforces its great applicability. Representative chromatograms containing real samples analyzed with this column in a fully automated multidimensional setup (extraction column – LC–MS/MS) are shown as example in the Fig. 6.

4. Concluding remarks

To the best of our knowledge this paper reports an study on the development and further optimization of packed GO-Sil columns as micro extraction devices for fully automated sample preparation on-line coupled with liquid chromatography - tandem mass spectrometry. The main goal was the evaluation of how the tube

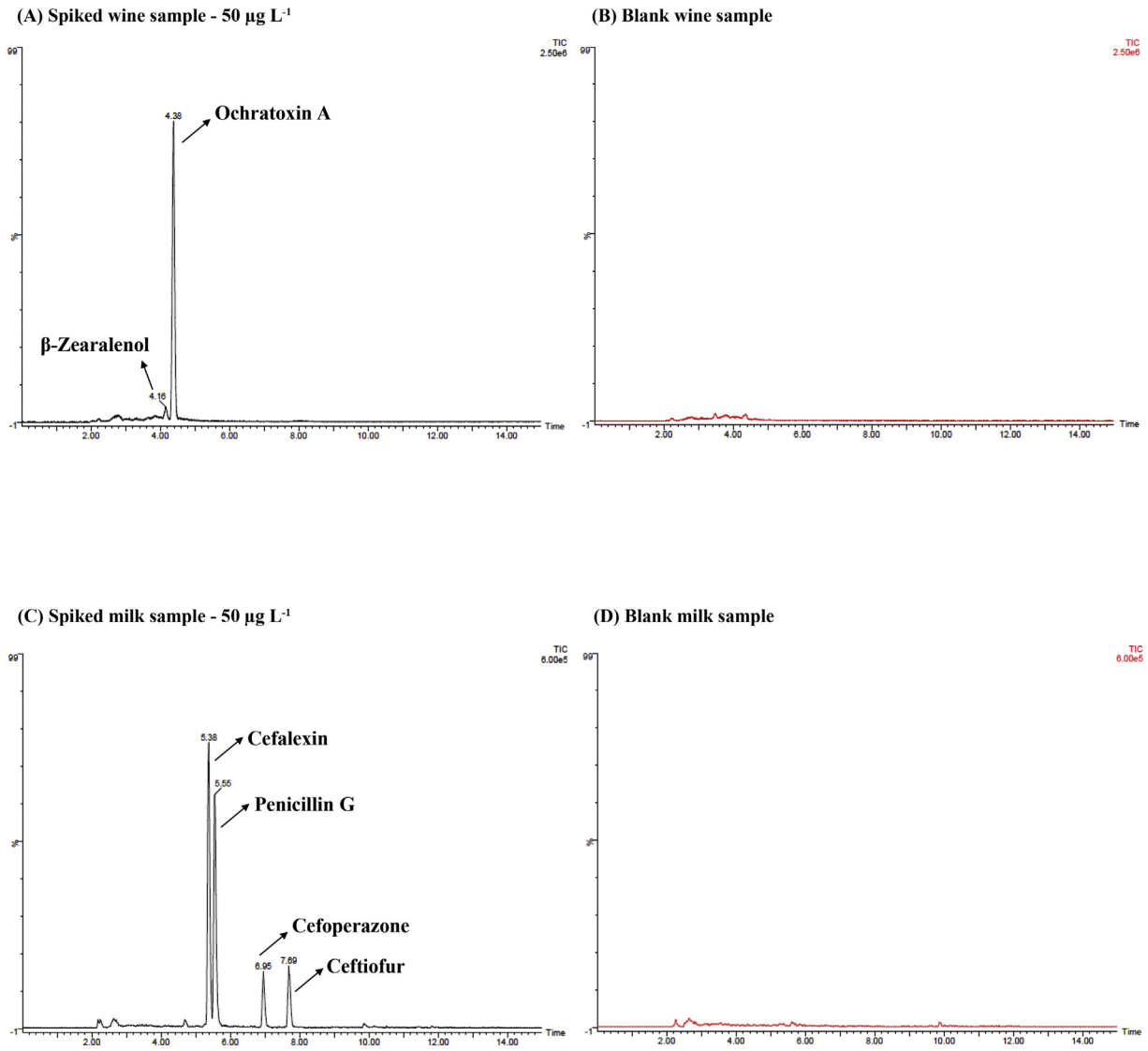

Fig. 6. Representative chromatograms of: spiked mycotoxins in wine sample (A); blank wine sample (B); β-lactams in milk sample (C) and (D) blank milk sample. Samples were analyzed by automated multidimensional liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Extraction column: GO-Sil (200 mm × 250 µm).

physical dimensions (length and internal diameter) as well as tube body construction material influences the extraction performance. Four analytes belonging to the β-lactam group were selected as model compounds to evaluate the extraction column properties.

From a univariate study it was found a linear correlation between column extraction efficiency and the quantity of GO-Sil sorbent employed as a function of length or i.d. selected; better performance was found for columns with longer lengths. When investigated tubes with different i.d. and equal lengths the results shown a similar trend: larger i.d. (500 µm) column showed higher extraction efficiency once more GO-Sil sorbent was packed into the column. Conversely, column's having close i.d.s (180 µm and 250 µm) generated similar results. Therefore, a new set of experiments was performed maintaining the GO-Sil volume constant while varying L and i.d. accordingly. The results revealed a close relation between column length and i.d., suggesting that higher extraction performance was achieved by using extraction columns of smaller internal diameter combined with longer lengths. In this case, transverse diffusion is improved as internal diameter decreases, which favor the analytes sampling all the available paths into the tube; longer lengths allow the analytes to spend more time interacting with GO-Sil. For this reason, column A (200 mm x 250 µm) showed the best extraction efficiency with great reproducibility. Analysis of different chemical classes (β-lactams, sulfonamides, triazines, parabens, cannabinoids and mycotoxins) using the same developed column A was successfully done. The results reveal a versatile performance as well as great robustness for column A, which still maintain its original performance even after more than 150 injections by comparison the chromatographic peak area and reproducibility between injections made during this study. In short, both internal diameter as well as tube lengths plays an important role in the extraction performance. This information contributes to the recent trend towards the miniaturization not only for analytical columns but also for extraction columns aiming improvements throughout the analytical system. The results reported in this work might contribute improving the production of new extraction columns as well as their applications on miniaturized multidimensional liquid chromatography.

Conflict of interest statement

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.03.023.

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CAPÍTULO 6

Multidimensional liquid chromatography employing a graphene oxide capillary column as the first dimension: Determination of antidepressant and antiepileptic drugs in urine.

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Multidimensional Liquid Chromatography Employing a Graphene Oxide Capillary Column as the First Dimension: Determination of Antidepressant and Antiepileptic Drugs in Urine

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Abstract: Human mental disorders can be currently classified as one of the most relevant health topics. Including in this are depression and anxiety, which can affect us at any stage of life, causing economic and social problems. The treatments involve cognitive psychotherapy, and mainly the oral intake of pharmaceutical antidepressants. Therefore, the development of analytical methods for monitoring the levels of these drugs in biological fluids is critical. Considering the current demand for sensitive and automated analytical methods, the coupling between liquid chromatography and mass spectrometry, combined with suitable sample preparation, becomes a useful way to improve the analytical results even more. Herein we present an automated multidimensional method based on high-performance liquid chromatography-tandem mass spectrometry using a lab-made, graphene-based capillary extraction column connected to a C8 analytical column to determined five pharmaceutical drugs in urine. A method enhancement was performed by considering the chromatographic separation and the variables of the loading phase, loading time, loading flow, and injection volume. Under optimized conditions, the study reports good linearity with $R^2 > 0.98$, and limits of detection in the range of 0.5–20 μg L^{-1}. Afterward, the method was applied to the direct analysis of ten untreated urine samples, reporting traces of citalopram in one of them. The results suggest that the proposed approach could be a promising alternative that provides direct and fully automated analysis of pharmaceutical drugs in complex biological matrices.

Keywords: liquid chromatography; mass spectrometry; sample preparation; automation; on-line; multidimensional; extraction column; urine; antidepressants; pharmaceutical drugs

1. Introduction

Diseases associated with human mental disorders can be currently classified as one of the most emergent topics in medicine. In this context are the widely known psychiatric illnesses called depression and anxiety. According to the World Health Organization, it is estimated that roughly 4.4% of the world population has already suffered from them. It is predicted that depression will be the second-most prevalent human disorder by 2030 [1].

In general, depression is considered a chronic disease that can arise in any stage of life, causing significant damage, including economic and social problems, and even leads to suicidal thoughts [2]. The most frequent symptoms of depression include unstable moods, fatigue, sadness, and insomnia. Additionally, anxiety can be considered another common type of psychiatric disorder that, when overlooked, leads to depression. In this case, arrhythmia, hyperventilation, sweating, racing thoughts,

and insomnia indicate anxiety. Taking into account the similarities, there is presumably a direct correlation in terms of medical interventions. The most popular treatments involve cognitive psychotherapy, and mainly the use of pharmaceutical antidepressants (ADs) [3]. Therefore, considering the present panorama of mental disorders frequently reported in the 21st century, it is also expected that there will be an increase in antidepressant uptake by people in future.

Typically, these pharmaceutical drugs are divided into four main classes: tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitor (SSRI), selective noradrenaline reuptake inhibitor (SNRI), and monoamine oxidase inhibitors (MOI) [4]. Although there are several different medicines commercially available, most of them have similar side effects (mainly in the early stages of administration), and a slow time to start acting on the human brain [5]. Besides these, other medications, such as antiepileptic drugs, can also be used to treat such disorders since they can act as mood stabilizers in some cases [6].

For these reasons, precise monitoring regarding their levels in the biological fluids is mandatory to guarantee therapeutic effectiveness and to diminish side effects. Moreover, the use of these drugs combined with other prescription medications may cause toxic problems, and, in the last few decades, their use for recreational purposes has concerned health organizations around the world [7,8]. Therefore, the development of analytical methods to determine the residues of ADs in human samples is very important in areas such as medicine and forensics. Several analytical techniques can be employed for these purposes, such as gas and liquid chromatography, capillary electrophoresis, and spectrophotometry, among others [1,9–11]. Considering the current demand for methods to be more sensitive and selective, the coupling between liquid chromatography and mass spectrometry becomes a useful way to improve the analytical results even more. Nonetheless, given the lower concentration levels of ADs and the complexity of biological samples, high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) is not enough to achieve such results; hence, a previous step called sample preparation is often required [12].

Generally, these procedures are focused on removing interferents from the matrix, and on extracting/pre-concentrating target analytes [13]. The most common sample preparation techniques are conventional solid-phase extraction (SPE) and liquid–liquid extraction (LLE), which were proposed more than 50 years ago. These traditional approaches have many disadvantages, including laborious and time-consuming steps, large amounts of sample and solvent requirements, and disposable hardware (especially SPE), among other restrictions [14]. In order to overcome these shortcomings, modern sample preparation techniques based on the principles of the precursor solid-phase microextraction (SPME) began to appear in the early 1990s [14]. Consequently, the current trends are mainly based on miniaturization, automation, and high-throughput analysis, which point out automated methods that integrate sample preparation and HPLC-MS/MS as a suitable combination [15].

In this context, herein we propose an automated multidimensional method employing two columns, where the first one is specifically used for sample preparation and the second performs the chromatographic separation followed by tandem mass spectrometry detection. It is noteworthy that our capillary extraction column was packed with a lab-made extractive phase consisting of graphene oxide supported on an aminopropyl silica surface (GO-Sil). This column is much cheaper than the commercially available ones and has a reported excellent performance and robustness [16]. Additionally, the capillary dimensions of the extraction column (200-mm length and 508-μm i.d.) allow for economies in quantities of solvent, sample, and extractive phase, which are under the principles of green chemistry, which is so important nowadays. Its excellent extractive performance is attributed mainly to the high surface area of the graphene oxide, together with the delocalized π-electron system, which suggests a good affinity with molecules containing aromatic rings like the pharmaceutical drugs herein analyzed. In this case, the π-π interaction is the main interaction mechanism responsible for selective extraction. Aiming to evaluate the system performance, we selected four antidepressant drugs (ADs) as chemical probes, namely carbamazepine, citalopram, clomipramine, and desipramine, and one anticonvulsant AC, namely sertraline.

2. Results and Discussion

2.1. Method Enhancement

2.1.1. Chromatographic Separation

During the early stages of this work, experiments were performed that aimed to optimize the analytes' chromatographic separation. Figure 1 illustrates the main results obtained by varying the mobile phase composition. As can be seen, our first attempt using isocratic mode (Figure 2E) reported a lower chromatographic resolution. However, as we were evaluating different combinations of mobile phases (D → B), improvements on the resolution were achieved. Finally, Figure 2A shows the best conditions regarding the separation of the five target analytes. In this case, an elution gradient employing ultrapure water and acetonitrile, both acidified with 0.2% formic acid, reported the best results. These gains in the resolution using the elution gradient might be due to the similarities in the analytes' chemical structure, which required subtle variations on the mobile phase elution strength, in order to separate one from another compound. Additionally, as our mass spectrometer operated in electrospray (ESI) positive mode, which is known to suffer from a matrix effect that might lead to ion suppression or enhancement, the acidification of the mobile phases could aid the analytes to be more ionizable, increasing the analytical signal.

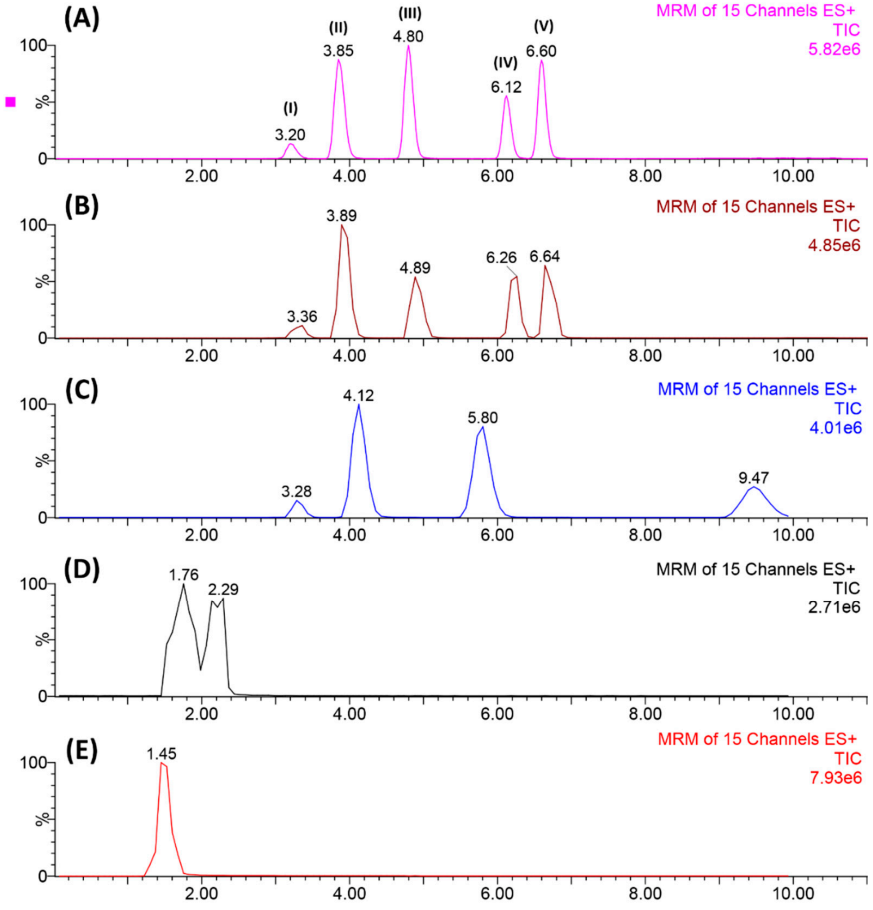

Figure 1. Representation of the chromatographic separation enhancement from E → A: (**A**) best condition applying elution gradient (H_2O/ACN + 0.2% formic acid), (**B**) satisfactory separation but the dwell-time was not adjusted, (**C**–**E**) mobile phase without acidification and mobile flow rate not adjusted. Elution order: (I) carbamazepine, (II) citalopram, (III) desipramine, (IV) sertraline, and (V) clomipramine.

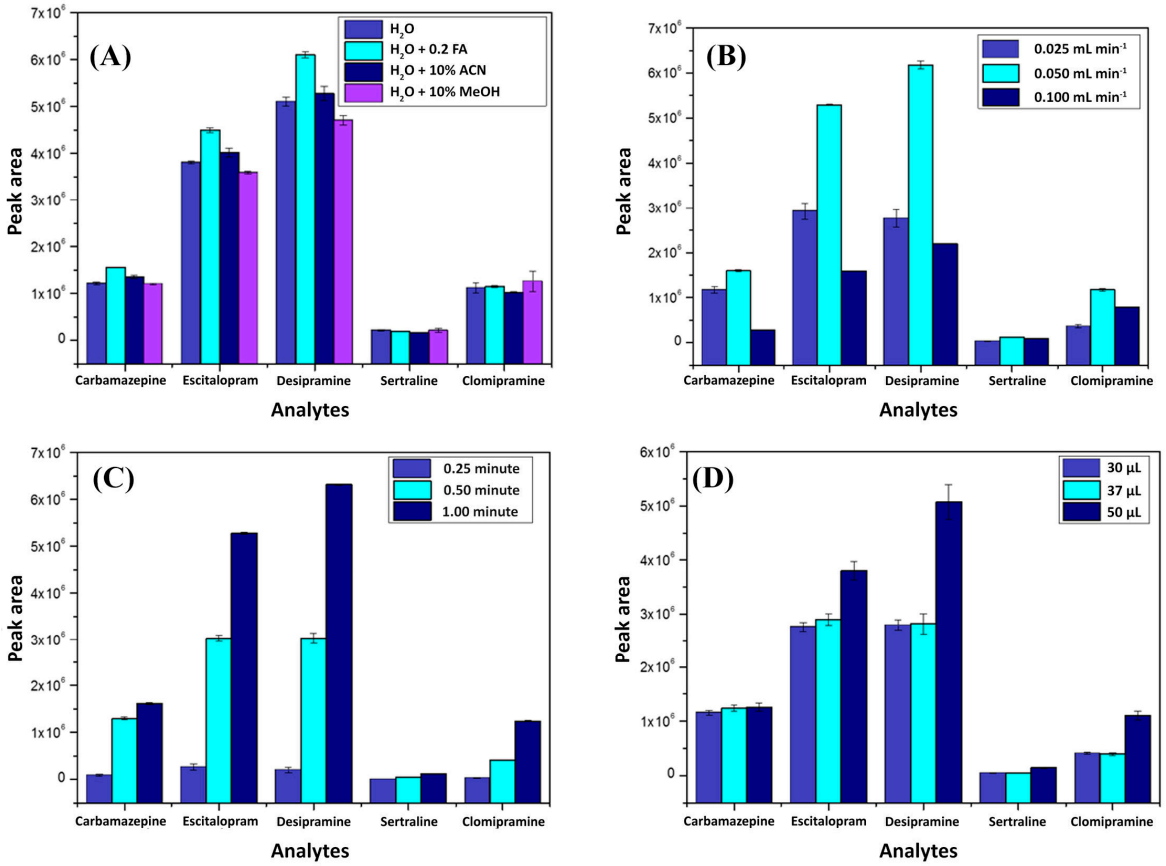

Figure 2. Method enhancement parameters obtained by univariate experiments considering the following parameters: (**A**) loading phase, (**B**) loading flow, (**C**) loading time, and (**D**) injection volume.

2.1.2. Multidimensional Automated Procedure

In the sequence, a batch of experiments aiming to achieve an ideal analytical condition for all other influential parameters was conducted. Figure 2 depicts the results obtained for each investigated variable through univariate experiments by considering the area under the chromatographic peak as the response variable. All parameters were studied using triplicate injections. It is important to emphasize that when a parameter was not being evaluated, it was kept in the following standard analytical conditions: loading phase, H_2O; loading flow, 0.05 mL min^{-1}; loading time, 0.5 min; and injection volume, 50 µL.

First, the best composition of the loading mobile phase was evaluated. As can be seen in Figure 2A, the best extraction performance was reported using ultrapure water with formic acid (0.2%). This behavior can be explained due to the lower pH (≈3.2) obtained when formic acid (FA) is used, which can favor the interactions between the analytes and the sorbent phase. In this pH range, most molecules are charged and consequently have more affinity for the polar oxygen groups present on the graphene oxide surface [17,18]. Apart from that, using methanol and acetonitrile in the loading phase is expected to produce a higher elution strength, which makes the sorption of the analytes in the extraction column difficult; they pass directly through it, going to waste. Sequentially, the loading flow was investigated using univariate experiments with three different values: 0.025, 0.050, and 0.100 mL min^{-1}. Figure 2B depicts the results using 0.050 mL min^{-1}, reporting the best performance for the majority of the analytes. As can be seen, the intermediate value had the best performance when comparing it with 0.025 mL min^{-1}. This fact can be explained by considering that the lower flow rate value might not be enough to ensure that all analytes had passed through the extraction column at the time the valve was switched to the elution position, causing analytes not to be sorbed into the extraction columns. Conversely, when considering 0.05 mL min^{-1}, a higher flow hampered the analytes since they were

desorbed due to a more diluted condition or due to the higher force that pushed them inside the extraction column, resulting in lower extraction performance.

After determining the best characteristic of the loading phase composition and flow rate, the other parameters were studied. Figure 2C shows that by increasing the loading time in which the analytes were pumped inside the extraction column, a better extraction performance was achieved. This effect is reasonable since a greater loading time implies more interaction between the analytes and the sorbent phase. Therefore, 1 min was fixed as the selected loading time. Furthermore, the volume of the sample injected into the system was varied to include these three values: 30, 37, and 50 µL. As can be expected, the larger sample volume (50 µL) resulted in better extraction performance since this is directly proportional to the number of analytes available to interact with the extraction column. For this reason, 50 µL was fixed as the injection volume.

2.2. Figures of Merit

The figures of merit herein evaluated were determined according to the International Conference on Harmonization (ICH) guidelines [19].

First, the method selectivity was evaluated by analyzing a sample obtained from a pool formed by blank urines, collected from consenting volunteers, which were compared with those obtained from the same sample after being spiked with a mixture containing the target analytes. As no peaks were observed in the multiple reaction monitoring (MRM) ion transition for each compound, the method was considered as being selective (Figure 3). In the sequence, the limits of detection and quantification were determined via successive injections of spiked urine samples until observing a signal to noise ratio near to 3:1 and 10:1, for LOD and LOQ, respectively. Therefore, the limits of detection ranged from 0.01–2.0 µg L^{-1} and the limits of quantification from 0.5–20 µg L^{-1}. The method linearity was determined considering six different concentration levels, with each one being evaluated on triplicate injections. The linear interval for each analyte was: 1–200 µg L^{-1} for carbamazepine, citalopram, and desipramine, and 20–200 µg L^{-1} for sertraline and clomipramine. As shown in Table 1, the method presented good linearity with correlation coefficients (R^2) higher than 0.985.

Table 1. Method linearity characteristics and its limits of detection (LOD) and quantification (LOQ).

Analytes	Linear Equation	R^2	LOD (µg L^{-1})	LOQ (µg L^{-1})
Carbamazepine	y = 1681.7 + 2835.6x	0.999	0.01	0.5
Citalopram	y = −2542.9 + 6904.3x	0.997	0.04	0.5
Clomipramine	y = −14200.5 + 1167.5x	0.985	0.5	25
Desipramine	y = −1593.3 + 7048.4x	0.994	0.01	0.5
Sertraline	y = −1614.1 + 128.4x	0.985	2.0	20

Afterward, the method accuracy, precision, and enrichment factor were all determined by considering three concentration levels (low, medium, and high) evaluated using injection triplicates. As can be seen in Table 2, the method presented good accuracy, with the values being between 83.2 and 117.6, which is considered acceptable according to the ICH guidelines (80–120%). Sequentially, the intra-day precision was determined on the same day of those other validation parameters, while the inter-day precision was evaluated on a subsequent day. Table 2 shows the obtained relative standard deviation (RSD) values, ranging from 1.4–13.6%, which were also per the ICH guidelines. Finally, as our analytical method was based on a multidimensional automated approach, it was essential to study the enrichment factor obtained by pushing the analytes through the extraction column before chromatographic analysis. In general, an increase in the analytical signal is expected when a pre-concentration step is carried out. Table 2 shows the obtained results for it, highlighting a good enrichment factor for all target compounds providing a signal enhancement varying from 4.7 to 59.4 when compared to the direct injection approach. Therefore, these results support the choice for a multidimensional and automated method to perform sample preparation and determination of

pharmaceutical drugs in complex samples as urine. Furthermore, it must be underscored that the exceptional robustness of the in-house prepared extractive phase GO-Sil packed into the capillary extraction column was used for more than 250 urine injections without losing its original performance.

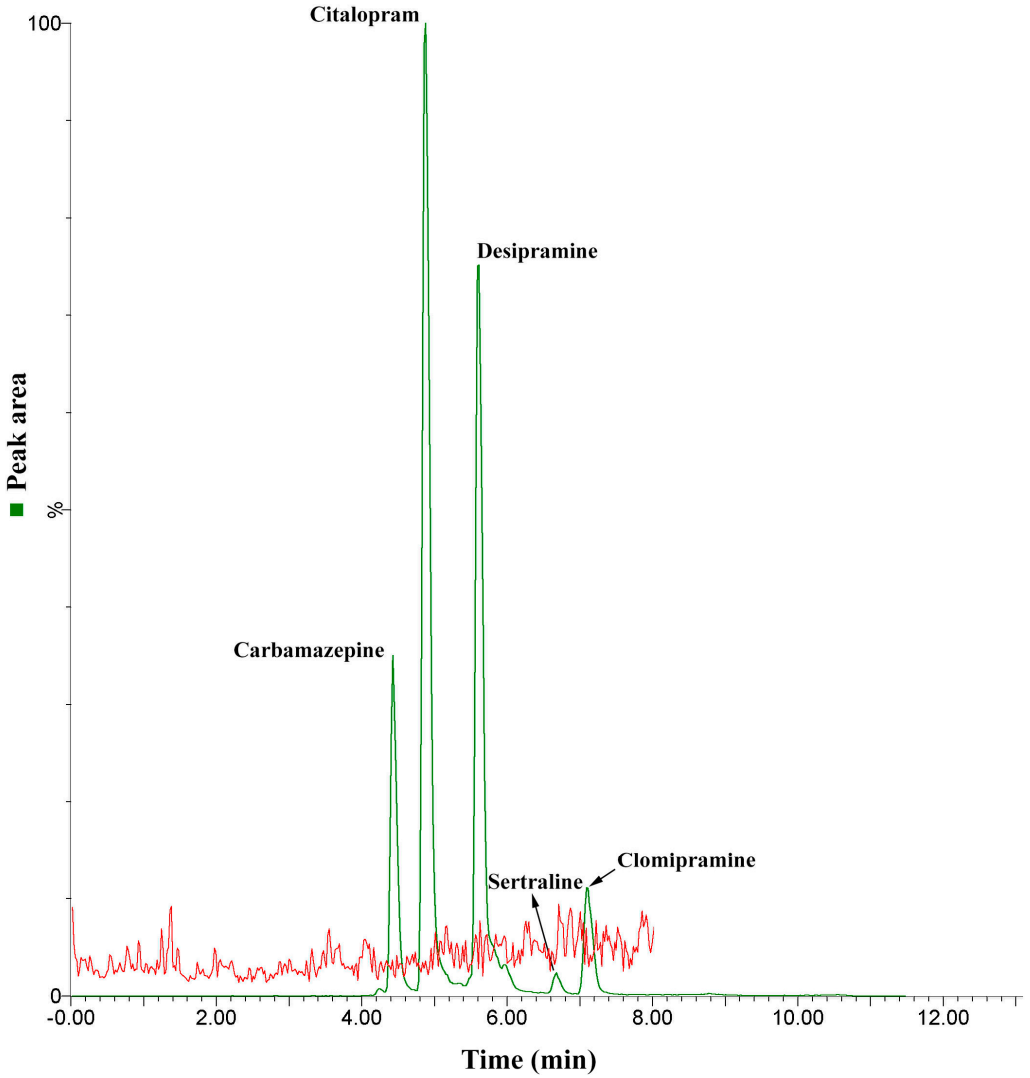

Figure 3. Chromatograms obtained by comparing a 100 μg L^{-1} spiked urine sample with an unspiked blank one in order to verify the selectivity of the proposed analytical method.

Table 2. Additional figures of merit including the method enrichment factor, accuracy, and precision. RSD: Relative Standard Deviation.

Analytes	Enrichment Factor			Accuracy (%)			Precision (% RSD)					
							Intra-Day			Inter-Day		
	L	M	H	L	M	H	L	M	H	L	M	H
Carbamazepine	4.7	5.3	5.1	83.2	95.8	98.8	12.3	2.3	3.6	13.6	2.1	1.4
Citalopram	6.8	7.6	7.0	125.3	89.7	99.1	2.0	1.9	3.2	6.8	2.9	5.5
Clomipramine	17.3	18.1	17.4	98.7	117.6	102.4	5.2	6.1	4.0	9.2	3.2	4.8
Desipramine	18.2	16.4	15.0	105.8	114.8	102.3	6.9	3.5	11.8	11.2	4.5	2.4
Sertraline	21.2	59.4	13.1	98.7	117.6	102.4	12.8	4.5	6.5	8.1	4.1	1.4

2.3. Overall Method Performance

When looking to compare our obtained results with other published papers in the literature, we can underscore some advantages, as well as limitations. First, as our paper presents the use of a synthesized graphene-based sorbent packed into a capillary extraction column, its robustness is noteworthy, as just described, given that it was applied to more than 250 injections. As examples, other recent works pinpoint their lab-made extractive hardware being re-used five and seventy times without losing its efficiency, respectively [20,21]. Likewise, our developed extraction column surpasses by far the commercially available SPE cartridges, which can be ideally used only once. Furthermore, considering our automated multidimensional approach using two columns, the system required only 50 µL of urine with reduced reagent consumption and consequent waste generation [4,22,23]. The lack of steps demanding operator intervention due to the automation can lead to remarkable gains in analysis time (≈8 min), while it also diminishes analytical errors resulting from sample handling [4,23]. Another great quality of it is the capacity to perform the analysis of antidepressants and antiepileptics in undiluted and unprecipitated urine. As highlighted by Cai et al. [24], several methods developed to analyze ADs in urine have been carried out by considering a dilution step due to the high complexity of the samples. Finally, the LODs and LOQs of the proposed approach are in a similar range with most published works; although some methods can be more sensitive, our results provide a suitable range for its main goal [25,26]. From the authors' point of view, the major limitation of this proposed methodology is in its system configuration, since it demands an auxiliary pump and a switching valve, which might consist of a restriction for some laboratories.

2.4. Method Application

Separately from the pool of blank samples used during the development step, the analytical methodology herein described was applied to the analysis of other urine samples collected from consenting volunteers. From ten samples analyzed for the target compounds, one presented traces of citalopram in a concentration estimated to be in the order of 150 µg L^{-1}. This result is probably due to the considerably widespread use of citalopram (SSRI) at present since it has a broad spectrum of action, treating not only depression, but also obsessive-compulsive disorder, panic disorder, and social phobia [26]. Figure 4 shows the results comparing the referred sample (red line) with a blank one fortified with the analytes in a concentration range that resulted in an area similar to that obtained for the unknown sample. As can be seen, the signals for citalopram were in similar magnitude; the MRM transitions, the relative ratio between the monitored ions, and similarity of the retention time verifies the observed results.

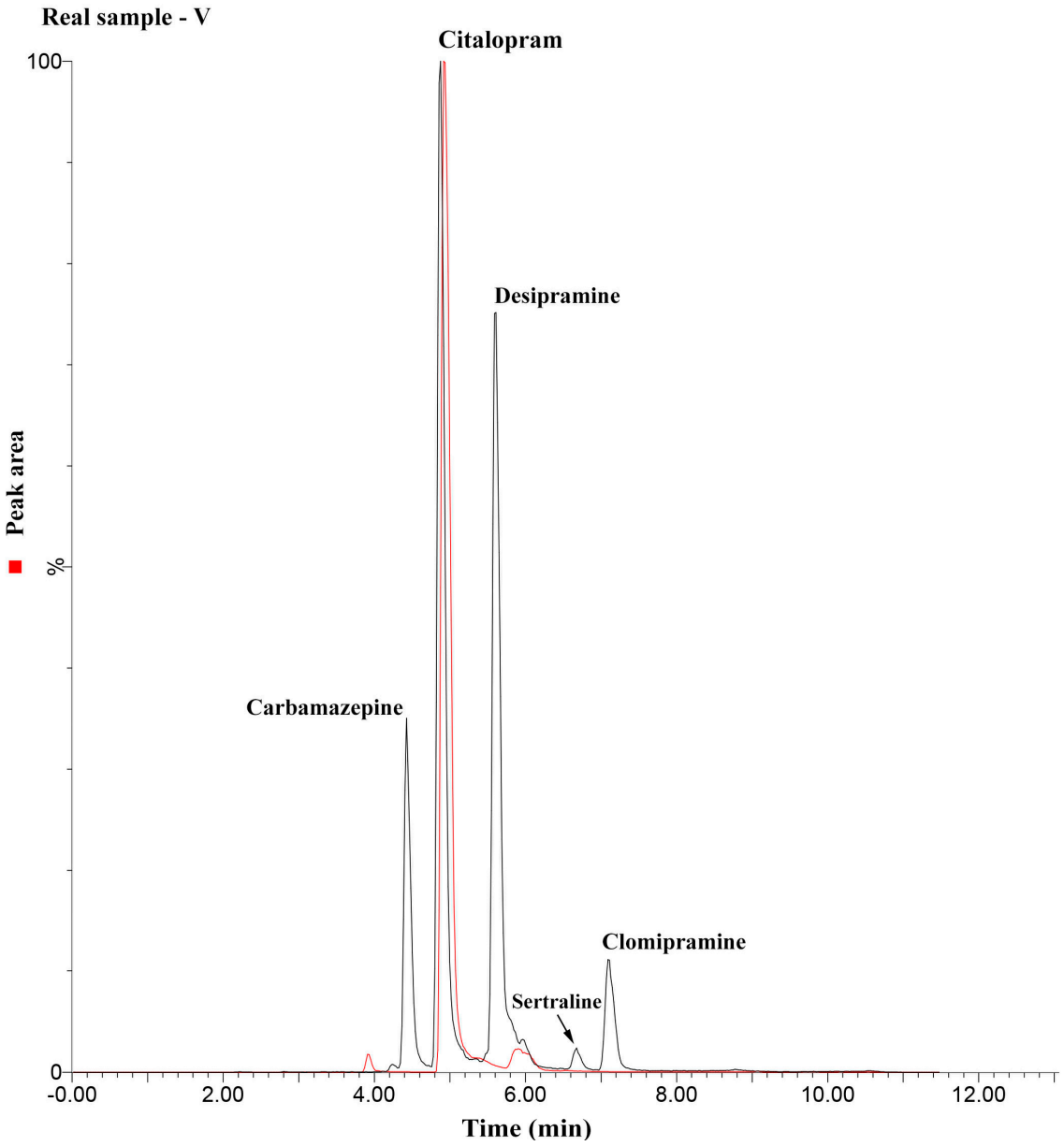

Figure 4. Chromatogram comparing a 150 μg L^{-1} spiked urine with a sample from a volunteer (red line) in which traces of citalopram were found.

3. Experimental

3.1. Reagents and Standard Solutions

High purity (99%) analytical standards of carbamazepine, citalopram, clomipramine, desipramine, and sertraline were all acquired from Fluka Analytical (St Louis, MO, USA). The analytes' stock solutions were all prepared in methanol at a concentration of 1000 mg L^{-1}, and subsequently diluted to 100 mg L^{-1}. The work solutions were prepared from the stock ones in a proper concentration by considering the goal of each experiment to be performed. It should be highlighted that all standard solutions were temperature-controlled (−30 °C) inside the amber flasks.

The HPLC grade solvents acetonitrile (ACN) and methanol (MeOH) were purchased from TEDIA (Farfield, OH, USA) and the ultrapure water was produced at our laboratory using a MILLI-Q purification system from Millipore (Burlington, MA, USA). Furthermore, MS grade formic acid (FA) acquired from Sigma-Aldrich (St Louis, MO, USA) was used to acidify the chromatographic mobile

phases. The GO-Sil extractive phase was synthesized and had already been used in previous works published by our research group [16,18].

3.2. Extraction Column Preparation

As our extraction column possessed capillary physical dimensions (200-mm length and 508-µm i.d.), our best choice to produce it was using the slurry packing procedure. In short, this consisted of using a high-pressure pump to push a suspension containing the stationary phase inside the column tubing, similar to that utilized in the production of HPLC and U-HPLC analytical columns. Therefore, the slurry packing system mainly consisted of a packing solvent, a slurry solvent to dissolve the stationary phase, a reservoir where the suspension was kept, and the column hardware often placed in the inferior part of the system.

In this work, a Haskell DSFH-300 hydropneumatic pump acquired from Haskel (Burbank, CA, USA) was employed as the pushing pump, while ultrapure water was used as the packing solvent. The suspension consisted of 10 mg of GO-Sil extractive phase dissolved in 700 µL of the slurry solvent (isopropanol/tetrahydrofuran; 6:1 *v*/*v*). The packing pressure was maintained at ≈600 bar during the procedure (≈60 min) in order to fill the column tubing. For more detailed information about the extraction column production, as well as for the GO-Sil extractive phase characterization assays (SEM and FTIR), please refer to a recent manuscript published by our research group [16].

3.3. Instrumentation

The analytical system was composed of an Acquity UPLC liquid chromatograph equipped with a binary solvent manager, and a sample manager coupled to a Xevo TQ S mass spectrometer using electrospray ionization, all from Waters (Milford, MA, USA). Moreover, a Shimadzu LC 10A*i* equipped with a degasser 10A from Shimadzu (Kyoto, JAP), and an electronically assisted switching valve from Supelco (St. Louis, MO, USA) were used to carry out the automated sample loading step, transferring the sample from its original vial to inside the first (extraction) column.

The chromatographic separations were achieved using a Poroshell 120 SB-C8 analytical column from Agilent (Santa Clara, CA, USA) (100 mm × 2.1 mm × 2.7 µm d_p) at a temperature of 40 °C. The mobile phase consisted of ultrapure water and acetonitrile (both acidified with 0.2% formic acid) at a flow rate of 0.20 mL min^{-1}, and the loading phase contained acidified ultrapure water (0.2% formic acid) at a flow rate of 0.05 mL min^{-1}.

The mass spectrometry parameters were optimized via direct infusion of each analyte in standard solutions at a concentration of 0.5 mg mL^{-1}, assisted by the IntelliStart optimization software (4.1) from Waters (USA). Under the optimized conditions, the detection method included a positive ESI, capillary voltage of 3.9 kV, source temperature of 150 °C, desolvation gas (N_2) temperature of 650 °C and flow of 1000 L h^{-1}, and collision gas (Ar) flow of 0.15 mL min^{-1}. In order to enhance the method selectivity, the MS/MS configuration operation in the multiple reaction monitoring (MRM) was chosen to be used. All the analytes' transitions used for identification/quantification, as well as its main detection parameters, can be found in Table 3.

3.4. Multidimensional Analytical Method

The multidimensional analytical method was composed of two columns (extraction and analytical) connected using the switching valve, which was responsible for steering the flow depending on the purpose. Figure 5 illustrates the configuration assembled to perform the automated analysis.

Before starting any analysis, the urine samples were simply filtered through a 0.22-µm cellulose membrane to avoid clogging the whole system.

During each analysis, the autosampler was responsible for controlling the chromatographic injection and the valve positions. This was done through a sequence of events scheduled in the software. First, the sample injection was performed with the valve set at the loading position (valve ports connected through the purple line; see Figure 5). Therefore, the LC 10A*i* auxiliary pump carried

the sample through the capillary extraction column, at a flow of 0.05 mL min^{-1}, in order to retain the analytes while the majority of interferents went to waste. Meanwhile, the HPLC binary solvent pump conditioned the analytical column with the initial composition of the elution gradient. After 1 min, the valve was switched to the eluting position (valve ports connected through the red dotted lines; see Figure 5). Thus, the chromatographic mobile phase was pumped inside the extraction column, at a flow rate of 0.2 mL min^{-1}, to desorb the analytes, shifting them to the analytical column and further to the mass spectrometer. In the sequence, the multidimensional system was washed and conditioned again to be ready for the next injection. Table 4 summarizes the main steps regarding the described analytical procedure.

Table 3. Analytes' multiple reaction monitoring (MRM) precursor and product ions and its main detection parameters.

Analyte	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Cone Voltage (V)	Collision Energy (V)	Dwell Time (ms)
Carbamazepine	253	152	24	42	0.075
		167	24	44	0.075
		180	24	32	0.075
Desipramine	267	72	22	14	0.075
		193	22	42	0.075
		208	22	24	0.075
Sertraline	306	123	16	48	0.075
		159	16	30	0.075
		275	16	14	0.075
Clomipramine	315	58	24	30	0.075
		86	24	18	0.075
		227	24	42	0.075
Citalopram	325	109	32	30	0.075
		234	32	26	0.075
		262	32	20	0.075

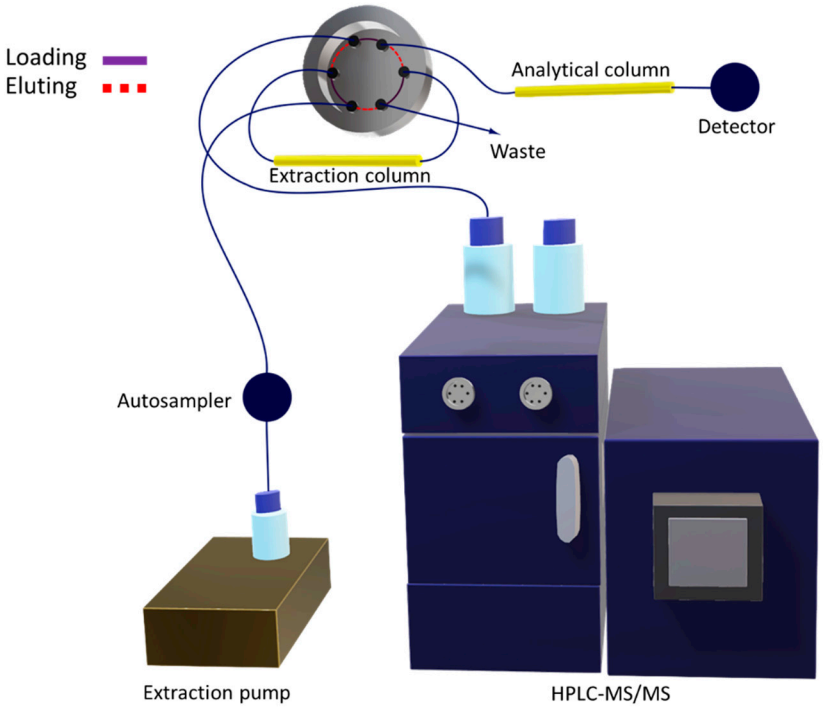

Figure 5. Illustrative drawing representing the multidimensional configuration, including the extraction column (first dimension) connected to the analytical column (second dimension) via a switching valve placed before the HPLC-MS/MS instrument.

Table 4. Analytical steps involved in the automated multidimensional extraction/determination of the analytes.

Event	Time (min)	Solvent Composition (Extraction Column)	Solvent Composition (Analytical Column)
Loading	0.00–1.00	H_2O + 0.2% FA	H_2O (A)/ACN (B) * (30%:70%)
Eluting	1.00–3.00	H_2O (A)/ACN (B) * (30%:70% → 35%:65%)	H_2O (A)/ACN (B) * (30%:70% → 35%:65%)
	3.00–6.00	H_2O (A)/ACN (B) * (35%:65% → 40:60%)	H_2O (A)/ACN (B) * (35%:65% → 40:60%)
	5.00–6.00	H_2O (A)/ACN (B) * (40%:60%)	H_2O (A)/ACN (B) * (40%:60%)
Cleaning	6.00–7.00	H_2O (A)/ACN (B) * (40%:60% → 50%:50%)	H_2O (A)/ACN (B) * (40%:60% → 50%:50%)
	7.00–7.66	H_2O (A)/ACN (B) * (50%:50% → 10%:90%)	H_2O (A)/ACN (B) * (50%:50% → 10%:90%)
	7.66–8.60	H_2O + 0.2% FA	H_2O (A)/ACN (B) * (10%:90%)
Conditioning	8.60–11.50	H_2O + 0.2% FA	H_2O (A)/ACN (B) * (30%:70%)

* Both mobile phases acidified with 0.2% formic acid.

3.5. Method Enhancement

In order to achieve a satisfactory sample clean-up (eliminating the majority of endogenous urine compounds) combined with a good chromatographic resolution and MS detectability, a batch of univariate experiments were performed. Therefore, the influences of the elution gradient, injection volume, loading flow, loading time, and loading phase composition were all investigated. These experiments were performed via injection of triplicates of blank urine samples spiked at 100 μg L^{-1}.

First, the chromatographic separation was studied by changing the mobile phase solvent composition as well as the pH. Three solvents were tested (MeOH, ACN, and H_2O), and formic acid was added to modify the pH. Sequentially, four parameters directly related to the extraction column were considered: (i) the loading phase composition: H_2O, H_2O (0.2% FA), H_2O/ACN, and H_2O/MeOH; (ii) the loading flow: 0.025, 0.05, and 0.1 mL min^{-1}; (iii) the loading time: 0.25, 0.5, and 1.0 min; and (iv) the injection volume: 30, 37, and 50 μL. The parameters and its evaluation conditions were chosen by considering our experience with such types of multidimensional configurations [16,25].

3.6. Figures of Merit

Afterward, a systematic study regarding the analytical figures of merit commonly considered for validation procedures was performed according to international guidelines [19]. Therefore, individual experiments were carried out by contemplating six different variables: linearity, accuracy, precision, limits of quantification and detection, pre-concentration factor, and selectivity. It is essential to highlight that the pool of urine samples used in this step was collected from consenting volunteers and previously tested to verify the absence of the analytes such that they could be considered blank samples that would not interfere with the spiked concentration levels.

The method linearity was studied through the matrix-matched calibration method by spiking urine samples at six different concentration levels: 1, 25, 50, 75, 100, 150, and 200 μg L^{-1} for carbamazepine, citalopram, and desipramine; 20, 40, 80, 100, 150, and 200 μg L^{-1} for sertraline; and 25, 50, 75, 100, 150, and 200 μg L^{-1} for clomipramine. Each concentration level was evaluated using triplicate extractions with the automated multidimensional approach. The limits of detection (LODs) and quantification (LOQs) were determined via comparison of the signal to noise ratio in blank samples and those spiked at known concentration levels. Determination of the LOD was chosen at a signal to noise ratio of 3:1, while for LOQ, a signal to noise ratio of 10:1 was considered. The selectivity was investigated via comparing the pool of "blank" urine with those spiked at known concentration levels to verify the absence of interferent signal on the compounds' retention time or MRM transitions. First, the accuracy was determined in three different concentrations via measuring the actual value obtained from the linearity equation (C_r) and comparing it with the theoretical concentration value of each spiking level on the analytical curve (C_t). Sequentially, precision was studied in terms of the relative standard deviation (RSD %) at three different levels of concentration, repeated in two consecutive days (intra- and inter-day assays). Finally, the pre-concentration factor (or enrichment factor) was evaluated

by performing several injections of spiked urine samples via employing the multidimensional system (passing through the extraction column), which were compared with those similarly spiked and were directly injected into the analytical column.

3.7. Method Application

Urine samples used in this work were collected from consenting volunteers. Part of it was prior analyzed for the presence of the target drugs; in its absence, they formed a pool of samples used as "blank samples" during all stages of the study development. Additionally, the other samples not tested were used to verify the method's applicability after the determination of the figures of merit. All aliquots were only filtered through 0.22 μm cellulose membrane prior injection into the automated multidimensional system.

4. Conclusions

Herein an online automated analytical method based on multidimensional liquid chromatography coupled to tandem mass spectrometry was developed to extract and determine four antidepressants and one antiepileptic drug in human urine. The approach was based on the interconnection between two columns being the first accountable to perform the analytes' extraction (first dimension) while the second worked as a chromatographic analytical column (second dimension). Our capillary extraction column was packed with a synthesized graphene-based sorbent that exhibits excellent extraction performance and robustness being used for more than 250 injections. The method takes roughly 8 min and used 50 μL of undiluted and unprecipitated urine, demanding only a simple filtration step before injection into the multidimensional system. Besides, essential parameters were investigated to find out an ideal analytical condition allowing the determination of some validation figures of merit: linearity, accuracy, precision, selectivity, enrichment factor, LOD, and LOQ. Afterward, all ten urine samples collected from the consenting individuals in the study were analyzed to verify the proposed procedure. The presence of citalopram residues at a concentration level of around 150 μg L^{-1} was found in one of the ten analyzed samples. Therefore, based on the results obtained and reported in this manuscript, the proposed multidimensional analytical method was revealed to be a promising way to perform rapid and effective trace analysis of antidepressant and antiepileptic drugs in urine that easily adaptable to work with other biological complex matrices, such as saliva and plasma, among others.

Author Contributions: E.V.S.M. wrote this version of the manuscript. E.V.S.M. and A.L.d.T. performed the synthesis of the extractive phase/produced the extraction column, supervised the method enhancement, and performed the validation/urine applications. A.L.d.T. processed the data. J.d.S.A. performed the method enhancement and wrote a Portuguese version of this manuscript. F.M.L. conceptualized and supervised the whole research project, provided all required facilities, and reviewed/edited this manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACN, acetonitrile; ADs, antidepressants; ESI, electrospray ionization; FA, formic acid; GO-Sil, graphene oxide supported onto aminosílica; HPLC, High-performance liquid chromatography; i.d., inner diameter; ICH, International Conference on Harmonization; LC, liquid chromatography; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MRM, multiple reaction monitoring; MS, mass spectrometry; RSD, relative standard deviation; SPE, solid-phase extraction; SPME, solid-phase microextraction; SSRI, selective serotonin reuptake inhibitor; UPLC, ultra-performance liquid chromatography.

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Sample Availability: Not available.

CAPÍTULO 7

Evaluation of Two Fully Automated Setups for Mycotoxin Analysis Based on Online Extraction-Liquid Chromatography-Tandem Mass Spectrometry.
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Article

Evaluation of Two Fully Automated Setups for Mycotoxin Analysis Based on Online Extraction-Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: Mycotoxins are secondary metabolites of fungi species widely known for their potentially toxic effects on human health. Considering their frequent presence in crops and their processed food, monitoring them on food-based matrices is now an important topic. Within such a context, the sample preparation step is usually mandatory before the chromatographic analysis, due to the complexity of matrices such as nuts, cereals, beverages, and others. For these reasons, we herein present the evaluation of two greener setups, based on the automation and miniaturization of the sample preparation step for mycotoxin analysis in different beverages. Firstly, we describe an analytical method based on a multidimensional assembly, coupling a lab-made microextraction column (508 µm i.d. × 100 mm) to a UPLC–MS/MS for the analysis of ochratoxin A in beverages. This configuration used a synthesized sorbent phase containing C18-functionalized graphene–silica particles, which exhibited excellent extraction performance, as well as being reusable and cheaper than commercially available extractive phases. Sequentially, a second setup, based on a multidimensional capillary LC coupled to MS/MS, was assessed for the same purpose. In this case, a graphene oxide-based capillary extraction column (254 µm i.d. × 200 mm) was used as the first dimension, while a C18 analytical capillary column performed the mycotoxin separation in beverages. Although this second one has similarities with the first, we focused mainly on the benefits related to the link between a miniaturized/automated sample preparation device with a capillary LC–MS/MS system, which made our analysis greener. Additionally, the chromatographic efficiency could even be enhanced.

Keywords: mycotoxins; liquid chromatography; sample preparation; automation; miniaturization

1. Introduction

Mycotoxins are toxic secondary metabolites produced by some filamentous fungi and whose presence has been detected mainly in agricultural products and processed food [1,2]. A recent review published by Eskola et al. [3] estimated that more than 60% of all crops around the world contain mycotoxins, and several classes can simultaneously be found in food. Contamination can happen at any stage of cultivation, harvesting, processing, or storage, when environmental conditions are favorable for the proliferation of these fungi, especially humidity and temperature [3]. Chemical and environmental factors, such as water content, pH, available nutrients, substrate, and the physical integrity of the plant and grain, also affect the growth of fungi and the mycotoxins [4]. Food contamination by fungi is an important issue in food safety, since the compounds produced by these microorganisms have severe consequences for human and animal health [3]. Mycotoxins present in food are usually produced by fungi of the genera *Aspergillus*, *Fusarium*, and *Penicillium*. Among the most relevant groups, with the highest occurrence and health risks, are aflatoxins (aflatoxin B_1, AFB_1), ochratoxins (ochratoxin A, OTA),

trichothecenes (deoxynivalenol, DON), fumonisins (fumonisin B_1, FB_1), and zearalenone (ZEA) [5]. The International Cancer Research Agency (IARC) classified the mycotoxins depending on their toxic effects: group 1 as proven carcinogen agents, group 2A as probable carcinogen agents, and group 2B as possible carcinogen agents to humans [6].

Aflatoxins are the most relevant and studied group, which comprises 20 different metabolites, among them: aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), M_1 (AFM1), and M_2 (AFM_2) [7]. In general, aflatoxins are extremely toxic, possessing carcinogenic metabolites (classified in group 1 by the IARC [6]) being also associated with hepatocellular carcinoma (HCC) or liver cancer. Aflatoxins are commonly reported in several types of cereals, nuts, and spices, as well as their metabolites (AFMs), which can be found in milk and dairy products [8,9]. Aflatoxins show a high resistance to sterilization, pasteurization, and thermal food treatments, being of the utmost importance for preventing food contamination, especially with AFB_1 [7].

In the same way, ochratoxins A, B, and C are produced by several species of the *Aspergillus* genus in tropical climates, as well as by the *Penicillium* genus in temperate climates. Among them, ochratoxin A (OTA) is the most toxic and frequently reported in foodstuffs. OTA has been found in a wide variety of foods, the main sources being cereals, wine, and coffee [10,11]. Usually, this mycotoxin is stable at high temperatures and in acidic environments [12]. Their most notable adverse effect is nephrotoxicity, associated with Balkan endemic nephropathy (BEN), which causes tubular degeneration and interstitial fibrosis. Additionally, OTA is a teratogenic and carcinogenic agent, classified by IARC in group 2B [7].

Zearalenone (ZEA), a metabolite from the *Fusarium* genus, and is currently classified as a potential estrogenic agent, due to its ability to bind to estrogen receptors in animals, causing hormonal deregulations leading to atypical fetal development in mammals and infertility [13]. Other effects include hepatic damages and renal lesions in rodents, since they are often peeking in farm warehouses where cereals are stored and, also, a decrease in cows' milk production due to its toxicity affecting the mammary glands [14]. Generally, ZEA can be found in rice, maize, nuts, and other foodstuffs, which show it to be a relevant chemical compound for analysis [14,15].

The analysis of mycotoxins can be done by qualitative methods such as thin-layer chromatography (TLC) and enzyme-linked immunosorbent assays (ELISA), or by quantitative ones, such as HPLC coupled to a fluorescence detector (FLD) or tandem mass spectrometry (MS/MS) [16]. The use of LC–MS/MS allows the unambiguous confirmation of mycotoxins and their metabolites by their mass spectra information. Nowadays, this is one of the most critical techniques that provides much information, allowing for the simultaneous detection of multi-present mycotoxins and emerging mycotoxins or masked metabolites [5]. However, before the instrumental analysis of mycotoxins can be carried out successfully, a previous sample preparation step is usually mandatory [17]. For this reason, mycotoxins are extracted from the food matrix and added to a more appropriate media or solvent [18]. After that, the extract is subjected to a cleaning step, usually employing liquid–liquid extraction (LLE) or solid-phase extraction (SPE) cartridges, preferably packaged with an immunoaffinity sorbent, which are known as immunoaffinity columns (IACs). IACs have been one of the most relevant achievements and the preferred method for mycotoxin analysis, being a highly selective sorbent due to the use of antibodies bound to a support material [17,19]. Among the advantages of the IAC employment in mycotoxin analysis are the attainment of clean extracts and low detection limits (0.1–0.2 ng g^{-1}), selectivity, smooth operation, and less organic solvent consumption. However, it has some disadvantages, such as the low number of antibodies in the phase and the high dependence on the extraction conditions, such as the aqueous media, controlled pH, ionic strength, and extract concentration. Besides, IACs have a short half-life, as antibodies biodegrade and are incompatible with some organic solvents that denature them [19].

Although a satisfactory sample preparation can be obtained by conventional SPE and LLE techniques, they are often related to high levels of reagent consumption, waste generation, high cost, and sample/stationary phase requirements. Therefore, novel methods to analyze mycotoxins have emerged, focusing on the automation and miniaturization of the sample preparation procedure towards

the development of greener approaches [20]. In this case, techniques such as online SPE [21–25] in-tube SPME (solid-phase microextraction) [26], turbulent flow chromatography (TFC) [17,27,28], and customized procedures derived from them [17,29–31], arose as suitable tools to perform such analyses while benefits emerging from automation/miniaturization could be reached. In this context, downsizing the sample preparation represents a promising way to be in accordance with green chemistry principles, as it is related to reduced consumption of chemicals/samples, and a reduced generation of toxic waste. Additionally, applying automation concepts helps to overcome the problems frequently related to the offline conventional sample preparation approaches, such as laborious and time-consuming procedures, the manual handling of samples, and in some cases, poor accuracy or precision [17]. Recent trends for mycotoxin analysis seem to prioritize the miniaturization and automation of the sample preparation approach, allowing them to obtain high-throughput analytical methods [20].

For these reasons, we herein present the evaluation of two multidimensional LC systems based on the automation and miniaturization of the sample preparation steps for mycotoxin analysis in different food matrices. First, an analytical method, based on the coupling of a lab-made microextraction column (508 µm i.d. × 100 mm) to a UPLC–MS/MS, was employed for the analysis of OTA in beverages. This approach uses a synthesized sorbent phase containing C18-functionalized graphene–silica particles. Sequentially, a modern setup using a multidimensional capillary LC coupled to MS/MS was assessed for the same purpose. In this case, a graphene oxide-based capillary column (254 µm i.d. × 200 mm) was used as the first dimension, while a C18 analytical capillary column performed the mycotoxin separation in beverages. Although both setups have similarities, we focused mainly on the benefits related to the link between a miniaturized/automated sample preparation device and a capillary LC–MS/MS system. This one made our mycotoxin analysis greener, whereas the chromatographic efficiency could have been enhanced. Ultimately, this work aims to gather the analytical results and prospects for these two fully automated and miniaturized setups, supporting them as greener alternatives specially developed to extract and analyze mycotoxins from complex samples such as wine, beer, and coffee.

2. Results and Discussion

2.1. Setup 1: Ochratoxin A Analysis by Packed Microextraction Column Online Coupled to UPLC–MS/MS

2.1.1. Chromatographic Conditions for the Analysis of Ochratoxin A

Initially, the microextraction column was online-coupled to the UPLC–MS/MS, using both loading and mobile phases acidified with 0.1% of formic acid to keep the OTA protonated and favoring its ionization. For the OTA analysis, a step-gradient elution program (Figure 1, see blue line) was used, which allowed the performance of the following steps: (1) the conditioning of the analytical column and extraction of OTA in the microcolumn, (2) the elution and determination of OTA, (3) clean-up, and (4) the conditioning of both columns. It is noteworthy that the selected gradients were optimized to reduce matrix interferents, to obtain the highest OTA analytical signal, and to avoid the carryover effects.

As previously observed by our research group, a mobile phase of H_2O:ACN (acetonitrile) 78:22 *v*/*v* was used from 0 to 7.63 min for conditioning the analytical column, as well as for loading the sample into the microextraction column [26]. In the second isocratic step, from 8.5 to 13.5 min (OTA elution), three ACN compositions (50%, 60%, and 70%) were tested. OTA's largest peak area was obtained with 60% ACN with an elution time of 11.2 min. Later, the clean-up step was verified by injecting an OTA standard solution followed by a blank of water. In this way, 95% of ACN for 6.30 min was enough to clean the analytical system. Finally, the chromatographic method returned to the initial gradient step for conditioning both columns for the next injection.

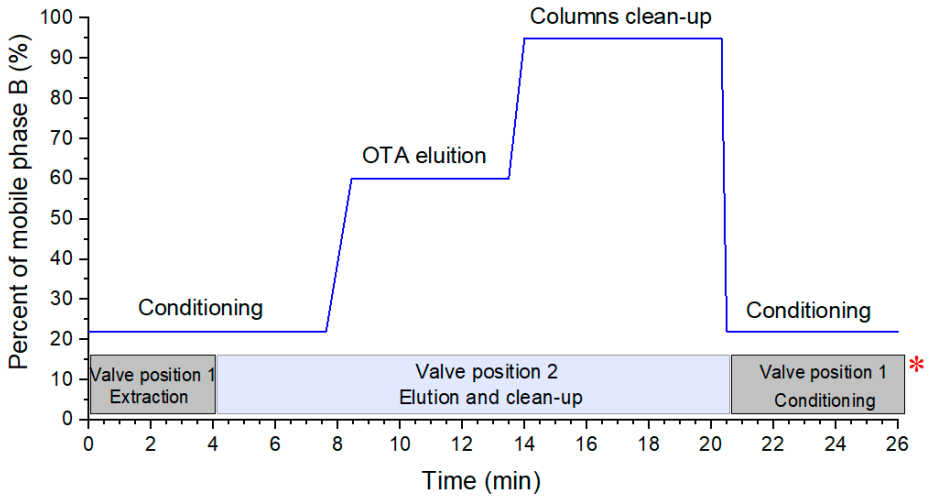

Figure 1. Step elution gradient for the analysis of OTA (Ochratoxin A) by an extraction microcolumn coupled to UPLC–MS/MS, indicating the mobile phase composition in the UPLC pumps, A: H_2O:0.1% formic acid and B: ACN (Acetonitrile): 0.1% formic acid (blue line). The box below the gradient indicates the configuration of the switching valve (*).

2.1.2. Extraction Valve Programming Events

The switching valve time events were programmed to determine the minimum extraction time and the percentage of ACN in the loading phase to retain the OTA in the microextraction column (see boxes in Figure 1). In these experiments, the loading phase (H_2O: ACN 78:22, *v*/*v*) flow rate was 0.100 mL min^{-1}. OTA standard solutions (20.0 µg L^{-1}) were consecutively injected, varying the time values from 6 to 1 min, aiming to determine the suitable loading time. These results (Figure S1) showed that by using a loading time from 1 to 4 min, the OTA area does not decrease significantly, unlike from 4 to 6 min, where a progressive reduction in the chromatographic peak was observed. For these reasons, 4 min was chosen as the loading time, because for more complex matrices, such as coffee, the use of longer loading times helped to eliminate more interfering analytes, avoiding the general contamination of the analytical system.

Sequentially, the loading phase composition was evaluated at four different levels: 5%, 10%, 15%, and 22% of ACN (flow rate, 0.100 mL min^{-1}; loading time, 4 min). The OTA peak areas obtained by using 5%, 10%, 15%, and 22% of ACN were: 9.91×10^4, 9.50×10^4, 9.29×10^4, and 7.98×10^4, respectively. Therefore, as the OTA area was not significantly affected by using 5% to 15% of ACN, the last was chosen as the loading phase composition, since fewer interferent signals were observed at this value.

Finally, the loading flow was evaluated at three levels: 0.100, 0.150, and 0.200 mL min^{-1}. The higher OTA response was achieved at a flow rate of 0.150 mL min^{-1}, possibly because at the lower flow of 0.100 mL min^{-1}, a satisfactory sorption interaction between the OTA and the sorbent phase was not provided, while at a higher flow of 0.200 mL min^{-1}, part of the OTA stayed in the mobile phase, going to waste. Thus, 0.150 mL min^{-1} was fixed as the optimal value.

Shortly, after all the tests, the optimal value for each parameter was: loading phase of H_2O:ACN (85:15, *v*/*v*), 4 min of loading time, and 0.150 mL/min loading flow rate. Within such a context, injections of instant coffee and wine spiked with OTA at 2.0 µg L^{-1} were performed to verify the system performance, and will be discussed in the following section.

2.1.3. Applicability of the Extraction Microcolumn Coupled to UPLC–MS/MS for the Analysis of OTA in Beverages

The proposed method was evaluated with samples of instant coffee, beer, and red wine, with OTA spiked at 2 µg L^{-1} to verify its applicability to them and the OTA response in each one. Table 1

shows the comparison of the signal-to-noise (S/N) ratio obtained from the different matrices at the same concentration, and the corresponding chromatograms are shown in Figure 2. The SRM (selected reaction monitoring) chromatograms (Figure 2), corresponding to the OTA's highly selective MS/MS transition, show that the method can be applied to OTA determination in instant coffee, beer, and wine. The OTA retention time signal was observed at 11.2 min, without any interferent peak being detected. The analysis of wine and beer was more straightforward than for coffee, as these samples were already in the liquid state, requiring just degassing and filtering before injection into the system. This reduced the sample preparation time compared to instant coffee, which needed to be dissolved in hot water, centrifuged, and filtered before the injection.

Table 1. Evaluation of the OTA *m*/*z* 404.1 → *m*/*z* 238.9 ion transition response as a function of the analyzed matrix.

Matrix	Spiked OTA (μg L^{-1})	S/N Ratio	LOQ (ng L^{-1})
Instant coffee	2.0	57.02	351
Malt beer	2.0	271.73	74
Lager beer	2.0	230.35	86
Red wine	2.0	294.72	68

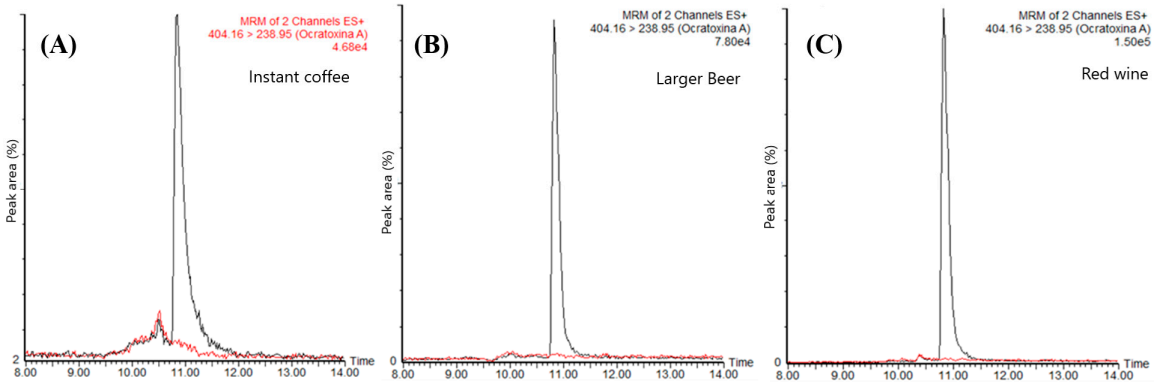

Figure 2. Superposition of the SRM (selected reaction monitoring) chromatograms of OTA ion transition (*m*/*z* 404.1 → *m*/*z* 238.9) from the analysis of (**A**) instant coffee, (**B**) beer, and (**C**) red wine samples fortified with OTA at 2.0 μg L^{-1} (black trace) and blank matrix sample (red trace).

A higher OTA response was obtained in beer and wine samples within such a context as in coffee since at the same spiked concentration, a higher S/N ratio was obtained in these two matrices (Table 1). For this reason, lower limits of quantification were achieved in beer (malt: 74 ng L^{-1}, lager: 86 ng L^{-1}) and wine (68 ng L^{-1}) when compared to instant coffee (351 ng L^{-1}). These values were determined considering a signal-to-noise ratio of 10:1 for each matrix. From the analyzed matrices, it can be inferred that the instant coffee sample was the most complex one, presenting more significant suppression of the OTA signal in the MS/MS detector (matrix effect). During the analyses, it was observed that the OTA response varied depending on the type of beer; a similar behavior was observed for different brands of instant coffee samples. This observed behavior suggests that the matrix effects (ME) should be carefully evaluated for each matrix before the routine use of the method to analyze OTA.

2.1.4. Method Overview

The proposed method, based on the first setup, was successfully implemented with potential applications in the analysis of OTA in wine, beer, and instant coffee. The employment of a non-selective sorbent for the OTA extraction proved to be advantageous, as this sorbent phase showed an excellent affinity for it, providing good retention with the initial extraction conditions evaluated. Additionally, it provided a good sensitivity for the OTA determination in beer, wine, and instant coffee samples, at spiked levels of 2 μg L^{-1}, and their determination below the legally allowed concentration

levels of OTA. Other advantages of this microextraction column were the reduced quantity of sorbent employed (20 mg) and its robustness, being re-used over 250 times without losing its original sorption characteristics.This sorbent was simple to synthesize from inexpensive raw materials (Mejía-Carmona et al. [32]) and easier to use compared to the immunoaffinity ones, which require special attention on the solvent composition and the use of buffer solutions [19]. Additionally, the high usability of this microextraction column shows the material's excellent stability and a possible reduction in the method costs.

Moreover, the full automation of the analytical method brings several perceived advantages. Among them, it should be highlighted that the excellent extraction was performed with suitable LOQs and the achievement of an effective clean-up, and the complete analysis was performed in a single analytical run with a considerable reduction in the sample preparation steps. As an example, for the wine and beer analysis, the samples were just degassed and filtrated before their direct injection into the discussed analytical system, thus reducing the sample handling and possible contamination. Besides, in this system, the solvent consumption and waste generation were minimized by operating both columns (extraction and analytical) at lower flow rates (<0.200 mL min^{-1}) than those utilized in most conventional systems.

2.2. Setup 2: Aflatoxins, Ochratoxin A, and Zearalenone Analysis by Multidimensional Capillary LC–MS/MS

2.2.1. Chromatographic Separation

Firstly, the liquid chromatographic separation between our two initial compounds (OTA and ZEA) was investigated by injections of a standard solution, containing both analytes, at a concentration level of 15 μg L^{-1}. Figure 3 depicts the obtained results (from A to D), through which the improvements obtained in the resolution, analysis time, as well as a reduction in peak band broadening, can be seen. In this case, the progress is attributed to the chromatographic parameters that were modified between these injections. The trace (A) in Figure 3 was conducted in isocratic mode using a mobile phase composition of ACN:H_2O (30:70, *v*/*v*), without being acidified. This isocratic elution mode can explain the higher elution time observed in this case. As it is known, electrospray ionization (ESI, in this case operating in positive mode) can be highly influenced by the pH or the presence of matrix interferents. Thus, the weak signal observed for ZEA (a small peak near 6 min) could be improved by decreasing the pH and favoring ionization, as observed in Figure 3B, where the ZEA signal was enhanced by the addition of 0.1% formic acid on both mobile phases. Additionally, the reduction in the retention times could be attributed to the use of the elution gradient instead of isocratic mode. The following step consisted of improving the resolution between the peaks, as well as fixing the subtle break on top of the OTA peak (Figure 3B,C). For this purpose, the mobile phase flow rate increased from 8 μL min^{-1} to 10 μL min^{-1} to diminish the peak width, while an elution gradient that was steeped less was applied to maintain the separation between the ZEA and OTA. Finally, the chromatogram showed in (D) was achieved by keeping the mobile phase flow rate at 10 μL min^{-1}. Additionally, the formic acid percentage was increased to 0.5%, and the elution gradient was slightly modified by enhancing the ACN percentage, while decreasing the gradient steepness curve (Figure S3, Supplementary Materials).

2.2.2. The Fully Automated Extraction Procedure

After that, with an excellent chromatographic separation already achieved, the other part of the multidimensional system was assessed. In this step, the main goal was to find an ideal condition for the sorption of ZEA and OTA into the capillary extraction column. Figure S4 (Supplementary Materials) shows the variation of the parameters' loading phase and loading time, commonly considered in automated sorption-based extraction procedures. As can be seen, the higher loading time (1 min) reported the best response for the total peak area of each analyte, which suggests a good sorption interaction between the target compounds and the GO-Sil extractive phase. As a result, 1 min was fixed as the loading time for the subsequent analysis. Moreover, the loading flow employed was

evaluated in three different values when the sample was pumped to the extraction column: 10, 25, and 50 μL min^{-1}. Figure S4 (Supplementary Materials) depicts the obtained results for each analyte's chromatographic peak area, indicating the intermediate value of 25 μL min^{-1} as the best among them. This trend indicates that a loading flow of 10 μL min^{-1} was probably insufficient to provide a satisfactory sorption interaction between the mycotoxins and the extractive phase. In contrast, 50 μL min^{-1} seemed to be too high, causing analytes to be more diluted when pumped inside the extraction column, which compromised the extraction performance. Additionally, for the loading solvent, an aqueous solution containing 22% ACN was chosen, taking into consideration previous experience from our research group [26].

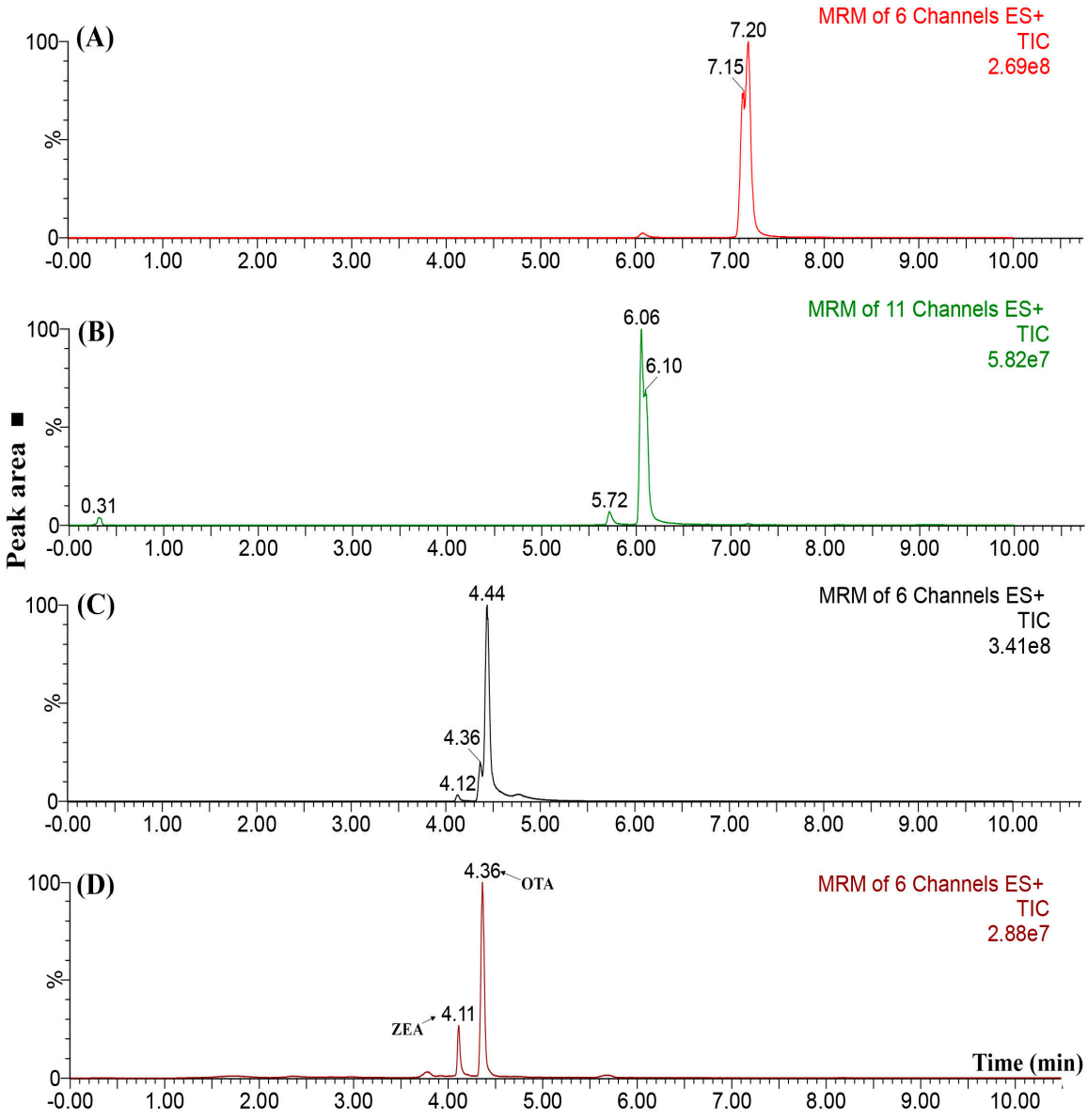

Figure 3. Chromatograms of a mixture containing OTA and ZEA (zearalenone) (15 μg L^{-1}) aiming to achieve a satisfactory liquid chromatography separation. (**A**) Isocratic elution employing ACN:H_2O (30:70, *v*/*v*); (**B**) acidification with 0.1% formic acid into mobile phases and the employment of an elution gradient; (**C**) enhancing the flow rate from 8 to 10 μL min^{-1} and (**D**) enhanced condition by fixing the flow rate at 10 μL min^{-1} and performing a less-steeped elution gradient.

2.2.3. Applicability of the Proposed Analytical Method in Wine Samples

After establishing the best analytical condition in system 2, three wine samples were tested for the presence of OTA and ZEA. These analyses were performed to evaluate the performance of the fully automated and miniaturized method in real samples at concentration levels usually considered in the literature.

The first test carried out consisted of injecting three spiked wine samples at 15 μg L^{-1} to verify the retention time (t_R) reproducibility. Figure S5 (Supplementary Materials) shows that the t_R for OTA (4.35 min) and ZEA (4.09 min) perfectly matched, considering the samples were just filtered by a cellulose membrane (0.22 μm) before the analysis. These results suggest that the method could have eliminated most of the matrix interferents that could have affected the analysis. Although a signal can be identified at 3.7 min, this is not a problem, as it does not overlap with any signal at the mycotoxins' retention time. In the sequence, blank wine samples were injected and compared with the chromatographic profiles previously acquired. The results obtained by employing the selected reaction monitoring (SRM) mode were compared by examining the total ion chromatogram (TIC) as well as each ion transition. As shown in Figure 4, there was no signal for OTA or ZEA in the blank sample (D), suggesting it was free of mycotoxin contamination. However, there is an interferent signal from the matrix that was observed at 3.74 min, related to a ZEA identification ion transition, which was not the most intense, and thus is not shown in Figure 4. Once no signal overlaps with those of OTA and ZEA are monitored, the analytical method can be considered selective.

In addition to the wine samples, coffee and almond liquors were analyzed. These samples were chosen due to the known possibility of the presence of mycotoxins, considering their raw material origin: almond and coffee. For this stage, apart from OTA and ZEA, four aflatoxins were also included in the method scope (AFB_1, AFB_2, AFG_1, and AFG_2). Figure S6 (Supplementary Materials) shows the total ion chromatogram (TIC) for the spiked almond liquor (A), spiked coffee liquor (B), and a spiked standard solution (C) containing the target analytes at 15 μg L^{-1}. As can be seen, the automated analysis, based on setup 2, can also be used to analyze other complex matrices potentially related to mycotoxin contamination. This versatility is an excellent characteristic of this automated system 2, when used for more than one matrix and several different target mycotoxins. Another essential characteristic is their short run time analysis, reinforcing the high throughput of the automated methods. In the proposed method, just a simple filtration with a cellulose membrane was carried out before the unattended analysis. In this way, several samples could be processed daily (ca. 6 samples per hour), enhancing productivity, while the use of miniaturized approaches allow a significant economy of solvents and samples (injection volume of 1 μL) while generating less waste.

For the last example, Figure 5 depicts the results of almond liquor analyzed using the selected reaction monitoring (SRM) mode, highlighting the more intense ion transition for each mycotoxin monitored. It is noteworthy that the AFB2 chromatographic peak (3.63 min) did not appear in the TIC (G), because it was much less intense, and thus was suppressed by the others. However, whenthe ion transition was isolated (E) we can see it properly. Additionally, the good overall selectivity obtained when using in-tandem MS as a detection method can help our system 2 to enhance its capacity even more to detect multi-mycotoxin signals at low concentration levels. Similarly to setup 1, the LOQs were determined by calculating the concentration values at a signal-to-noise ratio of 10 (S/N 10:1) for each analyte. In this way, the reported LOQs were: wine (OTA = 110 ng L^{-1}; ZEA = 380 ng L^{-1}), almond liquor (OTA = 320 ng L^{-1}; ZEA = 620 ng L^{-1}; AFG_1 = 350 ng L^{-1}; AFG_2 = 1110 ng L^{-1}; AFB_1 = 450 ng L^{-1}; AFB_2 = 1000 ng L^{-1}), and coffee liquor (OTA = 350 ng L^{-1}; ZEA = 780 ng L^{-1}; AFG_1 = 600 ng L^{-1}; AFG_2 = 540 ng L^{-1}; AFB_1 = 510 ng L^{-1}; AFB_2 = 1080 ng L^{-1}).

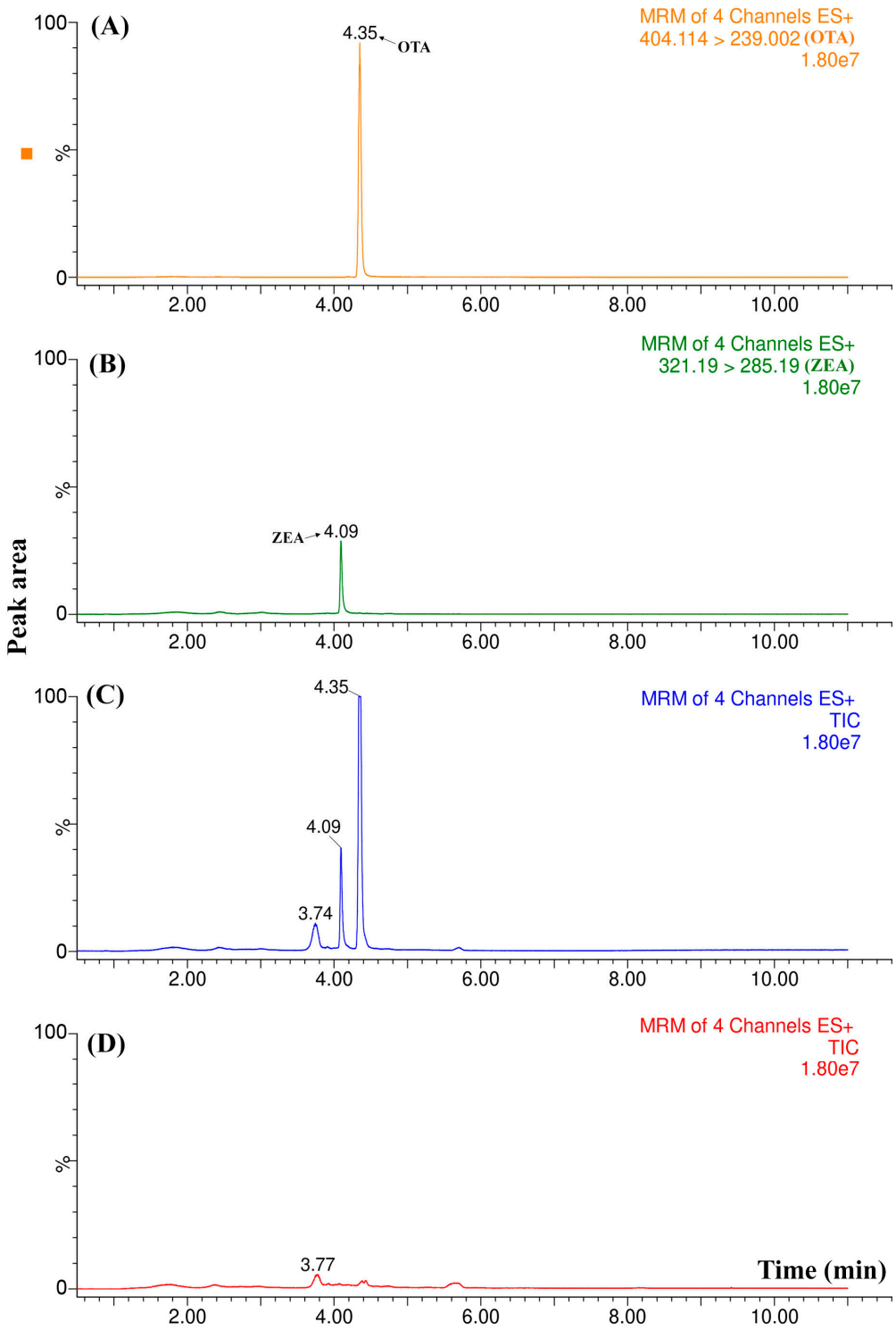

Figure 4. Representative chromatograms comparing a blank wine sample with one spiked at a concentration of at 15 μg L^{-1}. (**A**) OTA ion transition; (**B**) ZEA ion transition; (**C**) total ion chromatogram of the spiked wine; and (**D**) total ion chromatogram of the blank wine sample.

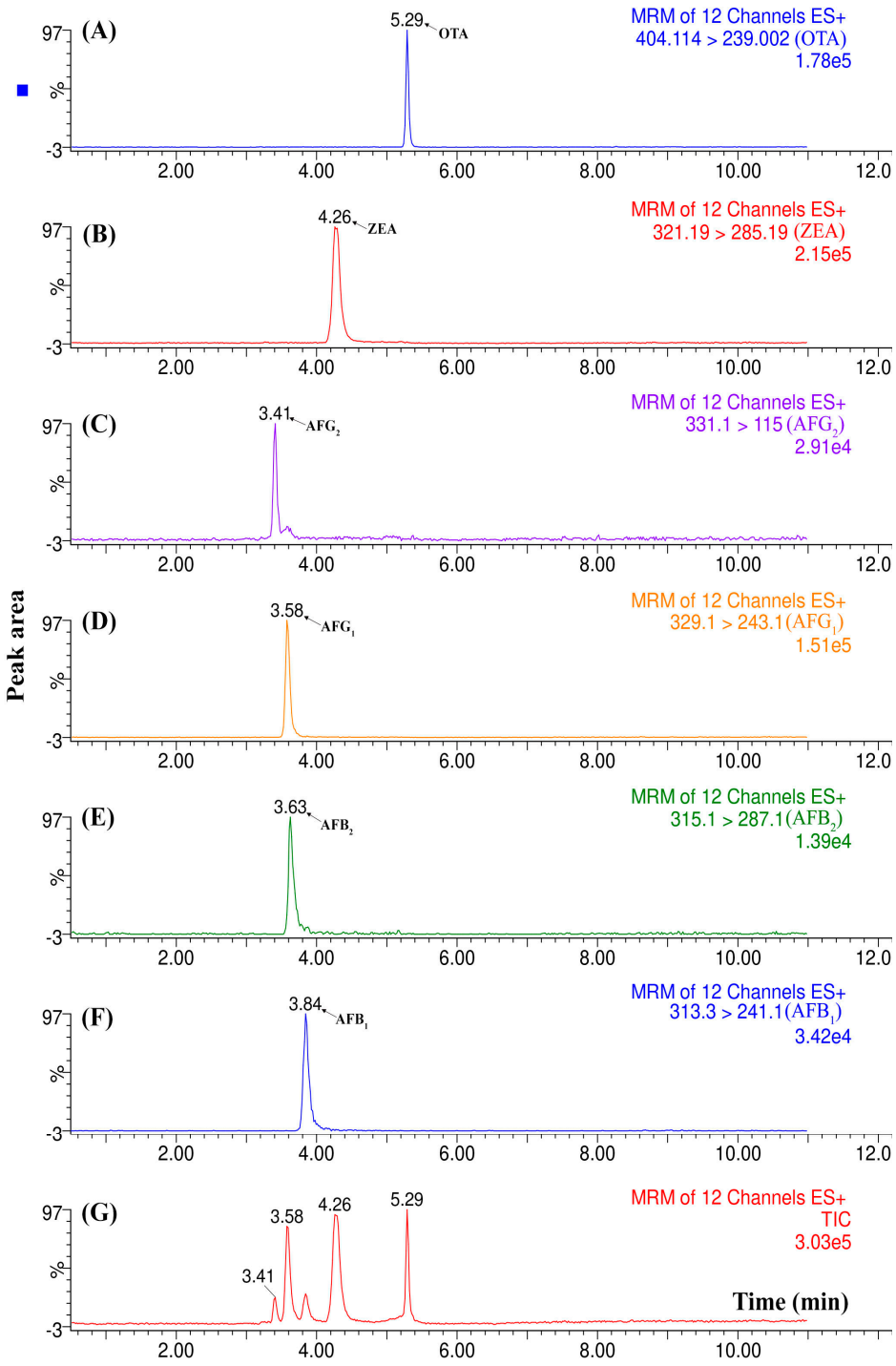

Figure 5. Total ion chromatogram (TIC) and selected reaction monitoring (SRM) of each mycotoxin analyzed in a spiked almond liquor sample. (**A**) OTA ion transition; (**B**) ZEA ion transition; (**C**,**D**) AFG_1 and AFG_2 ion transitions (Aflatoxin G_1, Aflatoxin G_2); (**E**,**F**) AFB_1 and AFB_2 (Aflatoxin B_1, Aflatoxin B_2) ion transitions; (**G**) TIC of the analyzed sample.

2.2.4. Method Overview

Despite some publications that report the use of automated or miniaturized methods for mycotoxin sample preparation, the field is still predominantly consists of non-automated and conventional approaches [15,21–23,25,29]. Additionally, from these publications, we can see the occurrence of sample preparation methods based on the association of automation and miniaturization is rare.

The proposed systems 1 and 2 aim to link the best of them (automation and miniaturization) for such purposes.

In this section, centered on system 2, some of its main characteristics should be highlighted. Looking at the miniaturization benefits, it is unarguably economical, regarding the reagent consumption and waste generation of this method. Comparing to other recently published approaches [21–23], our system 2 works with approximately a hundred times less mobile phase, as well as requiring less than 4% of "real" samples than the referred works. Likewise, methods requiring mycotoxin sample preparation could demand preliminary steps [33,34], such as centrifugation, dilution, shaking, or filtration. It is noteworthy that in this work, due to the process automation, wine samples should be filtered immediately before injection, allowing the analysis of up to six samples per hour. Another critical advantage that could be reinforced is the simple process of synthesizing and using our extractive phases based on graphene. Some published works report the use of immunoaffinity sorbents for mycotoxin sample preparation, which in general are more complex sorbents to work with, as well as being more expensive to acquire [24,35]. Besides, the proposed extraction column might be utilized for more than 250 analyses without modifying its original sorptive capacity.

From the automation viewpoint, system 2 also exhibits some favorable characteristics, especially emphasizing its more straightforward operation mode, since only a 0.22 µm sample filtration must be done before the injection. Considering that after this action, all analysis steps are controlled by the instrument software, it brings three significant features: (i) the possibility of remote-operated analyses, (ii) increasing analysis throughput, and (iii) low manual handling, which decreases the potential occurrence of analytical errors. Furthermore, considering the miniaturization of the extraction part and the whole analytical technique (including capillary LC), system 2 provides a high chromatographic peak profile and excellent signal intensity. This configuration might be a promising alternative to developing greener analytical methods without sacrificing the quality of the analytical results, which is within the current trends of green chemistry.

Finally, a brief comparison between some analytical characteristics of systems 1 and 2 is shown in Table 2. Following this, some observations can be highlighted: considering the purpose of these two systems, both exhibit good analysis time, less than 20 min in total. Another aspect to consider is the relation between the LOQs and injection volume. As can be seen, system 1 reached inferior LOQs compared to system 2, due to the large injection volume available on it (50 µL). This characteristic suggests it as a more suitable configuration for dealing with the lower concentration levels investigated in common matrices such as domestic coffee, beer, and wine samples. Conversely, when the analysis of a more expensive matrix is desirable (e.g., imported beverages like those liquors herein analyzed), system 2 seems more adequate, since it can achieve satisfactory LOQs by using around fifty times lower sample volumes. Indeed, when high throughput analysis is the main goal, system 2 arises as a promising configuration, as it put together all benefits of an online sample preparation approach with a good efficiency of capillary liquid chromatography, even in reduced sample volumes. Assuredly, both configurations generate small volumes of waste, which was one of our main goals: proposing methods for mycotoxin analysis without compromise with the environmental principles of green chemistry.

Table 2. Comparison between the main analytical characteristics of systems 1 and 2.

Parameter	System 1	System 2
Injected sample volume (µL)	50	1
Time per analysis (min)	19	10
Extraction flow rate (µL min^{-1})	150	25
Analysis flow rate (µL min^{-1})	200	10
Waste generated per analysis (mL)	6.65	0.12
LOQs (ng L^{-1}) [a]	68–86	110–1110

[a] range considering all analytes.

3. Experimental

3.1. Standards and Reagents

Ochratoxin A analytical standard (OTA) 99.5% and Zearalenone (ZEA) 99.5% were both purchased from Fluka-Analytical (St. Louis, MO, USA). A standard mix of aflatoxins (99.5%) containing AfB_1, AfB_2, AfG_1, and AfG_2 solubilized in benzene:acetonitrile (98:2) were acquired from Merck (Darmstadt, Germany).

HPLC grade acetonitrile (ACN) methanol (MeOH), and formic acid (FA) 98% grade AR, were purchased from Tedia (Fairfield, OH, USA), and ultra-high purity (UHP) water was supplied from a Milli-Q system (Burlington, MA, USA).

3.2. Mycotoxin Standard Solutions

OTA and ZEA stock solutions of 150 mg L^{-1} were made in methanol and stored at −18 °C in an amber vial. The aflatoxin stock solution was prepared from a standard mix dilution to a concentration level of 0.5 mg L^{-1}, this solution was used to prepare each work solution before chromatographic injections.

The exact concentration of the OTA stock solution was determined by UV–Vis spectrophotometry [11]. For this, 1.00 mL of stock solution was placed in a vial and dried with N_2, and then dissolved in 1.00 mL of toluene: acetic acid (99:1, *v*/*v*). This solution was transferred to a 1.0 mL quartz cell, and the concentration was determined at λ_{max} = 333 nm. Working solutions, prepared and used as needed, were made in the mobile phase solvent from the stock solution of 30 mg L^{-1} of OTA in MeOH.

3.3. Extraction Column Packing Procedure

First, a Haskel DSHF-122 hydropneumatic pump from Haskel (Burbank, CA, USA) was employed to pack both extraction columns utilized in setups 1 and 2. Although both extraction dispositive was packed by the same instrumentation, some differences in the operating procedure and the sorbent phase are stated next.

Setup 1 employed as a sorbent the GO anchored on aminopropyl silica particles and functionalized with octadecylsilane and trimethylsilane (SiGOC18ecap). The synthesis and characterization of this material are described by Mejía-Carmona et al. [32]. Approximately 20.0 mg of SiGOC18ecap was packed in a stainless steel capillary column (508 µm i.d. × 100 mm) by a slurry packing method using a solution of THF:IPA (1:6, *v*/*v*) as the slurry solvent to suspend the sorbent, and ultrapure water as the packing solvent.

The extraction column of setup 2 was produced by packing approximately 10.0 mg of sorbent (GO-Sil) into stainless steel tubing (254 µm i.d. × 100 mm) using the same slurry and packing solvents. The synthesis and characterization of the GO-Sil are described by Maciel et al. [36]. Figure S7 (Supplementary Materials) shows a picture of the hardware required to assemble the miniaturized extraction columns utilized in this work.

3.4. Setup 1: Assembly and General Characteristics

For this setup, an Acquity UPLC liquid chromatography from Waters (Milford, MA, USA) supplied with a binary solvent manager and autosampler was used, coupled to a XEVO TQ Mass Spectrometer with an electrospray ionization (ESI) interface, all from Waters (Milford, MA, USA). Chromatographic separation was performed in an analytical column Poroshell 120 SB-C8 (2.1 mm d.i. × 100 mm; 2.7 µm d.p.), used under the following conditions: column temperature, 40 °C; injector temperature, 15 °C; injection volume, 50 µL in full-loop mode; flow rate 0.200 mL min^{-1}, mobile phases A: H_2O 0.1% formic acid, and B: ACN 0.1% formic acid, in stepwise elution gradient mode (see blue line in Figure 1). The injector needle was washed between each injection with an additional washing cycle.

ESI conditions: positive ionization mode; capillary voltage, 3.0 kV; cone voltage, 16 V; source temperature, 150 °C; desolvation temperature, 400 °C; solvation carrier gas N_2, 800 L h^{-1}; collision gas flow Ar, 0.15 mL min^{-1}. Tandem MS/MS conditions were optimized by direct infusion of the OTA standard solutions at 100 µg L^{-1}, data were acquired in selected reaction monitoring

(SRM) mode by MassLynx 4.1 software from Waters (Milford, MA, USA), and dwell time, 0.62 s. Selected quantification OTA transition was *m*/*z* 404 → *m*/*z* 239 (cone voltage, 24 V; collision energy, 26 eV), and confirmation transition was *m*/*z* 404 → *m*/*z* 221 (cone voltage, 24 V; collision energy, 36 eV).

For assembling the multidimensional set up (Figure 6), the microextraction column was connected to the UPLC–MS/MS system using an external Supelpro® six-port valve (operated by the chromatograph software in two positions, 1: loading and 2: elution), and a Shimadzu LC-20AT auxiliary pump. The sample loading/extraction was made in the flow-through mode and the elution in the back-flush mode. In the loading position, 50 µL (full-loop mode) of the sample was injected and carried to the extraction microcolumn by the loading phase (H_2O:ACN (85:15, *v*/*v*) acidified with 0.1% formic acid at 0.150 mL min^{-1} flow rate) using the auxiliary pump, which was connected to the auto-injector. The sample was flushed for 4 min through the extraction microcolumn; the target compounds were retained, while matrix interferents were eliminated through the waste. Subsequently, the valve was switched to the elution position, and the UPLC mobile phase passed through the extraction microcolumn in a back-flush flow at 0.200 mL min^{-1}, over 16.5 min in a step gradient. The first solvent gradient (H_2O:ACN (40:60, *v*/*v*) 0.1% formic acid) transferred the OTA to the analytical column to be separated and quantified by the MS/MS detector. The second gradient (H_2O:ACN (5:95, *v*/*v*) 0.1% formic acid) did the clean-up of both columns (extraction and analytical). Finally, after 5 min of conditioning, system 1 was ready for other injections. The scheme of the time used to operate the valves is depicted in Figure 1 (see boxes).

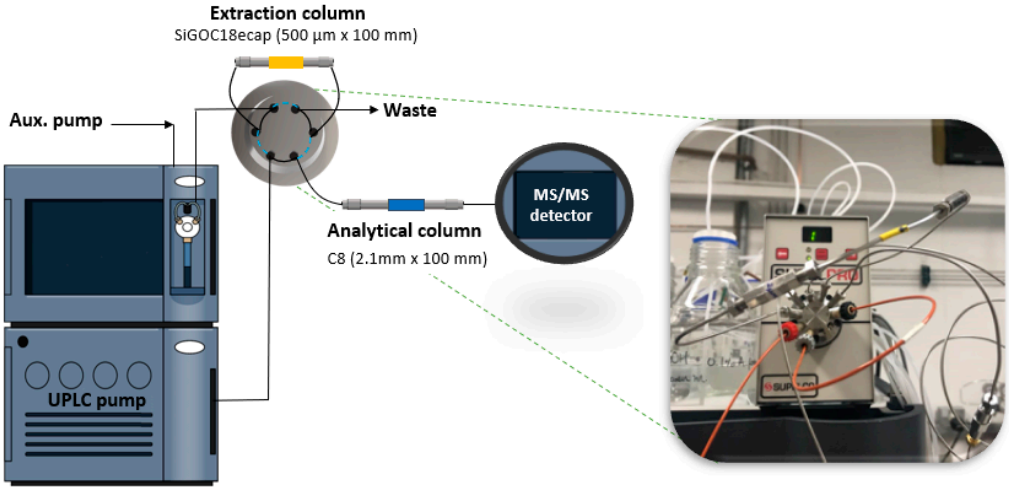

Figure 6. Representative scheme of setup 1, obtained by a coupling of the extraction microcolumn to a UPLC–MS/MS system, in flow-through mode. A six-port valve is shown in the loading (black trace) and elution (blue trace, back-flush mode) positions. In the inset, a photograph depicts the valve system.

3.5. Setup 2: Assembly and General Characteristics

In this case, setup 2 was composed of an Acquity M-Class for liquid chromatography supplied with a µ-Binary solvent manager (pumping system), a sample manager (injector), and a trap valve manager (switching valve) coupled to a Xevo TQ S Micro (MS/MS), the whole equipment setup was acquired from Waters (Milford, MA, USA). Figure 7 shows a representative scheme of the assembled system on the right, while an actual photograph of the capillary extraction column connected to the trap valve manager is enlarged on the left.

The extraction column used was a lab-packed GO-Sil with capillary dimensions (254 µm × 200 mm; 50 µm d.p.). Likewise, the chromatographic separation was performed using a C18 THS capillary column (300 µm × 100 mm; 1.7 µm d.p.), purchased from Waters (Milford, MA, USA), operating at a temperature of 35 °C with the mobile phase composed of ultrapure water (A) and acetonitrile (B), both 0.1% acidified with formic acid, under a flow rate of 10 µL min^{-1}. The in-tandem MS detection

was performed using the selected reaction monitoring mode (SRM) responsible for identifying the specific ion transitions for OTA, ZEA, and the four aflatoxins. The monitored transitions were selected by the direct infusion of a standard solution of each analyte (50 μg L^{-1}) using the assisting software IntelliStart 4.2, from Waters (Milford, MA, USA). The analyte transitions and their main detector parameters are presented in Table S1 (Supplementary Materials). Other important MS/MS detection parameters included: positive electrospray ionization; capillary voltage, 3.5 kV; source temperature, 150 °C; desolvation temperature, 350 °C; desolvation gas N_2, 900 L h^{-1}; and collision gas Argon (Ar).

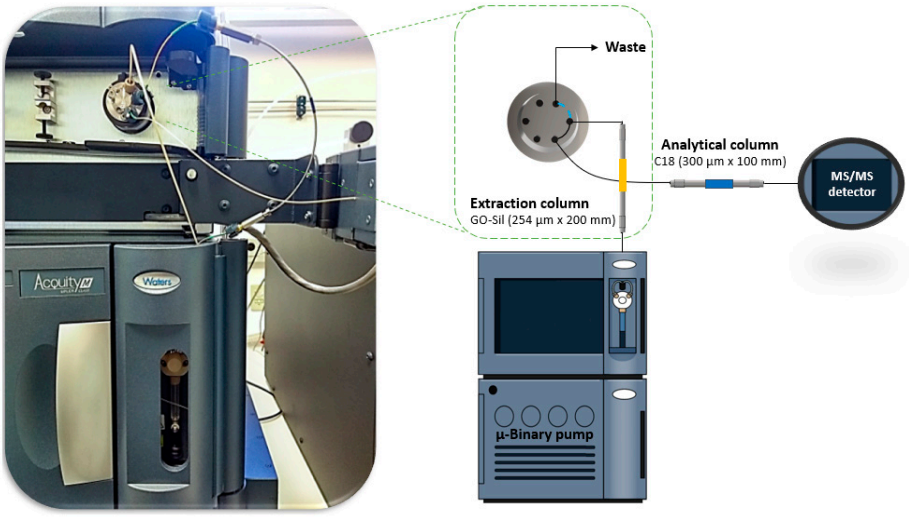

Figure 7. Representative scheme of the system 2 configuration. An actual photograph of the switching valve connections and the capillary extraction column is depicted in the left, while a drawing of the whole system is displayed on the right side of the figure.

After system 2's short description, an explanation about the working steps is presented. The mycotoxin automated analysis was performed in two different steps: (i) automated extraction and (ii) capillary LC–MS/MS analysis. The trap valve manager was responsible for controlling the mobile phase flow direction, alternating between loading and elution positions. Therefore, in the first step after the 1 μL sample injection by the equipment, only the GO-Sil extraction column received the mobile phase flow at 25 μL min^{-1}, composed of 0.2% FA acidified H_2O:ACN (78:22, *v*/*v*). During this, the μ-binary solvent manager pushed the sample from the loop (1 μL) into the extraction column, over 1 min, to load the target analytes in the extractive phase while most of the matrix interferents were eliminated through the waste. Subsequently, the valve was switched to the elution position, and then the μ-binary solvent manager pumped a flow of 10 μL min^{-1} into the extraction column connected in-line with the analytical column to separate and detect the target compounds. In this case, the mobile phase had the same composition as the extraction step, but an elution gradient was employed (Figure S3, Supplementary Materials). Finally, after ten minutes of analysis, system 2 was ready for the next injection.

3.6. Sample Preparation

For system 1, wine and beer samples were degassed in ultrasound for 5 min, then spiked with OTA at the specified concentration and filtered through a 0.22 μm cellulose membrane. Instant coffee samples were ground until a fine powder was obtained. One gram of the sample was dissolved with 100 mL of boiling water, shaken (1000 rpm, 10 min), and centrifuged (14,500 rpm, 10 min). An aliquot of the supernatant was spiked with OTA, at the specified concentration, and filtered through a 0.22 μm cellulose membrane before the analysis.

For system 2, the samples of wine, and almond and coffee liquors were just spiked with mycotoxins (OTA, ZEA, and AFAs) at the specified concentration and filtered through a 0.22 μm cellulose membrane.

4. Conclusions

This work presents two different setups to analyze residues of ochratoxin A, zearalenone, and four aflatoxins (B_1, B_2, G_1, and G_2) in complex matrixes. The focus was on the development and evaluation of greener analytical methods aimed to correlate the benefit of miniaturization and automatization for the field of mycotoxin analysis. In this context, both analytical systems (1 and 2) consisted of an automated sample preparation method followed by liquid chromatography coupled to in-tandem mass spectrometry. In short, system 1 consisted of a lab-made microextraction column packed with a new SiGOC18ecap sorbent phase, which presented excellent extraction performance, resulting in good analytical responses up to a concentration of 2 μg L^{-1}. In this case, a fully automated sample preparation of beer, wine, and instant coffee was performed before OTA identification. Another interesting point here is the cheap extraction hardware employed, which costs under US$50 and can perform more than 250 injections before losing its original working condition. Additionally, due to the microextraction column dimension, only 20 mg of the sorbent phase was packed into it, while the sample required for the method was only 50 μL, being much less than is used for conventional sample preparation techniques based on QuEChERS (Quick, Easy, Cheap, Rugged, and Safe) and SPE, among others [23,29,33,37]. Similarly, the method proposed in system 2 was primarily based on the benefits obtained by allying miniaturization and automation in one configuration. For this reason, a new assembly employing a 254 μm i.d. extraction capillary column packed with a graphene-based sorbent (GO-Sil), was employed in the fully automated extraction of several mycotoxins in wine samples, and almond and coffee liquors. The miniaturized sample preparation was further coupled to capillary LC–MS/MS to emphasize, even more, the impact of miniaturization on the economy of chemicals and samples. The estimated consumption of reagents and waste generation of system 2 is shown to be 100 times lower than in recently published works [21–23] while it also demands not more than 4% of the amount of sample commonly required [33,34]. Like in system 1, the in-lab made extractive phase showed good behavior, performing more than 250 injections. When looking at the robustness of the two lab-packed extraction columns, these methods showed a profit perspective, since commercially available SPE-based extraction devices are usually disposable. Another attractive characteristic was the absence of widely employed additional steps, such as centrifugation, dilution, and the shaking of samples before the extraction step. Due to this, the total analysis time was approximately ten minutes, and manual handling was kept to a minimum in both methods. By comparing them, system 1 seems to be more suited to the analysis of mycotoxins in common matrices (such as coffee and wine) when a larger volume of samples is available and lower limits of quantification are required. Contrariwise, when higher throughput is the main goal, and a lower reagent cost is desirable, system 2 might be a good configuration. As a drawback, to assemble the configurations used in these setups, a switching valve and an auxiliary pump must at least be present, beside the analytical equipment. However, the authors thought that the benefits rising from miniaturization and automation could make such a kind of instrumentation cheaper in the long run. For all these reasons, this manuscript aimed to present and evaluate through some analytical experiments on the performance of two fully automated and miniaturized different methods for mycotoxin analysis. From our point of view, this system exhibits excellent performance and represents a suitable alternative to achieve high-throughput methods that are environmentally friendly at the same time.

Supplementary Materials: The following are available online: Figure S1: Chromatograms obtained in SRM mode of the evaluation of the loading time from 1 to 6 min of a standard solution of OTA at 20 μg L^{-1} at 0.100 mL min^{-1} flow rate of H_2O:ACN (78:22, *v*/*v*) acidified 0.1% formic acid; Figure S2: Wasting fractions collected during the online extraction step by each minute from 1 to 4 min (during the sample loading time, 4 min) of (A) wine and (B) instant coffee samples, both spiked at 20 μg L^{-1} with OTA; Figure S3: Step elution gradient employed in the multi-mycotoxin analysis by multidimensional capillary LC–MS/MS. Mobile phases → A: H_2O: 0.1% formic acid and B: ACN: 0.1% formic acid (blue line). The box below the gradient indicates the configuration of the switching valve (*); Figure S4 Performance (peak area vs. analytes) of the analytical parameters considered in the extraction method enhancement, carried out by univariate experiments (n = 3). (A) Loading time; and (B) loading flow; Figure S5: Total ion chromatograms of three spiked wine samples (15 μg L^{-1}) to illustrate the retention

time reproducibility and chromatographic behavior profile; Figure S6: Representative chromatograms showing: (A) almond liquor, (B) coffee liquor, and (C) standard solution. All of them were spiked at a concentration of at 15 μg L^{-1}; Figure S7: Typical microcolumn hardware used as an extraction device in both systems; Table S1: Analytes' precursor and product-ion and its main detection parameters utilized in the selected reaction monitoring MS mode.

Author Contributions: E.V.S.M, and K.M.-C.; contributed equally to this work. Conceptualization, E.V.S.M., K.M.-C., and F.M.L.; Methodology, E.V.S.M., and K.M.-C.; Formal analysis, E.V.S.M., and K.M.-C.; Investigation, E.V.S.M., and K.M.-C.; Resources, F.M.L.; Writing—original draft preparation, E.V.S.M., and K.M.-C.; Writing—review and editing, E.V.S.M., K.M.-C., and F.M.L.; Visualization, E.V.S.M., and K.M.-C.; Supervision, F.M.L.; Project administration, F.M.L.; Funding acquisition, F.M.L. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

ACN	acetonitrile
AFAs	aflatoxins
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
ESI	electrospray ionization
FA	formic acid
GO-Sil	graphene oxide supported on amino silica
HPLC	high-performance liquid chromatography
IAC	immunoaffinity columns
i.d.	inner diameter
LC	liquid chromatography
LLE	liquid–liquid extraction
MeOH	methanol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
OTA	ochratoxin A
SiGOC18ecap	graphene oxide anchored to aminopropyl silica particles and functionalized with octadecylsilane and trimethyl silane
SRM	selected reaction monitoring
SPE	solid-phase extraction
SPME	solid-phase microextraction
UPLC	ultra-performance liquid chromatography
TFC	turbulent flow chromatography
ZEA	zearalenone

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Sample Availability: Samples of the compounds are not available from the authors.

CAPÍTULO 8

A cartridge-based device for automated analysis of solid matrices by online multidimensional sample-prep-capillaryLC-MS/MS.

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A cartridge-based device for automated analysis of solid matrices by online multidimensional sample-prep-capillaryLC-MS/MS

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From the desk of
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São Carlos, November 22nd, 2021.

To: Analytical & Bioanalytical Chemistry
Dr. Steffen Pauly
Editorial Director

Dear Dr. Pauly.

We are submitting to your attention an original research article entitled

" **A cartridge-based device for automated analysis of solid matrices by online multidimensional sample-prep-capillaryLC-MS/MS** "

by *Edvaldo Vasconcelos Soares Maciel, and Fernando Mauro Lanças* to be considered for publication in Analytical & Bioanalytical Chemistry.

We inform you that this research article is original and is not being submitted or published elsewhere.

The proposed approach is based on a cartridge packed with solids (soil samples) coupled with a capillaryLC-MS, combining sample preparation and analytical steps into a unique platform. As a proof-of-concept, nine pesticides used in sugarcane crops were extracted and analyzed by our proposed method. For optimization, a fractional factorial design (2^{5-1}) was performed with the following variables: aqueous dilution of the sample (V_1), extraction strength (V_2), matrix washing time (V_3), extraction flow (V_4), and analytical flow (V_5). After, the most influential ones (V_1, V_2, and V_3) were taken into a central composite design (2^3) to select their best values. Under optimized conditions, the

method reported linear ranges between 10 – 125 ng g^{-1} with $R^2 > 0.985$. Accuracy and precision were in according to the values established by the International Council for Harmonisation (Q2(R1)). Therefore, the proposed approach was effective in extracting and analyzing selected pesticides in soil samples. We also analyzed pesticides in honeybees to reveal the analytical method's potential application for other solid matrices.

We understand that this innovative approach will bring a great interest to the readers interested in the automated analysis of solid matrices, besides those interested in miniaturized analytical techniques.

The authors declared that they have no conflicts of interest to disclose.

Thank you for the consideration regarding this manuscript.

Sincerely,

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A cartridge-based device for automated analysis of solid matrices by online multidimensional sample-prep-capillaryLC-MS/MS

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Abstract

Sample preparation is an essential step focused on eliminating interfering compounds while pre-concentrating the analytes. However, its multiple steps are generally laborious, time-consuming, and a source of errors. Currently, automated approaches represent a promising alternative to overcome these drawbacks. Similarly, miniaturization has been considered an ideal strategy for creating greener analytical workflows. The combination of these concepts is currently high-desired by analytical chemists. However, most automated and miniaturized sample preparation techniques are primarily concerned with liquid samples, while solids are frequently overlooked. We present an approach based on a cartridge packed with solids (soil samples) coupled with a capillaryLC-MS, combining sample preparation and analytical steps into a unique platform. As a proof-of-concept, nine pesticides used in sugarcane crops were extracted and analyzed by our proposed method. For optimization, a fractional factorial design (2^{5-1}) was performed with the following variables: aqueous dilution of the sample (V_1), extraction strength (V_2), matrix washing time (V_3), extraction flow (V_4), and analytical flow (V_5). After, the most influential ones (V_1, V_2, and V_3) were taken into a central composite design (2^3) to select their best values. Under optimized conditions, the method reported linear ranges between 10 – 125 ng g^{-1} with $R^2 > 0.985$. Accuracy and precision were in according to the values established by the International Council for Harmonisation (Q2(R1)). Therefore, the proposed approach was effective in extracting and analyzing selected pesticides in soil samples. We also analyzed pesticides in honeybees to reveal the analytical method's potential application for other solid matrices.

Graphical abstract

Keywords: mass spectrometry, liquid chromatography, automation, miniaturization, sample preparation, and pesticides.

Abbreviations: **ACN**, acetonitrile; **C18**, octadecyl silane; **capillaryLC**, capillary liquid chromatography; **CCD**, central composite design; $\mathbf{C_{exp}}$, experimental concentration; $\mathbf{C_{tr}}$, theoretical concentration; **DoE**, design of experiment; **ESI**, electrospray ionization; **FA**, formic acid; **GO-Sil**, graphene oxide supported onto amino silica; **ICH**, International Council for Harmonisation; **LOD**, the limit of identification; **LOQ**, the limit of quantification; **MEPS**, microextraction by packed sorbent; **MS**, mass spectrometry; **MS/MS**, in-tandem mass spectrometry; **MSPD**, matrix solid-phase dispersion; **PSE**, pressurized liquid extraction; **QuEChERS**, Quick, Easy, Effective, Rugged and Safe; **RSD**, relative standard deviation; **SBSE**, stir-bar sorptive extraction; **SPME**, solid-phase microextraction; **SRM**, selected reaction monitoring; **TFC**, turbulent flow chromatography; **UPLC**, ultra-performance liquid chromatography;

1. Introduction

Over the years, analytical chemistry has evolved to become one of the most important branches of science responsible for monitoring and detecting chemical substances in several matrices. Its tools support academics and industries associated with pharmaceutical drugs, healthcare products, fuel, energy, and food production [1, 2]. In addition, other significant areas such as medicine, forensic, veterinary, and environment are also assisted by analytical methods [3]. In this context, the raw materials used in these niches, and ultimately the samples investigated by the analytical chemists are deemed complex due to the occurrence of endogenous and exogenous compounds, including the so-called target analytes and others, acting as interfering compounds in the analytical method [3, 4].

For these reasons, sample preparation is often required before executing any analysis. Ideally, this critical process eliminates the interfering compounds while isolating and pre-concentrating the target analytes [5]. However, this procedure usually possesses multiple steps, widely recognized as a potential source of analytical errors, being also considered laborious and time-consuming most times [6]. Thus, notwithstanding the technological advances on analytical instrumentation achieved during the last decades, the development of high-throughput and greener sample preparation persists among the most desired goals for analytical chemists [7, 8]. A promising strategy for meeting such requirements is automating the sample preparation process and hyphenating it with analytical techniques (e.g., gas and liquid chromatography [9, 10]. In this manner, automation might improve both productivity and accuracy by accelerating and simplifying the processes [11]. Likewise, miniaturization also represents a promising strategy to address these challenges once it is directly associated with lower demand for solvent and sample while reducing the chemical waste and the exposure of analysts to a toxic environment [12, 13]. Under this perspective, a combination of automation and miniaturization emerges as an appropriate strategy for developing modern sample preparation approaches [14].

One of the most common strategies to achieve such goals is by automating already well-established non-automated techniques; different methods have been reported over the last decades under this approach. They include the solid-phase extraction automation (online SPE) [5, 15]; adapting miniaturized techniques such as solid-phase microextraction (e.g., in-tube SPME) [16], microextraction by packed sorbent (MEPS) [17, 18], stir-bar sorptive extraction (SBSE) [19], and other approaches based on LC principles, such as turbulent flow chromatography (TFC) [20]. Although their effectiveness, they are most successfully employed for liquid samples while being a challenge to achieve equivalent results for solids and semi-solids (e.g., soil, sludge, insects, and others).

Usually, solid matrices must be previously processed by transferring the target analytes from the original raw material to an appropriate chemical media before the analysis, often a liquid solvent compatible with the analytical technique. For example, water, acetonitrile, or methanol are commonly employed in LC-based methods. Therefore, solid matrices are prepared by techniques such as MSPD (matrix solid-phase dispersion), QuEChERS ("Quick, Easy, Cheap, Effective, Rugged and Safe"), and pressurized liquid extraction (PSE) [21, 22]. Although their efficiency, they usually require multiple steps, which can be simplified by miniaturizing and automating the process. Also, the sample preparation procedures based on these techniques often require a substantial amount of reagents and samples available (regularly a few grams).

To date, only a few reports in the literature are focused on developing automated methods for solid matrices [23–29]. Therefore, this is an under-development topic that still requires more contribution by the analytical chemists involved with miniaturization and automation to fill some gaps. Considering this outlook, the authors of this work believe that coming up with an alternative sample preparation approach for solids, combining miniaturization and automation, could be a relevant contribution. We herein describe our first results on a multidimensional system focused on the automated sample preparation of raw-solid matrices followed by capillaryLC-MS/MS analysis. For this purpose, a stainless-steel tubular cartridge was designed and employed as a supporting device in which the solid sample was packed. It was connected in line with the capillary extraction column and the chromatographic one. Noteworthy, this system is fully miniaturized once a minimum amount of sample and reagents are used (approx. a few micrograms).

At last, according to the Green Chemistry concept, this work was developed to prioritize environmentally friendly characteristics, which is currently critically relevant. The approach was assessed in the automated sample preparation of sugarcane crop soils to analyze pesticides as a proof-of-concept. This choice is supported by several reasons: (i) sugarcane is one of the most relevant agricultural activities in Brazil; (ii) Brazil and India produce approximately half of the world demand for such commodity; (iii) sugarcane is a raw source for several products, including sugar, cachaça (sugarcane spirit), brown sugar candy (*rapadura*), sugarcane juice (garapa), ethanol, and several others; and (iv) due to the Brazilian climate conditions and the considerable demand for sugarcane, pesticides are heavily employed in such crop often leaving residues within [30]. As a complement in the proof-of-concept, lyophilized bodies of Africanized honeybees *(Apis mellifera)* were also evaluated, employing the proposed approach to verify its versatility. The same pesticides

used in sugarcane were targeted since these honeybees live near the sugarcane crops previously analyzed, being susceptible to such contamination.

2. Materials and methods

2.1 Chemicals

High purity (99%) analytical standards of imidacloprid, tebuthiuron, clomazone, carbofuran, diuron, hexazinone, simazine, cypermethrin, and atrazine were acquired from Fluka-Analytical (St Louis, USA). Conversely, thiacloprid was purchased from AccuStandard (New Haven, USA). The stock solutions were separately prepared in methanol at the concentration level of 1000 mg L^{-1}. Also, an intermediary solution mixing them was prepared in methanol at the concentration of 5 mg L^{-1} and subsequently diluted in an ultrapure aqueous solution suitable for each experiment, at concentration levels depending upon the primary goal.

HPLC grade acetonitrile (ACN) 99% was acquired from Tedia (Fairfield, USA), while MS grade formic acid (FA) was from Sigma-Aldrich (St Lous, USA). Deionized ultrapure water was supplied by a Milli-Q system from Millipore (Burlington, USA). Moreover, octadecyl silane (C18) and Florisil, both acquired from Merck (Darmstadt, DE) were employed as a sorbent during the study.

2.2 Cartridge description

Figure 1a shows an illustrative figure of the lab-made stainless-steel cartridge. Noteworthy, it was built based on devices often used as a guard or pre-concentration LC columns. The main differences are the physical dimensions and the frits' bed. The lab-made cartridge possesses 1.7 mm of internal diameter (i.d.) with 50 mm of length. It gently accommodates ca. 60 - 65 mg of the packed blended matrix without high-pressure procedures (bare soil samples and sorbent phase – **Figure 1b**). Conversely, a conventional HPLC pre-concentration hardware is slightly larger, possessing up to 50mm i.d. and 100 – 120 mm of length. Similarly, other approaches commonly employed for sample preparation of solids (e.g., QuEChERS or MSPD) also use much larger devices (e.g., centrifuge tubes) to accommodate quantities up to 10g of samples. In our case, the lab-made cartridge is proposed aiming to obtain similar performance using much fewer reagents and samples while maintaining or even enhancing the analytical throughput, as we will discuss in section 3. As the cartridge is coupled to the capillaryLC-MS/MS system, the frits' bed contains a metallic frit (2 µm porosity) and a cellulose-regenerated membrane (0.22 µm) to ensure that particles from the raw-solid matrix would not pass towards the capillaryLC system. The cellulose-regenerated membrane has approximately the same diameter as the yellow portion of the frits' bed (12mm), as shown in **Figure 1c**. It is worth mentioning that several other cartridge configurations were evaluated before; however, none of them showed the practical maneuverability of this cartridge.

<Insert Fig.1>

2.3. Solid sample processing

To evaluate the automated analysis of the raw-solid matrix through the lab-made cartridge, two different samples were evaluated: sugarcane crop soil and lyophilized bodies of honeybees *Apis*

mellifera L., with a focus on the first one. Before starting the packing process, the soil samples were passed through a molecular sieve (200 mesh). *In contrast, Apis mellifera L.* samples were received ready for analysis after a lyophilization process of the whole bees. In both cases, the cartridge packing process was similar: (i) weighting of 10mg of soil or 20mg of lyophilized honeybees; (ii) weighting of 50 mg of C18-bonded silica; (iii) blend them for 20 s; (iv) and finally pack the sample. A funnel-like plastic tool was employed to introduce the composite sample within the cartridge in a practical manner. In short, this whole procedure is like those used for MSPD-blending and packing into SPE-cartridge or disks [31]. It is worth mentioning that C18 is mixed with the solid sample for two reasons: (i) abrasive function for sample disruption; (ii) favor the transferring of the target compounds from the sample to the C18 surface aiming at enhancing extraction rate while achieving a good clean-up. Finally, the cartridge cap is threaded with the frits' bed suitably allocated, ready for the automated sample-prep-capillaryLC-MS/MS analysis.

2.4. Instrumental apparatus

The cartridge characteristics were already explained in section 2.2; only the other parts will be described next. Therefore, the modules responsible for sample clean-up and analysis include an Acquity M-class UPLC assisted by a μBinary solvent manager, a sample manager, a trap valve manager, an oven (set at 45 °C), the extraction and analytical columns. The employed columns were: (1) a capillary extraction column (200mm x 254 μm i.d.), in-house packed with graphene oxide supported onto aminosílica (GO-Sil) [32], and a Waters Acquity M-class BEH C_{18} capillary analytical column (150mm x 300 μm i.d.).

The chromatographic mobile phase was composed of a mixture containing ultrapure water (H_2O) and acetonitrile (ACN) operating at variable flow rates**,** depending on the analysis step. Besides the capillaryLC system, a Shimadzu LC 10A auxiliary pump, purged with ultrapure water, was used to perform an aqueous dilution of the mobile flow from the sample cartridge. This step is crucial once the high organic content (% ACN) mobile phase, used to extract the pesticides from the solid matrix, must be diluted before entering the extraction capillary column to enhance the sorption capacity in the GO-Sil sorbent phase. The information about mobile phase composition, time, and flow rate of each step used during analysis, are shown in **Table 1**.

<Insert Table 1>

Noteworthy, this capillaryLC was coupled to a Xevo TQ S Micro mass spectrometer, the whole system from Waters (Milford, USA). The MS parameters were optimized by direct infusion of pesticides` standard solutions (0.5 mg L^{-1}). The reported conditions include: ESI source operating at positive mode; capillary voltage of 3.6 kV; desolvation gas (N_2) temperature at 800 °C and flow at 800 L h^{-1}, source temperature at 150°C; and collision gas (Ar) flow at 0.15 mL min^{-1}. Furthermore, the MS was set to operate under the selected reaction monitoring mode (SRM) to achieve suitable selectivity and sensitivity for each analyte. In this case, the most intense ion transitions for each target analyte and its properties are shown in **Table A.1** at the supplementary material.

2.5. Multidimensional system operation

The multidimensional system herein employed is divided into three main parts: the sample cartridge, the extraction capillary column, and the analytical capillary column **(Figure 2).** They are interconnected through a specific module (trap valve manager).

In short, the stainless-steel sample cartridge (1st step) is accountable for the automated extraction of the pesticides from the raw-solid matrices. After this step, the pesticides are guided, through a valve, towards the extraction column (2nd step), being retained in the sorbent phase (GO-Sil), while most interfering substances go to the waste. Noteworthy, before the pesticides reach the GO-Sil extraction column, a mixing Tee positioned between the sample cartridge and extraction column performs an aqueous dilution to enhance the sorption propensity. In the sequence, the LC mobile phase elutes the pesticides from the extraction column, connected inline with the analytical one (3rd step), responsible for the chromatographic stage just before the mass spectrometer.

<Insert Fig.2>

2.6. Optimization of the analytical conditions

2.6.1. Fractional factorial design

Before the first chemometric optimization was executed, we conducted experiments to determine the exact mass of solid and co-sorbent packed within the cartridge. We tested two different co-sorbents: C18 and Florisil. The sample:co-sorbent mass ratios tested were 10:50, 15:45, and 25:35 mg.

Chemometric methods were used to simultaneously optimize the experimental variables that possibly affect the extraction of pesticides in soil samples using the proposed approach. The strategy consisted of first using a screening approach for preliminary evaluation of the most influential variables. For this step, a two-level fractional factorial design (2^{5-1}) was chosen. Once the main goal was to understand how each variable influences the method, the runs were duplicated with three central points. The variables chosen for this DoE were: %H_2O or aqueous dilution of the sample extract, %ACN or extraction strength, matrix washing time, extraction flow rate, and analytical flow rate. It must be highlighted that these variables were selected based upon reports from the literature and the authors' previous experience with similar analytical systems.

Table A.2 in the supplementary material illustrates the DoE and the values considered for each variable. Noteworthy, the experiments were randomly performed to mitigate other intrinsic effects which could arise during the study.

2.6.2. Central composite design

After determining the most influential variables through the $2^{(5-1)}$ fractional factorial design, a surface response method (the central composite design (CCD)) was employed for final optimization. This CCD comprises a two-level factorial design (2^3), a star design, and four central points. **Table A.3** (supplementary material) shows the variables considered and their values. Again, the experiments were randomly carried out. Following suggestions found in the literature, the central

composite design was chosen once it had already been successfully applied to analyze pesticides [33]. According to the obtained results from section 2.6.1, the variables considered in the CCD were: extraction flow, %H_2O or dilution of the sample extract, and %ACN or extraction strength.

2.7 Applications

2.7.1 Determination of nine pesticides in sugarcane crop soil

As mentioned in section 2.3, one of the solid matrices evaluated was sugarcane crop soil. We used such a matrix during the chemometric experiments and figures of merit evaluation, to assess the method's overall performance. The interest in it is due to the geographical location of São Carlos – SP – Brazil, surrounded by plenty of sugarcane crops, being it one of the most important agricultural activities of the state. It is worth mentioning that we got free-of-pesticides soil samples to use during the development stage when fortification with standard solutions was required.

After achieving an optimized condition through the chemometric experiments, the most critical analytical figures of merit were evaluated according to the International Council for Harmonisation (ICH) Q2(R1) guideline. The parameters studied were: limits of detection and quantification (LOD and LOQ), linearity, accuracy, and precision (intra- and inter-day). All experiments were performed using the matrix-matched method by applying blank soil samples previously tested for the absence of any signal related to the target pesticides. The LOQs and LODs were determined by utilizing the signal-to-noise ratio approach (LODs at concentration levels of 3:1 signal-to-noise ratio, while LOQs were considered at 10:1). The linearity was evaluated through the coefficient of determination (R^2) in five different concentration levels ranging from 10 ng g^{-1} to 125 ng g^{-1}. The experiments were conducted in triplicate (n=3) for each concentration level, being the calibration curve plotted as a function of the chromatographic peak area (y-axis) vs. its concentration levels (x-axis). Subsequently, the intra- and inter-day precision were evaluated by examining the relative standard deviations (%RSD) at three concentration levels (10, 50, and 125 ng g^{-1}) in two consecutive days. Also, the same concentration levels were used to assess the method accuracy, which was achieved by comparing the theoretical concentration levels (C_{tr}) to the experimental values (C_{exp}) obtained through the linearity equation for each pesticide.

In the end, the proposed analytical method was tested in soil samples collected from sugarcane crops of two different locations (Tatuí and São Carlos, both in the state of São Paulo – Brazil). Noteworthy, the sampling step was carried out in the middle of sugarcane crops by digging at a depth of around 15 cm. All these collected samples were previously processed according to section 2.3.

2.7.2 Other

In addition to the sugarcane crop soils, *Apis mellifera L.* Africanized honeybees were screened for the presence of the same pesticides. This idea arose because these insects are commonly found where the sugarcane crop soils were collected. So, there is a possibility of those individuals becoming contaminated by those pesticides upon pollinating such crops. Consequently, they would either transfer the pesticides to the honey and propolis or even die. Therefore, it has to be considered that in addition to being a relevant source of natural antibiotics and relevant foodstuffs, honeybees are

also an essential pollinating agent and environmental-quality indicator. So, the determination of pesticides in these biomarkers is of fundamental importance at this moment of intense and worldwide concern about environmental contamination. Of note, such an experiment was carried out only with qualitative purposes for testifying the proposed multidimensional system versatility to work with another complex solid matrix. Therefore, only a comparison between blank samples and spiked ones was evaluated at a concentration level of 100 ng g^{-1}.

3. Results and Discussion

3.1 Optimization of the analytical conditions

3.1.1 Fractional factorial design

Before the multivariate optimization, we carried out a series of univariate experiments to determine the exact mass ratio of solid matrix:co-sorbent. Also, we tested two different co-sorbents: C18 and Florisil. The choice for these co-sorbents is described in the literature for MSPD standard procedures [34]. Although Florisil reported satisfactory results, it showed the disadvantage of frequent cartridge clogging during the extraction step. So, since the first stages of this work, C18 was set for the study. Regarding the combination between solid:co-sorbent mass ratio, Figure A.1 in the supplementary material shows that 10:50 mg reported better analytical signals intensity and extraction efficiency for most pesticides. Additionally, the condition using 10:50 solid:co-sorbent reported cleaner chromatograms, suggesting that a more satisfactory clean-up was obtained when such a combination was employed.

The Fractional factorial design (2^{5-1}) was executed to investigate the ideal condition for the pesticides' extraction from soil samples. This DoE was first chosen to screen the most influential variables (V_x) related to the analytical system. The Pareto charts were used to illustrate the effects of $\%H_2O$ or dilution of the sample extract (V_1), %ACN or extraction strength (V_2), matrix washing time (V_3), extraction flow rate (V_4), and analytical flow rate (V_5). As shown in **Figure 3**, the most influential variables were V_1, V_2, and V_4, respectively. In this way, there was a positive effect on method performance when reducing the extraction flow and increasing both %ACN in the extraction step and the aqueous dilution of the sample extract ($\%H_2O$) before it enters the extraction column. The Pareto charts of the other pesticides reported a similar behavior – the same three variables as the most influential ones. Considering this 2^{5-1} DoE as only a part of the chemometric studies, these three most-significant variables (V_1, V_2, and V_4), were further evaluated by a surface response method (CCD). As the soil washing time and the analytical flow rate (V_3 and V_5) did not exhibit a substantial influence on the method performance, their values were chosen considering the fractional factorial design 2^{5-1}. Therefore, the following experiments were fixed in 10 µL min^{-1} for analytical flow rate and 3 minutes of soil washing time.

<Insert Fig.3>

3.1.2 Central composite design (CCD)

The final optimization step involved a CCD considering the three most influential variables reported from the first fractional factorial design (2^{5-1}). For this reason, this step included not only the default levels (low and high) but the center point (0) and "star" point (+ -) in order to achieve response surfaces suggesting the optimal condition for further experiments. The CCD response surface methodology measures the effect of one variable and its interactions with other ones. Within such a context, the response surfaces and the desirability function, both obtained from the CCD, were used to achieve the final optimized situation considering all pesticides involved.

As shown in **Figure 4a**, there is a positive correlation between medium values of the percentage of ACN and extraction flow, suggesting an optimal region around the middle portion of the response surface for such variables. In the sequence, **Figures 4b** and **4c** corroborate these prior results showing optimal regions when medium values of the percentage of ACN and extraction flow were combined with high aqueous dilution for the sample extract (%H_2O). This behavior can be explained by taking some considerations:

(i) Using high percentages of ACN (> 90%) in the cartridge containing the packed soil sample (first step) can be helpful to remove the pesticides from it. However, at the same time, it negatively interferes in the subsequent sorption of such analytes into the extraction column (second step). Considering this fact, 82% ACN was used in this step once it posses an adequate strength to remove the pesticides from the soil while not interfering so much in the subsequent sorption process.

(ii) A medium flow rate value during the extraction step seems to be more suitable, once higher values might increase the linear velocity and analytes' band dilution, which could negatively interfere with the sorption process inside the extraction column. A lower extraction flow rate could hinder the analytes from achieving a satisfactory interaction with the GO-Sil sorbent phase, thus decreasing the sorption process;

(iii) High aqueous dilution values (high %H_2O) for the sample extract eluted from the cartridge, was already expected to positively affect the results once it directly favors the sorption of the pesticides onto the GO-Sil extraction column.

Taking all these issues into consideration, the final optimized situation for the analytical procedure was: 35 μL min^{-1} of extraction flow, 82% of ACN in the cartridge-based extraction step, 42 μL min^{-1} of aqueous dilution of the sample extract (%H_2O), and the already optimized values of 10 μL min^{-1} for analytical flow rate and 3 minutes of soil washing time.

<Insert Fig.4>

3.2 Applications

3.2.1 Determination of nine pesticides in sugarcane crop soil

Figure 5 presents an illustrative chromatogram displaying a blank soil sample (last trace at the bottom) together with the SRM transitions referent to the individual pesticides spiked at concentration levels of 75 ng g^{-1}. As can be seen, no interfering chromatographic peaks overlap those of the analytes once monitoring specific MS/MS ion transitions for each pesticide. Therefore, the proposed analytical method can be considered selective. After testifying the method selectivity, the limits of quantification and detection were determined considering the signal-to-noise ratio of 3:1 and 10:1, respectively. So, the method reported a LOQ of 10 ng g^{-1} and a LOD of 7 ng g^{-1}. Subsequently to the determinations of these values, the linearity was assessed, considering the LOQ as the first point of concentration in the analytical curve.

<Insert Fig.5>

In the sequence, **Table 2** gathers the central values obtained from evaluating each figure of merit: linearity, coefficient of determination (R^2), linear equation, accuracy, and precision (intra- and inter-day). As illustrated, all pesticides have satisfactory performance, considering the R^2 values above 0.985 and their respective linear equations. Although most of the accuracy, intra-, and inter-day precision values have stood according to the criteria established by the ICH validation guideline (80-120% and RSD < 20%, respectively), some values have surpassed it. Nonetheless, as these results are related to a new strategy to achieve extraction, sample clean-up, and analysis in a unique multidimensional approach, they are satisfactory for this developmental stage and - in our opinion - promising. As this work focused on evaluating the cartridge-based solid sample preparation method, the study assessed some leading figures of merits. For this reason, we did not analyze a broad range of different soil samples even with the achieved results indicating the method as suitable for such purposes.

3.2.2 Other

To assess the cartridge-based sample preparation approach in other type of solid sample, lyophilized Africanized honeybee samples were also tested. **Figure A.2**, in the supplementary material, shows an illustrative chromatogram of a blank sample and the same sample spiked with the investigated pesticides at a concentration level of 75 ng g^{-1}. As can be seen from the chromatograms, there is a similarity between the chromatographic profile obtained from the analyzed soil samples (Figure 5) and the analyzed honeybee samples (Figure A.1). This result suggests that the proposed cartridge-based sample preparation approach might also be applied to other complex matrices, thus encouraging those researchers working with solid samples to search for additional solid matrix applications using the described or a similar system.

<Insert Table 2>

4. Concluding remarks

This paper describes an original approach to carry out sample preparation of raw solid matrices. So far, the main techniques used for such purposes are QuEChERS and MSPD, which have been reporting satisfactory results throughout the last years. Nonetheless, a widely known downside of them is the substantial amount of reagents and samples, in addition to steps, often required to perform standard procedures. For this reason, our idea was to present a greener alternative focusing on the reduction of reagents and samples required. In this manner, the proposed analytical method consumes approximately 490 uL of solvents per analysis, whereas it demands 10mg of soil or 20mg of lyophilized honeybees. For example, Acosta-Dacal [35] published a recent paper that monitors 218 pesticides in agricultural soil using QuEChERS combined with LC-MS/MS and GC-MS/MS. Despite its good performance, the greener characteristic presented in this work is evident once the economy on reagents is roughly 95% while using 90% less amount of soil. Likewise, Nedai et al. [36] reported an MSPD-based miniaturized approach to determine emerging pollutants in soil with excellent economies on samples, demanding only 20mg of samples. On the other hand, there is no significant economy of organic solvents once the authors have employed conventional HPLC, demanding higher mobile phase flow rates compared to our capillaryLC-based approach. Compared with the achieved LODs and LOQs presented in the proposed method, some of the publications just discussed have reached lower values. However, considering that our primary goal was to assess and propose a greener automated alternative, the proposed method – that still might be further optimized to achieve even lower LOQs (not the purpose of this report) can be considered a promising strategy to achieve such goals. Lastly, based on the progress carried out on this study and the additional analysis of honeybees briefly described here, we hope that these results encourage other studies focusing on the development of automated environmentally friendly sample preparation procedures especially designated for raw solid samples.

CRediT author statement

Edvaldo V. S. Maciel: Conceptualization, Methodology, Writing – Original Draft, Formal Analysis, and Investigation.

Fernando Mauro Lanças: Conceptualization, Methodology, Writing – Reviewing and Editing, Supervision, Resources, and Funding Acquisition.

Declaration of competing interest

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

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Figure Captions

Fig.1 Illustration of the stainless-steel cartridge used for packing the solid matrices: **(a)** general view of the assembled cartridge; **(b)** Internal parts highlighting the packed matrix together with co-sorbents (blend), and **(c)** heads of the device in which are located the metallic frits.

Fig.2 Schematic illustration of the multidimensional online sample-prep-capillaryLC-MS/MS system emphasizing its connections and fluidic paths. **(a)** Valve positions are set for the pesticides' extraction and sorption. **(b)** Valve positions set for the capillaryLC-MS/MS analysis of the pesticides.

Fig.3 Pareto charts obtained from the first fractional factorial design $2^{(5-1)}$.

Fig.4 Response surfaces obtained from the 2^3 central composite design optimization correlating the pesticides' extraction performance with the main variables (V_1, V_2, and V_3).

Fig.5 Illustrative chromatogram relating a soil blank sample (bottom trace) the SRM of the individual pesticides spiked at concentration levels of 75 ng g^{-1}.

Table Captions

Table.1 Experimental details related to each step of the multidimensional sample-prep-capillaryLC-MS/MS approach used to extract pesticides from soil samples.

Table.2 Some critical figures of merit considered during the study and their respective reported values for each pesticide.

Figure Captions

Fig.1 Illustration of the stainless-steel cartridge used for packing the solid matrices: **(a)** general view of the assembled cartridge; **(b)** Internal parts highlighting the packed matrix together with co-sorbents (blend), and **(c)** heads of the device in which are located the metallic frits.

Fig.2 Schematic illustration of the multidimensional online sample-prep-capillaryLC-MS/MS system emphasizing its connections and fluidic paths. **(a)** Valve positions are set for the pesticides' extraction and sorption. **(b)** Valve positions set for the capillaryLC-MS/MS analysis of the pesticides.

Fig.3 Pareto charts obtained from the first fractional factorial design $2^{(5-1)}$.

Fig.4 Response surfaces obtained from the 2^3 central composite design optimization correlating the pesticides' extraction performance with the main variables (V_1, V_2, and V_3).

Fig.5 Illustrative chromatogram relating a soil blank sample (bottom trace) the SRM of the individual pesticides spiked at concentration levels of 75 ng g^{-1}.

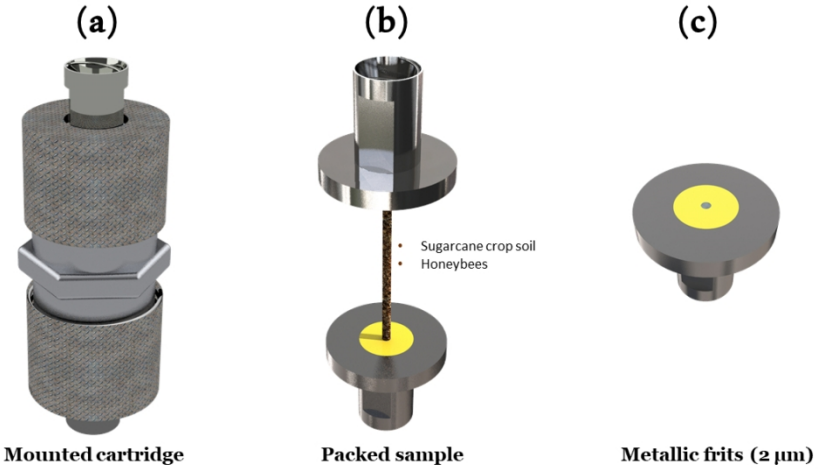
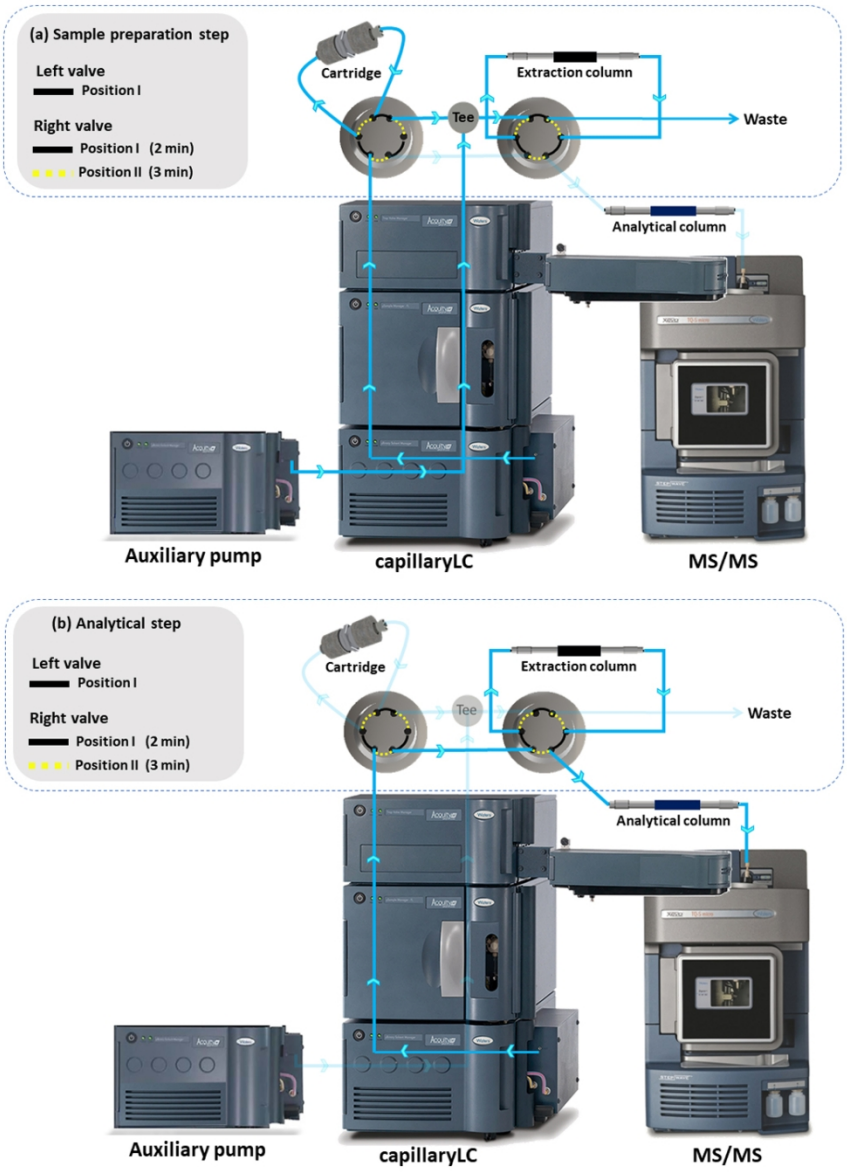


Fig.1 Illustration of the stainless-steel cartridge used for packing the solid matrices: (a) general view of the assembled cartridge; (b) Internal parts highlighting the packed matrix together with co-sorbents (blend), and (c) heads of the device in which are located the metallic frits.

338x190mm (96 x 96 DPI)



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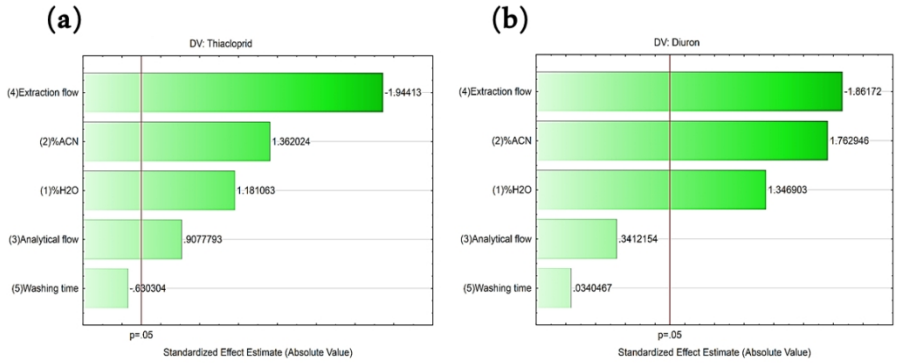

Fig.3 Pareto charts obtained from the first fractional factorial design 2(5-1).

338x190mm (96 x 96 DPI)

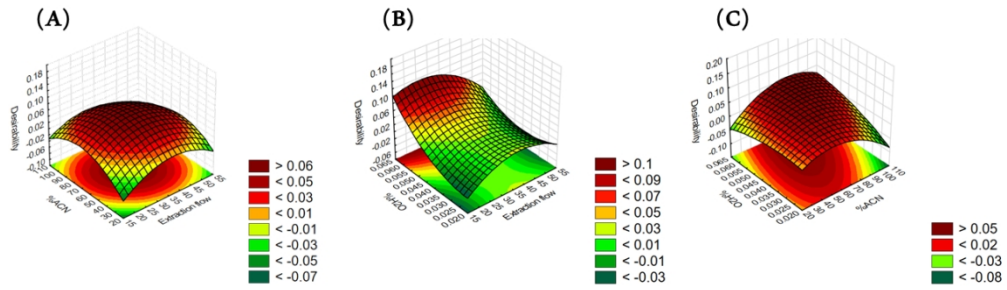

Fig.4 Response surfaces obtained from the 23 central composite design optimization correlating the pesticides' extraction performance with the main variables (V1, V2, and V3).

338x190mm (96 x 96 DPI)

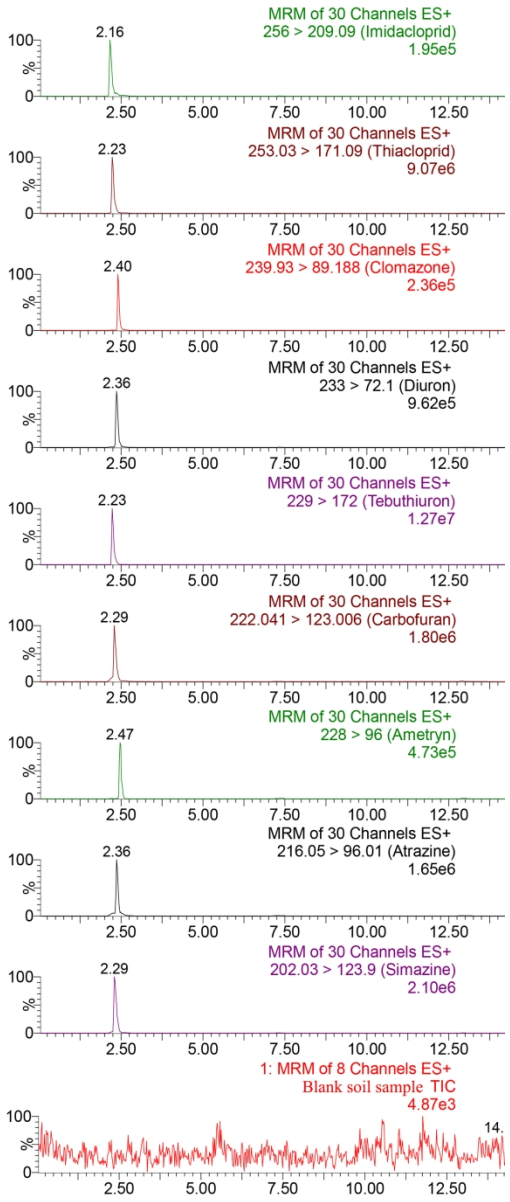

Fig.5 Illustrative chromatogram relating a soil blank sample (bottom trace) the SRM of the individual pesticides spiked at concentration levels of 75 ng g-1.

119x276mm (300 x 300 DPI)

Table.1 Experimental details related to each step of the multidimensional sample-prep-capillaryLC-MS/MS approach used in the extraction of pesticides from soil samples.

Event	Time (min)	Auxiliary pump (H_2O)	capillaryLC pump		
		Flow rate (µL min^{-1})	Flow rate (µL min^{-1})	H_2O (%)	ACN (%)
Washing	3.0	off	100	99.9	1.0
Extraction/sorption	3.0	42	35	18.0	82.0
Analysis*	0.0	off	10	70.0	30.0
	1.0		10	70.0	30.0
	4.0		10	0.1	99.9
	5.0		10	0.1	99.9
	5.5		10	70.0	30.0
	7.5		10	70.0	30.0

* For analysis event, please consider the time (min) as progressive because is an elution gradient instead of isocratic mode as in the washing and the extraction/sorption steps.

Table.2 Some important figures of merit considering during the study and their respective reported values for each pesticide.

Pesticide	Linear equation	R^2	Concentration (ng g^{-1})	Accuracy (n=3)	Precision (RSD%) (n=3)	
					day 1	day 2
Imidaclopri d	y = 3462.6x - 23880	0.9967	10	80.4	12.3	15.2
			50	106.5	17.8	16.1
			125	120.4	22.1	24.0
Thiacloprid	y = 20319x - 56298	0.990 9	10	80.1	2.8	5.7
			50	107.7	5.0	2.3
			125	121.7	18.6	17.2
Clomazone	y = 1379.1x - 17067	0.9971	10	124.5	11.7	13.7
			50	98.8	9.8	12.0
			125	124.7	24.6	18.8
Diuron	y = 4896.4x - 60503	0.9922	10	124.4	9.1	17.6
			50	96.6	13.6	17.3
			125	122.7	20.3	13.2
Tebuthiuron	y = 653.91x - 7597.3	0.9933	10	125.0	16.0	9.9
			50	104.6	19.8	21.1
			125	118.9	10.4	13.6
Carbofuran	y = 37658x - 474108	0.9927	10	126.6	8.8	11.3
			50	80.9	14.2	12.9
			125	123.4	19.71	20.1
Ametrine	y = 6861.5x - 70274	0.988 3	10	106.3	14.3	12.8
			50	114.2	15.2	17.9
			125	123.4	12.1	19.8
Atrazine	y = 3572.7x + 78966	0.9855	10	112.8	9.6	13.5
			50	94.1	10.0	6.5
			125	120.3	20.5	18.3
Simazine	y = 1272.9x - 11115	0.9901	10	105.6	15.4	10.3
			50	95.8	13.36	9.4
			125	121.2	13.5	15.2

Supplementary material

A cartridge-based device for automated analysis of solid matrices by online multidimensional sample-prep-capillaryLC-MS/MS

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Table A.1 – MS parameters related to the most intense ion transitions used for quantification and identification of each pesticide.

Pesticide	Precursor Ion (*m/z*)	Quantitative transition			Qualitative transition			
		Product Ion (*m/z*)	CE (V)*	Cone Voltage (V)	Product Ion (*m/z*)	t_R (min)	CE (V)	Cone Voltage (V)
Simazine	202.30	123.90	18	28	131.94	8.28	18	28
Atrazine	216.50	103.95	26	22	96.01	9.94	20	22
Carbofuran	222.04	123.01	10	12	165.09	9.41	34	12
Tebuthiuron	229.00	67.55	34	10	116.90	7.26	26	36
Diuron	233.00	46.30	14	34	72.10	10.16	18	34
Ametryn	228.00	96.00	28	18	67.90	10.60	36	18
Clomazone	239.93	89.19	46	50	124.96	10.68	16	50
Thiacloprid	253.03	70.99	28	14	171.09	7.48	14	14
Imidacloprid	256.00	209.14	19	24	209.09	5.66	14	54

*CE: Collision Energy

Table A.2 – Variables and experimental range considering in the DoE $2^{(5-1)}$ during the screening experiments for optimization of the method.

Standard Run	Fractional factorial design: $2^{(5-1)}$					
	Replica	% H_2O	%ACN	Analytical flow	Extraction flow	Washing time
1	1	0.300000	70.00000	6.00000	25.00000	5.000000
2	1	0.420000	70.00000	6.00000	25.00000	3.000000
3	1	0.300000	90.00000	6.00000	25.00000	3.000000
4	1	0.420000	90.00000	6.00000	25.00000	5.000000
5	1	0.300000	70.00000	10.00000	25.00000	3.000000
6	1	0.420000	70.00000	10.00000	25.00000	5.000000
7	1	0.300000	90.00000	10.00000	25.00000	5.000000
8	1	0.420000	90.00000	10.00000	25.00000	3.000000
9	1	0.300000	70.00000	6.00000	40.00000	3.000000
10	1	0.420000	70.00000	6.00000	40.00000	5.000000
11	1	0.300000	90.00000	6.00000	40.00000	5.000000
12	1	0.420000	90.00000	6.00000	40.00000	3.000000
13	1	0.300000	70.00000	10.00000	40.00000	5.000000
14	1	0.420000	70.00000	10.00000	40.00000	3.000000
15	1	0.300000	90.00000	10.00000	40.00000	3.000000
16	1	0.420000	90.00000	10.00000	40.00000	5.000000
17	2	0.300000	70.00000	6.00000	25.00000	5.000000
18	2	0.420000	70.00000	6.00000	25.00000	3.000000
19	2	0.300000	90.00000	6.00000	25.00000	3.000000
20	2	0.420000	90.00000	6.00000	25.00000	5.000000
21	2	0.300000	70.00000	10.00000	25.00000	3.000000
22	2	0.420000	70.00000	10.00000	25.00000	5.000000
23	2	0.300000	90.00000	10.00000	25.00000	5.000000
24	2	0.420000	90.00000	10.00000	25.00000	3.000000
25	2	0.300000	70.00000	6.00000	40.00000	3.000000
26	2	0.420000	70.00000	6.00000	40.00000	5.000000
27	2	0.300000	90.00000	6.00000	40.00000	5.000000
28	2	0.420000	90.00000	6.00000	40.00000	3.000000
29	2	0.300000	70.00000	10.00000	40.00000	5.000000
30	2	0.420000	70.00000	10.00000	40.00000	3.000000
31	2	0.300000	90.00000	10.00000	40.00000	3.000000
32	2	0.420000	90.00000	10.00000	40.00000	5.000000

Table A.3 – Variables and experiments considering in the central composite design 2^3 for the optimization of the method.

Standard Run	$2^{(3)}$ central composite design			
	Block	Extraction flow	%ACN	%H2O
8	1	45.00000	82.00000	0.050000
3	1	25.00000	82.00000	0.030000
10 (C)	1	35.00000	62.00000	0.040000
4	1	25.00000	82.00000	0.050000
9 (C)	1	35.00000	62.00000	0.040000
6	1	45.00000	42.00000	0.050000
7	1	45.00000	82.00000	0.030000
5	1	45.00000	42.00000	0.030000
2	1	25.00000	42.00000	0.050000
1	1	25.00000	42.00000	0.030000
16	2	35.00000	62.00000	0.057889
15	2	35.00000	62.00000	0.022111
11	2	17.11146	62.00000	0.040000
13	2	35.00000	26.22291	0.040000
17 (C)	2	35.00000	62.00000	0.040000
14	2	35.00000	97.77709	0.040000
12	2	52.88854	62.00000	0.040000
18 (C)	2	35.00000	62.00000	0.040000

Figure A.1 – Illustrative chromatograms obtained from the experiment to test sample:co-sorbent mass ratio (mg) (n=3): **(a)** 25:35 mg; **(b)** 15:45 mg; **(c)** 10:60 mg. As can be seen, the lower mass of sample combined with the larger amount of C18 reported the most intensity and "cleaner" chromatogram.

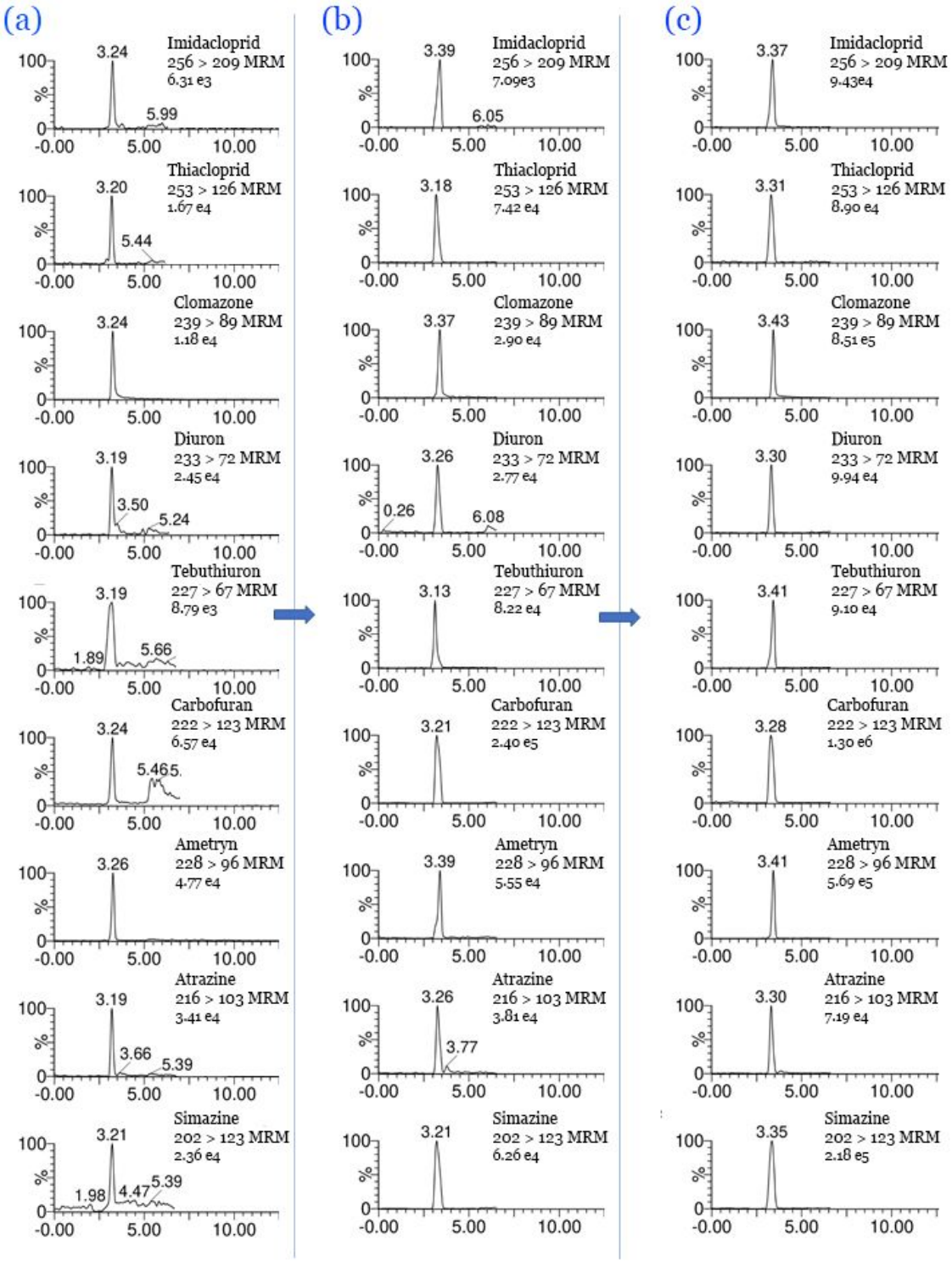
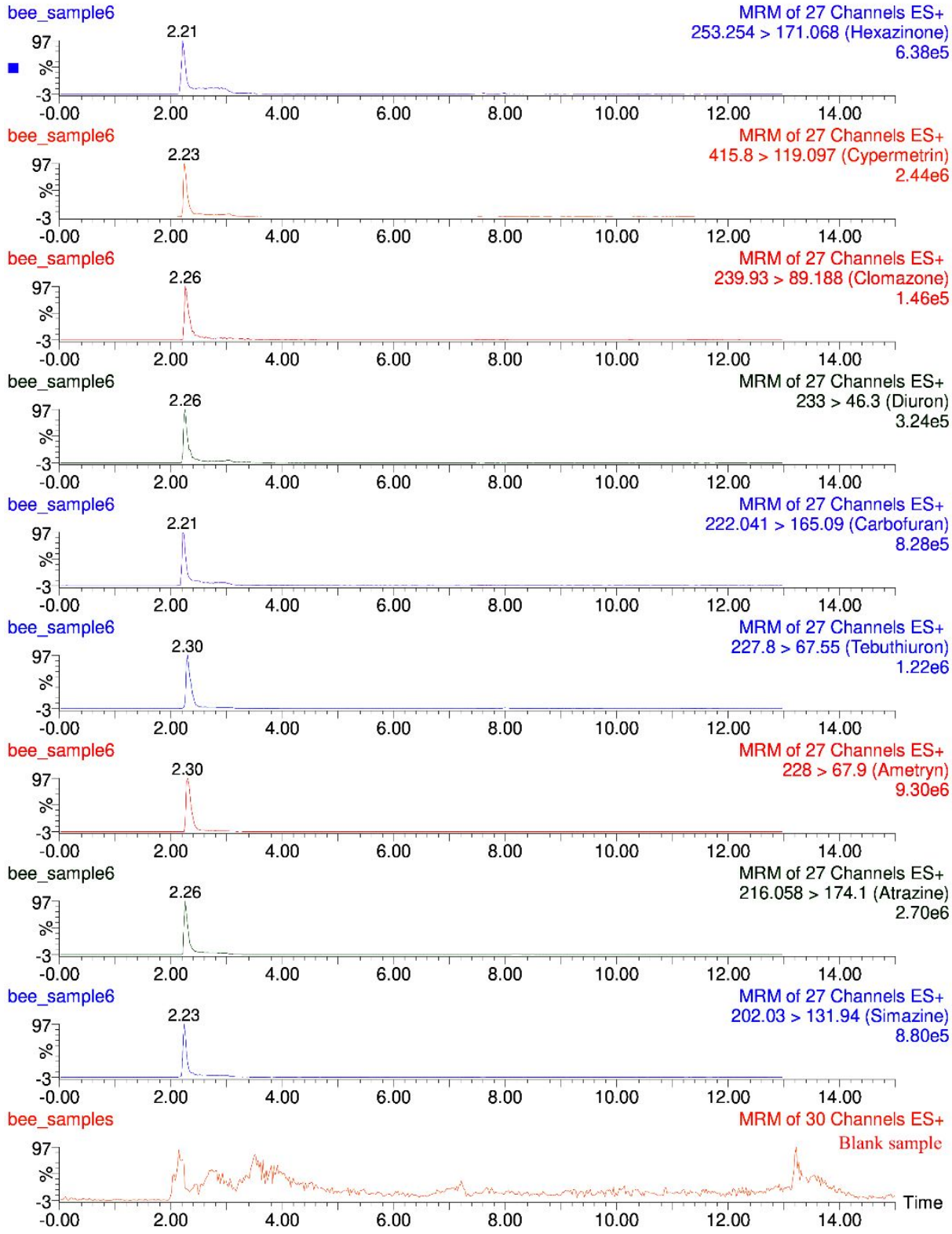


Figure A.2 – Illustrative chromatogram of a blank sample of Africanized honeybee and the same one spiked with the pesticides at a concentration level of 75 μg L^{-1}



CAPÍTULO 9

Conclusões e perspectivas futuras

Neste trabalho foram desenvolvidos mais de um tipo de método multidimensional para o preparo da amostra automatizado acoplado a cromatografia líquida e a espectrometria de massa. Especificamente, o autor reportou o desenvolvimento de método automatizado parcialmente miniaturizado – coluna de extração miniaturizada e coluna analítica convencional, e método automatizado totalmente miniaturizado – coluna de extração e coluna analítica miniaturiada. Para testar a versatilidade e eficiência dos métodos criados, estes foram utilizados para uma variedade de aplicações como a análise de fármacos em fluídos biológicos, e de micotoxinas e fármacos em bebidas alcólicas e não alcólicas.

Complementarmente, o autor reportou o desenvolvimento de um método parcialmente automatizado e totalmente miniaturizado para preparo de amostras sólidas, com posterior análise por cromatografia líquida e espectrometria de massas. Neste caso o intuito era iniciar o desenvolvimento de uma nova proposta para a extração, sorção, separação e detecção de analitos com o mínimo de interveção possível do analista – uma forma semi-automatizada de analisar amostras sólidas que são geralmente processadas via "*matrix solid-phase dispersion"* ou *"quick, easy, effective, rugger and safe, QuEChERS"*. Como prova de conceito, o sistema foi testado para a análise de nove pesticidas presentes em amostras de solo provenientes de região de cultivo de cana-de-açucar e em corpos de abelha retiradas das mesmas regiões.

Todos estes métodos apresentaram resultados satisfatórios em comparação com os métodos convencionais e não automatizados tradicionalmente utilizados. O ponto positivo a ser destacado, é a economia expressiva no uso de reagentes e amostra e a consequente diminuição na geração de resíduos e redução no tempo total de análise, resultando em aumento na produtividade analítica. Além disso, o fato do emprego da coluna de extração "isolar" o sistema analítico de um contato direto com a matriz, diminúi o efeito da matriz no resultado final. Isso é um fator importantíssimo em sistemas de LC-MS que utilizam fonte de ionização por electrospray. Um outro ponto importante é que para todos os métodos desenvolvidos, as colunas de extração foram produzidas pelo autor deste trabalho e são frutos de uma publicação inicial focada na otimização de produção dessas colunas (Capítulo 5).

O outro objetivo chave para o sucesso deste trabalho era a síntese de materiais a base de grafeno e o seu emprego como fase extratora na produção das colunas de extração. O autor conseguiu sintetizar e empacotar colunas de extração constituídas de

GO, SiGO, e SiGOC18$_{edc}$. Dentre estes materiais, os dois ultimos foram os que apresentaram melhor capacidade de extração para os analitos estudados neste projeto. Quando comparados com fases comerciais, os materiais sintetizados tiveram performance equiparável. No entanto, o simples processo de síntese com baixo custo, capacidade de funcionalização e customização, aliados a robustez do material, podem ser destacadas como vantagens frente aos comercialmente disponíveis. No caso da robustez, estima-se que a coluna de extração empacotada com SiGO com maior capacidade de extração e, consequentemente, utiliza com maior frequência durante o doutorado, tenha participado de aproximadamente 1000 análises incluindo injeção de padrões analíticos e amostras reais.

De modo geral, este trabalho reafirma o papel importante e atual que os métodos de preparo de amostra automatizados – neste caso baseados em "*column switching*" – podem exercer dentro de uma química analítica mais ecológicamente correta, equacionando de maneira efetiva o consumo e geração de resíduos. Como perspectivas futuras, o autor deste trabalho gostaria de destacar, resumidamente e de maneira sistemática, algumas das direções que vê como promissoras dentro do assunto foco:

(i) Após evidenciadas as características principais dos métodos analíticos aqui desenvolvidos, creio que a utilização dos mesmos para outros tipos de aplicações que este trabalho não abordou possa ser uma vertente. Aqui por exemplo, focamos mais na extração, separação/detecção de móleculas pequenas (resíduos e contaminantes), portanto abrindo oportunidade para outros pesquisadores focarem no emprego de tais sistemas dedicados a análise de macromoléculas.

(ii) Os materiais a base de grafeno sintetizados reportaram performance excelente como fase extratora. Desta forma, pode-se imaginar sua utilização, após pequenas adaptações, como fases estacionárias para cromatografia líquida.

(iii) Por fim, devido ao aprendizado e a paixão pela ciência que adquiri durante o doutorado, gostaria de salientar que como perspectivas futuras pessoais gostaria de continuar trabalhando no desenvolvimento de métodos multidimensionais automatizados para extração, sorção, separação e detecção de compostos em matrizes complexas. Devido a experiência adquirida com moléculas pequenas e materiais a base de grafeno, prevejo um futuro focado em outras classes de analitos – de maior peso molecular – e na síntese de outras fases seletivas para emprego como sorvente e também fase estacionária para cromatografia.