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Miniaturized Approaches for Sample Preparation in Hair Testing for Drugs of Abuse and Their Application in Clinical and Forensic Scenarios

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Dedication

Dedicated to my mother for letting me dream. And to my father for his unconditional support. It's been quite a ride.

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Preface

And those who were seen
dancing
were thought to be insane
by those
who could not hear
the music.

- Friedrich Nietzsche

WOULD YOU BELIEVE IN WHAT YOU BELIEVE IN
IF YOU WERE THE ONLY ONE WHO BELIEVED IT?
-KANYE WEST

Resumo alargado

A amostra de cabelo é uma das matrizes alternativas mais importantes. A sua análise foi relatada pela primeira vez no final dos anos 70, e desde então permitiu ajudar os toxicologistas nos mais diversos campos de atuação.

Uma vez que as drogas são bastante estáveis nesta amostra, a sua análise proporciona a avaliação de histórias de uso de drogas ocorridas há centenas de anos, com grande interesse antropológico, mas também pode ser importante na resolução de casos forenses em que o cabelo é a única amostra obtida a partir de um cadáver. Já no indivíduo vivo, esta amostra é atualmente bastante utilizada para detetar xenobióticos (drogas de abuso, produtos farmacêuticos, contaminantes ambientais, agentes dopantes, etc.) em âmbito forense, em situações de renovação da carta de condução, avaliação do cumprimento da terapia de substituição de drogas, medicina ocupacional, avaliação e documentação de situações de abuso de álcool, entre outras.

O facto de poder ser colhida sob supervisão com reduzida probabilidade de adulteração, e a elevada estabilidade são apontadas como as suas principais vantagens.

Tal como acontece com as matrizes convencionais, a preparação das amostras revela-se uma etapa importante para a eliminação de interferentes e pré-concentração de analitos, influenciando significativamente a confiabilidade e a precisão da análise. Relativamente a esta matriz, as técnicas de preparação de amostra usadas não diferem das adotadas para outras matrizes, restringindo-se às clássicas extração em fase sólida (SPE) e extração líquido-líquido (LLE). No meio académico, estas técnicas convencionais são atualmente consideradas métodos do passado.

Na última década testemunhou-se um rápido desenvolvimento de novas técnicas de preparação de amostras, existindo uma grande tendência para a miniaturização.

O uso de técnicas miniaturizadas para pré-concentração permite ainda automatização, desempenho de alto rendimento, acoplamento online com instrumentos analíticos, resultando em baixos custos por análise devido ao reduzido consumo de solventes. As técnicas de microextração, como a microextração em fase líquida e microextração em fase sólida, apresentam essas vantagens sobre as abordagens clássicas, no entanto, a sua aplicabilidade em amostras de cabelo ainda está pouco explorada.

Esta dissertação tem como objetivo demonstrar o estado atual das abordagens miniaturizadas para concentração de analitos em amostras de cabelo, as vantagens da

microextração em seringa empacotada (MEPS) em diferentes campos analíticos e a sua aplicabilidade prática em amostras de cabelo com três trabalhos diferentes: determinação de opioides; determinação de metadona e EDDP, determinação de cocaína e metabolitos usando cromatografia gasosa acoplada à espectrometria de massa em tandem (GC-MS/MS).

Relativamente ao estado atual das abordagens miniaturizadas aplicadas a amostras de cabelo, é possível afirmar que existiu um aumento na investigação envolvendo tanto a microextração em fase sólida (SPME) como em fase líquida (LPME), sendo que a SPME teve maior representatividade.

Nesta última abordagem, a técnica de microextração em fase sólida dispersiva (D-µ-SPE) surge como a mais explorada nos últimos 5 anos, se não considerarmos em conjunto as diferentes variantes da microextração com fibra, nomeadamente a de imersão direta (DI-SPME), *headspace* (HS-SPME) ou capilar (IT-SPME). Adicionalmente, a D-µ-SPE foi a que apresentou mais novidades relativamente a sorbentes sólidos, impulsionada por desenvolvimentos com nanotubos de carbono, grafeno, óxido de grafeno (GO), para além do uso de nanopartículas magnéticas modificadas (MNPs) e polímeros impressos com iões (IIPs).

No que se refere à LPME, o uso de fibra oca (HF)-LPME tem sido cada vez mais explorado em amostras de cabelo, revelando grande versatilidade em relação aos analitos alvo. Foram também observados aperfeiçoamentos através da funcionalização com GO e com a utilização de líquidos iónicos (IL). No entanto, se todas as variantes da microextração líquido-líquido dispersiva (DLLME) forem consideradas em conjunto, esta é sem dúvida a abordagem mais investigada. A inclusão de solidificação de gota orgânica flutuante (DLLME-SFO), solventes supramoleculares (SM-DLLME) e líquidos iónicos de temperatura controlada (TIL-DLLME) apresentaram como principal vantagem a menor toxicidade.

Dentro de todas as técnicas de microextração aplicadas a amostras de cabelo, a MEPS surge como pouco explorada. De facto, à data do início deste projeto, apenas um trabalho havia aplicado a MEPS para isolar compostos a partir de amostras de cabelo. Contudo, a MEPS tem sido implementada com sucesso para extrair uma ampla gama de compostos a partir de diferentes matrizes. A sua aplicabilidade está comprovada nas mais diversas áreas, tais como monitorização terapêutica, toxicologia forense, bem como em análises alimentares e ambientais.

Esta técnica é reconhecida como uma miniaturização da clássica SPE e foi desenvolvida em 2004 por Abdel-Rehim com o objetivo de reduzir o volume de amostra e solvente usados. Tornou-se ainda bastante atrativa por permitir a reutilização do material sorbente e proporcionar um procedimento automatizado através do fácil acoplamento

aos sistemas cromatográficos. Na MEPS o enchimento é reduzido (1-4 mg) e fica localizado numa micro-seringa em vez de num cartucho. Por sua vez, a amostra flui através do enchimento de forma bidirecional (aspirações), melhorando assim a eficiência do processo devido ao aumento do contacto entre a amostra e o sorbente.

Tendo em conta todas as potencialidades que a MEPS apresenta no âmbito de preparação de amostras, decidiu-se testar a sua utilidade para amostras de cabelo em três aplicações.

Na primeira procedeu-se ao desenvolvimento e validação de um método analítico para determinar tramadol (TRM), codeína (COD), morfina (MOR), 6- acetilcodeína (6-AC), 6-monoacetilmorfina (6-MAM) e fentanil (FNT) em amostras de cabelo com recurso à GC-MS/MS. Utilizando um sorbente M1 (4 mg; 80% C_8 e 20% SCX), o procedimento englobou os seguintes passos: (*i*) acondicionamento (3 x 250 µL de metanol e 3 x 250 µL de ácido fórmico 2%); (*ii*) passagem da amostra (15 x 150 µL); (*iii*) lavagem (150 µL de ácido fórmico a 3,36%); e (*iv*) eluição (8 x 100 µL de hidróxido de amónio 2,36% em metanol). Obteve-se linearidade para todos os compostos entre o limite inferior de quantificação (LLOQ) e 5 ng/mg, com coeficientes de determinação superiores a 0,99. Os LLOQs alcançados foram 0,01 ng/mg para TRM, COD e 6-AC e 0,025 ng/mg para MOR, 6-MAM e FNT. As recuperações variaram entre 74 e 90% (TRM), 51 e 59% (COD), 22 e 36% (MOR), 69 e 99% (6-AC), 53 e 61% (6-MAM) e 75 e 86% (FNT). O método revelou-se preciso e exato com coeficientes de variação tipicamente abaixo de 15% e erros relativos dentro de um intervalo de ± 15%, respetivamente.

Na segunda aplicação, desenvolveu-se um procedimento para a rápida concentração de metadona e do seu principal metabolito (EDDP). A abordagem miniaturizada foi acoplada a GC-MS/MS. A MEPS foi efetuada com um sorbente M1 (4 mg; 80% C_8 e 20% SCX) acondicionado com três ciclos de 250 µL de metanol e três ciclos de 250 µL de ácido fórmico a 2%. Posteriormente, a passagem da amostra fez-se com nove ciclos de 150 µL seguida de uma etapa de lavagem que envolveu três ciclos de 50 µL com ácido fórmico 3,36%. Para a eluição dos compostos, foram aplicados seis ciclos de 100 µL de hidróxido de amónio a 2,36% em metanol. O método foi linear de 0,01 a 5 ng/mg para ambos os compostos, apresentando coeficientes de determinação superiores a 0,99. As recuperações variaram entre 73 e 109% para metadona e 84 e 110% para EDDP. Por fim, a precisão e a exatidão estavam de acordo com os postulados das diretrizes internacionais para validação de métodos analíticos.

Na terceira aplicação foi desenvolvida um método, também com recurso ao sorbente M1 (4 mg; 80% C_8 e 20% SCX), para pré-concentração de cocaína (COC), benzoilecgonina (BEG), ecgonina metil éster (EME), norcocaína (NCOC), cocaetileno (COET) e anidroecgonina metil éster (AEME). A determinação dos compostos foi mais uma vez

realizada com recurso à GC-MS/MS. O procedimento final consistiu nos seguintes passos: (*i*) acondicionamento (250 µL de metanol e 250 µL de água desionizada); (*ii*) passagem da amostra (21 x 150 µL); (*iii*) lavagem (50 µL de água desionizada e 50 µL de tampão acetato pH 4); e (*iv*) eluição (3 x 100 µL de hidróxido de amónio a 2%em metanol). As recuperações obtidas foram consideradas aceitáveis para a maioria dos compostos, nomeadamente 44-64% para COC, 63-73% para COET, 21-28% para BEG e 36-44% para NCOC. Foram obtidas recuperações mais baixas para AEME (4-6%) e EME (1-3%). O método foi linear entre os LLOQs e 5 ng/mg, sendo que os LLOQs foram 0,010 ng/mg para a COC e COET, 0,025 ng/mg para a EME, BEG e NCOC e 0,150 ng/mg para a AEME. Por sua vez, o método foi considerado também preciso e exato com coeficientes de variação inferiores a 15%, e com um erro relativo médio dentro de ± 15% para todos os compostos, exceto para o LLOQ (20%).

Os três trabalhos tiveram uma etapa de otimização do procedimento de extração, a qual foi facilitada pelo uso do desenho experimental (DOE). O DOE é uma das principais ferramentas estatísticas com implementação na investigação e na indústria. Esta ferramenta permite o delineamento experimental, através de um número de ensaios definidos, com o objetivo de avaliar a influência de diversos fatores (variáveis) nas respostas obtidas de um processo. O DOE tornou-se vantajoso porque permitiu a otimização dos procedimentos analíticos através de um número reduzido de ensaios sem prejuízo da qualidade da informação obtida. Para além disso, permitiu ainda o estudo simultâneo das diferentes variáveis passiveis de afetar a MEPS.

Com a aplicação bem-sucedida da MEPS nos trabalhos supracitados, foi possível comprovar que esta técnica miniaturizada se torna uma excelente alternativa para análises toxicológicas em amostras de cabelo. A MEPS é bastante vantajosa, reduzindo o uso de solventes e permitindo a reutilização do sorbente (> 100 extrações), o que pode ser economicamente atraente para laboratórios.

Embora nos últimos cinco anos as abordagens miniaturizadas tenham despertado um grande interesse académico, poucas implementações têm sido observadas em laboratórios de análises de rotina. Com a relevância e aplicabilidades aqui descritas, esperamos que esse panorama mude num futuro próximo.

Palavras-chave

Cabelo; Drogas de abuso; Microextração; MEPS; Toxicologia.

Abstract

Hair is nowadays one of the most important alternative matrices that have attracted attention for the analysis of various drugs. The fact that it can be collected under supervision, the lower probability of tampering with, and the greater stability are cited as major advantages. As with conventional matrices, the preparation of hair samples is an important step for clean-up and pre-concentration of analytes, which significantly affects the reliability and accuracy of the analysis.

The use of miniaturized pre-concentration techniques, driven by the concept of "green chemistry", has minimized the waste usually associated to classical techniques, and microextraction techniques are known for using lower solvent volumes and for saving time; however, their applicability to hair samples is still poorly explored.

The aim of this dissertation is to discuss the status of miniaturized clean-up approaches for hair samples. In addition, the advantages of microextraction by packed sorbent (MEPS) in different analytical fields are addressed, and the practical applicability of this technique in hair samples is demonstrated by three different works using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS): determination of selected opioids; determination methadone and EDDP; determination of cocaine and metabolites. These novel methods were optimized and validated according to internationally accepted guidelines.

Regarding the current status of the application of miniaturized approaches to hair samples, an increased research has been observed in both solid-phase (SPME) and liquid phase microextraction (LPME), with SPME showing higher representativeness.

In this last approach, dispersive micro-solid phase extraction (D-μ-SPE) emerges as the most used in the last 5 years, if we do not include the different variants of fibre microextraction, namely the direct immersion (DI-SPME), headspace (HS-SPME) or in tube (IT-SPME) approaches. Moreover, D-μ-SPE was the one that showed more innovations in terms of solid sorbent material, driven by developments with carbon nanotubes, graphene, graphene oxide (GO), and the use of modified magnetic nanoparticles (MNPs) and ion-imprinted polymers (IIPs).

As for LPME, the use of hollow fibre (HF)-LPME has been extensively explored for hair samples, showing great versatility for target analytes. Improvements were also observed by functionalization with GO and by the use of ionic liquids (IL). However, when all variants of dispersive liquid-liquid microextraction (DLLME) are considered, this is undoubtedly the most researched approach. The inclusion of methods involving solidification of a floating organic drop (DLLME-SFO), supramolecular solvents (SM-

DLLME), and temperature-controlled ionic liquids (TIL-DLLME) were considered very beneficial due to their lower toxicity.

Of all the microextraction techniques applied to hair samples, MEPS seems to be little explored. In fact, before this project was initialized , there was only one paper that had applied MEPS to pre-concentrate analytes from hair samples. Nonetheless, MEPS has been successfully used to extract a variety of compounds from different matrices, and its applicability has been demonstrated in a number of areas, including therapeutic monitoring, forensic toxicology, and food and environmental analysis.

This technique is considered as a miniaturization of the classical solid phase extraction (SPE) and was developed in 2004 by Abdel-Rehim with the aim of reducing the volumes of both the sample and the solvents. It also became very attractive as it allows the reuse of the sorbent material and offers an automated procedure by easy coupling to chromatographic systems. In MEPS, the sorbent is reduced (1-4 mg) and is located in a micro-syringe instead of a cartridge. In turn, the sample flows bidirectionally through the sorbent (aspirations), improving the efficiency of the process due to the increased interaction with the sorbent.

Considering the potential of MEPS in the context of sample preparation, we decided to test its usefulness for hair samples in three applications.

In the first work, we present an analytical method which was developed and validated for the determination of tramadol (TRM), codeine (COD), morphine (MOR), 6-acetylcodeine (6-AC), 6-monoacetylmorphine (6-MAM) and fentanyl (FNT) using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). Using an M1 sorbent (4 mg; 80% C_8 and 20% SCX), the procedure included the following steps: (i) conditioning (3 x 250 µL of methanol and 3 x 250 µL of 2% formic acid); (ii) sample load (15 x 150 µL); (iii) washing (150 µL of 3.36% formic acid); and (iv) elution (8 x 100 µL of 2.36% ammonium hydroxide in methanol). Linearity was obtained for all compounds between the lower limit of quantification (LLOQ) and 5 ng/mg, with determination coefficients higher than 0.99. The obtained LLOQs were 0.01 ng/mg for TRM, COD and 6-AC and 0.025 ng/mg for MOR, 6-MAM and FNT. The recoveries ranged from 74 to 90% (TRM), 51 to 59% (COD), 22 to 36% (MOR), 69 to 99% (6-AC), 53 to 61% (6-MAM) and 75 to 86% (FNT). The method proved to be precise and accurate with coefficients of variation typically below 15% and relative errors within a range of ± 15%, respectively.

In the second work, a procedure was developed for the rapid concentration of methadone and its main metabolite (EDDP). The miniaturized approach was coupled to GC-MS/MS. MEPS was performed with an M1 (4 mg; 80% C_8 and 20% SCX) sorbent conditioned with three cycles of methanol (250 µL) and three cycles of 2% formic acid (250 µL). Subsequently, the sample was loaded through nine cycles of 150 µL followed by a washing

step that involved three cycles of 50 µL with 3.36% formic acid. For the elution of the compounds, six cycles of 100 µL with 2.36% ammonium hydroxide in methanol were used. The method was linear from 0.01 to 5 ng/mg for both compounds, with determination coefficients greater than 0.99. The recoveries ranged from 73 to 109% for methadone and from 84 to 110% for EDDP. Finally, precision and accuracy were in accordance with the international guidelines for analytical method validation.

In the third work, a MEPS technique, with M1 (4 mg; 80% C_8 and 20% SCX) sorbent, was developed for the pre-concentration of cocaine (COC), benzoylecgonine (BEG), ecgonine methyl ester (EME), norcocaine (NCOC), cocaethylene (COET) and anhydroecgonine methyl ester (AEME). The determination of the compounds was carried out using GC-MS/MS. The final procedure consisted of the following steps: (i) conditioning (250 µL of methanol and 250 µL of deionized water); (ii) sample load (21 x 150 µL); (iii) washing (50 µL of deionized water and 50 µL of acetate buffer pH 4); and (iv) elution (3 x 100 µL of 2% ammonium hydroxide in methanol). The obtained recoveries were considered acceptable for most compounds, namely 44-64% for COC, 63-73% for COET, 21-28% for BEG and 36-44% for NCOC. Lower recoveries were obtained for AEME (4-6%) and EME (1-3%). The method was linear between the LLOQs (0.010 ng/mg for COC and COET, 0.025 ng/mg for EME, BEG and NCOC and 0.150 ng/mg for AEME) and 5 ng/mg. In turn, the method was considered precise and accurate with coefficients of variation below 15%, and with an average relative error within ± 15% for all compounds, except for LLOQ (20%).

With the successful application of MEPS it has been demonstrated that this miniaturized technique is an excellent alternative for toxicological analysis in hair samples. MEPS has the advantage of reducing solvent use, and the sorbent may be reused (> 100 extractions), which can be economically attractive to laboratories.

Although there has been considerable academic interest in miniaturized clean-up approaches over the past five years, few implementations have been observed in routine laboratories. With the relevance and applicability herein described, we expect this panorama to change in the near future.

Keywords

Hair samples; Drugs of abuse; Microextraction; MEPS; Toxicology.

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List of Abbreviations

6-AC	6-Acetylcodeine
6-MAM	6-Monoacetylmorphine
AAFS	American Academy of Forensic Sciences
AAPTS	N-(2-aminoethyl)-3-aminopropyltrimethoxysilane
AEME	Anhydroecgonine methyl ester
APDC	Ammonium pyrroldinedithiocarbamate
APS	Amino-propyl silane
BEG	Benzoylecgonine
BPs	Bromophenols
BSA	Bovine serum albumin
CAR/DVB	Carboxen/Divinylbenzene
CAR/PDMS	Carboxen/Polydimentylsiloxane
CBs	Chlorobenzenes
CD-IMS	Corona discharge ion mobility spectrometry
CE	Capillary electrophoresis
CE-UV	Capillary electrophoresis - Ultraviolet detection)
CICS-UBI	Health Sciences Research Centre- University of Beira interior
CME	Capillary microextraction
CNTs	Carbon nanotubes
COC	Cocaine
COD	Codeine
COET	Cocaethylene
CPs	Chlorophenols
CR-SWCNTs	Congo red modified single wall carbon nanotubes
CTAB	Cetyl trimethylammonium bromide
CV	Coefficient of variation
CVAAS	Cold vapour atomic absorption spectrometry
DAD	Diode array detector
DDW	Double-distilled water
DES	Deep eutectic solvents
DI-SPME	Direct immersion - Solid Phase Microextraction
DIW	Deionized water
DLLME	Dispersive liquid-liquid microextraction
DLLME-SFO	Dispersive liquid-liquid microextraction with solidification of floating organic drop
DMA	Dimethylarsinic acid
DMBA	N,N-dimethlybenzylamide
DMIP	Dummy molecularly imprinted polymer
DMPAHPD	5-(2′, 4′-dimethylphenylazo)-6-hydroxypyrimidine-2, 4-dione
DMSO	Dimethyl sulphoxide
DOE	Design of experiments
DSDME	Directly suspended droplet microextraction
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimentylsiloxane
D-μ-SPE	Dispersive micro-solid phase extraction
EDDP	2-ethylidene-1,5-dimethyl-3,3- diphenylpyrrolidine

EI	Electron ionization
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	Ecgonine methyl ester
ESM	Electronic Supplementary Material
EtG	Ethyl glucuronide
ETAAS	Electro thermal atomic absorption
EU	European Union
EWDTS	European Workplace Drug Testing Society
FAAS	Flame atomic absorption spectrometry
FAEEs	Fatty acid ethyl esters
FCT	Portuguese Foundation for Science and Technology
FDA	Food and Drug Administration
FEDER	European Regional Development Fund
FID	Flame ionization detector
FLD	Fluorescence detector
FNT	Fentanyl
FTIR	Fourier transform infrared spectroscopy
GC	Gas Chromatography
GC-FID	Gas chromatography - Flame Ionization Detector
GC-MS	Gas chromatography - Mass spectrometry
GC-MS/MS	Gas chromatography - Tandem mass spectrometry
GC-PDOES	Gas chromatography - Point Discharge Optical Emission Spectrometer
GFAAS	Graphite furnace atomic absorption spectrometry
GHB	Gamma Hydroxybutyrate
GMA-DVB	Glycidyl methacrylate-co-divinyl benzene
GO	Graphene oxide
GRP	Gabinete de Relações Públicas
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HCAs	Heterocyclic amines
HDVB	Highly cross-linked polystyrene divinylbenzene
HF	Hydrofluoric acid
HFBA	Heptafluorobutyric anhydride
HFBCl	Heptafluorobutyric chloride
HF-LPME	Hollow fibre - Liquid Phase Microextraction
HF-SLPME	Hollow fibre - Solid–Liquid Phase Microextraction
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography - Diode array detector
HPLC-FLD	High performance liquid chromatography - Fluorescence detection
HPLC-HRMS/MS	High performance liquid chromatography - High resolution tandem mass spectrometry
HPLC-MS	High performance liquid chromatography - Mass spectrometry
HPLC-UV	High performance liquid chromatography - Ultraviolet detection
HRMS	High resolution mass spectrometry
HS-IMS	*Headspace* extraction - Ion mobility spectrometry
HS-SPME	*Headspace* - Solid Phase Microextraction
ICH	International Conference on Harmonization

ICP-MS	Inductively coupled plasma – Mass spectrometry
ICP-OES	Inductively coupled plasma – Optical emission spectrometry
IIP	Ion imprinted polymers
IL	Ionic liquid
IMS	Ion Mobility Spectrometry
IS	Internal standard
IT-SPME	In-tube - Solid Phase Microextraction
LC	Liquid Chromatography
LC-MS	Liquid Chromatography - Mass Spectrometry
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LC–QTOF-MS	Liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry
LDH	Layered double hydroxide
LLE	Liquid-liquid extraction
LLLME	Liquid–liquid–liquid microextraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LOQ	Limit of quantification
LVSS- CE-UV	Large volume sample stacking - Capillary electrophoresis – Ultraviolet detection
LPME	Liquid Phase Microextraction
MCX	Reversed phase + strong cation exchange
MDA	3,4-Methylenedioxy-amphetamine
MDEA	3,4-Methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxy-methamphetamine
ME	Matrix effect
MEPS	Microextraction by packed sorbent
MFCs	Magnetic metal-organic framework composites
MIPs	Molecular imprinted polymers
MMA	Monomethylarsonic acid
MMT/PS	Montmorillonite/Polystyrene
MNPs	Magnetic nanoparticles
MOFs	Metal-organic frameworks
MOR	Morphine
MPTS	3-mercaptopropyltrimethoxysilane
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPME	Magnetic solid-phase microextraction
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
MS-FAAS	Micro-sampling flame atomic absorption spectrometry
NCOC	Norcocaine
NPs	Nanoparticles
n.a.	Not applied
n.s.	Not specified
ODS	Octadecylsilane
OSA	Ammonium octadecyl sulfate
PA	Polyacrylate
PAHs	Polycyclic aromatic hydrocarbons

PAN	1-(2-Pyridylazo)-2-naphthol (2nd chapter) polyacrylonitrile (3rd chapter)
PAN/GO	Polyacrylonitrile/graphene oxide
PCBs	Polychlorinated biphenyls
PEG	Polyethyleneglycol
PDMS	Polydimentylsiloxane
PDMS/DVB	Polydimentylsiloxane/Divinylbenzene
PNIPA	Poly(Nisopropylacrylamide)
PP	Polypropylene
PPY	Polypyrrole
PS/DVB	Polystyrene/Divinylbenzene
PTFE	Polytetrafluoroethylene
PTh	Polythiophene
QC	Quality control
RAM	Restricted access material
RB	Rhodamine B
RE	Relative error
RSD	Relative standard deviation
RSM	Response surface methodology
RTIL	Room temperature ionic liquid
SAMHSA	Substance Abuse and Mental Health Services
SAX	Strong anion exchanger
SCX	Strong cation exchanger
SE-LPME	Surfactant enhanced liquid phase microextraction
SDS	Sodium dodecyl sulphate
SICAD	General-Directorate for Intervention on Addictive Behaviours and Dependencies
SM-DLLME	Supramolecular solvent-based dispersive liquid-liquid microextraction
SOFT	Society of Forensic Toxicologists
SoHT	Society of Hair Testing
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SQT-FAAS	Slotted quartz tube - Flame atomic absorption spectrometry
SS	Switchable solvent
SS-LPE	Switchable solvent based liquid phase extraction
Sulf-G/PANI	Sulfonated graphene/polyaniline
SUPRAS	Supramolecular solvents
SWGTOX	Scientific Working Group for Forensic Toxicology
TDM	Therapeutic drug monitoring
TEA	Triethylamine
THC	Tetrahydrocannabinol
THC-COOH	11-Nor-9-carboxy-Δ9-tetrahydrocannabinol
TIAFT	The International Association of Forensic Toxicologists
TIL-DLLME	Temperature controlled ionic liquids dispersive liquid-liquid microextraction
TMS	Trimethyl chlorosilane
TOF-MS	Time of flight – Mass spectrometry
TRM	Tramadol

UA-LDS-DLLME	Ultrasound assisted – Low density solvent - Dispersive liquid-liquid microextraction
UBI	University of Beira Interior
UHPLC	Ultra-high performance liquid chromatography
UHPLC-MS/MS	Ultra-high performance liquid chromatography–tandem mass spectrometry
UPLC-MS/MS	Ultra-performance liquid chromatography–tandem mass spectrometry
UNODC	United Nations Office on Drugs and Crime
UV/Vis	Ultraviolet-Visible spectrometer
WADA	World Anti-Doping Agency
[C4MIM][PF6]	1-butyl-3-methylimidazolium hexafluorophosphate
[HMIM][PF6]	1-Hexyl-3-methylimidazolium hexafluorophosphate

List of Scientific Publications

Publications related to this doctoral thesis

i. **Rosado, T.**, Barroso, M., Vieira, D. N., & Gallardo, E. (2019). Determination of selected opiates in hair samples using microextraction by packed sorbent: a new approach for sample clean-up. Journal of Analytical Toxicology, 43(6), 465-476 https://doi.org/10.1093/jat/bkz029

ii. **Rosado, T.**, Gallardo, E., Vieira, D. N., & Barroso, M. (2019). New approach for sample clean-up using microextraction by packed sorbent to determine methadone and EDDP in hair samples. Toxicologie Analytique et Clinique, 31(2), S82. https://doi.org/10.1016/j.toxac.2019.03.131

iii. **Rosado, T.**, Gallardo, E., Vieira, D. N., & Barroso, M. (2020). Microextraction by packed sorbent as a novel strategy for sample clean-up in the determination of methadone and EDDP in hair. Journal of Analytical Toxicology, 44(8), 840-850. https://doi.org/10.1093/jat/bkaa040

iv. **Rosado, T.**, Gallardo, E., Vieira, D. N., & Barroso, M. (2020). New miniaturized clean-up procedure for hair samples by means of microextraction by packed sorbent: determination of cocaine and metabolites. Analytical and Bioanalytical Chemistry, 412(28), 7963-7976. https://doi.org/10.1007/s00216-020-02929-6

v. Microextraction by Packed Sorbent. Microextraction techniques in Analytical Toxicology **T. Rosado**, E. Gallardo, D.N. Vieira, M. Barroso. (2021). In: Microextraction techniques in Analytical Toxicology, Chapter 6. Publisher: CRC Press, Taylor & Francis Group. (Accepted)

vi. **Tiago Rosado**, Mário Barroso, Duarte Nuno Vieira, Eugenia Gallardo. (2021). Trends in microextraction approaches for handling human hair extracts-A review. Analytica Chimica Acta. (in press). https://doi.org/10.1016/j.aca.2021.338792

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i. **Rosado, T.**, Gonçalves, J., Luís, Â., Malaca, S., Soares, S., Vieira, D. N., Barroso, M., Gallardo, E. (2018). Synthetic cannabinoids in biological specimens: A review of current analytical methods and sample preparation techniques. Bioanalysis, 10(19), 1609-1623. https://doi.org/10.4155/bio-2018-0150

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iii. The Role of Liquid Chromatography in Toxicological Analysis
T. Rosado, S. Soares, S. Malaca, J. Gonçalves, M. Barroso, E. Gallardo. In: High-Performance Liquid Chromatography: Types, Parameters and Applications, Chapter 1, Publisher: Nova Science Publishers, Inc., Editors: Ivan Lucero, 2018, pp.1-120 [ISBN: 978-1-53613-543-5].

iv. Prata, M., Ribeiro, A., Figueirinha, D., **Rosado, T**., Oppolzer, D., Restolho, J., Araújo, A., Costa, S., Barroso, M., Gallardo, E. (2019). Determination of opiates in whole blood using microextraction by packed sorbent and gas chromatography-tandem mass spectrometry. Journal of Chromatography A, 1602, 1-10. https://doi.org/10.1016/j.chroma.2019.05.021

v. Malaca, S., **Rosado, T**., Restolho, J., Rodilla, J. M., Rocha, P. M., Silva, L., Margalho, C., Barroso, M., Gallardo, E. (2019). Determination of amphetamine-type stimulants in urine samples using microextraction by packed sorbent and gas chromatography-mass spectrometry. Journal of Chromatography B, 1120, 41-50. https://doi.org/10.1016/j.jchromb.2019.04.052

vi. Caramelo, D., **Rosado, T**., Oliveira, V., Rodilla, J. M., Rocha, P. M., Barroso, M., Gallardo, E. (2019). Determination of antipsychotic drugs in oral fluid using dried saliva spots by gas chromatography-tandem mass spectrometry. Analytical and Boanalytical Chemistry, 411(23), 6141-6153. https://doi.org/10.1007/s00216-019-02005-8

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ix. Gonçalves, J., **Rosado, T**., Soares, S., Simão, A. Y., Caramelo, D., Luís, Â., Fernández, N., Barroso, M., Gallardo, E., Duarte, A. P. (2019). Cannabis and its secondary metabolites: Their use as therapeutic drugs, toxicological aspects, and analytical determination. Medicines, 6(1), 31. https://doi.org/10.3390/medicines6010031

x. Gonçalves, J., Ramos, R., **Rosado, T**., Gallardo, E., Duarte, A. P. (2019). Development and validation of a HPLC–DAD method for quantification of phenolic compounds in different sweet cherry cultivars. SN Applied Sciences, 1(9), 1-11. https://doi.org/10.1007/s42452-019-0680-4

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xv. The Role of Gas Chromatography in Clinical and Forensic Toxicology
Ana Y. Simão, Mónica Antunes, Joana Gonçalves, Sofia Soares, Teresa Castro, **Tiago Rosado**, Débora Caramelo, Mário Barroso, André R.T.S. Araújo, Jesus Rodilla, Eugenia Gallardo. In: Gas Chromatography: History, Methods and Applications. Chapter 1, Publisher: Nova Science publishers, 2019 [ISBN: 978-1-53617-350-5].

xvi. Ketamine and Other Phencyclidine Analogues: A Review of Their Use as Drugs of Abuse, Toxicological Aspects and Bioanalytical Approaches
Ana Sofia Vargas, Débora Caramelo, Ana Y. Simão, Sofia Soares, Joana Gonçalves, **Tiago Rosado**, Mónica Antunes, Mário Barroso, Eugenia Gallardo. In: Ketamine: History, Uses and Health Effects. Chapter 4, Publisher: Nova Science publishers, 2019 [ISBN: 978-1-53616-731-3].

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xxii. The Role of CYP2D6 in Opiates and Opioids Consumers and Its Implications in Clinical and Forensic Toxicology
Mónica Antunes, Ana Y. Simão, Suzana Fonseca, Joana Gonçalves, Sofia Soares Ema Almeida, Carina Gameiro, **Tiago Rosado**, Ana Paula Duarte, Mário Barroso, André R. T. S. Araújo, Jesus Rodilla, Eugenia Gallardo. In: Advances in Medicine and Biology. Volume 168. Chapter 1, Publisher: Nova Science publishers, 2020 [ISBN: 978-1-53618-454-9].

xxiii. Paroxetine in Human Biological Specimens: A Review of Current Sample Pretreatment and Bioanalytical Approaches
Sofia Soares, Joana Gonçalves, Ana Y. Simão, **Tiago Rosado**, Débora Caramelo, Nicolás Fernández, André R.T.S. Araújo, Mário Barroso, Eugenia Gallardo, Charles M. Lepkowsky. In: The Pharmacological Guide to Paroxetine. Chapter 4, Publisher: Nova Science publishers, 2020 [ISBN: 978-1-53618-045-9].

xxiv. *Salvia divinorum*: Beyond Its Therapeutic Uses

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Joana Gonçalves, Ana Y. Simão, Sofia Soares, Carina Gameiro, Ema Almeida, **Tiago Rosado**, Ângelo Luís, Eugenia Gallardo, Ana Paula Duarte. In: Prunus: Classification, Cultivation and Toxicity. Chapter 2, Publisher: Nova Science publishers, 2020 [ISBN: 978-1-53617-755-8].

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xxviii. Liquid chromatography–mass spectrometry as a tool to identify adulteration in different food industries.
Antunes, M., **Rosado, T.**, Simão, A.Y., Gonçalves, J., Soares, S., Barroso, M., Gallardo, E. In: Food Toxicology and Forensics. Chapter 5, Publisher: Academic Press, 2021 [ISBN: 978-0-12-822360-4]

List of Scientific Communications

Oral and Poster communications related to this doctoral thesis

i. DESENVOLVIMENTO E VALIDAÇÃO DE UM MÉTODO PARA A DETERMINAÇÃO DE OPIÁCEOS EM AMOSTRAS DE CABELO COM RECURSO Á MICROEXTRACÇÃO EM SERINGA EMPACOTADA E GC/MS-MS (Oral Communication)
Tiago Rosado, Hernâni Marques, Joana Gonçalves, Sofia Soares, Duarte Nuno Vieira, Eugenia Gallardo, Mário Barroso; 17.º Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra (Portugal), November 2018.

ii. MICROEXTRACTION BY PACKED SORBENT AS A NEW APPROACH FOR SAMPLE CLEAN-UP IN HAIR ANALYSIS FOR OPIATES
Rosado Tiago, Gallardo Eugenia, Vieira Duarte Nuno, Barroso Mário.; 56th The International Association of Forensic Toxicologists Annual Meeting, Gent (Belgium), August 2018

iii. MICROEXTRACTION BY PACKED SORBENT TO DETERMINE METHADONE AND EDDP IN HAIR SAMPLES: A NEW APPROACH FOR SAMPLE CLEAN-UP
T. Rosado, E. Gallardo, D.N. Vieira, M. Barroso.; 57th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT), Birmingham (United Kingdom), September 2019.

iv. OTIMIZAÇÃO DE UM TÉCNICA MINIATURIZADA PARA CLEANUP E DETERMINAÇÃO DE COCAÍNA E SEUS METABOLITOS EM AMOSTRAS DE CABELO
T. Rosado, H. Marques, M. Mota, A. Simão, M. Antunes, M. Barroso, D.N. Vieira, E. Gallardo.; 18º Congresso Nacional de Medicina Legal e Ciencias forenses, Coimbra (Portugal), November 2019 - ***(Honorable Mention in Forensic Toxicology Panel)***

v. HAIR ANALYSIS: THE USEFULNESS OF MICROEXTRACTION FOR SAMPLE CLEANUP (Oral Communication)
Rosado T., Barroso M., Vieira D.N., Gallardo E.; ANALÍTICA 2020 - 10.º Encontro da Divisão de Química Analítica da SPQ, Lisboa (Portugal), October, 2020.

vi. AND NOW FOR SOMETHING COMPLETELY DIFFERENT: HAIR TESTING FOR COCAINE AND METABOLITES USING MEPS (Oral Communication)
Tiago Rosado, Eugenia Gallardo, Hernâni Marques, Duarte Nuno Vieira, Mário Barroso.; 26th Annual SoHT Meeting, Santiago de Compostela (Spain), September 2021.

vii. NEW TRENDS IN HAIR ANALYSIS: THE USE OF MICROEXTRACTION APPROACHES FOR PRE-CONCENTRATION AND CLEAN-UP
Tiago Rosado, Ana Aysa Simão, Duarte Nuno Vieira, Eugenia Gallardo, Mário Barroso.; 26th Annual SoHT Meeting, Santiago de Compostela (Spain), September 2021.

Oral and Poster communications unrelated to this doctoral thesis

i. DETERMINATION OF METHADONE AND ITS METABOLITE EDDP IN ORAL FLUID USING THE DRIED SALIVA SPOT SAMPLING APPROACH AND GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY
Ribeiro Andreia, Prata Margarida, Vaz Cristiana, **Rosado Tiago**, Restolho José, Barroso Mário, Araújo André RTS, Gallardo Eugenia.; 56th The International Association of Forensic Toxicologists Annual Meeting, Gent (Belgium), August 2018

ii. DETERMINATION OF OPIATES IN WHOLE BLOOD USING MICROEXTRACTION BY PACKED SORBENT
Prata Margarida, Ribeiro Andreia, Figueirinha David, **Rosado Tiago**, Oppolzer David, Restolho José, Araújo André RTS, Costa Suzel, Barroso Mário, Gallardo Eugenia.; 56th

The International Association of Forensic Toxicologists Annual Meeting, Gent (Belgium), August 2018

iii. DETERMINATION OF AMPHETAMINES-TYPE STIMULANTS IN URINE SAMPLES USING MICROEXTRACTION BY PACKED SORBENT AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY (Oral Communication)
Sara Malaca, **Tiago Rosado**, José Restolho, Jesus Rodilla, Lucia Silva, Cláudia Margalho, Mário Barroso, Eugenia Gallardo.; 3rd Regional TIAFT Meeting, Cappadocia (Turkey), October 2018

iv. APLICAÇÃO DA MICROEXTRACÇÃO EM SERINGA EMPACOTADA PARA A DETERMINAÇÃO DE OPIÁCEOS EM AMOSTRAS DE SANGUE TOTAL POR CROMATOGRAFIA GASOSA ACOPLADA À ESPETROMETRIA DE MASSA TRIPLO QUADRUPOLO
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v. DETERMINAÇÃO DE METADONA E EDDP EM AMOSTRAS DE FLUIDO ORAL COM AMOSTRAGEM POR SALIVA SECA (Oral Communication)
Andreia Ribeiro, Margarida Prata, Cristiana Vaz, **Tiago Rosado**, Sofia Soares, Joana Gonçalves, José Restolho, Mário Barroso, André RTS Araújo, Eugenia Gallardo.; 17.º Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra (Portugal), November 2018

vi. MONITORIZAÇÃO DE FÁRMACOS E TÓXICOS: UMA FERRAMENTA ÚTIL NO DIAGNÓSTICO E SEGUIMENTO CLÍNICO (Oral Communication)
Tiago Rosado, Sofia Soares, Débora Caramelo, Joana Gonçalves, Mário Barroso, Eugenia Gallardo.; 1ªs Jornadas de Investigação Clínica do Centro Académico Clínico das Beiras, Covilhã (Portugal), November 2018

vii. DRIED SALIVA SPOTS: DEVELOPMENT AND VALIDATION OF A NEW METHOD FOR THE QUANTIFICATION OF DRUGS OF ABUSE IN ORAL FLUID BY GC-MS
Fernández Nicolás, Cabanillas Laura M, **Rosado Tiago**, Barroso Mário, Gallardo, Eugenia, Quiroga Patricia N.; XIV TIAFT Latin-American Regional Meeting I International meeting on NPS & Drug design, Buenos Aires (Argentina), November, 2018

viii. EVALUATION OF THE PHARMACEUTICAL POTENTIAL OF CHARACIOPSIS AQUILONARIS SKUJA ACOI 2424
Clara Martins, Octavio Ferreira, **Tiago Rosado**, Eugenia Gallardo, Samuel M. Silvestre, Lilia M A Santos.; PSE Meeting - Natural Products in Drug Discovery and Human Health, Lisboa (Portugal), July 2019

ix. STUDY OF BIOACCESSIBILITY AND BIOAVAILABILITY OF PHENOLIC CONTENT OF SWEET CHERRY (PRUNUS AVIUM L.) BY IN VITRO DIGESTION AND CELLULAR MODEL (Oral Communication)
Joana Gonçalves, Rodrigo Ramos, Sandra Rocha, **Tiago Rosado**, Eugenia Gallardo and Ana Paula Duarte.; the XII Iberoamerican Congress of Food Engineering CIBIA, Faro (Portugal), July 2019

x. SWEET CHERRIES (PRUNUS AVIUM L.): BIOACCESSIBILITY AND BIOAVAILABILITY OF THEIR PHENOLIC COMPOUNDS
J. Gonçalves, R. Ramos, Â. Luís, S. Rocha, **T. Rosado**, E. Gallardo, A. P. Duarte.; PSE Meeting - Natural Products in Drug Discovery and Human Health, Lisboa (Portugal), July 2019

xi. ANALYTICAL APPROACH TO DETERMINE ANTIPSYCHOTIC DRUGS IN ORAL FLUID BY DRIED SALIVA SPOTS
Débora Caramelo, **Tiago Rosado**, Victor Oliveira, Mário Barroso, Eugénia Gallardo.; 57th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT), Birmingham (United Kingdom), September 2019.

xii. CARATERIZAÇÃO DE INTOXICAÇÕES NO SERVIÇO DE URGÊNCIA DO HOSPITAL DE BRAGA
E. Lopes, S. Anjos, S. Dória, A. Y. Simão, **T. Rosado**, André R.T.S. Araújo, J. Teixeira, E. Gallardo.; 18º Congresso Nacional de Medicina Legal e Ciências forenses, Coimbra (Portugal), November 2019

xiii. DESENVOLVIMENTO E VALIDAÇÃO DE UMA METODOLOGIA PARA A DETERMINAÇÃO DE ANTIPSCÓTICOS COM AMOSTRAGEM POR DRIED SALIVA SPOTS (Oral Communication)
Caramelo, D; Simão, A; **Rosado, T**; Oliveira, V; Barroso, M; Gallardo, E.; 18º Congresso Nacional de Medicina Legal e Ciências Forenses, Coimbra (Portugal), November 2019

xiv. PROFILE OF INTOXICATIONS IN PORTUGAL BETWEEN 2014-2018
Beatriz Teles, Mário Barroso, **Tiago Rosado**, André RTS Araújo, Eugenia Gallardo.; SPF2020, Coimbra (Portugal), February 2020

xv. DRIED SALIVA SPOTS: OPTIMIZAÇÃO DE UMA TÉCNICA PARA A DETERMINAÇÃO DE MARCADORES TABACO EM FLUIDO ORAL
H. Marques, P. Cruz-Vicente, **T. Rosado**, A. Simão, S. Soares, J. Gonçalves, L. Passarinha, E. Gallardo.; XII Biochemistry Day (online event), April 2021

xvi. TOPICAL FORMULATION WITH AS1411-GOLD NANOPARTICLES AS CERVICAL CANCER DRUG DELIVERY SYSTEM
Jéssica Nunes, Ana S. Agonia, **Tiago Rosado**, Eugenia Gallardo, Rita Palmeira-de-Oliveira, Ana Palmeira-de-Oliveira, José Martinez-de-Oliveira, José Fonseca-Moutinho, Maria Paula Cabral Campello, António Paulo, Andrew D. Ellington, Carla Cruz.; Applied Nanotechnology and Nanoscience International Conference 2021 (Online event), March 2021

xvii. DESENVOLVIMENTO DE UM MÉTODO POR DRIED SALIVA SPOTS PARA A MONITORIZAÇÃO TERAPÊUTICA DE ANTIDEPRESSIVOS EM FLUIDO ORAL
S. Soares, **T. Rosado**, S. Costa, M. Barroso, E. Gallardo.; III Jornadas Ibéricas de Toxicologia (Online event), June 2021

xviii. VALIDAÇÃO DE UM MÉTODO ANALÍTICO PARA A DETERMINAÇÃO DE OPIÁCEOS EM URINA COM RECURSO À MICROEXTRAÇÃO EM SERINGA EMPACOTADA
Ana Y. Simão, Catarina Monteiro, Hernâni Marques, **Tiago Rosado**, Mário Barroso, Maristela Andraus, Eugenia Gallardo.; III Jornadas Ibéricas de Toxicologia (Online event), June 2021

xix. DRIED SALIVA SPOTS: OPTIMIZAÇÃO E VALIDAÇÃO DE UMA TÉCNICA PARA A DETERMINAÇÃO DE MARCADORES DE TABACO EM FLUIDO ORAL (Oral Communication)
H. Marques, **T. Rosado**, A.Y. Simão, L. Passarinha, E. Gallardo.; III Jornadas Ibéricas de Toxicologia (Online event), June 2021

Thesis overview

This thesis is divided into five chapters. The first chapter provides general information on the problem of drug abuse, drug analysis and hair testing for drugs. Two review papers are included in this chapter. The first is a critical review of the advances observed over the last 5 years in the use of miniaturised approaches for sample clean-up and drug pre-concentration in hair analysis (Paper I). The second discusses the pros and cons of MEPS for sample preparation, as well as the factors affecting its performance, configurations and commercially available sorbents, and applications with special focus in the fields of clinical and forensic toxicology (Paper II).

The second chapter focuses on the global aims of this thesis, while the third chapter refers to original research, and includes three published studies on drugs determination in hair using the MEPS approach for sample clean-up. The first published study (Paper III) describes the development and optimization of a method using MEPS for sample clean-up in the determination of tramadol, codeine, morphine, 6-monoacetylmorphine, 6-acetylcodeine and fentanyl in hair samples. The second published study (Paper IV) concerns the development and validation of an analytical method using MEPS to determine methadone and its main metabolite EDDP in hair samples. Lastly, the third published study (Paper V) reports the optimization and full validation of an analytical method to determine cocaine, ecgonine methyl ester, benzoylecgonine, norcocaine, cocaethylene and anhydroecgonine methyl ester in hair samples using MEPS as clean-up procedure.

The fourth chapter includes a discussion and final reflection concerning the five published papers, while the fifth chapter presents the conclusions regarding the entire study within the scope of this thesis.

Chapter 1 - Introduction

1.The problem of drug use

Drug use has always been a reality in our societies, whether for personal pleasure, pain relief, or for traditional, cultural, and religious reasons [1]. According to the 2019 drug report from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), it is estimated that 96 million adults (aged 15-64) in the European Union (EU) have used illicit drugs at least once in their lifetime, representing approximately 29% of adults [2]. Extending the analysis, in 2016 the United Nations Office on Drugs and Crime (UNODC) reports that globally, approximately 269 million people have used drugs at least once, representing 5.4% of the world's population aged 15–64 [3].

Although traditional plant-based substances such as cannabis, cocaine and heroin remain strong in the drug markets, growth in non-medical use of pharmaceutical drugs and in the synthetic drug market has been observed in recent years [3].

Globally, cannabis remains the most commonly used drug, with 192 million users in 2017, representing 3.9% of the global adult population (15–64) [3]. In the same year, an estimated 57.8 million used opioids globally, including those who used both opiates (30.4 million) and pharmaceutical opioids, representing a prevalence of 1.2% [3]. The third most commonly reported drug class was amphetamines, particularly methamphetamine, and pharmaceutical stimulants, with 27 million (0.5%) of the adult population using these substances [3]. To a lesser extent, 19 million (0.4%) of the global population aged 15–64 years were cocaine users in 2017 [3]. An increase in cocaine use has been observed in the Western and Central Europe while a mixed trend has been observed in the Americas [3].

As for the EU, the most commonly used drugs are not different from those reported by the UNDOC, with cannabis being used by 25.2 million adults (aged 15-64) in 2019, representing 7.6% of this age group [2]. Different from the global data is the cocaine use reported for the EU in the same year. Cocaine is reported as the second most commonly used illicit drug with 4.3 million (1.3%) estimation among adults (aged 15-64) [2]. With approximately the same prevalence, 3,4-methylenedioxy-methamphetamine (MDMA) and amphetamines are used by 2.7 million (0.8%) and 2.0 million (0.6%) adults (aged 15-64), respectively [2]. Finally, and also diverging from global data, the prevalence of high-risk opioid use among adults (15-64) is estimated to be 1.3 million high-risk opioid users in 2018, representing approximately 0.4% of the EU population [2].

In Portugal, the scenario is similar to the EU. The latest report from the General-Directorate for Intervention on Addictive Behaviours and Dependencies (SICAD) [4], which includes data from 2016 and 2017, shows an overall prevalence of 4.8% drug use in the general population (15-74 years old). Of this, cannabis accounts for 4.5%, followed by 0.2% for cocaine, 0.1% for MDMA and heroin[4].

Although the percentage of annual illicit drug use may seem small at first glance, this use poses a serious threat to human health and is becoming a nationwide problem [5], due to material and moral losses, reduction in national wealth, increased morbidity and mortality, and increase in

addiction related crimes [5,6]. It should also be remembered that drug use is the leading cause of disability and suffering, especially among adolescents and younger adults [7]. There is a strong association between drug use disorders and psychiatric comorbidities, apart from the negative health consequences such as non-fatal overdoses, infectious diseases and premature death. Moreover, an association between drug use disorders and social disadvantage, i.e., low educational level, financial instability, and poverty, is also observed [3].

In Europe, the mortality rate due to overdose in 2018 was estimated at 22.3 deaths per million in the population aged 15-64 years [2]. Opioids, such as heroin, were the leading cause of overdose deaths in the same year. Nevertheless, other opioids were also frequently mentioned in post mortem toxicology reports, mainly methadone, but also buprenorphine, fentanyl and tramadol [2]. Cocaine, amphetamines, MDMA and cathinones were associated to a smaller number of overdose deaths, with their importance varying across countries [2]. In Portugal, 55 drug related deaths were reported between 2018 and 2019, of which 52% were positive for cocaine, 44% for opiates, 27% for cannabis and 22% for methadone [4]. It has also been reported that more than one substance was detected in 92% of drug related deaths [4].

The drug market also constitutes an economic problem. The harmful effects of organized drug crime are widespread as illicit drug trafficking is an international business involving the transfer of large amounts of money [2]. As a result, huge profits are generated which need to be laundered before further use. This money laundering is usually done through the acquisition of high value assets or businesses, resulting in a distortion of the real economy and a competitive disadvantage for legitimate businesses and consumers [2].

On a social and political level, it is important to consider that many people use licit and/or illicit drugs, but only a minority use drugs in a problematic way [8]. The effects of drugs effects are regularly portrayed in the media as negative, which also leads to stigmatization of people who use drugs [8]. These negative portrayals can have a direct impact on clinical care, as the higher the stigma, the fewer treatment options and harm reduction are offered[8].

Hereupon, Portugal passed Law 30/2000, which came into force on July 1, 2001 and presented a novel legal system for drug users, which became the object of study by many countries [9]. With this law, drug use is still illegal, but is now regarded as an administrative offense and is no longer considered crime [9]. The latter is in line with the recognition of others life choices and social circumstances, respect for human dignity and respect for the right to health [9]. Decriminalizing drug possession and investing in harm reduction and treatment services can be beneficial for both public safety and health [1]. However, the term 'decriminalization' should not be confused with 'depenalization/legalization', as the possession, purchase and consumption of illicit drugs in amounts greater than those stipulated still remains a criminal offense [1].

The Portuguese policy is known worldwide as one of the most successful of its kind [10]. The number of infectious diseases among drug users decreased and the number of drug overdose deaths stabilized, with one of the lowest counts in the whole EU [10]. In addition, the number of drug users seeking medical treatment increased and the social cost of drug use decreased by 18%

since this policy of decriminalization has been pursued [10]. Moreover, use among young adults has also been found to be remarkably low [10].

The problem of drug abuse is a high yield issue with great impact worldwide. However, there is still a lack of agreement concerning the best approach to address it, from decriminalization to a complete ban of the substances [11].

2. Drug analysis

Forensic toxicology usually involves three main areas of action: workplace drug testing, human performance (e.g. driving under the influence of alcohol and/or drugs) and *post-mortem* [12].

As it is a broad field, several guidelines have been published to provide answers to specific questions, namely reference methods, technical competence, cut-offs, validity testing and environmental requirements [13]. The most important guidelines were developed by the Substance Abuse and Mental Health Services (SAMHSA) [14] and the European Workplace Drug Testing Society (EWDTS) [15–19]. However, other guidelines such as those of the Society of Forensic Toxicologists (SOFT) and the toxicology section of the American Academy of Forensic Sciences (AAFS) [20] recommend a supplementary set of guidelines [13]. For the general analytical aspects of drug analysis, The International Association of Forensic Toxicologists (TIAFT) [21], and specifically for doping detection in athletes, the World Anti-Doping Agency (WADA) [22] have developed guidelines [13].

All guidelines emphasise the paramount importance of proper sample selection to obtain accurate results, interpret them in a scientifically sound manner, and subsequently be useful in solving forensic cases [22]. A large number of biological samples can be analysed under the umbrella of forensic toxicology, but the most common are blood and urine. However, other samples may be used depending on the application [23].

Blood remains the preferred biological sample whenever available, and its collection should be close enough to the event in question [23]. In this sense, it provides the most direct evidence of a drug in the body and allows some correlation with its toxicological effects [23]. Moreover, blood is preferred over plasma or serum because most forensic data report results in blood [23]. Nonetheless, there are jurisdictions that prefer results in plasma or serum because they can be more easily compared to clinical data [23]. Blood and/or plasma samples are useful to assess recent and short-term exposure to drugs as it is possible to detect the parent compound, but one of the limitations is the low concentrations found for many basic drugs and other poisons [24,25]. In addition, interpretation of quantitative results from *post-mortem* blood can be challenging [24]. Indeed, *post-mortem* redistribution occurs as well as ongoing conversion of the parent compound to its metabolites [26].

Conversely, urine is the preferred sample for screening for the presence of drugs, as these substances and metabolites are usually present there in higher concentrations than in blood, and the available sample volume is often not limiting [23]. However, urine is a waste product stored in the bladder and for this reason it is not possible to infer a dose consumed or correlate with the effect of the drugs at the time of sampling [23]. No special equipment is required for urine collection, but appropriate facilities and procedures are needed to prevent sample tampering [24].

Several drugs undergo metabolism and are subsequently excreted in the urine both in the form of metabolites and unchanged; moreover, both the parent drug and metabolites may have

psychoactive and/or addictive properties, and therefore it is relevant that they are all detected [14]. For example, THC (cannabis) is rapidly metabolized and little to nothing is usually found in urine samples. Nevertheless, its main metabolite, 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), can be detected within hours of exposure and for more than three weeks in heavy users, so only the metabolite is required for detection in this sample [14]. The same is true for cocaine, which is also readily metabolized, with its major metabolite benzoylecgonine (BEG) being detectable in urine for periods ranging from a few days to three weeks in the case of heavy users. For this reason, positive cases may rely only in the detection of the metabolte [14]. As for codeine, it is metabolized to morphine and both can be detected in urine for several days [14]. Heroin, on the other hand, is undetectable after about 30 minutes after consumption. Therefore, the detection of its metabolite 6-monoacetylmorphine (6-MAM) in urine is considered evidence of illicit heroin use; however, its detection window is not very large, as it is usually only present up to 24 hours after heroin use [14].

In addition to blood and urine, there are other samples that can be collected and used depending on the application [24]. Excretions such as exhaled air and meconium (first faecal material of a neonate) or secretions such as saliva and sweat are usually less useful for the interpretation of quantitative data, but may be relevant for qualitative work [24]. In terms of *post-mortem* samples, blood and urine are also collected, but of great interest are gastric contents, liver and vitreous humour [23]. Gastric contents can be helpful in assessing the degree of oral administration when overdose is suspected and can complement blood data [23]. The liver is important when the informative value of the drug concentration in the blood is low, and more information is needed to determine the role of the drug in the death [23]. On the other hand, vitreous humour is a very pertinent sample when significant *post-mortem* changes or trauma have occurred [23]. Other samples such as lungs, kidneys, brain, bone and bone marrow can also be used for drug detection in post-mortem scenarios [23].

Immunoassays and some of the analytical instruments, namely liquid chromatography coupled to mass spectrometry (LC-MS), can cope with the direct use of urine samples or precipitated blood [12]. These are simple and rapid procedures, but immunoassays are not suitable for a large number of drugs, and the co-injection of a large amount of matrix can interfere with the ionization process in LC-MS [12]. For this reason, and in cases of complex matrices such as those mentioned above, the first step in any drug analysis is to isolate target analytes from the matrix [23]. Currently, a variety of isolation techniques have been described and used, depending on the sample, the analytes being screened and the detection method, but the most commonly used are liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

LLE is the oldest but still widely used technique. In this technique, an inert organic solvent immiscible with water at an appropriate pH is used to extract the analyte(s) from the biological material [23,24]. Solvent selection is an important task in method development, and in principle a less polar solvent will be more efficient in extracting the target analytes. However, the selection of a solvent with too much ‘extraction power’ can also increase the extraction of matrix interferences, resulting in dirtier extracts and reducing selectivity [23,24]. Commonly, the

procedure requires mechanical mixing of the aqueous and organic phases, after which they are separated by centrifugation [24].

On the other hand, SPE is based on the sorption of the analyte(s) to a solid support that allows selective binding [23]. The solid support consists of siliceous or other materials with relatively narrow particle size distribution in disposable cartridges that allow sequential extraction, clean-up and elution of the target analytes. This technique is still widely used, and the most common supports are alkyl-bonded silica mini-columns, such as C18 or mixed-phase columns [23,24]. SPE offers several advantages over LLE, namely easier batch processing and the fact that the extracts may contain fewer interfering compounds [24]. Since ionized compounds can be isolated by SPE, the methods do not require such extreme pH values as LLE [24]. The latter justifies the suitability of SPE for drugs that are unstable under these pH values [24].

Although LLE and SPE remain the most commonly used techniques, advances in sample preparation increased significantly in recent years [27]. There is a trend towards miniaturization of extraction techniques based on conventional methods, aiming to minimize the consumption of sample and organic solvents [27]. Other features of these microextraction techniques include the ability to reuse sorbents and automated online coupling to chromatographic systems [27].

GC and LC have become the mainstay of chromatographic methods [23]. Both qualitative and quantitative information can often be obtained in the same analysis using these two types of instruments [24]. Temperature programming in GC is analogous to gradient elution in LC, but it is much easier to execute and allows the analysis of drugs of different volatility in one analysis [24]. These drugs include cocaine, amphetamines, barbiturates, some opioids (e.g. codeine, heroin and methadone), some benzodiazepines and other hypnotics, most antidepressants and antipsychotics. In addition, highly polar compounds such as morphine, benzoylecgonine and some benzodiazepines can be made amenable to GC by suitable derivatization [23]. For the analysis of hydrophilic, thermally labile and/or polar compounds that are not amenable to GC, LC is the best option. In both cases, the detector of choice in forensic laboratories is the mass spectrometer (MS). The latter generates spectroscopic data of peaks that allow identification not possible with other detectors, where only retention time can be used to presume the presence of the drugs [23].

3. Hair testing for drugs

Hair has been used for years to assess and document human exposure to drugs [28]. In the 1960s and 1970s, this alternative sample was used to assess exposure to toxic heavy metals [29]. The use of hair for the determination of organic substances, especially drugs, was not possible at that time because the analytical methods were not sensitive enough [29]. Nowadays, with the improvements in analytical instrumentation, the coupling of MS with chromatographic methods became the first option for hair analysis. The latter is now routinely used to document drug exposure in forensic scenarios [29].

Although hair looks like a simple structure, it is actually a complex matrix [30]. The hair shaft is composed of three different cell types: an outer cuticle, an inner medulla, and a central cortex (Fig. 1) [30,31]. In addition, human hair is composed of approximately of 65-95% protein, 15-35% water, and 1-9% lipids. The mineral content of hair varies from 0.25 to 0.95% (on a dry weight basis) [31].

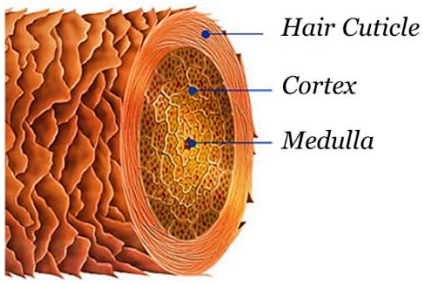

Figure 1. Schematic cross-section of a hair fibre [32].

There are three stages in the hair growth cycle. The first stage (anagen phase) is a long period of active hair growth, at a rate of approximately 0.6–1.4 cm per month [29,30]. Thereafter, the hair follicle enters a short transition stage during which cell division stops and the follicle begins to degenerate (catagen phase, lasting approximately 2 weeks) [29,30]. Following the transition phase, the hair follicle enters a resting period (telogen phase, lasting approximately 10 weeks), during which the hair shaft completely stops growing and hair growth begins to shut down [29,30]. On the adult scalp, approximately 85% of the hairs are in the anagen phase, while the remaining 15% are in the resting stage [29,30]. The growth rate depends on the anatomical location, race, sex and age [30].

The exact mechanism by which drugs are incorporated into the hair is still unknown, but three pathways have been proposed: (*i*) from the blood during hair development in the follicle; (*ii*) from sweat and sebum; and (*iii*) from the external environment (Fig. 2) [33,34]. The combination of these three pathways represents a model that seems to explain incorporation better than a passive model (transfer from the blood into the growing cells in the follicle itself). Several experimental findings are in favour of this three-pathway model; indeed, the drug/metabolite ratios in the

blood differ from those in hair, and the concentrations of drugs in the hair can be significantly different in individuals receiving the same dose. In addition, drugs and metabolites are present in high concentrations in sweat and sebum, and they persist longer in these secretions than in blood [29]. After incorporation, drugs can bind to the intracellular components of hair cells, such as melanin and sulphydryl- containing amino acids [29].

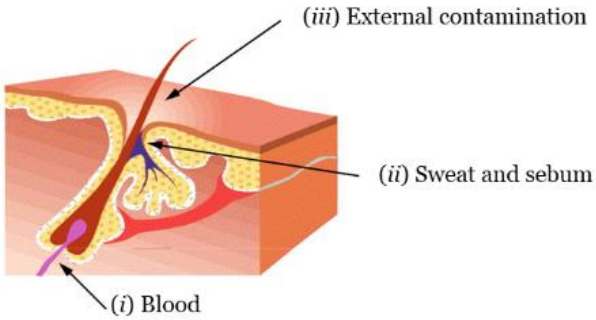

Figure 2. Possible routes of drug incorporation into hair [29].

The above characteristics have led to hair analysis for drugs gaining more and more attention and recognition [35]. Nowadays, hair is recognized as the third most important biological sample routinely used for the detection of xenobiotics (drugs of abuse, pharmaceuticals, environmental contaminants, hormones, etc.) in clinical and forensic toxicology, traffic medicine and occupational medicine [30,33]. In the field of toxicology, the main applications of hair analysis are validation of drug use history, diagnosis of uncontrolled alcohol use through determination of ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs), verification of doping practices, driver's license reinstatement, drug-facilitated crimes, and assessing *in utero* drug exposure [33,36].

The main advantage of hair analysis for drugs compared to urine and blood testing is the larger monitoring window (weeks-months or years, depending on hair shaft length, versus 2-4 days for most drugs) [29]. Analysis of blood and urine provides short-term information about an individual's drug use, whereas hair analysis allows access to long-term histories [29]. Another advantage of hair matrix over traditional matrices is the simpler and non-invasive sample collection, even when there is the necessity of a careful supervision by law enforcement officers to avoid the risk of sample tampering or substitution [33]. In addition, hair is considered a strong and stable tissue, yet it can be affected by cosmetic treatments such as bleaching or dyeing and perm application [29]. Long-term effects of weather (sun, rain, wind) may damage the hair shaft. These may have an effect on the concentrations of drugs found in the hair [29].

Hair sampling should be performed by competent personnel in a safe, contamination-free facility [37]. In general, a bunch of hairs of the thickness of a pencil (approximately 100–200 hairs) should be either plucked (in *post-mortem* cases) or tied off at the root end with cotton thread and then cut at the head vertex posterior as close to the scalp as possible [24]. This region is

considered the best for hair collection, since growth rate is less variable there, a more steady number of hairs is in the growth phase, and the hair is less affected by age- and sex-related factors [33]. Tying with a thread helps to keep the hairs aligned, which is necessary for segmental analysis [24]. In addition, the collected sample should be placed in aluminium foil in an aligned manner with the proximal end clearly identified (Fig.3) [24].

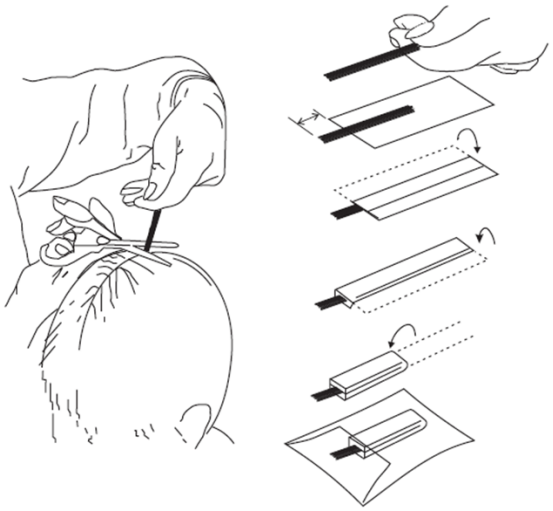

Figure 3. Collection of head hair [24].

When scalp hair is not available, or if head hair has been excessively bleached or permed, arm hair, axillary hair, and pubic hair may be other possible sources for drug detection. However, the ability for segmental analysis is lost and interpretation is quite arduous [24,33].

Guidelines for the collection procedure and all adopted procedures for drug testing in hair have been published by the Society of Hair Testing (SoHT) [37] and the European Workplace Drug Testing Society (EWDTS) [18,19], and are considered key references.

The preparation of hair samples involves a number of steps. In most laboratories, the first step is hair washing [33,35]. Washing hair samples prior to analysis has two main purposes, the first is to remove sweat, sebum or surface material (e.g. skin cells) and hair care products that may interfere with the analysis [37]. The second purpose is to remove externally deposited drugs, since if they are not removed, the analyst may be misleaded towards an incorrect finding of active drug use, instead of drug exposure [18,37]. One should keep in mind that there are no standardised washing procedures, but some recommendations are available [37]. It can be a combination of aqueous and/or organic solvents validated by the laboratory [18]. However, it is recognised that organic solvents such as methylene chloride or acetone only remove surface contaminants, while the use of aqueous solutions or methanol can swell the hair and additionally extract drugs from within the hair [37]. Wash residues should be stored for later analysis and compared with positive hair test results [18].

After hair samples have been washed, they should be dried and cut into small pieces or ground into a powder [37]. The amount of hair used for analysis varies from laboratory to laboratory and

each laboratory determines its requirements during method validation, but typically 10–50 mg of hair are accurately weighed [18,37].

The analytes must then be extracted from the matrix, a process commonly referred to as hair digestion or incubation. Several extraction approaches have been described: (*i*) methanolic incubation; (*ii*) acidic incubation; (*iii*) alkaline incubation; (*iv*) buffer incubation; and (*v*) enzymatic incubation [18]. Again, extraction procedures vary from laboratory to laboratory and each laboratory must validate its method before use [18].

Methanolic incubation (5–18 h) in an ultrasonic bath is compatible with almost all drugs [38]. It can be used to extract drugs that are sensitive to hydrolysis, such as heroin, and lipophilic drugs, such as tetrahydrocannabinol (THC) [38]. However, methanolic extracts have relatively high impurity levels and often result in incomplete and frequently low recoveries [38]. Acidic or buffer incubations are usually good options for basic drugs (e.g. opiates, cocaine and metabolites, amphetamines, methadone), and are commonly performed with aqueous 0.01 to 0.5 M HCl or phosphate buffer (pH 6.4 or 7.6) [38]. Nevertheless, it should be noted that partial hydrolysis of cocaine to BEG and 6-MAM to morphine may occur with acidic incubation [38]. As for alkaline incubation, it is usually performed with aqueous NaOH 1M for one hour at 80 °C, which is advantageous for nicotine, amphetamines, THC, antidepressants and neuroleptics [38]. Finally, enzymatic incubation offers the advantage of hair samples solubilization without degrading unstable compounds such as cocaine and heroin [30]. Enzymes such as pronase and Proteinase K can be used [38].

Regardless of the extraction method chosen, the resulting extract can be analysed directly using screening techniques or, in some cases, chromatographic techniques [38]. However, the SoHT recommends further clean-up with either LLE or SPE before confirmation by chromatographic techniques [38]. A positive result can be used to confirm whether an individual has been exposed to a drug or has used that drug frequently [18]. The SoHT recommends specific cut-offs and criteria for a positive drug test for hair in forensic cases [38]. With respect to workplace drug testing, the EWDTS recommends the same cut-off concentrations, although it adds other substances to the list (e.g. benzodiazepines and z-drugs) [18]. Table 1 summarises the cut-off concentrations recommended by the SoHT [38], EWDTS [18] and the expected concentrations to be found in positive cases [33].

Table 1. Cut-off concentrations and expected concentrations in hair samples.

		SoHT [38,39]		EWDTS [18]		Expected Concentrations [33]
Group	Target analytes	Screening (ng/mg)	Confirmation (ng/mg)	Screening (ng/mg)	Confirmation (ng/mg)	Confirmation
Alcohol (segment 0-3)	EtG	n.s.	0.03 (A) 0.005 (B)	n.s.	0.03	n.s.
	FAEE (EtPa)		0.35 (A) 0.12 (B)		n.s.	
	FAEE		n.s.		0.5	
Amphetamines	Amphetamine	0.2	0.2	0.2	0.2	0.5 – 50.0 ng/mg
	Methamphetamine		0.2		0.2	
	MDA		0.2		0.2	
	MDMA		0.2		0.2	
	MDEA	n.s.	n.s.		0.2	n.s.
Cannabinoids	THC	0.1	0.05	0.1	0.05	THC: 0.05 – 10 ng/mg, in most cases < 2 ng/mg THC-COOH: 0.2 – 50 pg/mg, in most cases < 5 pg/mg
	THC-COOH		0.0002		0.0002	
Cocaine	Cocaine	0.5	0.5	0.5	0.5	0.5 – 100 ng/mg, in most cases < 50 ng/mg, in crack abusers > 300 ng/mg is possible
	BEG		0.05		0.05	
	EME		0.05		0.05	
	NCOC		0.05		0.05	
	COET		0.05	n.s.	n.s.	
Opiates	Morphine	0.2	0.2	0.2	0.2	0.5 – 70 ng/mg, in most cases < 30 ng/mg
	Codeine		0.2		0.2	
	6-MAM		0.2		0.2	
Methadone	Methadone	0.2	0.2	0.2	0.2	0.2 - 50 ng/mg
	EDDP		0.05		0.05	
Buprenorphine	Buprenorphine	0.01	0.01	0.01	0.01	< 1 ng/mg
	Norbuprenorphine		0.01		0.01	
Ketamine	Ketamine	n.s.	n.s.	0.5	0.5	n.s.
	Norketamine		n.s.		0.1	
Benzodiazepines /z-drugs	n.s	n.s.	n.s.	0.05	0.05	n.s.

(A) chronic excessive alcohol consumption; (B) repeated alcohol consumption; COET (Cocaethylene); EDDP (2-ethylidene-1,5-dimethyl-3,3- diphenylpyrrolidine); EME (ecgonine methyl ester); EtG (Ethyl glucuronide); EtPA (Ethyl Palmitate) n.s. (not specified); MDA (3,4-Methylenedioxyamphetamine); MDEA (3,4-Methylenedioxy-N-ethylamphetamine); MDMA (3,4-Methylenedioxymethamphetamine); NCOC (norcocaine); THC-COOH (11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol).

It is important to remember that the use of hair samples for investigative analysis depends on whether the analytical procedure is sensitive enough to identify drug traces [34]. This is relevant when the subject's urine sample was positive and the hair analysis was negative [34]. For this reason, the consensus in the forensic community has been that a negative hair result cannot rule out specific drug use, just as a negative hair result should not override a positive urine result [34].

A negative hair result is also a result that can be interpreted in two different ways: (*i*) the subject did not use or was not exposed to the specific drug or (*ii*) the procedure is not sensitive enough to detect the drug [34].

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4. Paper I - Trends in microextraction approaches for handling human hair extracts - a review

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Review

Trends in microextraction approaches for handling human hair extracts - A review

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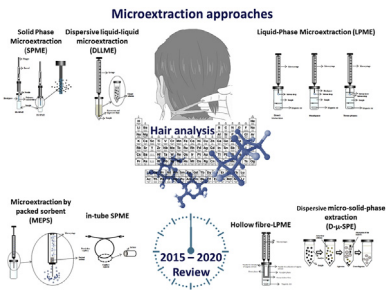
HIGHLIGHTS

- Current trends in microextraction techniques for hair analysis are discussed.
- Applicability of new microextraction techniques for hair analysis and outputs.
- New solid sorbent materials for species extraction/pre-concentration with improved recoveries are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

The complementary role of hair in testing scenarios has expanded across the spectrum of toxicological and clinical monitoring investigations and, over the last 20 years, hair analysis has gained increasing attention and recognition. Moreover, a great deal of attention has been paid to the miniaturisation of extraction procedures, minimising/eliminating toxic organic solvents consumption, making them user-friendly and rapid, in addition to maximising extraction efficiency. The aim of this work is to provide a critical review of the advances observed over the last 5 years in the use of miniaturised approaches for sample clean-up and drug pre-concentration in hair analysis. There have been major improvements in some well-established microextraction approaches, such as liquid phase microextraction, mainly through the use of supramolecular and ionic liquids. In addition, new developments have also been reported in solid phase microextraction, driven by d-SPE applications. In the last 5 years, a total of 69 articles have been published using some type of microextraction technique for hair specimens, thus justifying the relevance of a critical review of innovations, improvements and trends related to these miniaturised approaches for sample preparation.

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Contents

1. Introduction

Blood and urine samples have been used for several years in the determination of xenobiotics due to their main advantages, for instance the correlation of blood levels to the observed signals and symptoms, and the high concentrations usually detected in urine [1]. Nevertheless, alternative specimens such as hair are becoming more important in a variety of fields [2].

One of the most important alternative matrices in what concerns testing of human specimens is hair, which has been receiving increased attention [3]. The fact that the hair sample is collected in a less embarrassing fashion for the person to be tested, and its stability at room temperature (in contrast to what occurs with other body fluids and tissues) have been pointed as great advantages [3]. Notwithstanding, the most compelling advantage of hair is its extended window for xenobiotics detection (approximately 1 month per cm of hair), as it allows retrospective assessment of exposure to those compounds [4,5]. It is not, however, free from limitations, the most commonly mentioned of which are the variable availability/length of hair, the concerns of the examinee about cosmetic visibility upon sample collection, and the somewhat high relative cost [6]. In addition, there is still controversy about how to interpret the results, particularly regarding the occurrence of external contamination, the influence of cosmetic treatments, ethnical bias, and mechanisms of drug incorporation. In addition, there is uncertainty regarding the correlation between urine/serum and hair concentrations, which complicates conclusions regarding risk assessment. Nevertheless, most analytical problems concerning hair preparation and analysis are now dealt with adequately [7]. Several wide-ranging reviews have been published on this subject, namely those by Pragst [8], Kintz [9,10], Barroso [1,4] or Nakahara [11]. The basics of hair anatomy and physiology have also been made clear in journal papers, for instance those from Harkey [12], Huestis and Cone [13], and others [3].

While in the 1960s and 1970s hair analysis was mostly used to assess exposure to toxic elements, nowadays this type of analysis is directed towards other kinds of substances (such as drugs of abuse, pharmaceuticals, environmental contaminants and hormones, among others), in the context not only of clinical and forensic toxicology, but also of occupational and traffic medicine [10,14]. This extended scope is undoubtedly associated to the evolution of analytical methods, especially in terms of sensitivity [10]. Moreover, scientific research with hair is continuously growing, and published studies have been more focused on detailing drug incorporation mechanisms and drug behaviour in hair, rather than solely on drug detection [11].

As with conventional samples are involved, hair sample preparation is an important step in isolating the desired components from the matrix and markedly influences precision and accuracy [15]. Although testing hair for drugs can be considered a much more complex problem, it is technically no more difficult or challenging than testing other specimens [3,14]. According to the guidelines from the Society of Hair Testing (SoHT) for drug testing in hair [16], the process first involves a decontamination procedure, then a pre-treatment step is applied to release and isolate the drugs from the matrix, and finally the resulting extract can either be analysed directly or further purified before chromatographic analysis.

The most common clean-up procedures generally involve either liquid−liquid (LLE) or solid-phase extraction (SPE) approaches [16,17]. However, in order to minimise waste production, novel microextraction approaches have been introduced, and these are both less time- and labour-consuming [18]. The development of solid-phase microextraction (SPME) in 1990 [19] boosted this new era of greener techniques for analytical chemistry, briefly described as the three Rs (replace, reduce and recycle: replacement of hazardous organic by green solvents, reduction of solvent usage and of waste products, and solvent/sorbent recycling) [18]. Over the years SPME has gained considerable popularity, and several modifications or alternative approaches have been introduced [20]. In the same decade, special attention was also paid to liquid-phase microextraction (LPME), implemented for the use of small volumes of liquid samples [20]. Just like SPME, LPME has been subject to modifications and improvements up to date [20]. These sample preparation approaches may also be used in the determination of metals and non-metals, which are afterwards determined using atomic spectrometry techniques, while the determination of organic compounds requires chromatographic and mass spectrometric techniques. Two reviews on hair analysis were published in 2015 [21,22] and one in 2017 [23]. Baciu et al. [21] focused mainly

on analytical instrumentation and its advantages in terms of performance. Furthermore, clean-up procedures and their relevance were poorly addressed, and the clean-up procedures considered were the classical SPE and LLE. On the other hand, Vogliardi et al. [22] dedicated their review to hair sample pre-treatments, including a comprehensive review of basic, acidic, methanolic, acetonitrile and enzymatic extraction procedures. Extensive research on sample pre-treatments was conducted, but the conditions used (e.g., solvents, type of sorbent and steps) were poorly described. Additionally, results such as analytes recoveries and limits of detection (LODs) and quantification (LOQs) could have been cited in order to facilitate a critical comparison. The authors have addressed a few microextraction approaches, namely SPME, focusing particularly on *headspace* (HS)-SPME, and LPME, but few applications to hair analysis were described. More recently, He et al. [24] and Brandão da Costa et al. [25] reviewed microextraction approaches for samples analysed in forensic toxicology. Nevertheless, all mentioned reviews were dedicated to the determination of drugs of abuse. Outside the forensic field, the review by Pozebon et al. [23] focused on elemental analysis in hair. This is also of great interest since the determination of chemical elements exposure by hair analysis has increased in toxicological, clinical and environmental investigations. The authors addressed some miniaturised techniques used for the concentration of chemical elements before their determination by inductively coupled plasma-mass spectrometry (ICP-MS) due to its high sensitivity in trace elemental analysis [23].

The aim of the present paper is to review the application of microextraction approaches to hair testing, which have seen developments over the last 5 years. Regarding SPME, improvements and innovations have been reported, for instance in-tube (IT)-SPME. Additionally, the development of new fibre coatings for both direct immersion (DI)-SPME and HS-SPME is a novelty worth noting and addressing. Also, the application of microextraction by packed sorbent (MEPS) to clean-up hair digests is growing, providing great extraction efficiency for drugs of abuse. Lastly, dispersive micro-solid phase extraction (D-μ-SPE) has been boosted by the use of magnetic nanoparticles (MNPs), as well as of ion imprinted polymers (IIP).

There has also been increased research in LPME, mainly in what concerns dispersive liquid-liquid extraction (DLLME), with the use of supramolecular solvents and room temperature ionic liquids, which offer promising applications and meet the recommendations of green chemistry. Also, in this field of DLLME, the solidification of floating organic drop has been highlighted for a simple and fast procedure.

This review compiles all microextraction approaches that have been applied in the analysis of hair samples for clean-up purposes since 2015. More emphasis is given to new advances, solvents, sorbents and fibres, in procedures for hair analysis. More than 60 original research articles on the improvements in this field have been published since 2015 and, up to date, no review addressing in full microextractions applied to hair specimens, advantages and analytical results is available. This review also addresses several types of compounds in hair analysis, e.g., metals and non-metals, drugs of abuse, pharmaceutical drugs, and biological markers. In addition, recent developments, different formats and configurations and performances of miniaturised techniques are discussed.

1.1. Literature search criteria and overview

Two electronic databases were used for the systematic literature search: Medline and Google scholar. Search strings were “microextraction hair samples” (All Fields), and only papers from 2015 to the 31st of October 2020 were selected.

To be subject to review, the selected papers had to fulfil a number of criteria, and therefore papers involving (*i*) clinical studies and *(ii)* case reports in which hair analyses were associated to microextraction approaches; (*iii*) microextraction approaches coupled to analytical methods developed for hair analysis; (*iv*) drugs and chemical elements determined in hair samples using microextraction approaches were included in this review. Conversely, exclusion criteria included: (*i*) papers written in a language other than English; (*ii*) letters to the editor, opinions, reviews; and (*iii*) studies using animal hair.

A total of 69 articles were included out of 111 found, indicating that the use of microextraction approaches for the clean-up of hair samples is a growing field. Only 17 articles that met the inclusion criteria had been published before 2015. Both SPME and LPME approaches were extensively studied. SPME was the most reported due to new research on sorbent materials, but LPME investigation was not that farther away, which might be justified by the growing use of new solvents with lower toxicity, agreeing with recent concerns about costs and environmental hazards of solvent waste disposal (Fig. 1a).

Regarding the SPME approach (Fig. 1c), the D-μ-SPE technique seems to be the one for which more improvements were reported during the considered period, if we do not consider the different variants of fibre microextraction as a whole, namely DI-SPME, HS-SPME or IT-SPME. New improvements regarding solid sorbents have been published mainly for D-μ-SPE, and in addition to multi-walled carbon nanotubes, graphene, graphene oxide, there has been an increased interest in using modified MNPs and ion-imprinted polymers (IIPs). The fact that fibre-SPME can result in breakage, stripping of coatings, and possible bending of needles, may justify the great research made nowadays on D-μ-SPE also for the analysis of hair samples. Nevertheless, amongst fibre-SPME variants, HS-SPME still remains the most versatile in what concerns the analytes, which justifies the great number of applications of this technique published in the past five years, although with few modifications in the fibre type. A smaller number of applications to the analysis of hair specimens is observed with MEPS and with magnetic solid-phase microextraction (MSPME); however, both techniques are quite compelling.

Concerning LPME (Fig. 1b), the hollow fibre (HF)-LPME approach has been increasingly applied to hair samples, showing also great versatility regarding the analytes. This is also due to improvements in the technique, namely functionalization with graphene oxide (GO) and, in fewer cases, the use of ionic liquids. Nevertheless, when all variants of dispersive liquid-liquid microextraction (DLLME) are considered together, this is undoubtedly the most studied and applied LPME approach. The inclusion of solidification of floating organic drop (DLLME-SFO), supramolecular solvent-based (SM-DLLME), and temperature controlled ionic liquids (TIL-DLLME) has brought some advantages, such as lower toxicity. The use of TIL-DLLME to clean-up hair samples analysis has been widely reported over the past five years, with these reports focusing mainly on the pre-concentration of metals.

In general, analytes have not changed much from reports prior to 2015. Metals and other elements continue to be the most frequently determined species, followed by drugs of abuse and pharmaceutical drugs (Fig. 2a). Although many improvements and developments were observed, almost half of the reports focus on the determination of a single analyte, particularly concerning metals (Fig. 2b). This can be justified by selectivity issues. Additionally, most of the published multi-analyte determinations using microextraction approaches involved only one class of compounds (Fig. 2c).

Concerning elemental analysis, it is possible to observe that the dispersive approach has been tested most frequently, making D-μ-

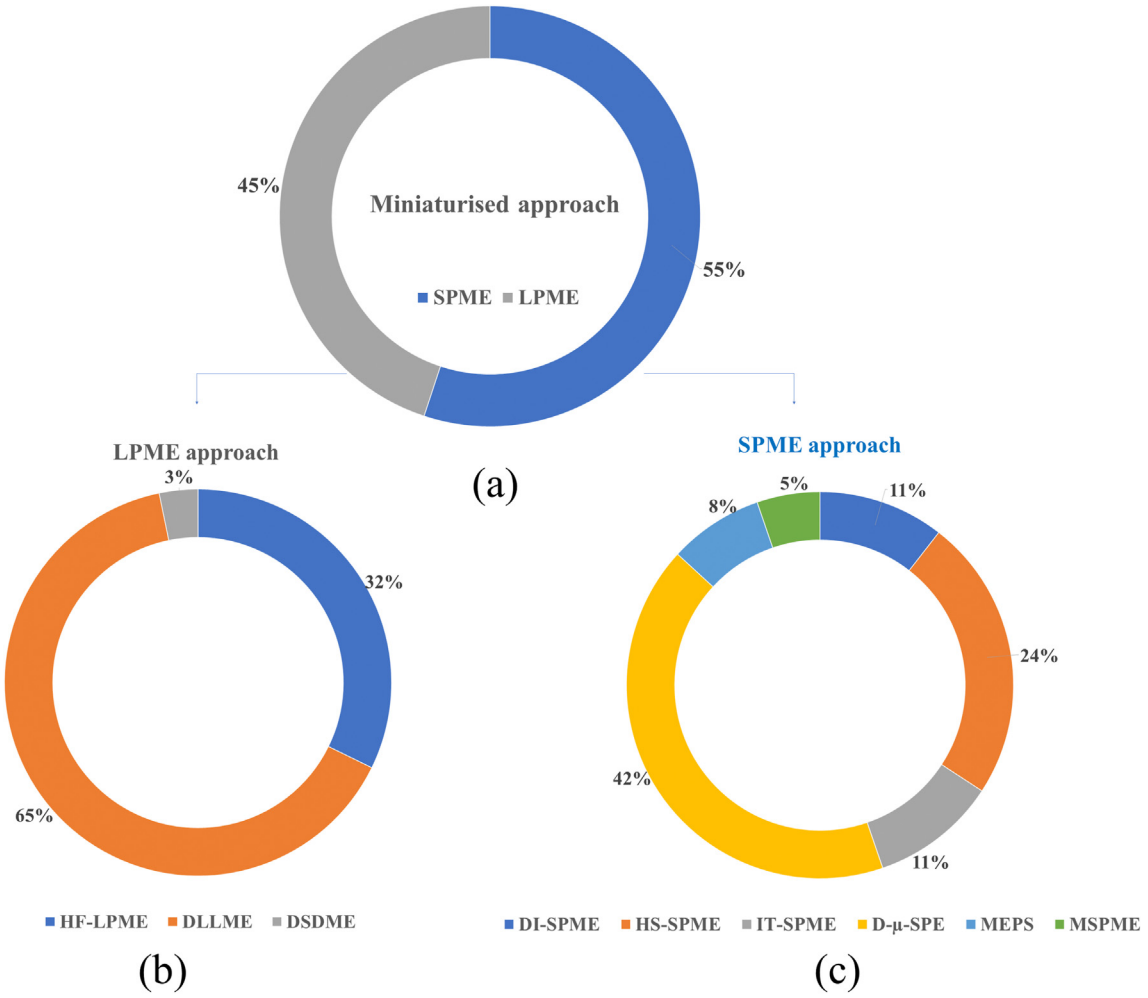

Fig. 1. Percentage of papers about approaches used for the clean-up of hair samples published since 2015: a) all miniaturised approaches; b) LPME approaches; c) SPME approaches.

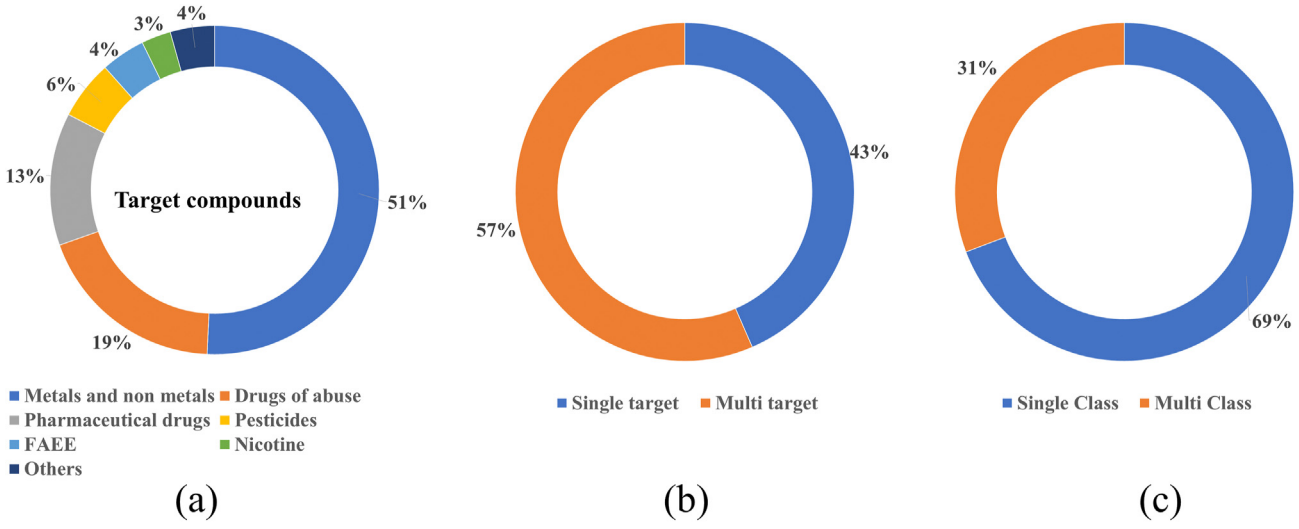

Fig. 2. Percentage of species and compounds determined using miniaturised cleanup techniques published since 2015: a) all target compounds; b) single target vs. multi target analysis; c) single class vs. multiclass analysis.

SPE and DLLME the most studied techniques (Fig. 3). In fact, most new developments in sorption materials applied in D-μ-SPE were used for the preconcentration of chemical elements. The subsequent elemental analysis after pretreatment of the samples by dispersive approach was performed by cold vapour atomic absorption spectrometry (CVAAS), which is commonly used for

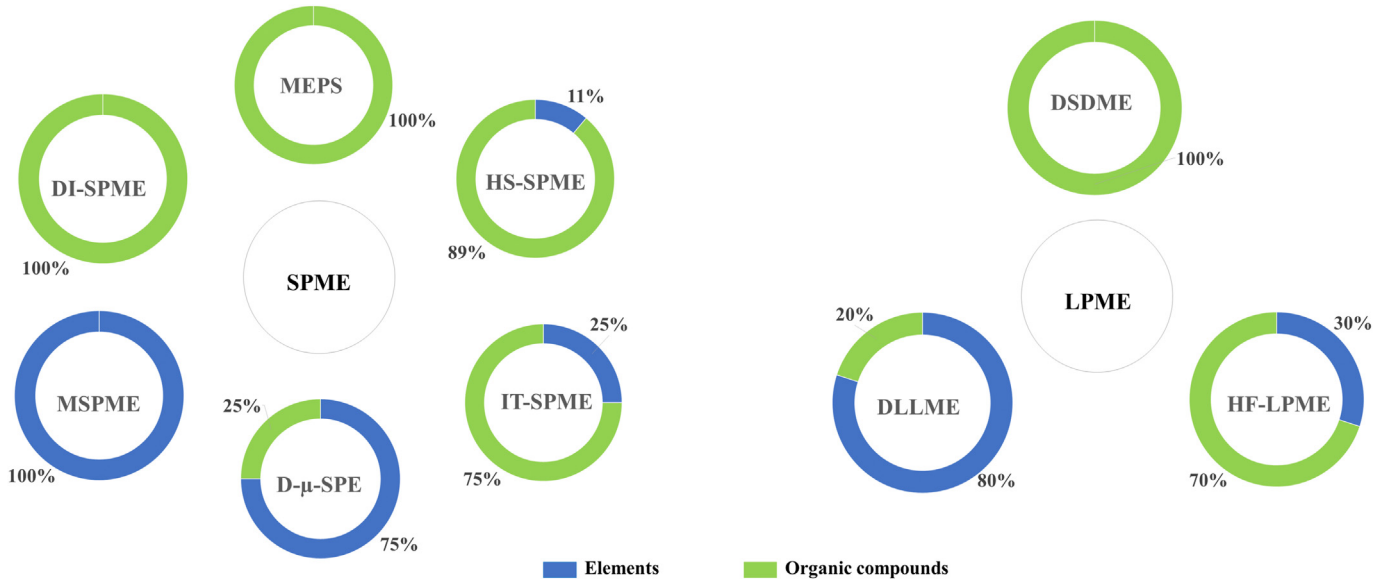

Fig. 3. Percentage of elements and organic compounds determined in each miniaturised cleanup technique published since 2015.

mercury determination, electro thermal atomic absorption spectrometry (ETAAS), or flame atomic absorption spectrometry (FAAS). Atomic absorption spectrometry appears, then, as the elected analytical instrumentation [26–46]. However, according to data presented in 2017 by Pozebon et al. [23], ICP-MS was predominantly used, followed by ICP-OES, while atomic absorption spectrometry was only used in a few applications, mainly for the determination of mercury. In this work, only a few articles report the use of ICP-MS, which could be justified by the fact that the summarised publications focus primarily on the preconcentration of elements and new extraction developments. Two works were reported for MSPME, and metals were the target analytes. Still on the SPME approach, no developments or research on the preconcentration of chemical elements using DI-SPME or MEPS have been observed in the last five years. Fig. 4 discriminates the approaches used in the last five years for each element and group of organic compounds, complementing the described information. The sorption materials developed for D-μ-SPE could either be packed in bins for MEPS or cover DI-SPME fibres, but in the first case the procedure would become laborious, and in the second case stability problems could arise.

DLLME has also been widely used in elemental hair analysis. Both supramolecular solvents and temperature controlled ionic liquids attracted great interest from the scientific community, as well as microextraction approaches used for the clean-up of hair extracts. In terms of hair analysis, in the last five years SM-DLLME and TIL-DLLME were only investigated for the isolation of chemical elements. This is not only justified by the lower toxicity of these solvents, but also by the great recoveries obtained with their use. The subsequent analysis is also predominantly performed by atomic absorption spectrometry.

2. SPME

Arthur and Pawliszyn [19] developed a new method called SPME, and it rapidly gained popularity [47]. In SPME, a small

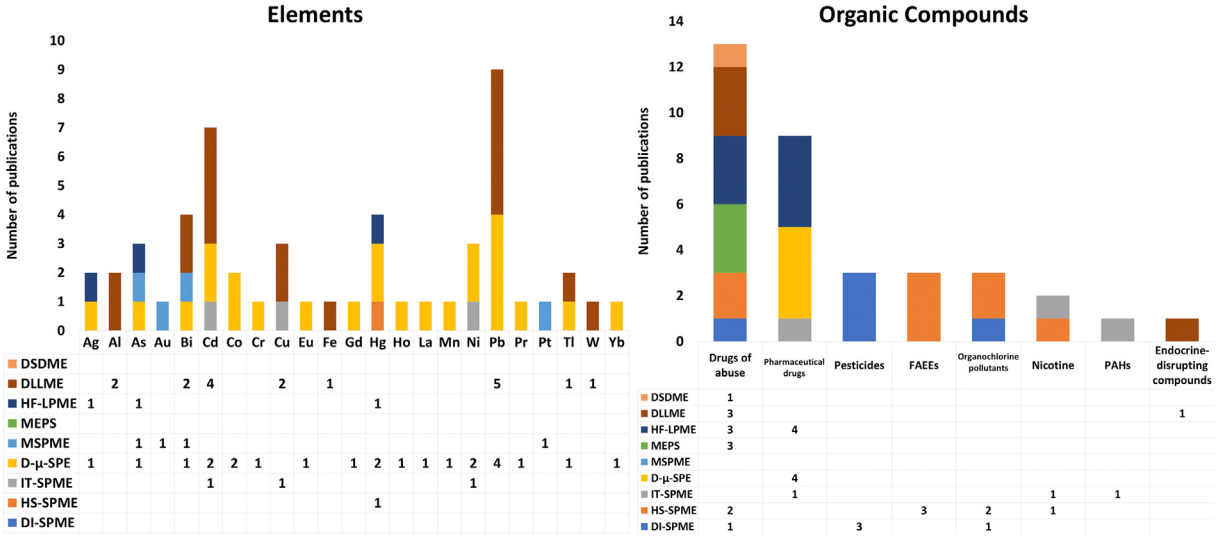

Fig. 4. Distribution of miniaturised approaches according to the type of analyte.

amount of extracting phase is placed on a solid support (fibre) that is exposed to the sample. After the partitioning of the compounds of interest between the sample and the extracting phase, the fibre is placed in a gas (GC) or liquid-chromatograph (LC) interface for desorption and subsequent analysis [48]. Usually, the solid support consists of a fused-silica fibre coated on the outside with an appropriate stationary phase [49]. The coatings are designed in order to be hydrophobic enough to be able to exclude water, a major component in most samples, but at the same time to be efficient for analyte extraction. In addition, the parameters affecting adsorption and desorption should be previously optimised for maximum sensitivity. Nevertheless, there is an engineering of coatings with higher selectivity, by varying their chemical composition or incorporating affinity moieties, which facilitates fast determinations by direct desorption of the SPME fibre in detector devices [50].

This sampling technique has many advantages over classical sample preparation techniques, being *(i)* rapid, simple, solvent free and sensitive, *(ii)* a simple, effective adsorption/desorption technique, *(iii)* compatible with GC or LC instrumentation, *(iv)* capable of providing linear and highly consistent results for a wide range of concentrations, and *(v)* small in size, which is convenient for portable devices used in field sampling [47].

Three types of SPME fibre can be used to clean-up in the analysis of hair samples for the determination of several substances: DI-SPME; HS-SPME and IT-SPME.

In addition to fibre SPME, new and increasingly popular solid-phase miniaturised techniques may be included in this category. For instance, D-μ-SPE has been recently proposed by several researchers for clean-up in hair samples analysis aiming at improving sensitivity. This approach involves adding small amounts of sorbent (in the low mg range) to the aqueous solution. After the compounds are retained, they are eluted, thermally desorbed or directly monitored by spectroscopic techniques [51]. In addition, these low amounts of sorbent material can be packed in a chip, and their usefulness for hair analysis has already been proven. Another miniaturised approach is MEPS, in which the sorbent (1–4 mg) is placed in a syringe barrel, acting as a plug, or between the needle and the barrel, acting as a cartridge. The cartridge bed can be packed or coated with several sorbents, providing selective and suitable sampling conditions [52]. One of its features is the possibility of reusing the sorbent several times and the ability of online coupling to LC–MS and GC–MS systems [52].

2.1. DI-SPME

DI-SPME involves a fibre, commonly a fused-silica needle coated with a stationary phase, fitted in a syringe-type holder [53,54]. The fibre is then directly exposed to the hair extract solution for concentration of the analytes.

There are several commercially available SPME fibres, and those for which more applications for hair extracts clean-up have been published are typically non-polar phases (polydimentylsiloxane, PDMS 100 μm), followed by bipolar phases (polydimentylsiloxane/divinylbenzene, PDMS/DVB 65 μm). The choice of these fibres depends on the target analyte, and while PDMS (30 or 100 μm) fibres have mainly been used to concentrate methadone, cannabinoids and cocaine [55–58], the PDMS/DVB 65 μm fibre was used for pesticides [59,60]. The use of this technique for the pre-concentration of elements has not been reported in the last five years. Although other fibres are commercially available, such as tripolar (divinylbenzene/carboxen/polydimentylsiloxane, DVB/CAR/PDMS 50/30 μm), bipolar (carboxen/polydimentylsiloxane, CAR/PDMS 75 μm), and polar (polyethylene glycol, PEG 60 μm, and polyacrylate PA 85 μm) phases, they have not been used yet for DI-SPME [61]. The adsorption of the target compounds will reflect the equilibrium partitioning between the coated fibre and the sample, after which the amount of each analyte will be directly related to its concentration in the aqueous sample [53]. Reports on DI-SPME are quite consensual regarding this process, stating that the whole extraction and analyte transfer steps usually take only a few minutes [53]; however, clean-up of hair extracts with this technique tend to require a minimum of 20 min equilibrium per sample, either at room or elevated temperature.

While published methods before 2015 were primarily developed aiming at determining a small number of analytes (usually 1 to 3) [55–58,62–64], which would require less exposure of the fibre to the sample, methods published in the last five years have been more ambitious in terms of the number of analytes. Indeed, recent methods reported DI-SPME for the determination of a larger number of compounds in hair, most of which involving pesticides and metabolites. The number of compounds was 56, including organochlorines, organophosphates, pyrethroids, carbamates, other pesticides and polychlorinated biphenyls (PCBs) [60], up to 140 pesticides and metabolites [65]. Béranger et al. [65,66] presented two reports using DI-SPME when, actually, being the second report essentially statistical analyses of 64 of the 140 compounds presented in the first report [65]. In order to obtain good extraction yields extraction times of 80 min at a temperature of about 60 °C were reported by the authors, and these conditions may not seem appealing to a routine analysis laboratory. Nevertheless, the choice of the SPME fibre is critical and should be evaluated carefully, as extraction yields may vary from 12 to 144%, depending on the affinity of the compounds with the fibre, as shown by Hardy et al. [60]. Table 1 summarises extraction, clean-up conditions and results in hair analysis obtained using DI-SPME in the last five years.

2.2. HS-SPME

The headspace sampling is a fundamental technique to characterise the volatile fraction of samples. However, regarding biological specimens, HS-SPME can be more advantageous than DI-SPME, since the influence of matrix is reduced, resulting in cleaner extracts [68]. In this variation of SPME, the concentration factor of a compound will depend on its structure and volatility, on the fibre coating, and on a few physical parameters, namely agitation, headspace equilibration temperature and time, salting out, and compound diffusion rate from the vapour phase to the surface of the fibre [69].

For hair specimens it is not different, and careful adjustments of extraction conditions may significantly enhance sensitivity, even enabling the determination of semi-volatile compounds [68]. In addition, in order to further improve sensitivity, derivatization procedures by acylation, alkylation and/or silylation reagents are common. HS-SPME is used wherever possible, as evidenced by the larger number of applications and analytes reported for hair (Table 2) when compared to DI-SPME, which may be due to the fact that in HS-SPME the fibre is not directly exposed to hair extracts. Although fibres have generally proven to be robust for DI-SPME applications, some authors point out the fact that, as gas is sampled in HS-SPME, more hostile (e.g., strong acid or alkaline) sample preparation and derivatization conditions may be used, which cannot be used in DI-SPME because of fibre damage [68].

HS-SPME has been reported for instance in the determination of organochlorine pollutants [70–72], metals [73], fatty acid ethyl esters (FAEE) [74–77], recreational drugs [78–89], pharmaceutical drugs [90,91], and nicotine [92,93]. This wide range of applications is linked to the use of different types of fibres. A polar phase PA 85 μm was used in the determination of nicotine in hair [93], while bipolar phases such as CAR/PDMS 75 μm have been described as

Table 1
Hair analysis using DI-SPME; extraction, clean-up conditions, instrumentation, LOD, LOQ and analytes recoveries.

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Fibre	Extraction/ desorption	Instrumentation	LOD	LOQ	Recoveries	Ref.
64 pesticides and metabolites	50 mg	SDS and then methanol (n.s.)	Overnight at 40 °C; 1 mL of acetonitrile	300 μL of extract and 7.6 mL of phosphate buffer at pH 7 (1 M)	n.s.	Fibre exposure: 60 °C for 80 min; desorption:260 °C for 10 min.	GC-MS/MS	0.003 −3.091 pg/ mg	0.02 −50 pg/ mg	n.s.	[66]
140 pesticides and metabolites	50 mg	SDS and then methanol (n.s.)	Overnight at 40 °C; 1 mL of acetonitrile	300 μL of extract and 7.6 mL of phosphate buffer at pH 7 (1 M)	n.s.	Fibre exposure: 60 °C for 80 min; desorption: 260 °C for 10 min.	GC-MS/MS	n.s.	0.02 −50 pg/ mg	n.s.	[65]
Norcocaine and cocaethylene	n.s.	n.s.	Overnight at 60 °C; 1 mL of methanol	Extract dried under a stream of nitrogen. Addition of phosphate buffer (pH 7.4)	30 μm PDMS	Fibre exposure: 80 °C for 1 h; desorption:2 min.	GC-MS	0.01 ng/ mg	0.02 ng/ mg	88−94%	[67]
56 compounds, including organochlorines, organophosphates, pyrethroids, carbamates, other pesticides and polychlorinated biphenyls (PCBs)	50 mg	Water for 2 min and acetonitrile for 2 min	Overnight at 40 °C; 1 mL of acetonitrile/ water (80:20, v/v)	300 μL of extract and 7.6 mL of phosphate buffer at pH 7 (1 M)	65 μm PDMS-DVB	Fibre exposure: 60 °C for 80 min; desorption: 260 °C for 10 min.	GC-MS/MS	n.s.	0.2 −5.5 pg/ mg	12−144%	[60]

GC-MS (Gas chromatography-mass spectrometry); GC-MS/MS (Gas chromatography-tandem mass spectrometry); LOD (Limit of detection); LOQ (Limit of quantification); n.s. (not specified); PDMS (Polydimethylsiloxane); PDMS/DVB (Polydimethylsiloxane/Divinylbenzene; SDS (Sodium dodecyl sulphate).

great fibre coatings for the determination of ethyl glucuronide [94], or as CAR/DVB 65 μm for pharmaceutical drugs in hair [90,91]. However, as also occurs for DI-SPME, the commonly described fibres for HS-SPME are PDMS 100 μm and PDMS/DVB 65 μm. While the PDMS 100 μm fibre was commonly used for hair analysis in the past, only one article describing its use has been published in the last five years. The most recent works mainly report the use of bipolar phases, probably due to the recommendations of the manufacturers for more volatile polar analytes (e.g., amines and alcohols) or the associated advantages, namely more efficient adsorption and faster release of the analytes when PDMS/DVB 65 μm coated fibre is used. This fibre was successfully applied to preconcentrate mercury from hair samples, and this was the only element determined using HS-SPME in the period covered in the present review.

In the past 5 years, some authors have developed and tested their own fibre coatings for specific analytes. This is the case of Ghiasvand et al. [92] who synthesised a thin film of sulfonated graphene/polyaniline (Sulf-G/PANI) nanocomposite for fibre coating. The authors used a vacuum-assisted HS-SPME variant to enhance nicotine pre-concentration from hair samples.

2.3. IT-SPME

IT-SPME, also named capillary microextraction (CME) by several authors, has been successfully used to clean-up hair extracts. Its application has been mainly in the determination of metals [95−101], although its use to pre-concentrate mutagenic and carcinogenic heterocyclic amines (HCAs) [102] and drugs of abuse as amphetamine, methamphetamine and their methylenedioxy derivatives [103] has also been described. This versatility is justified by the available types of coatings, as it also occurs with other SPME techniques. There has been an interest in polymers such as polypyrrole (PPY), and this has increased due to their great potential. New polymers have features of novel materials, namely ion exchangers, energy storage materials, corrosion-resistant coatings, catalysts, separation materials, actuators and chemical sensors [103]. Nevertheless, even with these novel materials, some of the commercially available capillaries did not allow good recovery of polar compounds and ionic species, mainly because of the less polar and non-ionic stationary phases used. The need to develop novel coating materials for IT-SPME has led Zheng and Hu and coworkers to dedicate great part of their research to this field. Coatings made of Congo red modified single wall carbon nanotubes (CR-SWCNTs) [96], sol−gel 3-mercaptopropyltrimethoxysilane (MPTS) modified silica [101], sol−gel coating of N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AAPTS)-silica [100], or poly(N-isopropylacrylamide) (PNIPA) gel [98] are examples of developments by these scientists, and have proven suitable for metals pre-concentration from human hair samples. The research continued in the past five years, with Hu et al. [95] preparing an amino group modified poly(glycidyl methacrylate-co-divinyl benzene) [poly(GMA-DVB-NH2)] monolithic capillary column for the selective extraction of metals from hair samples. Additionally, Yao et al. [104] proved that this technique can be expanded to pharmaceutical drugs, and developed a graphene-modified monolithic capillary column for the determination of benzodiazepines. The authors report that graphene forms π−π stacking interactions with benzene rings of poly(N-vinylcarbazole-divinylbenzene), resulting in improved efficiency for benzodiazepines, when compared to the neat polymer, providing additional chemical stability to the graphene monolith.

IT-SPME is considered by many authors as an ideal sample preparation technique due to its fastness, automation and also because it is solvent-free and inexpensive [105,106]. However, in the last five years, IT-SPME did not appear to be the most attractive miniaturised technique to use for clean-up in hair analysis. In fact, commercially available capillary columns do not guarantee good extraction efficiencies for many compounds, and developing modified monoliths is time consuming, which may justify their infrequent use. Table 3 summarises the developed methods using IT-SPME for hair analysis.

2.4. D-μ-SPE

One of the most popular miniaturised solid-phase based techniques used for pre-concentration, clean-up and extraction

Table 2
Hair analysis using HS-SPME; extraction, clean-up conditions, instrumentation, LOD, LOQ and analytes recoveries.

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Fibre	Extraction/ desorption	Instrumentation	LOD	LOQ	Recoveries	Ref.
Organochlorine pollutants	100 mg	2 × 5 mL water and 1 × 5 mL of hexane	n.s.	2 mL of NaOH 10 M; 1 mL of water and 0.3 g of NaCl	65 μm PDMS/DVB	Fibre exposure: 90 °C for 30 min at 250 rpm. Desorption: 270 °C for 5 min.	GC-MS	0.1 −6.1 pg/ mg	0.3 −20.3 pg/ mg	n.s.	[70]
Fatty acid ethyl esters	50 mg	2 × 8 mL n-heptane for 15 min by gently shaking in one-way aluminium beaker	15 h at 25 °C; 0.5 mL of DMSO and 2 mL of n-heptane	Cooled below 0 °C; n-heptane phase evaporated; 1 mL phosphate buffer (0.1 M, pH = 7.6) and 0.5 g NaCl added	65 μm PDMS/DVB	Fibre exposure: 90 °C for 30 min at 250 rpm. Desorption: 250 °C for 5 min.	GC-MS	n.s.	0.014 −0.020 ng/ mg	n.s.	[74]
Fatty acid ethyl esters	50 mg	n-heptane and dried in a sample concentrator for up to 30 min	12 h at 80 rpm; 0.5 mL of DMSO and 2 mL of n-heptane	Cooled below 0 °C; n-heptane phase evaporated; Phosphate buffer and NaCl (n,s) added	65 μm PDMS/DVB	Fibre exposure: 90 °C for 30 min at 500 rpm. Desorption: 260 °C for 0.5 min.	GC-MS/MS	3−10 pg/ mg	9−34 pg/ mg	n.s.	[75]
Hg and MeHg	100 −150 mg	DIW, detergent, DIW and acetone	12 h at 60 °C; 0.5 mL of DIW, 1.0 mL of 30% (v/v) HNO_3.	Cooled; Filtered; Neutralised with 1.5 M KOH; 5 mL of DIW; pH adjusted to 4.5 with acetate buffer. Derivatization: 0.2 mL of 1% (w) NaBEt4 solution (melted at 0 °C).	65 μm PDMS/DVB	Fibre exposure: 25 °C for 10 min. Desorption: 150 °C for 1 min.	GC-PDOES	0.35 −1 pg/mg	1.2 −3.3 pg/ mg	85−93%	[73]
Fatty acid ethyl esters	50 mg	2 × heptane (3 m Land 5 min of stirring each)	16−17 h; 2 mL of heptane and 0.5 mL of DMSO	Dried; 1 mL phosphate buffer 50 mM added	65 μm PDMS/DVB	Fibre exposure: 90 °C for 30 min. Desorption: 250 °C for 1 min.	GC-MS	n.s.	0.01 −0.09 ng/ mg	n.s.	[76]
Nicotine	n.s.	n.s.	n.s.	n.s.	Sulfonated graphene-polyanilinenanocomposite coated fibre	Fibre exposure: 60 °C for 10 min. Desorption: 280 °C for 2 min.	GC-FID	0.002 ng/ mg	n.s.	94−95%	[92]
JWH-073 and Cannabis Congeners	50 mg	n.s.	30 min at 80 °C; NaOH 1 M	Neutralised with HCl 10 M, LLE at pH 8.0 with hexane/ethyl acetate (9/1)	100 μm PDMS	Fibre exposure: 120 °C for 20 min. Desorption: 270 °C for 10 min.	GC-MS	0.01 ng/ mg	0.1 ng/mg	n.s.	[78]
PCBs, DDTs and HCB	n.s.	Hexane and water (n.s.)	NaOH (n.s.)	NaCl (n.s.)	n.s.	n.s.	GC-MS	n.s.	n.s.	n.s.	[72]
Methamphetamine and amphetamine	20 mg	3 × water and acetone. The hair was dried at 60 °C	30 min at 70 °C; 200 μL of 0.5 M NaOH.	Cooled to 40 °C. Derivatization with 50 μL of HFBCl:HFBA (8:2, v/v), combined with 1650 μL of 1 M K_2CO_3	65 μm PDMS/DVB	Fibre exposure: 90 °C for 10 min. Desorption: 250 °C for 5 min.	GC-MS	0.10 −0.15 ng/ mg	0.15 −0.20 ng/ mg	n.s.	[79]

DIW (Deionised water); DMSO (Dimethyl sulphoxide); GC-FID (Gas chromatography - Flame Ionization Detector); GC-MS (Gas chromatography - Mass Spectrometry); GC-MS/MS (Gas chromatography - Tandem Mass Spectrometry); GC-PDOES (Gas chromatography - Point Discharge Optical Emission Spectrometer); HFBA (Heptafluorobutyric anhydride); HFBCl (Heptafluorobutyric chloride); LOD (Limit of detection); LOQ (Limit of quantification); n.s. (not specified); PDMS (Polydimethylsiloxane); PDMS/DVB (Polydimethylsiloxane/Divinylbenzene; PTFE (Polytetrafluoroethylene).

Table 3
Hair analysis using IT-SPME; extraction, clean-up conditions, instrumentation, LOD, LOQ and analytes recoveries.

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Fibre	Extraction/ desorption	Instrumentation	LOD	LOQ	Recoveries	Ref.
Nicotine and cotinine	1 −2 mg	3 × 1 mL dichloromethane	30 min at 80 °C; 1 mL of distilled water	Volume made up to 1 mL with distilled water	Carboxen 1006 PLOTcapillary column (60 cm × 0.32 mm i.d.)	Load: 25 draw/ eject cycles of 40 μL sample at a flow rate of 200 μL/min; Desorption: 2.5 mM ammonium formate/methanol (25/75, v/v) at a flow rate of 0.2 mL/min.	LC-MS/MS	0.45 and 0.13 pg/ mL	n.s.	87−97%	[107]
Ni, Cu and Cd	50 mg	Shampoo (n.s.), 10 mL acetone for 30 min; 2 × water (n.s.).	Microwave digestion: 150 °C at 10 atm for 2 min, 180 °C at 18 atm for 2 min and then 200 °C at 25 atm for 3 min; 800 W; 3 mL concentrated HNO_3	Water to 50 mL; pH 8.0	Amino group modified poly (glycidyl methacrylate-co-divinyl benzene) [poly (GMA-DVB-NH2)] monolithic capillary column	Load: 1 mL sample at a 0.1 mL/min flow rate for 10 min. Desorption: 40 mL 0.2 mol/L HNO_3 at a 0.1 mL/min flow rate.	ICP-MS	1.5 −17.1 ng/ L	5 −50 ng/ L	85−112%	[95]
Benzodiazepines	40 mg	n.a.	30 min; sonication; 10 mL acetone	200 μL of methanol, diluted with 4 mL of water	A graphene monolithic column fabricated in a capillary using π-electron-rich poly(N-vinylcarbazole-divinylbenzene)	Load: 4 mL sample at a 0.1 mL/min flow rate. Desorption: 80 μL of methanol at a 0.01 mL/min flow rate.	HPLC-MS	n.s.	n.s.	87.2 −94.3%	[104]
18 Polycyclic aromatic hydrocarbons (PAHs)	2 −5 mg	3 × 1.0 mL n-hexane	60 min; sonicated; 0.4 mL of 50 mM NaOH in methanol.	0.05 mL of 0.2 M acetate buffer (pH 5.0). Diluted up to 0.5 mL with water	CP-Sil 19CB (14% cyanopropyl phenyl methylsilicone) capillary column (60 cm × 0.32 mm i.d., film thickness 1.0 m)	Load: 20 draw/ eject cycles of 40 μL sample at a flow rate of 150 μL/min. Desorption: (n.s.)	HPLC-FLD	0.5 −20.4 pg/ mL	n.s.	70−113%	[108]

HPLC-FLD (High performance liquid chromatography - Fluorescence detection); HPLC-MS (High performance liquid chromatography - Mass spectrometry); ICP-MS (Inductively coupled plasma - Mass spectrometry); LC-MS/MS (Liquid chromatography − Mass spectrometry); LOD (Limit of detection); LOQ (Limit of quantification); n.s. (not specified).

procedures involving human hair specimens is D-μ-SPE [109]. The sorbents are either micro- or nanoparticles (NPs), and as it happens with SPME fibre coatings, several commercial or synthetic nanomaterials are available, for instance functionalised silica, multi-walled carbon nanotubes, graphene, graphene oxide and modified magnetic NPs (MNPs) [109]. Yet, the described methods applied to hair seem to require larger amounts of sample when compared the previously described techniques.

Modified aluminium oxide [26] and titanium dioxide NPs [27] have been reported as good options for the pre-concentration of metals from hair. Aluminium oxide NPs were considered advantageous because they did not require analyte oxidation or reduction [26]. Still, the most commonly applied NPs for the analysis of hair samples are MNPs because they can effectively be separated under external magnetic field [110−116]. These MNPs can be represented by Fe3O4, as its versatility has allowed concentrating metals [111−113], antidepressant drugs [115] and benzodiazepines [114] from hair samples. However, other MNPs have been synthesised, namely honey coated magnetic multi-walled carbon nanotubes (Honey@magnetic-CNTs) for the determination of sunitinib [110] and Preyssler tungsten heteropolyacid, H14 [NaP5W30O110], immobilised on the surface of mesoporous nanomagnetite to recast nortriptyline [116].

Some authors also recognise that interfering ions might be co-extracted with the target ion on sorbents, including MNPs [38]. To overcome this lack of selectivity, they propose the use of ion-imprinted polymers (IIPs) [38] which have several advantages, such as high selectivity, low cost, ease of production, large surface area, stability at high temperature and pressure, durability and reusability [40]. Regarding application to hair, these polymers only demonstrated successful application to pre-concentrate metals, and the extraction yields obtained were quite comparable to those of MNPs [38,40].

The development of new NPs is still growing and it is possible that their use will soon be extended to other classes of compounds. For instance, Alawadi et al. [41] used a green method for the synthesis of silver nanoparticles (Ag-NPs), which was used for the pre-concentration of bismuth from hair. These NPs were synthesised by the authors from pistachio skin extract. Rajabi et al. [42] synthesised a novel nanosorbent [layered double hydroxide with 4-amino-5-hydroxyl-2,7-naphthalendisulfonic acid monosodium salt interlayer anion (Mg−Al-AHNDA-LDH)] which was used to pre-concentrate potentially toxic metals from hair. The fact that this nanosorbent is immediately dissolved at a pH lower than 4 and that the centrifugation step was unnecessary was a great achievement. Table 4 summarises the extraction and clean-up conditions, and

Table 4
Hair analysis using D-μ-SPE; extraction, clean-up conditions, instrumentation, LOD, LOQ and analytes recoveries.

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Extraction/ desorption	Instrumentation	LOD	LOQ	Recoveries	Ref.
Co, Ni, Mn and Cd	100 mg	n.s.	5 min at 180 °C; 3 mL concentrated HNO_3 and 2 mL H_2O_2	Dissolved with 0.1 M HNO_3	Magnetic ZnFe2O4 nanotubes. 20 mL sample at pH 7.0; 20 mg of adsorbent. Load: Vortex for 50 s. Desorption: 1 mL of 0.4 M HNO_3 sonicated for 40 s.	ICP-MS	n.s.	n.s.	n.s.	[111]
Bi	1 g	Water	2 h at 400 °C; 15 min at 70 °C; 2 mL concentrated HNO_3	Diluted to 25 mL with water; 2 mL diluted to 10 mL with water	Ag-NPs. 10 mL sample at pH 10; 10 mg of adsorbent. Load: stirred for 5.8 min at 400 rpm. Desorption: 100 μL of 3 M HCOOH, vortexed for 4.7 min at 2800 rpm.	ETAAS	0.09 ng/ mL	n.s.	95.6%	[41]
La, Pr, Eu, Gd, Ho and Yb	100 mg	n.s.	180 °C (ramp, 10 °C/min; hold, 15 min) with a power of 1.0 kW; 4 mL of HNO_3 and 2.0 mL of H_2O_2	Dissolved with 0.1 M HNO_3.	Magnetic ZnFe2O4 nanotubes. Sample (n.s.) at pH 8.0; 10 mg of adsorbent Load: sonication for 1.5 min. Desorption: 1 mL of 0.5 M HNO_3 sonicated for 1 min.	ICP-MS	0.01 −0.75 pg/ mL	n.s.	93−107%	[112]
Sunitinib	50 mg	20 mL dichloromethane, 15 mL acetone and 2 × methanol (15 and 10 mL) for 5 min.	5 h at 50 °C; 2 mL methanol pH 7.4 with phosphate buffer solution.	n.s.	Honey coated magnetic multi-walled carbon nanotubes (Honey@ magnetic-CNTs). 5 mL sample; 5 mg of adsorbent Load: stirred in 1 mL of ethanol for 5 min. Desorption: 3 mL of methanol, stirred for 5 min.	HPLC-UV	1.58 ng/ mL	5.28 ng/ mL	89−98%	[110]
Ag	500 mg	Acetone for 30 min, water.	30 mL of a mixture of $HClO_4$ and HNO_3 (1:8, v/v).	Several drops of H_2SO_4 (1:1, v/v); dissolution in water.	Ag(I)-ion imprinted polymer (IIP). 50 mL sample; 30 mg of adsorbent at pH 6 Load: stirred for 20 min Desorption: 1 mL of 2 M HNO_3, stirred for 5 min.	ETAAS	2.3 pg/mL	10 pg/mL	96%	[40]
Tl	500 mg	Acetone for 20 min, water.	30 mL of a mixture of $HClO_4$ and HNO_3 (1:8 v/v).	Several drops of H_2SO_4 (1:1, v/v); dissolution in water.	Nanocomposite of magnetite, halloysite nanotubes and dibenzo-18-crown-6. 10 mL sample pH 10; 40 mg adsorbent Load: sonicated for 4 min Desorption: 2 mL HNO_3 (3.0 M), sonicated for 5 min.	ETAAS	1.8 ng/L	6.0 ng/L	97−98%	[43]
Cd, Cr, Pb, Co, and Ni	2 g	3:1:20 (v/v) and 3:2:5 (v/v) of diethyl ether, acetone, and deionised water, for 1 h in ultrasonic bath. Water.	10 mL of concentrated HNO_3, evaporated, 2 mL of concentrated H_2O_2 (or $HCLO_4$), evaporated.	n.s.	Mg− Al-AHNDA-LDH nanosorbent. 10 mL sample pH 6; 25 mg of adsorbent Load: sonicated for 40 s, rapidly withdrawn and	MS-FAAS	0.6 −2.4 ng/ mL	n.s.	94−101%	[42]

Table 4 (*continued*)

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Extraction/ desorption	Instrumentation	LOD	LOQ	Recoveries	Ref.
					pushed out (10 times). Desorption: (n.s.)					
Pb	500 mg	1 × Water, 3 × acetone, 3 × water	100 °C for 45 min and then at 150 °C for 45 min; 12 mL concentrated HNO_3 and 2 mL concentrated $HClO_4$	Cooled to 70 °C, 5 mL 30% H_2O_2, evaporated, 20 mL 2 M HNO_3 at 100 °C for 1 h. Dissolved in water to 250 mL.	Pb(II)-ion imprinted polymer (IIP). 50 mL sample pH 5.5; 30 mg of adsorbent. Load: stirred for 8 min Desorption: 2 mL 2 M HNO_3, stirred for 4 min.	ETAAS	0.55 ng/L	1.0 ng/L	99.5%	[38]
Pb	50 mg	Acetone for 30 min, 3 × water (n.s.).	Low temperature (n.s.); 3.2 mL of a mixture solution of concentrated nitric acid and perchloric acid (1:8 v/v)	Several drops of H_2SO_4 (1:1 v/v); diluted with water.	Pb (II)-ion imprinted polymer (IIP). 50 mL of sample pH 6; adsorbent Load: shaken for 4 min Desorption: 1 mL of 0.1 M thiourea/ 0.1 M HCl for 5 min.	GFAAS	2.4 ng/L	n.s.	98.6%	[44]
Total Hg, CH_3Hg^+ and Hg^{2+}	100 mg	Non-ionic surfactant, water and acetone.	4 mL of 5 M HCl, sonicated for 30 min.	Diluted with water.	Fe3O4@SiO2@γ-MPTS MNPs. 50 mL sample pH 3.0; 10 mg of adsorbent Load: sonicated for 5min. Desorption: 1.5 M HCl containing 0.01% (m/v) thiourea for CH_3Hg^+; 1.5 M HCl containing 3% (m/v) thiourea for total mercury (THg).	ICP-MS	1.6 −1.9 ng/L	5−10 ng/ L	81.3 −99.6%	[113]
Total Hg, CH_3Hg^+ and Hg^{2+}	350 mg	n.s.	Microwave digestion: 1 mL of 30% H_2O_2; 5 mL of 65% HNO_3; 20 min at 300 W.	Diluted with water.	TiO2. 10 mL of sample pH 7.5; 10 mg of adsorbent Load: sonicated for 5 s. Desorption: 500 μL of 1 M HNO_3 sonicated for 5s.	CVAAS	0.004 ng/ mL	0.013 ng/ mL	n.s.	[27]
Pb	n.s.	n.s.	n.s.	n.s.	In capillary-Schiff's base functionalised magnetic nanoparticles. 10 mL of sample pH adjusted in the range of 4−11; 0.5 mL TX-114; 5.5 mg of adsorbent. Load: 60 μL of hydrophobic [C4MIM][PF6] Desorption: re-dispersed in 0.5 mL of methanol acidified with HCl, sonicated for 5min.	FAAS	n.s.	n.s.	97−106%	[45]
Diazepam, oxazepam, clonazepam, alprazolam, and midazolam	200 mg	20 mL dichloromethane sonicated for 10 min; 15 mL acetone sonicated for 20 min; 2 × 15 mL methanol sonicated for 10 min.	5 h at 50 °C; 2 mL methanol at pH 7.4; stirring	Sonicated for 5 h; filtered; evaporated; reconstituted in water	Fe3O4/SiO2 NPs. 30 mL of sample pH = 8.0; 50 mg of adsorbent; 2 mL the CTAB solution (15 mg/mL). Load: stirred for 30 min at room temperature.	HPLC-UV	9.7 −34 ng/ mL	n.s.	84.9 −90.5%	[114]

(*continued on next page*)

Table 4 (*continued*)

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Extraction/ desorption	Instrumentation	LOD	LOQ	Recoveries	Ref.
					Desorption: 500 μL methanol.					
As	1 g	n.s.	Microwave digestion: 6 mL of concentrated HNO_3 (65%) and 2 mL of concentration H_2O_2 (30%); 2 min for 250 W, 2 min for 0 W, 6 min for 250 W, 5 min for 400 W and 8 min for 550 W, ventilation: 8 min.	Cooled; Diluted to 500 mL with water.	Modified aluminium oxide nanoparticle. 20 mL of sample, 2 mL phosphate buffer 0.4 M (pH 1.5); 8 mg of adsorbent. Load: sonicated for 10 min Desorption: 100 μL of 1 M HCl; sonicated for 10 min.	GFAAS	1.81 ng/L	6.03 ng/L	96%	[26]
Venlafaxine	50 mg	20 mL dichloromethane, 20 mL acetone, and 15 mL methanol (n.s.)	5 h at 55 °C; 2 mL methanol.	n.s.	Fe3O4/KH- 570 MNPs. 4 mL of sample; 10 mg of adsorbent Load: shaken for 25 min. Desorption: 500 μL methanol.	GC-FID	0.2 μg/g	1 μg/g	89.4%	[115]
Nortriptyline	50 mg	20 mL dichloromethane, 15 mL acetone, and 2 × methanol (15 and 10 mL) for 5 min.	5 h at 50 °C; 2 mL methanol pH 7.4	Diluted with water.	Preyssler tungsten heteropolyacid, H14 [NaP5W30O110], immobilised on the surface of mesoporous nanomagnetite. 10 mL of sample; 5 mg of adsorbent in 1 mL of ethanol. Load: sonicated for 15 min. Desorption: 3 mL of methanol; sonicated for 15min.	HPLC-UV	7.9 ng/mL	26.4 ng/mL	93–105%	[116]

CVAAS (Cold vapour atomic absorption spectrometry); ETAAS (Electro thermal atomic absorption); FAAS (Flame atomic absorption spectrometry); GC-FID (Gas chromatography - Flame Ionization Detector); GFAAS (Graphite furnace atomic absorption spectrometry); HPLC-UV (High-performance liquid chromatography with ultraviolet detection); ICP-MS (Inductively coupled plasma mass spectrometry); LDH (Layered double hydroxide); LOD (Limit of detection); LOQ (Limit of quantification); n.s. (not specified); MS-FAAS (Micro-sampling flame atomic absorption spectrometry); PTFE (Polytetrafluoroethylene).

results obtained for D-μ-SPE applied in hair analysis over the last five years.

2.5. MSPME

Although MNPs have been mostly used for D-μ-SPE, their assembling in packed columns in a chip has good biocompatibility, large specific surface area and allows low sample consumption, they are also highly reproducible [117,118]. With this in perspective, a new miniaturised approach was created, the MSPME. Two MSPME applications to hair have been reported in the last five years, both by the same authors [117,118].

In the first application, a novel sorbent consisting of magnetite nanoparticles and 1,3,5-tris(4-aminophenyl) benzene has been described for MSPME in a chip-based array, for pre-concentration of platinum, gold and bismuth from hair [117]. The authors presented a method consisting of eight extraction columns packed with these MNPs merged on a microfluidic chip for micro-extraction. A solution of 12% (m/v) cysteamine hydrochloride (pH 8.0) was used for elution, and ICP-MS was used [117]. Nevertheless, this study was mainly targeted for urine and cell samples, and for this reason little information on hair pre-treatment conditions is provided, namely in what concerns the amount of sample or the washing procedure. The authors only reported results for bismuth, and on the good accuracy achieved.

The second application proposed magnetic metal-organic framework composites (MFCs) for array chip-based MSPME for arsenic species determination in hair samples, using ICP-MS [118]. While MFC with a great surface area and large pore volume was used as sorbent for most target arsenic species, a modified composite (MFCSH) was needed for As (III) extraction. In the proposed method, 0.01, 0.05 and 0.5 M $NH_3 \cdot H_2O$ were sequentially used for desorption of respectively monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and As (V), followed by As determination using ICP-MS. Additionally, 0.5 M HNO_3–2% thiourea (m/v) was used for As (III) desorption [118]. All steps were computer programmed, which allowed for easy automation. Once again, and as occurred in the former work, the target samples were cell cultures, hence poor information is given about hair pre-treatment conditions. With this, the accuracy of the method was verified by extraction and microwave assisted digestion of certified human hair (GSH-1), and good accuracy was reported.

2.6. MEPS

MEPS was developed by Abdel-Rehim et al. at AstraZeneca and it is basically a variation of SPE that has been miniaturised for sample volumes as small as 10 μL [119,120]. In MEPS the sorbent is packed

into a syringe that can be used multiple times, with the advantage of being an user friendly and rapid procedure [121]. Usually the aqueous sample (20–1000 μL) is drawn through the syringe by an autosampler (with up and down cycles) in order to promote adsorption of the analyte. This solid phase is then washed with appropriate solvents (50–150 μL) to eliminate interfering material. The analytes are then eluted directly into the injection port with an organic solvent or LC mobile phase (20–50 μL) [122].

This miniaturised technique is still little used in human hair analysis, although commercially available sorbents have basically the same nature as those used in SPE. Any sorbent material [e.g., silica based (C_2, C_8, C_{18}), strong cation exchanger (SCX), restricted access material (RAM), HILIC, carbon, polystyrene–divinylbenzene copolymer (PS-DVB) or molecular imprinted polymers (MIPs)] can be used, but regarding human hair clean-up only octadecylsilane (ODS) (C_{18}) and M1 (mixed-mode C_8/SCX) have been applied so far [119].

To date, only four analytical methods have been reported on MEPS, three of which were developed in the last five years. Rosado et al. [123–125] have been adopting MEPS as a clean-up approach for hair samples. Its application was successfully demonstrated, so far, for selected opiates, methadone, cocaine and metabolites. In the determination of selected opiates [124] a mixed mode sorbent was used, and the MEPs steps were conditioning (three cycles of 250 μL of methanol and three cycles of 250 μL of 2% formic acid), after which the sample was loaded using 15 cycles of 150 μL. A subsequent wash step was performed with 150 μL of 3.36% formic acid and the analytes were eluted through eight cycles of 100 μL of 2.36% ammonium hydroxide (in methanol). The authors stated that this technique was an excellent alternative to classic procedures, reducing the volumes of organic solvents required and allowing extraction efficiencies between 22 and 99% and obtaining LOQs of 0.025 ng/mg or lower. Following the previous successful application of MEPS, the authors felt compelled to adopt similar procedures for the determination of methadone and its major metabolite in hair [123]. In this case, the conditioning of the mixed mode sorbent included 3 cycles of 250 μL of methanol and 3 cycles of 250 μL of 2% formic acid, after which the hair extract was loaded with 9 cycles of 250 μL. In order to eliminate interferences, 150 μL of 3.36% formic acid was passed through the sorbent and the analytes were eluted using 6 cycles of 100 μL of 2.36% ammonium hydroxide (in methanol). The authors obtained a LOQ of 0.025 ng/mg and recoveries around 60% for both compounds. Over 100 extractions were performed using the same cartridge, resulting in a reduction in cost per analysis. Recently, a MEPS procedure was also developed and optimised to pre-concentrate cocaine and metabolites from 50 mg of hair [125]. The obtained acidic extracts were submitted to MEPS that consisted of mixed mode sorbent conditioned with 250 μL of methanol and 250 μL of deionised water. The extracts were passed through the sorbent in 21 draw-eject cycles of 150 μL, after which a washing step was performed with 50 μL of deionised water and pH 4 acetate buffer. The analytes (cocaine, benzoylecgonine, ecgonine methyl ester, norcocaine, cocaethylene and anhydroecgonine methyl ester) were eluted through three cycles of 100 μL of 2% ammonium hydroxide in methanol, derivatized and determined by GC-MS/MS. Overall, good LOQs were reported with the proposed method, namely 0.010 ng/mg for cocaine and cocaethylene, 0.025 ng/mg for ecgonine methyl ester, benzoylecgonine and norcocaine, and 0.150 ng/mg for anhydroecgonine methyl ester. However, the adopted MEPS procedure resulted in poor recoveries for ecgonine methyl ester (1–3%) and anhydroecgonine methyl ester (4–6%). Better recoveries were obtained, though, for the remaining analytes, between 21 and 73% [125].

Regarding elemental analysis, the great potential of MEPS has not been explored. The new synthesised NPs and IIPs can be incorporated into a MEPS BIN. However, MEPS might seem less user-friendly when compared to D-μ-SPE, with both techniques having the advantage of sorbent reusage.

3. LPME

Although the use of SPME fibres has become increasingly popular, they have significant drawbacks, such as the relatively low recommended operating temperature, their instability and swelling in the presence of organic solvents (which can be troublesome when using HPLC), fibre breakage, stripping of coatings, bending of needles and higher cost [60].

LPME is a miniaturised sample pre-treatment technique adapted from traditional LLE [126]. With this technique only a few microlitres of solvent are needed to extract analytes in comparison to the large amounts of toxic organic solvents required for traditional LLE. In addition, LPME is a faster, more environmentally friendly, and simpler technique, that is also compatible with GC, LC and capillary electrophoresis (CE) [47,127].

Generally, extraction takes place into a small volume of a water-immiscible solvent (acceptor phase) from an aqueous sample containing the analytes (donor phase), and may be divided into two main categories: membrane based extraction, generally called HF-LPME, in which the extraction solvent is supported and protected by a porous membrane; and dispersive liquid-liquid microextraction (DLLME), in which a tertiary component solvent system is used [126].

3.1. HF-LPME

HF-LPME was introduced by Pedersen-Bjergaard and Rasmussen [128], and has gained considerable interest in a broad field of hair analysis. This technique can be performed in either two-phase or three-phase microextraction modes. Regarding two-phase modes, substances are extracted from the aqueous sample into the organic phase immobilised in the pores and lumen of the hollow fibre. Concerning three-phase modes, compounds are extracted from the aqueous sample by organic solvent immobilised in the pores of the hollow fibre into another aqueous phase (acceptor phase) within the lumen of the hollow fibre [126].

Simplicity, speed, less sample manipulation, and low consumption of organic toxic solvents (in the low microlitre range) are some of the advantages of HF-LPME; this is particularly true for the three-phase model, which generally provides excellent clean-up and good enrichment factors, even when complex biological samples such as human hair are analysed [129,130].

This technique has been widely employed in hair analysis, mainly for pre-concentration of drugs of abuse (e.g., cannabinoids, cocaine and metabolites and amphetamine type stimulants) [131,132], pharmaceutical drugs (e.g., phenobarbital) [133] and metals [134]. However, certain parameters of the developed method should be carefully optimised. For instance, the hollow fibre should be hydrophobic in nature and compatible with the organic solvent being used. In addition, the fibre should have low water solubility and high boiling point to reduce its dissolution and the loss of analytes, while keeping them solubilised [126].

Polypropylene capillary membranes with an inner diameter of around 600 μm have been mostly used in hair analysis, allowing compatibility with volumes in the range of microlitres for the acceptor solution. In addition, wall thicknesses of 200 μm have also been described, as they provide excellent mechanical stability and simplify the extraction units [126,135]. Amongst polypropylene capillary membranes, PP Q3/2 Accurel KM was the most adopted [131–133], but others such as PP 50/280 Accurel [134] have also been described, with the main difference being their inner

Table 5
Hair analysis using HF-LPME; extraction, clean-up conditions, instrumentation, LOD, LOQ and analytes recoveries.

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Hollow fibre	Extraction/desorption	Instrumentation	LOD	LOQ	Recoveries	Ref.
Ag	500 mg	Acetone (n.s.).	Room temperature; 6 mL concentrated $HClO_4$ and 26 mL concentrated HNO_3	Several drops ofH_2SO_4 (1:1 v/v); heated to near dryness; 10 mL of water; filtered; Diluted to 100 mL	DES of choline chloride and thiourea immobilised on the surface GOnanosheets	50 mL sample pH 4.0. Fibre exposure: stirred for 15 min at 800 rpm. Desorption: 250 μL of nitric acid (1 M); sonicated for 5 min.	FAAS	0.2 ng/mL	n.s.	95–105%	[141]
Cocaine, Benzoylecgonine, Anhydroecgonine methyl ester, Cocaethylene	50 mg	Mild detergent and water (n.s.); 2 mL of dichloromethane for 15 min at 37 °C.	18 h at 50 °C; 2 mL of methanol	Derivatization: 100 μL acetonitrile, 2 μL pyridine and 2 μL butylchloroformate; sonicated for 6 min; pH adjusted to 9–10.	Q3/2 Accurel KM polypropylene (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size).	Immersion in dihexyl ether (organic phase) 15 s; Lumen filled with 50–70 μL of 0.05 M HCl (acceptor phase). Fibre exposure: 10 min at 2400 rpm Desorption: n.s.	GC-MS	0.03 –0.4 ng/mg	0.05 –0.5 ng/mg	n.s.	[131]
As (III) and As (V)	250 mg	n.s.	Microwave digestion: 10 mL aqua regia; 33% power for 3 min, 55% power for 5 min, 100% power (700 W) for 3 min, and 77% power for 3 min.	Appropriate amounts of APDC, RTILs, and Triton X-100. Diluted with water; pH 3.0.	Polypropylene hollow fibre (320 μm i.d., 50 μm wall thickness, 0.1 μm pore size)	100 mL sample; RTIL 0.01% (v/v) s and APDC 0.01% (m/v) added. Lumen filled with Triton X-100 (extractant and acceptor phase). Fibre exposure: 15 min at 1000 rpm 85 °C. Desorption: 65 μL 95% (v/v) alcoholic solution with 1% (v/v) HNO_3	FAAS	0,08 ng/mL	n.s.	n.s.	[142]
Ibuprofen and Naproxen	n.s.	20 mL dichloromethane, 15 mL acetone, and 2 × methanol (15.0 and 10.0 mL).	5 h at 50 °C; 2 mL methanol.	Filtered and diluted with water.	Hyperbranched polyglycerol/ graphene oxide (HBP/GO) nanocomposite reinforced.	Lumen filled with 2 μL functionalised GO in 1-octanol (acceptor phase). Fibre exposure: 20 min at 400 rpm. Desorption: 1 mL methanol; sonicated	HPLC-UV	n.s.	n.s.	n.s.	[136]
Methamphetamine	2 g	20 mL dichloromethane, 15 mL acetone, and 2 × methanol (15.0 and 10.0 mL)	20 min at 70 °C; 1 M NaOH	Cooled.	Q3/2 Accurel KM polypropylene (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size).	Lumen filled with 20 μL functionalised GO in 1-octanol (acceptor phase). Fibre exposure: stirred; time (n.s.). Desorption: n.s.	GC-FID	2.4 ng/mL	n.s.	95–98.5%	[143]
Phenobarbital	50 mg	15 min at 37 °C; 2 mL dichloromethane	15 min at 70 °C; 1 mL 1 M NaOH	n.s.	Q3/2 Accurel KM polypropylene (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size).	Lumen filled with NaOH 0.1 M (acceptor phase). Fibre exposure: sonicated for 10 min. Desorption: n.s.	GC-MS	0.1 ng/mg	0.25 ng/mg	n.s.	[133]
Methamphetamine, ephedrine and methadone	2 g	5 min with 20 mL dichloromethane, 15 mL acetone, 15 mL methanol and 10.0 mL methanol	20 min at 70 °C; 1 M NaOH	Cooled	Q3/2 Accurel KM polypropylene (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size).	HF dipped for 15 s in organic solvent. Lumen filled with 20 μL of HCl, 0.1 M (acceptor phase). Fibre exposure: stirred. Desorption: n.s.	CE-UV	3.03 –6.06 ng/mL	10 –20 ng/mL	58–70%	[132]
Mercury species including methyl-, ethyl-, phenyl- and inorganic mercury	250 mg	Water and acetone (n.s.).	10 min sonicated; 5 mL of 5 M HCl	Neutralised with 5 M NaOH, spiked with 18-crown-6 and NaCl, adjusted to pH 4.5, and diluted to 25 mL with water.	PP 50/280 Accurel polypropylene (i.d. 280 μm; wall thickness, 200 μm; pore size, 0.2 μm	HF in the middle of extraction vial with a U-shape. Chlorobenzene (extraction solvent); 0.4% (m/v) of 18-crown-6; pump rate 3 rpm; Fibre exposure: stirred for 25 min.	LVSS- CE-UV	n.s.	2.5 –5 ng/mL	90–104%	[134]

					Desorption: n.s.						
Naproxen	50 mg	20 mL dichloromethane, 20 mL acetone and 15 mL methanol	5 h at 55 °C; 2 mL methanol; pH 7.4.	n.s.	Carbon nanotubes modified with a Keggin polyoxometalate	Fibre exposure: stirred for 60 min. Desorption: 300 μL of methanol, sonicated for 10 min	FTIR	0.072 ng/mL	0.24 ng/mL	95–103%	[144]
Alprazolam, Lorazepam, Diazepam and Clonazepam	50 mg	20 mL dichloromethane, 20 mL acetone, 15 mL methanol and 10 mL methanol (n.s.).	5 h at 55 °C; methanol (n.s.)	Filtered, evaporated.	1-pentyl-3-methylimidazolium bromide coated titanium dioxide ([PMIM]Br@TiO2) reinforced	10 mL of sample pH 7 with 0.750 g NaCl. Fibre exposure: shacked at 500 rpm for 45 min. Desorption: 500 μL of methanol, sonicated for 10 min.	HPLC-UV	0.02–0.10 ng/mg	0.1–0.5 ng/mg	94.5–99.8%	[137]

APDC (Ammonium pyrroldinedithiocarbamate); CE-UV (Capillary electrophoresis with ultraviolet detection); DDW (Double-distilled water); DES (Deep eutectic solvents); FAAS (Flame atomic absorption spectrometry); FTIR (Fourier transform infrared spectroscopy); GC-FID (Gas chromatography - Flame Ionization Detector); GC-MS (Gas chromatography - Mass Spectrometry); GO (graphene oxide); HPLC-UV (High-performance liquid chromatography - ultraviolet detection); LOD (Limit of detection); LOQ (Limit of quantification); LVSS- CE-UV (Large volume sample stacking capillary electrophoresis with ultraviolet detection); n.s. (not specified); RTIL (Room-temperature ionic liquid).

diameter.

Another variation of HF-LPME was applied to extract ibuprofen and naproxen, as well as some benzodiazepines, from hair. In these cases, the membrane pores were filled with a solvent in which functionalised graphene oxide (GO) [136] or 1-pentyl-3-methylimidazolium bromide (ionic liquid) coated with TiO2 NPs [137] was dispersed. The technique was referred to as hollow fibre solid–liquid-phase microextraction (HF-SLPME) and it is usually characterised by its high selectivity and good extraction efficiency for the extraction of organic compounds in aqueous medium [138]. However, washing should be performed carefully when functionalised GO is used for HF-SLPME, since its excess may result in column damage during analysis [139]. Apart from fibre functionalization, the different modes of LPME were developed with the aim of reducing the use of organic solvents and replacing them with environmentally friendly alternative solvents [140]. An example is the deep eutectic solvents (DES) used by Karimi et al. [141], who used choline chloride and thiourea immobilised on the surface of GO nanosheets and reinforced inside the pores of the hollow fibre for the extraction of silver from hair. DES are a new generation of green solvents (biodegradable, biocompatible, non-toxic, non-flammable, and inexpensive), consisting of mixtures of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) that form liquids due to a large decrease in their melting points [140]. Some of their advantages are low volatility, thermal stability, high conductivity and tuneable miscibility, all of considerable interest for HF-SLPME, and demonstrated suitability for the pre-concentration of silver [140]. Table 5 summarises HF-LPME applications in hair analysis.

3.2. DLLME

DLLME was introduced by Assadi and co-workers [145] and consists of two steps. The first is the injection of an appropriate mixture of extracting and disperser solvents into an aqueous medium containing the target substances. The extracting solvent is dispersed as very fine droplets into the aqueous medium and the substances are concentrated therein. Due to the large surface area between the extracting solvent and the aqueous sample, equilibrium is readily achieved, and the extraction is not time dependent. The second step consists of centrifugation of the cloudy solution that forms, after which the analytes sediment and can be collected for determination [146].

A few considerations are necessary regarding DLLME: the dispersing solvent has to be completely miscible with the aqueous phase; the extracting solvent must have the potential for extracting the analytes, be soluble in the dispersing solvent and poorly soluble in water; and, in order to enable phases separation, the density of the extracting solvent has to differ greatly from that of water [147]. Nevertheless, full optimisation is usually recommended, namely in what concerns the amount of salt added to the sample (salting-out effect) and the pH of the samples, depending on the analytes [147]. This technique has been used in hair analysis mostly for the evaluation of exposure to metals [46,148,149]. Vicenti et al. [150] have extended its applicability to hair analysis by combining pressurised liquid extraction (PLE) with DLLME for the determination of sixty drugs of abuse in hair. The authors used 2-propanol as dispersing and chloroform as extraction solvents, and although the extraction yields varied widely depending on the target drugs, it was considered a great alternative to conventional LLE. The proposed procedure not only allowed reducing time and cost per analysis, but also reduced the sample amount required (10 mg), since a single analysis was performed for all analytes instead of one for each compound class [150].

Other modifications of DLLME have been proposed, and the

main reasons for their development are related to the fact that conventional DLLME requires solvents with higher density than water, which are often incompatible with the instruments, and are usually toxic [151]. In this sense, dispersive liquid-liquid microextraction based on DLLME-SFO, SM-DLLME, and TIL-DLLME have been developed and successfully applied to several biological specimens, including hair.

The DLLME-SFO approach was described by Leong and Huang in 2008 [152,153], and consists of a droplet of a water immiscible solvent with a melting point of 10–30 °C that floats on the surface of an aqueous sample containing the analytes. The mixture is agitated to maximise the contact area between the two solutions, after which the sample vial is placed in an ice bath to solidify the droplet for easy removal. The removed droplet containing the concentrated analytes is then allowed to melt for their further determination [151]. Two papers reported the application of this approach in hair analysis to access metals exposure, one using 1-dodecanol [28] and the other using 1-undecanol [151] as extraction solvents due to their low-toxicity. This variation of DLLME was also previously applied in the determination of endocrine-disrupting compounds in human hair [154], but the last reported application for this specimen relates to opium alkaloids [155] demonstrating its great versatility. Dehnavi et al. [155] used 1-undecanol and methanol as extraction and dispersive solvents, respectively, to pre-concentrate morphine and codeine from 20 mg of hair, obtaining pre-concentration efficiencies ranging from 97 to 110%. The authors concluded that the technique was fast, simple, repeatable, inexpensive, sensitive and environmentally friendly, making it a good alternative to conventional DLLME.

In order to reduce the use of toxic organic solvents, SM-DLLME has been pointed out as a suitable choice [156]. The concept of supramolecular solvent-based extraction was proposed by Ballesteros-Gómez and co-workers [157]. Supramolecular solvents (SUPRAS) are nano-structured liquids that consist of assemblies of amphiphiles dispersed in a continuous phase [29]. Due to their hydrophobic nature and hydrogen bonding interaction with chelates, the extraction efficiency may be high. However, when it comes to hair analysis, this efficiency has only been demonstrated for the determination of metals yet. Aydin et al. [29] proposed a method for the determination of copper in hair, involving the use of a supramolecular solvent in which reverse micelles of 1-decanol were dispersed in tetrahydrofuran. Also, Khan et al. [156] have described a method to determine aluminium in hair, for which a supramolecular solvent (undecanol–tetrahydrofuran) was used. Overall, the extraction efficiencies were good (higher than 90%), but no multi-analyte methods have yet been reported, which could change these figures.

Considerable interest has been observed in the use of room temperature ionic liquids (RTILs) to replace conventional organic solvents. Ionic liquids are good solvents for a wide range of materials, and are often composed of poorly coordinating ions; as such, they have the potential to be highly polar yet noncoordinating solvents, and since they are immiscible with a number of organic solvents they provide a nonaqueous, polar alternative for two-phase systems [158]. To date, elements from human hair have only been pre-concentrated using this approach. Arain et al. [30] reported the application of the RTIL-DLLME technique using the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate, [C4MIM][PF6] as extracting solvent in the determination of aluminium in hair samples from Alzheimer's disease patients. This RTIL has also been used in the determination of lead [31,32], and cadmium [33,34] in human hair. Another RTIL has been used, namely 1-Hexyl-3-methylimidazolium hexafluorophosphate [HMIM][PF6], which was applied to pre-concentrate tungsten from hair [159]. In this work the authors pointed out that the viscosity of this RTIL was high and its handling was difficult, so the working solution of [HMIM][PF6] had to be prepared in acetone. Previously, Arain et al. [30,31] had to dissolve the RTIL [C4MIM][PF6] in ethanol or acidic methanol before injection into the instrument for the same reason.

Two new approaches have been proposed for DLLME, namely ultrasound-assisted low-density solvent dispersive liquid-liquid microextraction (UA-LDS-DLLME) and switchable solvent based liquid phase extraction (SS-LPE). Meng et al. [160] implemented a UA-LDS-DLLME method for the determination of GHB in hair using GC-MS/MS. For this new approach, the authors suggested using an acidic aqueous solution and small amounts of low-density organic solvents during the grinding of the hair, justifying that the vibration of the ball mill during the grinding process would be able to disperse the organic solvents in the sample without the need for dispersants. Saturated ammonium dihydrogen phosphate solution and ethyl acetate were proposed and successfully applied for GHB extraction from hair. The latter solvent allowed reducing the time-consuming pre-treatment usually associated to digestion or long-term ultrasonic assisted treatments [160].

The SS-LPE method was developed by Atsever et al. [35] for iron determination in hair by slotted quartz tube-flame atomic absorption spectrometry (SQT-FAAS). The authors synthesised the switchable solvent (SS) from N,N-dimethlybenzylamide (DMBA) and ultrapure water with the addition of dry ice for protonation. Briefly, this method consisted of mixing a buffer solution with a complexing agent and the hair sample solution, to which an amount of SS was added, followed by vortex mixing. After a subsequent addition of NaOH to deprotonate DBMA and form a cloudy solution, the solution was centrifuged for phase separation and the upper phase was analysed [35]. Table 6 summarises the application of DLLME and its variations in hair analysis.

3.3. Directly suspended droplet microextraction (DSDME)

DSDME is another LPME variation consisting of a solvent microdroplet suspended on the top centre of an aqueous sample, consequently forming a self-stable single microdroplet system that is easy to handle and control [161]. In addition, internal recycling and intensification of mass transfer within the droplet can be achieved by its rotation around a symmetrical axis [161]. To date, only two DSDME methods were published for hair and only one of these in the last five years. These methods adopted a liquid–liquid–liquid microextraction (LLLME) approach, also called three-phase LLLME, which is based on the use of a small amount of a water-immiscible organic solvent and an aqueous phase containing the analytes. These analytes are then back-extracted into a third (aqueous) phase [162].

Kazemi and Alizadeh [163] were the only authors to publish a work using DSDME in the analysis of hair samples in the last five years. These authors applied this technique for the extraction and determination of methadone in 50 mg of hair. For this purpose, 5 mL of the hair extract was used and 350 μL of 1-octanol was added. After agitation, the acceptor phase (microdroplet of an acidic aqueous solution, pH = 1) was delivered to the top-centre position of the immiscible organic solvent and the mixture was agitated again. Methadone was extracted from the donor phase (hair extract) into an organic phase and then back-extracted into a directly suspended droplet. Extraction yields between 82 and 98% were reported, and it was concluded that DSDME is a rapid, sensitive, robust and reliable method for the determination of methadone in human hair specimens [163].

Table 6
Hair analysis using DLLME and variations; extraction, clean-up conditions, instrumentation, LOD, LOQ and analytes recoveries.

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Extraction	Instrumentation	LOD	LOQ	Recoveries	Ref.
60 drugs of abuse	10 mg	Phosphate buffer 0.25 mM pH 6.5, 2-propanol and acetone.	PLE: formate buffer 0.15 M (pH 3.5)/2-Propanol, 80/20 (v/v). T = 150 °C; P = 100 bar; preheat time = 1 min; heat time = 7 min; static time = 5 min; flush volume = 0%; purge time = 1 min.	Centrifuged for 5 min at 9000 rpm; 1.2 g of sodium chloride added.	DLLME. 5 mL sample, 10% isopropanol,. 20 μL of sodium hydroxide 1 M and 500 μL of carbonate buffer. Dispersing solvent:500 μL of 2-propanol Extraction solvent: 200 μL of chloroform Sonicated for 10 min at 120 W.	HPLC-HRMS/MS	0.1−5 pg/mg	2.5 −50 pg/mg	15−87%	[150]
Pb and Cd	200 mg	n.s.	4 mL of concentrated HNO_3; heating plate for 0.5 h; Microwave digestion: 150 °C and 18 atm for 4min, then 180 °C and 22 atm for 4 min.	Evaporated and diluted to 10 mL with water.	DLLME. 10 mL of aqueous sample and 0.4 g/L of APDC pH to 4. Dispersing solvent: 1 mL of NaOH (10 M) Extraction solvent: 2 mL of aqueous amine (P-TEA-C) Vortex mixed for 30 s at 2800 rpm .	GFAAS	3.9 −16 pg/mL	10 −40 pg/mL	94.9 −110%	[46]
Bi	250 mg	Water (n.s.).	150 °C; 30 mL of a mixture of $HClO_4$ and HNO_3 (1:8 v/v)	Evaporated and dissolved by drops of H_2SO_4 (1:1 v/v)	DLLME. Sample (n.s.); NaF, 0.1% m/v; pH 2 Dispersing solvent: 500 μL of methanol Extraction solvent: 150 μL of chloroform Manual shaking (n.s.)	Atomic fluorescence	0.16 ng/mL	0.53 mg/mL	95 −103.3%	[148]
Bi	500 mg	Acetone for 45 min; 1% neutral scouring agent for 3 min, water several times	30 mL of a mixture of $HClO_4$ and HNO_3 (1:8 v/v).	Evaporated and dissolved by drops of H_2SO_4 (1:1 v/v)	DLLME. Sample (n.s.) pH 5 using; Dispersing solvent: 500 μL of methanol Extraction solvent: 150 μL of CCl_4 Shaking 1 min	β-correction spectrophotometric method	0.54 ng/mL	1.80 ng/mL	96.8 −102.2%	[149]
Opium Alkaloids	20 mg	Detergent, dichloromethane, acetone and methanol (n.s.) for 5 min	5 h at 50 °C; Methanol pH 7.7	Cooled, filtered, evaporated and dissolved in 10 mL of water	DLLME-SFO. Sample (n.s.); pH 10.2; 1.3 mL potassium carbonate (10%, w/v) Dispersing solvent: 150 μL of methanol Extraction solvent: 40 μL of 1-undecanol n.s.	HPLC-UV	10 −17 ng/mL	20 −50 ng/mL	97−110%	[155]
Pb	50 mg	n.s.	Microwave digestion: 20 min at 180∘C; 6 mL HNO_3 (65% w/v), 3 mL H_2O_2 (30% w/v), and 1 mL HF.	n.s.	DLLME-SFO. 1 mL PAN (1×10^{-4} M), pH 9; NaCl Dispersing solvent: 200 μL of acetonitrile Extraction solvent: 100 μL of 1-undecanol n.s.	ETAAS	0.042 ng/mL	0.05 ng/mL	97%	[36]
Tl (III) and Tl(I)	10 g	Acetone, chloroform and water (n.s.),	15 min at 100 °C and then 15 min at 150 °C; 15 mL concentrated HNO_3	Evaporated; 10 mL of 0.1 M HNO_3 heated at 100 °C for several minutes; cooled; diluted to 50 mL with water.	DLLME-SFO. 3 mL of 0.1 M phosphate buffer pH 10.0, 0.2 mL of 1.5 mg/mL PAN and 3 mL of 10% (w/v) NaCl. Dispersing solvent: 2.5 mL of ethanol Extraction solvent: 100 μL of 1-dodecanol n.s.	FAAS	2.1 ng/mL	n.s.	96−104%	[28]
	100 mg			n.s.	DLLME-SFO.	LC-MS/MS	n.s.	n.s.	n.s.	[154]

(continued on next page)

Table 6 (*continued*)

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Extraction	Instrumentation	LOD	LOQ	Recoveries	Ref.
Bisphenol A, parabens, perfluoroalkyl compounds and a brominated flame retardant		Water, SDS (0.1% w/v) and water; sonicated for 5 min.	Overnight at 38 °C; 8 mL HCl 2 M and HCl 0.1 M.		Dispersing solvent: 500 μL of methanol Extraction solvent: 100 μL of 1-undecanol Sonicated for 10 min					
Cu	100 −250 mg	n.s.	20 mL concentrated nitric acid; heated until clear solution.	Filtered, diluted up to 10 mL with water.	SM-DLLME. 4-(2-thiazolylazo) resorcinol to chelate Cu (II). Dispersing solvent: n.s. tetrahydrofuran Extraction solvent: n.s. 1-decanol Sonicated for 5−8 min.	FAAS	1.13 μg/L	n.s.	n.s.	[37]
Cd	n.s.	Tap water, distilled water, 1% neutral detergents (Triton X-100), deionised water, and acetone (n.s.).	2 mL of concentrated HNO_3−H_2O_2 (2:1, v/v) for 10 min at room temperature; One-stage digestion program at 80% of total power (900 W), 5 −8 min	Cooled, evaporated and diluted to 10 mL with 0.1 M nitric acid;	SM-DLLME. 0.5 mL of 0.5% (w/v) APDC; pH adjusted to 6.0. Dispersing solvent: n.s. tetrahydrofuran Extraction solvent: n.s. 1-decanol (total 1 mL) Sonicated at 35 kHz for 5, 10, 15, 20, or 60 s at temperatures ranging from 30 to 70 °C.	FAAS	0.23 μg/L	n.s.	98−99%	[39]
Al	100 mg	n.s.	10 mL of concentrated HNO_3 at room temperature for 30 min, then 100 °C till dry; re-digested with 15 mL mixtures of HNO_3 and H_2O_2 (2:1 v/v).	2 mL of ammonia/ ammonium chloride buffer; pH adjusted to 8; Diluted to 10 mL water.	SM-DLLME. 0.3 mg 8-hydroxyquinoline for complex formation. Dispersing solvent: 0.1 mL tetrahydrofuran Extraction solvent: 0.1 mL undecanol Vortex for 2 min 40 × 100 rpm	Spectrophotometry	0.16 μg/L	n.s.	95−96%	[156]
Cu	10 mg	2 × acetone, water (n.s.).	5 mL concentrated HNO_3 (65%) and 10 mL H_2O_2 (30%) at 100 °C. Evaporated; 5 mL concentrated HNO_3/10 mL H_2O_2 added again and evaporated.	Dissolved up to 5 mL with water; pH adjusted to 6.	SM-DLLME. 0.5 mg of ammonium pyrrolidine dithiocarbamate for complex formation. Dispersing solvent: 600 μL tetrahydrofuran Extraction solvent: 150 μ undecanol Sonicated for 5 min.	FAAS	0.11 μg/L	0.34 μg/ L	95−96%	[29]
GHB	50 mg	1 × isopropanol and 2 × water (n.s.)	n.a.	n.a.	LDS-DLLME 1 mL saturated ammonium dihydrogen phosphate solution and 180 μL ethyl acetate added to the weighted hair; Ball mill for grinding; Sonicated for 3 min to form a cloudy suspension at 25 °C.	GC/MS/MS	0.01 ng/ mg	0.03 ng/ mg	71−89%	[160]
Fe	45 mg	n.s.	4 mL of 1.5 M NaOH heated up to 90 °C	Filtered; pH adjusted to pH 4.0; centrifuged and filtered again.	SS-LPE 8 mL of sample; 1.5 mL of pH 4.0 buffer solution and 0.75 mL of 0.015% complexing agent. 0.75 mL of SS added 1.25 mL of 1.50 M NaOH Vortexed for 30 s	SQT-FAAS	2.6 μg/L	8.6 μg/L	91−113%	[35]
Cd and Pb	500 mg				TIL-DLLME.	FAAS	n.s.	n.s.	99%	[33]

Table 6 (*continued*)

Analytes	Hair mass (mg)	Hair washing	Hair leaching	Hair extracts preparation	Extraction	Instrumentation	LOD	LOQ	Recoveries	Ref.
		Triton-X100, acetone and water (n.s.),	2 mL of mixture of concentrated HNO_3–H_2O_2 (2:1, v/v) for 10 min at room temperature; hot plate at 80 °C until clear solution.	Dissolved in 5 mL of 0.1 M HNO_3, filtered and diluted up to 10 mL with water.	0.2–1.0 M of L-cysteine (complexing agent) and pH adjusted from 6 to 10. 50–250 μL of [BMIM][PF6] and 0.25–2.0 mL of PF6 (0.5 mM) as ion-pair agent. Vortex mixed (20–100 s) and shaked (1–5 min).					
Pb	200 mg	n.s.	2 mL of mixture of concentrated HNO_3–H_2O_2 (2:1, v/v) for 10 min at room temperature; One-stage digestion heating at 80% total power (900 W) for 2–5 min.	Filtered; Diluted up to 10 mL with 0.2 M HNO_3	TIL-DLLME. 1 mL APDC complexing agent, 2 mL acetate–phosphate buffer, and 50–200 μL [C4MIM][PF6] Sonicated at 40–80 °C for 1–5 min. Pb-enriched phase treated with 0.5 mL (0.5–2 mol/L HNO_3) Sonicated 1–5 min.	FAAS	0.19 ng/mL	n.s.	98.7%	[32]
W	500 mg	Water, 3 × acetone and chloroform and again water (n.s.).	12 mL concentrated HNO_3 and 2 mL concentrated $HClO_4$; heated at 100 °C for 45 min and then at 150 °C for 45 min.	Cooled to 70 °C; 5 mL of 30% H_2O_2; heated to dryness at 200 °C; 20 mL of 1 M HNO_3 heated at 100 °C for 1 h; Dissolved up to 250 mL with n water	TIL-DLLME. 20 mL sample pH 7, 50 μL of a mixture (1.0×10^{-3} M of DMPAHPD, 0.04% v/v Triton X-100, 50 mg of [HMIM][PF6] (as extraction solvent) and 45 μL of sodium hexafluorophosphate; thermostatic bath at 45 °C for 5.0 min, after shaking.	Spectrophotometry	0.8 ng/mL	2.5 ng/mL	98–102%	[159]
Cd	200 mg	n.s.	2 mL of concentrated HNO_3–H_2O_2 (2:1, v/v) for 10 min at room temperature.	Semi-dried; Dissolved in 5 mL of water and filtered.	TIL-DLLME. 1 mL of 0.2% (m/v) 4-(2-pyridylazo) resorcinol, pH adjusted to 2–6. 20–90 mL of [BMIM][PF6]; heated from 20 to 60 °Cs; sonicated at 35 kHz 5–15 min.	FAAS	0.21 ng/mL	n.s.	92%	[34]
Pb	200 mg	n.s.	3 mL of a mixture of concentrated HNO_3–H_2O_2 (2:1, v/v) at room temperature for 10 min; Heated at 80% of total power (900 W) for 3–4 min.	Diluted up to 10 mL with 0.1 M concentrated HNO_3.	TIL-DLLME. pH was adjusted to 3–8; 1 mL of 0.1–0.5% (w/v) APDC; 500 μL of 0.025–0.20% (v/v) Triton X-114; 50–200 μL of [C4MIM][PF6] Agitated (n.s.)	FAAS	0.41 ng/mL	n.s.	97.8%	[31]
Al	200 mg	4 × 1:200 v/v dilution of Triton X-100; 3 × water and 2 × acetone (n.s.).	2 mL of concentrated HNO_3–H_2O_2 (2:1, v/v) for 10 min; One-stage digestion at 80% of total power (900 W), 4–5 min.	Cooled, evaporated and diluted to 10 mL with 0.1 M HNO_3	TIL-DLLME. 2 complexing reagents 0.1–0.5 mL of oxine (0.113%); and 0.1–0.5 mL of morin (0.125%); pH was adjusted to 4–8; 75 μL of [C4MIM][PF6]; Sonicated at 45 °C.	FAAS	0.56–0.64 ng/mL	n.s.	n.s.	[30]

APDC (Ammonium pyrroldinedithiocarbamate); DMPAHPD (5-(2′, 4′-dimethylphenylazo)-6-hydroxypyrimidine-2, 4-dione); FAAS (Flame atomic absorption spectrometry); ETAAS (Electro thermal atomic absorption); GC-MS/MS (Gas chromatography – Tandem Mass Spectrometry); GFAAS (Graphite furnace atomic absorption spectrometry); HF (Hydrofluoric acid); HPLC-HRMS/MS (High-performance liquid chromatography coupled to high resolution tandem mass spectrometry); HPLC-UV (High-performance liquid chromatography with ultraviolet detection); IL (ionic liquid); LC-MS/MS (Liquid chromatography - Tandem Mass Spectrometry); LOD (Limit of detection); LOQ (Limit of quantification); n.a. (not applied); n.s. (not specified); PAN (1-(2-Pyridylazo)-2-naphthol); RB (rhodamine B); SDS (Sodium dodecyl sulphate); SQT-FAAS (Slotted quartz tube-flame atomic absorption spectrometry).

4. Conclusions and future perspectives

In the present days, hair analysis is routinely used as a tool for the investigation of a large number of xenobiotics. This is due to the fact that drugs determination in hair is technically easier when compared to other matrices like blood and urine, using almost identical analytical methods and instrumental approaches.

However, in addition to the increasing number of techniques and procedures that have been reviewed, miniaturised clean-up approaches for hair samples are still rarely used or studied. Indeed, laboratories continue to use the common sample-preparation methods of SPE and LLE, because they are reportedly highly reproducible and allow high recoveries and throughput. Nevertheless, this review shows that miniaturised techniques can be just as reproducible and allow great enrichment factors like SPE and LLE. Besides this, the conscience on the implementation of sustainable development in the professional life of chemists on both laboratory and industrial scale is growing and demonstrated by the application of the green chemistry principles, mainly in the field of scientific research. This way, there is a constant search for reducing organic solvents (usually toxic) in classical techniques and development of fully automated techniques with the lowest number possible of analytical steps. These micro-scale techniques have attracted large academic interest but few implementations have been made in routine analysis laboratories, which is expected to change in the future.

The present review makes clear that among the several micro-techniques available nowadays for clean-up in hair analysis, those based on the use of sorptive materials are the most widely used. In addition, these materials have shown great versatility, in a wide range of applications (e.g., drugs of abuse, pharmaceuticals, metals, etc). The development of new materials should be pursued in order to achieve even better selectivity and higher adsorption capacity. This would result in the availability of more cost-effective sorbents in the future and in the improvement of their synthesis processes.

Regarding dispersive liquid–liquid microextraction, it seems that this technique has been little explored for hair. The published methods have been mainly applied to the determination of elements, although other fields exist where this approach can be successfully used. Concerning the new wave of research on ionic liquids, there is great potential for their application in these miniaturised approaches. They are beneficial on substituting traditional volatile organic solvents, reducing environmental contamination and toxicity. Moreover, ionic liquids are highly versatile solvents.

Lastly, it is important to consider the association of these miniaturised approaches with hyphenated techniques, still not widely used. The coupling of a micro-based extraction with more recent MS technology offers the possibility to overcome the limitations of multi-target screening. High-resolution and high-accuracy mass spectrometric techniques, such as linear ion trap, orbitrap and quadrupole time-of-flight mass analysers can enable accurate mass determinations of species obtained from drugs and can be extremely important in metabolites research. It would be desirable that the future of hair analysis laid in the combination of miniaturised sample clean-up approaches with these new instruments, paving the way to more environmentally friendly and sensitive procedures for drug detection in hair. Regarding elemental analysis it was possible to observe the preference for dispersive approaches within microextraction, however hair samples are commonly digested, and several elements determined using ICP-MS without analyte pre-concentration/clean-up and use of organic solvents. In this sense, Laser ablation (LA)-ICP-MS is an environmentally friendly and suitable technique for hair analysis, especially when spatial resolution is desired.

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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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5. Paper II - Microextraction by packed sorbent

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5

Microextraction by Packed Sorbent

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CONTENTS

5.1 Introduction: Fundamental Theory

Microextraction by packed sorbent (MEPS) is a sort of miniaturized solid-phase extraction (SPE) technique developed in 2004 by Abdel-Rehim et al. (Abdel-Rehim et al., 2004) and aimed at reducing both sample and solvent volumes, in order to provide an automated procedure by means of its easy coupling to chromatographic systems.

In this sampling approach the sorbent (from 1 to 4 mg) is located in a microsyringe rather than in an isolated extraction cartridge, as occurs in SPE (Figure 5.1).

Another difference relative to the latter, in MEPS the sample flows through the extracting device in a bidirectional fashion (aspirations or strokes), improving the process's efficiency due to the increase in the contact between the sample and the sorbent.

In order to increase the rate of mass transfer from the sample to the sorbent, both the extracting phase and particle size should be small. In addition, as close contact between the sorbent's surface and the sample is relevant, the amount of the sorbent, the loading volume, and the volume of the elution should be carefully optimized in order to avoid exceeding the method's breakthrough point (Abdel-Rehim 2011, 2004).

Activation of the extraction sorbent to facilitate the retention of analytes occurs at a first stage, for which an organic solvent, such as methanol, is used. After this step, the sample is withdrawn using the syringe, and several draw/eject cycles are usually needed in order to concentrate the target compounds in the sorbent. The sorbent is washed by rinsing with water, aiming at eliminating matrix constituents

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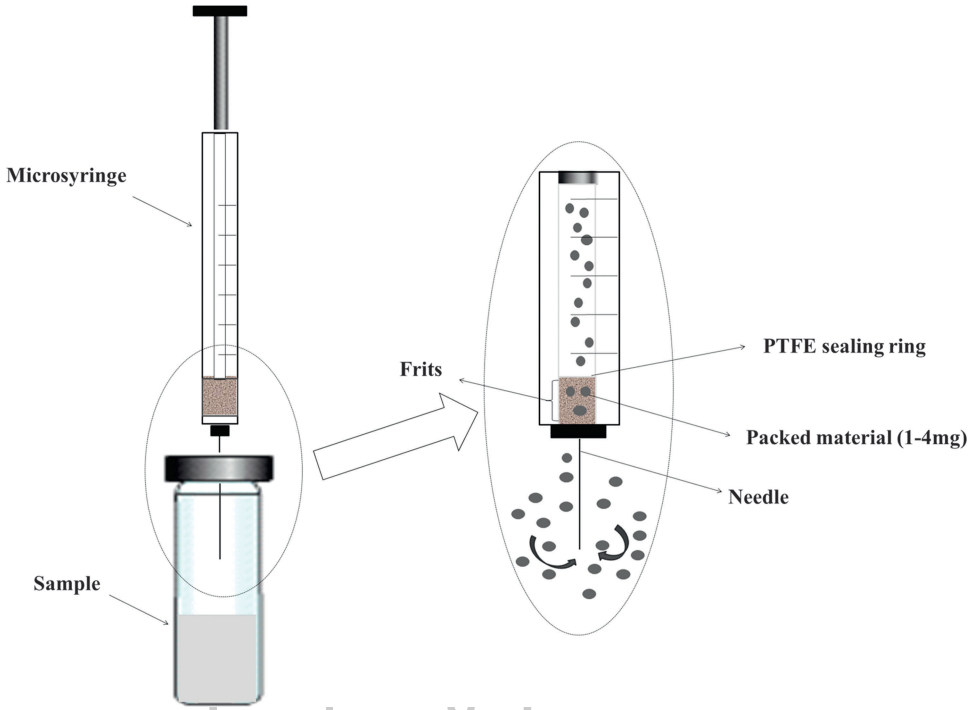

FIGURE 5.1 MEPS manual configuration.

(e.g., proteins). Finally, the analytes are eluted with an organic solvent (e.g., methanol or mobile phase) and directly injected in the analytical instrumentation.

These extraction cycles can be performed in two ways, either by drawing and ejecting several times in the same vial or by discarding the sample to waste after each draw of the syringe (Abdel-Rehim 2004). The whole procedure may be automated using some sort of autosamplers, or it can be connected directly to a gas chromatography (GC) injector using large volume injection approaches. Nevertheless, using liquid chromatography (LC) rather than GC is more prone to adequate automation, as small amounts of water may be introduced in chromatographic instruments due to the difficulty in drying adequately the sorbent prior to elution and to the relatively high polarity of the solvents normally used, which is in general not compatible with GC (Abdel-Rehim 2010,Abdel-Rehim 2011).

This technique is usually aimed at the preparation of liquid samples, so additional steps may be necessary for samples of tissues or hair. In those situations, an organic solvent (e.g., methanol) may be used in order to transfer analytes to the liquid phase prior to MEPS. Nevertheless, complex liquid matrices may also require pre-treatment in order to avoid sorbent clogging and allow extending its use. This is, in addition, important to extract and concentrate analytes present at lower concentrations, providing high sensitivity and selectivity. The influence of matrix interferences may be reduced by sample dilution (to decrease its viscosity, thus facilitating its passage through the sorbent), protein precipitation or filtration using selective filters. It is usually deemed necessary to proceed to pH adjustment to improve the analytes' interaction with the sorbent, and this is particularly important when ion exchange sorbents are involved. Other pre-treatment approaches for MEPS include sample homogenization, by vortex agitation, ultrasounds, or centrifugation (Yang et al. 2017).

Several parameters, namely volume and composition of washing and elution solutions, sorbent amount, and sorbent type, are capable of affecting MEPS performance (Yang et al. 2017). However, selecting the adequate extracting material is the most critical step in optimizing the whole procedure.

When compared to SPE or liquid-liquid extraction (LLE), the MEPS approach is very promising (Altun et al. 2004; Abdel-Rehim 2010), as it reduces sample preparation time and organic solvent consumption, and the cost of analysis is minimal (Abdel-Rehim 2011; Said et al. 2010). Even relative to

solid-phase microextraction (SPME), MEPS reduces sample preparation time (<1 min) and sample volume (10–1000 μL) and presents in general a much higher absolute recovery (>50%) (Abdel-Rehim 2011; Barroso et al. 2012; Moein et al. 2015b). Furthermore, the extraction cartridge can be used several times, and more than 50–100 extractions from plasma or 400 extractions from water samples have been described, whereas a conventional SPE column is used once and then discarded (Abdel-Rehim 2011; Barroso et al. 2012; Abdel-Rehim 2010; Altun and Abdel-Rehim 2008).

Although MEPS is a very simple and straightforward extraction technique, it is not free of disadvantages. When its application started increasing, some authors complained about the fact that the available sorbents were scarce, a problem that did not occur with traditional SPE (Páleníková and Hrouzková 2014). Nowadays, and especially in the last five years, a lot of research has been done, and a wide range of options have been developed in the field of solid packing material. These new sorbents have been successfully applied to MEPS syringes, but they seem to be limited to pre-concentrate a small group of analytes. Another disadvantage is the strong dependence of the analytes' recovery on the number of cycles (strokes) that the sample passes through the sorbent (Páleníková and Hrouzková 2014). Commonly, in order to achieve high recovery rates, multiple draw-eject cycles have to be applied, since the analytes' concentration in the sample will decrease after each cycle. Still, this cannot be accepted as a rule, since sorbents can reach a rapid saturation. The increasing number of draw-eject cycles will also increase the mechanical stress on the syringe plunger, resulting in a short life-time of the MEPS syringe (Páleníková and Hrouzková 2014). Another disadvantage, which is usually neglected, is related to solvents that might not be suitable for the procedure. During extraction optimization, it is common practice to mimic SPE procedures, including solvents applied, although reducing their volumes. Yet, it has been described that some solvents, such as dichloromethane and large amounts of isopropanol, can cause sorbent cavitation when passing through the BIN (Rosado et al. 2020a). One cannot forget that the amount of sorbent used in MEPS is around ten times lower than that used in SPE cartridges, and any sorbent loss (even at minimum amounts) can directly affect the extraction efficiency and BIN lifetime. Moreover, these solvents also appear to affect the plunger of the syringe over time.

5.2 Configurations and Sorbents

Several different sorbent materials are available for use in MEPS. These sorbents are essentially silica-based matrices (unmodified silica, C_2, C_8, and C_{18}), strong and weak cation and anion exchange functionalized C_{18} versions (SCX, SAX), and mixed-mode sorbents (80% C_8 and 20% SCX with sulfonic acid-bonded silica) (Table 5.1) (Yang et al. 2017). More recently, new sorbents have been made available, namely porous graphitic carbon and polymeric absorbent polystyrene-divinylbenzene copolymer (PV-DVB), either modified or functionalized, in order to present different retention capabilities for different target analytes (Abuzooda et al. 2015; Karimiyan et al., 2019; Altun and Abdel-Rehim 2008). Table 5.1summarizes the main types of commercialized sorbents.

A significant number of custom sorbents have been reported for use in MEPS, for instance molecular-imprinted polymers (MIPs), functionalized silica monoliths, based on cyanopropyl hybrid silica, and other restricted access materials (RAM) as well (Daryanavard et al. 2013; Ahmadi et al. 2017; Taghani et al. 2018; Bagheri et al. 2012a,b; Rahimi et al. 2013; Souza et al. 2015). These types of sorbents were developed for specific applications, and as such they are not commercially available. Their use is not yet widespread, but rather still limited to those proof-of-concept applications.

MEPS selectivity obviously depends on the type of sorbent, as different types of interaction (hydrophobic, polar, and ionic) between the analytes and the sorbent may occur (Yang et al. 2017; Pereira et al. 2019).

Particle size obviously influences MEPS performance. The most common particle size in conventional MEPS varies from 30 to 50 μm, but particle sizes of 140 or 3 μm have also been used (Yang et al. 2016; Porto-Figueira et al. 2015). These different sizes can be useful when complex matrices are involved, avoiding sorbent blocking and consequently erratic recoveries. Other formats of sorbents are available, namely graphene aerogel monolith, which does not have particles (Han et al. 2016; Yang et al. 2017).

Different modes are possible when operating MEPS, but the manual syringe is the most widely used format (Table 5.1).

TABLE 5.1

Main types of commercialized sorbents and modes of operation

Type of Sorbent	Characteristics
	Silica-based sorbents
Silica, C_2, C_4, C_8, C_{18}	The retention mechanism is based on normal and reverse phase separation. It is adequate for the extraction of both hydrophobic and hydrophilic analytes from aqueous matrices.
	Ion exchange materials
M1 (80% C_8 and 20% SCX with sulfonic acid-bonded silica), SCX, SAX	The retention mechanism is based on weak cation and anion exchange. It is applicable for easily ionized polar analytes.
Polystyrene copolymer (divinylbenzene, DVB; ENV +)	It is adequate for non-polar compounds.
Modes of Operation	**Characteristics**
Manual syringe	Simplicity, low cost, and ease of operation are the main factors responsible for its increasing popularity. It is a very repetitive process (Abdel-Rehim 2010).
Semi-automatic MEPS devices (e-Vol® syringes, and eXact3 Digital Syringe Driver)	It has sample enrichment and filtering in one single step. It is very easy to use, provides complete customization of extraction procedures, and allows greater precision. These devices could be used with μSPEed cartridges. The μSPEed cartridge design consists of a pressure-driven one-way check valve, allowing ultra-low dead volume connection; the samples and the solvents flow through the sorbent bed in a single direction in every step of the extraction. Therefore, aspiration occurs by pulling back the plunger and bypassing the sorbent when it is discarded. This version uses smaller sorbent particles (3 μm or even smaller, when traditional MEPS uses 50 μm diameter particles) in a small cartridge. These small particles provide a much bigger surface area, enhancing the contact between the sorbent and the analytes and improving a more efficient separation (Porto-Figueira et al. 2015; Pereira et al. 2019)
Automatic approaches	It has sample enrichment and filtering in one single step. It is very easy to use, provides complete customization of extraction procedures, and allows greater precision. These fully automated devices are still considerably expensive. Samples and solvents are loaded and discarded through the same channel, which may be of particular concern for those analytes presenting weak interactions with the sorbent. Indeed, they can be partially eluted and lost during extraction due to sample withdrawal and wash. Whereas it is possible to skip the washing step in a few situations, this strategy will impair selectivity and specificity for most applications, particularly when biological specimens are involved. To overcome this, a two-way valve laterally incorporated into the barrel of the syringe may be used. It is possible to use μSPEed cartridges (Moein et al. 2015b).

5.3 Sample Preparation Process

As already stated, the MEPS procedure usually follows a four-step approach, namely conditioning of the stationary phase, sample aspiration and ejection (strokes), interferences removal (washing), and analytes elution (Figure 5.2).

However, one should not be fooled by this apparent simplicity, as a wide range of optimization steps are deemed necessary in order to maximize extraction efficiency and sensitivity. For instance, selecting adequately the sorbent will be extremely important for a successful sample clean-up and also for analyte recovery.

It is possible to simplify or omit some of the steps depending on the target analytes and the desired degree of cleanliness of the extracts, bearing in mind that the ultimate goal of the procedure is to maximize efficiency.

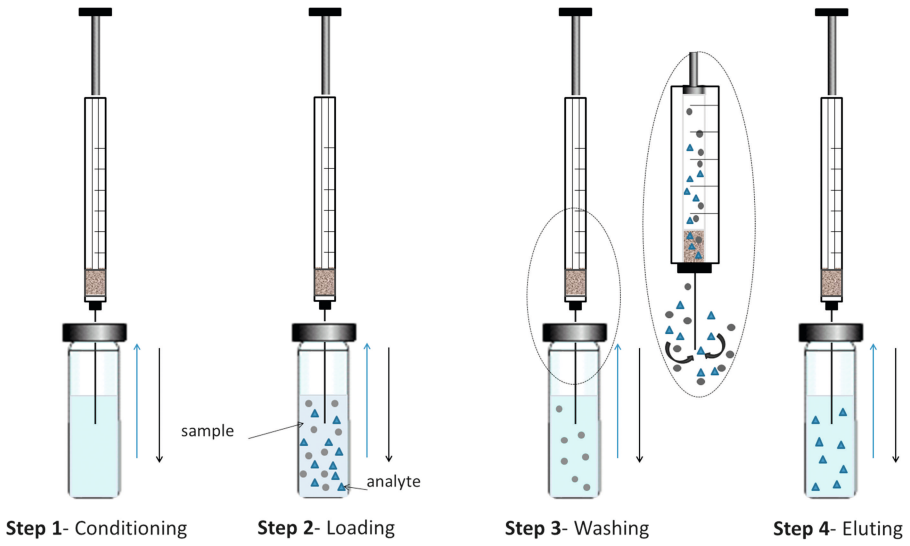

FIGURE 5.2 Operation steps (activation, sample loading, washing, and elution).

For instance, increasing the number of strokes will promote the contact time between the analytes and the sorbent. After the analytes are retained, a washing step is usually performed to remove undesired matrix constituents that are capable of interfering with the analysis. In most published MEPS applications, the wash solvent is the same as was used for sorbent conditioning; the choice of this solvent must be, however, careful and thoroughly optimized in order not to lose analytes in this step. Indeed, incrementing the amount of organic in the wash solvent is useful for efficient removal of matrix interferences, but it also is capable of weakening the analytes' interaction with the sorbent, promoting their early elution.

Analytes are eluted in the last step, which must also be critically optimized to allow their quantitative release from the sorbent in a solvent compatible with the analytical instrumentation that will be used. An organic solvent is generally used, and methanol, isopropanol, or acetonitrile, either by themselves or mixed with acidic or basic solutions (0.1–3%), have been described. In addition, the maximum amount of analyte should be eluted with low solvent volumes whenever possible, in order to increase the enrichment factor and allow direct injection into chromatographic systems if desired. Also, it facilitates the online coupling of extraction and instrumentation, with advantages concerning laboratorial throughput and cost per sample.

Abdel Rehim published in 2011 a tutorial paper on different protocols to use depending on the type of sorbents (Abdel-Rehim 2011). Figure 5.3 summarizes the main steps of MEPS procedures according to the type of sorbent.

Two approaches are usually seen in the optimization of these stages, either using the univariate (one factor is varied at a time) or the multivariate (with the aid of statistical tools allowing multiple factors to be varied simultaneously) ways. Examples of this last approach are the works from Rosado (Rosado et al. 2020b), Prata (Prata et al. 2019), or Oppolzer (Oppolzer et al. 2013), in which they managed to optimize the extraction process in different biological matrices (hair, blood, and urine) with a reduced number of experiments.

5.4 Applications in Toxicology

MEPS has been widely employed, not only in different fields of research, but also in routine analysis in many laboratories. MEPS applicability encompasses clinical, forensic toxicology, food, and environmental analysis applications, with successful implementations to extract a wide range of compounds from different matrices (Pereira et al. 2019).

Regarding clinical toxicology, this field is usually associated with therapeutic drug monitoring (TDM) at designated intervals in order to measure the concentration in the patient's bloodstream. However,

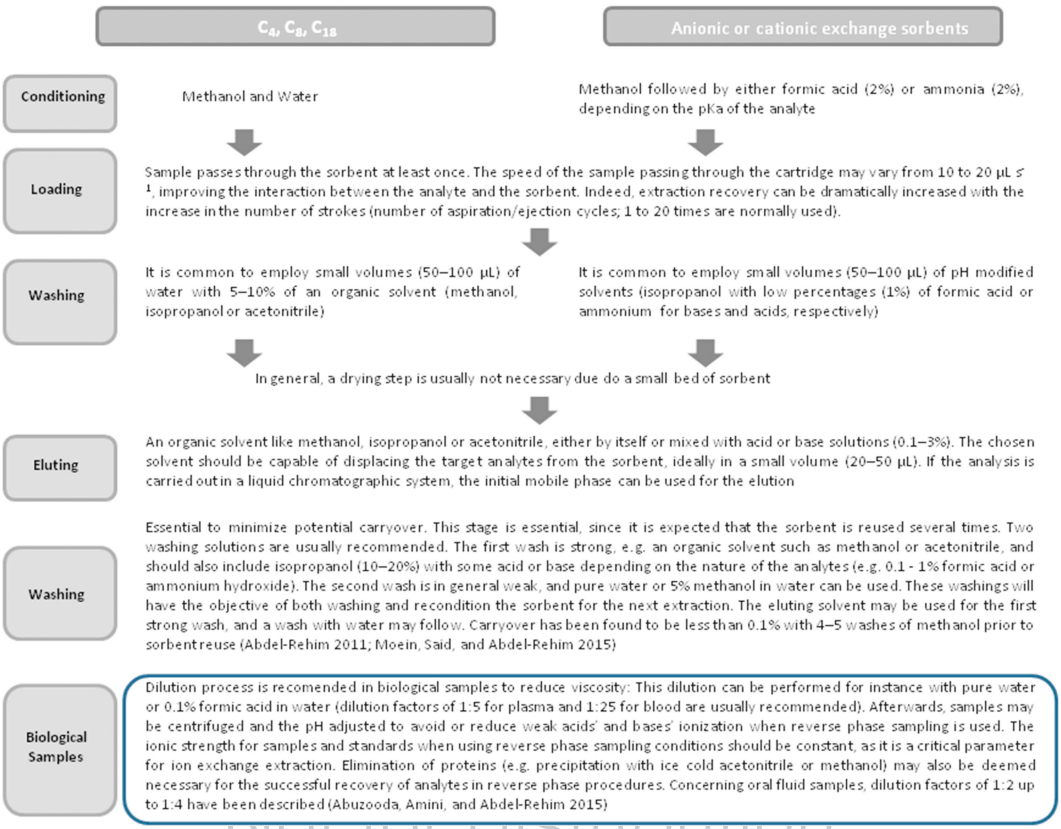

FIGURE 5.3 Main steps of MEPS procedures according to the type of sorbent.

clinical toxicology is a much broader field than just TDM, including catecholamines and metanephrine determination (Xiong and Zhang 2020b; Konieczna et al. 2016; Saracino et al. 2015), measurement of endocrine-disrupting chemical levels that result from human exposure (Silveira et al. 2020; Cristina Jardim et al. 2015), as well as polycyclic aromatic hydrocarbons (PAH) quantification due to their persistence in the environment (Martín Santos et al. 2020) and their effects on humans. Additionally, clinical toxicology has grown to the metabolomic field, and great research has been directed to the diagnostics of several diseases. Examples are the determination of sepsis biomarkers, such as aromatic microbial metabolites (Pautova et al. 2020c; Sobolev et al. 2017), and other biomarkers involved in the pathogenesis and pathophysiology of a wide range of diseases (Biagini et al. 2020; Berenguer et al. 2019). The most used specimen for the determination of this wide range of analytes is urine. Indeed, urine is usually available in sufficient amounts, and metabolites are available at greater concentrations than in other specimens, which makes it a great sample for metabolomics. Regarding sample preparation, proteins and cellular material are not present in urine at high levels, making laboratory analysis a simpler process (Rosado et al. 2017a). Before MEPS extraction, urine has a simple pre-treatment; commonly dilution, filtering, and occasionally a hydrolysis process might be adopted to liberate conjugates of the target analytes. Apart from urine, blood serum is also widely applied in the clinical toxicology field. Besides dilution, this specimen requires a much more thorough pre-treatment, namely centrifugation and/or protein precipitation, to avoid sorbent obstruction during extraction. The same happens with other alternative specimens cleaned up with MEPS for clinical purposes, namely saliva or central cerebrospinal fluid. For all specimens except urine, dilution is almost mandatory, but one should remember that the greater dilution is, the more draw-eject cycles need to be performed to obtain acceptable extraction efficiencies.

Regarding MEPS sorbents adopted in clinical toxicology, C_{18} was by far the most reported, as this sorbent solves problems related to the extraction of non-polar and low polar compounds containing

alkyl or aryl groups (Sobolev et al. 2017). Even for phenyl groups present in some aromatic microbial metabolites, this sorbent has proven its suitability (Sobolev et al. 2017). Nevertheless, other sorbents have been also adopted, and Konieczna et al. (Konieczna et al. 2016) stated that individual polar sorbents with surface-displayed amino groups (APS) might be more appropriate for all biogenic amines extraction, resulting in greater recoveries when compared to C_{18} (Konieczna et al. 2016). Most of the biogenic amines analyzed by the authors were very polar compounds; hence, the APS sorbent had the highest affinity (Konieczna et al. 2016). Table 5.2 describes MEPS procedures adopted in the last five years in the field of clinical toxicology.

Furthermore, in the sub-field of TDM, plasma samples are the most common, since therapeutic ranges for the drugs are usually determined in this specimen. Careful plasma pre-treatment will result in easier and faster MEPS procedures, as well as reduced matrix effects. The most adopted pre-treatment involved protein precipitation with trichloroacetic or perchloric acids followed by centrifugation. Once again, C_{18} sorbents were the most adopted for MEPS of fluoroquinolones (e.g., ciprofloxacin and levofloxacin), beta-lactam antibiotics (e.g., meropenem), imidazoles and triazoles, and non-steroidal anti-inflammatory drugs. Fuentes et al. (Fuentes et al. 2019) observed, however, that using a C_8–SCX mixed sorbent allowed better extraction efficiencies for most antidepressant drugs in patients urine samples. This would subsequently result in better sensitivity, allowing detecting lower concentrations.

Although plasma samples are the most used in TDM, other specimens have been cleaned up with MEPS. Locatelli et al. (Locatelli et al. 2015) extracted two fluoroquinolones from human sputum collected from cystic fibrosis patients. This non-conventional sample followed a similar pre-treatment than that of plasma, and even C_{18} has proven to be efficient for the same compounds in previous works. The authors achieved recoveries ranging between 60 and 80% (Locatelli 2015). Oral fluid samples diluted 1:4 were submitted to C_{18} MEPS to pre-concentrate metoprolol enantiomers, with recoveries ranging between 95 and 98% (Elmongy 2016). Aqueous humour has also been submitted to C_{18} MEPS for the determination of dexamethasone disodium phosphate and dexamethasone in patients with uveitis (Bianchi 2017). Although the authors have achieved great extraction efficiencies for both compounds, the sample load step seems quite laborious with 19 draw-eject cycles (Bianchi et al. 2017). Lastly, dialyzed samples were also used for the extraction of non-steroidal anti-inflammatory drugs, and MEPS has proved to be an excellent alternative to classical SPE (D'Archivio et al. 2016). All MEPS procedures reported in the last five years for TDM purposes are summarized in Table 5.3.

The forensic toxicology field can also be quite challenging. This is a multidisciplinary field that involves the determination and interpretation of the presence of drugs and other potential xenobiotics, usually in biological specimens. Although new MEPS sorbents have not been reported in the last five years in this field, there has been increasing research and application of this miniaturized technique to alternative specimens, especially hair samples. Hair matrix is advantageous due to its longer window for drug detection, hence allowing it to monitor past drug use and users under treatment programs (Rosado et al. 2020a). This represents a challenge in MEPS, since all mentioned biological specimens up to this point were fluid, and hair is solid. It is a very complex, strong, and stable matrix, and for this reason, an appropriate pre-treatment is required to remove the target analytes bound to its inner constituents. This pre-treatment is, actually, considered the extraction step from the sample, after which a further clean-up step can be adopted. Extraction may be carried out with organic solvents, usually methanol, or, depending on the target analytes, by means of weak acid (with hydrochloric acid) or alkaline (with sodium hydroxide) digestions. One should bear in mind, however, that methanol extractions can yield considerable interferences when compared to other procedures, and subsequently provide lower recoveries. After this first sample treatment, MEPS can be applied for the clean-up of the obtained hair extract.

In the last five years, three MEPS procedures have been reported for hair sample clean-up and determination of specific classes of drugs, namely cocaine and metabolites (Rosado et al. 2020b), selected opiates (Rosado et al. 2019), and methadone and EDDP (Rosado et al. 2020a). All three methods used a C_8–SCX mixed sorbent due to the different analytes' properties, and although the

TABLE 5.2

MEPS procedures in clinical toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Epinephrine (E) Norepinephrine (NE) Dopamine (D) Metanephrine (M) Normetanephrine (N) 3-methoxytyramine (3M)	Urine (0.1 mL)	Dilution with 100 µL of water and 100 µL of DPB buffer solution (pH 9.0)	C_{18} (n.s.); eVol®	Methanol and water (3 × 200 µL)	(7 × 100 µL)	2-amoniethyldip henylborinate buffer (1 × 100 µL) and water/methanol (1/1, v/v) (1 × 50 µL)	Water/ acetonitrile, 95/5, 0.25% formic acid (2 × 25 µL)	Water/ acetonitrile, 95/5, 0.25% formic acid (3 × 100 µL); methanol (1 × 100 µL), DPB buffer (1 × 100 µL), water/ acetonitrile, 95/5, 0.25% formic acid (1 × 100 µL), and water (1 × 100 µL)	LC-MS/MS	0.08 ng/mL (E) 0.30 ng/mL (NE) 0.53 ng/ mL (D) 0.176 ng/mL (M) 0.44 ng/mL (N) 0.176 ng/ mL (3M)	0.17 ng/mL (E) 0.65 ng/mL (NE) 1.53 ng/ mL (D) 1.34 ng/ mL (M) 3.43 ng/mL (N) 1.33 ng/ mL (3M)	n.s.	(Xiong and Zhang 2020b)
Methylparaben (MeP) Propylparaben (PrP) Bisphenol A (BPA) Bisphenol S (BPS) Benzophenone 1 (BP1) Triclocarban (TCC)	Urine (0.25 mL)	Buffered with 20 µL of 1M ammonium acetate (pH = 5) containing 50 unit of β-glucuronidase; incubated at 37 °C for 12 h; dilution with 250 µL of water	C_{18} (1 mg); Manual	Methanol and water (4 × 100 µL)	(5 × 100 µL)	10% methanol + 0.1% acetic acid (1 × 100 µL)	Methanol (1 × 30 µL)	Methanol and water (10 × 100 µL)	LC-MS/MS	0.10 ng/mL (MeP) 0.01 ng/ mL (PrP) 0.02 ng/mL (BPA) 0.01 ng/ mL (BPS) 0.005 ng/mL (BP1) 0.01 ng/ mL (TCC)	0.33 ng/mL (MeP) 0.03 ng/ mL (PrP) 0.07 ng/mL (BPA) 0.03 ng/ mL (BPS) 0.02 ng/mL (BP1) 0.03 ng/ mL (TCC)	96–104% (MeP) 98–116% (PrP) 104–110% (BPA) 97–105% (BPS) 99–113% (BP1) 101–116% (TCC)	(Silveira et al. 2020)
8-iso prostaglandin F2α (8-isoPGF2α) 8-iso prostaglandin E2 (8-isoPGE2) Prostaglandin E2 (PGE2)	Blood (0.05 mL in DBS)	Whole spot was cut; 350 µL of a methanol:water mixture (70:30 v/v) was added; evaporation down to 50 µL and dilution with water to 100 µL	C_{18} (4 mg); eVol®	Methanol and water (3 × 100 µL)	(5 × 100 µL)	Water:methanol (95:5 v/v) (1 × 100 µL)	Methanol: water (80: 20 v/v) (1 × 100 µL)	n.s.	LC-MS/MS	15 pg/mL (8-isoPGF2α) 20 pg/mL (8-isoPGE2) 15 pg/mL (PGE2)	n.s.	90–100% (8-isoPGF2α) 85–105% (8-isoPGE2) 90–110% (PGE2)	(Biagini et al. 2020)
Benzoic acid (BA) 3-phenylpropionic (PhPA) 3-phenyllactic (PhLA) 4-hydroxybenzoic (p-HBA) 4-hydroxyphenylacetic (p-HPhAA) 4-hydroxyphenylpropionic (p-HPhPA) homovanillic (HVA) 4-hydroxyphenyllactic (p-HPhLA)	Cerebrospinal fluid (0.04 mL)	Dilution with 40 µl of water, pH 7	C_{18} (4 mg); eVol	Methanol, water, 0.3 mM of formic acid (3 × 50 µL)	(20 × 50 µL)	0.3mM of formic acid (2 × 20 µL)	Diethyl ether (10 × 10 µL)	n.s.	GC-MS	0.1 µM (BA) 0.2 µM (PhPA) 0.2 µM (PhLA) 0.3 µM (p-HBA) 0.2 µM (p-HPhAA) 0.2 µM (p-HPhPA) 0.1 µM (HVA) 0.2 µM (p-HPhLA)	0.7 µM (BA) 0.5 µM (PhPA) 0.5 µM (PhLA) 0.6 µM (p-HBA) 0.5 µM (p-HPhAA) 0.5 µM (p-HPhPA) 0.4 µM (HVA) 0.4 µM (p-HPhLA)	60–90% (BA) 80–90% (PhPA) 70–80% (PhLA) 30–40% (p-HBA) 40–60% (p-HPhAA) 60–70% (p-HPhPA) 40–70% (HVA) 35–50% (p-HPhLA)	(Pautova et al. 2020b)

5-hydroxyindole-3-acetic acid (5HIAA) Indole-3-acetic acid (3IAA) Indole-3-carboxylic (3ICA) Indole-3-lactic acid (3ILA) Indole-3-propionic acid (3IPA)	Cerebrospinal fluid (0.04 mL) Serum (0.04 mL)	Dilution with 40 µl of water	C_{18} (4 mg); eVol®	Methanol, water, 0.3 mM of formic acid (3 × 50 µL)	(20 × 50 µL)	0.3 mM of formic acid (2 × 20 µL)	Diethyl ether (10 × 10 µL)	n.s.	GC-MS	0.3 µM (5HIAA) 0.2 µM (3IAA) 0.3 µM (3ICA) 0.2 µM (3ILA) 0.4 µM (3IPA) 0.4 µM (5HIAA) 0.2 µM (3IAA) 0.4 µM (3ICA) 0.3 µM (3ILA) 0.4 µM (3IPA)	0.4 µM (5HIAA) 0.4 µM (3IAA) 0.5 µM (3ICA) 0.4 µM (3ILA) 0.4 µM (3IPA) 0.4 µM (5HIAA) 0.4 µM (3IAA) 0.5 µM (3ICA) 0.4 µM (3ILA) 0.4 µM (3IPA)	40–80% (n.s) 40–60% (n.s)	(Pautova et al. 2020a)
Naphthalene (1) 2-methylnaphthalene (2) 1-methylnaphthalene (3) Biphenyl (4) Acenaphthylene (5) 3-phenyltoluene (6) Acenaphthene (7) Fluorene (8) Phenanthrene (9) Anthracene (10) Fluoranthene (11) Pyrene (12) Chrysene (13) Benzo (k) fluoranthene (14) Benzo(a) pyrene (15)	Oral fluid (1.5 mL)	Filtration through a 0.45 µm PTFE filter	C_{18} (n.s.); Automated	Ethyl acetate and water (1 × 500 µL)	(3 × 500 µL)	Water (1 × 250 µL)	Ethyl acetate (1 × 50 µL)	Water (3 × 500 µL) and Ethyl acetate (1 × 500 µL)	GC-MS	17 ng/L (1) 32 ng/L (2) 35 ng/L (3) 79 ng/L (4) 9.4 ng/L (5) 29 ng/L (6) 21 ng/L (7) 11 ng/L (8) 17 ng/L (9) 14 ng/L (10) 4.6 ng/L (11) 16 ng/L (12) 10 ng/L (13) 35 ng/L (14) 10 ng/L (15)	58 ng/L (1) 105 ng/L (2) 117 ng/L (3) 265 ng/L (4) 31 ng/L (5) 96 ng/L (6) 69 ng/L (7) 38 ng/L (8) 55 ng/L (9) 47 ng/L (10) 15 ng/L (11) 52 ng/L (12) 34 ng/L (13) 116 ng/L (14) 34 ng/L (15)	83% (1) 111% (2) 91% (3) 107% (4) 89% (5) 91%L (6) 88% (7) 78% (8) 120% (9) 119% (10) 88% (11) 98% (12) 104% (13) 114% (14) 108% (15)	(Martín Santos et al. 2020)
Vanillylmandelic acid (VMA)	Urine (0.010 mL)	Dilution with 90 µL of water	Anion exchange (AX) (n.s.); eVol®	Methanol and water (3 × 100 µL)	(7 × 100 µL)	5% ammonium hydroxide in water and 5% ammonium hydroxide in acetonitrile (1 × 100 µL)	Water/ acetonitrile 1:1 (v/v) (2% formic acid) (2 × 50 µL)	Water/ACN 1:1 (v/v) (2% formic acid) (4 × 100 µL) and water (1 × 100 µL)	LC-MS/MS	n.a.	0.5 µg/mL	58–71%	(Xiong and Zhang 2020a)
Leukotriene E (LTE4) Leukotriene B4 (LTB4) 11β-prostaglandin F2α (11β PGF2α)	Urine (n.s.)	pH of 5.1 (n.s.)	Retain anion exchange (R-AX) (n.s); eVol®	Acetonitrile and water at 0.1% formic acid (3 × 250 µL)	(10 × 250 µL)	0.1% formic acid (1 × 100 µL)	Methanol (2 × 50 µL)	Acetonitrile (3 × 250 µL) and water at 0.1% formic acid (1 × 250 µL)	UHPLC-DAD	0.16 ng/mL (LTE4) 0.04 ng/mL (LTB4) 1.12 ng/mL (11βPGF2α)	0.35 ng/mL (LTE4) 0.10 ng/mL (LTB4) 2.11 ng/mL (11βPGF2α)	84–91% (LTE4) 85–92% (LTB4) 96–100% (11βPGF2α)	(Berenguer et al. 2019)
Gamma-aminobutyric acid (GABA) Putrescine (Put) Cadaverine (Cad) L-ornithine (Orn) Spermidine (Spd)	Oral fluid (0.715 mL)	Centrifugation, dilution up to 5.0 mL with water	C_{18} (n.s.); Automated	Ethanol and water (1 × 100 µL)	(5 × 100 µL)	Water:methanol (80:20,v/v) (n.s.)	Ethanol (1 × 10 µL)	n.s.	GC-MS	3.12 ng/mL (GABA) 1.84 ng/mL (Put) 2.63 ng/mL (Cad) 5.15 ng/mL (Orn) 2.60 ng/mL (Spd)	10.40 ng/mL (GABA) 14.30 ng/mL (Put) 8.76 ng/mL (Cad) 17.20 ng/mL (Orn) 8.68 ng/mL (Spd)	89% (GABA) 94% (Put) 103% (Cad) 131% (Orn) 130% (Spd)	(Peña et al. 2019)

(Continued)

TABLE 5.2 ***(Continued)***

MEPS procedures in clinical toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Benzoic acid (BA) Phenylpropionic acid (PhPA) Phenyllactic acid (PhLA) 4-hydroxybenzoic acid (p-HBA) 4-hydroxyphenylacetic acid (p-HPhAA) 4-hydroxyphenylpropionic acid (p-HPhPA) Homovanillic acid (HVA) 4-hydroxyphenyllactic acid (p-HPhLA)	Serum (0.08 mL)	Concentrated sulphuric acid (2.5 μL) and water (70 μL) were added	C_{18} (4 mg.); n.s.	Methanol, water, 1% formic acid (3 × 50 μL)	(15 × 50 μL)	0.1% formic acid (2 × 20 μL)	Diethylether (10 × 10 μL)	n.s.	GC-MS	n.s.	160 ng/mL (BA) 60–100 ng/mL (PhPA) 60–100 ng/mL (PhLA) 60–100 ng/mL (p-HBA) 60–100 ng/mL (p-HPhAA) 160 ng/mL (p-HPhPA) 160 ng/mL (HVA) 160 ng/mL (p-HPhLA)	50% (BA) 70% (PhPA) 50% (PhLA) 30% (p-HBA) 40% (p-HPhAA) 50% (p-HPhPA) 50% (HVA) 45% (p-HPhLA)	(Pautova et al., 2019)
PS-18PS-19PS-20MOE-18MOE-19MOE-20	Serum (0.05 mL)	Deproteinization and dilution with 0.05 mL of ion pair reagent (IPR)	SDVB, C_8, and C_{18} (4 mg); eVol®	Methanol and water (1 × 100 μL); IPR (3 × 50 μL)	(5 × 100 μL)	IPR (1 × 100 μL)	Methanol:IPR (90:10) (1 × 100 μL)	Methanol/IPR 90/10 (3 × 100 μL) Methanol/ acetonitrile/ NH3 50/50/0.4 (3 × 100 μL) 2-propanol/ water/acid formic 90/10/ 0.1 (3 × 100 μL) Methanol (5 × 100 μL)	UHPLC-UV	n.s.	0.39 μM (PS-18) 0.39 μM (PS-19) 0.39 μM (PS-20) 0.39 μM (MOE-18) 0.39 μM (MOE-19) 0.39 μM (MOE-20)	85% (PS-18) 80% (PS-19) 75% (PS-20) 69% (MOE-18) 84% (MOE-19) 71% (MOE-20)	(Nuckowski et al. 2019)
BIFcis-DCCA trans-DCCA DBCA 3PBA	Urine (0.4 mL)	0.1 mL 1 M sodium acetate buffer (pH = 5.0) with β-glucuronidase; Incubated at 37 °C overnight (at least 12 h) before being acidified with 60 μL of formic acid	C_{18} (4 mg.); eVol®	Methanol (4 × 50 μL); 2% formic acid (3 × 20 μL)	(5 × 100 μL)	30% methanol in water (3 × 50 μL)	1,1,1,3,3,3-hexafluoroiso-propanol/ disopropylcar-bodiimide/ hexane mixture (1/2/97) (2 × 40 μL)	2-propanol (4 × 50 μL) and methanol (4 × 50 μL)	GC-MS	n.s.	0.06 ng/mL (BIF) 0.08 ng/mL (cis-DCCA) 0.08 ng/mL (trans-DCCA) 0.06 ng/mL (DBCA) 0.06 ng/mL (3PBA)	n.s.	(Klimowska and Wielgomas 2018)

Nitrobenzene (NB) 2-Nitrotoluene (2-NT) 3-Nitrotoluene (3-NT) 4-Nitrotoluene (4-NT) 2,6-Dinitrotoluene (2,6-DNT) 1,3-Dinitrobenzene (1,3-DNB) 2,4-Dinitrotoluene (2,4-DNT) 2,4,6-Trinitrotoluene (2,4,6-TNT) 1,3,5-Trinitrobenzene (1,3,5-TNB) 4-Amino-2,6-dinitrotoluene (4-Am-2,6-DNT) 2-Amino-4, 6-dinitrotoluene (2-Am-4,6-DNT) 2,4,6-Trinitrophenyl-Nmethylnitramine (Tetryl)	Plasma (1 mL)	n.s.	C_{18} (4 mg); Manual.	Methanol and water (1 × 100 µL)	(10 × 50 µL)	Water (1 × 50 µL)	Methanol (1 × 30 µL)	Methanol and water (3 × 100 µL)	GC-MS	0.014 to 0.828 ng/mL (n.s.)	0.046 to 2.732 ng/mL (n.s.)	90–95% (NB) 89–94% (2-NT) 90–94% (3-NT) 90–94% (4-NT) 89–93% (2,6-DNT) 91–94% (1,3-DNB) 88–92% (2,4-DNT) 89–94% (2,4,6-TNT) 88–92% (1,3,5-TNB) 88–90% (4-Am-2,6-DNT) 87 –90% (2-Am-4,6-DNT) 89–92% (Tetryl)	(Dhingra et al. 2018)
	Urine (1 mL)									0.014 to 0.828 ng/mL (n.s.)	0.046 to 2.732 ng/mL (n.s.)	92–96% (NB) 91–96% (2-NT) 90–97% (3-NT) 90–96% (4-NT) 90–93% (2,6-DNT) 93–97% (1,3-DNB) 90–93% (2,4-DNT) 90–95% (2,4,6-TNT) 91–95% (1,3,5-TNB) 89–91% (4-Am-2,6-DNT) 89 –91% (2-Am-4,6-DNT) 91–92% (Tetryl)	
trans,trans-muconic Acid (ttMA)	Urine (n.s.)	pH 7 (n.s.)	Quaternary ammonium ion exchange resin (SAX column) (4 mg.); n.s.	Methanol and water (3 × 100 µL)	(4 × 100 µL)	Water (3 × 100 µL)	10% (v/v) aqueous acetic acid (1 × 100 µL)	10% (v/v) acetic acid and water (4 × 150 µL)	HPLC - UV	0.03 µg/mL	0.1 µg/mL	93–99%	(Soleimani et al. 2017)
Benzoic acid (BA) 3-Phenylpropanoic acid (PhPA) 3-Phenylpropenoic acid (Cinnamic acid) 2-Hydroxy-3-phenylpropanoic acid (PhLA) 4-Hydroxybenzoic acid (HBA) 4-Hydroxyphenylacetic acid (HPhAA) 3-(4-Hydroxyphenyl) propanoic acid (HPhPA) 3-Methoxy-4-hydroxyphenylacetic acid (HVA) 3-(4-Hydroxyphenyl) -2-hydroxypropanoic acid (HPhLA)	Blood (0.08 µL)	n.s.	C_{18} (1 mg.); n.s.	Methanol, water, 1% of formic acid (3 × 50 µL)	(15 × 50 µL)	0.05% Sulphuric acid (2 × 20 µL)	Diethylether (10 × 10 µL)	n.s.	GC-MS	0.5 µM	n.s.	100% (BA) 102% (PhPA) 100% (Cinnamic acid) 58% (PhLA) 34% (HBA) 30% (HPhAA) 64% (HPhPA) 56% (HVA) 21%(HPhLA)	(Sobolev et al., 2017)

(*Continued*)

TABLE 5.2 ***(Continued)***

MEPS procedures in clinical toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Gamma-aminobutyric acid (GABA) Putrescine (Put) Cadaverine (Cad) L-ornithine (Orn) Spermidine (Spd)	Urine (0.715 mL)	Dilution 1:6 (n.s.)	C_{18} (4 mg.); automated	Ethanol and water (1 × 100 µL)	(5 × 100 µL)	3:1 v/v water:methanol (1 × 100 µL)	Ethanol (1 × 20 µL)	Ethanol (4 × 100 µL)	GC-MS	1.34 ng/mL (GABA) 1.81 ng/mL (Put) 1.10 ng/mL (Cad) 0.18 ng/mL (Orn) 2.70 ng/mL (Spd)	1.34 ng/mL (GABA) 1.81 ng/mL (Put) 1.10 ng/mL (Cad) 0.18 ng/mL (Orn) 2.70 ng/mL (Spd)	90–113% (n.s.)	(Casas Ferreira et al. 2016)
5-hydroxyindole-3-acetic acid (5-HIAA) Homovanilic acid (HVA) 3,4-dihydroxyphenylacetic acid (DOPAC) 3-methoxytyramine (3-MT) 5-hydroxytryptamine (5-HT) Dopamine (DA) Epinephrine (E) Tryptophan (Trp) Norepinephrine (NE) 5-hydroxytryptophane (5-HTrp) Tyrosine (Tyr) 3,4-dihydroxyphenylalanine (L-DOPA)	Plasma (0.1 mL)	0.1% formic acid added to pH 3	APS (4 mg); eVol®	Methanol and water (3 × 100 µL)	(8 × 100 µL)	0.1% formic acid (2 × 50 µL)	Methanol in 0.1% formic acid (3 × 50 µL)	Methanol and water (3 × 100 µL)	LC-MS	2 ng/mL (5-HIAA) 2 ng/mL (HVA) 2 ng/mL (DOPAC) 2 ng/mL (3-MT) 5 ng/mL (5-HT) 5 ng/mL (DA) 5 ng/mL (E) 2 ng/mL (Trp) 5 ng/mL (NE) 5 ng/mL (5-HTrp) 2 ng/mL (Tyr) 5 ng/mL (L-DOPA)	10 ng/mL (5-HIAA) 10 ng/mL (HVA) 10 ng/mL (DOPAC) 10 ng/mL (3-MT) 20 ng/mL (5-HT) 20 ng/mL (DA) 20 ng/mL (E) 10 ng/mL (Trp) 20 ng/mL (NE) 20 ng/mL (5-HTrp) 10 ng/mL (Tyr) 20 ng/mL (L-DOPA)	92% (5-HIAA) 98% (HVA) 94% (DOPAC) 90% (3-MT) 95% (5-HT) 104% (DA) 100% (E) 89% (Trp) 96% (NE) 91% (5-HTrp) 100% (Tyr) 88% (L-DOPA)	(Konieczna et al. 2016)
	Urine (0.05 mL)	0.1% formic acid added to pH 2								2 ng/mL (5-HIAA) 2 ng/mL (HVA) 2 ng/mL (DOPAC) 2 ng/mL (3-MT) 5 ng/mL (5-HT) 5 ng/mL (DA) 5 ng/mL (E) 2 ng/mL (Trp) 5 ng/mL (NE) 5 ng/mL (5-HTrp) 2 ng/mL (Tyr) 5 ng/mL (L-DOPA)	10 ng/mL (5-HIAA) 10 ng/mL (HVA) 10 ng/mL (DOPAC) 10 ng/mL (3-MT) 20 ng/mL (5-HT) 20 ng/mL (DA) 20 ng/mL (E) 10 ng/mL (Trp) 20 ng/mL (NE) 20 ng/mL (5-HTrp) 10 ng/mL (Tyr) 20 ng/mL (L-DOPA)	96% (5-HIAA) 99% (HVA) 97% (DOPAC) 92% (3-MT) 97% (5-HT) 92% (DA) 89% (E) 99% (Trp) 91% (NE) 84% (5-HTrp) 98% (Tyr) 84% (L-DOPA)	

Norepinephrine (NE) Epinephrine (E) Dopamine (DA)	Plasma (0.15 mL) Urine (0.01 mL)	Pippeted to FTA®cards; cutted; placed into a vial with 100 µL of ultrapure water and 240 µL of buffer solution; vortex agitation (2 min); centrifuged	C_{18} (4 mg); n.s.	Methanol and water (3 × 100 µL)	(12 × 100 µL)	buffer solution (1 × 100 µL) and water/methanol (50:50; v/v) (1 × 25 µL)	(2.5:97.5, v/v) of methanol, 30.0 mM citric acid in water and 0.5 mM OSA, adjusted to pH 2.92 (1 × 100 µL) (2.5:97.5, v/v) of methanol, 30.0 mM citric acid in water and 0.5 mM OSA, adjusted to pH 2.92 (2 × 100 µL)	Methanol and water (3 × 100 µL)	HPLC-ECD	0.03 ng/mL (NE) 0.03 ng/mL (E) 0.03 ng/mL (DA)	0.1 ng/mL (NE) 0.1 ng/mL (E) 0.1 ng/mL (DA)	91- 93% (NE) 91–95% (E) 85–90% (DA) 93–95% (NE) 90–95% (E) 86–89% (DA)	(Saracino et al. 2015)
Methyl paraben (MeP) Ethyl paraben (EtP) Propyl paraben (PrP) Butyl paraben (BuP) Benzyl paraben (BzP)	Urine (0.2 mL)	Dilution with 200 µL of phosphate buffer at pH 7.0	C_{18} (2 mg); manual	Methanol and water (1 × 250 µL)	(4 × 100 µL)	0.1% acetic acid in water (1 × 250 µL) and methanol/water (10:90, v/v) (1 × 100 µL)	Methanol/water (80:20, v/v) (1 × 50 µL)	Methanol and water (5 × 250 µL)	UHPLC-MS/MS	n.s.	0.5 ng/mL (MeP) 0.5 ng/mL (EtP) 0.5 ng/mL (PrP) 0.5 ng/mL (BuP) 0.5 ng/mL (BzP)	n.s.	(Jardim et al. 2015)

DAD (diode array detector); GC (gas chromatography); HPLC (high-performance liquid chromatography); LC (liquid chromatography); LOD (limit of detection); LOQ (limit of quantification); MS (mass spectrometry); MS/MS (tandem mass spectrometry); OSA (ammonium octadecyl sulfate); UHPLC (ultra high-performance liquid chromatography); UV (ultraviolet).

TABLE 5.3

MEPS procedures in drug monitoring, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Lumefantrine (L) desbutyl-lumefantrine (dL)	Plasma (n.s)	1:1 dilution and precipitation of plasma with 0.2% perchloric acid in acetonitrile. Supernatant collection (100 µL)	C_{18} (4 mg); n.s	n.s.	(10 × 70 µL)	Methanol: water (10:90) (n.s.)	Acetonitrile: 0.05% trifluoroacetic acid (90:10) (5 × 50 µL)	n.s.	HPLC-DAD	n.s.	50 ng/mL (L) 50 ng/mL (dL)	92–99% (L) 92–99% (dL)	(Siqueira et al. 2020)
Mirtazapine (MTZ) Venlafaxine (VLX) Escitalopram (ECIT) Fluvoxamine (FVX) Fluoxetine (FLX) Sertraline (SRT)	Urine (0.3 mL)	Dilution with 200 µL of phosphate buffer (0.05 M, pH 7)	Strong cation exchanger (C_8 + SCX) (4 mg); eVol®	Methanol and water (4 × 100 µL)	(10 × 100 µL)	Water (1 × 100 µL)	Methanol (1 × 50 µL)	Water, 0.1% formic acid and methanol (4 × 100 µL)	UHPLC-DAD	0.7 ng/mL (MTZ) 4.6 ng/mL (VLX) 1.8 ng/mL (ECIT) 3.8 ng/mL (FVX) 0.5 ng/mL (FLX) 7.0 ng/mL (SRT)	2.1 ng/mL (MTZ) 13.8 ng/mL (VLX) 5.4 ng/mL (ECIT) 11.4 ng/mL (FVX) 1.5 ng/mL (FLX) 21.0 ng/mL (SRT)	92–107% (MTZ) 80–102% (VLX) 92–112% (ECIT) 95–115% (FVX) 98–126% (FLX) 93–122% (SRT)	(Fuentes et al. 2019)
Meropenem (MERO) Levofloxacin (LEVO) Linezolid (LINZ)	Plasma (n.s.)	Dilution with water 1:2 (v/v)	C_{18} (4 mg); semiautomatic	n.s.	(10 × 150 µL)	Water (1 × 100 µL)	Methanol (1 × 50 µL)	Methanol and water (3 × 100 µL)	UHPLC-DAD	4 ng/mL (MERO) 4 ng/mL (LEVO) 7 ng/mL (LINZ)	20 ng/mL (MERO) 10 ng/mL (LEVO) 10 ng/mL (LINZ)	98–99% (MERO) 93–96% (LEVO) 96–97% (LINZ)	(Ferrone et al. 2017)
Ketoconazole (1) Terconazole (2) Voriconazole (3) Bifonazole (4) Clotrimazole (5) Tioconazole (6) Econazole (7) Butoconazole (8) Miconazole (9) Posaconazole (10) Ravuconazole (11) Itraconazole (12)	Plasma (0.17 mL) Urine (0.17 mL)	Dilution with trichloroacetic acid (20 mg/mL) in 1:0.5 (v:v)	C_{18} (n.s.); manual	Methanol and phosphate buffer (40 mM, pH 2.5) (3 × 150 µL)	(8 × 150 µL) (8 × 200 µL)	Phosphate buffer (40mM, pH 2.5): methanol (90:10, v-v) (1 × 200 µL)	Methanol (8 × 25 µL)	Methanol (5 × 200 µL)	HPLC-DAD	0.017 µg/mL (1) 0.070 µg/mL (2) 0.017 µg/mL (3) 0.017 µg/mL (4) 0.017 µg/mL (5) 0.017 µg/mL (6) 0.017 µg/mL (7) 0.017 µg/mL (8) 0.017 µg/mL (9) 0.017 µg/mL (10) 0.007 µg/mL (11) 0.017 µg/mL (12)	0.05 µg/mL (1) 0.2 µg/mL (2) 0.05 µg/mL (3) 0.05 µg/mL (4) 0.05 µg/mL (5) 0.05 µg/mL (6) 0.05 µg/mL (7) 0.05 µg/mL (8) 0.05 µg/mL (9) 0.05 µg/mL (10) 0.02 µg/mL (11) 0.05 µg/mL (12)	n.s.	(Campestre et al. 2017)
Dexamethasone (DEX) Dexamethasone disodium phosphate (DEX-SP)	Aqueous humour (0.05 mL)	n.s.	C_{18} (n.s.); eVol®	Methanol (4 × 50 µL) and water (2 × 50 µL)	(19 × 50 µL)	n.s.	Methanol (10 × 26 µL)	Methanol (10 × 50 µL)	LC-MS/MS	n.s.	0.5 ng/mL (DEX) 0.7 ng/mL (DEX-SP)	95–105% (DEX) 91–119% (DEX-SP)	(Bianchi et al. 2017)

Furprofen (FUR) Indoprofen (IND) Ketoprofen (KET) Fenbufen (FEN) Flurbiprofen (FLU) Indomethacin (INM) Ibuprofen (IBU)	Dialyzed samples (0.05 mL)	Centrifugation; dilution to 150 µL of water	C_{18} (n.s.); semi-automated	Methanol and water-methanol (95:5, v/v) (1 × 250 µL)	(10 × 50 µL)	10 mM phosphate buffer (pH 2.5) (1 × 100 µL)	Methanol and 1% sodium hydroxide in water 95:5 (v/v) (1 × 200 µL)	n.s.	UHPLC-DAD	8 ng/mL (FUR) 9 ng/mL (IND) 9 ng/mL (KET) 9 ng/mL (FEN) 9 ng/mL (FLU) 10 ng/mL (INM) 10 ng/mL (IBU)	25 ng/mL (FUR) 26 ng/mL (IND) 29 ng/mL (KET) 28 ng/mL (FEN) 27 ng/mL (FLU) 29 ng/mL (INM) 33 ng/mL (IBU)	94–99% (FUR) 94–99% (IND) 94–99% (KET) 98–99% (FEN) 94–99% (FLU) 97–99% (INM) 96–99% (IBU)	(D'Archivio et al. 2016)
Levofloxacin (1) Ciprofloxacin (2) Ulifloxacin (3) Moxifloxacin (4) Furprofen (5) Indoprofen (6) Ketoprofen (7) Fenbufen (8) Flurbiprofen (9) Indomethacin (10) Ibuprofen (11)	Plasma and Urine (0.18 mL)	Addition trichloroacetic acid (100 µL); centrifugation	C_{18} (n.s.); n.s.	Methanol and ammonium acetate buffer, 50 mM, pH 2.5 (3 × 150 µL)	(8 × 100 µL)	Ammonium acetate buffer: methanol (95:5, v-v) (1 × 150 µL)	Methanol (8 × 25 µL)	n.s.	HPLC -DAD	0.03 µg/mL	0.10 µg/mL	n.s.	(D'Angelo et al. 2016)
(R) -Metoprolol(S) -Metoprolol	Plasma (0.1 mL) Oral fluid (0.1 mL)	Dilution with water (1:4)	C_{18} (n.s.); n.s.	Isopropanol and water (1 × 100 µL)	(4 × 100 µL)	5% Methanol (2 × 100 µL)	Isopropanol (2 × 100 µL)	Isopropanol and water (4 × 100 µL)	LC-MS/MS	0.5 ng/mL (R) 0.5 ng/mL (S) 0.5 ng/mL (R) 0.5 ng/mL (S)	1.5 ng/mL (R) 1.5 ng/mL (S) 1.5 ng/mL (R) 1.5 ng/mL (S)	94–98% (R) 93–97% (S) 95–98% (R) 96–97% (S)	(Elmongy et al. 2016)
Betaxolol	Urine (0.25 mL)	Dilution 10 times with water and filtered	C_{18} (n.s.); automated	15% Acetonitrile (500 µL)	MEPS-SIC hyphenation (250 µL)	15% Acetonitrile (700 + 500 µL)	Acetonitrile: 0.5% of TEA in water, pH 4.5 (30:70) (2500 µL)	n.s.	HPLC-UV	1.5 ng/mL	5 ng/mL	100–108%	(Šrámková et al. 2015)
Ciprofloxacin (CIP) Levofloxacin (LEV)	Sputum (0.18 mL)	Trichloroacetic acid (20 mg/mL) in 1:0.5 ratio (v:v); centrifugation	C_{18} (n.s.); n.s.	Methanol (3 × 150 µL) and phosphate buffer (30 mM, pH 2.5) (3 × 150 µL)	(8 × 100 µL)	Phosphate buffer (30 mM, pH 2.5) and methanol (95:5, v-v) (1 × 150 µL)	Methanol (8 × 25 µL)	n.s.	HPLC-DAD	17 ng/mL	50 ng/mL	60% (CIP) 80% (LEV)	(Locatelli et al. 2015)

DAD (diode array detector); GC (gas chromatography); HPLC (high-performance liquid chromatography); LC (liquid chromatography); LOD (limit of detection); LOQ (limit of quantification); MS (mass spectrometry); MS/MS (tandem mass spectrometry); TEA (triethylamine); UHPLC (ultra high-performance liquid chromatography); UV (ultraviolet).

obtained recoveries were low for some markers of cocaine and opiates consumption, the limits of determination were comparable to those reported for SPE. This is explained not only by the analytical equipment used, but also by the selectivity of MEPS and the clean extracts obtained.

Another alternative specimen that was widely used in this field in the past years was oral fluid. The reported applications of MEPS for oral fluid clean-up and drug pre-concentration are noteworthy, since with a few microlitres and a rapid procedure it was possible to determine up to 30 different analytes (Rocchi et al. 2018). MEPS's potential is proven in this field due to the rapid extraction of a great number of xenobiotics from small amounts of samples. Moreover, its application to blood, plasma, and urine has continued to be reported in the last five years. The most described sorbent in the forensic toxicology field is not C_{18}, like in the previous fields, however. Instead, mixed-mode sorbent appears to be the most suitable to pre-concentrate multi-class drugs on a multi-method. Table 5.4 describes MEPS procedures adopted in the last five years in the field of forensic toxicology.

The field of analysis, for which more developments regarding sorbents were observed, is undoubtedly environmental toxicology. The samples' type do not vary much; all of them are water samples, except one which is soil, and the analytes to be determined are mainly polycyclic aromatic hydrocarbons, phenoxyacetic acid herbicides, other pesticides, endocrine-disrupting chemicals, and trace levels of a few pharmaceutical drugs. Another different aspect in this field is the volume of sample submitted to MEPS. In fact, while in other fields MEPS works with volumes in the order of microlitres, in environmental toxicology volumes of several millilitres are used. The latter is also justified by the fact that the new developed sorbents are packed in larger capacity syringes, commonly insulin syringes (1 mL). Most publications in this field did not specify sample pre-treatment before MEPS, perhaps because their major goal was sorbent development; however, a few mentioned centrifugation or filtration of the samples.

The use of soil as an environmental sample has further proven the great versatility of this miniaturized technique. Serenjeh et al. (Serenjeh et al. 2020) proposed a headspace approach of MEPS for the determination of volatile polycyclic aromatic hydrocarbons in soil. The authors used 2 mg of aminoethyl functionalized SBA-15 (SBA-15-NH_2) as sorbent, and after pre-heating the soil sample 15 min at 150 °C, the MEPS syringe sampled the air in the closed vial to concentrate the analytes (Serenjeh et al. 2020). Although the reported extraction efficiencies were not high, this approach appears as an excellent option for other solid samples. The latter procedure and other MEPS applications in the last five years for environmental toxicology are resumed in Table 5.5.

Finally, the food toxicology field has the most heterogeneous types of samples. Specimens used in this field can go from solid (e.g., fruits, flour) to liquid (e.g., milk, wine, juices), and MEPS applications have proven suitable for all of them. Even though many sorbent developments were made in the last five years concerning food toxicology analysis, C_{18} continues to be the most reported sorbent. Indeed, this sorbent has been applied to pre-concentrate fluoroquinolones from bovine milk (Aresta et al. 2019), polybrominated diphenyl ether (Souza et al. 2019), phthalates in cold drinks (Kaur et al., 2016), ochratoxin A and furanic derivatives in wines (Savastano et al. 2016; Perestrelo et al. 2015), and polychlorinated biphenyls in bovine serum (Yang et al. 2016), all of them resulting in recoveries above 70%. Poorer recoveries were reported for this sorbent when applied to pre-concentrate pesticides in sugarcane juice samples (27 to 65%) (Fumes et al. 2016). Noteworthy is the work reported by Di Ottavio et al. (Di Ottavio et al. 2017) that accomplished the extraction of 25 pesticide and fungicide residues in wheat flour. The target analytes are widely used in wheat and present different physico-chemical characteristics; hence, the authors opted for highly cross-linked polystyrene divinylbenzene (HDVB) sorbent.

Depending on the sample type, different pre-treatments should be adopted. For instance, milk and egg samples should undergo a protein precipitation step, whereas fruits and other solid samples should be crushed and solubilized under sonication. For all of them, a further centrifugation step and dilution should be employed to improve sorbent durability. The different procedures are summarized in Table 5.6.

TABLE 5.4

MEPS procedures in forensic toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent Re-use					
Cocaine (COC), Benzoylecgonine (BEG), Ecgonine methyl ester (EME), Norcocaine (NCOC), Cocaethylene (COET), Anhydroecgonine methyl (AEME)	Hair (50 mg)	1 mL of 0.1 M hydrochloric acid incubation overnight at 60 °C. Neutralization with 100 µL of 1M sodium hydroxide	M1 (80% C_8 and 20% SCX) (4 mg); Manual	Methanol and water (1 × 250 µL)	(21 × 150 µL)	Water and acetate buffer of pH 4 (1 × 50 µL)	2% ammonium hydroxide in methanol (3 × 100 µL)	1% ammonia in methanol–acetonitrile (50:50, v/v) and 1% formic acid in 2-propanol–water (10:90) (2 × 250 µL)	GC-MS/MS	0.010 ng/mg (COC) 0.025 ng/mg (BEG) 0.025 ng/mg (EME) 0.025 ng/mg (NCOC) 0.010 ng/mg (COET) 0.150 ng/mg (AEME)	0.010 ng/mg (COC) 0.025 ng/mg (BEG) 0.025 ng/mg (EME) 0.025 ng/mg (NCOC) 0.010 ng/mg (COET) 0.150 ng/mg (AEME)	44–65% (COC) 21–28% (BEG) 1–3% (EME) 36–44% (NCOC) 63–73% (COET) 4–6% (AEME)	Rosado et al. (2020b)
Methadone (MET), EDDP	Hair (50 mg)	1mL of 1M sodium hydroxide for 45 min at 50 °C; neutralization with 100 µL of 20% formic acid	M1 (80% C_8 and 20% SCX) (4 mg); Manual	Methanol and 2% formic acid (3 × 250 µL)	(9 × 150 µL)	3.36% formic acid (3 × 50 µL)	2.36% ammonium hydroxide in methanol (6 × 100 µL)	1% ammonia in methanol–aceto-nitrile (50:50, v/v) and 1% formic acid in 2-propanol–water (10:90) (4 × 250 µL)	GC-MS/MS	0.01 ng/mg (MET) 0.01 ng/mg (EDDP)	0.01 ng/mg (MET) 0.01 ng/mg (EDDP)	73–109% (MET) 84–111% (EDDP)	Rosado et al. (2020a)
Methylone	Oral fluid (0.5 mL)	10 µL of 1 M carbonate buffer at pH 9.0	C_{18} (4 mg); eVol®	2-propanol and water (1 × 100 µL)	(5 × 100 µL)	0.1 M carbonate buffer at pH 9.0 (1 × 100 µL)	2- propanol (5 × 100 µL)	n.s.	IMS	4 ng/mL	14 ng/mL	78–91%	Sorribes-Soriano et al. (2020)
Fentanyl (F), Sufentanil (SuF), Alfentanil (AlF), Acrylfentanyl (AcryF), Thiofentanyl (ThF), Valerylfentanyl (ValF), Furanylfentanyl (FuF), Acetyl fentanyl (AcetF), Carfentanil (CarF), Norfentanyl (NorF), Acetyl norfentanyl (AcetNorF)	Urine (0.2 mL)	Dilution with 0.6 mL water	C_{18} (n.s.); eVol®	Methanol and water (2 × 50 µL)	(8 × 50 µL)	Water and isopropyl alcohol (95:5 v/v) (2 × 50 µL)	Acetonitrile (1 × 50 µL)	Acetonitrile: methanol mixture (1:1 v/v) and water: methanol mixture (95:5 v/v) (4 × 50 µL)	LC-MS/MS	0.1 ng/mL (F) 0.11 ng/mL (SuF) 0.1 ng/mL (AlF) 0.1 ng/mL (AcryF) 0.1 ng/mL (ThF) 0.1 ng/mL (ValF) 0.1 ng/mL (FuF) 0.1 ng/mL (AcetF) 0.1 ng/mL (CarF) 1 ng/mL (NorF) 1 ng/mL (AcetNorF)	1 ng/mL (F) 1 ng/mL (SuF) 1 ng/mL (AlF) 1 ng/mL (AcryF) 1 ng/mL (ThF) 1 ng/mL (ValF) 1 ng/mL (FuF) 1 ng/mL (AcetF) 1 ng/mL (CarF) 1 ng/mL (NorF) 1 ng/mL (AcetNorF)	28–35% (F) 31–32% (SuF) 30–30% (AlF) 29–34% (AcryF) 27 –31% (ThF) 32–35% (ValF) 30–37% (FuF) 24–27% (AcetF) 31–34% (CarF) 13–14% (NorF) 5–6% (AcetNorF)	da Cunha et al. (2020)
Dichloropane	Oral fluid (0.09 mL)	pH adjusted with 10 µL phosphate buffer (1 M, pH 7)	C_8 (4mg); eVol®	2-propanol (3 × 100 µL) and water (2 × 100 µL)	(4 × 100 µL)	Water (4 × 100 µL)	2-propanol (10 × 50 µL)	n.s.	IMS	30 ng/mL	90 ng/mL	85–107%	Sorribes-Soriano et al. (2019)
									GC-MS	70 ng/mL	200 ng/mL		

(*Continued*)

TABLE 5.4 *(Continued)*

MEPS procedures in forensic toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent Re-use					
Tramadol (TRM), Codeine (COD), Morphine (MOR), 6-acetylcodeine (6-AC), 6-monoacetylmorphine (6-MAM), Fentanyl (FNT)	Hair (50 mg)	2 mL of methanol incubated overnight at 65°C; evaporation and reconstitution with 500 µL of 2% formic acid	M1 (80% C_8 and 20% SCX) (4 mg); manual	Methanol and 2% formic acid (3 × 250 µL)	(15 × 150 µL)	3.36% formic acid (3 × 50 µL)	2.36% ammonium hydroxide in methanol (8 × 100 µL)	1% ammonium hydroxide in acetonitrile:methanol (1:1) and 1% formic acid in isopropanol:water (10:90) (4 × 250 µL)	GC-MS/MS	0.010 ng/mg (TRM) 0.010 ng/mg (COD) 0.025 ng/mg (MOR) 0.010 ng/mg (6-AC) 0.025 ng/mg (6-MAM) 0.025 ng/mg (FNT)	0.010 ng/mg (TRM) 0.010 ng/mg (COD) 0.025 ng/mg (MOR) 0.010 ng/mg (6-AC) 0.025 ng/mg (6-MAM) 0.025 ng/mg (FNT)	74–90% (TRM) 51–59% (COD) 22–35% (MOR) 69–99% (6-AC) 53–61% (6-MAM) 75–86% (FNT)	Gallardo et al. (2019)
Codeine (COD), Morphine (MOR), 6-monoacetylmorphine (6-MAM)	Blood (0.25 mL)	Dilutionwith 0.4 mL of 0.1 M phosphate buffer(pH 6); protein precipitation with ice-cold acetonitrile; centrifugation, evaporation and addition of 8.5 mL of 2% formic acid	M1 (80% C_8 and 20% SCX) (4 mg); manual	Methanol and 2% formic acid (3 × 250 µL)	(20 × 250 µL)	3.36% formic acid (1 × 250 µL)	2.36% ammonium hydroxide in methanol (11 × 250 µL)	Methanol and water (3 ×250 µL)	GC-MS/MS	5 ng/mL (COD) 5 ng/mL (MOR) 5 ng/mL (6-MAM)	5 ng/mL (COD) 5 ng/mL (MOR) 5 ng/mL (6-MAM)	13–20% (COD) 6–8% (MOR) 14–20% (6-MAM)	Prata et al. (2019)
Amphetamine (AMP), Methamphetamine (MAMP), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylmethamphetamine (MDMA), 3,4-methylenedioxy-N-methyl-α-ethylfenilethylamine (MBDB), 3,4-methylenedioxy-N-ethylamphetamine (MDE)	Urine (0.2 mL)	Dilution with 0.1 mL of ammonium acetate (pH 6.7)	C_{18} (4 mg); manual	Methanol and water (1 × 250 µL)	(9 × 100 µL)	Water (1 × 150 µL) and water:methanol (95:5) (1 × 150 µL)	2% ammonium hydroxide in acetonitrile (4 × 100 µL)	Ammonium hydroxide in acetonitrile: methanol (1:1) and 1% formic acid in isopropanol:water (10:90) (4 × 100 µL)	GC-MS	n.s.	35 ng/mL (AMP) 25 ng/mL (MAMP) 50 ng/mL (MDA) 35 ng/mL (MDMA) 25 ng/mL (MBDB) 25 ng/mL (MDE)	32–49% (AMP) 19–38% (MAMP) 30–48% (MDA) 40–52% (MDMA) 34–50% (MBDB) 52–71% (MDE)	Malaca et al. (2019)

Azynphos-ethyl (AZP), Diazinon (DZN), Chlorpyrifos (CLP), Chlorfenvinfos (CLF), Parathion-ethyl (PRT), Quinalphos (QLP)	Blood (0.1 mL)	Dilution with 500 µL of ammonium acetate buffer (pH 4.9)	C_{18} (4 mg); manual	Methanol and water (4 × 250 µL)	(40 × 150 µL)	2-propanol (1.5%) in 0.1% formic acid in water (1 × 25 µL)	Methanol (4 × 110 µL)	Methanol (3 × 250 µL)	GC-MS/MS	n.s.	2.5 µg/mL (AZP) 0.5 µg/mL (DZN) 0.5 µg/mL (CLP) 0.5 µg/mL (CLF) 0.5 µg/mL (PRT) 0.5 µg/mL (QLP)	61–68% (AZP) 58–78% (DZN) 59–68% (CLP) 64–74% (CLF) 62–76% (PRT) 70–78% (QLP)	Santos et al. (2018)
Piperonyl piperazine (1) Methylone (2) 4-MEOPP (3) Dimethylcathinone (4) Buphedrone (5) Methedrone (6) Buthylone (7) Ethcathinone (8) Mephedrone (9) 4-MEC (10) Methoxetamine (11) alpha-PVP (12) 2C-B (13) 3,4-MDPV (14) AM-1220 (15) JWH-200 (16) AB-005 (17) JWH-018 N-pentanoic acid (18) JWH-018 N-(5-hydroxypentyl) (19) XLR-11 N-(4-hydroxypentyl) (20) MAM-2201 N-pentanoic acid (21) JWH-073 (22) WIN-55 (23) UR-144 N-(5-hydroxypentyl) (24) MAM-2201 (25) JWH-250 (26) XLR-11 (27) JWH-018 (28) JWH-081 (29) JWH-122 (30)	Oral fluid (0.09 mL)	Dilution with 30 µL of methanol and 60 µL of water (total volume 200 µL)	C_{18} (n.s.); n.s.	Methanol (3 × 250 µL); Water:methanol (75:25, v/v) (3 × 250 µL)	(5 × 250 µL)	Water:methanol (90:10, v/v) (3 × 200 µL)	10 mM formic acid (5 × 100 µL)	n.s.	UHPLC-MS/MS	0.850 ng/mL (1) 0.385 ng/mL (2) 0.830 ng/mL (3) 0.830 ng/mL (4) 0.045 ng/mL (5) 0.070 ng/mL (6) 0.230 ng/mL (7) 0.126 ng/mL (8) 0.050 ng/mL (9) 0.045 ng/mL (10) 0.012 ng/mL (11) 0.470 ng/mL (12) 0.050 ng/mL (13) 0.053 ng/mL (14) 0.005 ng/mL (15) 0.012 ng/mL (16) 0.020 ng/mL (17) 0.036 ng/mL (18) 0.045 ng/mL (19) 0.035 ng/mL (20) 0.093 ng/mL (21) 0.275 ng/mL (22) 0.005 ng/mL (23) 0.017 ng/mL (24) 0.030 ng/mL (25) 0.020 ng/mL (26) 0.009 ng/mL (27) 0.012 ng/mL (28) 0.060 ng/mL (29) 0.045 ng/mL (30)	2.600 ng/mL (1) 1.150 ng/mL (2) 2.500 ng/mL (3) 2.500 ng/mL (4) 0.135 ng/mL (5) 0.210 ng/mL (6) 0.700 ng/mL (7) 0.380 ng/mL (8) 0.150 ng/mL (9) 0.130 ng/mL (10) 0.035 ng/mL (11) 1.400 ng/mL (12) 0.150 ng/mL (13) 0.160 ng/mL (14) 0.015 ng/mL (15) 0.035 ng/mL (16) 0.055 ng/mL (17) 0.110 ng/mL (18) 0.135 ng/mL (19) 0.100 ng/mL (20) 0.280 ng/mL (21) 0.820 ng/mL (22) 0.015 ng/mL (23) 0.050 ng/mL (24) 0.090 ng/mL (25) 0.060 ng/mL (26) 0.025 ng/mL (27) 0.035 ng/mL (28) 0.170 ng/mL (29) 0.135 ng/mL (30)	49–53% (1) 68–77% (2) 85–96% (3) 73–85% (4) 41–46% (5) 40–46% (6) 45–49% (7) 48–55% (8) 31–40% (9) 33–36% (10) 33–38% (11) 65–72% (12) 67–96% (13) 70–84% (14) 63–70% (15) 49–52% (16) 67–68% (17) 78–92% (18) 68–91% (19) 58–68% (20) 75–80% (21) 65–74% (22) 70–89% (23) 72–95% (24) 87–96% (25) 60–84% (26) 78–89% (27) 73–86% (28) 61–69% (29) 76–93% (30)	Rocchi et al. (2018)
Tetrahydrocabinol (THC), 11-hydroxy-tetrahydrocababinol (11-OH-THC), 11-Nor-9-carboxy-tetrahydrocababinol (THC-COOH)	Plasma (0.25 mL)	Protein precipitation; dilution with 5 mL of 0.1 mM potassium phosphate buffer (pH = 6)	M1 (80% C_8 and 20% SCX) (4 mg); manual	Methanol and 0.1% formic acid (4 × 250 µL)	(26 × 250 µL)	3% acetic acid and 5% methanol (1 × 100 µL)	10% ammonium hydroxide in methanol (6 × 100 µL)	n.s.	GC-MS/MS	0.1 ng/mL (THC) 0.1 ng/mL (11-OH-THC) 0.1 ng/mL (THC-COOH)	0.1 ng/mL (THC) 0.1 ng/mL (11-OH-THC) 0.1 ng/mL (THC-COOH)	53–78% (THC) 57–66% (11-OH-THC) 62–65% (THC-COOH)	Rosado et al. (2017b)

(*Continued*)

TABLE 5.4 *(Continued)*

MEPS procedures in forensic toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent Re-use					
Cocaine (COC), Benzoylecgonine (BEG), Ecgonine methyl ester (EME)	Urine (0.2 mL)	Centrifuged at 4,500 rpm during 15 min; 100 µL of 0.1 mM potassium phosphate buffer	M1 (80% C_8 and 20% SCX) (4 mg); manual	Methanol and 0.1% formic acid (1 × 250 µL)	(6 × 150 µL)	0.1% formic acid (4 × 50 µL)	1% ammonium hydroxide in methanol (4 × 100 µL)	1% ammonia in methanol–acetonitrile (50:50, v/v) and 1% formic acid in 2-propanol–water (10:90) (4 × 100 µL)	GC-MS	25 ng/mL (COC) 25 ng/mL (BEG) 25 ng/mL (EME)	25 ng/mL (COC) 25 ng/mL (BEG) 25 ng/mL (EME)	67–83% (COC) 25–44% (BEG) 15–37% (EME)	Rosado et al. (2017a)
Morphine (1) Naloxone (2) Methylone (3) Flephedrone (4) Ethylcathinone (5) Ethylcathinone ephedrine (6) Scopolamine (7) 6-monoacetylmorphine (6-MAM) (8) Ethylone (9) Methylephedrine (10) Butylone (11) Mephedrone (12) Pentedrone (13) Benzoylecgonine (BEG) (14) Cocaine (15) Methylenedioxypyrovalerone (MDPV) (16) Cocaethylene (17) Pyrovalerone (18) EDDP (19) Buprenorphine (20) Methadone (21)	Oral fluid (0.3 mL)	200 µL of methanol; shaken; centrifugation; 300 µL of supernatant dilution with 200 µL of phosphate buffer (50 mM, pH 9)	M1 (80% C_8 and 20% SCX) (4 mg); eVol®	Methanol and water (1 × 100 µL)	(6 × 100 µL)	Water/ methanol 90:10 (v:v) (1 × 50 µL)	Dichloromethane/2-propanol/ ammonium hydroxide (78:20:2, v-v:v) (1 × 90 µL)	Eluent and methanol (1 × 100 µL); water, 0.1% formic acid and methanol (4 × 100 µL)	UHPLC-MS/MS	2.5 ng/mL (1) 0.5 ng/mL (2) 0.25 ng/mL (3) 0.25 ng/mL (4) 0.25 ng/mL (5) 0.25 ng/mL (6) 1 ng/mL (7) 1 ng/mL (8) 0.25 ng/mL (9) 0.25 ng/mL (10) 0.25 ng/mL (11) 0.25 ng/mL (12) 0.25 ng/mL (13) 0.5 ng/mL (14) 0.25 ng/mL (15) 0.25 ng/mL (16) 0.25 ng/mL (17) 0.25 ng/mL (18) 0.25 ng/mL (19) 0.25 ng/mL (20) 0.25 ng/mL (21)	10 ng/mL (1) 1 ng/mL (2) 0.5 ng/mL (3) 0.5 ng/mL (4) 0.5 ng/mL (5) 0.5 ng/mL (6) 2.5 ng/mL (7) 2.5 ng/mL (8) 0.5 ng/mL (9) 0.5 ng/mL (10) 0.5 ng/mL (11) 0.5 ng/mL (12) 0.5 ng/mL (13) 1 ng/mL (14) 0.5 ng/mL (15) 0.5 ng/mL (16) 0.5 ng/mL (17) 0.5 ng/mL (18) 0.5 ng/mL (19) 0.5 ng/mL (20) 0.5 ng/mL (21)	22–87% (1) 94–107% (2) 75–93% (3) 60–100% (4) 100–125% (5) 84–106% (6) 44–86% (7) 86–99% (8) 91–110% (9) 87–101% (10) 84–125% (11) 86–124% (12) 75–99% (13) 85–112% (14) 79–105% (15) 72–92% (16) 87–108% (17) 92–102% (18) 90–104% (19) 92–107% (20) 82–111% (21)	Ares et al. (2017)
Chlordiazepoxide (CLD), Medazepam (MDP), Lorazepam (LZP), Oxazepam (OZP), Diazepam (DZP)	Alcoholic beverage	Dilution with water in 1:5 ratio (v/v)	C_{18} (n.s.); eVol®	Acetonitrile and water (3 × 100 µL)	(6 × 100 µL)	n.s.	Acetonitrile and water 90:10 (v/v), both acidified with 0.1% formic acid (3 × 100 µL)	2-n-propanol (2 × 100 µL)	UHPLC-UV	1 µg/mL (CLD) 0.5 µg/mL (MDP) 0.5 µg/mL (LZP) 0.5 µg/mL (OZP) 0.5 µg/mL (DZP)	2 µg/mL (CLD) 1 µg/mL (MDP) 1 µg/mL (LZP) 1 µg/mL (OZP) 1 µg/mL (DZP)	61–64% (CLD) 61–67% (MDP) 81–87% (LZP) 88–91% (OZP) 70–72% (DZP)	Magrini et al. (2016)

Norketamine (NK), Ketamine (K)	Plasma (0.25 mL)	Dilution with 7 mL of phosphate buffer	M1 (80% C_8 and 20% SCX) (4 mg); manual	Methanol (5 × 250 µL) and water (4 × 250 µL)	(26 × 250 µL)	0.1% acetic acid (1 × 100 µL) and 10% methanol (1 × 100 µL)	6% ammonia in methanol (1 × 100 µL)	Methanol (5 × 250 µL) and water (4 × 250 µL)	GC-MS/MS	5 ng/mL (NK) 5 ng/mL (K)	10 ng/mL (NK) 10 ng/mL (K)	63–75%(NK) 73–89% (K)	Moreno et al. (2015)
	Urine (0.2 5mL)	Dilution with 0.25 mL of water			(8 × 250 µL)	5.25% acetic acid (1 × 250 µL) and 5% methanol in water (1 × 100 µL)	3% ammonia in methanol (1 × 100 µL)					73–76%(NK) 89–101% (K)	
Amphetamine (AMP) (1) Benzoylecgonine (BEG) (2) Buprenorphine (3) Cocaine (COC) (4) Codeine (COD) (5) Diacetylmorphine (6) Ecgonine methylester (EME) (7) EDDP (8) Ketamine (9) 3,4-methylenedioxyamphetamine (MDA) (10) Methyldiethanolamine (MDEA) (11) 3,4-methylenedioxyethylmethamphetamine (MDMA) (12) Mescaline (13) Methadone (14) Methamphetamine (MAMP) (15) Morphine (MOR) (16) Norbuprenorphine (17) Norcocaine (NCOC) (18) Phencyclidine (19)	Oral fluid (0.12 mL)	Dilution with 80 µL of water and 40 µL of 25 mM NH3 in methanol; sonication and centrifugation	C_{18} (n.s.); n.s.	Methanol and water/methanol (80:20, v/v) (2 × 250 µL)	(5 × 250 µL)	50 mM NH3 in water/methanol (90:10, v/v) (3 × 100 µL)	5 mM formic acid in methanol (5 × 100 µL)	n.s.	LC-MS/MS	1 ng/mL (1) 0.8 ng/mL (2) 2 ng/mL (3) 0.3 ng/mL (4) 2 ng/mL (5) 10 ng/mL (6) 1 ng/mL (7) 0.3 ng/mL (8) 0.5 ng/mL (9) 1 ng/mL (10) 0.5 ng/mL (11) 0.5 ng/mL (12) 2 ng/mL (13) 0.2 ng/mL (14) 1 ng/mL (15) 2 ng/mL (16) 2 ng/mL (17) 0.5 ng/mL (18) 0.2 ng/mL (19)	3 ng/mL (1) 2 ng/mL (2) 5 ng/mL (3) 2 ng/mL (4) 5 ng/mL (5) 30 ng/mL (6) 4 ng/mL (7) 0.8 ng/mL (8) 1.5 ng/mL (9) 3 ng/mL (10) 1 ng/mL (11) 1 ng/mL (12) 5 ng/mL (13) 0.5 ng/mL (14) 3 ng/mL (15) 5 ng/mL (16) 5 ng/mL (17) 1.5 ng/mL (18) 0.6 ng/mL (19)	90% (1) 25% (2) 67% (3) 82% (4) 100% (5) 101% (6) 18% (7) 75% (8) 79% (9) 81% (10) 85% (11) 90% (12) 78% (13) 74% (14) 88% (15) 70% (16) 70% (17) 88% (18) 71% (19)	Montesano et al. (2015)

DAD (diode array detector); GC (gas chromatography); HPLC (high-performance liquid chromatography); IMS (ion mobility spectrometry); LC (liquid chromatography); LOD (limit of detection); LOQ (limit of quantification); MS (mass spectrometry); MS/MS (tandem mass spectrometry); UHPLC (ultra high-performance liquid chromatography); UV (ultraviolet).

TABLE 5.5

MEPS procedures in environmental toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Naphthalene (N), Fluorene (F), Fluoranthene (FT), Phenanthrene (PT), Pyrene (PY)	Soil (10 g)	15 min pre-heating vial at 150 °C	Aminoethyl functionalized SBA-15 (SBA-15-NH_2) (2 mg); Temperature of 0 °C t	Methanol (10 × 100 µL)	Headspace (10 × 100 µL)	n.s.	Methanol (10 × 400 µL)	Methanol (10 × 100 µL)	HPLC -UV	0.083 ng/g (N) 0.025 ng/g (F) 0.014 ng/g (FT) 0.028 ng/g (PT) 0.043 ng/g (PY)	0.250 ng/g (N) 0.075 ng/g (F) 0.042 ng/g (FT) 0.084 ng/g (PT) 0.130 ng/g (PY)	25% (N) 8% (F) 72% (FT) 65% (PT) 78% (PY)	(Serenjeh et al. 2020)
Nitrobenzene (NB), 2-Nitrotoluene (2-NT), 3-Nitrotoluene (3-NT), 4-Nitrotoluene (4-NT), 2,6-Dinitrotoluene (2,6-DNT),1,3-Dinitrobenzene (1,3-DNB), 2,4-Dinitrotoluene (2,4-DNT), 2,4,6-Trinitrotoluene (2,4,6-TNT), 1,3,5-Trinitrobenzene (1,3,5-TNB), 4-Amino-2,6-dinitrotoluene (4-Am-2,6-DNT), 2-Amino-4,6-dinitrotoluene (2-Am-4,6-DNT), 2,4,6-Trinitrophenyl-Nmethylnitramine (Tetryl)	River water (1 mL) Ground water (1 mL)	Filtered n.s.	C_{18} (4 mg); Manual.	Methanol and water (1 × 100 µL)	(10 × 50 µL)	n.s.	Methanol (1 × 30 µL)	Methanol and water (3 × 100 µL)	GC-MS	0.014 to 0.828 ng/mL (n.s.) 0.014 to 0.828 ng/mL (n.s.)	0.046 to 2.732 ng/mL (n.s.) 0.046 to 2.732 ng/mL (n.s.)	93–96% (NB) 92–98% (2-NT) 93–98% (3-NT) 93–97% (4-NT) 93–96% (2,6-DNT) 93–96% (1,3-DNB) 91–96% (2,4-DNT) 92–96% (2,4,6-TNT) 92–94% (1,3,5-TNB) 90–92% (4-Am-2,6-DNT) 91–92% (2-Am-4,6-DNT) 92–95% (Tetryl) 94–98% (NB) 93–98% (2-NT) 93–98% (3-NT) 93–97% (4-NT) 94–98% (2,6-DNT) 94–98% (1,3-DNB) 94–96% (2,4-DNT) 94–96% (2,4,6-TNT) 91–95% (1,3,5-TNB) 90–93% (4-Am-2,6-DNT) 91–92% (2-Am-4,6-DNT) 95–97% (Tetryl)	(Dhingra et al. 2018)

tripropyl phosphate (TPP), tributyl phosphate (TBP), tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro2-propyl), phosphate (TCPP), tris (1,3-dichloro-2-propyl), phosphate (TDCPP), tris(2-butoxyethyl) phosphate (TBEP), triphenyl phosphate (TPhP), (2-ethylhexyl) -diphenyl phosphate (EHDPP), tris(2-ethylhexyl) phosphate (TEHP), tricresylphosphate (TCP)	Tap water (50 mL)	Filtrated through 0.45 µm filters	silica-DVB (4 mg); eVol®	Methanol and water (2 × 250 µL)	(4 × 500 µL)	n.s.	Acetonitrile (3 × 20 µL)	Acetonitrile (7 × 250 µL)	GC-MS/MS	2.7 ng/L (TPP) 11 ng/L (TBP) 12 ng/L (TCEP) 13 ng/L (TCPP) 22 ng/L (TDCPP) 87 ng/L (TBEP) 13 ng/L (TPhP) 23 ng/L (EHDPP) 26 ng/L (TEHP) 99 ng/L (TCP)	10 ng/L (TPP) 25 ng/L (TBP) 25 ng/L (TCEP) 25 ng/L (TCPP) 50 ng/L (TDCPP) 200 ng/L (TBEP) 25 ng/L (TPhP) 50 ng/L (EHDPP) 50 ng/L (TEHP) 200 ng/L (TCP)	88–94% (TPP) 66–79% (TBP) 76–97% (TCEP) 82–97% (TCPP) 82–92% (TDCPP) 71 –81% (TBEP) 71–79% (TPhP) 65–72% (EHDPP) 63 –67% (TEHP) 73–83% (TCP)	(Naccarato et al. 2017)
	River water (50 mL)									2.9 ng/L (TPP) 10 ng/L (TBP) 12 ng/L (TCEP) 13 ng/L (TCPP) 24 ng/L (TDCPP) 95 ng/L (TBEP) 12 ng/L (TPhP) 24 ng/L (EHDPP) 28 ng/L (TEHP) 97 ng/L (TCP)		81–87% (TPP) 76–88% (TBP) 68–92% (TCEP) 67–79% (TCPP) 72–78% (TDCPP) 67 –77% (TBEP) 74–80% (TPhP) 59–68% (EHDPP) 59 –64% (TEHP) 71–81% (TCP)	
	Wastewater (50 mL)									3.0 ng/L (TPP) 12 ng/L (TBP) 12 ng/L (TCEP) 13 ng/L (TCPP) 25 ng/L (TDCPP) 101 ng/L (TBEP) 13 ng/L (TPhP) 28 ng/L (EHDPP) 28 ng/L (TEHP) 107 ng/L (TCP)		68–72% (TPP) 62–87% (TBP) 64–78% (TCEP) 61–73% (TCPP) 71–82% (TDCPP) 63 –66% (TBEP) 66–68% (TPhP) 62–71% (EHDPP) 58 –63% (TEHP) 67–78% (TCP)	

GC (gas chromatography); HPLC (high-performance liquid chromatography); LOD (limit of detection); LOQ (limit of quantification); MS (mass spectrometry); MS/MS (tandem mass spectrometry); UV (ultraviolet).

TABLE 5.6

MEPS procedures in food toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent Re-use					
Acenaphthylene (1) Acenaphthene (2) Fluorene (3) Phenanthrene (4) Anthracene (5) Fluoranthene (6) Pyrene (7) Benz[a] anthracene (8) Chrysene (9) Benzo [b]fluoranthene (10) Benzo[a]pyrene (11) Indeno[1,2, 3-cd] pyrene (12) Dibenz[ah] anthracene (13) Benzo[ghi] perylene (14)	Apple (20 g)	Skin and pulp were crushed in a blender before the extraction procedure; 25 mL ethanol; water bath and sonication at 30 °C and 35 kHz for 15 min; 1.2 mL was used for MEPS	HyperSep Retain polar-enhanced polymer (PEP) consisting of a styrene divinylben-zene copolymer (PS/DVB) modified with urea functional groups (4 mg); manual	n.s.	(6 × 200 µL)	n.s.	Methylene chloride (1 × 50 µL)	n.s.	GC-MS	0.036 µg/Kg (1) 0.048 µg/Kg (2) 0.044 µg/Kg (3) 0.044 µg/Kg (4) 0.045 µg/Kg (5) 0.031 µg/Kg (6) 0.038 µg/Kg (7) 0.059 µg/Kg (8) 0.062 µg/Kg (9) 0.073 µg/Kg (10) 0.087 µg/Kg (11) 0.130 µg/Kg (12) 0.125 µg/Kg (13) 0.124 µg/Kg (14)	0.120 µg/Kg (1) 0.160 µg/Kg (2) 0.147 µg/Kg (3) 0.146 µg/Kg (4) 0.149 µg/Kg (5) 0.135 µg/Kg (6) 0.128 µg/Kg (7) 0.195 µg/Kg (8) 0.206 µg/Kg (9) 0.242 µg/Kg (10) 0.291 µg/Kg (11) 0.433 µg/Kg (12) 0.418 µg/Kg (13) 0.414 µg/Kg (14)	17% (1) 18% (2) 25% (3) 76% (4) 31% (5) 75% (6) 46% (7) 51% (8) 43% (9) 48% (10) 56% (11) 92% (12) 106% (13) 75% (14)	(Paris et al. 2019)
Marbofloxacin (M), Ciprofloxacin (C), Enrofloxacin €	Bovine Serum (0.225 mL)	Dilution 1:2 with a saturated ammonium sulphate solution and centrifugation	C_8 (n.s.); eVol®	Acetonitrile and water (4 × 50 µL)	(5 × 50 µL)	Water (1 × 50 µL)	0.4% formic acid and acetonitrile (50:50, v/v) (1 × 50 µL)	Acetonitrile (10 × 50 µL)	UHPLC-DAD	13 ng/mL (M) 6 ng/mL (C) 10 ng/mL (E)	43 ng/mL (M) 20 ng/mL (C) 32 ng/mL (E)	83–84% (M) 83–86% (C) 80–81% (E)	(Aresta et al. 2019)
	Bovine Milk (0.225 mL)	Dilution 1:2 with a saturated ammonium sulphate solution and centrifugation								10 ng/mL (M) 10 ng/mL (C) 15 ng/mL (E)	33 ng/mL (M) 32 ng/mL (C) 48 ng/mL (E)	79–80% (M) 83–86% (C) 79–80% (E)	
	Bovine Urine (0.225 mL)	Centrifugation								12 ng/mL (M) 2 ng/mL (C) 5 ng/mL (E)	43 ng/mL (M) 7 ng/mL (C) 17 ng/mL (E)	86–86% (M) 88–89% (C) 84–85% (E)	
BDE-28BDE-47BDE-99BDE-100BDE-153BDE-154	Egg samples (50 mg of lyophilized)	4 mL hexane, 15 min ultrasound; 2 mL of concentrated sulphuric acid to the supernatant, 15 min in ultrasound; separation of the organic phase, evaporation and reconstitution with 500 µL of acetonitrile	C_{18} (n.s.); eVol®	Acetonitrile (1 × 100 µL)	(4 × 100 µL)	n.s.	Isooctane (n.s.)	Isooctane (2 × 100 µL)	GC-MS	0.42 ng/g lw	1.40 ng/g lw	87–110%	(Souza et al. 2019)

5-hydroxymethyl-2-furfural (5HMF), 2-furfural (F), 2-furyl methyl ketone (FMK), 5-methyl-2-furfural (5 MF)	Wine (0.2 mL)	n.s.	C_8 (n.s.); n.s.	Methanol and 0.1% formic acid (1 × 250 µL)	(3 × 200 µL)	0.1% formic acid (1 × 100 µL)	Methanol (1 × 200 µL)	Methanol and 0.1% formic acid (1 × 250 µL)	UHPLC-DAD	n.s.	n.s.	n.s.	(Perestrelo et al. 2017)
Carbendazim (1) Thiabendazole (2) Dimethoate (3) Carbofuran (4) Acetamiprid (5) Tricyclazole (6) Pirimicarb (7) Aldicarb (8) Imazalil (9) Dichlorvos (10) Propoxur (11) Carbofuran (12) Malaoxon (13) Carbaryl (14) Fosthiazate (15) Isoprocarb (16) Methacrifos (17) Methidathion (18) Methiocarb (19) Malathion (20) Pirimiphos methyl (21) Coumaphos (22) Chlorpyrifos methyl (23) Benfuracarb (24) Chlorpyrifos ethyl (25)	Wheat flour (200 mg)	375 µL of 10 mM acetonitrile-acetate buffer pH = 5, 60:40 v/v; sonication for 5 min; incubation at 40 °C for 5 min under constant stirring; centrifugation	HDVB (n.s.); n.s.	Methanol (3 × 100 µL) and 90:10 (v/v) water–acetonitrile solution (2 × 100 µL)	(7 × 250 µL)	Water (1 × 100 µL)	Acetonitrile (3 × 100 µL)	n.s.	UHPLC-MS/MS	3 × 10−3 mg/kg (1) 3 × 10−3 mg/kg (2) 1 × 10−3 mg/kg (3) 3 × 10−3 mg/kg (4) 3 × 10−3 mg/kg (5) 5 × 10−4 mg/kg (6) 1 × 10−3 mg/kg (7) 5 × 10−4 mg/kg (8) 2 × 10−3 mg/kg (9) 3 × 10−4 mg/kg (10) 1 × 10−3 mg/kg (11) 3 × 10−4 mg/kg (12) 1 × 10−3 mg/kg (13) 3 × 10−3 mg/kg (14) 5 × 10−3 mg/kg (15) 5 × 10−4 mg/kg (16) 5 × 10−3 mg/kg (17) 5 × 10−3 mg/kg (18) 5 × 10−4 mg/kg (19) 5 × 10−3 mg/kg (20) 5 × 10−3 mg/kg (21) 3 × 10−4 mg/kg (22) 1 × 10−3 mg/kg (23) 5 × 10−3 mg/kg (24) 1 × 10−3 mg/kg (25)	7.5 × 10−3 mg/kg (1) 7.5 × 10−3 mg/kg (2) 3 × 10−3 mg/kg (3) 7.5 × 10−3 mg/kg (4) 7.5 × 10−3 mg/kg (5) 1.5 × 10−3 mg/kg (6) 3 × 10−3 mg/kg (7) 1.5 × 10−3 mg/kg (8) 5 × 10−3 mg/kg (9) 9 × 10−4 mg/kg (10) 2.5 × 10−3 mg/kg (11) 7.5 × 10−4 mg/kg (12) 3 × 10−3 mg/kg (13) 7.5 × 10−3 mg/kg (14) 1.5 × 10−2 mg/kg (15) 1.5 × 10−3 mg/kg (16) 1.5 × 10−2 mg/kg (17) 1.5 × 10−2 mg/kg (18) 1.5 × 10−3 mg/kg (19) 1.5 × 10−2 mg/kg (20) 1.5 × 10−2 mg/kg (21) 9 × 10−4 mg/kg (22) 2.5 × 10−3 mg/kg (23) 1.5 × 10−2 mg/kg (24) 3 × 10−3 mg/kg (25)	45% (1) 44% (2) 60% (3) 47% (4) 81% (5) 70% (6) 72% (7) 68% (8) 61% (9) 71%(10) 86%(11) 79% (12) 76% (13) 82%(14) 62%(15) 64%(16) 95%(17) 85% (18) 98% (19) 87% (20) 92% (21) 97% (22) 43% (23) 57% (24) 19% (25)	(Di Ottavio et al. 2017)
Diethyl phthalate (DEP), Dipropyl phthalate (DPP), Dibutyl phthalate (DBP), Benzylbutyl phthalate (BBP), Dicyclohexyl phthalate (DCHP)	Cold drinks (1 mL)	Degasification in ultrasonic bath; dilution 20 times with methanol; filtration	C_{18} (4 mg); n.s.	Methanol and water (1 × 100 µL)	(10 × 50 µL)	Water (1 × 50 µL)	Methanol (1 × 30 µL)	Methanol and water (3 × 100 µL)	GC-MS	0.012 ng/mL (DEP) 0.008 ng/mL (DPP) 0.003 ng/mL (DBP) 0.015 ng/mL (BBP) 0.011 ng/mL (DCHP)	0.039 ng/mL (DEP) 0.026 ng/mL (DPP) 0.009 ng/mL (DBP) 0.049 ng/mL (BBP) 0.036 ng/mL (DCHP)	94–99% (DEP) 92–96% (DPP) 96–99% (DBP) 94–96% (BBP) 94–97% (DCHP)	(Kaur et al. 2016)
Ochratoxin A (OTA)	Wine (5 mL)	Dilution 1:4 and 1:2 (v/v) with 2% aqueous acetic acid	C_{18} (4mg); eVol®	Acetonitrile, methanol and 2% aqueous acetic acid/ ethanol (88:12, v/v) (1 × 50 µL)	(7 × 50 µL)	2% aqueous acetic acid and 2% aqueous acetic acid/ methanol (60/40 v/v) (1 × 20 µL)	Acetonitrile/ 2% aqueous acetic acid (90/10, v/v) (2 × 25 µL)	Acetonitrile/ 2% aqueous acetic acid (90/10, v/v) (3 × 50 µL)	HPLC-FLD	0.09 ng/mL	0.28 ng/mL	90%	(Savastano et al. 2016)

(*Continued*)

TABLE 5.6 *(Continued)*

MEPS procedures in food toxicology, analytical instrumentation, limits, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent Re-use					
Tebuthiuron (TEB), Carbofuran (CAR), Atrazine (ATR), Metribuzine (MET), Ametryn (AME), Bifenthrin (BIF)	Sugarcane juices (n.s.)	Centrifugation	C_{18} (n.s.); n.s.	Ethyl acetate (4 × 250 µL) and water (3 × 250 µL)	(9 × 250 µL)	Water (6 × 100 µL)	Ethyl acetate (8 × 30 µL)	n.s.	GC-MS	1.5 ng/mL (TEB) 0.3 ng/mL (CAR) 0.3 ng/mL (ATR) 0.8 ng/mL (MET) 0.2 ng/mL (AME) 0.3 ng/mL (BIF)	10 ng/mL (TEB) 2 ng/mL (CAR) 2 ng/mL (ATR) 3 ng/mL (MET) 2 ng/mL (AME) 2 ng/mL (BIF)	44% (TEB) 65% (CAR) 49% (ATR) 60% (MET) 45% (AME) 27% (BIF)	(Fumes et al. 2016)
PCB 28PCB 52PCB 101PCB 118PCB 138PCB 153PCB 180	Bovine Serum (0.1 mL)	Protein precipitation; centrifugation; dilution30 times	C_{18} (2 mg); manual	Methanol and water (1 × 100 µL)	(2 × 100 µL)	20% acetonitrile (1 × 100 µL)	Ethyl acetate (1 × 50 µL)	Ethyl acetate (8 × 50 µL)	GC-MS	0.06 ng/mL (PCB 28) 0.11 ng/mL (PCB 52) 0.28 ng/mL (PCB 101) 0.36 ng/mL (PCB 118) 0.53 ng/mL (PCB 138) 0.38 ng/mL (PCB 153) 0.49 ng/mL (PCB 180)	0.20 ng/mL (PCB 28) 0.37 ng/mL (PCB 52) 0.93 ng/mL (PCB 101) 1.20 ng/mL (PCB 118) 1.77 ng/mL (PCB 138) 1.27 ng/mL (PCB 153) 1.63 ng/mL (PCB 180)	87–89% (PCB 28) 79–91% (PCB 52) 66–88% (PCB 101) 60–82% (PCB 118) 69–72% (PCB 138) 61–65% (PCB 153) 66–74% (PCB 180)	(Yang et al. 2016)
5-hydroxymethyl-2-furfural (5HMF), 2-furfural (F), 2-furyl methyl ketone (FMK), 5-methyl-2-furfural (5 MF)	Wine (0.2 mL)	n.s.	C_{18} (4 mg); eVol®	Methanol and 0.1% formic acid (1 × 250 µL)	(3 × 200 µL)	0.1% formic acid (1 × 100 µL)	Methanol (1 × 200 µL)	Methanol and 0.1% formic acid (1 × 250 µL)	UHPLC-DAD	129.3 ng/L (5HMF) 25.8 ng/L (F) 4.5 ng/L (FMK) 21.3 ng/L (5 MF)	431.0 ng/L (5HMF) 86.1 ng/L (F) 14.9 ng/L (FMK) 70.8 ng/L (5 MF)	86–99% (5HMF) 84 –98% (F) 74–96% (FMK) 90–99% (5 MF)	(Perestrelo et al. 2015)

DAD (diode array detector); FLD (fluorescence detector); GC (gas chromatography); HPLC (high-performance liquid chromatography); LC (liquid chromatography); LOD (limit of detection); LOQ (limit of quantification); MS (mass spectrometry); MS/MS (tandem mass spectrometry); UHPLC (ultra high-performance liquid chromatography).

5.5 New Developments

The interest in new materials to be used in sample preparation is not new, and this aims at obtaining greater specificity and selective enrichment (da Silva and Lanças 2020). Finding the suitable sorbent to extract compounds that present different polarities (high polarity and non-polar) can be a big challenge (Mehrani et al. 2020). For this reason, different strategies have been developed to try to solve this problem, and several new solid pack materials have been reported in the last five years (da Silva and Lanças 2020; Mehrani et al. 2020).

Carbon nanomaterials are by far those for which greater interest was observed in the development of new sorbents for MEPS. This is justified by their unique physical and chemical properties, namely the large specific surface area, chemical and thermal stabilities, and excellent mechanical strength (Amiri and Ghaemi 2017). Graphene (G) is a two-dimensional carbon nanomaterial widely applied as sorbent, exhibiting a π-electron-rich structure, allowing strong hydrophobic and π-stacking interactions with many molecules (Sun et al. 2019). Nevertheless, the direct use of graphene as a sorbent is not practical since its large surface area may lead to irreversible binding caused by van der Waals interactions (Sun et al., 2019). This will generate a large backpressure during MEPS and may lead to syringe obstruction (Vasconcelos Soares Maciel et al. 2018; Sun et al. 2019). Furthermore, graphene oxide (GO), a precursor of graphene, presents many polar groups in its chemical structure and can be modified with other materials resulting in improved selectivity and better analyte recovery (Karimiyan et al. 2019; da Silva and Lanças 2020).

Ahmadi et al. (Ahmadi et al. 2018) used GO as MEPS sorbent for the extraction of local anesthetics from plasma and saliva. The authors justified the successful application of the sorbent with its high adsorption capacity for aromatic compounds (Ahmadi et al. 2018). On the other hand, Sun et al. (Sun et al. 2019) developed a sorbent consisting of GO coated with ZnO (GO–ZnO) for the extraction of carbamate pesticides from juice samples. This coating not only prevented graphene aggregation, but also provided hydrophilic surfaces for effective adsorption of water-soluble analytes (Ahmadi et al. 2018). Another way of preventing this problem with graphene was adopted by Vasconcelos et al. (Vasconcelos Soares Maciel et al. 2018), who bonded the GO onto a silica surface with its subsequent transformation to reduced graphene (G-Sil). With this sorbent, the authors improved the extraction of tetracyclines residues from milk samples (Vasconcelos Soares Maciel et al. 2018). Similar development was reported by Fumes et al. (Fumes and Lanças 2017), but using supported graphene on aminopropyl silica for the extraction of parabens from water samples. A different strategy was presented by Karimiyan et al. (Karimiyan et al. 2019), who used polyacrylonitrile/graphene oxide (PAN/GO) nanofibers, and successfully applied them for the pre-concentration of several drugs and metabolites from human plasma samples. It was also shown that ionic liquids (ILs) could be used for the extraction of chlorobenzenes (CBs), chlorophenols (CPs), and bromophenols (BPs) from water samples (Darvishnejad and Ebrahimzadeh 2020). These analytes are environmentally disrupting chemicals, and their pre-concentration was accomplished with a graphitic carbon nitride-reinforced polymer IL nanocomposite, a MEPS sorbent developed by Darvishnejad and Ebrahimzadeh (Darvishnejad and Ebrahimzadeh 2020). Recently, new composite graphitic materials have been made commercially available (CarbonX®) and are produced by coating stable substrates with graphene; these materials have been successfully applied to extract β-blockers from human plasma samples (Abuzooda et al. 2015). Further, a new type of graphitic sorbent (Carbon X-COA) was evaluated for the extraction of the local anesthetics lidocaine and ropivacain from plasma samples (Iadaresta et al. 2015).

Also widely explored, although not that novel, are MIPs. MIPs are provided, stereochemically, with specific recognition sites that are either shaped from a template molecule, such as the target analyte, or from dummy template molecules, such as analytes analogues (de Oliveira 2019). These have the advantage of a high recognition ability for the target analytes, to which the extraction becomes very selective (Meng and Wang 2019). Over the last five years many MIPs have been synthetized for MEPS application. Their synthesis commonly occurs by a complex formation between the functional monomer and template molecule (de Oliveira et al. 2019). Oliveira et al. (de Oliveira et al. 2019)

employed a new restricted-access MIP for the determination of estrone and estriol in urine samples based on a crosslinking reaction with BSA to obtain surface protein encapsulation of the MIP. In the same year, Meng et al. (Meng and Wang 2019) proposed the use of MIPs for the determination of levofloxacin from plasma samples, using deep eutectic solvents (DESs) as porogen for MIPs preparation to be applied on MEPS syringe. The DESs choice was based on its non-toxic, low cost, and inertness properties. Earlier, Soleimani et al. (Soleimani et al. 2018) reported the use of MIPs as MEPS sorbents for the pre-concentration of mandelic acid from urine samples. The same authors had previously reported MIPs' successful application to extract trans, trans-muconic acid from the same specimen (Soleimani et al. 2017). A different approach of MIPs was, however, developed by Moein et al. (Moein et al. 2015a). These authors used the dummy molecularly imprinted polymer (DMIP) method and obtained good results with its application for sarcosine extraction from both plasma and urine samples (Moein et al. 2015a).

Conducting polymers (π-conjugated polymers), such as polythiophene, polyaniline, and polypyrrole, are also considered promising sorbent materials to be used in MEPS (Florez et al. 2020; Abolghasemi et al. 2018). They present good environmental stability and nontoxicity and are easy to prepare with low cost (Florez et al. 2020). One of the most studied materials is polythiophene (PTh), gathering qualities as hydrophilic stability, redox activity, and an excellent interaction with aromatic groups (Florez et al. 2020). Florez et al. (Florez et al. 2020) reported PTh as an highly efficient sorbent for MEPS, and used it for the pre-concentration of steroids from bovine milk samples. Previously, Abolghasemi et al. (Abolghasemi et al. 2018) reported a nanostructured star-shaped polythiophene dendrimer as an highly efficient sorbent to extract clofentezine from milk and juice samples. The authors claimed that star-shaped and dendritic conductive polymers are great options due to their unique three-dimensional shape and physicochemical properties (Abolghasemi et al. 2018). In addition to the previous, the development of a nanocomposite consisting of polydopamine, silver nanoparticles, and polypyrrole has been described with great application for the microextraction of antidepressant drugs from urine samples (Bagheri et al. 2016).

Nanoclays are promising sorbent materials as well. Although their hydrophilic nature might turn them unsuitable for the extraction of organic compounds, methods such as cation-exchange reactions with alkyl ammonium, phosphonium, and/or imidazolium compounds may change this (Saraji et al. 2018). Montmorillonite (nanoclay) presents an elevated adsorption capacity, surface area, porosity, and swelling behavior (Saraji et al. 2018). Saraji et al. (Saraji et al. 2018) modified nanoclays with cetyltrimethylammonium bromide (CTAB) using a cation exchange reaction, with further modification by alkoxysilanes, and used it as MEPS sorbent to extract diazinon from water samples. More recently, a reinforced montmorillonite into polystyrene (MMT/PS) was prepared and coated onto cellulose filter paper to pre-concentrate fluoxetine from similar environmental samples (Matin et al. 2020).

Other sorbent materials with great potential due to their unique properties are metal-organic frameworks (MOFs) (Jiang et al. 2020). These consist of porous crystal material generated by the self-assembly of metallic ions (or clusters) with a bi- or multipodal organic linker (Jiang et al. 2020). Although MOFs have shown some drawbacks related to SPE applications, producing high resistance because of their sub-micron to micron size, their unique features enable them to be used in small amounts in MEPS (Jiang et al. 2020). Jiang et al. (Jiang et al. 2020) used a MOF to extract parabens from vegetable oils and obtained satisfactory adsorption capacities. Previously, Jiang et al. (Jiang et al. 2018) had already applied a MOF-MIL-101 (Cr) for semi-automated MEPS of six triazine herbicides from corn samples. Among the reported MOFs, MOF-5 is one of the most studied, and this was coated by amino-functionalized Fe_3O_4 and silica mesoporous (SBA-15) and used as MEPS sorbent to determine mandelic acid in urine samples for the first time by Rahimpoor et al. (Rahimpoor et al. 2019). More recently, the same research team successfully applied a MOF of MIL-53-NH_2 (Al) as MEPS sorbent to pre-concentrate urinary methylhippuric acids (Pirmohammadi et al. 2020).

The latest research on sorbent material applied to MEPS has been boosted by the use of natural compounds, hence called green sorbents. Rasolzadeh et al. (Rasolzadeh 2019) described the use of a biosorbent consisting of *Chlorella vulgaris*, a unicellular green microalgae, for the determination of

TABLE 5.7

New sorbents developed for MEPS, procedures applied in analytical toxicology, analytical instrumentation, limits of determination, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Clinical Toxicology													
Benzoic acid (BA), Phenylpropionic acid (PhPA), CinnamicPhenyllactic acid (PhLA), 4-hydroxybenzoic acid (p-HBA), 4-hydroxyphenylaceticacid (p-HPhAA), 4-hydroxyphenylpropionic acid (p-HPhPA), Homovanillic acid (HVA), 4-hydroxyphenyllactic acid (p-HPhLA)	Serum (80 µL)	Dilution with 70 µL of water and 2.5 µL of concentrated sulphuric acid	Hypercrosslinked polystyrene (HCLPS) (2 mg); n.s.	Acetone and water (2 × 80 µL)	(2 × 80 µL)	0.3 mM formic acid solution (1 × 80 µL)	Diethyl ether (3 × 80 µL)	n.s.	GC-MS	n.s.	0.5 µM	45% (BA) 50% (PhPA) 35% Cinnamic 70% (PhLA) 45% (p-HBA) 45% (p-HPhAA) 40% (p-HPhPA) 40% (HVA) 20% (p-HPhLA)	Pautova 2020c)
2-methylhippuric acid (2-MHA), 3-methylhippuric acid (3-MHA), 4-methylhippuric acid (4-MHA)	Urine (n.s.)	Adjusted to pH 2.0	Metal-organic frameworks (MOFs); MIL-53-NH2 (Al) (4 mg);	Methanol–water (1:1, v/v); (3 × 100 µL)	(4 × 100 µL)	Methanol–water (1:1, v/v); (1 × 150 µL)	Acetic acid–methanol 1:9 (4 × 80 µL)	n.s.	HPLC-UV	0.115 µg/mL (2-MHA) 0.005 µg/mL (3-MHA) 0.005 µg/mL (4-MHA)	0.380 µg/mL (2-MHA) 0.016 µg/mL (3-MHA) 0.016 µg/mL (4-MHA)	61–98% (2-MHA) 63–99% (3-MHA) 63–99% (4-MHA)	Pirmohammadi et al. (2020)
Estrone (E1), Estriol (E3)	Urine (0.2 mL.)	10 mL urine added to 150 µL of hydrochloric acid (1.0 M); 1 h at 65 °C, then the pH was adjusted to 10; centrifugation	Restricted molecularly imprinted polymer (RAM-MIP) (3 mg); n.s.	water (1 × 100 µL)	(1 × 200 µL)	Water (1 × 200 µL)	Methanol: acetic acid (9:1, v/v) (1 × 200 µL)	n.s.	HPLC-UV	n.s.	100 ng/mL (E1) 100 ng/mL (E3)	74–84% (E1) 67–70% (E3)	de Oliveira et al. (2019)
Mandelic acid (MA)	Urine (0.15 mL)	Centrifugation	MOF-5 @ Fe3O4-NH2 (n.s.); n.s. MOF-5 @ SBA-15 (n.s.); n.s.	n.s.	(4 × 100 µL)	Water (1 × 100 µL)	Methanol-nitric acid 8:2 (4 × 80 µL)	n.s.	HPLC-DAD	0.05 µg/mL	0.1 µg/mL	95% 90%	Rahimpoor et al. (2019)

(*Continued*)

TABLE 5.7 *(Continued)*

New sorbents developed for MEPS, procedures applied in analytical toxicology, analytical instrumentation, limits of determination, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Mandelic acid (MA)	Urine (n.s.)	Acidified and adjusted to pH 2.0	MIP (4 mg); manual	Methanol and water (3 × 100 μL)	(8 × 100 μL)	Water (1 × 100 μL)	Methanol–acetic acid (8:2, v/v) (2 × 100 μL)	Methanol–acetic acid (8:2, v/v) and water (4 × 150 μL)	HPLC-UV	n.s.	0.2 μg/mL	92%	Soleimani et al. (2018)
trans,trans-Muconic acid (tt-MA)	Urine (n.s.)	pH 2.0, centrifugation	MIP (4 mg); manual	Ethanol and water (3 × 100 μL)	(5 × 100 μL)	Water (1 × 100 μL)	Ethanol-acetic acid (80:20, v/v) (2 × 100 μL)	Ethanol–acetic acid (8:2, v/v) and water (3 × 150 μL)	HPLC-UV	0.015 μg/mL	0.05 μg/mL	90–92%	Soleimani et al. (2017)
Sarcosine	Plasma and urine (0.1 mL)	n.s.	Dummy molecularly imprinted polymer (DMIP) (2 mg); automated	Water (1 × 100 μL)	(200 μL n.s.)	Water/HCl (0.1 M) (80:20) (1 × 100 μL)	Acetonitrile/ Water (80:20) (1 × 100 μL)	Water/HCl (0.1 M) (80:20) (200 μL n.s.)	LC-MS/MS	1 ng/mL	3 ng/mL	87–89%	Moein et al. (2015a)
Drug monitoring													
Chlorpromazine (CLOR), Clozapine (CLOZ), Olanzapine (OLA), Quetiapine (QUET)	Plasma (0.1 mL)	Dilution with 400 μL of borate buffer solution (10 mM, pH 9)	Restricted access carbon nanotube (RACNT) (n.s.); manual	Acetonitrile and water (2 × 100 μL)	(3 × 100 μL)	Water (1 × 150 μL)	Acetonitrile (2 × 100 μL)	Acetonitrile and water (2 × 100 μL)	UHPLC-MS/MS	n.s.	10 ng/mL (CLOR) 10 ng/mL (CLOZ) 10 ng/mL (OLA) 10 ng/mL (QUET)	34% (CLOR) 69% (CLOZ) 58% (OLA) 28% (QUET)	Cruz et al. (2020)
o-Toluidine (TOL), Prilocaine (PRL), 2,6-Xylidine (XYL), Lidocaine (LID)	Plasma (0.2 mL)	Dilution (1:5) (n.s.)	Polyacrylonitrile/ Graphene Oxide (PAN/GO)	1% formic acid in methanol (5 × 100 μL) and water (3 × 100 μL)	(5 × 100 μL)	5% methanol in water (2 × 100 μL)	1% formic acid in methanol (3 × 100 μL)	1% formic acid in methanol (5 × 100 μL) and 1% formic acid in water (3 × 100 μL)	LC-MS/MS	1.25 nmol/L (TOL) 0.50 nmol/L (PRL) 2.50 nmol/L (XYL) 0.25 nmol/L (LID)	10 nmol/L (TOL) 2 nmol/L (PRL) 10 nmol/L (XYL) 2 nmol/L (LID)	69% (TOL) 96% (PRL) n.s. (XYL) 93% (LID)	Karimiyan et al. (2019)
Levofloxacin	Plasma (0.1 mL)	Addition of 300 μL of methanol; vortexed; centrifugation; supernatant was filtered	Deep eutectic solvents - molecularly imprinted polymers (DESs-MIPs) (4 mg); n.s.	Methanol and water (1 × 1 mL)	(20 × 400 μL)	Water-methanol (50: 50, v/v) (1 × 200 μL)	Acetonitrile/ ammonia (95/5, v/v) (1 × 400 μL)	n.s.	UHPLC-DAD	0.012 μg/mL	0.04 μg/mL	95–100%	Meng and Wang (2019)
Nitrofurantoin (NFT)	Urine (0.3 mL)	Filtration; pH 8	Dried *C. vulgaris* biomass (4 mg); n.s.	Water (2 × 100 μL)	(14 × 300 μL)	Water (1 × 100 μL)	30% acetone: water (6 × 150 μL)	30% acetone: water (3 × 100 μL)	Spectrophotometry	0.039 μg/mL	0.5 μg/mL	98%	Rasolzadeh et al. (2019)

Lidocaine (LID), Prilocaine (PRL), Ropivacaine (ROP)	Saliva (0.2 mL) Plasma (0.2 mL)	Dilution with 200 µL water; protein precipitation; centrifugation	Reduced graphene oxide (RGO) (2 mg); n.s.	Methanol and water (1 × 200µL)	(6 × 200 µL)	Water: methanol (95:5, %v/v) (1 × 200 µL)	Methanol: formic acid (90:10, %v/v) (2 × 100 µL)	n.s.	LC-MS/MS	n.s.	4 nM (LID) 4 nM (PRL) 2 nM (ROP)	97–106% (LID) 95–106% (PRL) 99–106% (ROP) 99–105% (LID) 97–107% (PRL) 98–100% (ROP)	Ahmadi et al. (2018)
Dexamethasone (DEX), Carbamazepine (CBZ), Naproxen (NPX)	Urine (1 mL)	1 mL urine diluted to 5 mL with water; adjusted at pH 7.5	Imprinted interpenetrating polymer network (IPN) (2 mg); n.s.	Methanol and water (3 mL)	(20 × 1 mL)	n.s.	Methanol (230 µL)	Methanol (4 × 250µL) and water (6 × 250 µL)	HPLC-UV	1.3 ng/mL (DEX) 1.5 ng/mL (CBZ) 1.4 ng/mL (NPX)	4.2 ng/mL (DEX) 5.0 ng/mL (CBZ) 4.7 ng/mL (NPX)	83% (DEX) 91% (CBZ) 89% (NPX)	Asgari et al. (2017)
Amitriptyline (AMP), Imipramine (IMP), Citalopram (CIT)	Urine (1 mL)	Dilution with 4 mL water; pH 5	Nanocomposite consisting of polydopamine, silver nanoparticles and polypyrrole (PDA-Ag-PPy) (2 mg); n.s.	Methanol, acetonitrile and water (1 mL)	(25 × 1 mL)	Water (1 × 1mL)	Acetonitrile (3 × 100 µL)	Acetonitrile and water (5 × 250 µL)	GC-MS	0.03 ng/mL (AMP) 0.05 ng/mL (IMP) 0.05 ng/mL (CIT)	0.10 ng/mL (AMP) 0.20 ng/mL (IMP) 0.15 ng/mL (CIT)	91–104% (AMP) 88–96% (IMP) 88 –94% (CIT)	Bagheri et al. (2016)
Haloperidol (HAL), Olanzapine (OLZ), Clonazepam (CLN), Mirtazapine (MTZ), Paroxetine (PXT), Citalopram (CIT), Sertraline (SRT), Chlorpromazine (CHP) Imipramine (IMP), Clomipramine (CLO), Quetiapine (QTP), Diazepam (DZP), Fluoxetine (FLX), Clozapine (CLZ), Carbamazepine (CBZ), Lamotrigine (LMT)	Plasma (0.2 mL)	Vortexed, centrifugation, the supernatant was diluted with 300 µL of ammonium acetate (5 mM)	Hybrid silica monolith (n.s.); n.s.	Methanol/ acetonitrile mixture (50:50 v/v) and water (4 × 200 µL)	(4 × 100 µL)	Water (1 × 150 µL)	Methanol/ acetonitrile (50:50 v/v) (1 × 100 µL)	Methanol/ acetonitrile mixture (50:50 v/v) and water (4 × 200 µL)	LC-MS/MS	n.s.	0.05 ng/mL (HAL) 0.05 ng/mL (OLZ) 0.10 ng/mL (CLN) 0.05 ng/mL (MTZ) 0.05 ng/mL (PXT) 1 ng/mL (CIT) 0.05 ng/mL (SRT) 0.10 ng/mL (CHP) 0.05 ng/mL (IMP) 0.10 ng/mL (CLO) 0.05 ng/mL (QTP) 0.05 ng/mL (DZP) 0.05 ng/mL (FLX) 0.05 ng/mL (CLZ) 0.10 ng/mL (CBZ) 1 ng/mL (LMT)	n.s.	de Souza et al. (2015)
Metoprolol (MET), Acebutolol (ACE)	Plasma (0.5 mL)	Centrifugation; dilution by water 4 times	New graphitic material (Carbon-XCOS) (2 mg); n.s.	n.s.	n.s.	Water (1 × 250 µL)	0.1% formic acid in methanol (n.s.)	n.s.	LC-MS/MS	n.s.	10 nM	80–90% (all)	Abuzooda et al. (2015)
Lidocaine (LID), Ropivacaine (ROP)	Plasma (0.2 mL)	Dilution with 0.1% formic acid in water (0.8 mL); centrifugation	Graphitized carbon (CarbonX®COA) (2 mg); n.s.	0.1% formic acid in acetonitrile and water (5 × 250 µL)	(4 × 250 µL)	Water (2 × 200 µL)	0.1% formic acid in acetonitrile (1 × 200 µL)	0.1% formic acid in acetonitrile and water (5 × 250 µL)	LC-MS/MS	1 nM	5 nM	79–82% (all)	Iadaresta et al. (2015)

(*Continued*)

TABLE 5.7 ***(Continued)***

New sorbents developed for MEPS, procedures applied in analytical toxicology, analytical instrumentation, limits of determination, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Environmental toxicology													
Naphthalene (Naph), Anthracene (A), Acenaphthene (Ace), Phenanthrene (Phe), 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA)	Environmental, farm, and industrial water samples (n.s.)	n.s.	Rosin/PAN and aloin/PAN electrospun nanofibers (n.s.); n.s.	2-propanol:methanol (50:50) and water (5 mL)	MEPS Hyphenation 12 adsorption cycles (12 min for 20 mL of sample solution)	Water (2 mL)	3 min for 600 µL of 2-propanol: methanol (50:50)	n.s	GC-FID	0.1–0.3 ng/mL (Naph) 0.1–0.3 ng/mL (A), 0.1–0.3 ng/mL (Ace), 0.1–0.3 ng/mL (Phe) 0.3–0.5 ng/mL (2,4-D) 0.3–0.5 ng/mL (MCPA)	0.33–0.99 ng/mL (Naph) 0.33–0.99 ng/mL (A), 0.33–0.99 ng/mL (Ace), 0.33–0.99 ng/mL (Phe) 0.99–1.65 ng/mL (2,4-D) 0.99–1.65 ng/mL (MCPA)	85–96% (Naph) 85–96% (A), 85–96% (Ace), 85 –96% (Phe) 86–98% (2,4-D) 86–98% (MCPA)	Mehrani et al. (2020)
4-Nitrophenol (1) 2-Bromophenol (2) 2-Chlorophenol (3) 1,4-Dichlorobenzen (4) Pentachlorophenol (5) 1,2,3-Trichlorobenzen (6)	Tap water, river water, well water and a sample of cave water (10 mL)	Filtration	Graphitic carbon nitride/polymer ionic liquid connected to halloysite nanotubes (g-C3N4-IL@HNT) (10 mg); n.s.	n.s.	(10 × 1 mL)	Water (1 × 1 mL)	Acetonitrile (n.s.)	Methanol (2 × 2 mL) and water (2 × 5 mL)	HPLC-UV	1 ng/mL (1) 1 ng/mL (2) 0.5 ng/mL (3) 0.5 ng/mL (4) 0.5 ng/mL (5) 0.5 ng/mL (6)	4 ng/mL (1) 4 ng/mL (2) 2 ng/mL (3) 2 ng/mL (4) 2 ng/mL (5) 2 ng/mL (6)	82–97% (1) 82–99% (2) 77–104% (3) 81–94% (4) 95–101% (5) 76–98% (6)	Darvishnejad and Ebrahimzadeh (2020)
La3+Tb3+	Sea water, Power plant water, Industrial wastewater and Radiological wastewater (50 mL)	Filtration	Gelatin/ sodium triphosphate hydrogel nanofiber mat (GT/STP HNFM) (12 mg)	1M nitric acid, methanol and water (1 × 2 mL)	50 mL (n.s.)	n.s.	1M nitric acid (1 × 1 mL)	n.s.	ICP-OES	0.1 ng/mL (La3+) 0.2 ng/mL (Tb3+)	0.3 ng/mL (La3+) 0.6 ng/mL (Tb3+)	85–102% (La3+) 85–100% (Tb3+)	Moradi et al. (2020)
Fluoxetine	Wastewater, river and dam water samples (10 mL)	n.s.	montmorillonite-polystyrene nanocomposite coated on cellulosic paper (MMT/PS/Cell)	Methanol and water (n.s.)	(5 × n.s.)	n.s.	Methanol (25 × 300 µL)	Methanol and water (20 × 300 µL)	Fluorescence spectroscopy (FL)	2 ng/mL	7 ng/mL	76–10/%	Matin et al. (2020)
2-Methyl-4-chlorophenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D)	Farm water samples (2 mL)	n.s.	Bifunctional periodic mesoporous organosilica with imidazolium framework (BFPMO-IL) (4 mg)	Methanol and water pH2 (1 × 300 µL)	(5 × 2 mL)	Water (3 × 50 µL)	Methanol (1 × 150 µL)	Methanol and water (3 × 300 µL)	GC-FID	0.1 ng/mL (MCPA) 0.5 ng/mL (2,4-D)	0.5 ng/mL (MCPA) 1.2 ng/mL (2,4-D)	62% (MCPA) 67% (2,4-D)	Mousavi et al. (2019)

Diazinon (DZN)	Agricultural wastewater, river water and well water (2 mL)	n.s.	Montmorillonite (nanoclay) modified by cetyltrimethylammonium bromide (CTAB) (2 mg); n.s.	Methanol and water (1 × 200 µL)	(10 × 200 µL)	Water (1 × 200 µL)	Methanol (7 × 50 µL)	Water and metanol (7 × 200 µL)	CD-IMS	n.s.	0.2 ng/mL	95–106%	Saraji et al. (2018)
Chlorpyrifos (CLP), Fenthion (FEN), Fenitrothion (FET), Ethion (ETN), Edifenphos (EDI), Phosalone (PSL)	River water, dam water and tap water samples (1 mL)	n.s.	Graphene oxide reinforced polyamide nanocomposite (GO/PA NC) (6 sorbent layers, n.s.); manual	n.s.	(5 × 1 mL)	n.s.	Methanol (25 × 150 µL)	n.s.	GC-FID	0.3 ng/mL (CLP) 0.3 ng/mL (FEN) 1 ng/mL (FET) 0.2 ng/mL (ETN) 0.3 ng/mL (EDI) 0.3 ng/mL (PSL)	1 ng/mL (CLP) 1 ng/mL (FEN) 3 ng/mL (FET) 1 ng/mL (ETN) 1 ng/mL (EDI) 1 ng/mL (PSL)	96–113% (CLP) 88–111% (FEN) 78–99% (FET) 90–113% (ETN) 85–98% (EDI) 102–105% (PSL)	Ayazi et al. (2018)
Diazinon (DZN), Malathion (MLT), Ethion (ETN)	Water samples (10 mL)	n.s.	Natural nanoperlite (n.s.); n.s.	Methanol/acetone (1:1) and water (2 or 3 × 2 mL)	(10 × 1 mL)	Water (1 × 1 mL)	Dichloromethane (1 × 100 µL)	n.s.	GC-MS	0.07 ng/mL (DZN) 0.38 ng/mL (MLT) 0.13 ng/mL (ETI)	0.2 ng/mL (DZN) 0.2 ng/mL (MLT) 0.4 ng/mL (ETI)	85–96% (DZN) 81–92% (MLT) 94–103% (ETI)	Taghani et al. (2018)
Methyl paraben (MeP), Ethyl paraben (EtP), Propylparaben (PrP), Butyl paraben (BuP), Benzyl paraben (BeP)	Lake water, domestic wastewater, a swimming pool and tapwater	Centrifugation	Graphene supported on aminopropyl silica (Si-G) (7 mg); (n.s.)	n.s.	(6 × 600 µL)	n.s.	Acetonitrile (10 × 100 µL)	Acetonitrile (4 × 500 µL) and water (4 × 1 mL)	LC-MS/MS	0.06 ng/mL (MeP) 0.06 ng/mL (EtP), 0.06 ng/mL (PrP), 0.06 ng/mL (BuP) 0.09 ng/mL (BeP)	0.2 ng/mL (MeP) 0.2 ng/mL (EtP), 0.2 ng/mL (PrP), 0.2 ng/mL (BuP) 0.3 ng/mL (BeP)	n.s.	Fumes and Lanças (2017)
Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Di-isobutyl phthalate (DIBP), Di-n-butyl phthalate (DnBP), Di-2-ethylhexyl phthalate (DEHP)	Tap water, river water and mineral water (8 mL)	n.s.	Hydroxyapatite [HAP] (2 mg); manual	Methanol and water (1 × 0.5 mL)	8 mL (n.s.)	Water (1 mL)	Dichloromethane (1 × 60 µL)	Methanol (1 × 200 µL) and water (1 mL)	GC-FID	0.02 ng/mL (DMP) 0.05 ng/mL (DEP) 0.04 ng/mL (DIBP) 0.05 ng/mL (DnBP) 0.1 ng/mL (DEHP)	0.07 ng/mL (DMP) 0.15 ng/mL (DEP) 0.14 ng/mL (DIBP) 0.1 ng/mL (DnBP) 0.25 ng/mL (DEHP)	88–99% (DMP) 88–98% (DEP) 87–99% (DIBP) 88–99% (DnBP) 86–97% (DEHP)	Amiri et al. (2017)
Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Di-isobutyl phthalate (DIBP), Di-n-butyl phthalate (DnBP), Di-2-ethylhexyl phthalate (DEHP)	Tap water, river water and mineral water (10 mL)	n.s.	3D carbon nanotube/carbon nanofiber–graphene nanostructures (CNT/CNF–G) (2 mg); manual	Methanol and water (1 × 1 mL)	(70 × 1 mL)	Water (1 × 1 mL)	Methanol (0.75 mL)	Methanol (3× 100 µL) and water (1 mL)	GC-FID	0.006 ng/mL (DMP) 0.01 ng/mL (DEP) 0.007 ng/mL (DIBP) 0.001 ng/mL (DnBP) 0.005 ng/mL (DEHP)	0.02 ng/mL (DMP) 0.03 ng/mL (DEP) 0.02 ng/mL (DIBP) 0.003 ng/mL (DnBP) 0.015 ng/mL (DEHP)	93–97% (DMP) 90–99% (DEP) 92 –97% (DIBP) 92–98% (DnBP) 94–99% (DEHP)	Amiri and Ghaemi (2017)
Food toxicology													
Progesterone (PGN), Prednisolone (PRE), Estradiol (ESD)	Bovine milk (0.25 mL)	5 mL of bovine milk added to 10 mL of acetonitrile; centrifugation; dilution with 25 mL water	Polythiophene (PTh) (4 mg); n.s.	Water (1 × 250 µL)	(2 × 250 µL)	Water (1 × 100 µL)	Methanol: formic acid (7: 3, v/v) (1 × 200 µL)	n.s.	HPLC-DAD	n.s.	16 ng/mL (PGN) 16 ng/mL (PRE) 16 ng/mL (ESD)	99% (PGN) 88% (PRE) 97% (ESD)	Florez et al. (2020)

(Continued)

TABLE 5.7 *(Continued)*

New sorbents developed for MEPS, procedures applied in analytical toxicology, analytical instrumentation, limits of determination, and recoveries (2015–2020)

Analytes	Sample (Volume/ Weight)	Sample Preparation	MEPS Sorbent	MEPS Steps					Analytical Instrumentation	LOD	LOQ	Recoveries	Ref
				Conditioning	Load	Wash	Elution	Sorbent re-use					
Methyl 4-hydroxybenzoate (MP), Ethyl 4-hydroxybenzoate (EP), Propyl 4-hydroxybenzoate (PP)	Vegetable oil samples (1 mL)	Dilution with 5 mL of n-hexane	Metal-organic frameworks (MOFs); HKUST-1 (20 mg); semi-automated	n.s.	Sonication in the syringe for 11 min at room temperature	Hexane (1 mL)	Methanol (1.1 mL)	n.s.	LC-MS/MS	n.s.	1.97 ng/mL (MP) 3.03 ng/mL (EP) 4.57 ng/mL (PP)	82–112% (MP) 74–102% (EP) 87–120% (PP)	Jiang et al. (2020)
Metolcarb (MCB), Carbaryl (CBL), Isoprocarb (ICB), Diethofencarb (DCB)	Juice samples (5 mL)	Centrifugation; filtration; NaCl (0.75 g)	Reduced graphene oxide coated with ZnO (RGO–ZnO) (5 mg); manual	Acetonitrile (2 mL) and water (4 mL)	(5 × 1 mL)	Acetonitrile/ water (5:95, v/v); (1 × 1 mL)	Acetonitrile (6 × 200 µL)	n.s.	HPLC-UV	0.45 ng/mL (MCB) 0.23 ng/mL (CBL) 1.21 ng/mL (ICB) 1.14 ng/mL (DCB)	1.45 ng/mL (MCB) 0.75 ng/mL (CBL) 3.52 ng/mL (ICB) 3.46 ng/mL (DCB)	97% (MCB) 95–99% (CBL) 93–102% (ICB) 98–102% (DCB)	Sun et al. (2019)
Prometryn (P), Atrazine (A), Terbumeton (T), Secbumeton (S)	Maize powder (1 g)	5 mL of n-hexane and sonication; centrifugation	Au/LDH nanohydrobrids (60 mg); n.s.	n.s.	4 mL of supernatant, addition 60 mg of Au/LDH nanohybrids; ultrasound and the mixture was drawn into a 5 mL syringe	n-hexane (800 µL)	Ethyl acetate (400 µL)	n.s.	HPLC-DAD	0.035 ng/g (P) 0.056 ng/g (A) 0.085 ng/g (T) 0.108 ng/g (S)	0.12 ng/g (P) 0.19 ng/g (A) 0.28 ng/g (T) 0.36 ng/g (S)	92–99% (P) 92–100% (A) 94–100% (T) 94–101% (S)	Li et al. (2018)
Clofentezine	Milk and juice samples (0.4 mL)	Adjusted pH 4	Nanostructured star-shaped polythiophene dendrimer (S-PTh dendrimer) (3 mg); manual	Methanol, acetone and acetonitrile (n.s.) and 5 mL water	n.s.	Water (2 × 100 µL)	Methanol (4 × 100 µL)	Acetonitrile (6 × 100 µL)	HPLC-DAD-UV	2 ng/mL	n.s.	n.s.	Abolghasemi et al. (2018)
Chlortetracycline (CTC), Tetracycline (TTC), Oxytetracycline (OXT), Doxycycline (DOX)	Milk samples (5 mL)	5 mL of milk sample and 2 mL of trifluoracetic acid 20% (v/v); 20 mL of a McIlvaine buffer solution/ EDTA and centrifugation	Graphene particles supported on silica (G-Sil) (7 mg); n.s.	Methanol (8 × 0.5 mL) and McIlvaine/EDTA buffer (2 × 1 mL)	(6 × 1 mL)	McIlvaine/ EDTA buffer (2 × 1 mL)	Methanol (9 × 100 µL)	n.s.	LC-MS/MS	0.03 ng/mL (CTC) 0.13 ng/mL (TTC) 0.11 ng/mL (OXT) 0.21 ng/mL (DOX)	0.05 ng/mL (CTC) 0.42 ng/mL (TTC) 0.95 ng/mL (OXT) 0.69 ng/mL (DOX)	n.s.	Vasconcelos Soares Maciel et al. (2018)

Triazine herbicides(n.s.)	Corn samples (1 g)	5 mL of Acetonitrile; sonicated; centrifuged; 4 mL supernatant was used	Metal-organic frameworks (MOFs); MIL-101(Cr) (9 mg); semi-automated	n.s.	Supernatant was transferred to tube containing sorbent under sonication at room temperature for 9 min	n-hexane (1 mL)	Acetonitrile (2 mL)	n.s.	LC-MS/MS	0.01–0.12 ng/g (n.s.)	0.05–0.35 ng/g (n.s.)	73–107% (n.s.)	Jiang et al. (2018)
Norfloxacin (NOR), Ofloxacin (OFL), Fleroxacin (FLE), Ciprofloxacin (CIP), Danofloxacin (DAN), Lomefloxacin (LOM), Enrofloxacin (ENR), Orbifloxacin (ORB)	Milk samples (0.01 g)	Protein precipitation; centrifugation; interlayer was used	Immunoaffinity; glass beads bound with QN monoclonal antibodies (0.2 g)	n.s.	Interlayer was passed through	Water (3 × 1 mL) and	Methanol-PBS solution (9:1, v/v) (1 × 600 µL)	n.s.	HPLC-FLD	0.1 ng/g (NOR) 0.1 ng/g (OFL) 0.1 ng/g (FLE) 0.1 ng/g (CIP) 0.05 ng/g (DAN) 0.1 ng/g (LOM) 0.1 ng/g (ENR) 0.1 ng/g (ORB)	0.3 ng/g (NOR) 0.3 ng/g (OFL) 0.3 ng/g (FLE) 0.3 ng/g (CIP) 0.15 ng/g (DAN) 0.3 ng/g (LOM) 0.3 ng/g (ENR) 0.3 ng/g (ORB)	63% (NOR) 73% (OFL) 91% (FLE) 58% (CIP) 86% (DAN) 63% (LOM) 67% (ENR) 54% (ORB)	Zhang et al. (2017)

CD-IMS (Corona discharge ion mobility spectrometry); DAD (diode array detector); FID (flame ionization detector); FLD (fluorescence detector); GC (gas chromatography); HPLC (high-performance liquid chromatography); ICP-OES (inductively plasma optical emission spectrometry); LC (liquid chromatography); LOD (limit of detection); LOQ (limit of quantification); MS (mass spectrometry); MS/MS (tandem mass spectrometry); UHPLC (ultra high-performance liquid chromatography); UV (ultraviolet).

nitrofurantoin in urine samples. Not fully green, but still pertinent, was the work published by Mehrani et al. (Mehrani et al. 2020) in which natural compounds extracted from aloe vera plants and gum of pine trees were used to synthesize the sorbents. These compounds were aloin (polar compound) and rosin (non-polar compound). After their coupling with polyacrylonitrile (PAN), aloin and rosin formed aloin/PAN and rosin/PAN nanofibers used as sorbents to pre-concentrate polycyclic aromatic hydrocarbons and phenoxyacetic acid herbicides from water samples (Mehrani et al. 2020).

Over the last five years, MEPS applicability has been greatly explored in all fields of analytical toxicology (Table 5.7). These new sorbent developments have represented the majority of the published articles regarding MEPS, justifying the importance of the solid material packed in the syringe to improve method selectivity.

5.6 Perspectives and Future Challenges

MEPS emerged in accordance with green chemistry principles and aimed to improve the sustainable development for chemists in both the research and routine analysis fields. Although MEPS is still limited to research, the last five years have been very productive, with a large number of new sorbents developed and new approaches tested, but their application for routine analysis at an industrial scale remains scarce. Therefore, it is urgent to implement techniques such as MEPS that provide great enrichment factors, are rapid and automated, minimize sample volumes required, and reduce toxic wastes.

The commercially available sorbents do not seem to cover all necessities, hence the constant look for new solid materials. Nevertheless, new solid materials developed and reported are restricted to few classes of target analytes and are not suitable for a multi method approach. Interesting enough is all the new research dedicated to green sorbents, namely microalgae and vegetable materials. More studies should be performed in this field, including sorbent stability and broader application. Ion liquids continue being explored in this matter and appear as a great option for future sorbent developments, revealing low toxicity and wide applicability.

Finally, MEPS coupling with more recent MS technology should be considered. Over the last five years no linear ion trap, orbitrap, and quadrupole time-of-flight mass analyzers were described with MEPS. The coupling with the mentioned mass analyzers would offer the possibility to surpass the limitations of multi-target screening.

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Chapter 2 - Global aims

Global aims

Substance use and/or abuse is still a major problem worldwide. Both licit (e.g., alcohol) and illicit drugs (e.g., cocaine, opiates and prescribed/misused drugs) are associated with high morbidity and mortality. This is no different in Portugal, and recent data show that drug related deaths are mainly associated with cocaine use. Opiates and methadone also account for a large proportion of drug related deaths in this country. Nevertheless, morbidity and mortality are not the only problems resulting from drug use. It also leads to psychological, economic and sociological problems both in the individual and in the society.

Testing for drug abuse has therefore become an important tool in combating the above problem. Testing in the workplace and in roadside traffic controls has been considered very useful. Recently, this area has improved with the development of new drug testing methods for the determination of drugs of abuse in alternative biological samples, highlighting hair as one of the most important.

Hair analysis is recognised for providing evidence of the use, reduction, abstinence, or lack of use of drugs of abuse. It also allows estimating the extent and timing of drug exposure. For this reason, hair analysis results are now increasingly used as evidence in court and are generating considerable interest in the field of forensic toxicology. Hair samples offer a wider window of detection, allow differentiating the source of some drug exposures, and are more difficult to tamper with when compared to a urine test.

As with other biological samples, hair preparation is a critical step in isolating target analytes from the complex matrix. The new trends in sample preparation techniques are towards miniaturisation, automation and online coupling, low cost, high efficiency and reduction in solvent consumption. These have been widely used for common samples such as blood/plasma/serum and urine, but for hair samples these miniaturised approaches are still little explored. Among the latter, microextraction by packed sorbent (MEPS), has proven successful in many areas of toxicology (e.g., clinical, forensic, food, environmental). MEPS is a simple, fast and robust sample preparation technique and is in line with new trends, but, until the start of the present work, only one application for hair samples has been reported.

In this sense, the first goal of this work was to evaluate, in the form of a critical review, the status and recent advances in the use of miniaturised approaches for sample clean-up and drug pre-concentration in hair analysis. In addition, our aim was to discuss the advantages and disadvantages of the MEPS technique and the factors affecting its performance. We aimed at compiling the current applications of MEPS, focusing particularly on those in the field of clinical and forensic toxicology. Finally, our aim was to prove the suitability of MEPS for hair samples clean-up, through three practical works in which MEPS was used for sample clean-up in the determination of some of the drugs most commonly involved in drug-related deaths.

Chapter 3 - Results

Paper III - Determination of selected opiates in hair samples using microextraction by packed sorbent: a new approach for sample clean-up

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Determination of Selected Opiates in Hair Samples Using Microextraction by Packed Sorbent: A New Approach for Sample Clean-up

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Abstract

In this article the development and validation of an analytical method using microextraction by packed sorbent (MEPS) to determine tramadol (TRM), codeine (COD), morphine (MOR), 6-acetylcodeine (6-AC), 6-monoacetylmorphine (6-MAM) and fentanyl (FNT) in hair samples by gas chromatography coupled to tandem mass spectrometry (GC–MS-MS) is presented. The MEPS used a mixed mode sorbent, and the steps for sample cleanup were conditioning (three cycles of 250 μL of methanol and three cycles of 250 μL formic acid 2%); sample load (15 cycles of 150 μL); wash (150 μL of 3.36% formic acid); and elution (eight cycles of 100 μL of ammonium hydroxide 2.36% (in methanol)). Linearity was obtained from the lower limit of quantitation (LLOQ) up to 5 ng/mg, with all target compounds revealing determination coefficients >0.99. The LLOQs achieved were 0.01 ng/mg for TRM, COD and 6-AC, and 0.025 ng/mg for MOR, 6-MAM and FNT. The recoveries ranged from 74 to 90% (TRM), 51 to 59% (COD), 22 to 36% (MOR), 69 to 99% (6-AC), 53 to 61% (6-MAM) and 75 to 86% (FNT). Precision and accuracy revealed coefficients of variation typically below 15% and relative errors within a ±15% interval, respectively. This new approach has proven to be an excellent alternative to classic procedures, reducing the volumes of organic solvents required.

Introduction

Nowadays, the prevalence of both natural and synthetic opioids use and abuse is still considered a social problem (1, 2). These drugs have efficient analgesic activity but can also be addictive (3). In Europe, it was last estimated that opioid users are ~1.3 million (1, 4), and these figures continue to represent a serious public health issue, with the associated health and social costs (5).

Hair has been demonstrated as a fundamental biological specimen for drug testing besides blood/plasma and urine (6), since it is a strong and stable tissue, which is less affected by adulterants or short-term abstinence (7). In addition, its wide window of detection is pointed as one of the biggest advantages when using this sample (7).

The determination of opioids in hair samples has been widely reported with great applications in forensic toxicology and drug abuse studies (6). The progress in separation techniques, detection methods, as well as the improvement in the extraction and cleanup procedures applied to hair samples allowed the determination of opioids at the levels of pico-mole/mg (6). In the late 90s, the analytical method most frequently used to determine these drugs in human hair was gas chromatography coupled to mass spectrometry (GC–MS) (8–15). The application of tandem mass spectrometry coupled to a gas chromatographer (GC–MS-MS) was also described during this period, improving sensitivity (16–18). Both analytical methods have also, in the following years, been employed to determine opioids in

hair samples (5, 19–46), with literature presenting great accuracy and sensitivity in the obtained results. More recently, the necessity of a multi-drug analytical method development, in which GC-unstable compounds need to be determined, justified the increase in the applications of liquid chromatography–tandem mass spectrometry (LC–MS-MS) in hair testing procedures (3, 47–64). Other approaches use high resolution mass spectrometry (HRMS), such as time of flight-mass spectrometry (TOF-MS) (65–71), which allowed the improvement of accurate mass measurements.

Nevertheless, the progress in hair analysis is also related to advances in sample preparation techniques (34). Solid-phase extraction (SPE) is the traditional and most commonly used method for sample cleanup after extraction (5, 26–28, 30–33, 42–47, 53, 54, 56, 58, 65, 72), overall resulting in great recoveries for opioid drugs. Also, liquid–liquid extraction (LLE) has proven to be an efficient option regarding opioid pre-concentration and extraction from hair samples (29, 48, 49, 51, 55, 73). However, the increasing search for techniques that are simple, inexpensive, with reduced organic solvent requirements, associated to good recoveries and adequate selectivity, has led to the application of miniaturized procedures in this biological specimen. Regarding opiates, the use of headspace solid-phase microextraction (HS-SPME) (19, 34, 36) and surfactant enhanced liquid-phase microextraction (SE-LPME) (74) have been described.

Microextraction by packed sorbent (MEPS) is a miniaturization of the conventional SPE technique (75). The ability to be totally automated, the need for lower solvent volumes, the ease and low cost have made this technique an excellent choice to extract an array of drugs from different matrices (76–80). Despite all the proven potential, up to date few applications of MEPS have been reported for hair samples. To the best of our knowledge, there is only one application of MEPS to hair specimens, which was described by Miyaguchi *et al.* (81), for the determination of amphetamines. Applications of MEPS to a wide range of specimens have been published recently, namely in blood/plasma or serum (82–84), oral fluid (52, 85–87) and urine (88, 89), and proven to be successful in toxicology.

This paper describes the development and optimization of a method using MEPS-GC–MS-MS for sample clean-up in the determination of tramadol, codeine, morphine, 6-monoacetylmorphine, 6-acetylcodeine and fentanyl in hair samples. This is the first time that a MEPS procedure is used for sample clean-up for opiates in hair testing scenarios.

Materials and Methods

Reagents and standards

The analytical standards of tramadol (TRM), codeine (COD), 6-acetylcodeine (6-AC), morphine (MOR), 6-monoacetylmorphine (6-MAM) and fentanyl (FNT), as well as the internal standards (ISs) codeine-d3 (COD-d3), morphine-d3 (MOR-d3) and 6-acetylmorphine-d3 (6-MAM-d3) were all obtained from Sigma-Aldrich (Lisbon, Portugal). Methanol (Merck Co, Darmstadt, Germany), isopropanol (Fischer chemical, Loughborough, UK) and acetonitrile (Prolabo, Lisbon, Portugal) were all of analytical grade. Deionized (DI) water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA). Formic acid (Panreac Química SA, Barcelona, Spain) and ammonium hydroxide (J.T. Baker, Deventer, Holland) were also used with pro-analysis grade.

N-methyl-*N*-(trimethylsilyl) trifluoroacetamide and trimethyl chlorosilane (TMS) were acquired from Macherey-Nagel (Düren, Germany), and the microwave used for the derivatization process was purchased from Samsung (Lisbon, Portugal).

A MEPS syringe (250 µL) and M1 cartridges (4 mg; 80% C8 and 20% SCX), both SGE Analytical Science, Australia, were used.

Working solutions were prepared by proper dilution of stock solutions with acetonitrile to the final concentrations of 2.5 and 0.25 µg/mL for all analytes, and a working solution of ISs at 0.5 µg/mL was also prepared with acetonitrile. All stock and working solutions were stored in the absence of light at 4°C.

Hair samples

Opioid-free hair samples used for all experiments were provided by laboratory staff (CICS, Covilhã, Portugal). Authentic hair samples were obtained from opioid addicts under supervision at the Centro de Atendimento ao Toxicodependente—Casas de Santiago (Belmonte, Portugal), and were sent to the Farmaco-toxicology laboratory from UBImedical (Covilhã, Portugal).

Gas chromatography and mass spectrometry conditions

Chromatographic analysis was performed with an HP 7890A gas chromatography system equipped with 7000B triple quadrupole mass spectrometer, both from Agilent Technologies (Waldbronn, Germany), coupled to a MPS2 autosampler and a PTV-injector from Gerstel (Mülheim an der Ruhr, Germany). The separation of the opioid drugs was achieved with a capillary column (30 m × 0.25-mm I.D., 0.25-µm film thickness) with 5% phenylmethylsiloxane (HP-5 MS), supplied by J & W Scientific (Folsom, CA, USA).

The initial oven temperature was held at 90°C for 2 min, then raised to 300°C at 20°C/min (held for 3 min), giving a total run time of 15.5 min. The temperatures of the injection port and the transfer line were set at 240 and 280°C, respectively. The sample was introduced into the gas chromatograph by splitless injection mode and the flow of helium (carrier gas) was held constant at 0.8 mL/min.

The mass spectrometer operated with a filament current of 35 µA and electron energy of 70 eV in the positive electron ionization mode, and the flow rate of the collision gas (nitrogen) was set at 2.5 mL/min.

Data was acquired in the multiple reaction monitoring (MRM) mode using the MassHunter WorkStation Acquisition Software Rev. B.02.01 (Agilent Technologies), and the tandem mass spectrometry conditions were optimized by injecting a derivatized standard solution at different collision energies and dwell times. Finally, the transitions were chosen based on selectivity and abundance in order to maximize the signal-to-noise ratio in matrix extracts (Table I).

Sample Preparation

Hair decontamination and extraction

In order to remove possible external contamination or unnecessary dirt from the outer surface, hair samples were sequentially washed with dichloromethane, deionized water and methanol. The last wash was stored for further analysis, and the hair samples were left to dry at room temperature.

Each hair sample was cut into fragments of <1 mm, and 50 mg was weighed into glass tubes. Then, 2 mL of methanol was added and the tubes were tightly closed. The tubes were slightly agitated in a vortex-mixer and incubated overnight at 65°C. The digested hair samples were afterwards centrifuged at 3,500 rpm for 15 min and the methanolic phase was transferred into new glass tubes, 20 µL of ISs working solution was added, and the extracts were evaporated

Table I. Retention times and selected transitions for the identification of analytes

Opioid	Retention time (min)	Quantifying transition (m/z)	Qualifying transition (m/z)	Collision energy (eV)	Dwell time (μs)
TRM	10.84	334.0–84.1	334.0–210.1	20 (5)	50
COD	13.15	371.0–234.0	371.0–343.0	10 (10)	50
COD-d3	13.13	374.0–374.0	–	5	50
MOR	13.38	429.1–236.1	429.1–287.2	10 (20)	50
MOR-d3	13.36	432.0–432.0	–	5	50
6-AC	13.51	341.0–282.0	341.0–229.0	10 (10)	50
6-MAM	13.74	399.0–287.3	399.0–340.3	15 (15)	50
6-MAM-d3	13.72	402.4–402.4	–	5	50
FNT	14.62	244.0–146.1	244.0–189.2	15 (10)	50

[a]() The collision energy used for the qualifying transition is presented between brackets.

to dryness under a stream of nitrogen. The extracts were reconstituted with 500 μL of 2% formic acid.

Microextraction by packed sorbent

The sample cleanup procedure was previously optimized, and the final conditions were as follows.

The M1 MEPS cartridge was previously conditioned with three cycles of 250 μL of methanol and three cycles of 250 μL of 2% formic acid in water. Sample loading was performed with fifteen cycles of 150 μL of the reconstituted sample. Subsequently, a washing step was applied with 150 μL of 3.36% formic acid in water to remove endogenous interferences from the sorbent. The retained compounds were eluted from the sorbent with eight cycles of 100 μL of 2.36% ammonium hydroxide in methanol, which was afterwards evaporated to dryness under a stream of nitrogen. Finally, and in order to re-use the sorbent, two solutions were sequentially used: 1% ammonium hydroxide in acetonitrile:methanol (1:1) and 1% formic acid in isopropanol:water (10:90) (four cycles of 250 μL each).

The dry extracts were derivatized with 50 μL of MSTFA with 5% TMS, in a microwave oven for two minutes at 800 W, and a 2 μL aliquot of the resulting solution was injected onto de chromatographic system.

Validation procedure

The developed analytical method was fully validated according to the guiding principles of the Food and Drug Administration (FDA) (90), the International Conference on Harmonization (ICH) (91) and the Scientific Working Group for Forensic Toxicology (SWGTOX) (92). The parameters selectivity, linearity and limits, intra- and inter-day precision and accuracy, recovery and autosampler stability were evaluated in a 5-day validation protocol.

Results and Discussion

Optimization of the cleanup procedure

In order to maximize cleanup efficiency, it is important to adequately evaluate the solvents and sorbent to apply. The MEPS technique is no different. Initially, an evaluation of the procedures reported in the literature (data not shown), allowed the selection of the most suitable solutions for each step.

Once the mixed mode sorbent (anion–cation exchange) appeared as the most appropriate, according to the MEPS tutorial by Abdel-Rehim (78), methanol followed by formic acid were chosen as conditioning solvents.

Design of experiments (DOE) is a statistical tool that may be used to rapidly evaluate, in a multivariate fashion, the critical factors that have a significant impact on the extraction procedure. A two-level full factorial design (2k) was applied and five factors were studied, each one at two levels (low-high): number of sample load strokes (5–15 × 150 μL), number of washes (1–3 × 50 μL), percentage of formic acid (1–3%) in the washing solution, ammonium hydroxide percentage (1–3%), and number of elution cycles (4–8 × 100 μL).

This study was performed with blank hair samples spiked at 5 ng/mg; the ISs were added after extraction.

By evaluating the obtained pareto charts (data not shown), none of the variables under study was considered significant in terms of response, and therefore a response surface methodology (RSM) was used; based on this RSM approach, the best conditions for sample clean-up were as follows: conditioning (three cycles of 250 μL of methanol and three cycles of 250 μL formic acid 2%); sample load (15 cycles of 150 μL); wash (3 × 50 μL of 3.36% formic acid); and elution (eight cycles of 100 μL of ammonium hydroxide 2.36% (in methanol)).

Method Validation

Selectivity

The method's selectivity was evaluated by the analysis of 10 blank hair specimens from different origins. These specimens were searched for eventual interferences at the retention times and selected transitions of the target compounds. The identification criteria for positivity, as well as to guarantee a suitable confidence in identification, the maximum allowed tolerances for the relative ion intensities between the transitions (as a percentage of the base peak) followed the WADA statements (93). Figure 1 represents a chromatogram of a blank hair sample, while Figure 2 represents a chromatogram obtained at the lower limit of quantification (LLOQ). The present method was considered selective, once no compound could be identified in the blank hair specimens by means of those criteria.

Calibration curves and limits

The method was found linear between 0.010 and 5 ng/mg for TRM, COD and 6-AC, and in the range of 0.025–5 ng/mg for MOR, 6-MAM and FNT ($n = 5$). The calibration curves were obtained by plotting the peak area ratio between each analyte and the IS against analyte concentration. A determination coefficient (R^2) value of at least 0.99 and the calibrators' accuracy within a ±15% interval

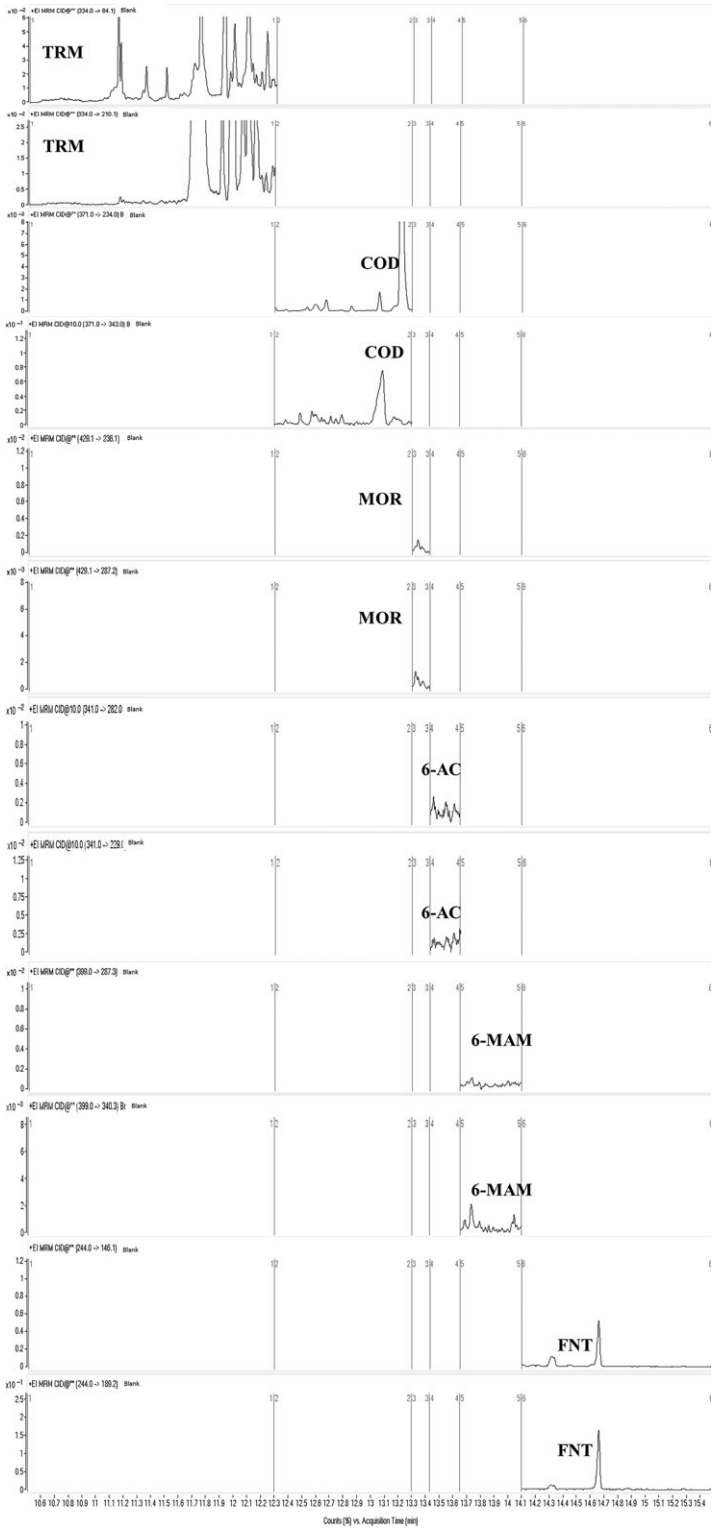

Figure 1. Chromatogram of a blank hair specimen.

from the nominal value (except at the LLOQ, ±20%), were adopted as acceptance criteria. Weighted least squares regressions ($1/x$) were adopted to compensate for heterocedasticity.

The LLOQ was considered the lowest concentration of each opioid that could be measured with a coefficient of variation (CV, %) of <20% and a relative error (RE, %) within ±20% of the nominal concentration. The LLOQ obtained was 0.010 ng/mg for TRM, COD and 6-AC and 0.025 ng/mg for MOR, 6-MAM and FNT. The method's limits of detection (LOD) were not systematically evaluated, and were considered to be equal to the concentrations adopted for the LLOQ, since values below that concentration are reported as negative. Table II resumes linearity data.

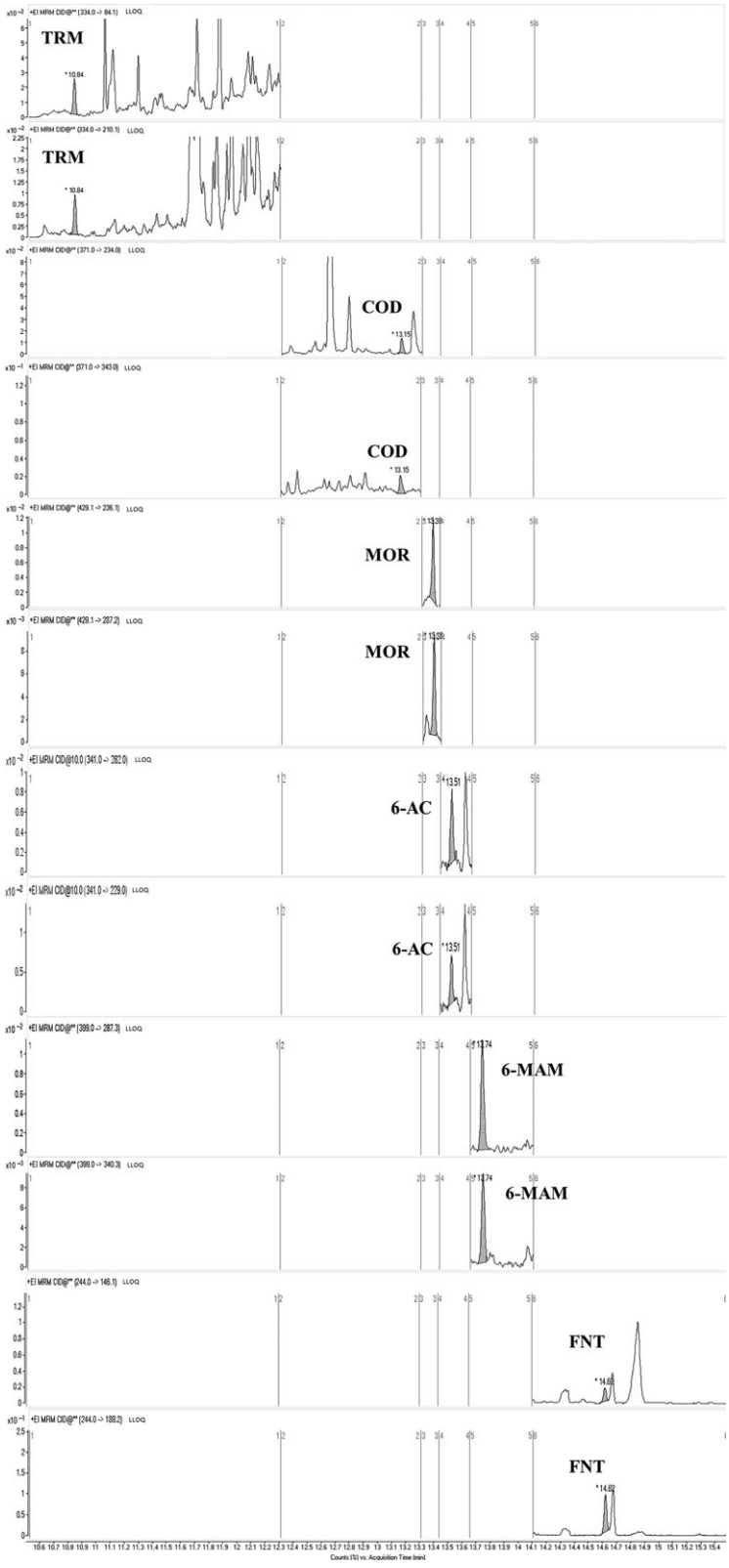

Figure 2. Chromatogram of a hair specimen spiked at LLOQ.

The limits described in the present method can be considered quite satisfactory, especially when compared to the available literature on the subject. Verri *et al.* (56) reports the same LLOQ for TRM when extracting also 50 mg of hair, however, using SPE Strata XC cartridges and an LC–MS-MS system. The present method also reports a lower LLOQ for TRM than that described by Maublanc *et al.* (55) that applies LLE as sample clean up procedure and LC–MS-MS. Overall, and regarding COD, MOR, 6-AC and 6-MAM,

the herein described procedure results in lower LLOQs than those applying SPE MCX (5, 28, 54), Bond Elut (32, 43, 44), Chromabond (26, 27) and ZSDAU020 (47, 72) cartridges on approaches for multi-analyte determinations in hair. SPE is a traditional and widely implemented cleanup procedure for hair specimens, however, the great volumes of organic solvents usually required with this technique might be considered a pitfall. The MEPS procedure here presented can be considered as an excellent alternative, since it greatly reduces the volumes of organic solvents used.

Many authors adopt the hair incubation as a unique sample preparation procedure, not considering the additional cleanup of the resultant extract. This results in lower losses of the analytes, but also in dirtier extracts, hence compromising chromatographic analysis. Usually these methods are described for LC–MS-MS. Regarding opioids in hair samples and including FNT, MEPS allowed also lower LLOQs than those reported by authors who do not use cleanup post extraction (23, 25, 50, 57, 60, 61, 64, 66, 67).

Few miniaturized procedures are described to pre-concentrate opioids from hair samples. Aleska *et al.* (36) report a LOQ of 0.6 ng/mg for COD, MOR and 6-MAM using HS-SPME coupled to a GC–MS system. Sporkert and Pragst (19) achieved a LOD of 0.5 ng/mg for TRM, also using HS-SPME coupled to a GC–MS system, and Yazdi and Es'haghi (74) reported other type of miniaturized technique, SE-LPME, with LOQs of 17 and 66 ng/mL for COD and MOR, respectively. To the best of our knowledge, and regarding the application of these miniaturized procedures to hair specimens, the present method is only surpassed by that from Moller *et al.* (34), who have used 10 mg of hair with HS-SPME coupled to GC–MS and achieved a LOQ of 0.005 ng/mg for COD and 0.01 ng/mg for MOR and 6-MAM. Nevertheless, in the herein described MEPS procedure a higher number of compounds and metabolites have been included.

Regarding MEPS application to determine opiates, few reports are available, and most of them are multi-analyte procedures, and not specifically related to opiate drugs. These multi-methods consider mainly MOR, COD and 6-MAM among other drugs of abuse. Ares *et al.* (86) have used MEPS also with an M1 sorbent to determine MOR and 6-MAM in oral fluid samples, achieving LLOQs ranging from 2.5 to 10 ng/mL. Fernández *et al.* (83) used an M1 sorbent to determine MOR in plasma samples achieving a LLOQ of 50 ng/mL. Lower LLOQs were achieved by Montesano *et al.* (94) using C18 sorbent to determine MOR, COD and 6-MAM in oral fluid samples, and ranged from 2 to 5 ng/mL.

Intra-day, inter-day and intermediate precision and accuracy

The evaluation of the inter-day precision and accuracy was performed within a 5-day period at a minimum of seven concentration levels. These concentrations were the same applied to build the calibration curve. The coefficients of variation (CVs) obtained were typically lower than 14% for all target compounds at the tested concentration levels with an accuracy within a ±12% interval. Regarding intra-day precision and accuracy, this was evaluated by the analysis, on the same day, of six replicates of blank hair spiked at a minimum of four concentration levels, assuring that the LLOQ was included. The observed CVs were lower than 14% at all studied concentrations, with a mean RE within ±9%. Additionally, intermediate (combined intra- and inter-day) precision and accuracy were also studied using the quality control (QC) samples at three concentration levels (0.035, 0.35 and 3 ng/mg). These QC samples were prepared ($n = 3$) and analyzed simultaneously with the calibration curves on 5 different days ($n = 15$). The obtained CVs were typically lower than 15% and accuracy within ±6% interval. All data are presented in Table III.

Recovery

In order to study recovery of the cleanup step, two groups of samples ($n = 3$) were prepared by spiking blank hair at three concentration levels: 0.035, 0.5 and 4 ng/mg. The first group of samples was spiked after MEPS (representing 100% recovery), while the second group of samples was spiked before MEPS. The ISs were added to both groups only after MEPS procedure. Recoveries were calculated by comparison of the relative peak areas obtained in group 2 with those obtained for group 1 (Table IV).

Overall, the recoveries obtained for most target opioids were more than satisfactory, namely, for TRM (74–90%), 6-AC (69–99%) and FNT (75–86%). The only analyte for which a recovery below 50% was obtained was MOR; however, a LLOQ of 0.025 ng/mg was comfortably achieved.

Regarding the available literature, the results obtained with this MEPS technique are similar to those presented by Ramírez *et al.* (53), who used SPE with a MCX sorbent and describe recoveries ranging from 57 to 74%, and also to those presented by Mussoff *et al.* (26), who have used a SPE chromabond cartridge and report recoveries from 62 to 97%. Greater recoveries have been obtained by Barroso *et al.* (5), however, using SPE for sample clean-up. These findings are normal, taking into account that miniaturized procedures often present poorer recoveries, as usually lower amounts of sorbent are used. Regarding miniaturized techniques, Aleska *et al.* (36) used HS-SPME and obtained recoveries below 68%, while Yazdi and Es'haghi (74) used SE-LPME and presented recoveries ranging from 58 to 86%. The recoveries obtained with these miniaturized procedures can also be considered similar to those reported for MEPS in this work.

Stability

It is well established that hair specimens present great stability. Hair is a strong tissue, being less affected by adulterants, an advantage over traditional matrices (7). Once the drug is incorporated into the

Table II. Linearity data ($n = 5$)

Opioid	Weight	Linear range (ng/mg)	Linearity		R^{2a}	LLOQ (ng/mg)
			Slope[a]	Intercept[a]		
TRM	1/*x*	0.010–5	0.0006 ± 0.0003	0.0173 ± 0.0254	0.9982 ± 0.0010	0.010
COD	1/*x*	0.010–5	0.0007 ± 0.0001	0.0094 ± 0.0089	0.9976 ± 0.0015	0.010
MOR	1/*x*	0.025–5	0.0011 ± 0.0005	0.0290 ± 0.0413	0.9980 ± 0.0019	0.025
6-AC	1/*x*	0.010–5	0.0012 ± 0.0003	0.0093 ± 0.0133	0.9968 ± 0.0022	0.010
6-MAM	1/*x*	0.025–5	0.0034 ± 0.0011	0.0242 ± 0.0009	0.9979 ± 0.0018	0.025
FNT	1/*x*	0.025–5	0.0089 ± 0.0039	0.0118 ± 0.0748	0.9964 ± 0.0018	0.025

[a]Mean values ± standard deviation.

Table III. Inter-, intra-day and intermediate precision and accuracy

		Inter-day ($n = 5$)			Intra-day ($n = 6$)			Intermediate ($n = 15$)		
Opioid	Spiked	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)
TRM	0.01	0.0098 ± 0.0008	8.26	−1.89	0.0102 ± 0.0012	12.37	2.34			
	0.025	0.0245 ± 0.0017	6.80	−2.11	0.0242 ± 0.0014	5.70	−3.24			
	0.035							0.0338 ± 0.0024	7.02	−3.50
	0.05	0.0495 ± 0.0059	11.88	−1.07	0.0496 ± 0.0047	9.55	−0.85			
	0.2	0.1995 ± 0.0025	12.44	−0.24	0.1911 ± 0.0118	6.16	−4.44			
	0.35							0.3701 ± 0.0038	1.03	5.76
	1	1.0653 ± 0.0767	7.20	6.53						
	2	1.9817 ± 0.0782	3.94	−0.91						
	3							2.9545 ± 0.3342	11.31	−1.52
	3.5	3.5305 ± 0.0075	0.21	0.87						
	5	4.9394 ± 0.1087	2.20	−1.21	5.3608 ± 0.2610	4.87	7.22			
COD	0.01	0.0108 ± 0.0001	9.14	8.02	0.0108 ± 0.0010	9.30	8.02			
	0.025	0.0234 ± 0.0021	8.90	−6.45	0.0251 ± 0.0028	10.98	0.32			
	0.035							0.0330 ± 0.0023	6.85	−5.82
	0.05	0.0500 ± 0.0029	5.79	0.01	0.0498 ± 0.0028	5.52	−0.43			
	0.2	0.1848 ± 0.0109	5.89	−7.59	0.1863 ± 0.0143	7.69	−6.86			
	0.35							0.3424 ± 0.0346	10.11	−2.18
	1	1.1023 ± 0.0314	2.85	10.22						
	2	1.9997 ± 0.1220	6.10	−0.01						
	3							2.8463 ± 0.1973	6.93	−5.12
	3.5	3.3908 ± 0.1966	5.80	−3.12						
	5	4.9688 ± 0.0373	0.75	−0.62	5.1497 ± 0.3869	7.51	2.99			
MOR	0.025	0.0234 ± 0.0020	8.04	−6,49	0.0238 ± 0.0025	10.57	−4.77			
	0.035							0.0371 ± 0.0023	6.20	5.95
	0.05	0.0511 ± 0.0041	7.95	2,15	0.0516 ± 0.0039	7.61	3.28			
	0.2	0.1930 ± 0.0195	10.08	−3,49	0.1998 ± 0.0198	9.92	−0.11			
	0.35							0.3339 ± 0.0193	5.78	−4.60
	1	0.9776 ± 0.0554	5.67	−2,24						
	2	1.9395 ± 0.1156	5.96	−3,03						
	3							3.0929 ± 0.1094	3.54	3.10
	3.5	3.6622 ± 0.0167	0.46	4,63						
	5	5.0245 ± 0.1738	3.46	0,49	5.1901 ± 0.4049	7.80	3.80			
6-AC	0.01	0.0093 ± 0.0013	13.87	−6.84	0.0108 ± 0.0011	10.26	7.82			
	0.025	0.0222 ± 0.0017	7.49	−11.13	0.0248 ± 0.0028	11.45	−0.73			
	0.035							0.0345 ± 0.0039	11.25	−1.42
	0.05	0.0522 ± 0.0047	9.02	4.44	0.0493 ± 0.0039	7.95	−1.50			
	0.2	0.1997 ± 0.0127	6.36	−0.16	0.2153 ± 0.0147	6.83	7.66			
	0.35							0.3433 ± 0.0493	14.37	−1.91
	1	1.0477 ± 0.1358	12.96	4.77						
	2	2.0414 ± 0.0724	3.55	2.07						
	3							3.1202 ± 0.2201	7.05	4.01
	3.5	3.4179 ± 0.0482	1.41	−2.35						
	5	4.9515 ± 0.2202	4.45	−0.97	5.0054 ± 0.4657	9.30	0.11			
6-MAM	0.025	0.0256 ± 0.0029	11.21	2.25	0.0243 ± 0.0022	8.87	−2.63			
	0.035							0.0359 ± 0.0028	7.92	2.45
	0.05	0.0513 ± 0.0041	7.88	2.66	0.0476 ± 0.0049	10.35	−4.90			
	0.2	0.2026 ± 0.0219	10.82	1.31	0.1967 ± 0.0257	13.08	−1.64			
	0.35							0.3799 ± 0.0151	3.98	8.55
	1	1.0260 ± 0.0581	5.66	2.60						
	2	2.0662 ± 0.0952	4.61	3.31						
	3							3.0911 ± 0.3656	11.83	3.04
	3.5	3.5771 ± 0.1160	3.24	2.20						
	5	4.8586 ± 0.1140	2.35	−2.83	5.4809 ± 0.2888	5.27	9.62			
FNT	0.025	0.0232 ± 0.0020	8.74	−7.38	0.0256 ± 0.0024	9.47	2.22			
	0.035							0.0344 ± 0.0034	9.89	−1.69
	0.05	0.0493 ± 0.0044	8.87	−1.36	0.0500 ± 0.0039	7.73	0.00			
	0.2	0.2034 ± 0.0186	9.13	1.70	0.1960 ± 0.0240	12.23	−2.02			
	0.35							0.3374 ± 0.0339	10.05	−3.61
	1	0.9950 ± 0.1263	12.70	−0.50						
	2	2.1233 ± 0.0549	2.58	6.16						
	3							2.9537 ± 0.4053	13.72	−1.54
	3.5	3.3134 ± 0.1526	4.61	−5.33						
	5	4.9737 ± 0.1993	4.01	−0.53	4.5250 ± 0.2973	6.57	−9.50			

All concentrations in ng/mg. CV, coefficient of variation; RE, relative error [(measured concentration-spiked concentration/spiked concentration)] × 100. Mean values ± standard deviation.

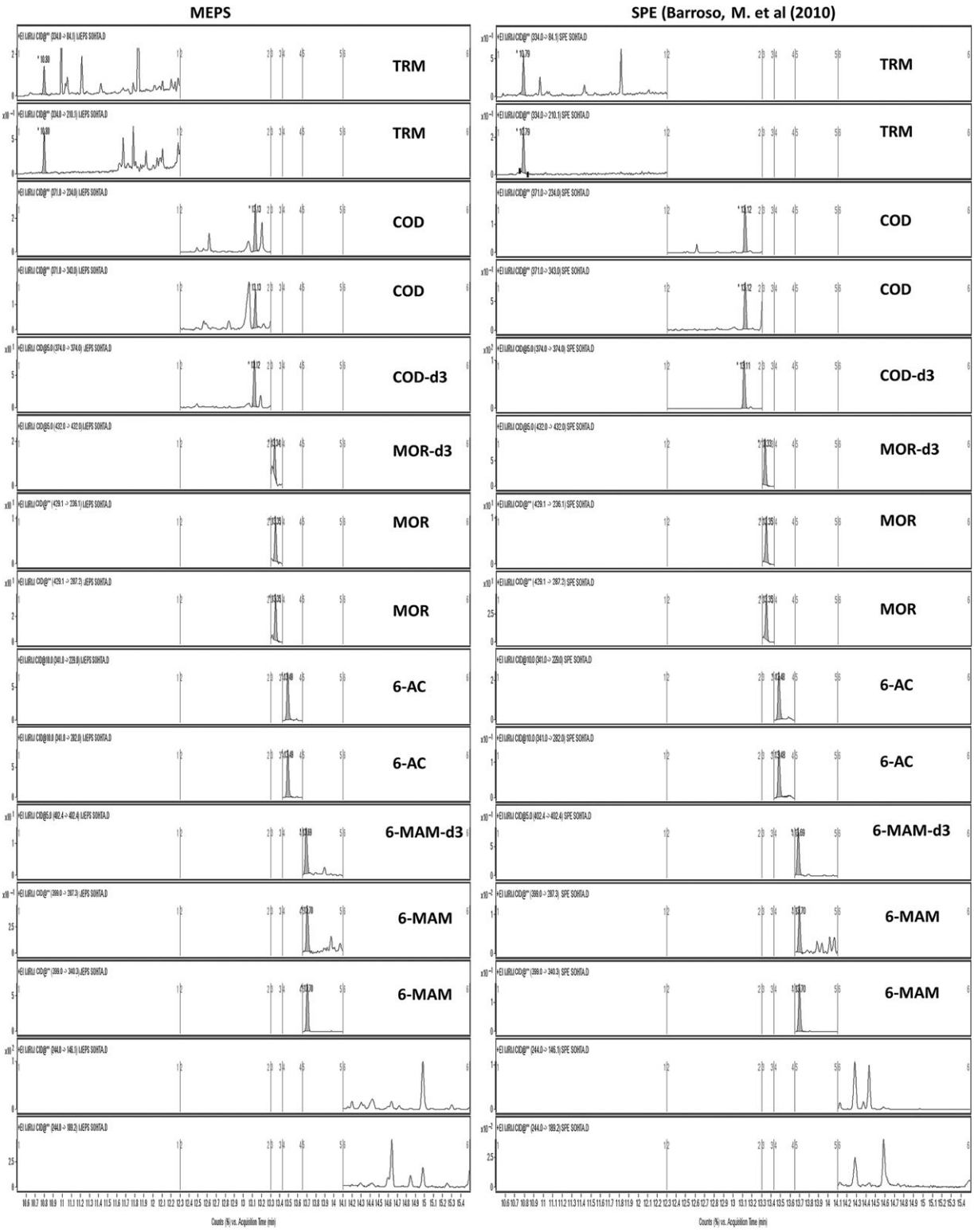

Figure 3. Chromatograms of an authentic hair sample positive for TRM, COD, MOR, 6-AC and 6-MAM, using both procedures for sample clean-up.

hair it is fixed, remaining fixed as hair grows (33). The specimen can be collected and stored at room temperature.

However, after submitted to extraction, MEPS procedure and derivatization, the extracts should demonstrate enough stability over the anticipated run time for batch size. For this reason, the stability of processed samples was evaluated at the QC concentration levels (n = 3), in which previously analyzed samples were re-analyzed after 24 h in the autosampler. Their concentrations were

Table IV. Recovery (%) of the target opioids under the optimized MEPS procedure ($n = 3$)

Opioid	Concentration (ng/mg)		
	0.035[a]	0.5[a]	4[a]
TRM	73.60 ± 10.83	76.06 ± 7.28	89.99 ± 5.44
COD	51.28 ± 8.29	55.87 ± 3.42	58.58 ± 2.55
MOR	35.65 ± 2.26	35.10 ± 5.01	22.34 ± 0.19
6-AC	68.66 ± 4.15	84.42 ± 8.23	99.19 ± 11.25
6-MAM	60.97 ± 4.74	52.57 ± 4.68	60.88 ± 8.32
FNT	75.46 ± 3.88	86.03 ± 10.60	79.69 ± 10.83

[a]Mean values ± standard deviation.

Table V. Analyte stability in processed samples ($n = 3$)

Opioid	Spiked	Measured	CV (%)	RE (%)
TRM	0.035	0.0362 ± 0.0019	5.36	3.31
	0.35	0.3690 ± 0.0406	10.99	5.42
	3	3.0525 ± 0.3570	11.70	1.75
COD	0.035	0.0301 ± 0.0006	2.04	−13.88
	0.35	0.3301 ± 0.0250	7.57	−5.70
	3	2.7780 ± 0.0914	3.29	−7.40
MOR	0.035	0.0347 ± 0.0051	14.70	−0.91
	0.35	0.3443 ± 0.0283	8.22	−1.62
	3	2.7849 ± 0.1123	4.03	−7.17
6-AC	0.035	0.0337 ± 0.0019	5.76	−3.70
	0.35	0.3815 ± 0.0045	1.17	8.98
	3	3.0581 ± 0.0335	1.10	1.94
6-MAM	0.035	0.0351 ± 0.0046	13.00	0.21
	0.35	0.3574 ± 0.0364	10.18	2.13
	3	3.1529 ± 0.3449	10.94	5.10
FNT	0.035	0.0360 ± 0.0033	9.07	2.96
	0.35	0.3655 ± 0.0193	5.27	4.42
	3	3.0466 ± 0.3980	13.06	1.55

All concentrations in ng/mg. CV, coefficient of variation; RE, relative error [(measured concentration-spiked concentration/spiked concentration)] × 100. Mean values ± standard deviation.

determined on the basis of a new calibration curve prepared on the day of re-analysis.

The CVs obtained were typically lower than 15% with RE within a ±14% interval, confirming the possibility of a re-analysis after 24 h in the autosampler with no significant change in the concentration of the target compounds (Table V).

Method applicability

Method applicability was verified by analysis of 16 authentic hair samples obtained from current or ex-opioid consumers under supervision of Centro de Atendimento ao Toxicodependente—Casas de Santiago (Belmonte, Portugal). As example, Figure 3 represents the chromatogram obtained from the analysis of sample 11, positive for TRM, COD, MOR, 6-AC and 6-MAM. The results obtained for all hair specimens are summarized on Table VI. All authentic samples analyzed were negative for fentanyl, and therefore the method's applicability in its determination was not fully herein demonstrated.

The present MEPS cleanup procedure was also compared to the cleanup procedure described by Barroso *et al.* (5) and the measured concentrations on authentic samples were very similar, resulting in a coefficients of variation typically lower than 15% for TRM, 12%

Table VI. Results obtained from authentic hair samples obtained from current or ex-opioid consumers—comparison between the two clean-up techniques

	MEPS[a]						SPE[a]					
Sample no.	TRM	COD	MOR	6-AC	6-MAM	FNT	TRM	COD	MOR	6-AC	6-MAM	FNT
1	0.56	1.62	0.40	2.32	4.52	Negative	0.59	1.47	0.38	2.21	4.93	Negative
2	Negative	Negative	0.04	0.06	1.42	Negative	Negative	Negative	0.04	0.06	1.62	Negative
3	0.18	Negative	Negative	0.04	0.18	Negative	0.16	Negative	Negative	0.04	0.20	Negative
4	0.30	Negative	Negative	0.07	0.40	Negative	0.34	Negative	Negative	0.07	0.43	Negative
5	0.14	Negative	Negative	Negative	0.03	Negative	0.15	Negative	Negative	Negative	0.03	Negative
6	1.84	1.30	0.45	1.57	5.59	Negative	1.89	1.45	0.49	1.49	6.21	Negative
7	0.12	Negative	Negative	Negative	Negative	Negative	0.11	Negative	Negative	Negative	Negative	Negative
8	0.19	0.37	0.11	0.42	2.50	Negative	0.21	0.40	0.12	0.42	2.17	Negative
9	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
10	0.08	Negative	Negative	Negative	Negative	Negative	0.09	Negative	Negative	Negative	Negative	Negative
11	0.19	0.13	0.14	0.14	0.84	Negative	0.17	0.11	0.13	0.15	0.91	Negative
12	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
13	Negative	0.07	0.08	0.08	0.57	Negative	Negative	0.08	0.09	0.08	0.63	Negative
14	0.19	0.16	0.08	0.16	1.30	Negative	0.16	0.17	0.08	0.16	1.47	Negative
15	0.05	Negative	Negative	0.02	0.23	Negative	0.05	Negative	Negative	0.02	0.20	Negative
16	0.29	Negative	0.11	0.71	3.70	Negative	0.33	Negative	0.10	0.73	3.94	Negative

[a]All concentrations in ng/mg; mean values.

for COD, 10% for MOR, 5% for 6-AC and 14% for 6-MAM (Table VI). A chromatogram of sample 11 using that procedure is also shown in Figure 3. This way, it was possible to confirm that the herein described procedure is an excellent alternative to SPE.

Conclusions

The herein described method for the simultaneous determination of tramadol, codeine, morphine, 6-acetylcodeine, 6-monoacetylmorphine and fentanyl in hair samples using MEPS coupled to GC–MS-MS system was fully validated. The entire procedure has proven to be simple with an ease and fast operation, sensitive, selective, precise and accurate.

The analytical method was linear within the adopted ranges for all opioids with a LLOQ of 0.010 ng/mg for tramadol, codeine and 6-acetylcodeine and 0.025 ng/mg for morphine, 6-monoacetylmorphine and fentanyl.

The present work is the first coupling MEPS to GC–MS-MS to determine opioids in hair samples, and to best of our knowledge, the second applying MEPS as sample cleanup to hair specimens. This technique results in a great alternative to the classic cleanup techniques, with low consumption of organic solvents, also allowing the re-utilization of the sorbent (over 100 extractions) and thus reducing the cost per analysis.

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Paper IV - Microextraction by packed sorbent as a novel strategy for sample clean-up in the determination of methadone and EDDP in hair

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Article

Microextraction by Packed Sorbent as a Novel Strategy for Sample Clean-Up in the Determination of Methadone and EDDP in Hair

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Abstract

A microextraction by packed sorbent (MEPS) procedure for rapid concentration of methadone and its primary metabolite (EDDP) in hair samples was developed. The miniaturized approach coupled to gas chromatography with tandem mass spectrometry (GC–MS-MS) was successfully validated. Hair samples (50 mg) were incubated with 1 mL of 1 M sodium hydroxide for 45 min at 50°C, time after which the extract was neutralized by adding 100 µL of 20% formic acid. Subsequently, MEPS was applied using a M1 sorbent (4 mg; 80% C8 and 20% strong cation-exchange (SCX)), first conditioned with three 250-µL cycles of methanol and three 250-µL cycles of 2% formic acid. The extract load occurred with nine 150-µL cycles followed by a washing step involving three 50-µL cycles with 3.36% formic acid. For the elution of the analytes, six 100-µL cycles of 2.36% ammonium hydroxide in methanol were applied. The method was linear from 0.01 to 5 ng/mg, for both compounds, presenting determination coefficients greater than 0.99. Precision and accuracy were in accordance with the statements of international guidelines for method validation. This new miniaturized approach allowed obtaining recoveries ranging from 73 to 109% for methadone and 84 to 110% for EDDP, proving to be an excellent alternative to classic approaches, as well as other miniaturized procedures.

Introduction

Methadone, (+) -6-dimethylamino-4,4-diphenylheptan- 3-one, is a synthetic narcotic analgesic commonly used for the treatment of heroin and morphine addiction (1). The basic prerequisite for admittance in methadone programs is both detoxification and long-term maintenance therapy (2). Nevertheless, a risk of overdose exists, possibly leading to fatal outcomes, and for that reason, monitoring is necessary in order to detect substance misuse and prevent illicit diversion of prescribed opiates (2, 3).

Although urine is usually analyzed from patients undergoing these programs, hair analysis may be an useful alternative to verify drug history and compliance (2, 3). In fact, hair samples present limited possibility of tampering with, becoming more difficult to hide drug intake when compared with urinalysis. Additionally, the hair sampling method is non-invasive, while urine sample collection, which should be performed with strict supervision, may be considered embarrassing for both the individual being tested and supervisor (4).

Regarding hair testing in patients under methadone-maintenance programs, methadone and its primary metabolite 2-ethylidine-1,5-

dimethyl-3,3-diphenyl-1-pyrrolidine (EDDP) are usually detected (5). However, this specimen is a very complex matrix, since the drugs are strongly bound to inner-hair constituents, and as such the analysis involves, at a first stage, an initial sample pre-treatment step, commonly named incubation or extraction, that will allow the release and solubilization of drugs (6, 7). The resultant extract can be either directly analyzed (3, 8–16) or may require subsequent clean-up, for which liquid–liquid extraction (LLE) (17–22) and solid-phase extraction (SPE) (4, 18, 23–32) are the most commonly used approaches.

The pre-treatment of complex matrices by using miniaturized sample preparation methods remains of great interest in the research field (33–35). Indeed, they feature advantages when compared with classical techniques, such as the higher speed of analysis with the higher associated efficiency, low cost of operation due to lower solvents consumption, environmental friendly and highly selective analysis (36). Microextraction techniques, such as liquid-phase microextraction (LPME) (37) and solid-phase microextraction (SPME) (5, 38–42), present these advantages and have been successfully applied as clean-up procedures for the determination of methadone and EDDP in hair.

Less explored, concerning drug analysis in hair, is the microextraction by packed sorbent (MEPS) approach, which is a miniaturization of the conventional SPE packed bed cartridges, allowing reducing bed volumes from the millilitre to the microlitre ranges (43–47). MEPS emerged in 2004, developed by Abdel-Rehim (48) and has been accepted as an attractive miniaturized option and powerful sample-preparation technique, because it is fast, simple and requires very small volumes of samples and solvents, resulting in results comparable to those of SPE (43–47). Additionally, MEPS allows full automation, including the sample processing, extraction and injection steps as an online sampling device using the same syringe (43–47). Up until now, and concerning hair testing, this technique has only been applied in the determination of methamphetamine and amphetamine (49) and a number of selected opiate compounds (50).

The aim of this work was the development and validation of an analytical method using MEPS to determine methadone and its main metabolite EDDP in hair samples.

Materials and Methods

Reagents and standards

Methadone and EDDP analytical standards, as well as the internal standards (ISs), methadone-d_3 and EDDP-d_3, were obtained from Sigma-Aldrich (Lisbon, Portugal). The working solutions of both methadone and EDDP were prepared by proper dilution of stock solutions with methanol to the final concentrations of 5 and 0.25 µg/mL, while a working solution of the two ISs was also prepared in methanol at a concentration of 1 µg/mL. All stock and working solutions were stored at −20°C.

Formic acid (Panreac Química SA, Barcelona, Spain), ammonium hydroxide (J.T. Baker, Deventer, Holland), methanol (Merck Co, Darmstadt, Germany), isopropanol (Fischer chemical, Loughborough, UK) and acetonitrile (Prolabo, Lisbon, Portugal) were all pro-analysis grade. Deionized (DI) water was obtained from a Milli-Q System (Millipore, Billerica, MA, USA).

The MEPS syringe (250 µL) and M1 cartridges (4 mg; 80% C8 and 20% SCX), both from SGE Analytical Science, were acquired from VWR international (Alfragide, Portugal).

Statistical analyses used for optimization were carried out with Minitab Statistical Software version 17 and SPSS version 25.

Hair samples

Blank hair samples for methadone and EDDP were provided by laboratory staff (CICS, Covilhã, Portugal) and were used for MEPS optimization and method validation. Authentic hair samples were obtained from individuals undergoing methadone treatment program at Centro de Atendimento ao Toxicodependente—Casas de Santiago (Belmonte, Portugal) and, subsequently, sent to the health sciences research center (CICS, Covilhã, Portugal). Both blank and authentic samples were stored in paper envelopes in a dry, dark environment at room temperature, away from direct sunlight.

Gas chromatographic and mass spectrometric conditions

An HP 7890A gas chromatography (GC) system coupled to a 7000B triple quadrupole mass spectrometer (MS), both from Agilent Technologies (Waldbronn, Germany), was used for analysis. Automated injections were performed with an MPS2 auto-sampler and a PTV-injector from Gerstel (Mülheim an der Ruhr, Germany). Methadone and EDDP chromatographic separation was possible using a capillary column (30 m × 0.25-mm I.D., 0.25-µm film thickness) with 5% phenylmethylsiloxane (HP-5 MS), supplied by J & W Scientific (Folsom, CA, USA).

The oven gradient of temperatures started at 150°C for 2 min, after which it was raised to 300°C at 20°C/min and was held at that temperature for 3 min, originating a total run time of 12.5 min. The temperatures of the injection port and transfer line were 220 and 280°C, respectively. The sample extract (2 µL) was injected into the GC in the splitless mode, and helium was used as a carrier gas at a constant flow of 0.8 mL/min.

The MS operated with a filament current of 35 µA and an electron energy of 70 eV in positive electron ionization mode. Nitrogen was used as a collision gas with a flow rate set of 2.5 mL/min. All data were acquired in the multiple reaction monitoring mode with the help of the MassHunter WorkStation Acquisition software rev. B.02.01 (Agilent Technologies). The injection of methanolic standard solutions of methadone and EDDP at different collision energies and dwell times contributed for the optimization of the final MS conditions. The transitions were chosen based on selectivity and abundance in order to maximize the signal-to-noise ratio in hair extracts (Table I).

Sample preparation

Hair decontamination and extraction

The washing procedure adopted for all hair samples prior to analysis involved a sequential soaking in dichloromethane, DI water and methanol, 15 min each, at room temperature with agitation. This procedure allowed the removal of hair care products, sweat, sebum or surface material that could interfere with the chromatographic analysis and/or reduce extraction recovery. This step also becomes important to remove potential external drug contamination. For that reason, the last wash was stored for further analysis, in order to check for the presence of the target compounds. After the decontamination procedure, hair samples were left to dry at room temperature.

After completely dried, each hair sample was cut into fragments of less than 1 mm, and 50 mg was weighed into glass tubes. Then, 1 mL of 1 M sodium hydroxide was added and the tubes were tightly closed. The tubes were vortex-mixed and incubated for 45 min at 50°C. After digestion of the hair samples, these were neutralized by adding 100 µL of 20% formic acid in water, vortex-mixed and centrifuged at 3500 rpm for 15 min. The extracts were transferred into new glass tubes, and 25 µL of ISs working solution was added.

Table I. Retention times and selected transitions for the identification of analytes

Analyte	Retention time (min)	Quantifying transition (m/z)	Qualifying transition (m/z)	Collision energy (eV)	Dwell time (μs)
EDDP	8.17	275.4–232.3	275.4–247.2	20 (15)[a]	50
EDDP-d_3	8.16	237.2–220.2	–	10	50
MTD	8.68	222.1–105.1	222.1–117.0	20 (20)[a]	50
MTD-d_3	8.67	297.0–297.0	–	5	50

[a]Collision energy used for the qualifying transition.

Microextraction by packed sorbent

The MEPS procedure used for sample clean-up was optimized, resulting in the following final conditions. The MEPS cartridge was previously conditioned with three 250-μL cycles of methanol and three 250-μL cycles of 2% formic acid in water. Sample load was performed with nine withdraw–dispense cycles of 150 μL. A subsequent washing step was performed by three cycles with 50 μL of 3.36% formic acid in water, time after which the retained analytes were eluted from the sorbent with six 100-μL cycles of 2.36% ammonium hydroxide in methanol. The eluted solution was, then, evaporated to dryness under a stream of nitrogen. Since the same extraction cartridge is reused for the clean-up of several samples, the sorbent was sequentially washed with 1% ammonium hydroxide in acetonitrile:methanol (1:1) and 1% formic acid in isopropanol:water (10:90) (four cycles of 250 μL each) before each new sample extraction cycle.

The dry extracts were reconstituted with 50 μL of methanol, and a 2-μL aliquot of the resulting solution was injected onto the chromatographic system.

Validation procedure

The full validation of the developed analytical method followed the guiding principles of the Food and Drug Administration (51), the International Conference on Harmonization (52) and the Scientific Working Group for Forensic Toxicology (53). A 5-day validation protocol was adopted, and the evaluated parameters were selectivity, linearity and limits, intra- and inter-day precision and accuracy, recovery and auto-sampler stability.

Results and Discussion

Optimization of the MEPS procedure

Although simplicity is a feature usually associated to MEPS, this clean-up procedure should follow a range of optimization steps in order to obtain a fine tuning of extraction efficiency (46, 54). For instance, the appropriate selection of the sorbent is of extreme importance to obtain satisfactory clean-up and analyte recovery (46, 54).

As previously mentioned, MEPS is a miniaturization of SPE, and the sorbent selection was based on the available literature that applied the classic procedure to pre-concentrate EDDP and methadone from hair extracts. Moreover, Agilent Bond Elut Certify sorbent that consists of a nonpolar C8 sorbent and an SCX has been the most described sorbent (4, 24, 29, 30). Also, Oasis® MCX cartridges, with a mixed-mode polymeric sorbent, have been reported because they present high selectivity and sensitivity to extract basic compounds with cation-exchange groups (25, 27). In addition, Phenomenex® Strata X polymeric sorbent, ideal for clean-up of neutral, acidic or basic small molecule compounds, was successfully applied for methadone, amongst other drugs (18, 26). However, Phenomenex® Strata X was only used after an initial LLE of the hair extract. ISOLUTE® HCX, a mixed-mode sorbent, was efficiently used for methadone and EDDP determination in hair from human subjects following a maintenance program (31), and SPE Cationic Exchange from StepBio was also described for a multimethod that included both target analytes (28). To the best of our knowledge, only one work has reported octadecyl-modified silica phase sorbent to pre-concentrate these compounds from hair extracts (23), and for this reason, the microextraction procedure was carried out using mixed mode sorbent containing a mixture of 80% C8 and 20% SCX, labeled as M1 on the MEPS BIN.

Depending on the compounds to extract, some steps can be simplified or skipped (46, 54). Nevertheless, the number of sample extraction cycles, also known as strokes, the solvents used in the washing and elution steps, as well as their volumes, can be optimized for each application, leading to greater recoveries (46, 54). Considering a previous successful MEPS application from the same work group to determine selected opiates in hair samples, 3.36% formic acid in water and 2.36% ammonium hydroxide in methanol were selected as washing and elution solvents, respectively (50). Three cycles with 250 μL of methanol and 250 μL 2% formic acid were maintained for the sorbent conditioning step.

A two-level full factorial design with three factors (2^3) was developed in order to study the effect they had on the methadone and EDDP recoveries. The studied factors were the number of sample load strokes (3–9 × 150 μL), the number of washes (1–3 × 50 μL) and the number of elution cycles (2–6 × 100 μL). This study was performed using the Design of Experiments statistical tool, which rapidly evaluates, in a multivariate fashion, the critical factors that may have a significant impact on compounds' recoveries. A central point ($n = 3$) was added to the design matrix for precision evaluation. The evaluation was performed with blank hair samples spiked at 1 ng/mg. The ISs were added only after extraction.

According to the pareto charts obtained from the experimental design (Figure 1), the only factor that revealed a significant influence on both methadone and EDDP recoveries was the number of sample load strokes. Through the main effect plots (Figure 1), it is possible to observe a greater response when a higher number of strokes, in this case nine cycles, were adopted. The other two factors under study resulted in a very low response when compared with the sample strokes number, and therefore, those conditions that originated an apparent better response (considering the main effects plots) were chosen: number of washes (3 × 50 μL) and number of elution cycles (6 × 100 μL). The monitoring of the experimental design through the central point resulted in relative standard deviations (RSDs) of 5.9 and 3.2% for EDDP and methadone, respectively.

Once the number of withdraw–dispense cycles appeared as a significant factor, it seemed pertinent to evaluate if the increment of these cycles number above nine would result in the improvement of the recoveries. A subsequent study was made, in which the number

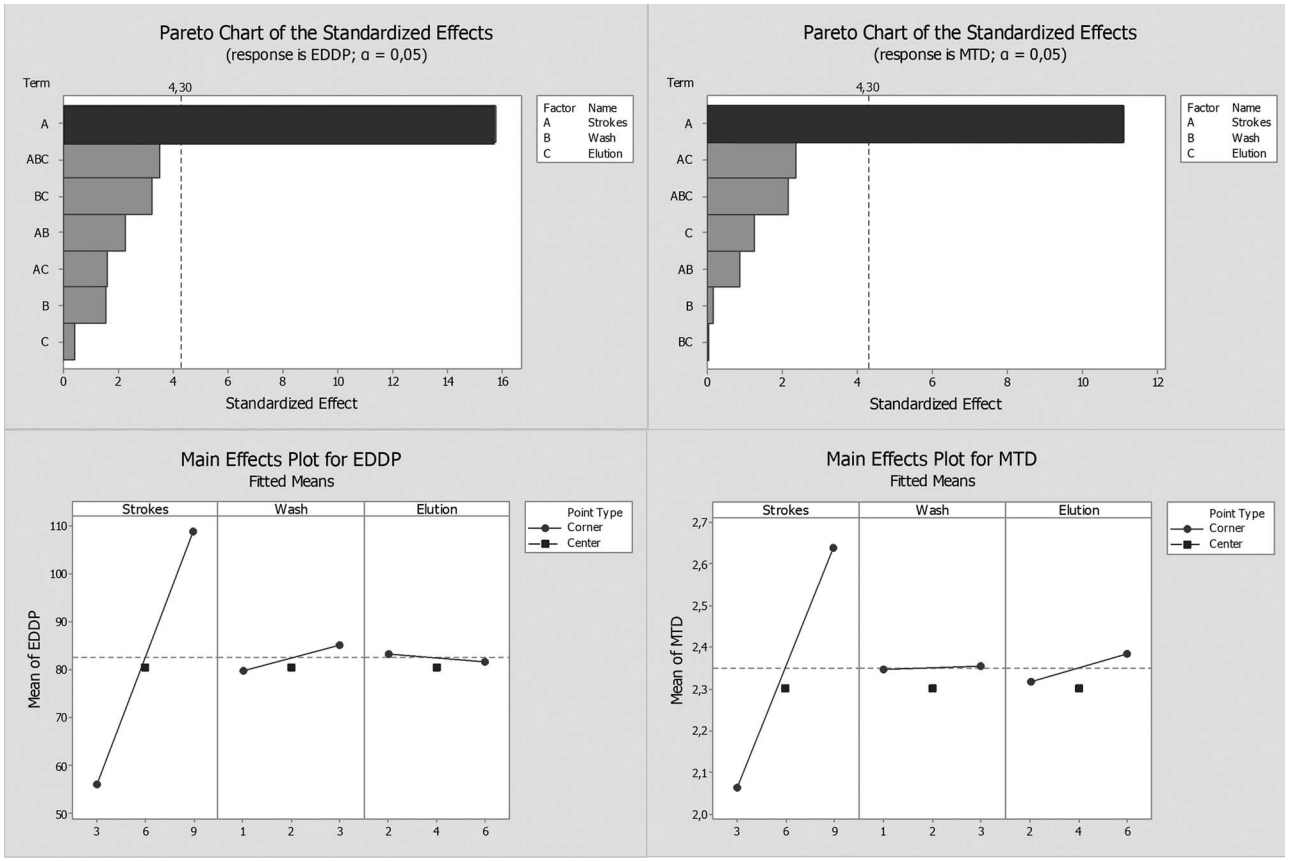

Figure 1. Pareto charts and main effects plots obtained for EDDP and methadone after experimental design.

of strokes was increased up to 18, while the other conditions were kept unchanged.

Figure 2 shows the graphical representation of the obtained results, when 9, 12, 15 and 18 strokes were applied on the sample load step ($n = 3$). One observes that, although recoveries increased with the number of strokes, the related-samples Friedman's two-way analysis of variance by ranks gave no statistical difference for either EDDP ($P = 0.068$) or methadone ($P = 0.060$).

After the MEPS steps optimization, and according to the reported results, the final procedure to clean-up hair extracts in order to determine EDDP and methadone was obtained.

Method Validation

Selectivity

Selectivity of the method was evaluated by the analysis of blank hair samples from 10 different origins (laboratory staff). These samples were analyzed and checked for possible interferences at the retention times and selected transitions of the analytes. Identification criteria for positivity included the use of ion ratios, retention times and signal-to-noise evaluation. The maximal accepted deviations for the studied parameters were those specified in the World Anti-Doping Agency's document (55). By using these criteria, no analyte could be identified in any of the analyzed samples. Figure 3 represents a chromatogram of a blank hair sample (on the left) and a chromatogram of a sample spiked at 0.01 ng/mg—the lower limit of quantification (LLOQ)—(on the right). By means of these criteria, the method was considered selective, since no compound could be identified in the blank hair specimens.

Calibration curves and limits

Calibration curves were constructed by plotting the ratio between each target compound and the respective IS peak areas against compound concentration. As acceptance criteria, a determination coefficient (R^2) of at least 0.99 and the calibrators' accuracy within a ±15% interval from the nominal value (± 20%, for the LLOQ) were adopted. Method linearity was, then, obtained in the range of 0.010–5 ng/mg for EDDP and methadone ($n = 5$). However, variational approach was adopted relying on a weighted least squares criterion (1/x) to compensate for heterocedasticity.

The LLOQ was considered the lowest concentration that could be measured with an RSD equal or lower than 20% and a relative error (RE, %) within ±20% of the nominal concentration. Considering the above, 0.010 ng/mg was obtained as the LLOQ for both EDDP and methadone with the present analytical method. Table II resumes calibration data.

The LLOQs reached with the present method can be considered good, comparing with the available literature that had the same goals. For instance, Concheiro et al. (26) used the same amount of hair, 50 mg, to determine cocaine, heroin and methadone to evaluate *in utero* drug exposure and reported an LLOQ of 0.020 ng/mg for methadone. The authors used both classic clean-up procedures, first LLE and then SPE, and analysis was carried out with liquid chromatography coupled to tandem mass spectrometry (LC–MS-

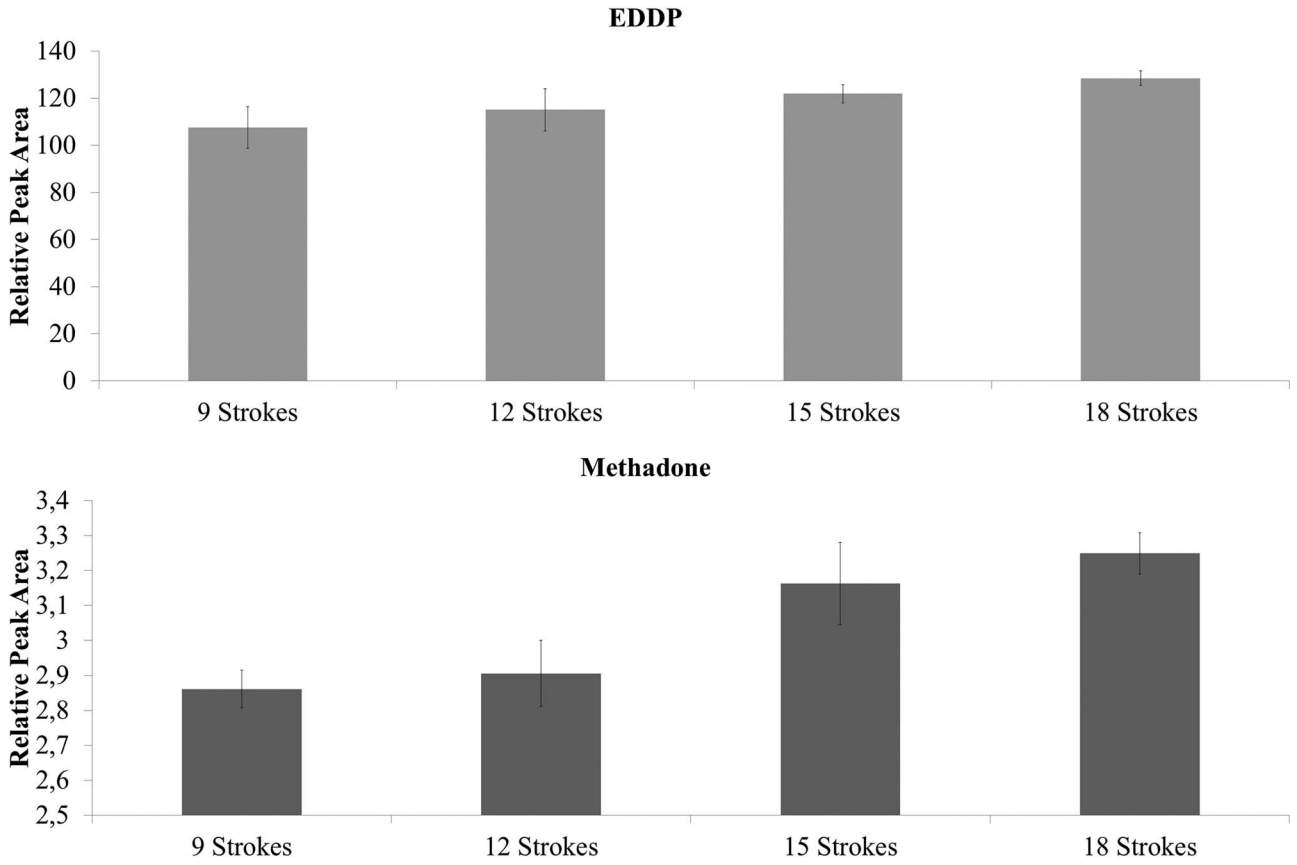

Figure 2. Graphical representation of number of strokes influence on EDDP and methadone recoveries ($n = 3$).

Table II. Linearity data ($n = 5$)

Analyte	Weight	Linear range (ng/mg)	Linearity slope*	Intercept*	R^2*	LLOQ (ng/mg)
EDDP	1/x	0.010–5	0.159 ± 0.0109	0.017 ± 0.7882	0.9965 ± 0.0025	0.010
Methadone	1/x	0.010–5	0.001 ± 0.0006	0.001 ± 0.0145	0.9970 ± 0.0012	0.010

*Mean values ± standard deviation.

MS). The same working group had already developed a multimethod for target screening and confirmation of 35 drugs and metabolites in 50 mg of hair by LC–MS-MS, again using LLE followed by SPE, and reported identical LLOQ for methadone (18). Additionally, De la Torre et al. (28) developed a high throughput analysis of drugs of abuse in 50 mg of hair, using SPE as clean-up technique and GC–MS, obtaining an LLOQ of 0.1 ng/mg for both EDDP and methadone. Skender et al. (30) performed a quantitative determination of amphetamines, cocaine and opiates in 50 mg of hair using SPE followed by GC–MS and achieved a limit of detection (LOD) of 0.3 ng/mg. Girod and Staub (31) determined methadone and EDDP in 50 mg of hair from human subjects following a maintenance program. The authors used SPE for sample clean-up with analysis by GC–MS and reported LLOQs of 0.05 ng/mg for methadone and 0.2 ng/mg for EDDP. Bermejo et al. (21) had the goal to simultaneously determine methadone, heroin and metabolites in 50 mg of hair using LLE followed by GC–MS and obtained an LLOQ of 0.13 ng/mg for methadone.

All these authors used a classical clean-up procedure after hair samples incubation, but several other researchers excluded this additional step, still achieving higher LLOQs than those obtained in the herein described method. Di Corcia et al. (11) published the simultaneous determination of multiclass drugs of abuse, also in 50 mg of hair, by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS-MS). These authors used no clean-up procedure after hair incubation and achieved an LLOQ 0.03 ng/mg for methadone. Musshoff et al. (14), with no clean-up added after incubation of 50 mg of hair, had the goal to determine opioid analgesics using LC–MS-MS with application to patients under palliative care and achieved an LLOQ of 0.03 ng/mg for methadone. Kelly et al. (15) used a greater amount of hair, 75 mg, to perform a chiral analysis of methadone and major metabolites by LC–MS-MS. The authors also described a sample preparation that consisted only of incubation and no additional clean-up procedure, obtaining LLOQs of 0.05 and 0.03 ng/mg for methadone and EDDP, respectively.

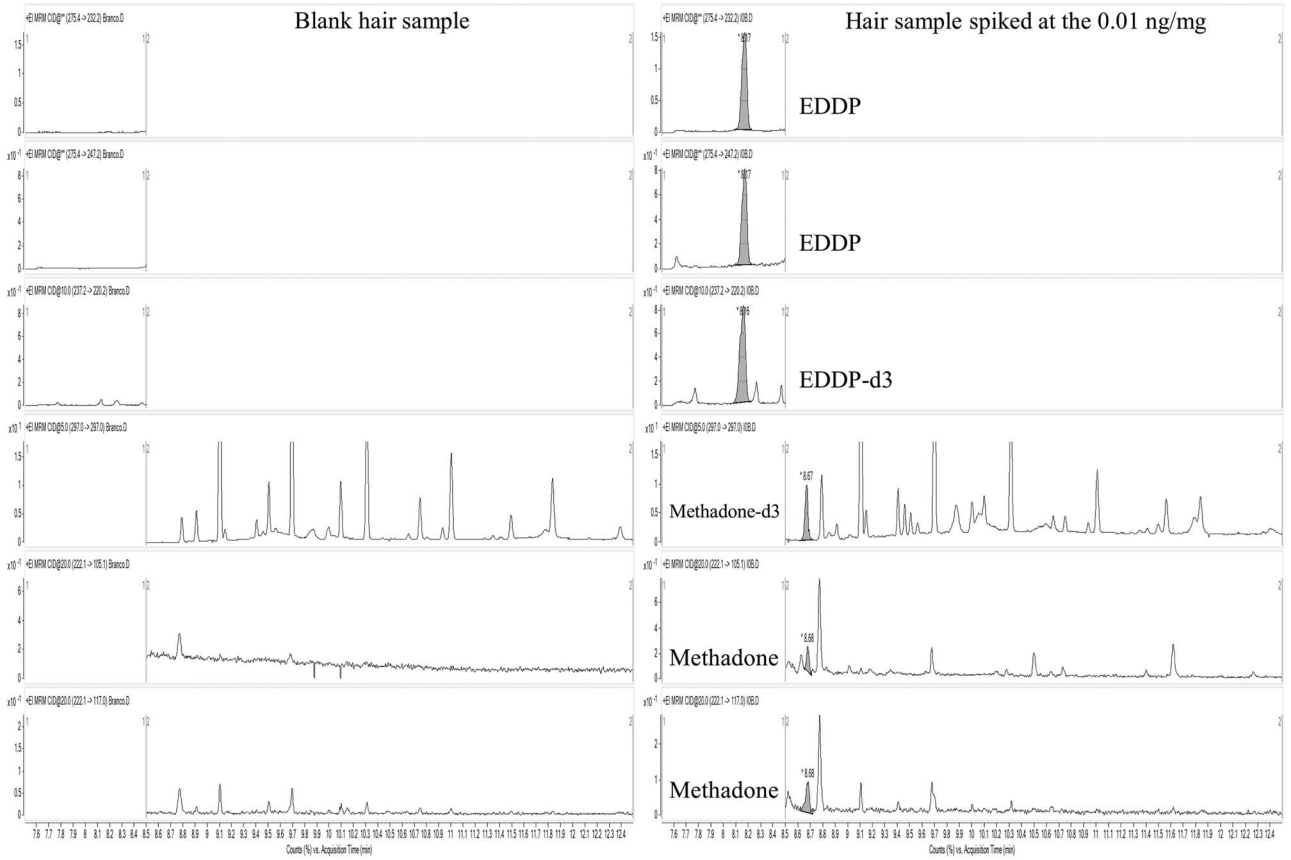

Figure 3. Chromatogram of a blank hair specimen and a hair specimen spiked at 0.01 ng/mg.

It is consensual that the amount of hair sample used may influence the limits of determination of an analytical method. Although the amount of 50 mg is widely applied, nowadays, there is a tendency for the reduction of the sample weight. Tournel et al. (17) used 20 mg of hair to determine methadone exposure in pediatric deaths with LLE and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS), reporting a LOD of 0.1 ng/mg for both compounds. Also, Fernández et al. (25) used 20 mg of hair for the simultaneous analysis of 33 basic drugs in hair. These authors used SPE as clean-up procedure and UHPLC–MS-MS for analysis reaching an LLOQ of 0.03 ng/mg. These limits are also higher than those presented in this work. However, a method development for methadone and other illegal drugs in 20 mg of hair from children with parents under maintenance treatment in a German community was performed by Pragst et al. (56) and should be highlighted. These authors used a liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry and obtained LLOQs of 0.001–0.003 ng/mg for the target compounds, values much lower than the ones achieved with the present work. The greater sensitivity was achieved with the use of a high-resolution detector.

Nevertheless, the amount of 10 mg of hair sample is also extensively applied for the accurate measure of these analytes (9, 12, 20, 27, 57) with LLOQs reported in the range of 0.01–0.1 ng/mg, values equal or greater than those herein described. To the best of our knowledge, the fewer amounts of hair samples used to determine methadone are described by three authors. Leung et al. (8) performed a surveillance of drug abuse in Hong Kong by the analysis of 5 mg of hair using LC–MS-MS, obtaining an LLOQ of 0.04 ng/mg for methadone. Sheibani et al. (58) determined methadone in 2 mg of human hair by headspace extraction and ion mobility spectrometry with an LLOQ of 0.03 ng/mg. Lastly, and of great interest, is the work by Zhu et al. (10) that presents a microfluidic chip based on nano liquid chromatography coupled to tandem mass spectrometry (nano-HPLC–Chip-MS-MS) for the determination of abused drugs and metabolites in 2 mg of human hair, reporting an LLOQ of 0.0005 ng/mg.

Regarding miniaturized procedures, only SPME and LPME have been described in order to determine EDDP and methadone in hair specimens, all of them resulting in greater LLOQs than ours. SPME differs from MEPS on the diffusion of the analytes mediated by stirring, in the first, or flow-through, in the second (54). Aleksa et al. (38) proposed the simultaneous detection of 17 drugs of abuse and metabolites in 10 mg of hair using headspace SPME (HS-SPME) and GC–MS achieving an LLOQ of 0.6 ng/mg for methadone. The authors used the most commonly applied 100-µm polydimethylsiloxane (PDMS) fiber, a non-polar fiber adding yet another level of selectivity and a reduction in background noise. Merola et al. (39) proposed the same miniaturized technique and fiber to determine different recreational drugs in 10 mg of hair with GC–MS obtaining an LLOQ of 0.16 ng/mg. Also, Musshoff et al. (5) with the same technique and fiber studied the dose–concentration relationships of EDDP and methadone in 10 mg of hair belonging to patients on a maintenance program. The authors reported LLOQs of 0.05 and 0.3 ng/mg for EDDP and methadone, respectively, using a GC–MS. Gentili et al. (40) aimed at a rapid screening procedure based on HS-SPME (100-µm PDMS) and GC–MS for the detection of many

recreational drugs in 20 mg of hair, achieving an LLOQ of 1.05 ng/mg for methadone. A different fiber was, however, used by Sporkert and Pragst (42) for the determination of methadone and EDDP in 10 mg of human hair by HS-SPME and GC–MS. The authors described a clean-up procedure using a 65-µm PDMS/divinylbenzene fiber, obtaining an LLOQ of 0.16 and 0.1 ng/mg for EDDP and methadone, respectively. Lucas et al. (41) used another variant of SPME with direct immersion (DI-SPME) for the determination of methadone and EDDP in 50 mg of human hair by GC–MS. The fiber used was, again, a 100-µm PDMS, achieving LLOQs of 0.36 ng/mg for EDDP and 3.46 ng/mg for methadone. When compared with MEPS, the SPME approach has often been mentioned as hardly suitable for high-throughput applications, mainly because of the long time required to establish equilibrium and the resultant low absolute recoveries obtained. The lower recoveries might justify the greater LLOQs obtained in the works described (54, 59).

Other adopted miniaturized technique was the surfactant-enhanced (SE) LPME, described by Yazdi and Es'haghi (37) to help determine basic drugs of abuse in 50 mg of hair. With this technique, the analytes were concentrated through an aqueous solution (donor phase) into an organic liquid immobilized within the pores of 2.0-cm length of polypropylene hollow fiber before they were trapped with the aqueous acceptor phase, contained within the lumen of the porous hollow fiber. The authors reported an LLOQ of 16 ng/mL for methadone when 2 mL of methanol was added to 50 mg of hair in incubation.

Intra-day, inter-day and intermediate precision and accuracy

The evaluation of the inter-day precision and accuracy was performed within a 5-day period at eight concentration levels, the same levels applied to build the calibration curve. The obtained coefficients of variation (CVs) were typically lower than 8% for methadone at the tested concentration levels with an accuracy within a ±12% interval, except for the LLOQ, for which an accuracy within a ±20% interval was obtained. Regarding EDDP, the CVs observed were typically lower than 11%, with exception of LLOQ (lower than 16%). The accuracy was within a ±5% interval for all tested concentration levels.

The intra-day precision and accuracy were evaluated by the analysis, on the same day, of six replicates of blank hair samples spiked at four concentration levels, LLOQ included. The observed CVs were lower than 9 and 13% for EDDP and methadone, respectively, at all studied concentrations, both analytes measurements resulting in a mean RE within ±9%. Lastly, intermediate (combined intra- and inter-day) precision and accuracy were also evaluated with the help of quality control (QC) samples at three concentration levels (0.035, 0.75 and 3.5 ng/mg). This study involved the preparation of the QC ($n = 3$) samples and their simultaneous analysis with the calibration curves on the 5-day period ($n = 15$). The measurement of EDDP QCs resulted in CVs typically lower than 10% and accuracy within ±10% interval. Regarding methadone, the obtained CVs were usually equal or lower than 9% with an accuracy within ±11% interval. All data are shown in Table III.

Recovery

Recoveries of the clean-up step for EDDP and methadone were studied by comparing two sets of samples. The first set involved blank hair extracts spiked with the target compounds, prior to MEPS procedure, while in the second set of samples, the spike only occurred after the MEPS procedure. This study was performed at three concentration levels: 0.035, 0.75 and 4 ng/mg ($n = 3$). The ISs were added to both sets only after MEPS procedure. The ratio of the relative peak areas obtained in the first set with those obtained for the second set allowed the calculation of the mean recoveries. Overall, the recoveries obtained with the MEPS procedure were very good, ranging from 84 to 110% for EDDP and 73 to 109% for methadone (Table IV).

The recovery values are comparable to those reported by Concheiro et al. (26) who used LLE followed by SPE with Strata X cartridges and obtained recoveries greater than 85% for methadone. The same extraction techniques were adopted by Lendoiro et al. (18) resulting in recoveries ranging from 102 to 106% for methadone. Girod et al. (31) used the classic technique SPE with Isolute HCX cartridges for hair analysis of human subjects following methadone maintenance program, reporting recoveries between 80 and 86% for both methadone and EDDP. Also, Barroso et al. (27) presented recoveries from 90 to 99% for both compounds, using SPE Oasis® MCX cartridges. Slightly lower recoveries, even still comparable, were those presented by Moeller et al. (23) with SPE Chromabond C_{18} cartridges, 70–80% for EDDP and methadone. Regarding LLE extractions, similar results to the herein presented were obtained by Bermejo et al. (21), who used ToxiTubes A® for the simultaneous determination of methadone, heroin and metabolites in hair, obtaining recoveries of 85% for methadone. The same technique was used by Wilkins et al. (22) for a quantitative analysis of methadone and two major metabolites in hair, resulting, however, in lower recoveries, 70% for both compounds.

Considering the above, it is fair to assume that the proposed MEPS procedure is a quite efficient technique to be applied on routine hair samples clean-up. It can undoubtedly assure great recoveries for methadone and EDDP, being in accordance with green chemistry standards, such as the environmentally friendly procedure due to lower organic solvents consumption.

If compared with other miniaturized techniques described for the same goal, the present work results in similar recoveries to those shown by Gentili et al. (40) who used HS-SPME with PDMS 100-µm fiber and obtain recoveries of 98% for methadone. Additionally, Lucas et al. (41) adopted DI-SPME for the determination of methadone and EDDP in human hair and achieved recoveries between 102 and 107% for EDDP and methadone, respectively. Also, Yazdi and Es'haghi (37) proposed a clean-up method for basic drugs of abuse in hair with SE-LPME and showed recoveries from 89 to 93% for methadone. However, as previously mentioned, SPME has also been known for its resultant low absolute recoveries. This is observed in the work of Merola et al. (39) that applied HS-SPME to determine different recreational drugs in hair reporting a recovery of 9.5% for methadone. Sporkert and Pragst (42) also used HS-SPME, although with a different fiber, aiming at the determination methadone and its metabolites in human hair, and achieve recoveries of 10.5–14.5% for both EDDP and methadone. Lastly, Lachenmeier et al. (57) described a method with headspace solid-phase dynamic extraction for the determination of drugs of abuse in hair samples and obtained recoveries of 16 and 23.5% for EDDP and methadone, respectively.

When comparing the proposed MEPS method in this work with other miniaturized techniques, specially SPME, the present work can be associated with good recoveries, high sensitivity, low carry over and low cost. On the other hand, SPME is commonly known for its low recovery, low sensitivity, high carry over and great costs associated (54).

Table III. Inter- and intra-day and intermediate precision and accuracy

Analyte	Spiked	Inter-day precision and accuracy ($n = 5$)			Intra-day precision and accuracy ($n = 6$)			Intermediate precision and accuracy ($n = 15$)		
		Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)
EDDP	0.01	0.01 ± 0.002	16.00	4.08	0.01 ± 0.004	4.72	−8.84			
	0.025	0.025 ± 0.003	10.27	0.61	0.026 ± 0.002	6.91	5.91			
	0.035							0.035 ± 0.003	9.54	0.72
	0.05	0.05 ± 0.002	3.85	−4.25						
	0.1	0.10 ± 0.003	2.63	−3.49						
	0.5	0.51 ± 0.015	3.01	1.25	0.48 ± 0.035	7.33	−4.49			
	0.75							0.75 ± 0.064	8.45	0.54
	1	1.03 ± 0.008	0.77	3.40						
	2.5	2.46 ± 0.262	10.67	−1.78						
	3.5							3.83 ± 0.193	5.05	9.24
	5	5.01 ± 0.259	5.18	0.19	5.13 ± 0.424	8.27	2.58			
Methadone	0.01	0.01 ± 0.000	10.58	19.55	0.01 ± 0.001	12.09	2.24			
	0.025	0.026 ± 0.002	7.38	5.99	0.026 ± 0.002	9.32	2.95			
	0.035							0.034 ± 0.003	9.00	−4.01
	0.05	0.05 ± 0.002	4.72	−8.98						
	0.1	0.09 ± 0.004	4.97	−11.28						
	0.5	0.51 ± 0.009	1.74	2.56	0.54 ± 0.039	7.25	8.63			
	0.75							0.67 ± 0.023	3.42	−10.57
	1	0.92 ± 0.034	3.65	−7.77						
	2.5	2.42 ± 0.055	2.29	−3.24						
	5	5.16 ± 0.035	0.69	3.16	5.37 ± 0.260	4.83	7.49	3.58 ± 0.203	5.66	2.34

All concentrations in ng/mg; CV—Coefficient of variation; RE—Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; Mean values ± standard deviation.

Table IV. Recovery (%) of EDDP and methadone under the optimized MEPS procedure ($n = 3$)

Analyte	Concentration (ng/mg)		
	0.035[a]	0.75[a]	4[a]
EDDP	84.22 ± 7.74	99.33 ± 6.44	110.69 ± 10.63
MTD	84.16 ± 8.42	73.17 ± 7.31	109.21 ± 9.43

[a]Mean values ± standard deviation.

Stability

Hair samples differ from other human specimens, such as blood or urine, used for toxicological analysis, due to its solid and durable nature, strong tissue, being less affected by adulterants (6, 60). Once drugs are incorporated into the hair, they remain fixed as hair grows, and for this reason, the sample can be collected and stored at room temperature (61). Nevertheless, after hair samples extraction through incubation and after the adopted clean-up MEPS procedure, the extracts should reveal enough stability over the anticipated run time for batch size. In this sense, it is important to evaluate the so-called stability of processed samples, also known as auto-sampler stability. This study was performed at the QC concentration levels ($n = 3$) by the re-analysis of these samples after a period of 24 h unassisted in the auto-sampler. Their concentrations were determined on the basis of a newly prepared calibration curve on the day of re-analysis. Both EDDP and methadone presented a good stability in the extracts over the period of 24 h. The results obtained on the stability assay are shown on Table V. The CVs obtained were typically lower than 10% with RE within a ±7% interval for EDDP, while methadone is measured with CVs commonly lower than 14% and with an RE within ±8% interval. The latter assures the possibility of a re-analysis after 24 h in the auto-sampler with no significant change in the concentration determination of the two target compounds.

Method applicability

Method applicability was verified by the analysis of authentic hair samples obtained from two individuals undergoing methadone treatment program at Centro de Atendimento ao Toxicodependente—Casas de Santiago (Belmonte, Portugal). As example, Figure 4 represents the chromatogram obtained from the analysis of one of those samples, positive for EDDP and methadone with measured concentrations of 0.11 and 0.37 ng/mg, respectively.

The same two specimens were additionally assessed as described by Barroso et al. (27) who used a mixed-mode SPE for hair samples clean-up to determine EDDP and methadone. The results obtained by the reproduction of Barroso et al. method were, then, compared with those obtained in the present work, and the obtained concentrations were similar (the resulting CVs were lower than 5% for EDDP and 9% for methadone). Thus, the proposed MEPS procedure may be considered as a great alternative to SPE, due to lower solvent volumes

Table V. Analyte stability in processed samples ($n = 3$)

Analyte	Spiked	Measured	CV (%)	RE (%)
EDDP	0.035	0.037 ± 0.003	9.56	5.70
	0.75	0.700 ± 0.069	9.92	−6.61
	3.5	3.693 ± 0.286	7.76	5.51
Methadone	0.035	0.037 ± 0.003	6.94	5.98
	0.75	0.692 ± 0.059	8.50	−7.73
	3.5	3.505 ± 0.464	13.25	0.14

All concentrations in ng/mg; CV—Coefficient of variation; RE—Relative error [(measured concentration-spiked concentration/spiked concentration)] x 100; Mean values ± standard deviation.

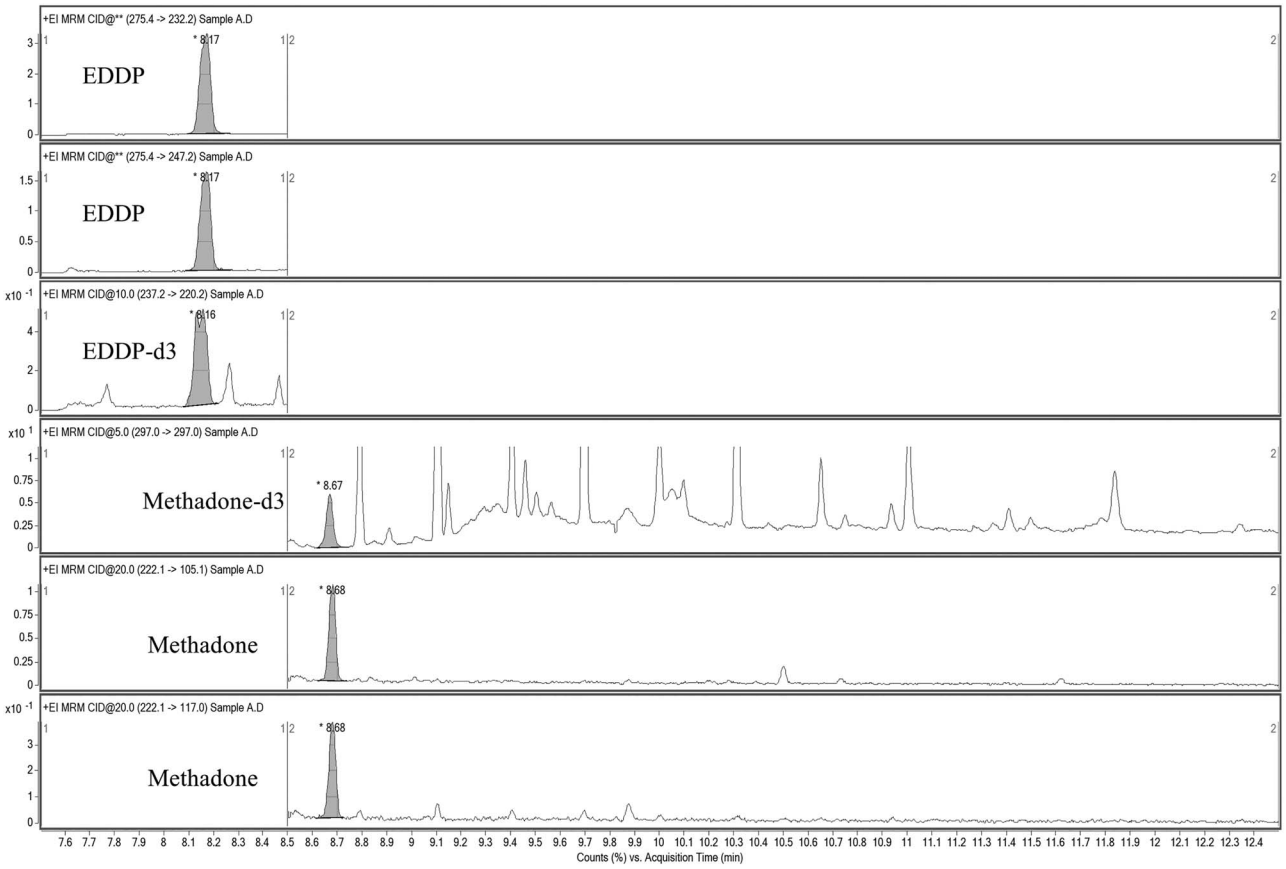

Figure 4. Chromatogram of authentic hair sample positive for EDDP and methadone.

consumption and the possibility of sorbent reusage (approximately 100 samples clean-up).

Conclusions

This work describes the application of MEPS for the determination of methadone and EDDP in hair by GC–MS-MS. The MEPS procedure was optimized, and the analytical method fully validated. Overall, the procedure has proven to be simple with an ease and fast operation, selective, precise and accurate. Additionally, MEPS resulted in great recoveries (73–110%), high sensitivity, low carry over and low cost. The method was linear between 0.010 and 5 ng/mg for both compounds, with the LLOQ assured at 0.010 ng/mg, and was successfully applied to the analysis of hair samples from patients undergoing a methadone maintenance program.

The developed method results in the first work to couple MEPS to GC–MS-MS for the determination of methadone and EDDP in hair samples, resulting in a great alternative to the classic clean-up techniques, such as LLE and SPE.

Acknowledgments

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Paper V - New miniaturized clean-up procedure for hair samples by means of microextraction by packed sorbent: determination of cocaine and metabolites

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RESEARCH PAPER

New miniaturized clean-up procedure for hair samples by means of microextraction by packed sorbent: determination of cocaine and metabolites

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Abstract

Cocaine is still one of the most commonly used illicit substances worldwide, with an estimated 4 million users in Europe in the last year. Hair samples have been widely used for the determination of episodic or repeated consumption of this substance, but the use of miniaturized techniques for hair sample clean-up has been challenging due to the sample complexity. Despite hair's complex matrix, MEPS provides a method that is fast, reduces the volume of extraction solvents used, and offers low-cost options (since extraction beds may be reused several times). Microextraction by packed sorbent using a mixed-mode sorbent was optimized for hair sample clean-up in order to determine cocaine, benzoylecgonine, ecgonine methyl ester, norcocaine, cocaethylene and anhydroecgonine methyl ester by gas chromatography coupled to tandem mass spectrometry. The method was fully validated according to internationally accepted criteria, presenting good linearity between the limits of quantification (0.01–0.15) and 5 ng/mg. Precision and accuracy resulted in coefficients of variation typically lower than 15%, with mean relative errors within ±15% for all compounds, except for the limit of quantification (±20%). The present work describes the first application of microextraction by packed sorbent for the concentration of cocaine and metabolites extracted from hair samples.

Keywords Microextraction by packed sorbent · Cocaine and metabolites · Hair testing

Introduction

According to the latest report of the European Monitoring Centre for Drugs and Drug Addiction [1], the cocaine (COC) market represents the second largest illicit drug market in Europe, with an estimated 4 million having used cocaine within the last year. Although COC in the form of hydrochloride has been around for decades, commonly used through nasal insufflation, free-basing crack cocaine as a smokable form has been associated with a younger and socioeconomically marginalized population [2].

Hair samples have been considered useful alternatives for drug monitoring in both forensic and workplace testing programs. This matrix presents advantages such as a longer window for drug detection when compared to urine. The extended window affords the possibility to document past drug use, and to monitor users under treatment programs [3, 4]. Additionally, the collection of hair is non-invasive, and is easily supervised, with minimal risk of sample adulteration; hair is also a matrix that offers drug stability and is easily transported and stored. Depending on the hair length, segmental analysis may be used to determine approximately when drug exposure occurred [3–7].

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Substances presumed to be specific markers for COC consumption are its hydrolytic metabolites, benzoylecgonine (BEG) and ecgonine methyl ester (EME). However, these metabolites may also be produced by spontaneous hydrolysis, and therefore several further metabolites may be used to adequately suggest the actual ingestion of the drug. Norcocaine (NCOC) is a metabolite originating from enzymatic demethylation of COC. Cocaethylene (COET) is a transesterification metabolite when ethanol and cocaine are ingested together, and anhydroecgonine methyl ester (AEME) is a specific pyrolysis product that is formed when COC is smoked, and may also be detected [8–10].

These substances are most commonly isolated from hair matrix by direct extraction with organic solvents, usually methanol [10, 11], or acid digestion with hydrochloric acid [9, 10, 12–14]. However, methanol extractions can provide lower recoveries and yield considerable interferences when compared to other procedures [5, 15, 16]. Many authors opt to analyze the resulting extract directly, trusting in the method's sensitivity, but the Society of Hair Testing (SoHT) guidelines [16] recommend that for confirmation procedures, further clean-up should be applied. The most widely adopted clean-up procedures are the classic solid-phase extraction (SPE) and traditional liquid–liquid extraction (LLE) [10]. In recent years, modern sample clean-up procedures have trended toward miniaturization, reducing the amount of organic solvents, sample, and steps required, and if possible, using automation and online coupling [17]. Several miniaturized clean-up procedures have been published, but only a few have been applied to COC hair analysis. The most frequently reported method is solid-phase microextraction (SPME) [18–21], although it is not free of drawbacks, such as solvent instability, low operating temperatures, and stripping of coatings [22]. Miniaturization for this purpose was also successfully accomplished with hollow-fiber liquid-phase microextraction (HF-LPME) [23].

Microextraction by packed sorbent (MEPS) is a miniaturized format for SPE, using the same commercially available sorbents. The sorbent bed is incorporated into a syringe, enabling manipulation of small amounts of sample. This technique is known to be simple and fast, requires low solvent volumes, and can be coupled online. Economically, the major advantage is the possibility for sorbent reuse [24]. To date, only three hair clean-up procedures have used the MEPS technique, all of them reporting its usefulness in hair analysis and successful application to authentic samples [25–27]. None of these worked with COC and its several metabolites.

The aim of this work was to optimize and validate a MEPS clean-up procedure for the determination of COC, BEG, EME, NCOC, COET and AEME in hair samples by GC-EI-MS/MS.

Materials and methods

Reagents and standards

Analytical standards of COC, BEG, EME, NCOC, COET and AEME, and internal standards (ISs) COC-d_3, BEG-d_3 and EME-d_3 were acquired from Sigma-Aldrich (Lisbon, Portugal). The analytical standards were obtained as 1 mg/mL solutions, except the IS (0.1 mg/mL). Working solutions of analytical standards were prepared by dilution with acetonitrile to the final concentrations of 10 and 0.5 μg/mL, while IS was diluted to 0.5 μg/mL working concentration. Both stock and working solutions were stored at −20 °C in the absence of light. Methanol (Merck Co, Darmstadt, Germany), acetonitrile (Prolabo, Lisbon, Portugal), 2-propanol (Fischer Chemical, Loughborough, UK), hydrochloric acid (Panreac Química SA, Barcelona, Spain), formic acid (Panreac Química SA, Barcelona, Spain) and ammonium hydroxide (J.T. Baker, Deventer, Netherlands) were pro-analysis grade. Deionized (DI) water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Potassium acetate was from Sigma-Aldrich (Lisbon, Portugal), and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and trimethyl chlorosilane (TMS) were purchased from Macherey-Nagel (Düren, Germany). The MEPS syringe (250 μL) and M1 cartridges (4 mg; 80% C8 and 20% SCX), all from SGE Analytical Science, were acquired from ILC (Porto, Portugal). A Samsung microwave oven (800 W; Lisbon, Portugal) was used for derivatization. Lastly, during optimization parameter evaluation, Minitab Statistical Software version 17 and SPSS version 25 were used.

Hair samples

For MEPS procedure optimization and method validation, blank hair samples for the target substances were used. These were kindly donated by laboratory staff of the Health Sciences Research Centre (CICS, Covilhã, Portugal). Authentic hair samples were obtained from drug users [Centro de Atendimento ao Toxicodependente - Casas de Santiago (Belmonte, Portugal)] and from proficiency testing schemes. Prior to any procedure related to the proposed analytical method, all samples were stored in tracing paper envelopes, and kept at room temperature away from direct light.

Gas chromatographic and mass spectrometric conditions

An HP 7890A gas chromatography system coupled to a 7000B triple quadrupole mass spectrometer, both from Agilent Technologies (Waldbronn, Germany), was used to separate and determine COC and metabolites. The injection was performed using a Gerstel MPS2 autosampler and PTV

injector (Mülheim an der Ruhr, Germany). A capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) with 5% phenylmethylsiloxane (HP-5 MS) from J&W Scientific (Folsom, CA, USA) was used. The injection port and the transfer line temperatures were set to 240 °C and 280 °C, respectively. The oven temperature started at 90 °C for 2 min, after which it was increased at a rate of 20 °C/min rate to 300 °C, and held for 3 min. The total run time was 15.5 min. Sample extracts were injected in the splitless mode, and the carrier gas (helium) had a constant flow of 0.8 mL/min.

For analyte determination, the mass spectrometer was operated in positive EI mode with a filament current of 35 μA and electron energy of 70 eV. The flow rate of nitrogen (collision gas) was set at 2.5 mL/min. Data acquisition was performed in multiple reaction monitoring (MRM) mode using MassHunter WorkStation acquisition software version B.02.01 (Agilent Technologies). Optimization of tandem mass spectrometry conditions was performed by injection of derivatized standard solutions at different collision energies and dwell times. Transitions were selected based on both selectivity and abundance, so that the maximum signal-to-noise ratio would be observed in hair extracts. The final acquisition conditions are listed in Table 1.

Sample preparation

Hair decontamination and extraction

The hair outer surface was cleaned of dirt and external contamination by sequentially washing samples with dichloromethane, deionized water and methanol, 3 mL each for 5 min with agitation. Subsequently, the samples were left to dry at room temperature, and the last wash solvent was stored for further analysis.

Hair samples were cut into approximately 1-mm fragments, and 50 mg was weighed into glass tubes. Acid digestion was performed by adding to each tube 1 mL of 0.1 M hydrochloric acid, followed by vortexing and incubation overnight at 60 °C. After cooling, neutralization was achieved by the addition of 100 μL of 1 M sodium hydroxide, again with vortex mixing. The samples were subsequently centrifuged at 3500 rpm for 15 min, and the extracts were decanted to new clean glass tubes, to which 25 μL of IS working solution was added.

Clean-up procedure by MEPS

The extract clean-up procedure was performed with MEPS technique, after which optimization (see results and discussion section) resulted in the following sequential steps: conditioning of the M1 sorbent with 250 μL of methanol followed by 250 μL of deionized water; sample loading was performed with 21 draw-eject cycles of 150 μL; the sorbent was rinsed with 50 μL of deionized water and acetate buffer of pH 4; sorbent was dried using three 50-μL draw-ejects of air; and the analytes were eluted with three cycles of 100 μL of 2% ammonium hydroxide in methanol. The eluted clean extracts were evaporated to dryness under a gentle stream of nitrogen, after which 50 μL of MSTFA with 5% TMS was added to the dry extracts, and microwave-assisted derivatization was performed at 800 W for 2 min. A 2-μL aliquot of the solution was injected into the chromatographic system.

To reuse the sorbent, after extract clean-up, sequential washing steps were performed with two cycles (250 μL) of each of the following solvents: 1% ammonia in methanol–acetonitrile (50:50, *v*/v) and 1% formic acid in 2-propanol–water (10:90).

Validation procedure

The analytical method was fully validated following a 5-day protocol. The evaluated parameters included selectivity,

Table 1 Retention times and selected transitions for the identification of COC and metabolites in hair samples

Analyte	Retention time (min)	Quantitative transition (m/z)	Qualitative transition (m/z)	Collision energy (eV)	Dwell time (μs)
AEME	7.38	151.5–92.0	180.5–152.1	20 (15)	50
EME	8.50	271.5–83.1	181.3–82.0	5 (10)	50
EME-d3	8.50	274.5–86.1	–	10	50
COC	11.97	182.5–82.2	182.5–150.1	10 (5)	50
COC-d3	11.96	184.1–85.0	–	10	50
COET	12.21	196.5–82.0	196.5–150.2	10 (5)	50
BEG	12.25	239.6–82.2	239.6–122.2	15 (15)	50
BEG-d3	12.24	241.9–85.1	–	15	50
NCOC	12.34	178.1–105.1	178.1–135.1	15 (10)	50

*() collision energy used for the qualitative transition

linearity and limits, intra- and inter-day and intermediate precision and accuracy, recovery, matrix effects and autosampler stability. All validation parameters were assayed according to the guiding principles of the US Food and Drug Administration (FDA) [28] and the Scientific Working Group for Forensic Toxicology (SWGTOX) [29], also taking into account the particular characteristics of the hair specimen.

Results and discussion

Optimization of the MEPS procedure

Optimizing the MEPS procedure is one of the most important evaluations to perform prior to analysis. A full optimization will lead to better extraction efficiencies, which will furthermore allow the detection of the compounds in low concentrations, such as those commonly found in hair samples. This group previously reported an optimized MEPS technique to determine COC, BEG and EME in urine [30]; however, the hair matrix is much more complex, and when this method developed for urine was directly applied, several interferences were found, impairing adequate detection. In addition, the present method adds three new consumption markers, AEME, NCOC and COET, for which a MEPS procedure had not been optimized.

Based on the literature, the SPE approach by Cognard et al. [31] appeared to be the most promising starting point after conducting a few tests. Additionally, the method involved some of the new metabolites for which MEPS had not been used. These authors used a mixed-mode sorbent, Isolute HCX, conditioned with methanol and water. After sample loading, the cartridge was washed sequentially with water, pH 4 acetate buffer and methanol. The sorbent was dried under vacuum, and compounds were eluted with a mixture of dichloromethane–isopropanol–ammonia hydroxide (80:20:2). This approach was tested in MEPS with miniaturized volumes, also using a mixed-mode sorbent, 250 μL for conditioning, 150 μL cycles for sample load, 150 μL for washing and 100 μL cycles for elution. Nonetheless, we soon realized that methanol had to be removed from the washing step due to significant loss of the target analytes. Also, from our own experience, dichloromethane and large amounts of isopropanol passing through the barrel insert and needle (BIN) cause sorbent cavitation.

In this sense, the final solvents used for the proposed MEPS technique were methanol and deionized water for conditioning, water and pH 4 acetate buffer for washing, and 2% ammonium hydroxide in methanol for elution.

A subsequent design of experiments (DOE) analysis was adopted. DOE rapidly evaluates, in a multivariate fashion, the critical factors that can have a significant influence on the analyte recoveries. For this, a two-level full factorial design with three factors (2^3) and a central point ($n = 3$) was performed. The assayed factors were as follows: number of sample draw-eject cycles, also known as strokes (5 to 15 × 150 μL); volume of washing solvents (50 to 150 μL); and number of elution cycles (3 to 9 × 100 μL). The DOE was evaluated with blank hair samples spiked with the compounds to a final concentration of 1 ng/mg, and ISs were added after extraction.

By observation of the DOE results (see Electronic Supplementary Material (ESM) Fig. S1), it is possible to conclude from the pareto charts that the number of strokes was the only parameter with a significant impact on the recoveries of COC, COET and BEG. The main effects charts of these compounds showed greater responses when 15 draw-eject cycles of 150 μL were used for sample loading. Regarding AEME and EME, the pareto charts show that the volume of washing solvent was the only parameter with a significant influence on the response. The main effects charts present greater recoveries for AEME and EME when 50 μL of each solvent (water and pH 4 acetate buffer) was used in the washing step. Moreover, and considering all target compounds, the washing step appeared to negatively affect recoveries. Nevertheless, it is important to reduce matrix interferences; hence the minimum volume of solvent provided by the DOE matrix was selected.

Lastly, the pareto chart analysis revealed that all three evaluated parameters significantly influenced the recovery of NCOC. An overall optimal response was observed for all six compounds when 15 draw-eject cycles of 150 μL were adopted for sample loading, 50 μL for washing, and three elution cycles applied. Only AEME had a slightly better response when five strokes were used for sample loading, but there was no statistical difference between extreme points. The precision of the DOE test was considered acceptable with variation coefficients ranging from 10.8 to 15.0%.

Once the number of draw-eject cycles appeared as the most significant parameter for the majority of the studied analytes, and the maximum number resulted in better responses, it seemed pertinent to evaluate the impact it would have if we increased it. Hence, a subsequent evaluation of the number of the sample loading strokes was performed, in a univariate fashion, keeping the remaining parameters unchanged. The studied numbers of draw-eject cycles were 15, 18 and 21 ($n = 3$).

The results of this univariate assay are shown in ESM Fig. S2, and a statistical hypothesis test and related-samples Friedman's two-way analysis of variance by rank revealed no significant difference when we increased the number of strokes to 21 for AEME ($p = 0.097$), EME ($p = 0.097$), COC ($p = 0.264$), COET ($p = 0.264$) and BEG ($p = 0.097$). A statistical difference was observed, however, for NCOC when comparing 15 and 21 load draw-eject cycles ($p = 0.043$). Indeed, evaluating the graphic in ESM Fig. S2, it is possible to observe better recovery of NCOC when 21 cycles were applied, and

for this reason, validation of the analytical method proceeded with this number of strokes.

The final optimized MEPS technique was as follows: conditioning of the M1 sorbent with 250 μL of methanol and 250 μL of deionized water; the sample passed by the sorbent with 21 cycles of 150 μL; the sorbent subsequently washed with 50 μL of deionized water and 50 μL of pH 4 acetate buffer; and finally, the analytes eluted with three cycles of 100 μL of 2% ammonium hydroxide in methanol. The total extraction time commitment was about 5 min per sample.

Method validation

Selectivity

To evaluate the analytical method selectivity, ten blank hair samples belonging to different donors were analyzed. This evaluation consisted of a search for possible interferences at the selected transitions for all compounds and ISs, at the respective retention times. Positivity identification criteria followed the World Anti-Doping Agency statements [32] for maximum allowed tolerance in relative ion intensities between transitions (as a percentage of the base peak), to guarantee unequivocal identification with suitable confidence. A chromatogram with the selected extracted transitions obtained at the lower limit of quantification (LLOQ) is shown in Fig. 1, while a chromatogram of a blank hair sample is presented in ESM Fig. S3. It was possible to prove the selectivity of the proposed analytical method, since by means of those criteria no target analyte could be identified in blank hair samples.

Calibration curves and limits

The method linearity was established, through five curves, on spiked hair samples extracted and analyzed according to the proposed method in the range of 0.010–5 ng/mg for COC and COET, 0.020–5 ng/mg for EME, BEG and NCOC, and 0.15–5 ng/mg for AEME. The calibration curves were obtained from the plot between peak area ratio (ratio between analyte and the ISs) and analyte concentration. Since only three ISs were used for the six compounds, EME-d_3 was used for AEME and EME, COC-d_3 was used for COC, COET and NCOC, and finally, BEG-d3 was used for BEG. This choice was based on analyte/IS structural similarity and chromatographic behavior, ensuring none of the ISs contributed to the respective analyte signals.

The criteria for acceptance included a determination coefficient (R^2) of at least 0.99 and calibration accuracy within ±15%, with the exception of the LLOQ (for which an accuracy within ±20% was considered acceptable). Nonetheless, 1/x weighted least square regressions had to be adopted to compensate for heteroscedasticity. The LLOQ was defined as the lowest concentration calibrator that presented adequate precision and accuracy, i.e., coefficient of variation (CV) lower than 20% and a measured relative error (RE) within ±20% of the target concentration. This analytical method presented LLOQs of 0.010 ng/mg for COC and COET, 0.025 ng/mg for EME, BEG and NOC, and 0.15 ng/mg for AEME. The limit of detection (LOD) was considered equal to the concentrations adopted for the LLOQ, since it was not systematically evaluated. Table 2 presents the calibration data. These LLOQs are in accordance with the recommendations of SoHT [16] and the European Guidelines for Workplace Drug Testing [33]. None of these guidelines recommend confirmation cut-offs for AEME.

The proposed method demonstrated LLOQs that can be considered satisfactory, especially when compared to other analytical methods that adopt miniaturized clean-up procedures for hair samples. Pego et al. [23] employed HF-LPME with a Q3/2 Accurel KM polypropylene hollow fiber for clean-up of 50-mg hair extracts, reporting LLOQs of 0.5 ng/mg for COC and AEME, and 0.05 ng/mg for BEG and COET. The authors used a GC-EI-MS technique that is not as sensitive as the GC-EI-MS/MS in the present work. Apart from HF-LPME, the most widely reported extraction technique was SPME. Fucci et al. [19] applied a direct-immersion (DI) SPME with a 30-μm polydimethylsiloxane-coated fiber for clean-up of 20-mg hair extracts and reported LLOQs of 0.2 ng/mg for COC, COET and NCOC. The lower amount of hair sample used, together with a methanolic hydrolysis, which is known to yield dirtier extracts, might have contributed to this lower sensitivity. Another SPME approach is headspace (HS) sampling, and this was adopted by Poon et al. [20] for clean-up of 10-mg extracts, obtaining a LLOQ of 0.40 ng/mg for COC, BEG and NCOC. The same LLOQs were reported for COC, COET, BEG and NCOC by Aleksa et al. [21] when the same technique was applied to 10 mg of hair. Additionally, Merola et al. [34] used HS-SPME for 10-mg hair extracts and obtained LLOQs of 0.09 ng/mg for COC and 0.01 ng/mg for COET. Gentili et al. [35] reported a LOD of 0.35 ng/mg for COC on 20 mg of hair using their HS-SPME procedure. Schiavone et al. [36] opted, however, for a DI-SPME approach for clean-up of 25-mg hair extracts and obtained a LLOQ of 0.5 ng/mg for COC. These authors compared DI-SPME with supercritical fluid extraction (SFE) and concluded that the results were quite comparable, but still more advantageous then the classical LLE approach. Lastly, Bermejo et al. [37] used the same SPME approach for 50 mg of hair, and reported LLOQs of 0.4 ng/mg for both COC and COET. All the above-mentioned SPME methods applied a polydimethylsiloxane-coated fiber and a GC-EI-MS system, presenting LLOQs greater than those with our method. SPME is still successfully used for the pre-concentration of a wide range of drugs, but presents certain drawbacks, such as solvent instability and stripping of the coatings [22]. MEPS may offer some advantages in comparison, namely the possibility of

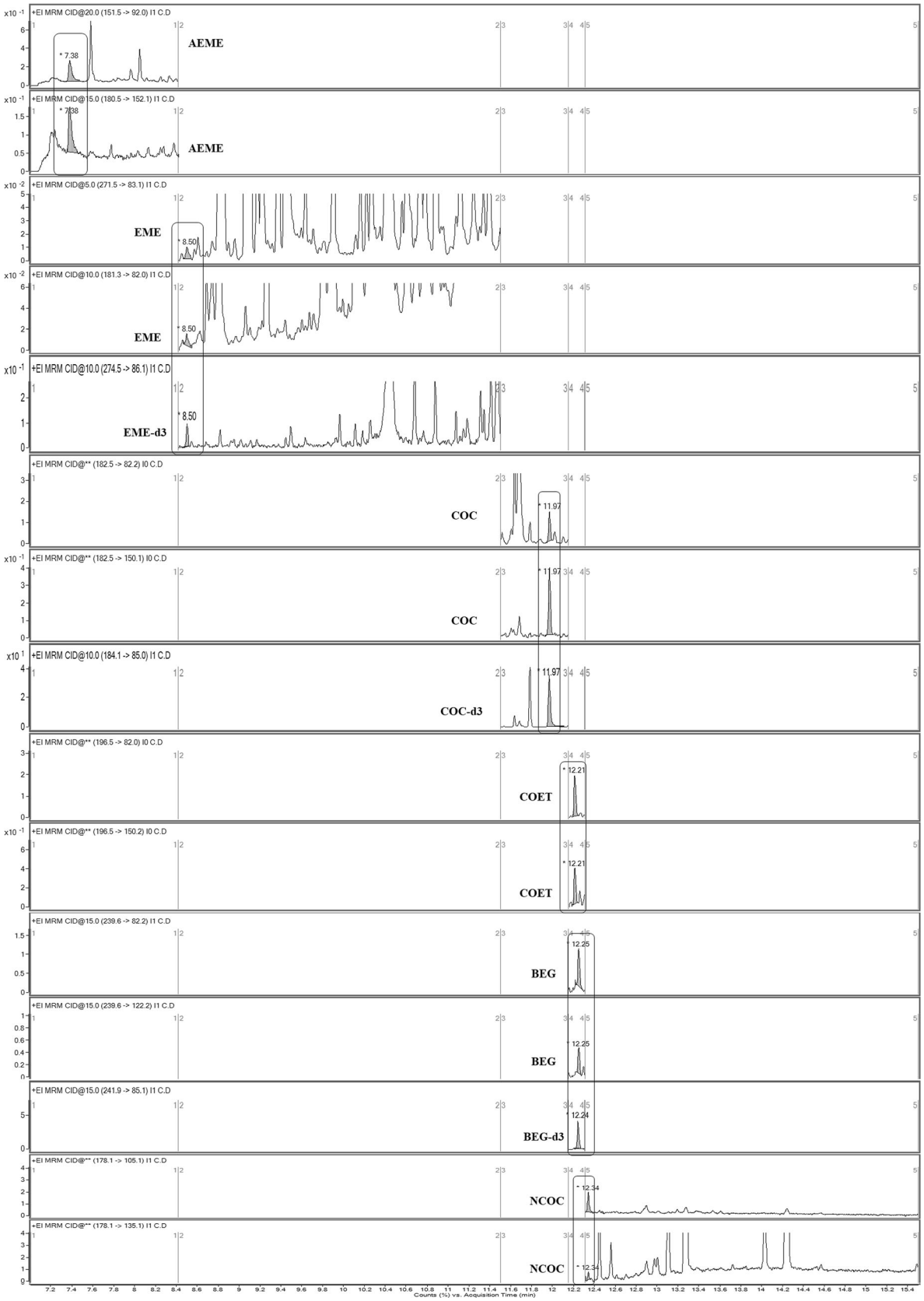

Fig. 1 Chromatogram of a hair specimen spiked at the LLOQ

Table 2 Linearity data ($n = 5$)

Analyte	Weight	Linear range (ng/mg)	Linearity		R^{2*}	LLOQ (ng/mg)
			Slope*	Intercept*		
AEME	1/x	0.15–5	0.0797 ± 0.0400	−6.1249 ± 11.0778	0.9962 ± 0.0015	0.15
EME		0.025–5	0.0038 ± 0.0011	0.2970 ± 0.0768	0.9961 ± 0.0005	0.025
COC		0.01–5	0.0033 ± 0.0006	0.0081 ± 0.0102	0.9989 ± 0.0011	0.01
COET		0.01–5	0.0056 ± 0.0050	0.0001 ± 0.0139	0.9989 ± 0.0004	0.01
BEG		0.025–5	0.0112 ± 0.0108	−0.0736 ± 0.0811	0.9962 ± 0.0008	0.025
NCOC		0.025–5	0.0021 ± 0.0005	−0.0270 ± 0.0124	0.9972 ± 0.0011	0.025

*Mean values ± standard deviation

reusing the BIN (the same BIN was used in the whole validation) and the rapid procedure.

Regarding the GC-EI-MS/MS used in the method proposed here, this surely contributed to the lower LLOQs obtained. Nonetheless, the application of this system to COC and metabolite determination in hair is scarce. Gambelunghe et al. [38] developed a GC-EI-MS/MS method for the determination of anabolic steroids in hair specimens, and included COC and BEG in the method. The authors did not adopt any clean-up procedure post-hydrolysis, and reported greater LLOQs (0.1 ng/mg for both compounds) [38]. Uhl [39] described tandem mass spectrometry as a helpful tool for the determination of drugs in hair samples and presented a GC-EI-MS/MS method in which COC, COET and BEG were included. The authors used 10 mg of hair and reported LLOQs of 0.25 ng/mg for the three analytes [39]. Also, Pichini et al. [40] reported a GC-EI-MS/MS method for the determination of COC, COET, BEG and some opiates. The authors used 100 mg of hair and obtained LLOQs of 0.02 ng/mg for COC and 0.01 ng/mg for COET and BEG. Kidwell et al. [41] performed a population study by hair analysis using 10 mg and the same analytical equipment, recording LLOQs of 0.05 for COC and BEG. It should be mentioned that none of these GC-EI-MS/MS works used any clean-up procedure after hydrolysis of hair samples.

Nowadays, LC-MS/MS systems are the most commonly used, and some authors still achieve LLOQs very close to ours [11, 42]. One example is a multi-component method developed by Fernández et al. [43] that included COC, COET and BEG. The authors used 20 mg of hair samples and mixed-mode SPE, obtaining a LLOQ of 0.031 ng/mg for the three analytes. Still, it should be pointed out that LC-MS/MS is one of the most sensitive instrumentations, allowing the determination of COC and several metabolites in concentrations lower than 5 pg/mg [44, 45].

Intra-day, inter-day and intermediate precision and accuracy

Inter-day precision was assayed at a minimum of seven calibrator concentrations within a 5-day period, while intra-day precision was evaluated by the analysis of six replicates on the same day. A minimum of four concentration levels were used for the six replicates. The concentrations were chosen to support the LLOQ. For the analytes AEME, COC and COET, it was possible to reach lower LLOQs than initially expected. Additionally, intermediate precision was evaluated using four quality control (QC) levels ($n = 3$) analyzed simultaneously with the calibration curves on the five different days. The four QC levels included the LLOQ, low (0.05 ng/mg), medium (0.8 ng/mg) and high (3.5 ng/mg) concentrations for all target analytes, except for AEME (0.3, 1.0 and 4.0 ng/mg were adopted as low, medium and high QC levels, respectively). The accuracy of the methods was characterized in terms of the mean RE between the measured and nominal concentrations, and the accepted limit was 15% for all concentrations, except at the LLOQ, where 20% was accepted. Concerning precision, this was evaluated by the coefficient of variation (CV) between measured concentrations, with an acceptable limit of 15%, except for the LLOQ, which should be lower than 20%.

Regarding inter-day precision, the observed CVs were lower than 14.5% for all compounds at the studied concentrations, with exception of AEME, which presented a CV of 18.71% at the LLOQ. The accuracy revealed a maximum mean RE within ±14.5%. For intra-day precision, the CVs were typically lower than 11.5% for all analytes and tested levels, and accuracy assay showed a maximum RE within ±15% with exception of NCOC that presented a mean RE of 15.91% at the LLOQ. Lastly, the intermediate precision resulted in CVs lower than 14% for all tested substances and concentrations, while the accuracy revealed a maximum RE within ±12.5%, with the exception of NCOC at LLOQ with a mean RE of 15.34%. Table 3 presents all data.

Recovery

To evaluate the recoveries obtained with the optimized MEPS procedure, two sets of hair samples ($n = 3$) were prepared at the low, medium and high QC levels. Set 1 represented the elution solution obtained from blank hair samples and spiked

Table 3 Inter-day, intra-day and intermediate precision and accuracy

Analyte	Spiked	Inter-day ($n = 5$)			Intra-day ($n = 6$)			Intermediate ($n = 15$)		
		Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)
AEME	0.15	0.15 ± 0.03	18.71	0.86	0.17 ± 0.006	3.34	14.59	0.17 ± 0.004	2.21	12.50
	0.20	0.21 ± 0.004	2.04	7.30	0.22 ± 0.004	1.87	8.49			
	0.30							0.31 ± 0.04	12.12	1.85
	0.40	0.39 ± 0.01	2.42	−2.31	0.40 ± 0.03	8.51	0.47			
	0.80	0.77 ± 0.09	11.80	−4.15						
	1.00							1.00 ± 0.10	9.96	0.46
	1.20	1.14 ± 0.13	11.00	−4.79						
	2.40	2.48 ± 0.05	2.03	3.24	2.34 ± 0.23	9.50	−0.48			
	3.60	3.56 ± 0.30	8.36	−0.97						
	4.00							4.22 ± 0.41	9.64	5.46
	5.00	5.04 ± 0.15	2.91	0.83	5.23 ± 0.40	7.62	4.32			
EME	0.025	0.024 ± 0.002	6.77	−5.62	0.025 ± 0.002	8.76	−1.51	0.026 ± 0.002	7.81	2.37
	0.05							0.05 ± 0.01	13.64	1.06
	0.10	0.10 ± 0.01	7.58	1.64	0.10 ± 0.01	9.33	4.57			
	0.25	0.26 ± 0.03	10.62	4.73						
	0.50	0.45 ± 0.02	5.18	−9.11						
	0.80							0.72 ± 0.04	5.22	−9.91
	1.50	1.67 ± 0.03	1.95	11.58	1.50 ± 0.17	11.08	−0.16			
	3.00	2.97 ± 0.18	6.12	−1.02						
	3.50							3.46 ± 0.25	7.18	−1.16
	5.00	4.89 ± 0.11	2.19	−2.19	4.89 ± 0.34	7.00	−2.14			
COC	0.01	0.01 ± 0.001	9.19	8.00	0.011 ± 0.001	6.67	6.12	0.01 ± 0.001	8.18	4.57
	0.025	0.026 ± 0.001	4.17	5.58	0.023 ± 0.002	8.49	−0.53			
	0.05							0.052 ± 0.004	7.21	3.58
	0.10	0.09 ± 0.002	1.91	−13.58	0.09 ± 0.006	6.40	−5.74			
	0.25	0.24 ± 0.03	13.36	−3.84						
	0.50	0.52 ± 0.03	5.36	3.33						
	0.80							0.84 ± 0.07	8.68	4.10
	1.50	1.53 ± 0.02	1.57	2.23	1.59 ± 0.09	5.83	6.12			
	3.00	2.91 ± 0.08	2.69	−2.85						
	3.50							3.68 ± 0.15	4.08	5.22
	5.00	5.06 ± 0.06	1.13	1.12	5.47 ± 0.28	5.15	9.43			
COET	0.01	0.011 ± 0.001	8.34	9.86	0.01 ± 0.001	6.29	8.99	0.01 ± 0.001	7.62	8.92
	0.025	0.025 ± 0.001	5.69	0.39	0.024 ± 0.002	8.30	−4.65			
	0.05							0.05 ± 0.006	11.77	−0.71
	0.10	0.09 ± 0.001	1.28	−13.47	0.09 ± 0.003	3.92	−10.47			
	0.25	0.27 ± 0.005	2.09	4.30						
	0.50	0.48 ± 0.005	1.16	−4.20						
	0.80							0.76 ± 0.06	7.64	−4.56
	1.50	1.58 ± 0.005	0.30	5.04	1.55 ± 0.07	4.73	3.61			
	3.00	2.94 ± 0.09	3.21	−2.14						
	3.50							3.54 ± 0.19	5.36	1.07
	5.00	5.01 ± 0.11	2.18	0.22	5.33 ± 0.23	4.39	6.51			
BEG	0.025	0.026 ± 0.001	2.71	3.16	0.027 ± 0.002	8.41	6.85	0.027 ± 0.002	8.70	7.77
	0.05							0.05 ± 0.005	10.45	2.26
	0.10	0.09 ± 0.002	2.60	−13.13	0.092 ± 0.006	6.34	−7.98			
	0.25	0.25 ± 0.02	6.41	−3.29						

Table 3 (continued)

Analyte	Spiked	Inter-day ($n = 5$)			Intra-day ($n = 6$)			Intermediate ($n = 15$)		
		Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)
	0.50	0.55 ± 0.01	2.00	9.86						
	0.80							0.80 ± 0.05	6.76	−0.50
	1.50	1.64 ± 0.08	4.70	9.19	1.66 ± 0.06	3.80	10.65			
	3.00	2.81 ± 0.07	2.37	−6.15						
	3.50							3.65 ± 0.18	4.99	4.26
	5.00	5.02 ± 0.14	2.80	0.36	5.33 ± 0.30	5.54	6.59			
NCOC	0.025	0.03 ± 0.001	4.27	14.48	0.029 ± 0.001	3.06	15.91	0.029 ± 0.001	3.83	15.34
	0.05							0.056 ± 0.001	2.34	11.53
	0.10	0.09 ± 0.001	0.95	−13.93	0.09 ± 0.07	7.29	−4.63			
	0.25	0.22 ± 0.01	4.13	−10.10						
	0.50	0.51 ± 0.04	8.25	2.90						
	0.80							0.81 ± 0.02	2.62	1.34
	1.50	1.62 ± 0.07	4.48	8.15	1.61 ± 0.12	7.45	7.18			
	3.00	3.04 ± 0.14	4.55	1.31						
	3.50							3.78 ± 0.16	4.32	7.90
	5.00	4.86 ± 0.03	0.71	−2.81	4.96 ± 0.33	6.71	−0.80			

All concentrations in ng/mg; *CV* coefficient of variation; *RE* relative error [(measured concentration − spiked concentration/spiked concentration)] × 100; mean values ± standard deviation

with the analytes after the clean-up procedure (representing 100% recovery), and set 2 consisted of blank hair samples spiked with analytes before the clean-up procedure. The ISs were added to both sets only after the elution step. Recoveries were calculated by comparing the relative peak areas obtained in set 2 with those of set 1, and Table 4 summarizes the obtained data. Overall, the best recoveries were obtained for COC (44–64%) and COET (63–73%), which possibly justifies the fact that these compounds were those presenting lower LLOQs. BEG and NCOC presented lower recoveries, 21–28% and 36–44%, respectively. Regarding AEME, the recoveries ranged between 4 and 6%, and this could also explain why the LLOQ is the highest when compared to the remaining compounds. This is in agreement with some of the few publications that include AEME in the analytical method. They also report AEME as the analyte with the highest LLOQ for determining cocaine and metabolites [8, 23, 31, 46], although these authors do not present recovery data. Interesting enough are the very low recoveries obtained for EME with the optimized MEPS procedure, since they ranged between 1 and 3%. However, these low recoveries still allowed for the comfortable LLOQ of 0.025 ng/mg. The MRM detection sensitivity was crucial for this.

Table 4 Recovery (%) of the compounds under the optimized MEPS procedure ($n = 3$)

Analyte	Low QC	Medium QC	High QC
AEME	4.2 ± 0.44	5.6 ± 0.48	6.3 ± 0.85
EME	2.9 ± 0.41	1.7 ± 0.18	0.8 ± 0.05
COC	50.3 ± 2.05	64.5 ± 8.45	44.0 ± 2.22
COET	65.3 ± 8.43	73.2 ± 7.31	62.7 ± 11.25
BEG	24.5 ± 1.39	28.2 ± 1.92	20.9 ± 1.64
NCOC	36.4 ± 4.52	43.9 ± 5.03	40.5 ± 5.23

*Mean values ± standard deviation

Moreover, EME recoveries may be in agreement with previous literature, since many authors report poorer recoveries for EME compared to the parent drug and other metabolites [47–51]. Nonetheless, the recoveries of these authors are greater than ours, due to either using SPE clean-up procedures or no clean-up procedures at all. Additionally, after an extended review of the developed analytical methods, it is possible to conclude that many authors do not include EME as a consumption marker, which further complicates this discussion.

When compared to other microextraction techniques, the majority of the recoveries obtained in this work are considered satisfactory. Merola et al. [34] used a HS-SPME procedure to determine several recreational drugs in hair and reported recoveries of 12.3 and 11.5% for COC and COET, respectively. However, Gambelunghe et al. [18] used DI-SPME, reporting greater recoveries for all compounds, but EME was not included in the method. Gentili et al. [35] adopted HS-SPME for pre-concentration of several drugs, but only included COC in the method, resulting in a recovery of 101.5% for this substance. Aleksa et al. [21] applied HS-SPME, reporting a wide range of recoveries (2–68%) for the 17 drugs tested, but these

recoveries were not discriminated, and again, EME was not included. Finally, Toledo et al. [52] described a DI-SPME procedure for COC, BEG and COET, obtaining recoveries greater than 75% for all. The other miniaturized hair clean-up procedures, applied to determine COC and metabolites, do not report any obtained recoveries [19, 20, 23, 36, 37, 53].

Matrix effects

The matrix effects (ME) were evaluated using the post-extraction spike method. Hence, a comparison was performed between the response of the target analytes in neat solution and the response of the target analytes spiked at same concentrations into the blank matrix sample that underwent the MEPS procedure. ME were assayed at the low, medium and high QC levels ($n = 3$) by using the formula ($Mean_{After} - Mean_{Neat}$ / $Mean_{Neat}$) × 100. All results are presented in Table 5. The observed ME are in accordance with international recommendations [28], with average variation in chromatographic signals ranging from –10 to +15%, and CVs typically lower than 12% for all compounds. Hence the quantification of the target analytes is not significantly affected by matrix constituents.

Stability

Although hair specimens present high stability and can be collected and stored at room temperature [16], it is important to evaluate whether the extracts demonstrate stability in the autosampler, after the MEPS and derivatization procedure. In this study, QC concentration levels ($n = 3$) were analyzed and re-analyzed after a 24-h period in the autosampler. Their concentrations after 24 h were measured with a newly prepared calibration curve on the day of re-analysis.

The obtained CVs were typically equal to or less than 15%, with RE within a ±10% interval (Table 6). The results confirm that all target analytes were stable in the derivatized extracts for at least 24 h, confirming the possibility of a re-analysis after 24 h in the autosampler with no significant change in the concentration.

Table 5 Matrix effects (%) and CV (%) of the compounds under the optimized MEPS procedure ($n = 3$)

Analyte	Low QC		Medium QC		High QC	
	ME ± SD	CV	ME ± SD	CV	ME ± SD	CV
AEME	111.1 ± 9.0	8.1	99.9 ± 8.8	8.8	103.3 ± 10.3	10.0
EME	103.8 ± 11.8	11.4	106.5 ± 3.1	2.9	90.6 ± 7.7	8.5
COC	96.1 ± 5.4	5.6	92.9 ± 0.9	1.0	94.2 ± 7.0	7.4
COET	109.6 ± 4.2	3.8	94.4 ± 12.7	13.4	85.4 ± 6.6	7.7
BEG	105.4 ± 7.9	7.5	97.2 ± 10.9	11.2	94.6 ± 4.0	4.2
NCOC	94.6 ± 10.8	11.4	93.7 ± 9.0	9.6	90.5 ± 6.8	7.5

Method applicability

The method was applied to authentic samples belonging to individuals consuming cocaine, which were sent to our laboratory for analysis, and also from proficiency testing schemes. Figure 2 shows the chromatogram obtained from one authentic sample analyzed, according to the optimized and validated method. It was possible to observe that both COC and BEG were above the upper limit of quantification after a preliminary analysis; therefore, this particular sample had to be properly diluted (20 mg) in order to allow quantification by interpolation in the calibration curve.

Additionally, the same samples were analyzed by the method published by Barroso et al. [54] in 2008, using mixed-mode SPE and GC-EI-MS, but now improved with new COC metabolites. The mean CVs obtained between the two analysis methods were 12.4% for COC, 3.8% for COET, 2.7% for BEG and 14.3% for NCOC. Neither AEME nor EME was included in the GC-EI-MS; hence, no data was sent for us to compare.

Table 6 Stability of samples in the autosampler for 24 h (n = 3)

Analyte	Spiked	Measured	CV (%)	RE (%)
AEME	0.3	0.31 ± 0.05	14.97	4.14
	1.0	1.09 ± 0.09	8.05	9.41
	4.0	4.02 ± 0.36	9.01	0.56
EME	0.05	0.05 ± 0.002	4.19	2.54
	0.8	0.73 ± 0.06	8.23	−8.91
	3.5	3.49 ± 0.54	15.38	−0.25
COC	0.05	0.05 ± 0.004	6.48	10.00
	0.8	0.87 ± 0.05	5.76	8.53
	3.5	3.73 ± 0.12	3.11	6.57
COET	0.05	0.05 ± 0.003	4.75	7.80
	0.8	0.77 ± 0.05	7.09	−3.27
	3.5	3.29 ± 0.20	6.12	−6.01
BEG	0.05	0.05 ± 0.002	4.66	9.00
	0.8	0.87 ± 0.05	5.74	9.04
	3.5	3.70 ± 0.08	2.12	5.77
NCOC	0.05	0.05 ± 0.003	5.92	8.85
	0.8	0.87 ± 0.07	7.68	8.60
	3.5	3.77 ± 0.25	6.63	7.66

All concentrations in ng/mg; *CV* coefficient of variation; *RE* relative error [(measured concentration − spiked concentration/spiked concentration) × 100]; mean values ± standard deviation

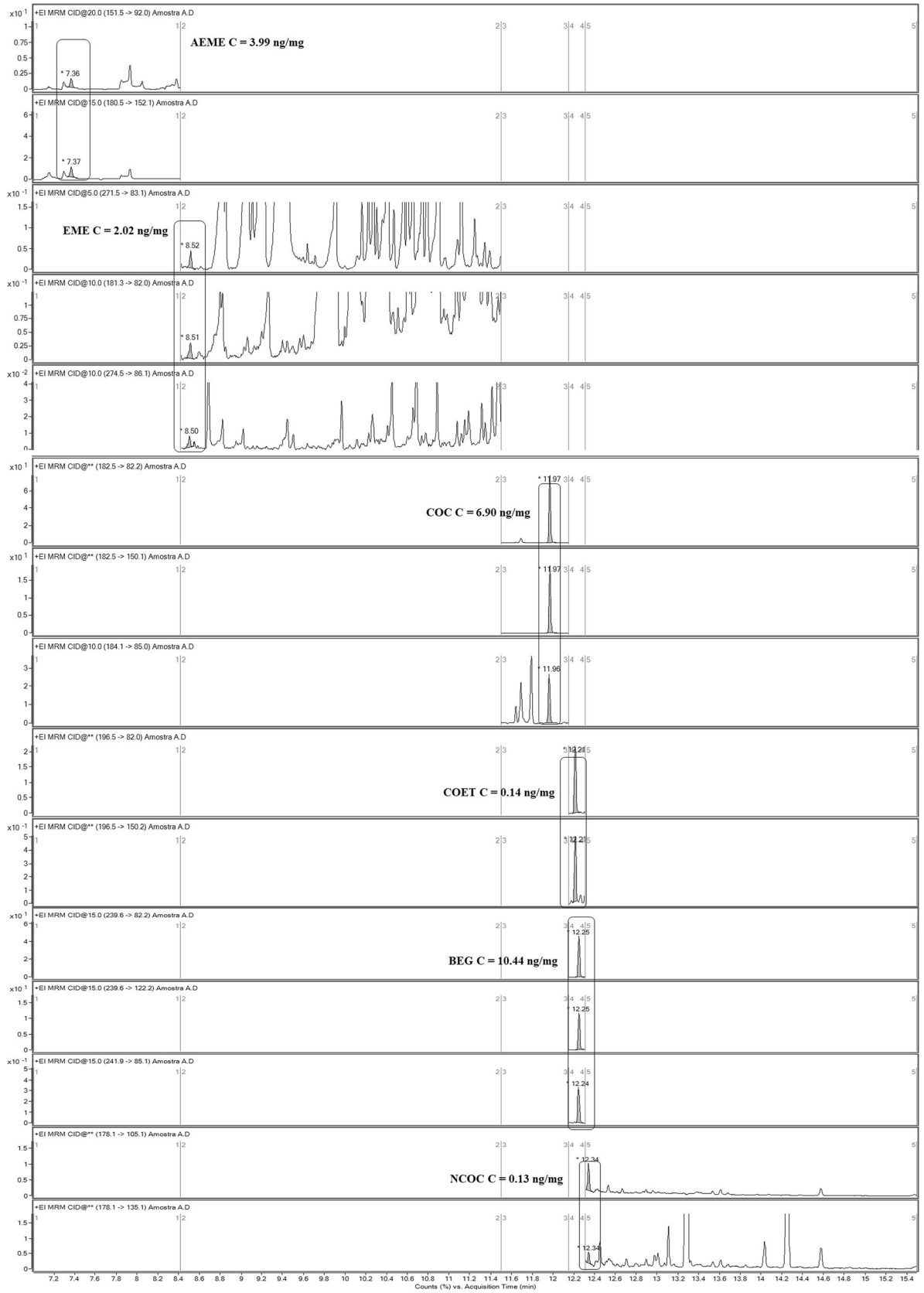

Fig. 2 Chromatogram of authentic hair sample

Conclusions

The present work describes the optimization and full validation of an analytical method to determine cocaine, ecgonine methyl ester, benzoylecgonine, norcocaine, cocaethylene and anhydroecgonine methyl ester in hair samples using MEPS as clean-up procedure and GC-EI-MS/MS. The combination of this microextraction technique for clean-up with tandem mass spectrometry proved to be a simple and rapid procedure, resulting in a sensitive, selective, precise and accurate method that can provide an excellent alternative for toxicological analysis.

Linearity was obtained within the adopted ranges, achieving LLOQs of 0.010 ng/mg for cocaine and cocaethylene, 0.025 ng/mg for ecgonine methyl ester, benzoylecgonine and norcocaine, and 0.150 ng/mg for anhydroecgonine methyl ester, a crack-cocaine-specific consumption marker that is not commonly included in hair analysis.

The method described herein is the first to use MEPS aimed at pre-concentration of cocaine and metabolites extracted from hair samples. This technique was shown to be quite advantageous when compared to the classic SPE and LLE approaches, reducing organic solvent volumes and enabling sorbent reuse (over 100 extractions), which might be economically appealing to laboratories. Moreover, the MEPS procedure is faster and can be automated.

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Compliance with ethical standards

Ethics declarations This study was conducted according to ethical standards and was approved by the ethics committee from Casas de Santiago. All analyses were carried out according to the ethical standards of the institution, and the analyzed samples were obtained from individuals who provided informed consent for their use (including the drug-free samples used in the validation experiments, provided by laboratory staff).

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 4 - Final reflection

Final reflection

Drug use has been and still is a reality in our society. While cannabis remains the most commonly used drug worldwide, the use of opiates still prevails in drug related deaths, followed by other opioids such as methadone and, to a lesser extent, cocaine. In Portugal, cocaine and opiates are also the most commonly reported drugs in *post mortem* toxicological cases. Based on these current data, we considered it important to develop analytical methods for the determination of selected opiates, methadone and cocaine and their metabolites in hair samples. Due to the relevance, we also have in mind the future development of a method for the determination of cannabis.

Hair samples are nowadays the most important alternative matrices with respect to human samples and have received a great deal of attention for both forensic and workplace drug testing. Their major advantage is the extended detection window (approximately 1 month per centimetre of hair), which also allows retrospective evaluation of drug exposure. This is not possible with the classical samples, blood/plasma and urine. In addition, hair has other advantages, namely non-invasive collection, the possibility of supervision to reduce the opportunity of tampering with, and great stability at room temperature. We decided to focus on hair analysis in this dissertation because, apart from the above advantages, it is well regulated by two entities: (*i*) the Society of Hair Testing (SoHT) and (*ii*) the European Workplace Drug Testing Society (EWDTS). This is not the case for other alternative samples. Moreover, hair analysis is not more difficult or demanding than testing classical samples. Nevertheless, we must admit that highly sensitive instrumentation is important to detect the presence of drug traces, which are common in this sample. Very little drug amounts incorporate the hair matrix, and there is a large influence of external agents (e.g., cosmetics) that can further reduce this amount. For this reason, cautious interpretation of negative results is advisable.

Just as important as the sensitive instrumentation is the sample clean-up technique used to pre-concentrate the target analytes and improve their detection. The use of novel microextraction techniques has proven quite advantageous, as they allow the amount of samples and organic solvents to be reduced, thus minimising waste production. Other advantages, such as reduced time and labour, reduced cost per analysis, and online coupling with analytical instruments, were found to be appealing and justified the trend towards their investigation. We conducted a comprehensive review of the application of microextraction techniques to hair samples. Although in the last five years the number of research articles on this topic has increased by more than 100%, their applicability to hair is still relatively low compared to other biological samples. This could be due to the fact that many hair analysts do not use clean-up procedures, especially when LC-MS instruments are used. However, the SoHT recommends a clean-up procedure for confirmation purposes. Another aspect is the low recoveries of drugs obtained with these microextraction techniques, which can be considered a disadvantage, especially taking into account the trace amounts to be determined. These low recoveries were most commonly observed with multi-target methods, so new developments in microextraction applied to hair samples are

directing most efforts toward new sorbent materials or solvents that will improve efficiency. The use of microextraction techniques for hair testing seems not to allow a large reduction in the amount of sample, since the amount of hair required in most reported microextraction developments is actually larger than that used in classic clean-up procedures (SPE and LLE).

Among the microextraction techniques reviewed, microextraction by packed sorbent (MEPS) appeared to be the least explored. MEPS is a miniaturized approach of classical SPE in which the sorbent is packed into a syringe. In addition to the advantages mentioned above, MEPS offers the possibility of sorbent reuse. This can be very attractive in the analytical field, as the cost per analysis decreases significantly. In light of this, we felt compelled to use MEPS as a clean-up technique for hair extracts. Until the start of this project, there was only one work reporting the use of MEPS use in hair samples to pre-concentrate amphetamine and methamphetamine. We were very pleased to see that our work was recognized and that we are the only research group that has successfully applied this miniaturized technique to hair samples in the last 10 years.

The MEPS procedure involves the same steps as SPE, and one might think that a validated SPE method could be directly converted into a MEPS method by simply reducing solvent volumes. However, this assumption is completely wrong, as some solvents commonly used in SPE are not suitable for MEPS syringe. Careful optimization of all procedural steps was needed in order to maximize recoveries and remove most interferences. Using the Design of Experiments (DOE) approach helped to perform this optimization faster and with reliable results.

Although we expected that recoveries of some analytes would be lower than those obtained with SPE, in a few cases (e.g., EME and AEME) these values were extremely low. By coupling MEPS with a highly sensitive GC-MS/MS instrument, the obtained limits of quantitation were well below the recommended cut-offs and even below those reported by some methods using classical clean-up procedures.

The work described here has demonstrated the successful application of microextraction techniques for the clean-up of hair samples. Focusing on MEPS procedures, we can state that they are an excellent alternative to classical techniques, as they are reproducible and provide good enrichment factors. We believe that miniaturized approaches should not only be a trend in the research field, but should be implemented in routine analytical laboratories. Awareness of the implementation of sustainable development and green chemistry principles should be expanded. Most laboratories continue to use classical techniques, although these miniaturized approaches are more user-friendly, easier to automate, reduce toxic wastes and are economically more advantageous.

Chapter 5 - Conclusions

Conclusions

In addition to the increasing number of techniques and procedures discussed in this thesis, miniaturized clean-up approaches for hair samples are still rarely used or studied. The review in Chapter 1 shows that miniaturized techniques can also be reproducible and have large enrichment factors, as do classical approaches.

To date, there has been considerable academic interest in these micro-scale techniques, but few implementations in routine analysis laboratories; this is expected to change in the future.

Among the various micro-scale techniques available today for the clean-up of hair samples, those based on the use of sorptive materials are the most commonly used. Moreover, these materials have shown great versatility, with a wide range of applications. The development of new materials should be further explored to achieve even better selectivity and higher adsorption capacity.

It is important to consider the combination of these miniaturized procedures with hyphenated techniques, still poorly applied. The coupling of a micro-based extraction with the newer MS technology offers the possibility to overcome the limitations seen in multi-target screening.

Although the application of MEPS is still limited to research, a large number of new sorbents have been developed and new approaches have been tested in the last five years.

The commercially available sorbents do not seem to cover all needs, hence the continuous search for new solid materials. The newly developed and reported solid materials are limited to a few classes of target analytes and are not suitable for multi method approaches.

We have developed optimized and validated three methods that demonstrate the successful application of MEPS for hair samples clean-up.

The first method was applied to the simultaneous determination of tramadol, codeine, morphine, 6-acetylcodeine, 6-monoacetylmorphine and fentanyl in hair samples using MEPS coupled to GC–MS/MS. The analytical method was linear within the adopted ranges for all opioids with a LLOQ of 0.010 ng/mg for tramadol, codeine and 6- acetylcodeine and 0.025 ng/mg for morphine, 6-monoacetylmorphine and fentanyl.

The second method was used for the simultaneous determination of methadone and EDDP in hair using MEPS coupled to GC–MS/MS. The method was linear between 0.010 and 5 ng/mg for both compounds, with the LLOQ assured at 0.010 ng/mg.

Finally, the third method was developed for the simultaneous determination of cocaine, ecgonine methyl ester, benzoylecgonine, norcocaine, cocaethylene and anhydroecgonine methyl ester in

hair samples using MEPS coupled to GC-MS/MS. Linearity was achieved within the adopted ranges, and LLOQs of 0.010 ng/mg for cocaine and cocaethylene, 0.025 ng/mg for ecgonine methyl ester, benzoylecgonine, and norcocaine, and 0.150 ng/mg for anhydroecgonine methyl ester were obtained.

All methods were found to be simple and quick to perform, selective, precise and accurate. Moreover, this technique was shown to be quite advantageous compared to the classical SPE and LLE approaches, as it reduces the volumes of organic solvents and allows the reuse of sorbents (over 100 extractions), which could be economically appealing to laboratories.

Annex I

Determination of selected opiates in hair samples using microextraction by packed sorbent: a new approach for sample clean-up.

1. DOE Output

1.1.Tramadol (TRM)

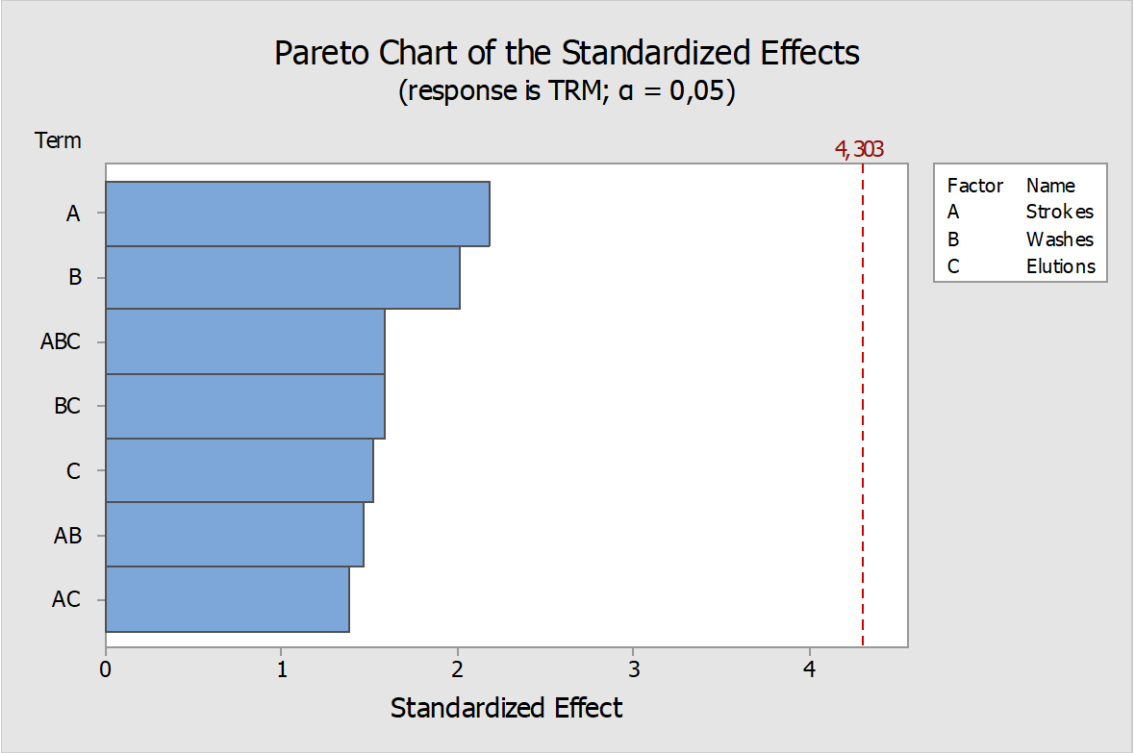

Figure 1. Pareto chart of the Standardized effects obtained for TRM.

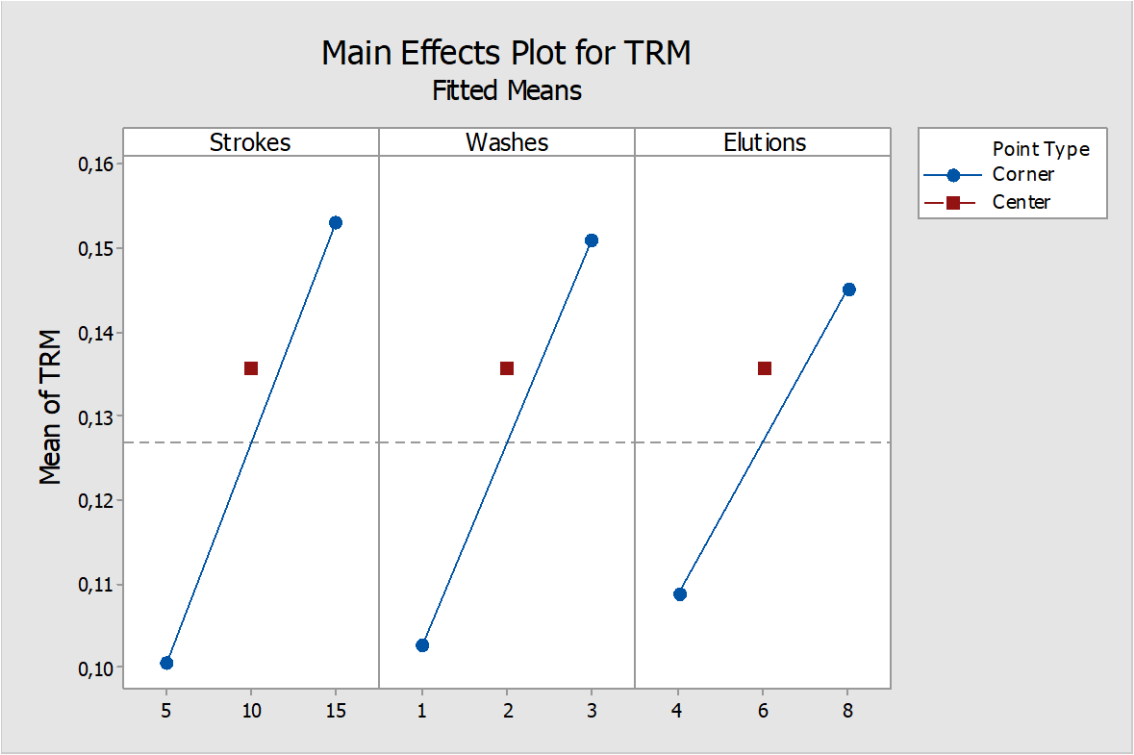

Figure 2. Main effects plot obtained for TRM.

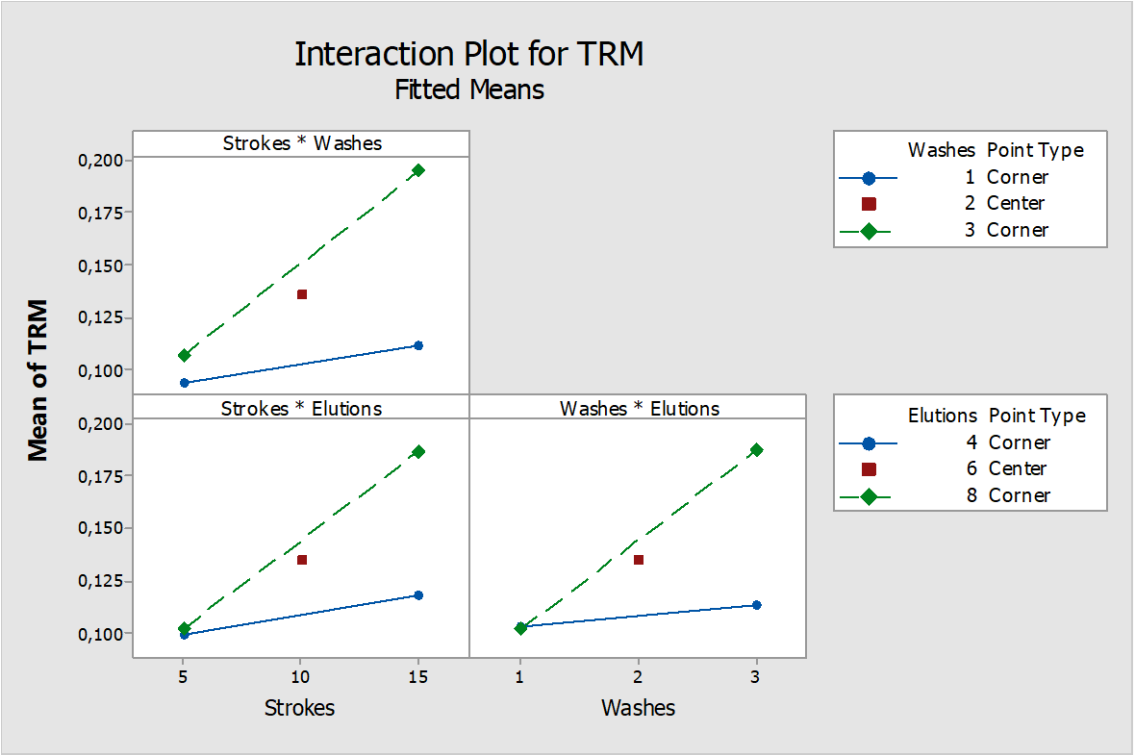

Figure 3. Interaction plot obtained for TRM.

1.2. Codeine (COD)

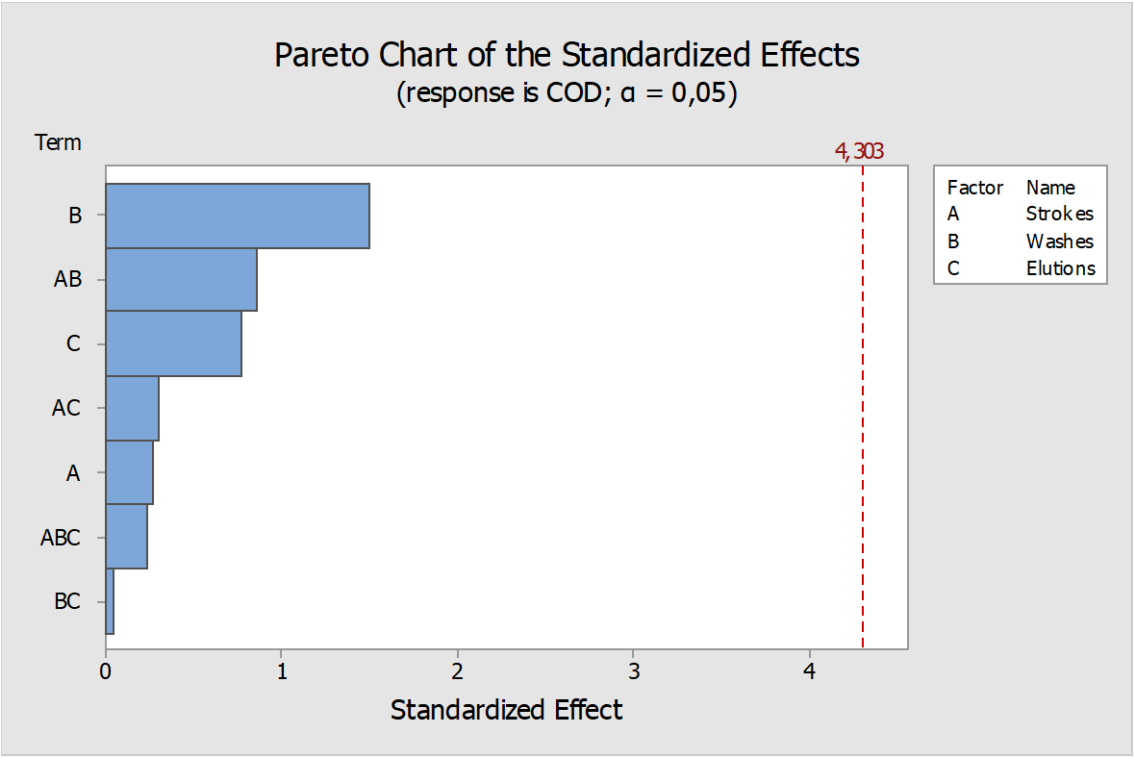

Figure 4. Pareto chart of the Standardized effects obtained for COD.

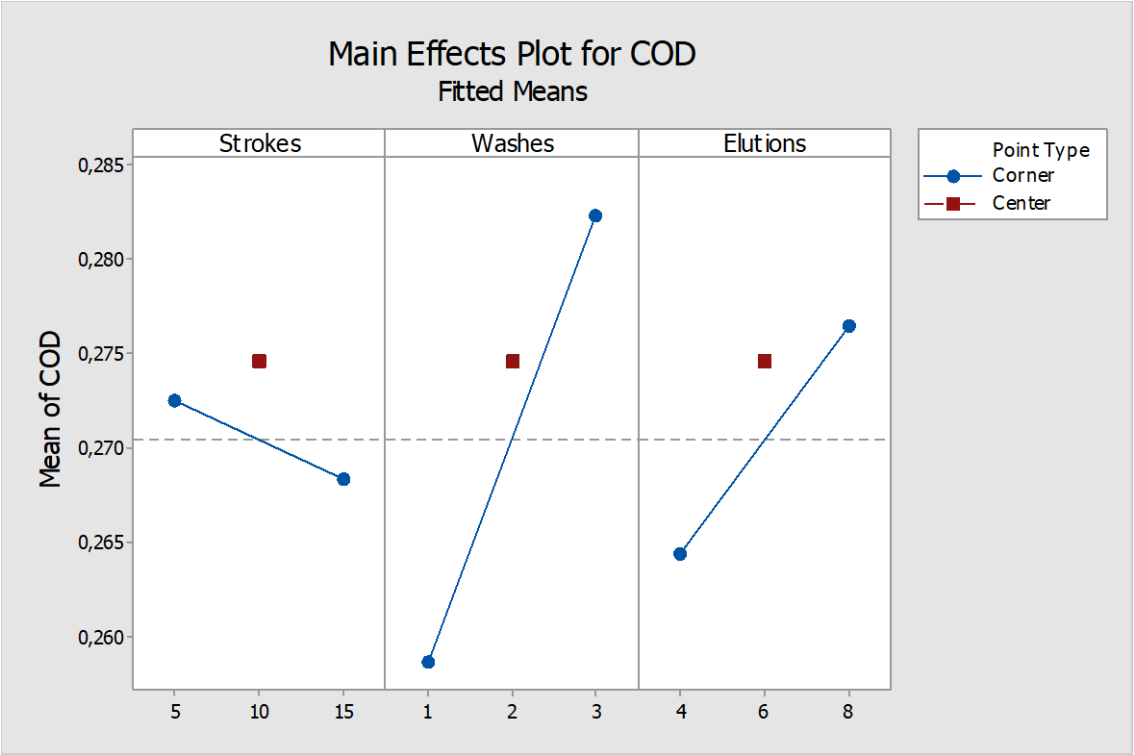

Figure 5. Main effects plot obtained for COD.

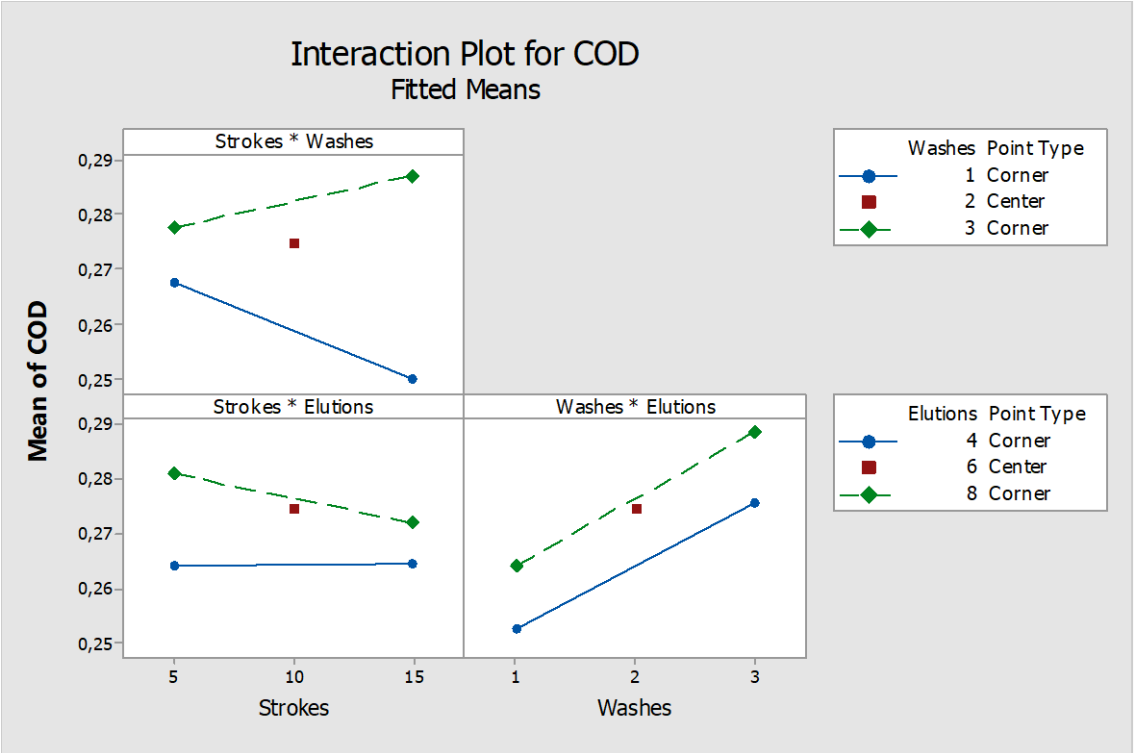

Figure 6. Interaction plot obtained for COD.

1.3. Morphine (MOR)

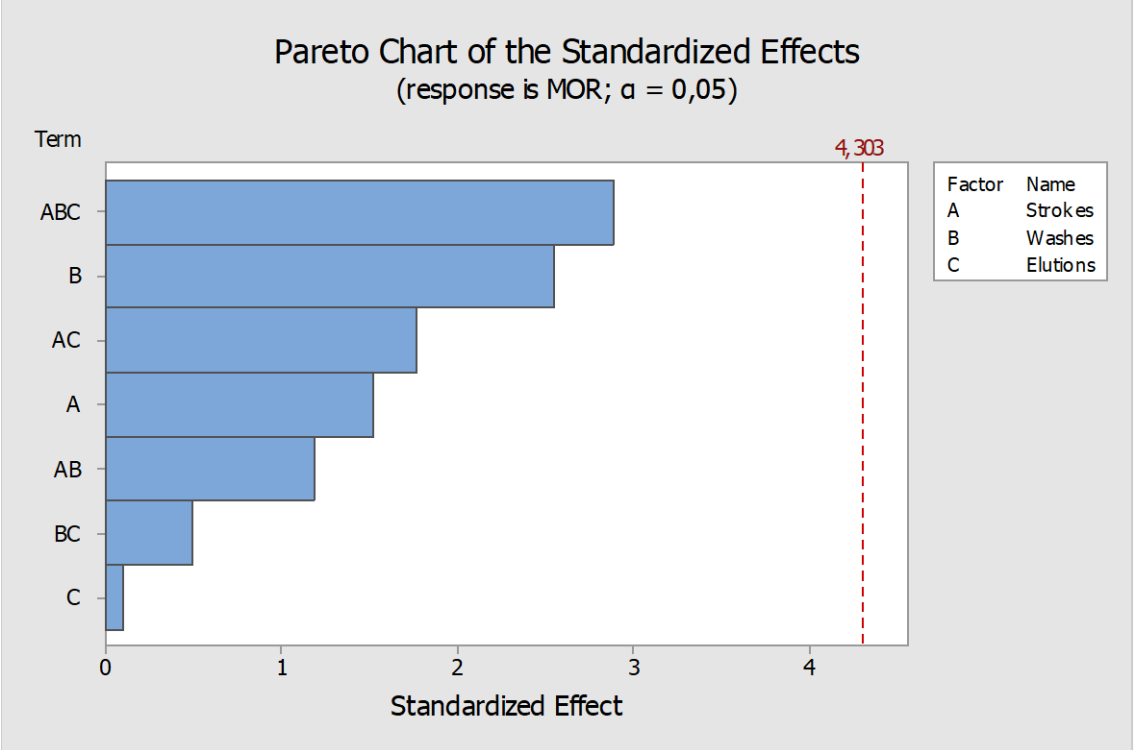

Figure 7. Pareto chart of the Standardized effects obtained for MOR.

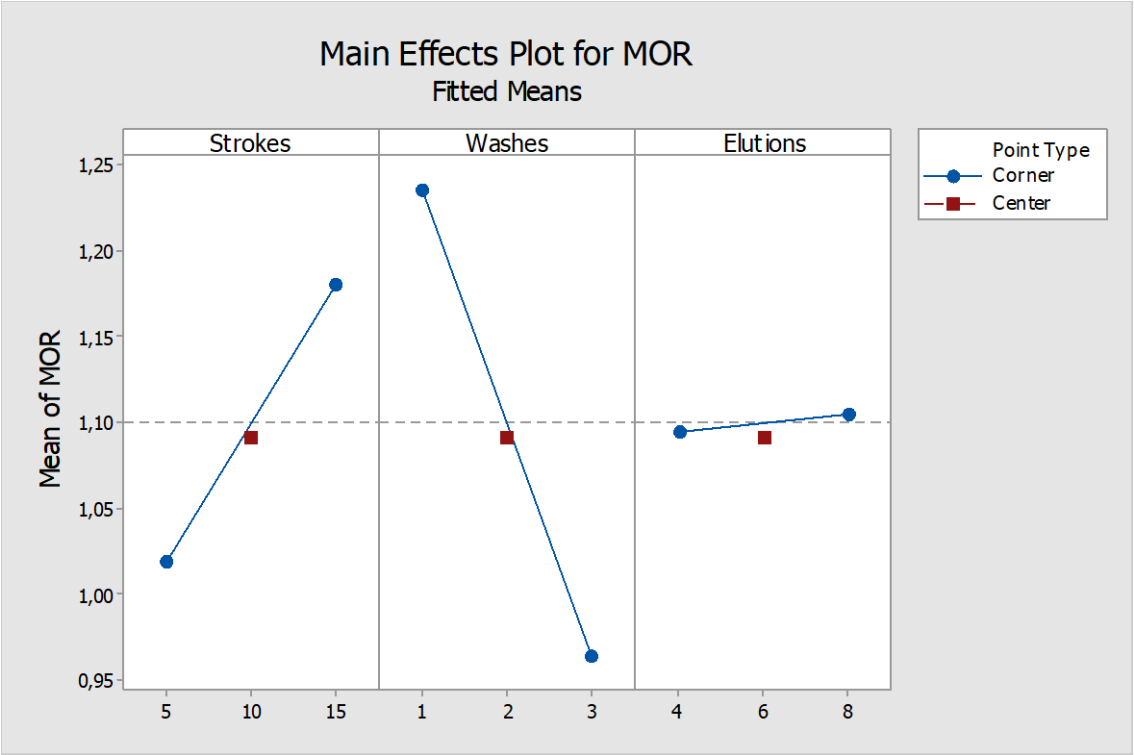

Figure 8. Main effects plot obtained for MOR.

Figure 9. Interaction plot obtained for MOR.

1.4. 6-Acetylcodeine (6-AC)

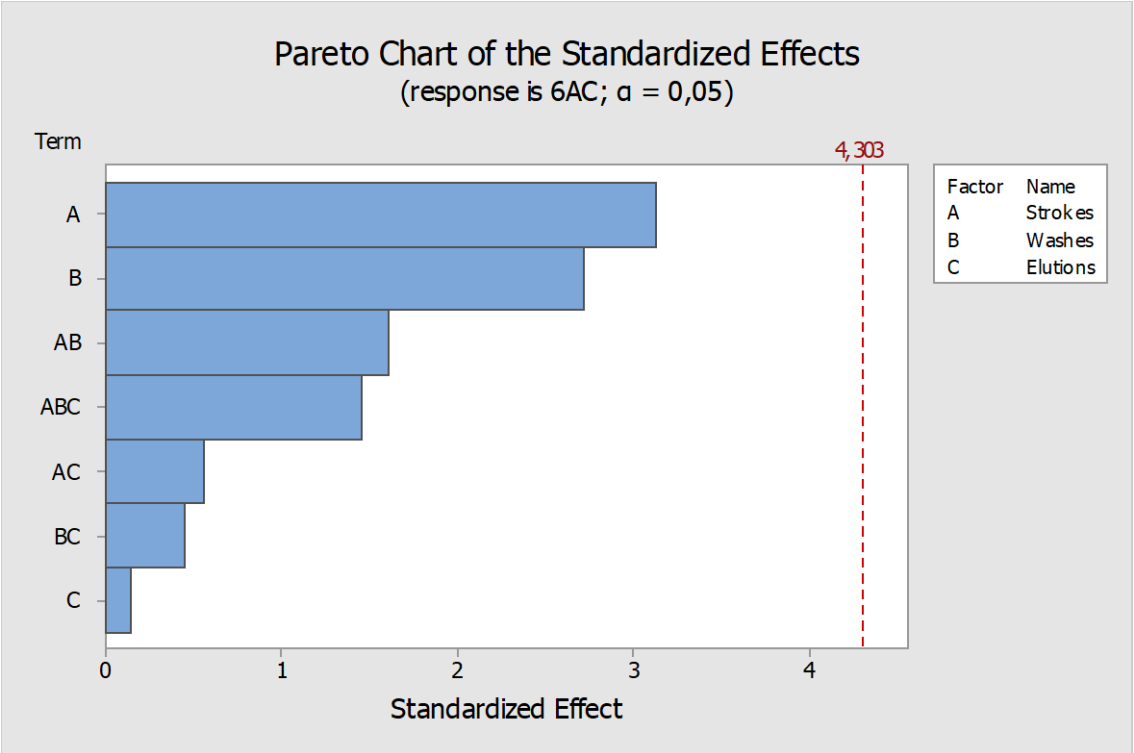

Figure 10. Pareto chart of the Standardized effects obtained for 6-AC.

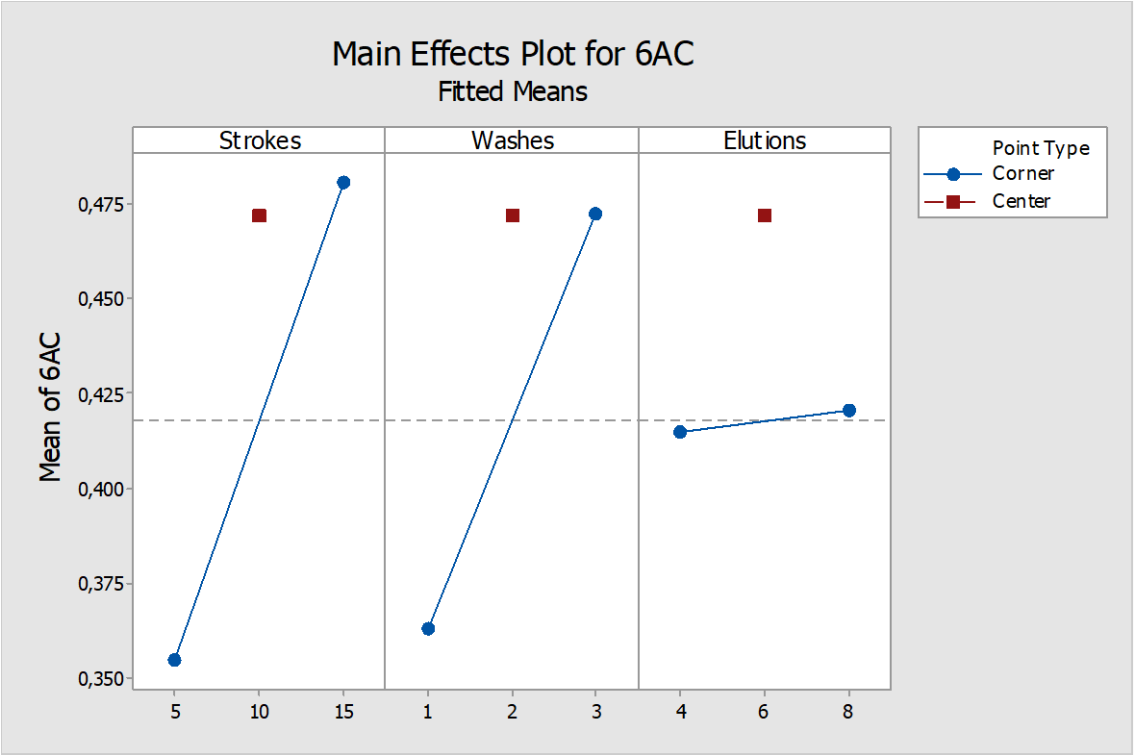

Figure 11. Main effects plot obtained for 6-AC.

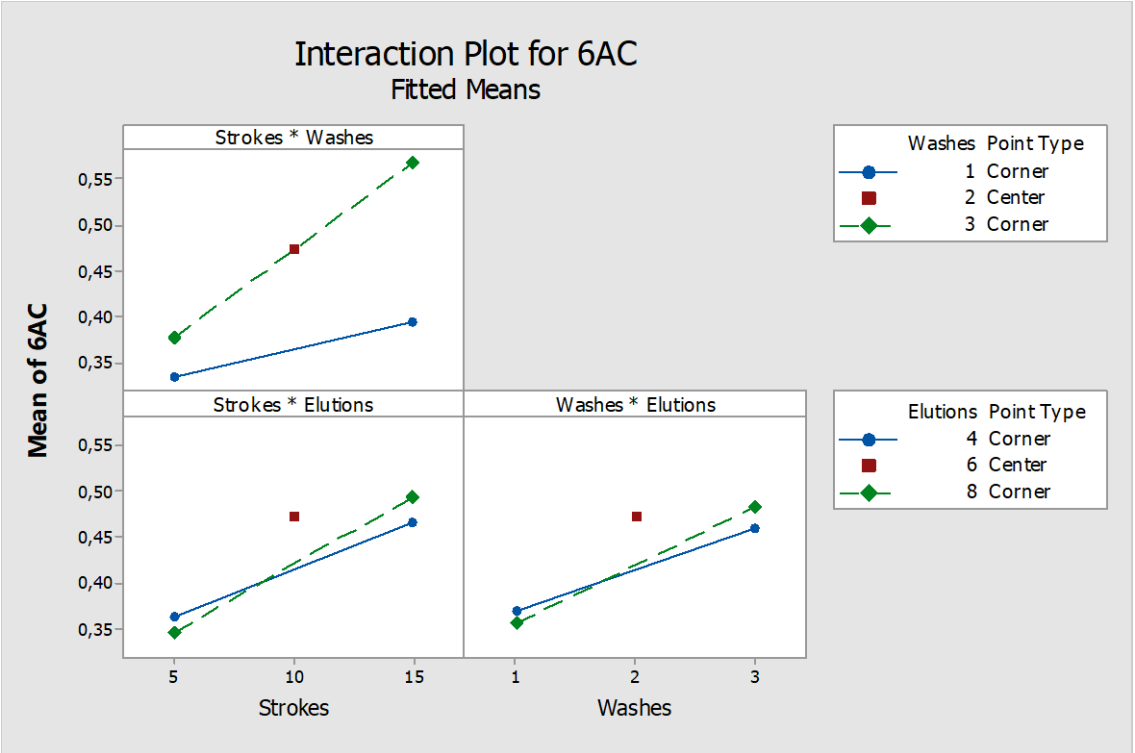

Figure 12. Interaction plot obtained for 6-AC.

1.5. 6-monoacetylmorphine (6-MAM)

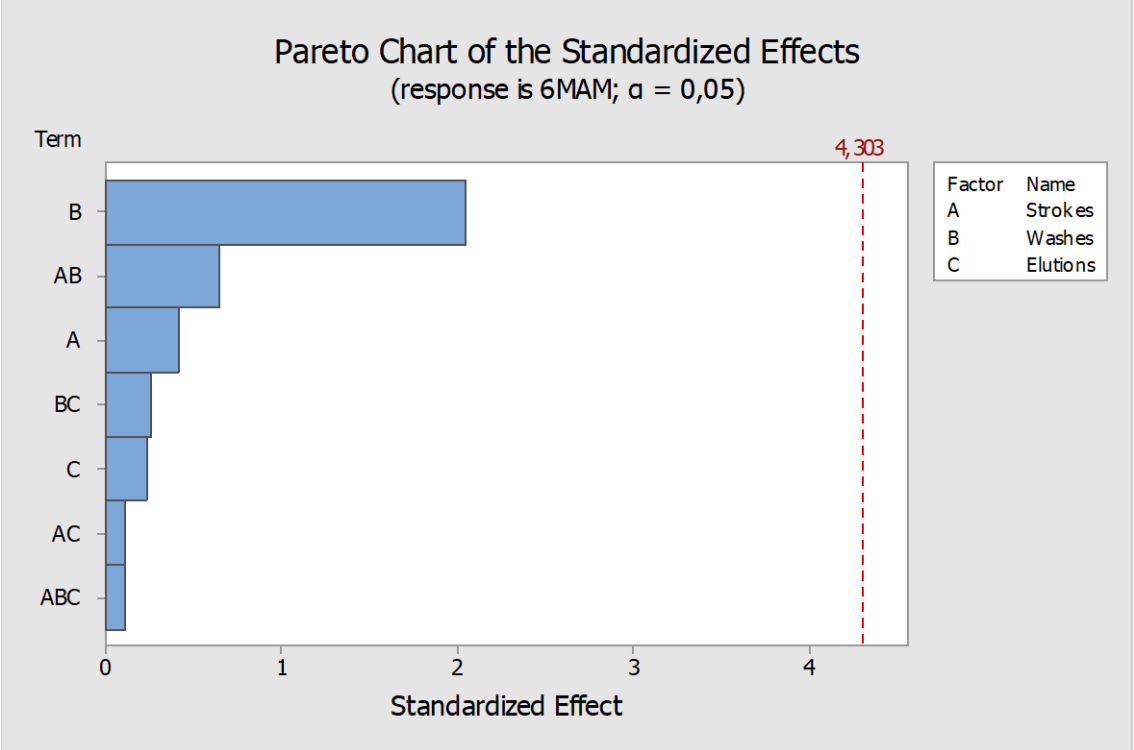

Figure 13. Pareto chart of the Standardized effects obtained for 6-MAM.

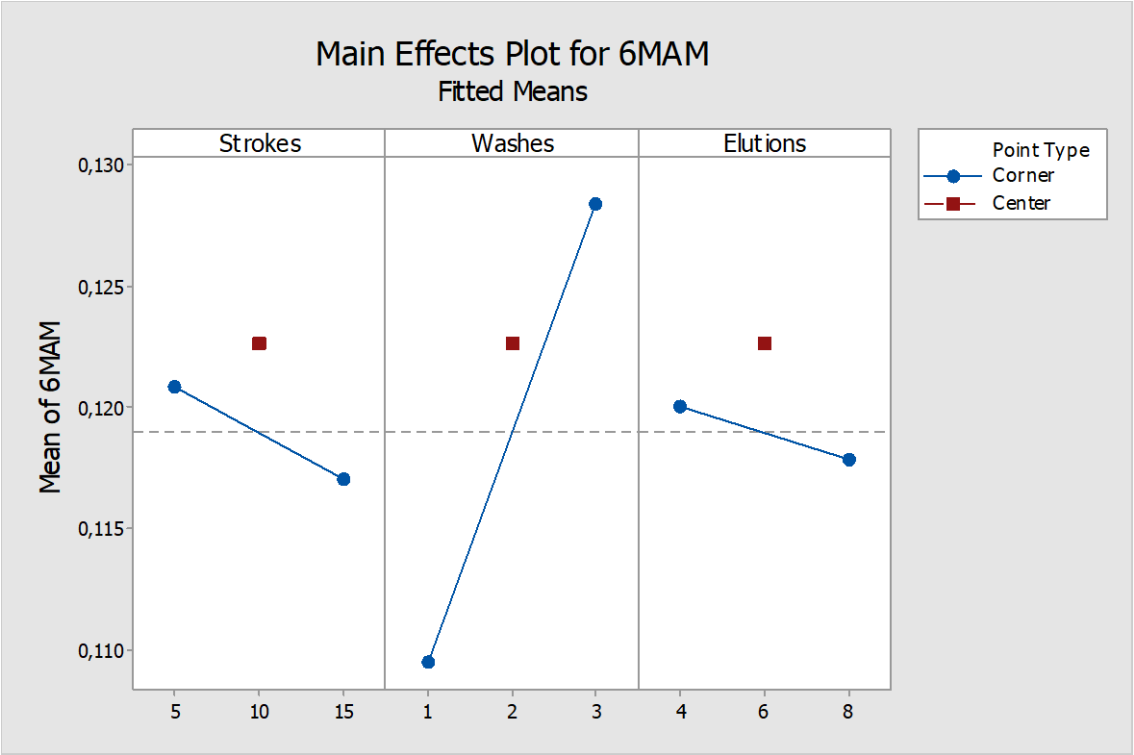

Figure 14. Main effects plot obtained for 6-MAM.

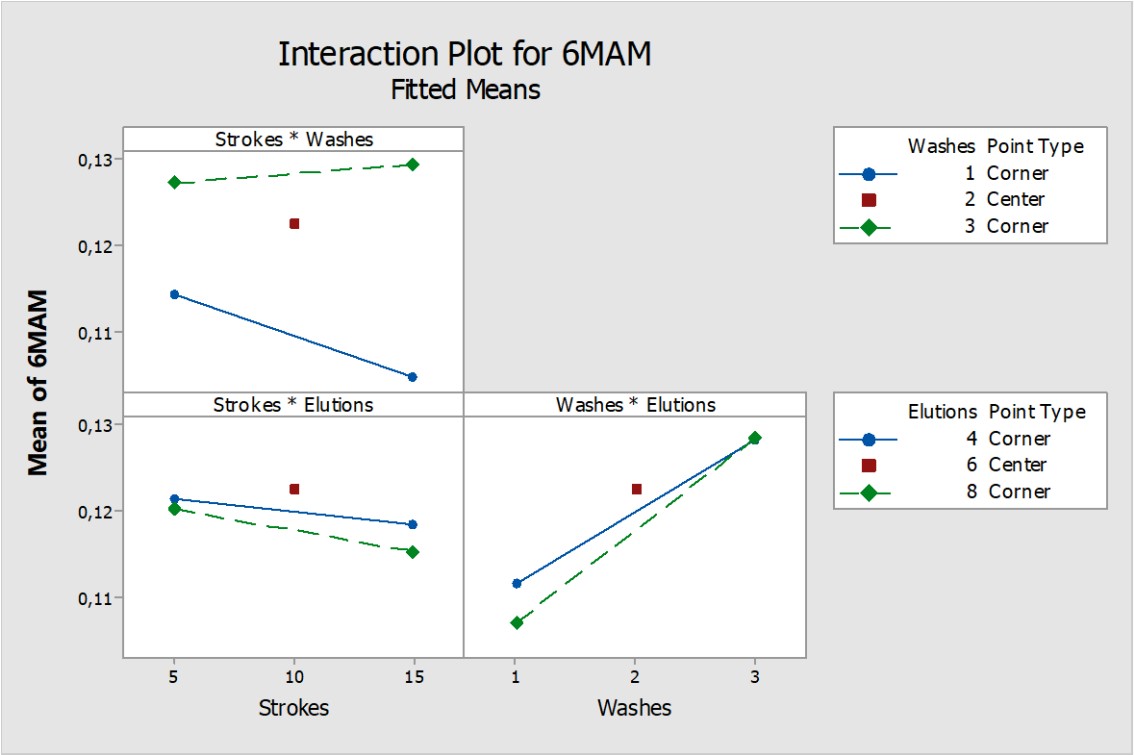

Figure 15. Interaction plot obtained for 6-MAM.

1.6. Fentanyl (FNT)

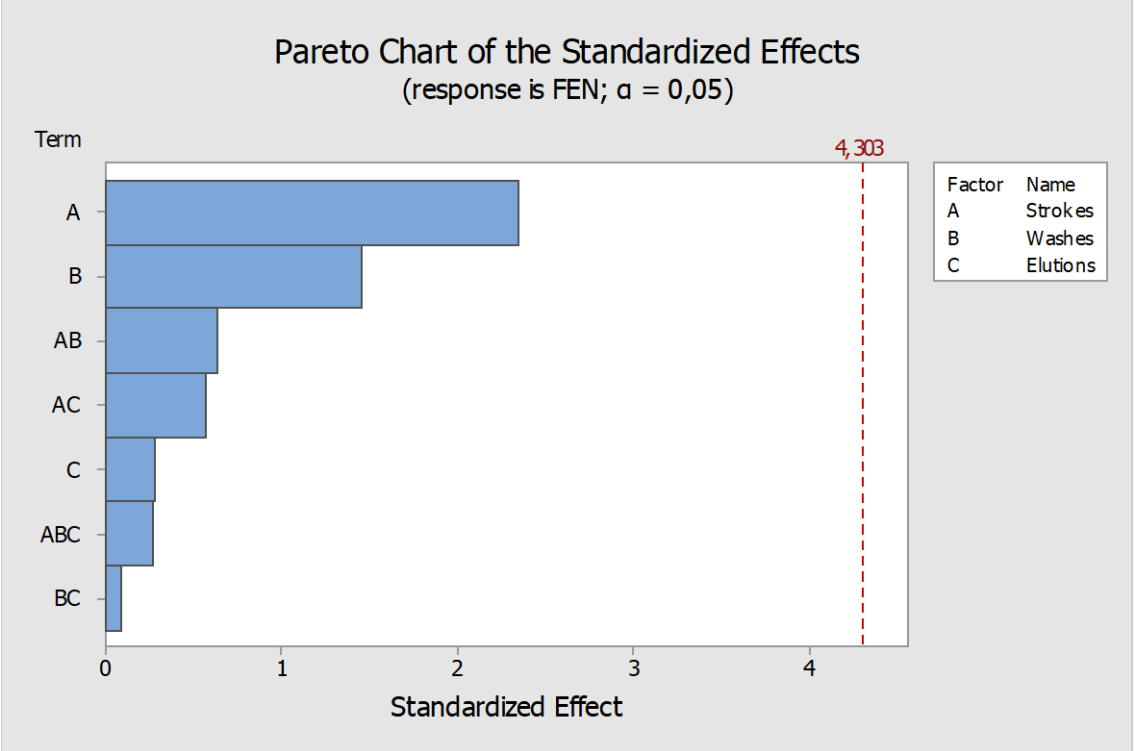

Figure 16. Pareto chart of the Standardized effects obtained for FNT.

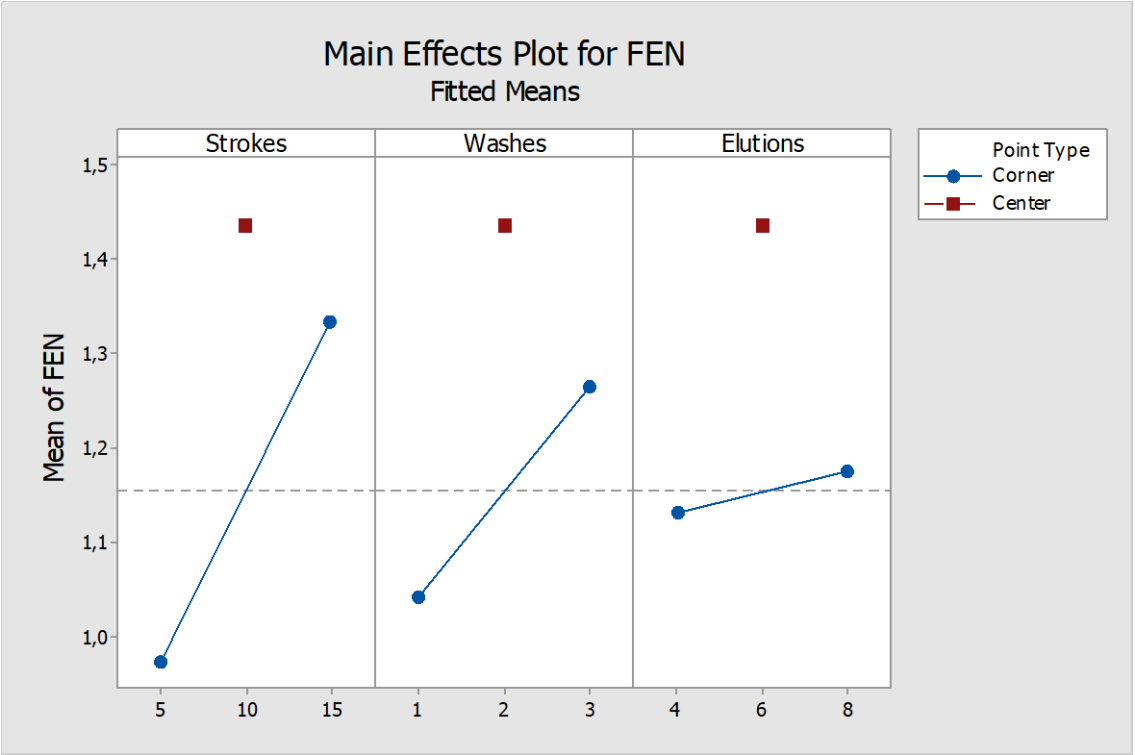

Figure 17. Main effects plot obtained for FNT.

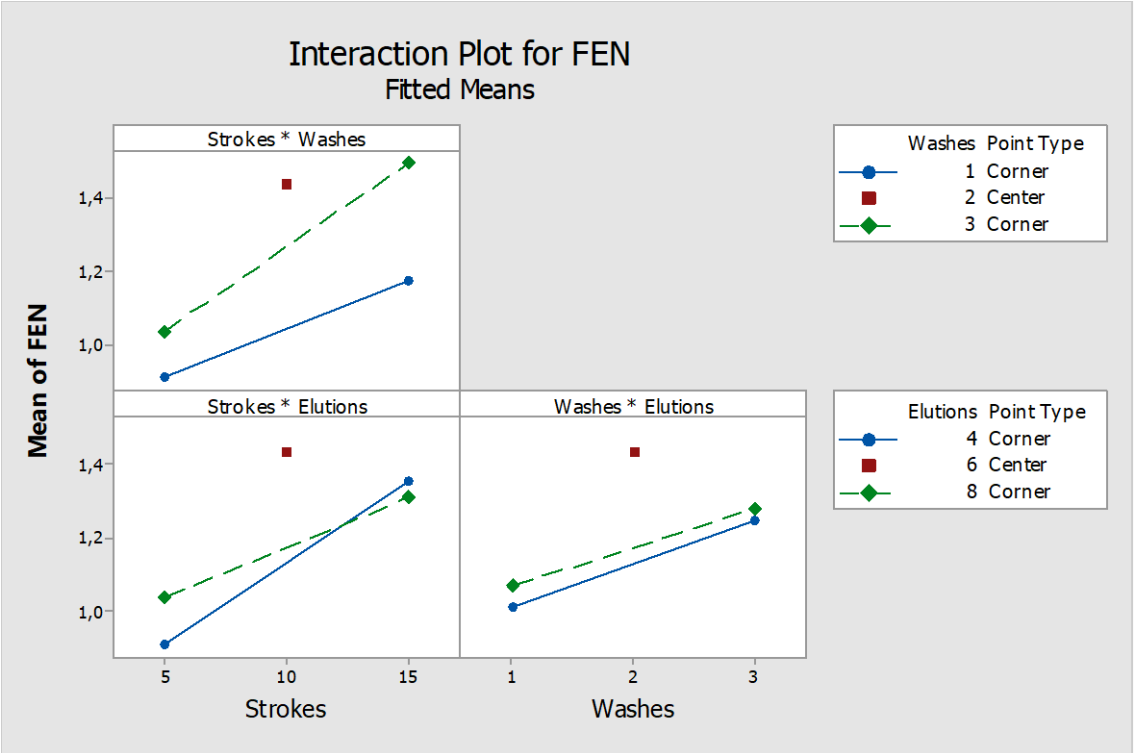

Figure 18. Interaction plot obtained for FNT.

Annex II

Microextraction by packed sorbent as a novel strategy for sample clean-up in the determination of methadone and EDDP in hair.

1. Related-Samples Friedman's Two-Way Analysis of Variance by Ranks.

1.1.2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)

Table 1. Peak integration data obtained in the univariate study of EDDP

9 Strokes				12 Strokes				15 Strokes				18 Strokes			
	A EDDP	A EDDPd3	RA		A EDDP	A EDDPd3	RA		A EDDP	A EDDPd3	RA		A EDDP	A EDDPd3	RA
1	218244	1981	110,1686	**1**	202966	1619	125,365	**1**	309152	2454	125,9788	**1**	313491	2374	132,0518
2	165647	1695	97,72684	**2**	226391	2056	110,1124	**2**	283997	2402	118,2336	**2**	308022	2436	126,4458
3	212102	1845	114,9604	**3**	379586	3459	109,7387	**3**	281119	2313	121,5387	**3**	471304	3712	126,9677
X	198664,33	1840,3333	107,6186	X	269647,67	2378	115,072	X	291422,67	2389,667	121,917	X	364272,3	2840,667	128,4884
SD	28758,29	143,0571	8,895276	SD	95927,111	961,33397	8,915979	SD	15421,338	71,30451	3,886462	SD	92732,47	755,2333	3,096988
CV	14,48%	7,77%	8,27%	CV	35,57%	40,43%	7,75%	CV	5,29%	2,98%	3,19%	CV	25,46%	26,59%	2,41%

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of 9 Strokes, 12 Strokes, 15 Strokes and 18 Strokes are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,042	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 1. Hypothesis test summary for EDDP

Each node shows the sample average rank.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
9 Strokes-12 Strokes	-,333	1,054	-,316	,752	1,000
9 Strokes-15 Strokes	-1,667	1,054	-1,581	,114	,683
9 Strokes-18 Strokes	-2,667	1,054	-2,530	,011	,068
12 Strokes-15 Strokes	-1,333	1,054	-1,265	,206	1,000
12 Strokes-18 Strokes	-2,333	1,054	-2,214	,027	,161
15 Strokes-18 Strokes	-1,000	1,054	-,949	,343	1,000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is , 05.
Significance values have been adjusted by the Bonferroni correction for multiple

Figure 2. Pairwise comparisons for EDDP

1.2. Methadone (MTD)

Table 2. Peak integration data obtained in the univariate study of MTD

9 Strokes				12 Strokes				15 Strokes				18 Strokes			
	A MTD	A MTDd3	RA		A MTD	A MTDd3	RA		A MTD	A MTDd3	RA		A MTD	A MTDd3	RA
1	18337	6340	2,892271	**1**	25309	8791	2,878967	**1**	36354	11715	3,103201	**1**	37219	11387	3,268552
2	21323	7619	2,798661	**2**	30885	10259	3,010527	**2**	39005	12635	3,08706	**2**	39530	12419	3,183026
3	24201	8369	2,891743	**3**	30049	10627	2,827609	**3**	38543	11685	3,298502	**3**	49118	14905	3,295404
X	21287	7442,6667	2,860892	X	28747,667	9892,3333	2,905701	X	37967,333	12011,667	3,162921	X	41955,667	12903,67	3,248994
SD	2932,17	1025,929	0,053894	SD	3007,1657	971,36879	0,094344	SD	1416,1548	540,03086	0,117694	SD	6309,4726	1808,385	0,058686
CV	13,77%	13,78%	1,88%	CV	10,46%	9,82%	3,25%	CV	3,73%	4,50%	3,72%	CV	15,04%	14,01%	1,81%

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of 9 Strokes, 12 Strokes, 15 Strokes and 18 Strokes are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,060	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 3. Hypothesis test summary for MTD

Annex III

New miniaturized clean-up procedure for hair samples by means of microextraction by packed sorbent: determination of cocaine and metabolites

1. Related-Samples Friedman's Two-Way Analysis of Variance by Ranks.

Table 1. Peak integration data obtained in the univariate study of COC and metabolites

	A AEME	A EME d3	AR	A EME	A EME d3	AR	A COC	A COC d3	AR	A COET	A BEG d3	AR	A BEG	A BEG d3	AR	A NCOC	A BEG d3	AR
15A	55689	97419	0,571644	3179	97419	0,032632	687399	646616	1,063071	779199	131951	5,905215	337427	131951	2,557214	133099	131951	1,0087
15B	42853	71148	0,602308	2204	71148	0,030978	462590	555732	0,832398	529196	115648	4,57592	235203	115648	2,033784	886110	115648	0,766204
15C	37584	82581	0,455117	2330	82581	0,028215	382965	452832	0,845711	404345	85628	4,722112	176033	85628	2,055788	693999	85628	0,810471
X	453753,33	837116	0,543023	2571	837116	0,030608	510984,7	551726,7	0,913727	570913,3	111075,7	5,067749	249554,3	111075,7	2,215595	970396	111075,7	0,861792
SD	9312,324	131722,23	0,077657	530,299	131722,23	0,002232	157881,4	96954,07	0,129508	190877,3	23497,55	0,728941	81648,49	23497,55	0,296055	326755,23	23497,55	0,129137
CV	21%	16%	14%	21%	16%	7%	31%	18%	14%	33%	21%	14%	33%	21%	13%	34%	21%	15%
	A AEME	A EME d3	AR	A EME	A EME d3	AR	A COC	A COC d3	AR	A COET	A BEG d3	AR	A BEG	A BEG d3	AR	A NCOC	A BEG d3	AR
18A	90434	120823	0,748483	5749	120823	0,047582	856463	725608	1,180338	970185	155942	6,221448	425141	155942	2,726276	187820	155942	1,204422
18B	76892	118132	0,650899	4083	118132	0,034563	667747	647493	1,031281	751520	134011	5,607898	319499	134011	2,384125	172757	134011	1,289126
18C	34356	53131	0,646628	1957	53131	0,036833	531082	585217	0,907496	642485	127718	5,030497	279837	127718	2,191054	112265	127718	0,879007
X	67227,33	973632	0,682003	3929,667	973632	0,03966	685097,3	652772,7	1,039705	788063,3	139223,7	5,619947	341492,3	139223,7	2,433818	157614	139223,7	1,124185
SD	292601,58	383288,79	0,057613	1900,644	383288,79	0,006954	163382,9	70344,26	0,136616	166878,4	148106,46	0,595567	75107,21	148106,46	0,27105	399809,03	148106,46	0,216513
CV	44%	39%	8%	48%	39%	18%	24%	11%	13%	21%	11%	11%	22%	11%	11%	25%	11%	19%
	A AEME	A EME d3	AR	A EME	A EME d3	AR	A COC	A COC d3	AR	A COET	A BEG d3	AR	A BEG	A BEG d3	AR	A NCOC	A BEG d3	AR
21A	29364	59664	0,492156	2386	59664	0,039991	461424	443241	1,041023	514246	95125	5,406003	231663	95125	2,435353	145233	95125	1,52676
21B	48513	77864	0,623048	2733	77864	0,0351	516533	586858	0,880167	729361	149374	4,882784	324225	149374	2,170558	222607	149374	1,490266
21C	65711	108307	0,606711	5075	108307	0,046858	661032	659214	1,002758	786117	151699	5,182084	328982	151699	2,16865	208460	151699	1,374169
X	478632,67	819445	0,573972	3398	819445	0,040649	546329,7	563104,3	0,974649	676574,7	132066	5,156957	294956,7	132066	2,258187	192100	132066	1,463731
SD	181822,22	245766,95	0,071324	1462,651	245766,95	0,005907	103086	109928,4	0,084031	143416,4	32012,96	0,262513	54865,5	32012,96	0,153433	411997,77	32012,96	0,079681
CV	38%	30%	12%	43%	30%	15%	19%	20%	9%	21%	24%	5%	19%	24%	7%	21%	24%	5%

1.1.Anhydroecgonine methyl ester (AEME)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of Strokes15, Strokes18 and Strokes21 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,097	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 1. Hypothesis test summary for AEME.

1.2. Ecgonine methyl ester (EME)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of Strokes15, Strokes18 and Strokes21 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,097	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 2. Hypothesis test summary for EME.

1.3. Cocaine (COC)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of Strokes15, Strokes18 and Strokes21 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,264	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 3. Hypothesis test summary for COC.

1.4. Cocaethylene (COET)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of Strokes15, Strokes18 and Strokes21 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,264	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 4. Hypothesis test summary for COET.

1.5. Benzoylecgonine (BEG)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of Strokes15, Strokes18 and Strokes21 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,097	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 5. Hypothesis test summary for BEG.

1.6. Norcocaine (NCOC)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of Strokes15, Strokes18 and Strokes21 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	,050	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 6. Hypothesis test summary for NCOC.

Each node shows the sample average rank.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Strokes15-Strokes18	-1,000	,816	-1,225	,221	,662
Strokes15-Strokes21	-2,000	,816	-2,449	,014	,043
Strokes18-Strokes21	-1,000	,816	-1,225	,221	,662

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.
Significance values have been adjusted by the Bonferroni correction for multiple

Figure 7. Pairwise comparisons for NCOC.

Annex IV

Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

New miniaturized clean-up procedure for hair samples by means of microextraction by packed sorbent: determination of cocaine and metabolites

Tiago Rosado, Eugenia Gallardo, Duarte N. Vieira, Mário Barroso

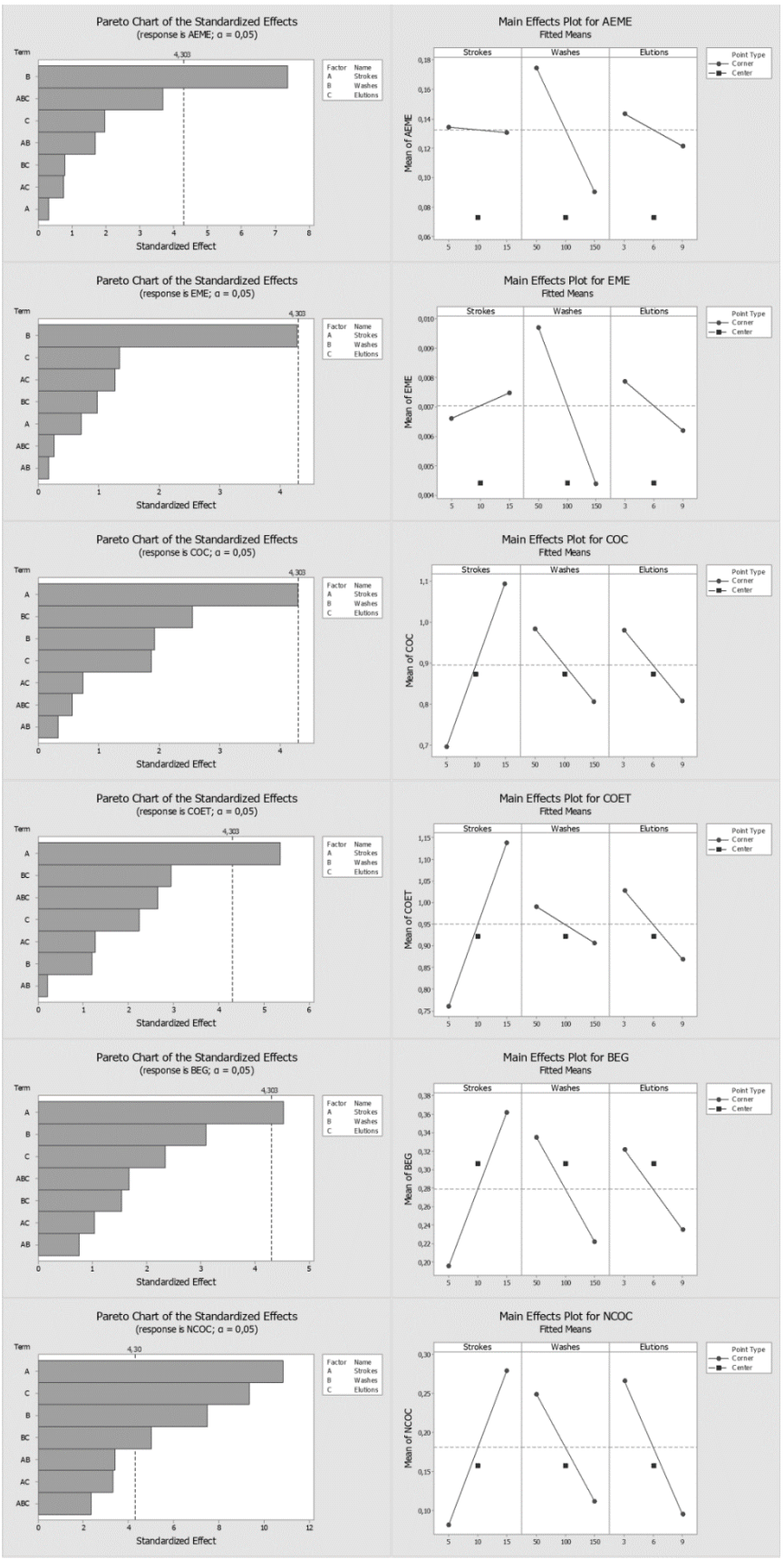

Figure 1. S1 Pareto charts and main effects plots obtained for COC and metabolites after experimental design.

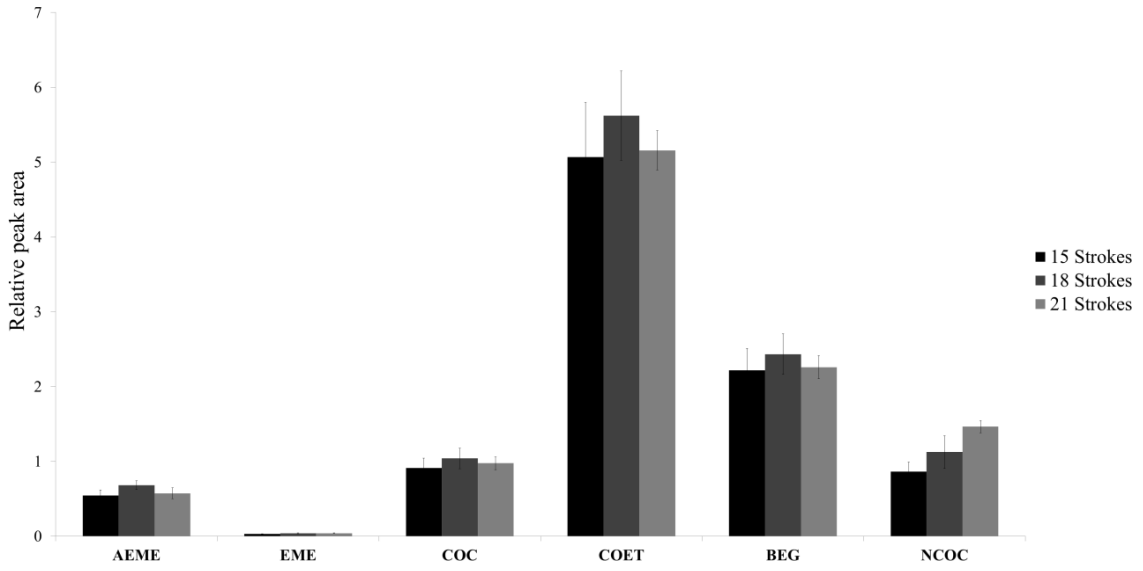

Figure 2. S2 Graphical representation of number of strokes influence on COC and metabolites recoveries (n=3).

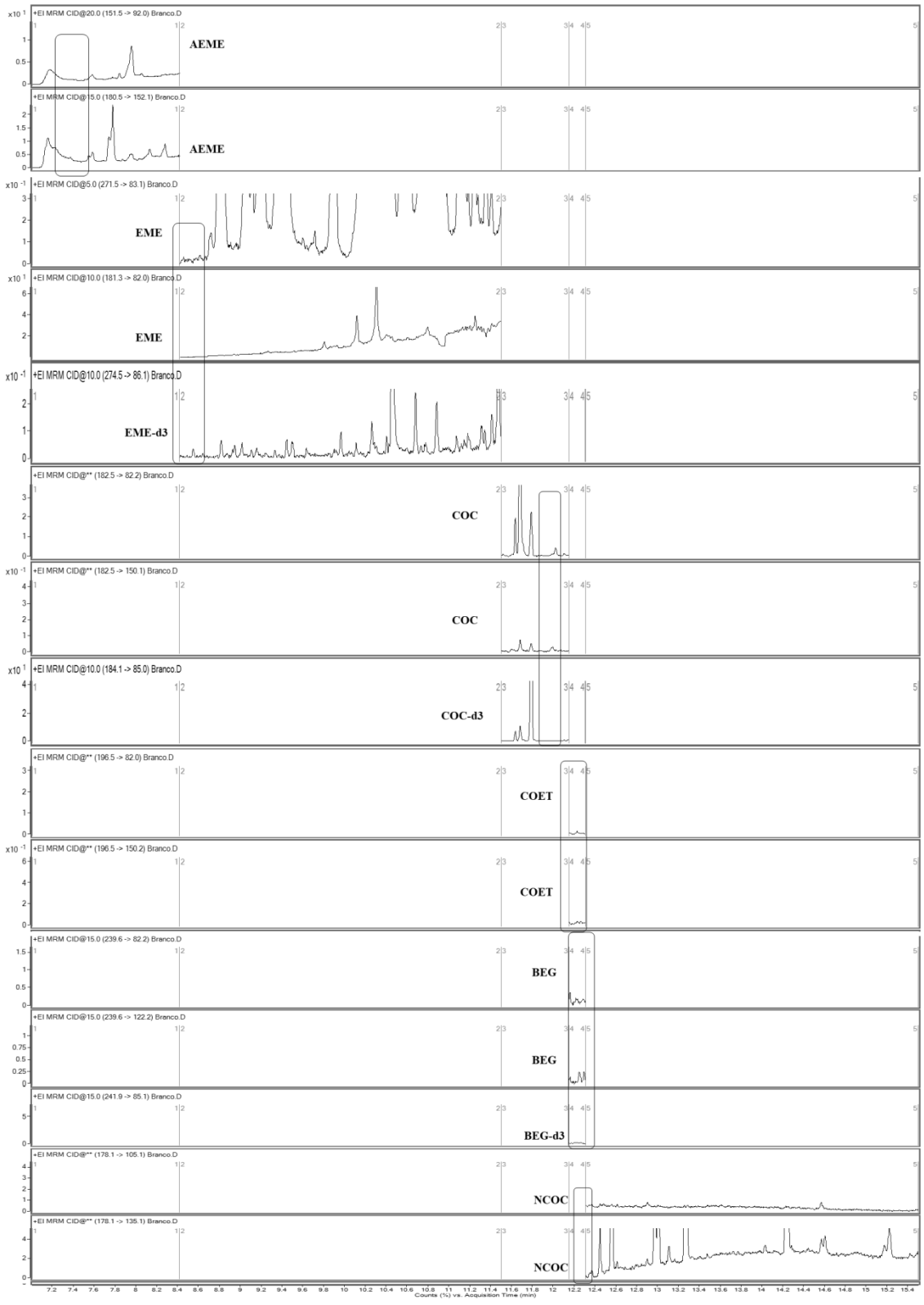

Figure 3. S3 Chromatogram of a blank hair specimen