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ÁREA DE TOXICOLOGIA

André Luis Fabris

Aplicação da Toxicologia Analítica Verde no desenvolvimento de metodologias baseadas em LC-MS para detecção de Novas Substâncias Psicoativas em amostras biológicas

São Paulo
2024

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4º Examinador

São Paulo, 20 de março de 2024

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“Ninguém se torna iluminado por imaginar figuras de luz, mas sim por tornar consciente a escuridão.”

Carl Jung

RESUMO

FABRIS, A. L. Aplicação da Toxicologia Analítica Verde no desenvolvimento de metodologias baseadas em LC-MS para detecção de Novas Substâncias Psicoativas em amostras biológicas. 2024. 152f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2024.

No início dos anos 2000, as Novas Substâncias Psicoativas (NPS) emergiram de forma sem precedentes causando uma drástica mudança no mercado de drogas sintéticas mundial. Estas substâncias são sintetizadas para fins ilícitos e mimetizam o efeito psicoativo das drogas tradicionais. Até o momento, mais de 1000 substâncias foram reportadas mundialmente, representando um grande problema de saúde pública principalmente associado ao desconhecimento das suas propriedades toxicológicas. Por este motivo, métodos analíticos para detectar e quantificar estas substâncias em materiais biológicos são importantes nos casos de toxicologia analítica e forense. Contudo, a tendência de reduzir o impacto ambiental destas metodologias tem ganhado popularidade com a Toxicologia Analítica Verde (GAT). Portanto, o objetivo do presente trabalho foi desenvolver novas técnicas analíticas para analisar as principais classes de NPS em amostras biológicas enquanto aplicando os princípios sustentáveis estabelecidos pela GAT. Os resultados obtidos no presente trabalho são apresentados como coletânea de artigos científicos publicados em revistas. Estes estão descritos nos capítulos 4 a 8. No capítulo 4, uma revisão sobre os desafios no desenvolvimento de técnicas de preparo de amostra para fins forenses é abordada com foco no uso das matrizes secas. No Capítulo 5, está descrito a aplicação da microextração líquido-líquido dispersiva para catinonas sintéticas em amostras de sangue total e urina. No capítulo 6, o artigo descreve o desenvolvimento da técnica microextração líquido-líquido homogênea com solventes de hidrofilicidade comutável para canabinoides sintéticos em amostras de plasma. No capítulo 7, a microextração em fase líquida em placas de 96 poços, cunhada extração paralela em membranas artificiais líquidas, foi desenvolvida para diferentes classes de drogas de abuso, incluindo NPS. O capítulo 8 mostra o desenvolvimento de uma extração por eletromembrana também no formato de placa de 96 poços para catinonas sintéticas em amostras de sangue total. Em todos os trabalhos, as técnicas de extração foram desenvolvidas, otimizadas e validadas. Os princípios da GAT foram aplicados de diferentes formas, como reduzindo o volume de amostra, simplificando os procedimentos, evitando o uso de solventes orgânicos, dentre outros. Assim, alternativas mais sustentáveis para a análise de drogas de abuso em amostras biológicas foram apresentadas e estas ajudam a consolidar e difundir o conceito do desenvolvimento de métodos analíticos com consciência ambiental além de fornecer ferramentas para auxiliar o controle das NPS no país.

Palavras-chaves: Novas substâncias psicoativas, Drogas de abuso, Toxicologia Analítica Verde, Toxicologia Forense, LC-MS/MS.

ABSTRACT

FABRIS, A. L. Application of Green Analytical Toxicology to the development of LC-MS-based methodologies for the detection of New Psychoactive Substances in biological samples. 2024. 152f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2024.

In the early 2000s, New Psychoactive Substances (NPS) emerged and unprecedentedly changed the illicit drug market. These substances are synthesized for illicit purposes and mimic the psychoactive effect of traditional drugs of abuse. To date, more than 1000 substances have been reported worldwide, representing a major public health problem mainly associated with their mostly unknown toxicological properties. In this context, analytical methods able to detect and quantitate these new drugs in biological specimens are important in cases of analytical and forensic toxicology. However, reducing the environmental impact of these methodologies has recently gained popularity with Green Analytical Toxicology (GAT). Therefore, the aim of this work was to develop new analytical techniques to analyze the main classes of NPS in biological samples while applying the environmentally friendly principles established by GAT. The results obtained throughout the development of the present work were split into four papers (chapters 4-8). In chapter 4, a review of common challenges faced during the development of new sample preparation techniques for forensic applications is described focusing on the use of dried matrices. In chapter 5, the application of dispersive liquid-liquid microextraction for synthetic cathinones in whole blood and urine samples is described. In chapter 6, the application of the somewhat recent switchable hydrophilicity solvent-based homogenous liquid–liquid microextraction to synthetic cannabinoids in plasma samples is reported. In chapter 7, liquid-phase microextraction in the 96-well plate format, termed parallel artificial liquid membrane extraction, for different classes of drugs of abuse, including NPS, in plasma samples is presented. In chapter 8, an electromembrane extraction in the 96-well plate format for synthetic cathinones in whole blood samples was developed. In this work, sample preparation techniques were developed, optimized and validated. The principles of sustainable chemistry in method development were applied in different ways, such as reducing the sample volume, simplifying procedures, avoiding the use of organic solvents, among others. Thus, greener alternatives were presented for the analysis of drugs of abuse in biological samples and contribute to consolidate and spread this trend of environmental consciousness during method development. Additionally, valuable techniques that can be used in the combat against NPS were provided.

Keywords: New psychoactive substances, drugs of abuse, Green Analytical Toxicology, Forensic Toxicology, LC-MS/MS.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

5-HT	Serotonina
CB_1	Receptores de canabinoides do tipo 1, do inglês *Cannabinoid Receptor type 1*
CB_2	Receptores de canabinoides do tipo 2, do inglês *Cannabinoid Receptor type 2*
DA	Dopamina
DLLME	Microextração Líquido-Líquido Dispersiva, do inglês *Dispersive Liquid-Liquid Microextraction*
DMT	N, N-dimetiltriptamina
EMCDDA	Observatório Europeu de Monitoramento de Drogas e Toxicodependência, do inglês *European Monitoring Centre for Drugs and Drug Addiction*
EME	Extração por Eletromembrana, do inglês *Electromembrane Extraction*
GAC	Química Analítica Verde, do inglês *Green Analytical Chemistry*
GAT	Toxicologia Analítica Verde, do inglês *Green Analytical Toxicology*
GC	Cromatografia Gasosa, do inglês *Gas Chromatography*
LC	Cromatografia Líquida, do inglês *Liquid Chromatography*
LC-MS/MS	Cromatografia Líquida Acoplada a Espectrometria de Massas em *Tandem*, do inglês *Liquid Chromatography Tandem Mass Spectromtry*
LLE	Extração Líquido-Líquido, do inglês *Liquid-Liquid Extraction*
LPME	Microextração em Fase Líquida, do inglês *Liquid-Phase Microextraction*
LSD	Dietilamida do ácido lisérgico, do inglês *Lysergic acid diethylamide*
MDMA	3,4-metilenodioximetanfetamina
NA	Noradrenalina
NPS	Novas Substâncias Psicoativas, do inglês *New Psychoactive Substances*

PALME	Extração paralela em membranas artificiais líquidas, do inglês *Parallel Artificial Liquid Membrane Extraction*
SHS-HLLME	Microextração Líquido-Líquido Homogênea Com Solvente De Hidrofilicidade Comutável, do inglês *Switchable Hydrophilicity Solvent-Based Homogenous Liquid–Liquid Microextraction*
SNC	Sistema Nervoso Central
SPE	Extração em Fase Sólida, do inglês *Solid-Phase Extraction*
SPME	Microextração em Fase Sólida, do inglês *Solid-Phase Microextraction*
THC	Δ^9-Tetrahidrocanabinol
UNODC	Escritório das Nações Unidas sobre Drogas e Crime, do inglês *United Nations Office on Drugs and Crime*

SUMÁRIO

1. INTRODUÇÃO

1.1 As Novas Substâncias Psicoativas

Nos últimos anos, o mercado de drogas de abuso mundial sofreu uma drástica mudança devido o aparecimento das Novas Substâncias Psicoativas (NPS). Oficialmente, são definidas como “moléculas desenhadas, em sua maioria, para fins ilícitos e com o objetivo de evadir as medidas de controle nacional e internacionalmente aplicadas às substâncias já controladas, das quais derivam ou mimetizam os efeitos”. Estes compostos não são controlados pela Convenção Única de Entorpecentes (1961) ou pela Convenção de Substâncias Psicotrópicas (1971) que regulam as drogas de abuso tradicionais, como a cocaína, anfetaminas e canabinoides. Assim, a definição de “NPS” foi adotada pelo Escritório das Nações Unidas sobre Drogas e Crime (UNODC) para melhor classificá-las. Contudo, diferente do que a nomenclatura sugere, estas drogas não são necessariamente “novas”, pois várias delas foram sintetizadas em meados do século passado, mas se tornaram disponíveis no mercado de drogas sintéticas somente a partir do início dos anos 2000 (EMCDDA, 2022; UNODC, 2022).

Desde o começo do fenômeno das NPS, centenas de compostos inéditos foram reportados, assim causando grande preocupação às autoridades responsáveis pelo controle destas substâncias. De acordo com a UNODC, um total de 1.232 moléculas foram identificadas mundialmente até novembro de 2023, como pode ser observado na Figura 1. Além disso, este massivo número de compostos está associado a grande diversidade e complexidade química. Isto acontece, pois estas substâncias são normalmente desenhadas em semelhança às drogas de abuso tradicionais visando mimetizar seus efeitos psicoativos, como estimulantes do sistema nervoso central, alucinógenos, etc. Para tanto, modificações químicas são realizadas nas estruturas de certas moléculas a fim de criar análogos ou derivados e, desta forma, um grande número de compostos com características químicas diversas é criado.

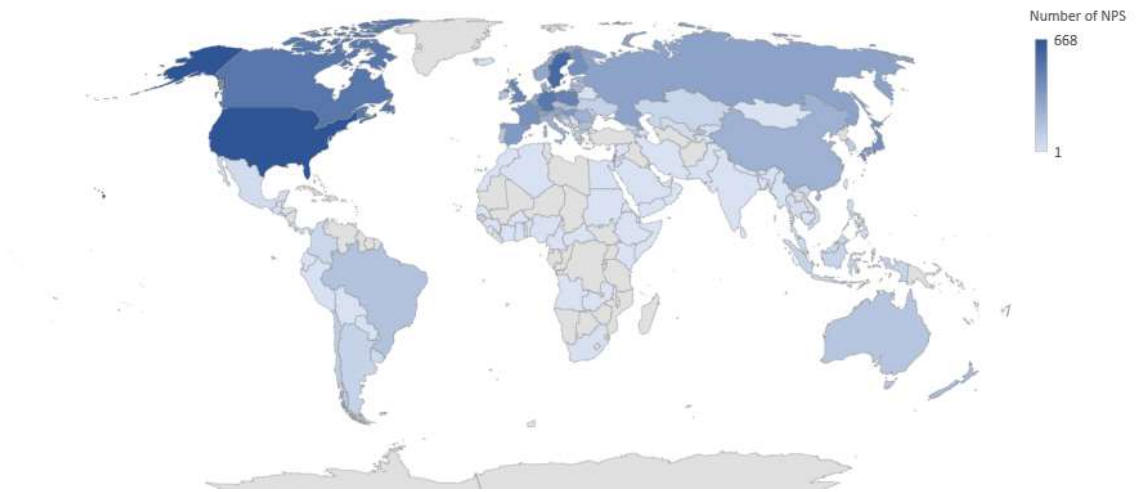

Figura 1. Emergência global das NPS: Número de NPS reportadas à UNODC até novembro de 2023. (Figura adaptada da UNODC - *Early Warning Advisory on NPS*, 2023).

Justamente pelo fato de as NPS possuírem estruturas químicas inéditas, estas comumente escapam do controle legal por não estarem inclusas em legislações vigentes e assim são consideradas lícitas até que sejam proscritas. Esta particularidade facilitou consideravelmente o seu comércio, que por este motivo também são referidas como "*legal highs*". Assim, as NPS ganharam grande popularidade e adesão entre os usuários, pois foram tidas como alternativas legais às drogas tradicionais (EMCDDA, 2022). Contudo, estas estruturas químicas inéditas possuem propriedades farmacológicas desconhecidas, muitas vezes sendo mais potentes e tóxicas do que as drogas convencionais. Por este motivo, diversos casos de intoxicações fatais foram reportados e estudos apontam que, embora muitas NPS produzam efeitos psicoativos semelhantes às drogas clássicas, existe maior risco de intoxicações letais, dependência química ou desenvolvimento de problemas de saúde associado ao seu uso (HERIAN; SWIT, 2023; KROTULSKI; PAPSUN; CHRONISTER; HOMAN *et al.*, 2021; PELLETIER; LE DARE; LE BOUEDEC; BOURDAIS *et al.*, 2023; SHOPAN; SCOLNIK; HASSOUN; FIRSOW *et al.*, 2023; WACHHOLZ; CELINSKI; BUJAK-GIZYCKA; SKOWRONEK *et al.*, 2023). Assim, as NPS se tornaram um problema de saúde pública mundial e o controle desta difusão é uma das prioridades das autoridades responsáveis. Contudo, esta é uma tarefa extremamente desafiadora devido à grande quantidade de moléculas, sua diversidade química e também sua natureza dinâmica de aparecerem e desaparecerem do mercado rapidamente.

O Observatório Europeu de Monitoramento de Drogas e Toxicodependência (EMCDDA) estima um aumento no total de apreensões de NPS de 929 em 2005 para mais de 46 mil em 2016, que é tido como o período de pico destas novas drogas na Europa. Posteriormente, este número sofreu uma queda para 21 mil no ano de 2020 e os últimos dados disponíveis indicam 29 mil apreensões em 2021. Em termos de quantidade de material apreendido, estes números equivalem a aproximadamente 950 kg em 2009, mais de 3 toneladas em 2016 e 8,5 toneladas em 2021 (EMCDDA, 2022). Quanto às classes de NPS, o EMCDDA aponta que foram apreendidas anualmente mais de 70 catinonas sintéticas e canabinoides sintéticos desde 2014, assim sendo consideradas as mais populares entre os usuários europeus. Estes grupos são seguidos pela classe das fenetilaminas, com cerca de 45 substâncias apreendidas anualmente desde 2014 (Figura 2). Atualmente, um total de 930 NPS é monitorada somente na Europa, sendo que 41 destas foram reportadas pela primeira vez em 2022, das quais mais da metade (24) foram canabinoides sintéticos (EMCDDA, 2022).

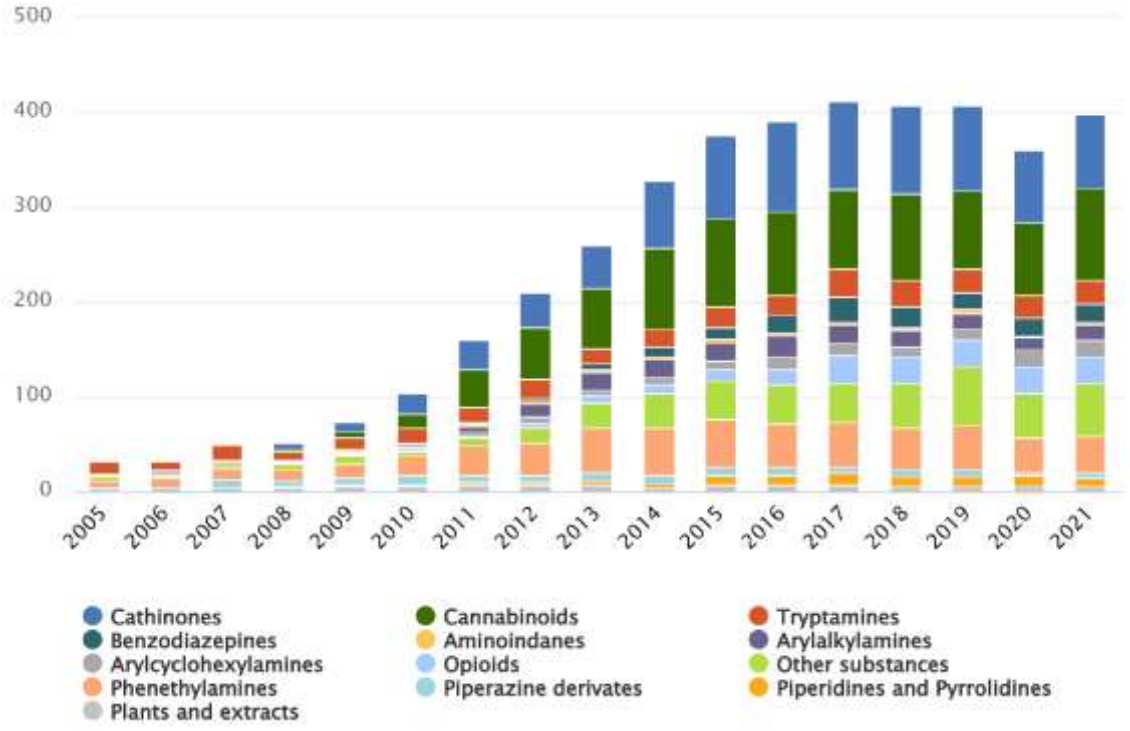

Figura 2. Número de NPS reportadas anualmente na União Europeia de 2005 a 2021. Figura disponível em EMCDDA - *European Drug Report 2023: Trends and Developments*).

No Brasil, as NPS também se difundiram expressivamente, contudo de forma ligeiramente distinta do que foi visto internacionalmente. De 2007 a 2015, a classe mais apreendida foi a das fenetilaminas. Em contrapartida, o grupo dos

canabinoides sintéticos teve apenas 10 substâncias apreendidas pela Polícia Federal em 2014 e 8 em 2015, enquanto o maior número de apreensões de catinonas sintéticas foi de apenas 7 compostos em 2015. Um aumento nestes números também foi visto no país no mesmo período do pico de NPS na Europa: somente entre 2014 e 2017, mais de 100 NPS foram identificadas, 38 destas apenas em 2017. Neste ano, uma mudança no perfil destas drogas ocorreu no Brasil, sendo que as catinonas sintéticas foram as NPS mais detectadas (46%), seguidas das fenetilaminas (26%), enquanto pouquíssimas detecções de canabinoides sintéticos foram feitas (6%) (ANVISA, 2017). Entre 2017 e 2018, as apreensões de catinonas sintéticas foram as maiores no país, totalizando aproximadamente 67% de todos os casos, enquanto o número das fenetilaminas caiu para 15% em 2018. Esta queda também foi observada no número de apreensões de canabinoides sintéticos, de aproximadamente 20% em 2016 para 2% em 2018 (PF, 2020). Embora relatórios oficiais mais recentes não tenham sido publicados pela Polícia Federal ou outros institutos de perícias do país, artigos científicos que contam com a participação de peritos e também comunicação interpessoal indicam que os canabinoides sintéticos são as NPS mais apreendidas após 2020, enquanto as catinonas sintéticas e outras classes estão mais escassas (SAR, 2023). Além disso, casos curiosos envolvendo NPS também foram registrados no país. Por exemplo, canabinoides sintéticos foram encontrados impregnados em papel de cartas enviadas a detentos em presídios brasileiros (RODRIGUES; SOUZA; DE MELO BARBOSA; DE CARVALHO PONCE *et al.*, 2022). De Araujo e colaboradores descreveram a apreensão de 53 casos contendo um canabinoide sintético que tem ação exclusivamente agonista de receptor endocanabinoide do tipo 2 (CB_2), o qual não produz o efeito psicoativo clássico da maconha (DE ARAUJO; FABRIS; NEVES JUNIOR; DE CARVALHO PONCE *et al.*, 2023). Este composto denominado BZO-HEXOXIZID teve pouquíssima popularidade em outros países e seu papel como droga de abuso ainda não foi esclarecido. Houve também o caso da pró-droga de LSD (dietilamida do ácido lisérgico), acetil-LSD, que é uma outra forma de evadir a legislação vigente e facilitar o comércio de NPS (JUNIOR; FABRIS; BARBOSA; DE CARVALHO PONCE *et al.*, 2022). Mais recentemente, peritos da Polícia Científica de São Paulo informaram um aumento sem precedentes na apreensão de opioides, principalmente da classe dos nitazenos (dados não publicados).

Curiosamente, a vasta maioria destes compostos foram apreendidos em amostras de erva, forma semelhante a como os canabinoides sintéticos são comercializados. Esta onda crescente dos novos opioides também foi observada internacionalmente, mas o abuso destas drogas nunca teve papel significativo no Brasil, surpreendendo os profissionais da área e alarmando órgãos de saúde sobre o início de uma possível epidemia de óbitos causados por substâncias opioides.

Em conjunto, os dados de apreensão de NPS mostram que estas drogas continuam a se reinventar e tem característica bastante dinâmica e imprevisível. Desta forma, acompanhar e prever as tendências no mercado de drogas de cada país ou região tem dificultado o trabalho das autoridades legais; fato este que causou mudanças nas políticas antidroga. Por exemplo, o sistema de listagem genérica para a proscrição de novas substâncias foi adotado na legislação brasileira em 2016 para complementar o sistema de listagem nominal que era utilizado exclusivamente até então. No sistema genérico, a definição química de uma família de substâncias e possíveis substituições moleculares são estabelecidas, permitindo a adoção de medidas de controle para todos os compostos que se enquadrem no que foi pré-estabelecido. Desta forma, é permitido às autoridades responsáveis certa antecipação ao surgimento destas novas moléculas e maior eficiência no combate às NPS. Por exemplo, de todas as NPS identificadas no território brasileiro, a maioria está listada nominalmente no Anexo I da Portaria SVS/MS nº 344/98, que controla as substâncias proscritas no país. Contudo, parcela considerável está listada por classes estruturais genéricas (ANVISA, 2017; PF, 2020). Além desta, o Subsistema de Alerta Rápido sobre Drogas foi implementado no país em 2021 para coletar e produzir dados e informações sobre drogas, além de detectar, avaliar e responder às ameaças sociais e à saúde pública. Desta forma, este sistema recentemente criado tem auxiliado na identificação e classificação de drogas emergentes e também difundindo informações ou alertas aos órgãos competentes acerca destas novas substâncias químicas (SAR, 2023).

De maneira geral, o fenômeno das NPS está presente há aproximadamente duas décadas e, embora os órgãos responsáveis estejam cada vez mais preparados para combater estas substâncias, moléculas inéditas continuam sendo criadas e inseridas no mercado de drogas ilícitas até os dias atuais.

1.2 Classificação das NPS

A UNODC classifica as NPS de acordo com as propriedades químicas e os efeitos farmacológicos. Atualmente, são 15 grupos, sendo eles os aminoindanos, benzodiazepínicos, canabinoides sintéticos, catinonas sintéticas, fenciclidina, fenetilaminas, fenidatos, fenmetrazinas, lisergamidas, triptaminas, nitazenos, análogos do fentanil, substâncias do tipo piperazinas, substâncias de origem natural e outras substâncias (UNODC, 2022). Esta classificação é constantemente atualizada ao passo que novas moléculas são reportadas ou grupos de NPS são originados.

Como brevemente abordado anteriormente, a classe dos canabinoides sintéticos, catinonas sintéticas e fenetilaminas costumam estar entre as mais populares em vários países, inclusive no Brasil. Em contrapartida, a difusão de outras classes dentre os usuários, como a dos opioides sintéticos e *designer* benzodiazepínicos, é altamente dependente de diversos fatores culturais, das medidas legais vigentes, dentre outros. Portanto, focaremos a seguir nas três classes de NPS com maior relevância no território brasileiro.

1.2.1 Canabinoides sintéticos

O Δ^9-tetrahidrocanabinol (THC) é o principal constituinte psicoativo presente nas plantas do gênero *Cannabis sp*. e tem ação agonista parcial dos receptores de canabinoides do tipo 1 (CB_1) e CB_2. O exato funcionamento do sistema endocanabinoide ainda não foi totalmente esclarecido, contudo sabe-se que o CB_1 é predominantemente expresso no sistema nervoso central (SNC), enquanto o CB_2 é majoritariamente expresso em órgãos do sistema imunológico e em menor quantidade no SNC. Ambos estes receptores são acoplados à proteínas G (GPCR) e, uma vez ativados, produzem efeitos fisiológicos distintos. Por exemplo, o THC se ligando ao CB_1, presente em neurônios pré-sinápticos nas regiões do córtex, amígdala, hipocampo, gânglios da base e cerebelo, causando relaxamento, analgesia, catalepsia, aumento do apetite, euforia, diminuição da atividade motora e da capacidade de raciocínio, dentre outros (ALVES; GONCALVES; AGUIAR; TEIXEIRA *et al.*, 2020; LU; MACKIE, 2016; SIMON; TOTH; HECKMANN; MAYER *et al.*, 2023). Por outro lado, a ativação do CB_2 não produz efeito psicoativo, embora alguns estudos apontem que este é rapidamente expresso em células nervosas em alguns quadros fisiológicos,

como doenças e abuso de substâncias psicoativas (LU; MACKIE, 2016). Neste contexto, a ativação do CB_2 leva à diminuição da síndrome de abstinência e, portanto, tem efeito antagônico ao CB_1. Assim, o resultado da interação do THC com o CB_1 é o responsável pelo vasto uso da cannabis por várias pessoas que buscam obter tais efeitos (LU; MACKIE, 2016).

O interesse em entender o sistema endocanabinoide e eventualmente utilizar os efeitos fisiológicos dos canabinoides para fins terapêuticos resultou na criação de análogos sintéticos do THC que visavam minimizar os efeitos psicoativos enquanto a ação terapêutica era mantida. Esta abordagem deu origem a classe dos canabinoides sintéticos, como a nabilona, o HU-210, JWH-018, dentre outros (Figura 3) (ALVES; GONCALVES; AGUIAR; TEIXEIRA *et al.*, 2020; ANDREWS; JORGE; CHRISTIE; GALLEGOS, 2022; KROTULSKI; CANNAERT; STOVE; LOGAN, 2021). Entretanto, foi somente no início dos anos 2000 que os canabinoides sintéticos ganharam popularidade no mercado ilícito de drogas de abuso sendo divulgados como alternativas legais e seguras à maconha, além de não serem detectados em testes tradicionais. Em 2008, no início do fenômeno das NPS, o primeiro canabinoide sintético oficialmente reportado neste contexto foi o JWH-018 após ter sido apreendido em alguns países europeus (ANDREWS; JORGE; CHRISTIE; GALLEGOS, 2022). Entre 2008 e 2011, muitos dos canabinoides sintéticos descritos previamente por cientistas foram encontrados sendo comercializados no mercado de drogas. Assim, após o ano de 2012, esta classe de NPS começou a de diversificar quimicamente ao ponto que esta é a classe de NPS com maior variedade química até o momento (DEVENTER; VAN UYTFANGHE; VINCKIER; RENIERO *et al.*, 2022; KROTULSKI; CANNAERT; STOVE; LOGAN, 2021).

A classe dos canabinoides sintéticos se diversificou consideravelmente e esta tendência continua, tornando bastante desafiadora a tarefa de incluir todas as informações referente à estes compostos. De maneira geral, é possível dividir os canabinoides sintéticos em 2 grandes grupos, aqueles que mantém semelhança com os canabinoides naturais e aqueles que não são derivados dos fitocanabinoides e por conseguinte possuem particularidades químicas mais distintas do THC. O primeiro grupo é dividido em clássicos e não-classicos, enquanto o segundo contém os aminoalquilindóis e os mais recentes OXIZIDs (Figura 3) (POTTS; CANO; THOMAS; HILL, 2020).

A maioria dos chamados canabinoides clássicos foram sintetizados na década de 1940 e apresentam estrutura tricíclica característica dos canabinoides naturais, como o homólogo do THC com 6 carbonos na cadeia lateral. O HU-210, por sua vez, tem sua síntese datada em 1988 com 100 a 800 vezes maior atividade em CB_1 do que o THC, sendo o canabinoide sintético mais potente descrito até o momento (Figura 3). Por outro lado, os canabinoides não-clássicos são análogos bicíclicos ou tricíclicos dos canabinoides clássicos, como o CP-47,497 e CP-55,940, sintetizados entre os anos 1970 e 1980 (Figura 3) (KROTULSKI; CANNAERT; STOVE; LOGAN, 2021; POTTS; CANO; THOMAS; HILL, 2020).

O segundo grande grupo de canabinoides sintéticos contém majoritariamente os aminoalquilindóis. Os primeiros compostos desta classe química foram sintetizados na década de 1980, como a pravadolina (WIN 48,098) e alguns análogos. Anos mais tarde, a série JWH e AM (JWH-073, JWH-018, AM-2201, etc.) foi sintetizada com o intuito de estudar o receptor CB_1 e identificar as propriedades químicas necessárias para gerar resposta agonista. Como ilustrado na Figura 3, este grupo possui diferenças químicas mais acentuadas se comparados aos fitocanabinoides, mas continuam exercendo atividade canabimimética. Os aminoalquilindóis podem ser subdivididos dependendo do tipo de grupos funcionais presentes na estrutura; por exemplo, os naftilindóis, como o JWH-015 e JWH-018, os adamantoilindóis, com o ACHMINACA e APINACA, dentre outros (ALVES; GONCALVES; AGUIAR; TEIXEIRA *et al.*, 2020; ANDREWS; JORGE; CHRISTIE; GALLEGOS, 2022; KROTULSKI; CANNAERT; STOVE; LOGAN, 2021). Mais recentemente, o subgrupo dos OXIZIDs tem chamado atenção. Estes diferem dos aminoalquilindóis devido a presença de uma acetohidrazida na região ligante da estrutura (Figura 3). Alguns países reportaram a apreensão destas NPS como droga de abuso. Curiosamente, grandes quantidades do BZO-CHMOXIZID foram apreendidas no Brasil; composto este que possui ação agonista seletiva CB_2 e irrisória em CB_1, intrigando os profissionais do uso de tal composto como entorpecente (DE ARAUJO; FABRIS; NEVES JUNIOR; DE CARVALHO PONCE *et al.*, 2023; DEVENTER; VAN UYTFANGHE; VINCKIER; RENIERO *et al.*, 2022).

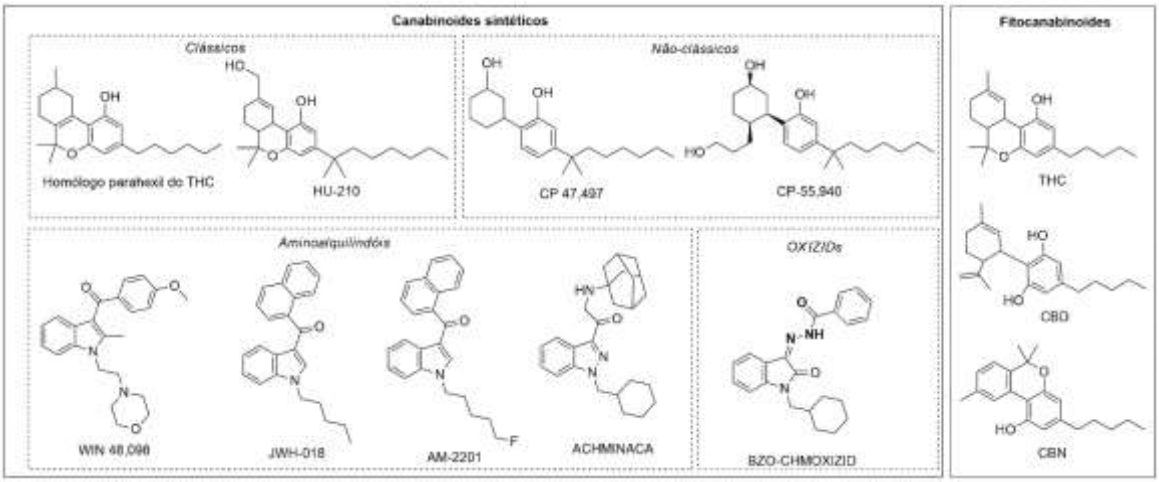

Figura 3. Ilustração de diferentes grupos de canabinoides sintéticos e comparação com os canabinoides naturais ou fitocanabinoides. Esta classe de NPS possui grande diversidade química, podendo ser dividida em dois grandes grupos que são subsequentemente divididos em subgrupos. Estão ilustrados exemplos das principais subclasses de canabinoides sintéticos e os fitocanabinoides estão dispostos apenas para comparação. THC: Δ9-tetrahidrocanabinol; CBD: canabidiol; CBN: canabinol.

Os canabinoides sintéticos são popularmente conhecidos ou denominados *Spice*, K2 ou K3, e são comercializados em folhas de plantas secas nas quais uma solução contendo a substância é borrifada para que então sejam fumadas pelos usuários. Esta via de administração é a mais semelhante à da maconha e por este motivo foi mais amplamente difundida no início do fenômeno das NPS. Entretanto, formas alternativas, como infusões para serem ingeridas, comprimidos, gomas de mascar, incensos herbais, pós e tiras orodispersíveis, também eram utilizadas (EMCDDA, 2017b; 2023; UNODC, 2022). Todavia, uma forma de uso dos canabinoides sintéticos que chamou atenção nos últimos anos foi a impregnação destes em papéis comuns ou selos do tipo LSD (NORMAN; WALKER; MCKIRDY; MCDONALD *et al.*, 2020; RODRIGUES; SOUZA; DE MELO BARBOSA; DE CARVALHO PONCE *et al.*, 2022). Esta apresentação teve bastante popularidade em presídios onde o acesso à tais substâncias por parte dos presidiários é facilitado e não deixa suspeitas, pois estas podem ser borrifadas em cartas, bilhetes, etc., e passadas por visitantes, além de não produzirem cheiro. Digno de nota, há relatos do uso destes papeis pela via ocular (NORMAN; WALKER; MCKIRDY; MCDONALD *et al.*, 2020).

Embora o THC seja uma substância com faixa terapêutica segura, isso não se aplica à maioria dos canabinoides sintéticos. Especialmente em virtude das várias vias de administração que estes podem ser consumidos (CANNAERT; SPARKES; PIKE; LUO *et al.*, 2020). Dependendo das substituições de

grupamentos funcionais que foram realizadas nas moléculas, os canabinoides sintéticos exercem diferentes efeitos. Por exemplo, o HU-210 é um potente agonista CB_1 e também tem afinidade pelo CB_2. Similarmente, o JWH-018 também tem alta potência em ambos CB_1 e CB_2. Em consequência, estes compostos causam maiores efeitos indesejáveis após o uso, como tremores, náusea, dispneia, agitação e convulsões – efeitos estes que não são experienciados com o uso da cannabis. Além destes, casos mais graves também foram reportados, como falência renal, rabdomiólise, coma e óbito (GIORGETTI; CECCHI; AUWARTER, 2023; GROTH; ROIDER; ANGERER; SCHAPER *et al.*, 2023; SIMON; TOTH; HECKMANN; MAYER *et al.*, 2023).

1.2.2 Catinonas sintéticas

A catinona é um alcaloide encontrado naturalmente nas folhas da planta *khat* (*Catha edulis*), nativa do Leste da África e da Península Arábica, e tem ação estimulante no SNC. Culturalmente, os nativos da região mascam as folhas da *khat* para obter os efeitos estimulantes da catinona. Sua estrutura química, com esqueleto β-ceto-feniletilamina, é semelhante às drogas tipo anfetamina, sendo também conhecidos como análogos β-ceto da anfetamina (Figura 4) (ALMEIDA; SILVA; PINHO; REMIAO *et al.*, 2022; KUROPKA; ZAWADZKI; SZPOT, 2023). Embora alguns compostos deste grupo tenham sido sintetizados em meados do século XX, como a metcatinona em 1928, mefedrona em 1929, o 3,4-metilenodioxipirovalerona em 1967, e, mais recentemente, a metilona em 1996, a catinona foi isolada da planta *khat* somente na década de 1970 (Figura 4) (GERMAN; FLECKENSTEIN; HANSON, 2014; HYDE; BROWNING; ADAMS, 1928). A síntese destes compostos na época tinha o objetivo de encontrar novos medicamentos. Por exemplo, a dietilpropiona ou anfepramona e a bupropiona eventualmente foram aprovados para uso humano e são utilizados até os dias atuais como inibidor do apetite e antidepressivo, respectivamente. Entretanto, muitos destes outros compostos que não tiveram o mesmo fim passaram a ser comercializados no mercado de drogas no início dos anos 2000 (SOARES; COSTA; BASTOS; CARVALHO *et al.*, 2021). Por exemplo, a metilona, em 2004, foi uma das primeiras catinonas sintéticas a ser comercializada pela internet e lojas físicas; em 2007, o uso da mefedrona começou a surgir em diversos países e outras catinonas sintéticas passaram a ganhar espaço no mercado, como a

butilona e a 3,4-metilenodioxipirovalerona (KUROPKA; ZAWADZKI; SZPOT, 2023). Juntamente ao aumento do uso dos compostos desta classe, uma queda na qualidade e disponibilidade do *ecstasy* (3,4-metilenodioximetanfetamina ou MDMA) e cocaína impulsionou ainda mais o uso das catinonas sintéticas em 2009, que continuou a crescer nos anos subsequentes (EMCDDA, 2017a; UNODC, 2013). Usuários buscavam alternativas à estas drogas e encontraram nas catinonas sintéticas, particularmente na mefedrona, efeito bastante semelhante (LOI; CORKERY; CLARIDGE; GOODAIR *et al.*, 2015). Além disso, a praticidade em obter estes compostos pela internet associada com o menor preço comparado à outras drogas, a maior potência e a condição lícita destes compostos também foram responsáveis por aumentar a sua difusão e adesão pelos usuários. Consequentemente, a crescente popularidade da mefedrona resultou na sua proibição em 2010, o que dificultou sua aquisição, mas não impediu completamente seu uso (EMCDDA, 2010). Assim, novas moléculas, usando o esqueleto químico da catinona como base, continuaram a ser desenvolvidas diversificando ainda mais esta classe de drogas de abuso (Figura 4).

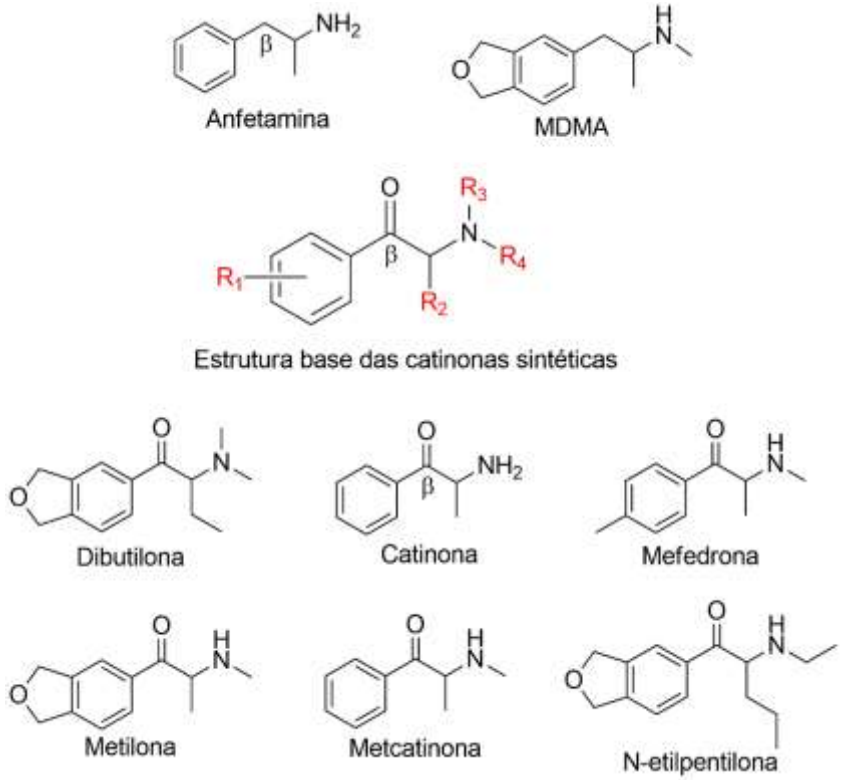

Figura 4. Representação da semelhança entre a estrutura química das catinonas sintéticas com as anfetaminas. A molécula da catinona é tida como o esqueleto base para a obtenção de derivados ou análogos. Algumas catinonas sintéticas estão dispostas como exemplos e a

anfetamina e o MDMA foram incluídos para comparação. O “β” indica o carbono β no qual um grupo cetona está presente na estrutura da catinona e seus derivados sintéticos.

O uso de estimulantes psicomotores, como a metanfetamina e cocaína, continua sendo um problema de saúde público mundialmente. Como os análogos da catinona possuem estrutura química bastante semelhante às das anfetaminas, efeito psicoativo similar também é produzido. Estas drogas causam o acúmulo dos neurotransmissores dopamina (DA) e serotonina (5-HT) na fenda sináptica, e da noradrenalina (NA) em menor proporção. O aumento na concentração destes pode acontecer por dois mecanismos: i) aumento na liberação de vesículas intracelulares contendo estes neurotransmissores, localizadas nos neurônios pré-sinápticos e ii) inibição da recaptação destas moléculas da fenda sináptica após terem sido liberadas. Este segundo mecanismo é a principal para terminar a transmissão nervosa das monoaminas, portanto perturbação desta via causa efeitos bastante pronunciados. Assim, o resultado do acúmulo destes neurotransmissores na fenda sináptica é o estímulo excessivo dos receptores pós-sinápticos causando os sintomas estimulantes e/ou alucinógenos experimentados após o uso das anfetaminas ou compostos semelhantes, especialmente no núcleo *accumbens* (ALMEIDA; SILVA; PINHO; REMIAO *et al.*, 2022; EMCDDA, 2021; LOI; CORKERY; CLARIDGE; GOODAIR *et al.*, 2015).

As catinonas sintéticas são comumente vendidas como sais de banho, adubo para plantas, dente outros, nas formas de pó cristalino, cápsulas ou tabletes. São tipicamente consumidas por insuflação nasal, mas podem ser ingeridas ou injetadas via intramuscular ou intravenosa, aumentando consideravelmente o risco de intoxicação (EMCDDA, 2021). Seu uso é mais popular entre jovens de ambos os sexos em ambientes de casas noturnas, bares e *raves*, justamente por conta do seu efeito estimulante e/ou empatogênico (ALMEIDA; SILVA; PINHO; REMIAO *et al.*, 2022; KUROPKA; ZAWADZKI; SZPOT, 2023; UNODC, 2022). Além destes efeitos, há relatos de várias reações adversas, como agressividade, hipertermia, psicose, paranoia, dor torácica, dentre outros (LOI et al., 2015). Contudo, também há relatos de efeitos mais graves, como falência de órgãos, convulsões e óbito, tanto se usadas individualmente ou associadas à outras drogas, como etanol, benzodiazepínicos e cocaína (EMCDDA, 2021; LOI;

CORKERY; CLARIDGE; GOODAIR *et al.*, 2015; WARRICK; WILSON; HEDGE; FREEMAN *et al.*, 2012).

1.2.3 Fenetilaminas

Os compostos chamados de alucinógenos ou psicodélicos causam alterações na percepção, humor e cognição e este efeito psicoativo é mediado principalmente por meio da interação com receptores serotoninérgicos (DE GREGORIO; AGUILAR-VALLES; PRELLER; HEIFETS *et al.*, 2021; GERWE; HE; POTTIE; STOVE *et al.*, 2022). Este grupo de moléculas alucinógenas é dividido de acordo com sua estrutura química em duas grandes classes, sendo elas as indoleamidas e as fenilalquilaminas. A primeira inclui a subclasse das ergolinas, como a dietilamida do ácido lisérgico (LSD), e das triptaminas, como a N,N-dimetiltriptamina (DMT) presente na ayahuasca. Similarmente, a classe das fenilalquilaminas também é dividida em duas subclasses de acordo com a estrutura química, sendo elas as fenilisopropilaminas, como os chamados compostos da série D, e fenetilaminas, como a mescalina e os compostos da série 2C ou 2C-x (Figura 5) (GEYER; NICHOLS; VOLLENWEIDER, 2017; HALBERSTADT, 2017; HERIAN; SWIT, 2023; PALAMAR; ACOSTA, 2020).

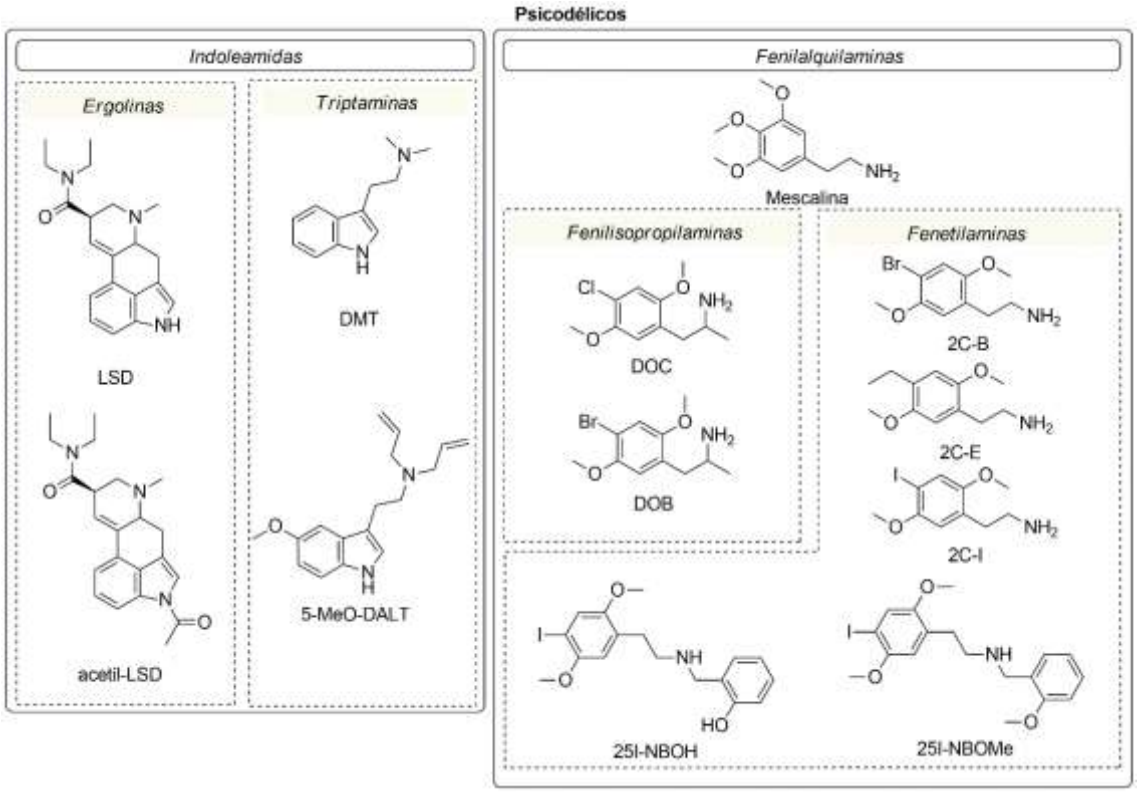

Figura 5. Ilustração dos diferentes grupos de alucinógenos. Os psicodélicos são divididos em dois grandes grupos, indoleamidas e fenilalquilaminas, e em dois subgrupos cada.

Historicamente, as fenetilaminas foram estruturalmente inspiradas na molécula da mescalina, um composto natural encontrado no cacto peiote e que é a única fenetilamina alucinógena de origem natural. Esta, por sua vez, possui baixa potência, necessitando de uma dose de 250 a 500 mg para produzir o efeito psicoativo (GEYER; NICHOLS; VOLLENWEIDER, 2017; HALBERSTADT, 2017). Entretanto, a estrutura química deste composto serviu como base para a criação de diversas fenetilaminas sintéticas por meio de modificações químicas. Por exemplo, os compostos “2C” são assim chamados devido aos dois átomos de carbono que separam o grupo amina do anel aromático (Figura 5). O primeiro composto desta classe foi sintetizado em 1974 pelo Alexander Shulgin, o 2C-B, mas décadas mais tarde seria encontrado no mercado de drogas em meio ao cenário das NPS. De forma semelhante, outras moléculas também foram produzidas por meio da substituição dos grupos químicos ligados ao anel aromático, como o 2C-E e 2C-I que contém um grupo etila e iodo, respectivamente, na posição 4. Além destes, um grupo particular de compostos 2C ganhou bastante destaque após 2010, os NBOMes. Estes contêm um grupamento metóxi-benzil ligado na amina presente na cadeia carbônica lateral (Figura 5). O primeiro NBOMe a aparecer no mercado ilícito foi o 25I-NBOMe, seguido do 25B-NBOMe e 25C-NBOMe. Desde então, vários outros NBOMes surgiram e eventualmente deram lugar aos NBOHs, que possuem um grupo hidróxibenzil ligado na amina. De forma similar, a denominada “série D” também foi resultado de modificações na molécula da mescalina. Contudo, estes possuem uma metila na cadeia carbônica lateral e, portanto, também são conhecidos como “anfetaminas alucinógenas” devido a semelhança estrutural com a anfetamina; não surpreendentemente, estas fenetilaminas também interagem com transportadores de monoaminas. Exemplos deste grupo são o 2,5-Dimetóxi-4-cloroanfetamina (DOC) e o 2,5-Dimetóxi-4-bromoanfetamina (DOB) (Figura 5) (HALBERSTADT, 2017; HERIAN; SWIT, 2023).

Estudos descrevem que os efeitos alucinógenos são mediados pela ativação de receptores serotoninérgicos, especialmente o subtipo 2A. Curiosamente, as indoleamidas são agonistas não-seletivos dos receptores serotoninérgicos, possuindo afinidade tanto pelo subtipo 1 quanto pelo subtipo 2, enquanto as

fenilalquilaminas são altamente seletivas para o subtipo 2. Entretanto, estas interações ligante-receptor dependem das substituições feitas nas moléculas, podendo resultar em ação agonista ou antagonista em determinados receptores com maior ou menor afinidade. De maneira geral, as fenetilaminas podem induzir a liberação de NA e DA ou inibir a recaptação da 5-HT, NA e DA resultando em acúmulo destes neurotransmissores na fenda sináptica. Desta forma, causam a super excitação de receptores presentes na membrana de neurônios pós-sinápticos resultando em efeitos estimulantes e/ou alucinógenos (GEYER; NICHOLS; VOLLENWEIDER, 2017). Por exemplo, o 25I-NBOMe foi descrito como tendo potência próxima do LSD, com doses na faixa de 0,5 a 1 mg (HALBERSTADT, 2017; PALAMAR; ACOSTA, 2020).

As fenetilaminas são comercializadas em forma de pó, cápsulas, líquidos ou em selos do tipo LSD, podendo ser ingeridas ou insufladas e são predominantemente usadas em ambientes de festas noturnas e bares (GERMAN; FLECKENSTEIN; HANSON, 2014; GEYER; NICHOLS; VOLLENWEIDER, 2017; PROSSER; NELSON, 2012). Dos efeitos adversos mais comuns causados por estes compostos, estão vômitos e diarreia, depressão, tontura, dores de cabeça e confusão. Entretanto, como esta classe de NPS é comumente usada alternativamente ao LSD e *ecstasy* e justamente por ser normalmente menos potente e/ou mais tóxica, vários casos de intoxicações letais já foram reportados, principalmente associados ao uso de NBOMes (HERIAN; SWIT, 2023; PROSSER; NELSON, 2012).

Atualmente, a classe dos canabinoides sintéticos é a mais popular, tanto na União Europeia quanto no Brasil. O grupo das catinonas sintéticas se encontra em segundo lugar; inclusive, o EMCDDA alerta em seu relatório mais recente sobre o aumento na apreensão de compostos desta classe de estimulantes e expressa preocupação sobre uma possível crise a ser causada pelas catinonas sintéticas futuramente. Por fim, embora as fenetilaminas sejam menos frequentes ou populares do que as duas outras classes de NPS e, dependendo do país, os números relacionados a estes compostos sejam baixos, estes alucinógenos ainda possuem relevância e trazem riscos à saúde pública, principalmente no Brasil (ANVISA, 2017). Assim, em virtude de novos compostos destas três principais classes continuarem emergindo no mercado de drogas brasileiro, se faz necessário manter um cuidado adicional a elas. Neste sentido,

uma ferramenta importante para o controle e combate às NPS é o uso das metodologias analíticas que são empregadas para detectá-las e quantifica-las em diferentes tipos de amostras. Contudo, com o ritmo frequente e alarmante no qual as NPS continuam surgindo e se diversificando, tais metodologias precisam ser constantemente revisadas, atualizadas e aprimoradas, portanto, é sobre elas que discutiremos na sequência.

1.3 Métodos analíticos para determinação das NPS

O contínuo surgimento de novas drogas sintéticas torna a tarefa de detectá-las e identificá-las bastante desafiadora. Isto ocorre, pois os testes rápidos utilizados na rotina, como imunoensaios e ensaios colorimétricos, não são destinados às NPS e normalmente somente às drogas clássicas. O massivo número destes compostos inéditos associado à sua grande diversidade química limita o uso destes testes e os torna rapidamente obsoletos para trabalhar com as NPS (SWORTWOOD; HEARN; DECAPRIO, 2014). Assim, técnicas analíticas mais sofisticadas, como a cromatografia e a espectrometria de massa, se tornaram o padrão ouro para determinar as NPS em diferentes casos na toxicologia analítica. Se destacam, por exemplo, a cromatografia em fase gasosa acoplada à espectrometria de massa (GC-MS) e cromatografia em fase liquida acoplada à espectrometria de massa (LC-MS), pois oferecem a especificidade e sensibilidade necessária para identificar e quantificar drogas de abuso em diversos tipos de amostra. Estas propriedades são fundamentais para trabalhar com as NPS, contudo, estes equipamentos são custosos e delicados, tornando necessário o uso de algum tipo de processamento de amostra para purificar estas matrizes antes de serem analisadas por estes instrumentos analíticos.

A literatura dispõe de vários trabalhos que descrevem diferentes técnicas de preparo de amostra, ou técnicas de extração, para analisar NPS em diferentes matrizes, como droga bruta e amostras biológicas diversas (KNEISEL; AUWARTER, 2012; LAU; CONCHEIRO; COOPER, 2020; ODOARDI; FISICHELLA; ROMOLO; STRANO-ROSSI, 2015; SANCHEZ-GONZALEZ; ODOARDI; BERMEJO; BERMEJO-BARRERA *et al.*, 2018; TANG; CHING; LEE; LAM *et al.*, 2014; TOMAI; GENTILI; CURINI; GOTTARDO *et al.*, 2021). Assim, o preparo destes diferentes tipos de amostra depende do nível de complexidade do material em questão. Por exemplo, as drogas brutas apreendidas, como pós e selos do tipo LSD, podem ser simplesmente diluídas em solvente orgânico e

posteriormente injetadas em um GC-MS (DE ARAUJO; FABRIS; NEVES JUNIOR; DE CARVALHO PONCE *et al.*, 2023; NORMAN; WALKER; MCKIRDY; MCDONALD *et al.*, 2020; RODRIGUES; SOUZA; DE MELO BARBOSA; DE CARVALHO PONCE *et al.*, 2022). Isto é possível principalmente devido as substâncias psicoativas estarem presentes neste material em altas concentrações, assim também dispensando a necessidade de equipamentos altamente sensíveis, como o LC-MS. Em contrapartida, matrizes biológicas, como sangue e urina, demandam de um processo normalmente mais elaborado e trabalhoso (DA CUNHA; OLIVEIRA; HUESTIS; COSTA, 2020; LAU; CONCHEIRO; COOPER, 2020; ODOARDI; FISICHELLA; ROMOLO; STRANO-ROSSI, 2015; SCHEID; ELLER; OENNING; CARASEK *et al.*, 2022). Neste caso, este tipo de material apresenta diversos compostos que interferem e/ou são incompatíveis com os instrumentos analíticos e precisam ser removidos, por exemplo, proteínas, lipídios, carboidratos, células, dentre outros. Além disso, a concentração dos analitos de interesse nestas amostras também é um desafio, pois costuma ser extremamente baixa. Por exemplo, a concentração plasmática de algumas NPS, como os canabinoides sintéticos, está na ordem de picograma por mililitro (ADAMOWICZ, 2021). Em contraste, amostras biológicas sólidas, como cabelo, tem complexidade ainda maior, exigindo etapas adicionais no processamento, como a lavagem da matriz para remoção de contaminantes externos e a digestão da fibra para liberar as concentrações baixíssimas de analitos que estão impregnados no seu interior (GOTTARDO; SORIO; MUSILE; TRAPANI *et al.*, 2014; RUBICONDO; SCUFFI; PIETROSEMOLI; MINEO *et al.*, 2023). Assim, o LC-MS costuma ser indispensável para analisar casos de NPS nestes tipos de amostras, principalmente devido sua alta sensibilidade e eficiência na separação de vários compostos com alta semelhança química.

A extração em fase sólida (SPE) e a extração líquido-líquido (LLE) são técnicas bastante consolidadas para processar amostras simples e complexas, como sangue, urina, saliva e cabelo, para fins toxicológicos (DA CUNHA; OLIVEIRA; HUESTIS; COSTA, 2020; LAU; CONCHEIRO; COOPER, 2020; ODOARDI; FISICHELLA; ROMOLO; STRANO-ROSSI, 2015). Entretanto, a crescente busca por alternativas mais sustentáveis resultou em outras abordagens sendo propostas para processar estes tipos de matrizes. Esta tendência teve início com a Química Verde, conceito introduzido por Anastas e

Warner em 1998 foi definido como “uso de técnicas e metodologias químicas que reduzem ou eliminam o uso ou geração de matérias-primas, produtos, subprodutos, solventes, reagentes etc., que são perigosos para a saúde humana ou para o meio ambiente” (ANASTAS, 1999; ANASTAS; WARNER, 1998). Posteriormente, este conceito foi adaptado para a Química Analítica Verde (GAC), que é uma aplicação da Química Verde nas áreas da Química Analítica. A GAC, por conseguinte, também consiste em desenvolver métodos mais rápidos, baratos e menos nocivos ao meio ambiente por meio do uso de menores volumes de solventes tóxicos – ou substituição destes solventes por opções mais sustentáveis –, assim diminuindo o desperdício de insumos e reduzindo os resíduos produzidos (GAŁUSZKA; MIGASZEWSKI; NAMIEŚNIK, 2013). Eventualmente, outras adaptações e ramificações surgiram, como a *Green Toxicology*, *Green Analytical Toxicology* (GAT), *White Analytical Chemistry* e *Green Sample Preparation* (DE PAULA MEIRELLES; FABRIS; FERREIRA DOS SANTOS; COSTA *et al.*, 2023; KREBS; MCKEAGUE, 2020; LÓPEZ-LORENTE; PENA-PEREIRA; PEDERSEN-BJERGAARD; ZUIN *et al.*, 2022; NOWAK; WIETECHA-POSŁUSZNY; PAWLISZYN, 2021). Todas estas têm o objetivo em comum de reduzir o impacto ambiental causado por determinadas práticas nas áreas da Química Orgânica e Analítica. A GAT, por sua vez, teve origem recente, em 2022, e estabeleceu diretrizes para o desenvolvimento de práticas sustentáveis no campo da Toxicologia Analítica (DE PAULA MEIRELLES; FABRIS; FERREIRA DOS SANTOS; COSTA *et al.*, 2023). A Figura 6 ilustra um resumo dos princípios estabelecidos pela GAT e técnicas de extração que se adequam a eles de alguma forma.

Figura 6. Resumo dos princípios da GAT e técnicas que atendem alguns deles. Os conceitos da GAT estão dispostos no centro enquanto técnicas que atendem alguns destes princípios de sustentabilidade estão dispostas nos círculos ao redor. SPME: microextração em fase sólida; SBSE: extração sortiva em barra de agitação; DLLME: microextração líquido-líquido dispersiva; QuEChERS: *Quick, Easy, Cheap, Effective, Rugged and Safe*; EME: extração por eletromembrana; PALME: e*xtração paralela em membranas artificiais líquidas*; LPME: microextração em fase líquida; SHS-HLLME: microextração líquido-líquido homogênea com solventes de hidrofilicidade comutável.

Diversos trabalhos mostram o desenvolvimento de técnicas analíticas destinadas a análise de drogas de abuso, tanto na forma bruta quanto em amostras biológicas, aplicando os princípios do desenvolvimento sustentável. Dentre as técnicas que tiveram maior difusão e popularidade, pode-se citar a microextração em fase sólida (SPME), microextração em fase líquida (LPME), microextração líquido-líquido dispersiva (DLLME), extração por eletromembrana (EME), dentre outras (LUIZ OENNING; BIRK; ELLER; FRANCO DE OLIVEIRA *et al.*, 2020; PILAROVA; SULTANI; ASK; NOVAKOVA *et al.*, 2017; SCHEID; ELLER; OENNING; CARASEK *et al.*, 2022; SILVEIRA; LOURENCO; FONSECA PEGO; GUIMARAES DOS SANTOS *et al.*, 2021). Estas técnicas oferecem vantagens interessantes em comparação às tradicionais SPE e LLE, como a redução do volume de amostra e de solventes tóxicos, maior rapidez e simplicidade na execução destas práticas, além da redução dos custos, dentre outros (DE PAULA MEIRELLES; FABRIS; FERREIRA DOS SANTOS; COSTA

et al., 2023; LÓPEZ-LORENTE; PENA-PEREIRA; PEDERSEN-BJERGAARD; ZUIN *et al.*, 2022). Interessantemente, o Brasil se encontra no terceiro posto entre os países que mais contribuem com a redução do impacto ambiental causado por estas práticas (GAMA; MELCHERT; PAIXAO; ROCHA, 2019). Parte deste trabalho tem sido feito por nosso grupo de pesquisa que, nos últimos anos, vem propondo técnicas e aplicações mais sustentáveis para a análise de drogas de abuso em diversas matrizes (BAIRROS; LANARO; ALMEIDA; YONAMINE, 2014; SILVEIRA; LOURENCO; FONSECA PEGO; GUIMARAES DOS SANTOS *et al.*, 2021). Inclusive, a GAT teve origem em nosso grupo com a colaboração de outros pesquisadores (DE PAULA MEIRELLES; FABRIS; FERREIRA DOS SANTOS; COSTA *et al.*, 2023). Portanto, embora vários trabalhos descrevam a análise de NPS em amostras biológicas, a sustentabilidade de seus métodos é muitas vezes negligenciada.

2. OBJETIVO

O presente projeto de pesquisa buscou desenvolver novas metodologias analíticas para identificar e quantificar as classes de NPS mais relevantes em território nacional, especialmente no estado de São Paulo, em amostras biológicas. Para tanto, os princípios da GAT foram considerados e implementados no desenvolvimento destas metodologias.

3. APRESENTAÇÃO DOS DADOS

Os dados obtidos no presente trabalho serão apresentados como uma coletânea de artigos científicos que foram publicados em revistas científicas internacionais. Assim, o Capítulo 4 é sobre artigo de revisão *Dried matrix spots in forensic toxicology* publicado na revista *Bioanalysis* e aborda desafios no desenvolvimento e adaptação de técnicas de preparo de amostras para analisar novos compostos. O Capítulo 5 é referente ao artigo *Development of a dispersive liquid-liquid microextraction for synthetic cathinones in biological fluids based on principles of Green Analytical Toxicology* publicado na revista *Journal of Analytical Toxicology*. O Capítulo 6 apresenta o artigo *A new application of the Switchable Hydrophilicity Solvent-Based Homogenous Liquid–Liquid Microextraction to analyze synthetic cannabinoids in plasma by LC-MS/MS* publicado na revista *Journal of Pharmaceutical and Biomedical Analysis*. O Capítulo 7 traz o artigo *Solvent-free parallel artificial liquid membrane extraction of drugs of abuse* publicado na revista *Analytica Chimica Acta*. Por fim, o Capítulo 8 mostra o artigo *An efficient and green method for the analysis of synthetic cathinones in whole blood using 96-well electromembrane extraction and LC-MS/MS* publicado na revista *Sustainable Chemistry and Pharmacy*. Os capítulos estão dispostos em ordem cronológica de submissão e publicação.

4. ARTIGO I

Dried matrix spots in forensic toxicology

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Dried matrix spots in forensic toxicology

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Dried matrix spots (DMS) has gained the attention of different professionals in different fields, including toxicology. Investigations have been carried out in order to assess the potential of using DMS for the analysis of illicit substances, the main interest of forensic toxicologists. This technique uses minimal volumes of samples and solvents, resulting in simple and rapid extraction procedures. Furthermore, it has proved to increase analyte stability, improving storage and transportation. However, DMS presents some limitations: the hematocrit influencing accuracy and inconsistencies regarding the means of spotting samples and adding internal standard on paper. Thus, we provide an overview of analytical methodologies with forensic applications focusing on drugs of abuse and discussing the main particularities, limitations and achievements.

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Keywords: amphetamines • cannabis • cocaine • dried blood spots • dried matrix spots • forensic toxicology • gamma-hydroxybutyric acid • new psychoactive substances • opioids • volumetric absorptive microsampling

Reported by Ivar Bang in 1913 [1], blood was the first matrix in the dried matrix spot (DMS) format, which eventually would be known as dried blood spots (DBS). Furthermore, in the 1960s Guthrie and Susi widened its applicability, using it to perform the measurement of phenylalanine for the early diagnosis of phenylketonuria in infants, becoming particularly important in newborn screening until today [2]. Currently, DBS is used in newborn screening and has been extensively applied in different fields to determine sexually transmitted diseases, biomarkers of inherited generic disorders, medications, drugs of abuse, etc. [3–10]. In order to obtain a DMS, a small drop of a biofluid (5–40 µl) is absorbed onto a filter paper and then is dried at room temperature for a couple of hours; blood, for instance, is collected by a finger- or heel-prick instead of venipuncture. Alternatively, sampling can be performed by spotting a drop of a previously collected sample. This concept offers a simple, noninvasive and cost-effective sample collection compared with in-tube samples, which require trained personnel to sample and process the specimen. Furthermore, the compact size of Guthrie cards combined with the absence of water improves the stability of analytes and facilitates storage and transportation [11–15]. Conversely, some limitations are also present when working with dried matrices, such as variations of hematocrit (HCT) among patients resulting in loss of accuracy [16,17].

Professionals of different medical sciences have directed their attention to the idea of replacing conventional liquid samples for dried matrices in daily analysis. This trend has resulted in a variety of studies investigating other biofluids, such as urine, serum/plasma, saliva, sweat and cerebrospinal fluid (CSF), in the DMS format. However, blood remains the most popular dried matrix accounting for the majority of works published so far [11,18–22]. In 1993, Henderson *et al.* published the first work using a dried matrix to analyze drugs of abuse [23]. Considering that the main interest of forensic toxicology is illicit substances, toxicologists did not take long to show further interest in DMS and have thus contributed greatly with investigational studies [12,24–27]. One of the main benefits DMS offers in this field is the improvement of analyte stability, allowing shipment and storage of samples for extended periods of time for counterproof purposes [28]. In addition, forensic toxicologists work mostly with biological samples, which usually offer challenges due to the presence of natural occurring interferents [29]. Thus, a sample preparation step prior to analysis is imperative and evidence has shown DMS as a valuable alternative for simplifying this step [30,31].

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Currently, a substantial number of validated methods have successfully detected several drugs of abuse on dried matrices, even including the novel synthetic ones [26,32,33]. The technique that started in 1913 and branched to forensic sciences in 1993 has recently achieved a landmark: the World Anti-Doping Agency (WADA) announced in 2019 that it would collaborate with other organizations in order to develop the use of DBS for sport drug testing. Their goal is to implement its use for the 2022 Winter Olympic and Paralympic Games in Beijing, China – which has been recently confirmed [25]. In virtue of the course, DMS has taken ever since it was first reported and where it has currently arrived in forensic toxicology, we provide an overview of the analytical methodologies reported so far. Here, a discussion highlighting the benefits, limitations, improvements and accomplishments will be carried out.

Analyte stability

The improvement in analyte stability is surely one of the main benefits of using dried matrices and allows its application on different contexts. For example, in sport drug testing, the increased analyte stability associated with the compact size of Guthrie cards provides a considerable wider window of analysis for confirmatory or complementary tests [34]. In these cases, urine is the matrix of choice, thus replacing it with dried urine spots (DUS) would enable the storage for extended periods of time occupying less physical space. With this in mind, considering WADA will adopt DBS for drug testing, another possibility is to store the urine samples collected from athletes as DUS [34–36]. Similarly, in driving under the influence of drugs (DUID) investigations, dried matrices also could be of help. In such cases, however, saliva is established as the matrix of choice in many countries and is collected from drivers during roadside testing. Nonetheless, controversies exist regarding the correlation between drug concentrations in blood and oral fluid, while adulterations might also occur [37]. Thus, DBS could be rapidly and easily obtained by finger-pricking drivers facilitating the work of police officers. In addition, blood is the recommended matrix to indicate whether one is under the effect of any illicit drug. Using dried oral fluid spots (DOFS) is also an alternative which should authorities choose to remain with oral fluid as the standard matrix for these cases instead of adopting DBS.

Another common practice in daily forensic analysis is investigations involving suicides or homicides. In this context, the use of DBS obtained with postmortem blood at the crime scene or autopsy has been described [23,24,38–40]. These reports are few, thus most questions regarding the use of postmortem blood as dried matrix are still unanswered. For example, how would the sample behave on paper considering the natural occurring effects after death, such as cell lysis and clotting? Would it result in improper diffusion on paper or troublesome sample treatment? Nevertheless, employing dried matrices would allow the better storage of evidence for longer periods of time enabling more reliable counterproof analysis. Interestingly, an alternative for such cases is the use of CSF. Although collection of this matrix is quite complex and invasive, this is not a limitation in cases where the subject is deceased, becoming a valuable specimen for forensic analysis. One study correlated the concentrations of some drugs between postmortem blood and CSF, indicating a consistent partitioning of the analytes [19]. Moreover, CSF has been recently investigated as a dried matrix alternative for medications, although results are still preliminary and drugs of abuse have not yet been addressed [41]. With this in mind, it is possible that CSF could replace postmortem blood, even as a DMS, although further investigations are required.

Investigations regarding the impact of the absence of water on DMS have contributed to better understand the underlying features of the technique. Nevertheless, although the overall stability is improved, each chemical has its own behavior after spotting on paper. For example, Chepyala *et al.* observed that both amphetamine (AMP) and methamphetamine (METH) have poor stability even on DBS, as less than 50% of the initial concentration was found after storing these samples for 1 month at room temperature [42]. However, this stability was greatly improved when stored under refrigeration. Moreover, Pablo *et al.* found lower concentrations of AMP in urine samples than on DUS and attributed this to a decreased analyte stability [11]. AMPs are a major class of abused stimulants known to be less stable than other analytes in biological samples; however, by sampling as DMS, this limitation is mitigated [42–45]. Similarly, the chemical stability of synthetic cathinones (SCAT) on biosamples is also substantially reduced. Reports suggest some SCAT degrade greatly after a few hours, in other words, they do not survive the transportation time to the laboratory. Interestingly, a comparative study showed that SCAT are more stable on DBS than in whole blood, considering that analytes are still detected after 90 days on samples stored at room temperature, although at low concentrations [46,47]. Similarly, other new psychoactive substance (NPS) has also been observed as being more stable in the absence of water. For example, *N*-(2-methoxybenzyl)phenethylamines (NBOMes) remained mostly undegraded (<20%) for 180 days at room temperature when stored as DBS [32,33,47,48]. Furthermore, synthetic

tryptamines were also more stable at room temperature when stored as DMS [33,46,49]. Notwithstanding this, while information regarding the stability of NPS on conventional liquid samples is easily found in the literature, studies that investigate NPS on dried matrices are scarce. We have found no works assessing the stability of other classes, such as synthetic cannabinoids, aminoindanes, phencyclidyne-type substances, etc., on DMS.

The increase of analyte stability on DMS is virtually universal, especially if refrigerated. Ambach *et al.* showed that cocaine (COC) and metabolites remain stable for 262 days at -80 and -20°C on DBS [17]. Interestingly, Lizot *et al.* simulated transportation conditions and observed that COC, benzoylecgonine (BZE), cocaethylene (CE) and norcocaine (NCOC) were stable for at least 14 days even at 45°C on DBS, while ecgonine methyl ester (EME) was not [16]. Sadler *et al.* reported that AMPs, COC, BZE and morphine were stable for 8 months stored under refrigeration, except for 6-acetylmorphine (6-AM) [50]. Furthermore, Garcia Boy *et al.* reported that concentrations of 6-AM decreased much faster in whole blood stored at 4°C than on DBS [14]. In this case, the problem is that this compound rapidly turns into morphine, a common metabolite of other substances, even prescribed medications, such as codeine, and thus cannot be used as a marker for heroin use [14,44,50]. Complementary data report that heroin was not stable on paper, further highlighting the importance of improving benchtop stability of 6-AM enabling a wider window of reliable and selective analysis [26].

After the sample is spotted and dried, the absence of water inhibits enzymatic and chemical reactions, avoiding analyte breakdown, biotransformation, etc. [15,16,51]. In addition, it has been reported that the paper itself has stabilizing properties for some analytes, accounting in favor of preventing chemical degradation [14]. The overall improvement of stability has major forensic benefits; for instance, when a sample is collected in a remote area, there is a lack of facilities and equipment to perform analysis, thus storage and transportation are crucial. Also, the compact size of DMS cards allows more samples to be stored in the same room and would reduce transportation costs. Even in the absence of refrigeration, which occasionally is the case, analytes are more stable on DMS than in liquid samples, ensuring analytical reliability.

Sampling challenges

The improvement in analyte stability granted by DMS is accompanied by some important limitations that require careful consideration. For instance, the process of sampling the matrix onto the paper and adding the internal standard (IS) lacks consensus of the scientific community and will be discussed next.

In practice, samples should be spotted directly onto the paper, for example, blood or saliva; however, during method development, the matrix is added by alternative means, such as pipetting. In of itself, this procedure is used to standardize the sampling step, however, might cause analytical imprecisions and eventually failing to be applicable in authentic cases. Reports of lack of precision or accuracy during the development of a DMS method are common, which might be related to how matrices were sampled. Still, no thorough investigation has been carried out in this regard. Notwithstanding, when working with blood, the HCT poses an additional challenge to standardize sampling, thus contributing to quantitation inconsistencies: the HCT effect. A remarkable work published by Lizot *et al.* assessed COC and some of its metabolites on DBS and carefully investigated the influence of different HCT values and sample volume spotted on paper. The authors reported that low HCT interfered in accuracy, while normal values did not cause major impairment in the determination of the metabolites analyzed [16]. This was attributed to the low HCT reducing blood viscosity, leading to altered liquid dispersion on paper along with uneven analyte distribution in the specimen. On the other hand, analyte recovery was not affected by the HCT and pipetting different volumes of blood had no significant impact on accuracy. This investigation was grounded on the fact that in authentic circumstances, DBS would be obtained from capillary blood (e.g., finger-prick), thus each spot would contain different sample volumes. Furthermore, although HCT is a limitation exclusive to DBS cases, other matrices could be affected by the concentration of other endogenous components resulting in altered dispersion of the liquid on paper. With this in mind, the vast majority of methods developed with any dried matrix usually pipette fixed volumes of the liquid onto paper in order to bypass this intrinsic variability. In addition, some authors have chosen to use the whole spot instead of punching a disk to further avoid inconsistencies in spot volumes. However, these approaches are not faithful to daily DMS sampling, reducing reproducibility. Moreover, whether the location where the spot is punched influences the analysis leads to further debate in the literature. For instance, Ambach *et al.* investigated the impact of punching a disk in different locations of the spot and showed that the outer edges had higher concentrations of some of the analytes analyzed in their work [17]. Furthermore, it was also reported that low HCT affected the analytical quantitation of these analytes, corroborating previous studies [12,16,17,52]. Moreover, Ingels *et al.* showed no significant differences in gamma-hydroxybutyric acid (GHB)

concentrations when punching a disk on alternating locations of the spot, which is in agreement with other works [53,54]. Furthermore, the volume of samples yielding better results was found to be from 20 to 35 µl and agrees with other reports in which HCT values influenced the accuracy of the analysis [16,52,55].

Another practice that usually contributes to great variability is adding the IS during DMS analysis. Some authors choose to add the IS directly onto the spotted matrix, while others add it during extraction [11,20,33,40,50]. In this context, Mommers *et al.* investigated different means of adding the IS on paper and the influence of blood spotting for morphine analysis [54]. When the whole filter paper was immersed in an impregnation solution containing the IS, imprecision of less than 6% was observed even when punching different locations of the spot, suggesting IS was distributed uniformly on paper. This might aid in the search for better precision and accuracy during method development, although the setback with this strategy is the use of high amounts of IS. Furthermore, the work also shows that pipetting and directly dropping the sample on paper results in higher recovery than using a microcapillary, and no significant difference was observed when 10–70 µl of blood was spotted. However, a higher concentration of the analyte was found when punching from the outer borders compared with the center of the spot [54]. Although the HCT effect was not assessed by the authors, their work provided valuable results aiding the elucidation of which factors impact the analysis of dried matrices. The process of sampling, punching and adding the IS might impact significantly the analysis, which should be reliable and free of bias, especially for forensic purposes. Thus, further investigations are required to find optimal conditions when working with DMS.

Methods for the analysis of multiple classes of illicit substances

Together with the increased analyte stability, the simplification of overall sample preparation is another benefit of using dried matrices. In addition, a great portion of the methods designed to analyze drugs of abuse on DMS attempt to optimize the analytical run by assessing different classes simultaneously. The main advantage of this approach is the reduction in time and resources, while efficiency is maintained. Such methods are particularly suited to investigate polydrug usage, optimizing daily analysis and cases where little information about the patient is available, and facilitating diagnosis.

Sample volume

The usual volume employed to obtain a DMS varies from 5 to 100 µl, although higher volumes have been studied by some authors [56]. This small amount of sample suffices to identify and quantitate several substances with high accuracy and precision. For instance, Thomas *et al.* and Versace *et al.* were able to identify more than 15 drugs of abuse with an automated method using only 5 µl of DBS [40,57]. Similarly, Ambach *et al.* published a screening method for 64 drugs of abuse, including AMPs, SCAT and cocainics, with only 10 µl of DBS [33]. In contrast, Kacargil *et al.* quantitated some AMPs, cocainics and opioids with high sensitivity, although needed a 100-µl DBS, while the method proposed by Stoykova *et al.* was limited for screening purposes and required a 250-µl DOFS. In terms of sensitivity, all methods achieved acceptable limits of detection (LOD) and limits of quantification (LOQ), while linearity range was within clinical concentrations (Table 1). Furthermore, despite the remarkable results obtained, it is important to consider that volumes higher than 100 µl are not easily obtained by heel- or finger-prick – or even by swab collection in cases where oral fluid is intended. Moreover, the volume spotted on paper will impact the drying time [14,20]. The microliters used in DMS are considerably lower than the volumes used in in-tube samples (>5 ml), although methods that need higher volumes have an additional step or limitation, as samples will have to be collected by conventional means, with those naturally obtained DMS not being suited for such analytical methods. These and other works are listed in Table 1 for comparison.

DMS extraction

After the biofluid is spotted on paper and dried, a sample preparation step must be performed prior to analysis as with conventional matrices. However, the difference of using DMS instead of liquid samples lies in the simplicity and straightforwardness of the process: the paper containing the spot is submersed in an extraction solvent enabling the migration of analytes from the aqueous to the organic layer (Figure 1). Hence, extraction depends mainly on the chemical properties of the substances of interest and might be achieved by employing different solvents or mixtures that have affinity and/or selectivity for the target analytes, usually avoiding highly polar biomolecules, such as proteins [42,43]. After solvent-based extraction, the sample preparation step of DMS is usually followed by centrifugation, evaporation of the organic solvent that was used for extraction, and reconstitution of the dried analytes with solutions compatible with the equipment. For instance, the work developed by Ambach *et al.*

Table 1. Summary of developed methods for multiple classes of drugs of abuse using dried matrices.

Analyte	Instrument	Matrix	Sample volume (µl)	LOD (ng/ml)	LOQ (ng/ml)	Recovery (%)	Linearity (ng/ml)	ME (%)	CV (%)	Ref.
AMP, METH, MDMA, MDA, COC, BZE, morphine, 6-AM	LC-MS/MS	DBS	50	0.5–5	1–5	57–90	–	-42 to 22	4.7–12	[58]
AMP, METH, MDMA, MDEA, MDA, COC, BZE, THC-COOH, 6-AM	LC-MS/MS	DUS	10	5–150	–	–	1–300	–	13.9–15	[11]
AMP, METH, COC, heroin, THC-COOH, THCA-A, THC, CBD, NPS	LC-MS/MS	DBS, DUS	20	–	–	–	5–500; 25–1000	–	4.8–22.3	[24]
AMP, METH, MDMA, MDEA, MDA, MBDB, COC, BZE, EME, CE, morphine, 6-AM, THC, THC-OH, THC-COOH	LC-MS/MS	DBS	5	<1–100	1–>100	20–48; 10–60[1]	1–100	0–114; 0–166[1]	–	[48]
AMP, METH, MDMA, MDEA, MDA	LC-MS/MS	DBS	100	2.18–3.32	6.54–15.97	80–120	5–200	80–110	5.75–7.68	[88]
AMP, METH, BZE, KET, Mitragyna	LC-MS/MS	DOFS	50	0.5–5	0.2	>80	0.2–50	78.2–95.9	4.7–8.9	[43]
AMP, MDMA, COC, BZE, CE	LC-MS/MS	DOFS	50	–	40	–	40–500	<25	6.24–14.75	[20]
AMP, METH, MDMA, MDEA, MDA, BZE	LC-MS/MS	DUS	–	–	10	73.3–112.7	0–1000	–	1.2–12.8	[35]
AMP, morphine, NPS	LC-MS/MS	DUS	20	200	–	34–126	1–10,000	73–133	–	[32]
AMPs, COC, DMT, KET, NPS	LC-MS/MS	DBS	10	1–5	–	57.1–98.3	–	–	–	[33]
AMP, METH, MDMA, MDEA, MDACOC, BZE, EME, CE, KET, NKET, LSD, morphine, 6-AM	LC-MS/MS	DBS	Three droplets of blood	0.5–1	0.2–2	24–54	2–500	47–89	8–15	[39]
AMP, METH, MDMA, MDEA, MDA, COC, BZE, EME, CE, morphine, M3G, M6G, 6-AM	LC-MS/MS	DBS	30	1–5	–	78.9–129.5	5–200	-3.5 to 1.9	0.8–8	[44]
MDMA, MDA, COC, THC, THC-COOH	LC-MS/MS	DBS	20	0.05–0.25	0.25–1	19–78	0–20	–	8–25	[38]
COC, BZE, morphine, 6-MAM	GC-MS	DBS		10–40	30–130	98–103	20–2000[2]	–	<12	[30]
Morphine, morphine-glucuronide, THC, THC-OH, THC-COOH, THC-COOH-glucuronide	LC-MS/MS	DBS	5	10	–	–	–	–	–	[57]
AMP, MDA, METH, MDMA, COC, BZE, morphine	LC-MS/MS	DBS	–	0.1–6.52	0.4–7.18	96.6–118	2.5–500	86–119	4.4–28.4	[45]
AMP, METH, MDMA, MDEA, MDA, PMMA, PMA, COC, KET, NKET, LSD, morphine, PCP, NPS	LC-QTOF-MS	DBS	25	0.3–21.4	–	67.6–104.4	–	70.9–96.4	2.38–11.4	[42]
AMP, METH, MDMA, MDA, morphine, 6-AM, COC, BZE, THC, THC-OH, THC-COOH	LC-MS/MS	DOFS	30	1.5–4.5	5–15	78.3–99.8	5–500	87.9–104.2	3.1–11.9	[61]
AMP, METH, MDMA, COC, morphine, THC	GC-MS	DUS	250	–	–	>70%	–	–	–	[56]
COC, BZE, morphine, 6-AM	GC-MS	DBS	20	0.26–2	0.87–6.7	95–101	20–2000[3]	–	5–8	[31]

[1]Corresponds to BP and HAL, respectively.
[2]Corresponds to µg/ml. Only analytes considered at ALOL are represented even if the method has been validated for other substances.
[3]Corresponds to µg/ml. Only analytes considered at ALOL are represented even if the method has been validated for other substances.
6-AM: 6-Acetylmorphine or 6-monoacetylmorphine; AMP: Amphetamine; BZE: Benzoylecgonine; CBD: Cannabidiol; CE: Cocaethylene; COC: Cocaine; CV: Coefficient of variation; DBS: Dried blood spot; DMT: *N*, *N*-Dimethyltryptamine; DOFS: Dried oral fluid spot; DUS: Dried urine spot; EME: Ecgonine methyl ester; GC-MS: Gas chromatography coupled with mass spectrometry; HPLC-MS/MS: High-performance liquid chromatography coupled with tandem mass spectrometry; HPLC-QTOF-MS: High-performance liquid chromatography coupled with Quadrupole Time of Flight mass spectrometer; KET: Ketamine; LOD: Limit of detection; LOQ: Limit of quantification; LSD: Lysergic acid diethylamide; M3G: Morphine-3β-glucuronide; M6G: Morphine-6β-glucuronide; MBDB: *N*, 3-Benzodioxolyl-*N*-methylbutanamine; MDMA: 3,4-Methylenedioxymethamphetamine; MDEA: 3,4-methylenedioxyethylamphetamine; ME: Matrix effect; METH: Methamphetamine; NKET: Norketamine; NCOC: Norcocaine; NPS: New psychoactive substance; PCP: Phencyclidine; PMA: Paramethoxyamphetamine; PMMA: Paramethoxymethamphetamine; THCA-A: Δ-9-tetrahydrocannabinolic acid A; THC-COOH: 11-Nor-9-carboxy-Δ-9-tetrahydrocannabinol; THC-OH: 11-Hydroxy-Δ-9-tetrahydrocannabinol

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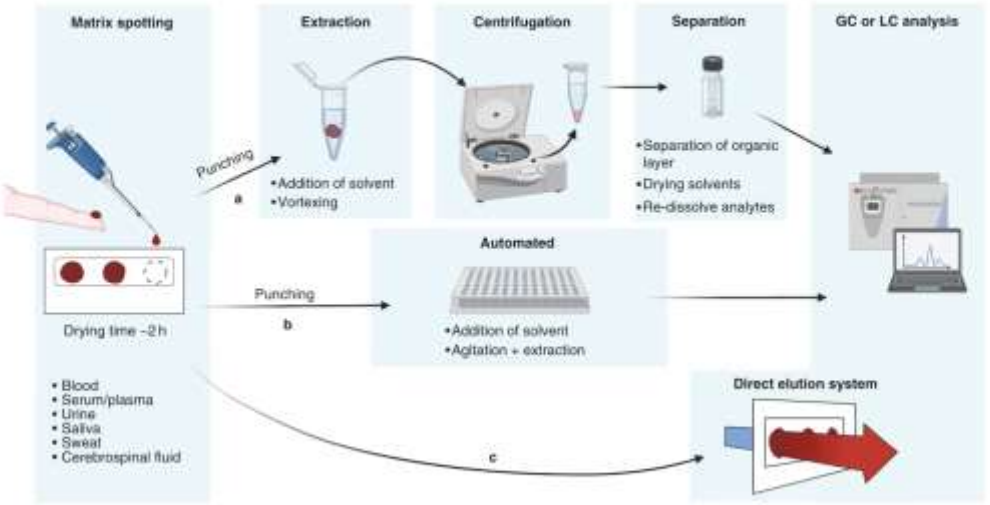

Figure 1. Overview of dried matrix spot analytical pipeline, from sampling to analysis. Matrix spotting is achieved by either direct or capillary drop of a biofluid on paper. After dried, samples undergo either **(A)** nonautomated or **(B)** automated sample preparation. In fully online automated systems, the DMS card is placed directly on the autosampler, disregarding manual punching **(C)**.
DMS: Dried matrix spot; GC: Gas chromatography; LC: Liquid chromatography.

consisted of extraction with 500 µl of methanol (MeOH) and subsequent vortexing for 15 min [53]. Next, 10 µl of MeOH + HCl was added and the whole solution was dried under a nitrogen stream. The dried extracts were redissolved with 100 µl of mobile phase and the solution was injected into the LC–MS/MS. In contrast to nonautomated methodologies, Versace *et al.* developed an automated screening method for 22 substances, including cocainics, opioids and AMPs, using an online desorption procedure with a 5-µl blood spot [40]. In their work, a 96-well plate containing the spots was employed and the extraction was achieved under 2 min with the addition of 100 µl of MeOH. Then, still in the autosampler, plates were mixed and injected into the LC–MS/MS system. Furthermore, Gaugler *et al.* reported a fully automated method in which 28 substances were analyzed in both DBS and DUS with no human interaction whatsoever after placing the samples in the autosampler [26]. In their method, each sample took only 5 min of total time-to-result and it was possible to further optimize time by using an alternative database proposed in the same work. The use of this approach allows the screening of over 1200 compounds in a 20-min run, in which AMPs, opioids, cocainics, cannabinoids and even synthetic cannabinoids were assessed (Table 1). The most common solvents used in DMS extraction are MeOH, acetonitrile (ACN) and aqueous buffers, although there are reports considering the limitations of using the latter. The main drawback is associated with the high polarity that results in extraction of undesirable polar biomolecules [42]. Moreover, the volumes employed in the majority of methods did not exceed 1 ml and achieved high analyte recovery (Table 1). Both automated and online extraction methods are attractive to any operator, as they simplify even further the overall DMS extraction procedure together with the reduction of human errors, although such approaches require very costly investment. Notwithstanding this, nonautomated methodologies have proven to achieve acceptable sensitivity and recovery comparable to their automated counterparts (Table 1). Figure 1 gives an overview of the main differences between automated and nonautomated sample processing.

Direct DMS extraction versus traditional extraction techniques

The possibility of avoiding more complex or time-consuming extraction techniques surely is a benefit for forensic analysis. However, it is important that the results are reliable considering they may be used in court. With that in mind, some authors have carried out comparative studies between traditional extraction techniques and direct DMS

extraction in order to evaluate their equivalence. For instance, Chepyala *et al.* compared the extraction of a spot using a solution of ACN to a solid-phase extraction (SPE) and the screening method yielded an average recovery of 80% for all analytes, high sensitivity and precision [42]. Furthermore, Gorziza *et al.* compared between a direct extraction to a WAX-S tips methodology [43]. Direct DOFS extraction yielded higher sensitivity for all analytes associated with high precision and accuracy. Moreover, recoveries were very similar in both methods (≈80%); except BZE which was better recovered from DOFS when directly extracted than by using WAX-S tips (82 vs 60%, respectively). In addition, in WAX-S tips extraction, a higher matrix effect (ME) was observed (>90%). Altogether, these data suggest that simple extractions suffice to achieve great analyte recovery, completely dismissing complex procedures, such as SPE, and reducing associated costs (Table 1). Other studies further endorse these findings [30,51]. Needless to say, direct DMS extraction was simpler and faster than other methods with a similar sample preparation step, as briefly described above. With this in mind, these comparative studies show that the response of both methods employed for analyte extraction was similar and, therefore, could be interchanged (Table 1).

Methods singularly analyzing specific classes of illicit substances

In contrast to methods designed to analyze multiple classes of drugs of abuse, which have some advantages as discussed in the previous sections, methodologies aimed at specific molecules or classes have proved to possess promising benefits. With this in mind, we will focus on such methods and their main achievements and particularities in the following sections.

Amphetamines

AMP-like substances are a class of abused chemicals mainly because of their stimulant properties and consist of different synthetic compounds, such as methylenedioxymethamphetamine (MDMA) and METH. This major group with forensic interest has been used illicitly for decades; however, only few authors have developed methods aiming exclusively at this class. To the best of our knowledge, Jantos *et al.* have published the only two works we could find in our extensive search of the literature. In their work, a method was developed in order to investigate the concentrations of some AMP-type drugs in whole blood and on DBS. In the first work, only MDMA and 3,4-methylenedioxyamphetamine (MDA) were analyzed; although both methods were proven equivalent, as similar concentrations were quantitated and extraction recovery did not differ, higher sensitivity for both analytes was found on DBS [58]. Meanwhile, the second work showed that concentrations found on both matrices were equivalent as well for AMP [28]. Furthermore, the comparison between both methods was acceptable in terms of ME, analyte recovery and stability. Of note is that the authors have reported slightly less distinctive ME was observed on DBS when compared with whole blood (Table 2). Needless to say, higher volumes were required to perform the analytical method with whole blood than DBS (500 vs 100 µl, respectively). For comparison's sake, a quick literature survey showed that methods designed for the determination of AMPs in liquid samples achieved similar sensitivity and recovery, further endorsing the relevance of the results obtained by Jantos *et al.* [59,60]. Even though such methods yielded lower LODs and LOQs, the use of a simple and rapid approach, as the one proposed by Jantos *et al.* with DBS instead of whole blood, is still valid, especially considering practical applications. For instance, the concentrations of MDMA found in blood samples in cases of driving under the influence of drugs (DUID) ranges from 50 to 500 ng/ml. Therefore, the DBS method would suffice for this application. Moreover, reports indicate that AMPs extract well from filter papers, therefore do not offer great challenges for methods with dried matrices, even those that analyzed different drug classes [20,35,50,61].

Cannabinoids

Similar to AMPs, there is a paucity of works focusing on the analysis of Δ9-tetrahydrocannabinol (THC) and metabolites in methodologies designed specifically for this class. In fact, the only method we could find in the literature was published by Mercolini *et al.* [62]. In their work, a usual sample preparation for DMS was carried out: a 10-µl DBS was used and extraction was performed with 1 ml of MeOH, followed by centrifugation, solvent drying and redissolution with mobile phase for LC injection. The only particularity was that the drying of spots was achieved by microwave treatment (700 W, 1.5 min) instead of room temperature. With this in mind, when comparing this work to other methodologies assessing cannabinoids exclusively in liquid samples, it is possible to note important particularities. First, the sample volume employed is usually higher and the following extraction step is considerably more complex and time consuming than it is for DMS; second, these methods have achieved slightly higher sensitivity; finally, and perhaps the main contrast between methodologies, was regarding the target analytes, as

Table 2. Summary of class-exclusive methods for drugs of abuse using dried matrices.

Class	Analyte	Instrument	Matrix	Sample volume (µl)	LOD (ng/ml)	LOQ (ng/ml)	Recovery (%)	Linearity (ng/ml)	ME (%)	CV (%)	Ref.
Amphetamines	AMP, MDMA, MDA	LC–MS	DBS	100	0.1–1.6	0.4–5.7	–	2.5–400	–	1.9–3.1	[28]
	MDMA, MDA	LC–MS/MS	DBS	100	0.12–1.6	0.4–5.7	–	–	–	2.29–4.99	[58]
	METH, PMMA, PMA	LC–MS	DOFS	10	3.3–8.3	10–25	28–65	25–100	–	4–13	[89]
Cocaines	BZE	LC–MS/MS	DBS	–	5	10	–	25–1000	–	13	[23]
	BZE	GC–MS	DBS	12	–	–	–	1–400	–	3.4–23.5	[88]
	COC, BZE, CE	LC-DAD	DBS	10	4–7	12–20	92–93	12–1000	–	4.1–4.7	[52]
	COC	LC–MS/MS	DBS	100	0.74	2.46	97	5–200	–	10.2	[13]
	BZE	LC–MS/MS	DBS	12	2	–	–	1–100	–	–	[67]
	COC, BZE, EME, NCOC, CE	LC–MS/MS	DBS	50	0.5	–	90.1–107.8	5–500	-3.23 to 1.94	1.27–6.74	[16]
	COC, BZE, EME, NCOC, MHBZE, CE	LC–MS/MS	DBS	25	–	1–5	≥20.9	1–500	40.4–57.7	≤13.4	[17]
	COC, BZE, EME, CE	LC–MS/MS	DBS	85	0.5–1	2–5	82.4–100.4	10–500	38–118.8	5.1–28.4	[38]
Cannabinoids	THC, THC-OH, THC-COOH	LC–MS/MS	DBS	10	0.10–0.20	0.25–0.50	>81	0.25–200	91–98	5.7–6.2	[62]
Opioids	Morphine	LC–MS/MS	DBS	–	–	–	–	0.4–1000[2]	–	5.1–6.3	[54]
	Morphine, 6-AM	LC–M/SMS	DBS	100	0.4–0.8[1]	1.4–2.7[2]	99.6–102.8	5–50	–	4.8–9	[14]
	Morphine, M3G, M6G	LC–MS/MS	DBS	20	0.25–0.5	1–2.5	95.6–108.3	1–1000	–	5.6–14.7	[89]
	Morphine, fentanyl	LC–MS/MS	DBS	10	–	0.1–1	73.5–78.1	0.1–500	<15	3.5–6.7	[55]
	Morphine	GC–MS	DUS	20	100	–	99.4[1]	1–100[1]	–	1.02–2.78	[51]
GHB	GHB	LC–MS/MS	DBS	–	–	8[3]	>70	8–1014[3]	<19	12	[90]
	GHB	GC–MS	DBS	50	1[1]	2[1]	–	2–100[1]	–	<15	[70]
	GHB	GC–MS	DBS	10	1[1]	2[1]	–	2–100[1]	–	<15	[53]
	GHB	GC–MS	DBS	10	1[1]	2[1]	–	2–100[1]	–	<15	[71]
	GHB	CE-DAD	DBS, DUS	100	0.210[1]	0.625[1]	87.9–93.6	0.625–125[1]	–	4.86–5.98	[72]
NPS	SCAT	LC–MS/MS	DBS	15	10	25	–	25–1000	9–16.6	2.2–7.1	[47]
	SCAT	LC–MS/MS	DBS	30	0.5–2	2–10	15.05–102.64	2–200	17.21–88.89	<20	[46]
	NBOMes	LC–MS/MS	DBS	15	0.05	0.1	–	0.1–10	-11.4 to 64.6	3.5–20	[48]
	5-MeO-DIPT	LC–MS/MS	DUS	10	0.1	0.2	78.8–82.8	0.2–100	107.6–168.7	2.7–8.5	[49]

[1]Corresponds to ppb.
[2]Corresponds to ng/100 µl.
[3]Corresponds to mg/ml.
¶Corresponds to nM. Only analytes considered as illicit are represented even if the method has been validated for other substances.
5-MeO-DIPT: 5-Methoxy-*N*,*N*-diisopropyltryptamine; 6-AM: 6-Acetylmorphine; AMP: Amphetamine; BZE: Benzoylecgonine; CBD: Cannabidiol; CE: Cocaethylene; CE-DAD: Capillary electrophoresis with diode array detector; COC: Cocaine; CV: Coefficient of variation; DBS: Dried blood spot; DOFS: Dried oral fluid spot; DUS: Dried urine spot; EME: Ecgonine methyl ester; GC–MS: Gas chromatography coupled with mass spectrometry; GHB: γ-Hydroxybutyric acid; HPLC–DAD: High-performance liquid chromatography with diode array detector; LC–MS/MS: High-performance liquid chromatography coupled with tandem mass spectrometry; KT: Ketamine; LOD: Limit of detection; LOQ: Limit of quantification; LSD: Lysergic acid diethylamide; M3G: Morphine-3β-glucuronide; M6G: Morphine-6β-glucuronide; MDA: 3,4-Methylenedioxyamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; ME: Matrix effect; METH: Methamphetamine; NCOC: Norcocaine; NKET: Norketamine; NPS: New psychoactive substances; OH-BZE: Phenol-hydroxylated benzoylecgonine; PMA: Paramethoxyamphetamine; PMMA: Paramethoxymethamphetamine; SCAT: Synthetic cathinone; THCA-A: Δ-9-Tetrahydrocannabinolic acid A; THC-COOH: 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol; THC-OH: 11-Hydroxy-Δ9-tetrahydrocannabinol.

more THC metabolites were evaluated using liquid samples, such as cannabinol and cannabidiol (Table 2) [26,63,64]. For example, the method of Pichini *et al.* employed only 100 µl of oral fluid, serum and urine, followed by a simple analyte extraction. The only downside is that urine samples had to undergo hydrolysis, which is not needed when using DUS. Moreover, cannabinoids were analyzed even in sweat samples with a similar extraction procedure [64]. In contrast, Mercolini *et al.* only used DBS and analyzed THC, 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH), and 11-Nor-9-carboxi-Δ^9-tetrahydrocannabinol (THC-COOH), while the other metabolites were not mentioned. This leaves room for speculation about whether attempts were made to include the remaining analytes and whether limitations complicated the analysis.

Cannabinoids have been described as troublesome during analyte extraction, which are limitations that do not seem to be a problem when using liquid samples. In the process of extracting different drug classes from filter papers in a single procedure, their chemical properties pose a particular challenge. In this context, several authors have reported not only low recovery of cannabinoids from DMS but also lack of sensitivity in their methods [11,26,36,40,57,61]. On the one hand, Pablo *et al.* reported that a low signal for THC-COOH was observed in latter vials within the same run, which has led the authors to investigate the stability of these analytes. Data showed that this cannabinoid is only stable for 3 h after being spotted on paper and dried [11]. Other authors have reported similar findings that could justify this limitation when analyzing cannabinoids on DMS [26,36,40,57,61]. On the other hand, Mercolini *et al.* showed that THC, THC-OH and THC-COOH were stable on the spot after being stored for 3 months at room temperature, with a recovery loss less than 10% compared with the initial concentrations. Furthermore, when comparing plasma and DBS analysis, levels of all analytes were found to be equivalent for both matrices [62]. Curiously, the authors reporting low stability of cannabinoids on DMS also employed solvents for extracting different classes of abused substances in the same procedure [11,26,36,40,57,61]. In addition, Mercolini *et al.* aimed specifically at cannabinoids and achieved high analyte recovery [62]. Perhaps, the microwave treatment in their work resulted in a fast-drying process and improved analyte stability. However, some authors attribute the extreme lipophilicity of cannabinoid molecules as being responsible for binding to active surfaces or matrices, such as cellulose, which the filter paper is made of, resulting in poor extraction from the DMS [36,63,66]. In this context, Stoykova *et al.* described that when developing a method to investigate drugs of abuse on DOFS, THC could not be detected unless a pretreatment (MeOH + HCl) was performed. Furthermore, even when DOFS underwent the pretreatment step, THC recovery was still low (45%) but detectable, while all other analytes had improved recovery (>70%) [56]. Therefore, it can be speculated that stability is not the limiting factor, but the high affinity of cannabinoids for the matrix paper leading to poor recovery. Nevertheless, the lack of consensus associated with scarce data makes difficult the understanding of the main causes responsible for the low extraction of cannabinoids from DMS. The consequence is that professionals who currently work with dried matrices or intend to adopt this microsampling technique to analyze THC and metabolites still need to be enlightened about strategies to overcome this limitation. Interestingly, another point of consideration is that Mercolini *et al.* applied a conversion factor in order to correct the influence of HCT on accuracy [62]. This measure was also adopted by the group in other works and it will be discussed with details in the next sections [52].

Cocainics

Perhaps the first work ever determining drugs of abuse on DMS was published by Henderson *et al.* in 1993 using GC/MS as confirmation for radioimmunoassay screening. In their work, a quite complex extraction procedure was used and BZE was the only analyte investigated [25]. Furthermore, Sosnoff *et al.* published a method for the same analyte with a similarly complex sample preparation, although higher analyte recovery was achieved. The main difference, however, consisted of the system they used for analysis, as LC–MS/MS was chosen over a GC/MS yielding higher sensitivity [67]. Henderson *et al.* then used this pre-established LC methodology in an epidemiology study to investigate prenatal exposure to COC by analyzing the presence of BZE on DBS of newborns [68]. Moreover, Alfazil and Anderson proposed a specific COC method using DBS with further improvements in analyte extraction; however, a SPE was employed after the drug was extracted from the paper in order to purify the extract [13]. Similar LOQ was achieved by this methodology compared with previous works investigating COC exposure using DMS (Table 2). Nonetheless, Mercolini *et al.* published a method aimed at determining not only COC but also two metabolites on DBS. Also in the same work, DBS and serum extractions were compared, which consisted of a simple solvent-based extraction and a SPE, respectively [52]. Analyte recovery both matrices was greater than 90%, although a three- to fourfold increase in sensitivity was achieved in serum (Table 2). They employed an arbitrary correction for HCT variability, thus obtaining acceptable consistency between the concentrations with serum and

with DBS. The effect of the HCT on DBS analysis has been extensively discussed in other works, therefore we shall not detail it here [12,15].

Opposing to the use of spiked samples collected from living people in order to validate the methods and perform the analysis, Moretti *et al.* used authentic postmortem blood instead and provided important data for discussion [38]. The authors chose to use a fixed volume of 85 µl and the whole spot was used in order to reduce analytical variabilities. The method was designed to analyze cocainics and achieved high sensitivity and specificity; however, precision for EME was not acceptable for international guidelines. Interestingly, in one case, blood samples were collected directly from the corpse at a crime scene and COC concentration was more than threefold higher on DBS collected at the crime scene than on DBS obtained by cardiac blood during autopsy, which the authors attributed to possible environmental contamination [38]. Nevertheless, the method has proved to be suitable for analyzing either whole blood or DBS, despite the HCT effect. The number of works proposing methods for the analysis of COC and metabolites are limited, however, the ones published so far have presented acceptable sensitivity, selectivity and analyte recovery, with the linear range being within clinical concentrations (Table 2). In addition, HCT effect, volume spotted and means of spotting have been considered in these works. Altogether, DBS has proved to be a good candidate for routine forensic applications involving analysis of COC and metabolites.

Opioids

Opioids are a class of therapeutic pain relievers (e.g., codeine) and also include heroin, a classic abused substance. These chemicals share morphine as a major metabolite, which is a prescription medication. Thus, the determination of the intermediary heroin metabolite 6-AM is essential to distinguish between the use of prescription opioids from heroin. Garcia Boy *et al.* published a thorough investigation of morphine and 6-AM on DBS in which samples underwent different drying times at different temperatures [14]. It was observed that 1 h at 40°C was sufficient to dry the spots, while analyte decomposition remained at a minimum. Furthermore, ethyl acetate yielded higher recovery than other organic solvents. Still, extraction efficiency was twofold higher for 6-AM than for morphine and no ME was observed. The method presented high sensitivity and efficiency at detecting both analytes in whole blood and on DBS, while the concentrations found on these matrices were similar. Furthermore, 6-AM has a short half-life and DBS improves its stability even at room temperature; thus, it is more feasible to use it over whole blood, as the window of detection is wider reducing the chance of false-negative results for heroin intake. This becomes especially important in cases where venous blood is not available or when time is crucial, such is the case of roadside screening. The method developed by Garcia Boy *et al.* also investigated the feasibility of using DBS over whole blood and showed promising results, although only morphine and 6-AM were analyzed. Furthermore, Clavijo *et al.* analyzed two other metabolites, morphine-3β-glucuronide (M3G) and morphine-6β-glucuronide (M6G), in whole blood and on DBS, comparing both matrices [69]. In this case, water yielded higher analyte recovery from DBS than other organic solvents and were equivalent between both matrices (>93%), while ME was not observed (Table 2) [69]. Even though validation parameters were acceptable for international guidelines, the authors described a higher sensitivity for morphine than for the other metabolites [69]. Interestingly, it has been reported that anticoagulants can increase the concentration of some analytes, such as morphine, and thus are not recommended when analyzing this substance [13]. Perhaps, this was the case, considering plasma with EDTA was used and LOQ for morphine was lower (Table 2). Jain *et al.* have also evaluated different extraction solvents for different conditions and concluded that water yields better analyte recovery from DUS (99.4%), corroborating data from Clavijo *et al.* [51,69]. In contrast, Verplaetse and Henion developed a fully automated SPE–LC–MS/MS method for the quantitative determination of opioids on DBS [55]. Sensitivity, linear range and recovery (>70%) sufficed for the intended concentrations on biosamples within a 5-min run. In addition, their method presented a high-throughput alternative capable of analyzing a superior number of samples over time than nonautomated methodologies. On the other hand, the method could not distinguish heroin users from prescription medications. Clearly, the intended applications of the authors were to monitor the therapeutic use of opioid medications. Moreover, another important drawback faced during opioid analysis is related to analyte stability [51,69]. For example, lower sensitivity and recovery have been reported for opioids in contrast to other compounds analyzed by the same methodology, which stability investigations showed signals of both morphine and 6-AM increasing over time [50]. This could be the same case with cannabinoids, in which a method aimed at this group of chemicals achieves better results (Tables 1 & 2) [69]. The determining features causing poor recovery of some opioids from DMS are still unclear and the investigations carried out so far could not fully elucidate this, thus additional studies are required. Nevertheless, it has been

established that the determination of morphine and 6-AM from whole blood and DBS are equivalent, therefore could be applied in forensic cases.

Gamma-hydroxybutyric acid

A commonly abused drug by teens and young adults at parties, GHB is also placed in alcoholic beverages of unsuspecting victims in order to facilitate sexual assaults. Thus, the importance of analyzing this chemical is imperative to aid this type of investigation. Curiously, GHB was only addressed in methods designed exclusively for its analysis and there can be different explanations for this, as will be discussed in this section.

The first work analyzing GHB on dried matrices reported poor recovery due to the chemical properties of the analyte. With this in mind, an ingenious maneuver was adopted to bypass this limitation, the 'on-spot derivatization' [70]. Briefly, a derivatization solution was added to a vial containing the whole DBS or a punched disk, followed by 5 min of sonication. Next, the solution was evaporated and DBS was redissolved with 200 µl of ethyl acetate, sonicated and centrifuged. Finally, the extraction solvent was transferred to another vial, which was injected into a GC–MS. By employing this strategy, analyte extraction was successfully achieved with acceptable sensitivity and accuracy [70]. In the following years, the group has improved this methodology and a following publication by showed that similar concentrations of GHB were found when analyzing whole blood and DBS, therefore being a reliable alternative [53,71]. Moreover, Saracino *et al.* proposed a method for GHB analysis on both DBS and DUS in which the authors chose to use a capillary electrophoresis with a diode array detector (CE-DAD) instead of GC–MS as an alternative to derivatization procedures [72]. Indeed, high analyte recovery (>90%) and lower LOD and LOQ were achieved. Nevertheless, even with higher sensitivity than the methods of Ingels *et al.*, the endogenous and therapeutic ranges were far superior and thus more than enough for GHB analysis (Table 2) [72]. Moreover, although a SPE was employed, the extraction was faster because no derivatization was required.

Although scarce, the works published so far show the potential of dried matrices in forensic investigations where GHB is suspected. For example, in cases of drug-facilitated sexual assault (DFSA), some of the drugs used in these crimes are naturally endogenous in low concentrations resulting in a challenge for toxicologists to properly undertake investigations, which is the case of GHB. In addition, the report of the assault is often delayed by the victim, thus prompt sampling guarantees that the target analytes are able to be identified within the window of detection. In this context, the use of dried matrices, such as DBS, would avert these limitations and enable simple and rapid blood collection without a phlebotomist. Hence, police officers, for instance, could perform the sampling upon suspicion. Another advantage of using dried matrices in these circumstances is that a more discreet alteration in GHB concentrations is observed on DBS compared with a significant increase in whole blood over time due to improvement in analyte stability [53,70,71]. This particular feature regarding the stability of compounds on DMS will be discussed in further sections.

New psychoactive substances

New psychoactive substances (NPS) emerged in the early 2000s and have dramatically changed the synthetic drug market since then. According to the United Nations Office on Drugs and Crime (UNODC), over 120 countries have reported at least one NPS in their territory and approximately 950 substances have already been reported worldwide [73]. In this context, methodologies have been reported to determine NPS on DMS. For instance, Wang *et al.* reported a simple analysis of 37 SCAT by LC–MS/MS achieving low LODs and LOQs for most analytes. Interestingly, even though recovery of analytes varied greatly (12–104%), it was concluded that ethyl acetate was the best solvent to extract this class of substances when compared with MeOH and ACN. In comparison, the method developed by Ambach *et al.* yielded similar LODs and targeted considerably more compounds, although it is only applicable for screening, thus needing further experiments and validation in order to be suited for quantitation [33]. Both works used simple and fast solvent-based extraction achieving high analyte recovery (Tables 1 & 2). Moreover, Yan *et al.* published a method for the quantitation of 5-methoxy-*N*,*N*-diisopropyltryptamine (5-MeO-DIPT), a synthetic psychedelic, on DUS. By aiming exclusively at this compound, higher sensitivity was achieved compared with other works, while precision and analyte recovery were also within acceptable ranges (Table 2) [33,66,69]. Needless to say, this method is restricted for specific circumstances because it aims only at 5-MeO-DIPT; however, expansion of the method to encompass other analytes is possible and encouraged, although would require revalidation. In a similar work, Cunha *et al.* aimed at the evaluation of seven different NBOMes on DBS. The results achieved are comparable to other publications in terms of sensitivity, accuracy and precision (Table 2) [68].

The number of works proposing analytical methods for assessing NPS on DMS is still scarce. Although great attention has been given to these compounds in the last few years, the majority of methodologies chose to use liquid samples. Furthermore, while SCAT usually draws more attention for toxicologists mainly due to its high popularity among drug abusers, other classes are neglected. UNODC classifies NPS in nine different groups and only the SCAT group was extensively addressed. Meanwhile, only some compounds of the phenethylamines and tryptamines class have been assessed by the methods using DMS as far as we are concerned. By aiming to analyze multiple compounds with different properties in a single run, several drawbacks are faced similar to with traditional drugs of abuse. Nevertheless, different authors accomplished to develop methodologies with acceptable results for screening and analysis of NPS on dried matrices (Table 2). Thus, this approach is encouraged rather than focusing at a single analyte due to the inherent characteristic of the ever-growing numbers of NPS.

Analytical instruments

The task of screening and analyzing drugs of abuse in biological samples, which is usually present in low concentrations, is one of the main goals of forensic laboratories and becomes even more challenging when only microliters are available. This is the case of DMS and such a limitation is only possible to overcome due to the existence of highly sensitive detection techniques, such as GC–MS or LC–MS. However, these types of equipment are expensive and warrant skilled operators; nevertheless, they provide very sensitive and accurate results, essential for forensic investigations. The overall process of DMS analysis is uncomplicated; still, ingenious adaptations have been developed in order to further facilitate these procedures, for example, the online and automated approaches (Figure 1) [11,40,44,55,74]. By employing such techniques, it becomes possible to use 96-well plates instead of vials, for instance, which grant high throughput for massive routine analysis. In addition, the human handling of specimens is limited to placing them on the autosampler, thus contributing to reducing operator errors and increasing reproducibility. Furthermore, the more recent concept of direct elution also improves DMS analysis [75]. The card is put directly into the equipment eliminating the need for punching the spots and manual extraction (Figure 1). Although it has not been applied to the analysis of drugs of abuse yet, reports show promising results, such as the increase in sensitivity and elimination of the HCT effect [75]. Nevertheless, these setups usually require modified instruments in order to perform the online or automated analysis, which are expensive and limit its acquisition and further applicability.

The majority of methods developed for drug analysis on DMS employed LC–MS systems, while only the minority used GC–MS or other instruments. This could be explained by the versatility of liquid chromatography associated with the gain in sensitivity, as authors suggest [11,25,30,31,43,67]. Moreover, the requirement of a derivatization step is an important disadvantage of GC–MS-based analysis; for example, a more complex DBS and DUS extraction with SPE followed by CE-DAD was faster and yielded higher sensitivity than a GC–MS method mainly because no derivatization was necessary [72]. Interestingly, in GHB analysis, the prevalence was GC–MS over LC; the authors had reported several setbacks in order to analyze this substance using liquid chromatography, thus GC was preferred [53,70,71].

Another particularity between the GC and LC systems is the ME. Although this challenge is only faced with LC and not a problem for GC analysis, less ME was reported when using DMS instead of liquid samples [14,28]. This could be explained by the sample treatment that dried matrices undergo which results in cleaner extracts with less interferents. Nevertheless, not all works aimed at investigating this feature in their methods, thus data are scarce (Tables 1 & 2). Hence, a more thorough investigation to understand the critical factors of ME on DMS is warranted.

Despite the relevance of GC for the analysis of several compounds of forensic interest, such as ethanol and GHB, the use of LC systems was more explored with DMS. Furthermore, evidence shows that the agreement of results obtained by LC–MS with GC–MS systems are higher than 80%; thus, traditional methodologies for drug analysis can be adapted to LC–MS systems, should the chemical properties of the analytes allow [31,35]. In addition, other reports demonstrate the interchangeable features of both systems and that LC–MS/MS yields higher specificity and sensitivity than GC–MS, although it tends to be more expensive [30,31]. Moreover, chromatographic techniques are preferred over immunoassays due to several limitations, such as low specificity, sensitivity and cross-reactivity between analytes. Hence, it remains to the operator to choose which instrument, technique and sample to use depending on the purpose.

Volumetric absorptive microsampling

As discussed in previous sections, one of the main challenges of using DBS is the HCT effect. The volume occupied by red blood cells in whole blood leads to alterations of sample viscosity; hence, the distribution of blood on paper together with the homogeneity of soluble molecules, such as the analytes of interest, will be affected. Other discussions have taken place regarding the volume of sample spotted on paper and how the spotting is performed, as it has been shown to have an impact on some analytical parameters [76–78]. In this context, a recent microsampling technique with the promise of overcoming the main limitations of conventional dried matrices, such as DBS, has been studied and applied in different bioanalytical analysis. The volumetric absorptive microsampling (VAMS™) technique consists of a plastic device with a hydrophilic polymer on one of the ends. This tip containing a porous material is capable of absorbing fixed volumes of blood; thus, it is not influenced by the HCT and avoids the risk of oversampling. VAMS, therefore, possesses the advantages of DMS techniques, while avoiding some of the main limitations [79].

Even though, VAMS was introduced recently to the scientific community, different applications have been reported, for medications, proteins, drugs of abuse, etc. For example, Mercolini *et al.* published a study evaluating the use of VAMS with conventional biological matrices, such as blood, urine and oral fluid [80]. The goal was to investigate the applicability of this approach with different matrices, while standardizing other features, such as sample volume absorbed by the device, exposure time, drying time, best solvent for extraction, stability, feasibility for its use with drugs of abuse, etc. Only 10 µl of biofluid was used and MeOH was chosen as the best solvent for extraction of the analytes thereby tested. For all samples, an addition of 500 µl of MeOH was followed by simultaneous ultrasound agitation and vortexing. Next, the extracts were dried and reconstituted with 100 µl of mobile phase for LC–MS/MS injection [80]. Needless to say, the overall sample preparation of VAMS is similar to traditional DMS and studies have already showed both techniques are equivalent alternatives, as similar concentrations of analytes on both [80]. The same is applied to accuracy, recovery and stability studies, as analytes were found to be more stable on VAMS than on liquid samples.

The overall characteristics of this technique have been discussed with details elsewhere and will not be addressed in the present review [79]. However, it is worth mentioning that VAMS has growing potential in forensic cases for combining the advantages of DMS while avoiding the main limitations [80–85]. Automation was also shown to be possible and even a novel spray ionization technique was developed – the sponge-spray ionization [86]. In this setup, a corona needle applies a high voltage discharge on the VAMS tip containing the sample, which is placed at the ion source. In the meantime, analytes are desorbed by a solvent flow forming a spray and entering the MS. This approach is similar to paper-spray ionization, which has been elegantly reviewed elsewhere, and enables a myriad of future applications in the forensic toxicology [87].

Conclusion

The interest of the scientific community provided valuable data showing that DMS could be a plausible alternative in the forensic toxicology to analyze drugs of abuse. While the main benefit is the improvement of analyte stability, the simplification of sampling and analyte extraction are also convenient and these methodologies are compatible with both gas and liquid chromatography. Nevertheless, a limitation of using dried matrices is the HCT effect that can cause loss of sensitivity and increased variability, reducing precision. Moreover, there is a lack of consensus regarding the means of spotting the biofluid and adding the IS on paper, the ideal volume to spot and where to punch the disk that will be used in analysis. Further investigations are still required in order to elucidate the limiting factors of DMS and how to overcome them.

Future perspective

Considering over a century has passed since it was created by Ivar Bang, the application of dried matrices in forensic toxicology is somewhat recent. Although toxicologists worldwide have published remarkable findings employing this technique to detect and quantitate illicit substances, the implementation in forensic routine is still modest. With the recent announcements of the WADA adopting DBS for drug testing in a global-scale event, perhaps the landscape will change. Dried matrices are very promising and could bring great benefits, especially regarding overall sample stability allowing storage and transportation for extended periods of time, while enabling reliable analysis. As technology advances, automation and higher analytical efficiency become more accessible, certainly favoring the development of methodologies with DMS. The experimentation of other biofluids in the DMS format also contributes to the development of this sampling technique. In regard to the main limitations

of DMS, VAMS is showing even more promising potential to overcome such drawbacks, while improving the existing advantages. Furthermore, novel ionization techniques, such as sponge-spray ionization and desorption electrospray ionization, have showed to be particularly suitable for analyzing dried matrices, thus facilitating even further practical applications of DMS. Altogether, dried matrices have the potential to become protagonists in different medical fields, including the forensic toxicology.

Executive summary

- Dried matrix spots (DMS) is a simple and inexpensive technique, which attracted the attention of different professionals, including toxicologists.
- One of the main advantages of DMS is easy sampling and improved analyte stability, essential for forensic purposes.
- Toxicologists have successfully applied dried matrices to the analysis of illicit substances, contributing greatly to the development of this sampling technique.
- Despite of the promising features of DMS, this technique is rarely implemented in authentic forensic routines, which might change in the near future considering World Anti-Doping Agency announced that DBS will be employed in the Olympic and Paralympic games of 2022.
- After years of studies and development of DMS techniques, the beginning of its use in forensic routines could finally be comming.
- Furthermore, the volumetric absorptive microsampling and other modern ionization techniques, such as paper spray, are suggestive of the further development of dried matrices approaches.

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5. ARTIGO II

Development of a dispersive liquid-liquid microextraction for synthetic cathinones in biological fluids based on principles of Green Analytical Toxicology

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OXFORD

Development of a Dispersive Liquid–Liquid Microextraction for Synthetic Cathinones in Biological Fluids Based on Principles of Green Analytical Toxicology

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Abstract

The present work describes a practical application of Green Analytical Toxicology (GAT) during the development of an eco-friendly dispersive liquid–liquid microextraction (DLLME) avoiding the use of highly toxic chlorinated solvents that are commonly used in this type of the technique. The purpose was to further consolidate GAT guidelines during method development. Thus, a full method optimization using a multivariate statistical approach and validation were performed. To that end, synthetic cathinones (SCs), one of the major classes of new psychoactive substances, were the target analytes due to their relevance and chemical diversity. Furthermore, whole blood and urine samples were the matrices of choice due to their clinical relevance. The sample preparation step prior to DLLME consisted of protein precipitation of whole blood samples, while urine specimens were centrifuged and diluted with ultrapure water. Then, borate buffer, sodium chloride and ethyl acetate:acetonitrile were added and vortexed. Finally, vials were centrifuged and the organic layer was transferred to autosampler vials, evaporated to dryness and resuspended with mobile phase prior to injection into the ultra-high performance liquid chromatography–tandem mass spectrometry system. Once optimized, the proposed DLLME was fully validated: 0.2 and 1 ng/mL as the limit of detection and 1 and 10 ng/mL as the limit of quantitation for urine and blood samples, respectively. The linear range was established as 1–100 and 10–1,000 ng/mL for urine and blood samples, respectively ($r^2 > 0.99$), while the bias and precision were within acceptable limits (≥80%). The matrix effect was of 1.9–260.2% and −12.3–139.6%; while the recovery was of 27.4–60.0% and 13.0–55.2%; the process efficiency ranged from 46.0% to 192.0% and 17.9% to 58.4% for whole blood and urine, respectively. Finally, the method was applied to real case samples as proof of applicability. Thus, a simple, cheap and fast eco-friendly technique to analyze SCs in two biological specimens was described.

Introduction

Qualitative and quantitative analyses of illicit substances in biological fluids have applications for different purposes in analytical toxicology, such as in human performance drug testing where these substances can be used for altering the athlete's performance, in roadside drug testing to monitor drivers driving under the influence of substances or in post-mortem forensic toxicology to assist in death investigations. In that context, blood and urine are conventional specimens with which it is possible to establish the cause–effect relationship and recent exposure. However, assessing drugs of abuse in these biofluids is a challenging task considering the naturally occurring interferents, such as cells, proteins and phospholipids. In addition, highly potent drugs, such as the new psychoactive substances (NPSs), are usually found at low concentrations (in the order of picograms or nanograms per milliliter) (1); thus, it is imperative to use a sample preparation step that enables the detection of such drugs in biological specimens. To that end, solid-phase extraction (SPE) and liquid–liquid extraction (LLE) are well-established tools that have been used for decades to analyze not only drugs of abuse but also a myriad of other chemicals (2–6). Nevertheless, as efficient as these techniques may be, they do have limitations, especially regarding the use of large volumes of samples and highly toxic solvents (2, 4, 5). In this context, alternative techniques have been proposed to overcome such limitations of classic SPE and LLE and improve the overall process. For example, the solid-phase microextraction (SPME) is a miniaturized derivation of SPE, while hollow fiber liquid-phase microextraction (LPME) and dispersive liquid–liquid microextraction (DLLME) are liquid-based techniques considered miniaturized derivations of LLE (7–9).

The growing interest in alternative tools for drug analysis follows a trend that originated in the late 1990s by Anastas and Warner, Green Chemistry (10). This concept aims at the use of more eco-friendly approaches in organic synthesis and it received great attention, thus spreading this idea to other fields of science and originating similar concepts. The Green Analytical Toxicology (GAT) is one of such concepts that were recently introduced and establish guidelines for the development of more environmentally friendly approaches specifically for toxicological analyses, such as Forensic Toxicology (11). Therefore, in the search for greener alternatives,

these miniaturized sample preparation techniques are valuable tools that have been widely explored by different research groups. As toxicologists routinely have to detect or quantitate substances at low concentrations in biological specimens, finding a proper substitute for conventional extraction techniques in order to meet GAT principles is not a simple task.

Rezaee and colleagues introduced DLLME in 2006 as a promising alternative for LLE (9). In this technique, a dispersant is used to increase the contact surface between the aqueous and organic phases. This dispersive agent, which is usually an organic solvent, is miscible in both phases. In contrast, the extraction solvent is immiscible in the aqueous sample (e.g., blood or urine), exactly like in LLE. Thereafter, by adding a dispersant, droplets of the organic solvent are dispersed into the aqueous phase, thus forming a cloudy solution (i.e., emulsion). As a result, the surface of contact is greatly increased facilitating analyte partitioning from the sample to the organic solvent, thus making this extraction process faster than LLE. Once the emulsion is formed, the organic phase can be easily collected after centrifugation. Among its main advantages, DLLME is a fast, simple and inexpensive procedure that requires low volumes of organic solvents and samples—all features aimed by GAT principles—thus, it is considered greener than LLE. Furthermore, although variations of DLLME have been proposed to avoid the use of organic solvents as dispersants, a limitation yet to be overcome is the use of highly toxic chemicals, such as chlorinated solvents (8). These commonly used reagents in DLLME pose a serious threat to both the environment and the operator; thus, their replacement is strongly encouraged by GAT. In fact, most published methods using DLLME make use of chlorinated solvents, such as chloroform ($CHCl_3$) or dichloromethane (12). As a result, the aim of the present study was to develop and validate a DLLME entirely avoiding the use of such highly toxic reagents to further encourage the search for more eco-friendly alternatives during method development. Despite that, it was demonstrated in the present study that it is feasible to adhere to GAT principles such as replacing common solvents that yield satisfactory results, but without sacrificing efficiency. Thus, a method to analyze synthetic cathinones (SCs), one of the most popular groups of NPSs, was proposed.

The choice of analyzing NPSs is plenty fold. First, the rising of these compounds in the early 2000s has drastically changed the illicit drug market as they usually are not under international control. These substances were sold as alternatives for traditional substances of abuse, such as cocaine and amphetamines, and have gained a great deal of attention. In addition, the hundreds of chemically diverse NPSs that flooded the drug market posed analytical challenges for forensic toxicologists. As consequence, existing methodologies for drug analysis became obsolete and had to be updated—or new ones had to be developed and validated in order to be fit for NPS assessment. In this context, SC is a major group of NPSs with stimulant properties. Figure 1 depicts the chemical structures of the 15 cathinones included in the present study. Hence, as these compounds are still a challenge for law enforcement and forensic toxicologists, this study describes the development and validation of a green DLLME for SC in whole blood and urine samples.

Experimental

Standards and chemicals

All analytes and internal standards (ISs) were acquired from Cerilliant Corporation (Round Rock, TX, USA). The stock solutions for all analytes were available at 1.0 mg/mL in methanol (MeOH), while for amphetamine-d_5 and 3,4-methylenedioxymethamphetamine-d_5 (MDMA-d_5) were at 100 µg/mL in MeOH. The standard solutions were diluted with MeOH to obtain working solutions at concentrations of 10 µg/mL. All standard solutions were stored in the freezer at −20°C until use.

MeOH, acetonitrile (ACN), ethyl acetate (EA), hexane, $CHCl_3$, tetrahydrofuran (THF), sodium tetraborate (borax), sodium chloride (NaCl), ammonium formate and formic acid were purchased from Merck (Darmstadt, Germany). All solvents used in the procedures were high-performance liquid chromatography grade, and ultrapure water was produced using a Milli-Q system (Millipore, Billerica, MA, USA).

Liquid chromatography–tandem mass spectrometry conditions

Chromatographic analyses were performed with a Waters Acquity ultra performance liquid chromatography (UPLC) system equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm). This system was coupled to a Quattro Premier XE mass spectrometer (Waters Corporation, Milford, MA, USA). A buffer solution of ammonium formate 1 mM with 0.1% formic acid was used as Mobile Phase A and MeOH with 0.1% formic acid as Mobile Phase B at a constant flow rate of 0.35 mL/min and a column oven temperature at 40°C. The chromatographic run time was 14.0 min with the following gradient elution: 0–2.0 min, 10% B as the starting gradient; 2.0–8.5 min, 10–25% B; 8.5–11.0 min, 25–100% B; 11.0–11.5 min, 100% B; 11.5–12.3 min, 100–10%B and 12.3–14.0 min, 10%B at the end of the run as post-run. The chromatographic separation of all SCs is shown in Figure 2. Multiple reaction monitoring (MRM) was used for all analyses with three transitions for each analyte. The mass spectrometer was equipped with an electrospray ionization source which operated in the positive ion mode for all analyses. Other mass spectrometer settings were established as follows: desolvation gas flow rate, 1100 L/h; cone gas flow rate, 200 L/h; desolvation temperature, 450°C; source temperature, 120°C and capillary voltage, 2 kV. The capillary voltage, collision energy, *m/z* transitions used as quantifiers and qualifiers for each analyte and retention times are displayed in Table I.

Sample preparation

Prior to extraction, both samples underwent a simple sample preparation step. A 200-µL aliquot of whole blood was precipitated with 200 µL of ACN, vortexed for 10 s and centrifuged for 5 min at 6,000 rpm. Then, the supernatant was transferred to a thin glass vial in which the ACN was evaporated under a gentle N_2 stream. On the other hand, urine samples were centrifuged for 5 min at 4,000 rpm and diluted at 1:10 with ultrapure water. At this step, both samples were spiked with working solutions containing all analytes and ISs in order to obtain the desired concentrations.

DLLME procedure

After evaporation of the ACN from the supernatant of whole blood in the glass vial, 200 µL of ultrapure water, 100 µL of

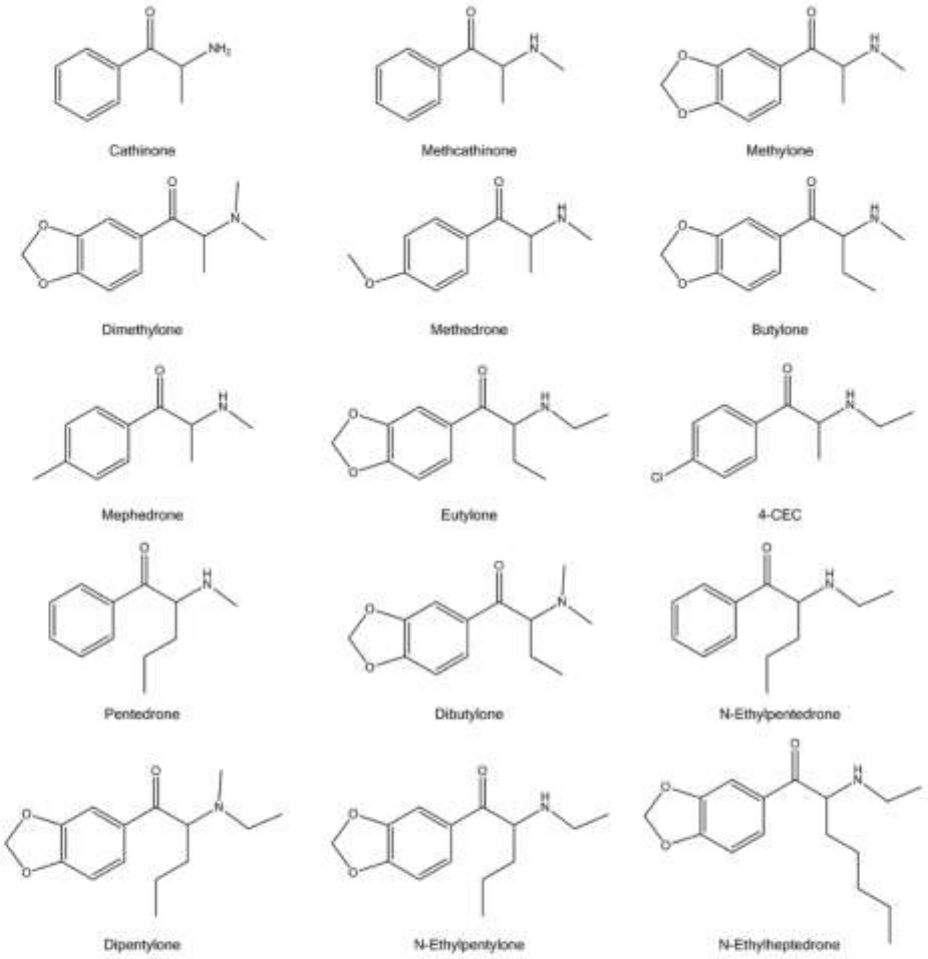

Figure 1. SC analyzed by the proposed DLLME. 4-CEC: 4-chloroethcathinone.

0.1 M borate buffer pH 8.0, ±60 mg of NaCl and 200 µL of a mixture of EA:ACN 1:2.5 were added. For urine samples, 200 µL of the previously diluted urine was placed in a thin glass vial, and 100 µL of 0.1 M borate buffer pH 8.0, 60 mg (± 10 mg) of NaCl and 200 µL of EA:ACN 1:2.5 were added. Next, all vials were vortexed for 30 s to form a cloudy solution and centrifuged for 5 min at 6,000 rpm, and the organic layer was transferred to an autosampler vial and evaporated to dryness. Finally, 50 µL of Mobile Phase A was used for resuspension of the dried extracts, and 5 µL was injected into the UPLC–tandem mass spectrometry system.

Method optimization

Univariate and multivariate statistical designs were used to optimize the method. For univariate studies, a two-way analysis of variance (ANOVA) was used, and experiments were performed in triplicate. As for multivariate designs, full or fractional factorial screenings (2^k or 2^{k-1}, respectively) and central composite design (CCD) were used. In both statistical approaches, significance was considered when $P < 0.05$, and the adjusted coefficient of determination (r^2) obtained by ANOVA served to determine the percent of data variance explained by the model. All data were processed

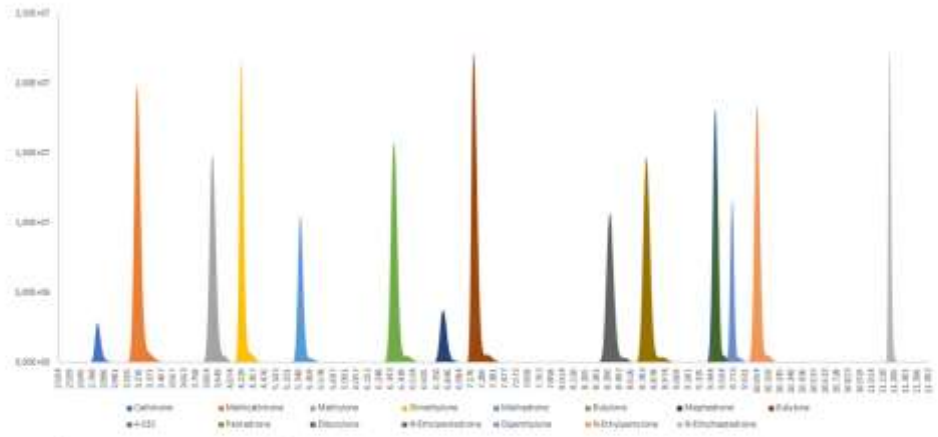

Figure 2. Chromatographic separation of all SCs. The gradient elution used to achieve the separation of all analytes is described in the "Liquid chromatography–tandem mass spectrometry conditions" section.

using GraphPad Prism® 8, Statistica® 10 and Minitab® 19 software.

Method validation

The method was fully validated according to the ANSI/ASB Standard 036, First Edition 2019 guide for method validation in Forensic Toxicology and supporting literature (13, 14).

Linearity, limit of detection and limit of quantitation

The limit of detection (LOD) was defined as a decision point concentration in which SCs are usually found in biological fluids (1–7, 15, 16). At this specific concentration, all identification criteria should be met: retention time and a signal-to-noise ratio of at least 3:1 achieved in a minimum of three different runs with at least three matrices from different sources.

The limit of quantitation (LOQ) was defined as the first point of the calibration curve for each matrix. In order to establish these values as the LOQ, three samples from different sources were analyzed in three different runs where all identification and quantitation criteria were met.

Linearity was established in a single run with six-point calibration curves for each matrix. Both curves were performed in quintuplicates for each calibration point, and the coefficient of variation (CV) should be $\leq$20% for the first and $\leq$15% for the other calibration points. A linear regression model was used, and r^2 was $\geq$0.99 for all analytes. In addition, heteroscedasticity on concentration range was investigated, and the proper weighting factor was applied whenever necessary.

Bias and precision

Bias and precision studies were performed by analyzing fortified specimens at three quality control (QC) levels in triplicates for each level during five different runs. One-way ANOVA was used to calculate combined within-run and between-run precision, and results are expressed as % CV. Acceptance criteria for both within-run and between-run precision were CV $\leq$ 20%.

Matrix effect, recovery and process efficiency

The matrix effect (ME), recovery (RE) and process efficiency (PE) were measured based on the work of Matuszewski et al. (14). Briefly, three experimental sets with three QC levels in each one were used. In Set 1, neat standards were simply diluted in the mobile phase and injected into the instrument; in Set 2, samples were spiked after the extraction procedure and in Set 3, samples were spiked before the extraction procedure. All sets were performed in quintuplicates at each QC level, but for both Sets 2 and 3, each of the replicates was collected from different subjects. Finally, absolute peak areas were used for estimating ME (Set 2/Set 1), RE (Set 3/Set 2) and PE (Set 3/Set 1).

Interference studies

Three different types of interference studies were performed to assess the selectivity of the method. First, 10 blank specimens, each from different subjects, were analyzed with the developed method to evaluate the interference of endogenous compounds. Next, common exogenous substances that may interfere with the method, such as prescription medicines and drugs of abuse, were analyzed by the method at a concentration of 10 µg/mL. A solution containing the following substances was prepared in the mobile phase and injected into the instrument: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, paroxetine, fluoxetine, norfluoxetine, sertraline, amitriptyline, nortriptyline, imipramine, desipramine, midazolam, nitrazepam, nordazepam, oxazepam, flunitrazepam, bromazepam, alprazolam, clonazepam, diazepam, phenobarbital, secobarbital, pentobarbital, Δ^9-tetrahydrocannabinol, cannabidiol, cannabinol, nor-9-carboxy-Δ^9-tetrahydrocannabinol, 11-hydroxy-Δ^9-tetrahydrocannabinol, cocaine, benzoylecgonine, cocaethylene, anhydroecgonine methyl ester,

Table I. MRM Transitions for All Aimed SCs

Analyte	Precursor ion (m/z)	Cone (V)	CE (V)	Product ions (m/z)	RT (min)
Cathinone	150.1	25	18	104.8	2.82
			18	116.7	
			30	77.1	
Methcathinone	163.9	18	10	146.1	3.26
			18	130.7	
			18	104.7	
Methylone	208.1	24	10	189.7	3.95
			20	159.7	
			28	131.7	
Dimethylone	222.0	30	16	146.9	4.25
			36	90.5	
			14	71.6	
Methedrone	194.2	20	12	175.9	5.38
			22	160.7	
			26	45.7	
Butylone	222.0	20	14	203.9	6.38
			22	174.1	
			36	130.7	
Mephedrone	178.1	30	10	160.1	6.85
			28	144.3	
			34	102.6	
Eutylone	235.9	24	12	217.9	7.23
			16	187.7	
			28	173.3	
4-CEC	212.1	22	14	193.7	8.55
			18	158.8	
			26	143.6	
Pentedrone	191.9	28	8	173.6	8.90
			14	131.8	
			22	90.5	
Dibutylone	236.0	24	12	217.9	9.55
			20	187.6	
			40	131.1	
N-ethylpentedrone	206.0	28	12	187.6	9.61
			18	145.3	
			28	129.8	
Dipentylone	250.0	18	22	174.8	9.77
			26	148.8	
			20	134.9	
N-ethylpentylone	250.2	24	12	231.9	10.10
			16	202.2	
			30	173.3	
N-ethylheptedrone	234.1	26	10	216.1	11.18
			18	145.9	
			24	90.5	
Amphetamine-d_4	141.2	17	11	95.9	4.44
			14	124.1	
MDMA-d_5	199.3	20	13	165.1	5.33
			21	135.3	
			25	107.1	

m/z: mass-to-charge ratio; CE: collision energy; RT: retention time; 4-CEC: 4-chloroethcathinone.

morphine, 6-acetylmorphine, codeine, caffeine, nicotine and cotinine. Finally, considering amphetamine-d_4 and MDMA-d_5 were used as ISs, it was investigated if any non-labeled amphetamine-d_5 or MDMA-d_5 that may be present as an impurity would interfere with the analytes of interest. Thus, a single sample was spiked with both labeled ISs, while another was spiked only with the SC of interest at the highest concentration of the calibration curve. Then, samples underwent the whole process of extraction and were injected into the instrument. For the three interference studies carried out, no peaks should be visualized in the analyte's detection window.

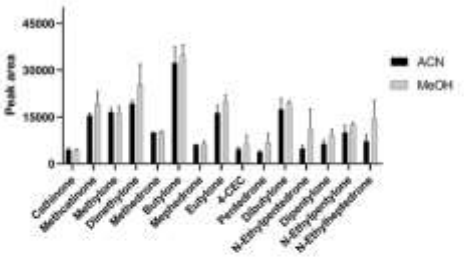

Figure 3. Comparison of solvents for protein precipitation. Absolute peak areas of each SC are being shown after whole blood samples were precipitated with ACN or MeOH. No statistical difference was observed between both solvents ($P > 0.05$).

Carryover

After an injection with all analytes at a high concentration (1,000 ng/mL), a blank run was analyzed for the presence of any peak of the analytes of interest in their respective detection windows to confirm carryover. These runs were performed in triplicate following ANSI/ASB Standard 036 recommendations.

Authentic samples

The study was approved by the Ethics Committees of both the University of São Paulo and the University of Campinas (CAAE: 46404121.8.3001.0067). Blood and urine samples were collected at the Laboratory of Analytical Toxicology from the Toxicological Assistance and Information Centre located at the University of Campinas.

Results and Discussion

Protein precipitation in whole blood samples

Before optimizing the DLLME, the protein precipitation by two different organic solvents was investigated in terms of analyte recovery. As a result, ACN was compared with MeOH and no statistical difference between these solvents was observed (Figure 3), indicating both reagents yield equivalent analyte recovery. However, ACN was chosen due to its higher protein precipitation efficiency in a 1:1 ratio compared to MeOH, even though the latter is a slightly greener reagent (17–19).

Extraction and dispersive solvent selection

The selection of organic solvents was based on the most common reagents used in DLLME, affinity for the analytes, solubility in water and greenness (12, 17, 20). A full factorial design with three and two levels for extraction and dispersive solvents, respectively, was performed to screen possible combinations of organic solvents. The following mixtures were tested: $CHCl_3$:MeOH, $CHCl_3$:THF, EA:ACN, EA:THF, Hexane:MeOH and Hexane:THF (Figure 4). The Pareto chart in Figure 4a shows that not only the extraction and dispersive solvents were significantly important for analyte recovery, but also the interaction between them. This information is more evident in the graphs depicted in Figure 4b and c, which show the combination of EA with ACN yielding the highest analyte

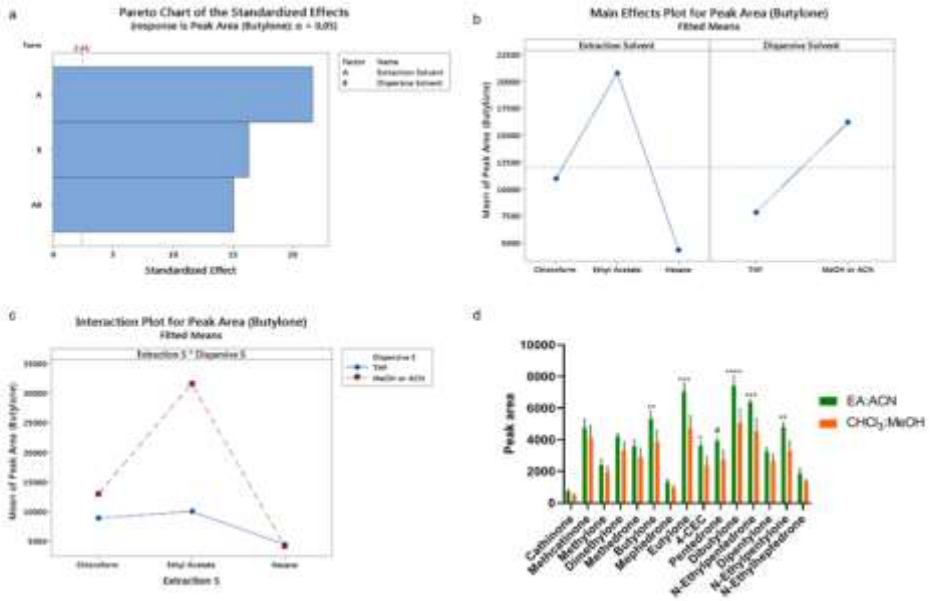

Figure 4. Investigation of possible solvent combinations for DLLME. (a) The Pareto chart showing statistical significance for the extraction solvent (Factor A), dispersive solvent (Factor B) and the interaction between them (Factor AB) (95% confidence interval). (b, c) Interaction plots showing that EA with ACN yielded higher SC recovery than the other combinations of solvents. (d) two-way ANOVA comparing the EA:ACN mixture with $CHCl_3$:MeOH. Data in a, b and c are representative of all analytes. #$P = 0.054$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

recovery compared to other mixtures. Afterward, a confirmation study of whether EA:ACN would stand out in terms of SC recovery when compared with $CHCl_3$:MeOH, a common choice for DLLME, was carried out (12, 16, 21–23). Figure 4d depicts the higher recovery of some SCs with the mixture EA:ACN, while no statistical difference for the remaining SC indicates similar recovery values. Based on these findings, EA:ACN proved to be a promising combination to be used in DLLME and thus was chosen for method optimization and validation.

Surface response design

First, a CCD was chosen to study other variables of the technique that could affect analyte recovery. This design consists of a 2^k factorial run, with $2k$ axial and center points. We assessed the volume (100–300 µL) and ratio (1:2.5–1:6) of the EA:ACN mixture together with the pH of extraction (6.0–10.5). The response of cathinone and mephedrone was considered since the sensitivity for both was the lowest among all SCs (Figure 4d). While the volume of the EA:ACN mixture was not significant for analyte recovery, the EA:ACN ratio and the pH of extraction showed to be statistically significant (ANOVA, $P < 0.05$) (Figure 5a and b). In this regard, by plotting a desirability surface graph, it is evident that the pH yielding maximum response for these SCs was around 8.0 (Figure 5c and d). Furthermore, in terms of the EA:ACN ratio, the response increased as the ratio diminished, indicating only EA should be used. Nevertheless, considering the dispersive solvent is fundamental to increase the miscibility of extraction solvent droplets into the aqueous phase in a DLLME, it was not feasible to use the extreme of this condition (i.e., solely EA). Thus, the 1:2.5 ratio of EA:ACN, respectively, was chosen as this value is still in the red zone of the surface graph, indicating a good response, and was close to the ratio limit (a higher ratio would decrease the response) (Figure 5c and d). The use of a dispersive solvent is paramount for DLLME and it is what mainly differentiates it from LLE. As a result, increasing the volume of ACN, the dispersive solvent used in the present technique would also increase analyte partitioning to EA, thus improving analyte recovery; hence, the 1:2.5 ratio was chosen. Moreover, ANOVA data of this CCD show a reasonable fit of the experimental data (adjusted $r^2 \geq 85\%$), indicating the proposed model was adequate to explain the variabilities observed on each factor studied. In this context, despite this caveat regarding the EA:ACN ratio in which the indicated desirability by the surface response graph was not feasible, this model was well suited for the other variables studied herein (solvent volume and pH).

Although there are reports of developed and validated DLLME for SC, only few of them fully optimized the technique with a multivariate approach. For example, Meng et al. compared LPME with DLLME in regard to SC

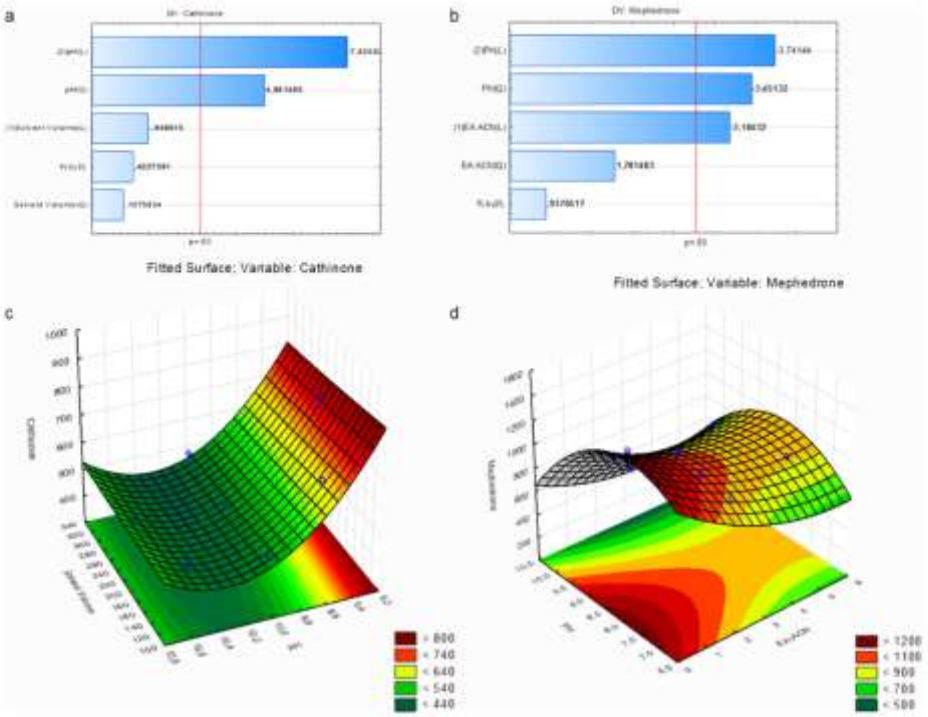

Figure 5. Optimization of DLLME with CCD. (a, b) Pareto charts expressing the relevance of pH of extraction and the EA:ACN ratio to the desired response (analyte recovery) with a 95% confidence interval. (c) Surface response graph showing the volume of EA:ACN did not affect analyte recovery in a statistically significant manner, while SC recovery increased lowering the pH <9.5 (red). (d) Surface response graph showing the maximum analyte recovery in pH ranging from 7.0 to 8.5 and as the EA:ACN ratio reduces (red). ANOVA, $P < 0.05$; adjusted $r^2 \geq 85\%$.

extraction using only a univariate optimization for different types of solvents in LPME (8). Furthermore, in the technique proposed by Fernandez et al., a thorough solvent comparison was performed, albeit only chlorinated solvents were included ($CHCl_3$, dichloromethane, dichloroethane, chlorobenzene, ethylene tetrachloride and carbon tetrachloride) (22). Notwithstanding that, they have also shown that low dispersant volume provided better extraction of methylone from oral fluid samples (22). In contrast, Airado-Rodríguez et al. showed that increasing the volume of dispersive solvents yields a higher recovery of MDMA from urine samples (24). In addition, in the univariate optimization described by Ahmadi-Jouibari et al. using ACN as a dispersant, it was concluded that low volumes of this reagent prevent the formation of a proper cloudy solution, which is paramount for DLLME. On the other hand, if the solvent volume is largely increased, analyte solubility in the aqueous phase increases as well, thus reducing extraction efficiency (25). In that regard, Akramipour et al. showed similar findings also using ACN as a dispersive solvent (26). Moreover, similar to the CCD performed herein, Kahl et al. applied this tool to study the ratio of organic solvents in DLLME for amphetamines and reported statistical differences between the minimum and maximum volumes tested (50–150 and 100–250 µL for extraction and dispersive solvents, respectively); nevertheless, as satisfactory sensitivity was achieved to extract the aimed analytes from oral fluid, they chose the 1:2 ratio of extraction and dispersive solvents ($CHCl_3$:ACN) (27). These data support our findings, as a narrow EA:ACN ratio to work with was achieved (Figure 4). However, increasing the volume of ACN to a 1:2.5 ratio sufficed for the applicability of the method. Altogether, elegant optimizations have been reported for DLLME, especially for SC, and the results of the present study are consistent with data previously reported. Nevertheless, the use of the EA:ACN mixture and a comparison proving it is more efficient to extract SC from blood and urine samples than $CHCl_3$:MeOH were shown for the first time (Figure 3d). This is especially important when method development is aimed toward greener alternatives.

Another variable that merits discussion is the pH value of the sample solution, considering it determines whether basic drugs, such as SC, are protonated or not. This influences the partitioning of the substance to the extraction solvent and might reduce the recovery efficiency of the target

analytes. Previous works using DLLME to extract SC and/or other stimulants used pH values >10.0, with the exception of Fernandez et al. that used pH 8.0—although in his work opioids, cocainics and other substances were included (8, 16, 21–23). Among the variables studied, they showed that medium to high pH yielded higher extraction of methylone (22). It is worth mentioning, however, that some of these methods aimed at amphetamines as well—which have similar chemical structures to SCs, although some particularities are observed. In addition, in methods for multiple classes of drugs, it is difficult to establish an optimal extraction pH for all analytes, considering their diverse chemical properties. In this context, the optimum pH value for SC extraction found in the study was around 8.0 (Figure 4), although there were some SCs that showed to be better extracted from slightly lower or higher pH values (data not shown). Hence, this pH proved to be suited for all SCs included in the proposed method, and the DLLME was thus fully optimized.

Method validation

The present method was validated with linearity from 1 to 100 ng/mL for whole blood and 10 to 1,000 ng/mL for urine samples ($r^2 > 0.99$) (Table II). In addition, the application of the *F*-test to the linearity data revealed method heteroscedasticity, which was corrected by the application of $1/x^2$ weighting factor for all SCs. According to previous reports in the literature that analyzed this group of NPS in blood and urine samples, this linearity range should suffice for most cases (1–7, 15, 16).

DLLME is faster and usually yields higher analyte recovery than LLE. In addition, as this technique is miniaturized, low volumes of samples and solvents are used. These features are in agreement with GAT guidelines, making DLLME a promising technique in that regard. Nevertheless, a common drawback when using low sample volumes is to achieve the intended LOD and LOQ, especially for potent substances that are found at low concentrations in biological fluids, such as SCs. In this context, solvents known to yield satisfactory results regarding drug extraction, such as $CHCl_3$, are usually chosen during method development. In contrast, the DLLME herein reported used no chlorinated solvents and achieved satisfactory LOD and LOQ values when compared with previous works that analyzed SCs in blood and urine (1–5). The values established as LOD and LOQ for all analytes were 0.2 and 1 ng/mL for whole blood and 1 and 10 ng/mL for urine, respectively (Table III). In addition, only 200 µL of the samples and 200 µL of the EA:ACN mixture were required. Odoardi et al. achieved 0.2 and 1 ng/mL for SC using 500 µL of whole blood and 350 µL of $CHCl_3$:MeOH (21); Mercieca et al. reported LODs of 1–2 and 2–10 ng/mL for SC using 2 mL of urine or blood, respectively, and 700 µL of $CHCl_3$:MeOH (16); Fisichella et al. achieved 0.5 and 2 ng/mL as LOD and LOQ, respectively, with 500 µL of whole blood and 350 µL of $CHCl_3$:MeOH (23); Meng et al. achieved LOD and LOQ of 1 ng/mL for methcathinone using 1 mL of blood and urine with 100 µL of toluene associated with 3 min of sonication, avoiding the use of a dispersive solvent (8). Of note, the aforementioned techniques used larger volumes of harmful chemicals in comparison to the DLLME herein described, although similar LOD and LOQ values were achieved. Furthermore, as for other types of techniques, such as SPE, these values are similar to those achieved by the proposed DLLME (2–5, 15, 28). Curiously, Khalilian and Razaee coupled SPE to DLLME for the analysis of amphetamines in plasma and urine (28). Needless to say, this procedure is time-consuming, more expensive and more complex than using solely DLLME. In addition, larger volumes of samples (>1 mL) and organic solvents were employed, which goes against GAT principles (28). In contrast, Alsenedi and Morison developed an SPME for amphetamines and SC using 1 mL of urine samples achieving 5–25 ng/mL as LOD and 25–100 ng/mL as LOQ, showing an interesting alternative technique to analyze these substances (7). Taken together with data from previous reports, the LOD established for blood samples provides a reasonable window of detection in case the plasmatic concentrations of the SC are diminishing. In contrast, as SCs tend to be more concentrated in urine samples, a higher LOD is recommended to analyze these substances in this biological fluid (Tables III and IV). Thus, similar to blood, the LOD of 1 ng/mL in urine also gives a reasonable window of detection in cases where the concentrations are diminishing due to the time of drug intake or the urine is diluted. As for quantitative analyses, the LOQs established by the proposed method also prove to suffice for the intended purposes (Tables III and IV).

Table II. Linear Range and Coefficient of Determination (r^2) Adjusted with Weighting Factor $1/x^2$ for All SCs

	Whole blood		Urine	
Analyte	Linear range (ng/mL)	r^2 ($1/x^2$ weighting factor)	Linear range (ng/mL)	r^2 ($1/x^2$ weighting factor)
Cathinone	1–100	0.9974	10–1,000	0.9939
Methcathinone	1–100	0.9999	10–1,000	0.9997
Methylone	1–100	0.9967	10–1,000	0.9957
Dimethylone	1–100	0.9952	10–1,000	0.9941
Methedrone	1–100	0.9993	10–1,000	0.9991
Butylone	1–100	0.9959	10–1,000	0.9947
Mephedrone	1–100	0.9972	10–1,000	0.9968
Eutylone	1–100	0.9961	10–1,000	0.9948
4-CEC	1–100	0.9995	10–1,000	0.9999
Pentedrone	1–100	0.9955	10–1,000	0.9971
Dibutylone	1–100	0.9932	10–1,000	0.9952
N-ethylpentedrone	1–100	0.9908	10–1,000	0.9973
Dipentylone	1–100	0.9967	10–1,000	0.9973
N-ethylpentylone	1–100	0.9928	10–1,000	0.9959
N-ethylheptedrone	1–100	0.9987	10–1,000	0.9971

4-CEC: 4-chloroethcathinone.

The within-run precision of the method varied from 2.4% to 17% and 2.8% to 12.5% for blood and urine, respectively. As for between-run precision, it varied from 3.3% to 18.7% and 3.7% to 18.2% for blood and urine samples, respectively (Tables II and III). These values are accepted according to international guidelines for method validation in Forensic Toxicology that recommend both types of precision should be <20% (13). The overall CV values were <10% for most analytes at the three QC levels. However, some SCs at specific QC levels showed higher values (e.g., dimethylone at the 20-ng/mL QC for urine); regardless of these few singularities in CV, all were below the 20% limit (Tables II and III). On the other hand, bias varied from −14.5% to 10% and from −15.4% to 10.7% for blood and urine, respectively. The overall bias variability was ±10% for most SCs apart

Table III. LOD, LOQ, Bias and Precision Obtained during Method Validation for Whole Blood Samples

Analyte	LOD (ng/mL)	LOQ (ng/mL)	QC (ng/mL)	Bias (%)	Precision (%)	
					Within-run	Between-run
Cathinone	0.2	1	2	4.9	4.7	8.0
			40	1.8	4.0	4.5
			80	−4.1	4.2	5.4
Methcathinone	0.2	1	2	−2.0	4.5	15.2
			40	8.9	3.5	3.3
			80	4.0	4.6	5.3
Methylone	0.2	1	2	−14.5	6.4	6.8
			40	−0.7	2.4	4.2
			80	−6.8	5.1	5.5
Dimethylone	0.2	1	2	−7.9	7.0	18.7
			40	0.2	2.7	3.6
			80	−6.5	5.3	5.4
Methedrone	0.2	1	2	−1.8	5.1	8.7
			40	2.5	5.1	4.3
			80	1.8	5.5	7.5
Butylone	0.2	1	2	−3.5	8.1	9.1
			40	3.8	2.8	4.1
			80	−4.2	2.4	4.2
Mephedrone	0.2	1	2	4.0	9.9	8.2
			40	4.7	3.2	4.4
			80	−1.1	2.7	5.2
Eutylone	0.2	1	2	−2.0	6.3	9.9
			40	4.7	7.1	6.2
			80	0.8	4.8	6.5
4-CEC	0.2	1	2	0.7	7.8	7.3
			40	5.0	7.9	8.6
			80	2.2	7.1	8.5
Pentedrone	0.2	1	2	−2.3	6.6	6.8
			40	7.8	7.3	6.9
			80	2.4	5.9	6.2
Dibutylone	0.2	1	2	−0.2	6.4	6.7
			40	5.0	7.4	6.5
			80	−4.0	5.4	5.5
N-ethylpentedrone	0.2	1	2	2.4	11.2	11.9
			40	6.4	9.6	8.3
			80	−1.6	7.2	7.5
Dipentylone	0.2	1	2	−2.4	6.6	6.8
			40	5.4	8.6	7.6
			80	0.4	6.9	7.2
N-ethylpentylone	0.2	1	2	−0.7	6.5	6.7
			40	6.6	10.2	8.8
			80	0.2	6.9	7.6
N-ethylheptedrone	0.2	1	2	7.8	15.8	17.6
			40	10.0	17.0	16.5
			80	−0.4	12.6	14.6

4-CEC: 4-chloroethcathinone.

from some exceptions: for example, methylone had −19.5% at QC of 2 ng/mL and *N*-ethylpentedrone had 15.4% at QC of 800 ng/mL (Tables II and III).

ME, RE and PE were performed according to Matuszewski et al., and the results are shown in Table V (14). The overall ME ranged from 1.9% to 260.2% and from −12.3% to 139.6% for whole blood and urine, respectively. Curiously, expressive values of ME were observed for some SCs, especially in whole blood. In contrast, these values were lower in urine samples, which indicates that the urine dilution in a 1:10 ratio prior to extraction could have contributed to decrease the interference exerted by matrix constituents. There are some cases of intense ME for some SCs described in the literature, but not as much as the values found with the DLLME presented in this study. Odoardi et al., for instance, reported ME ranging from 73% to 110% in whole blood, while the work of Fisichella et al. showed 80–104% of ME in the same matrix (21, 23). Nevertheless, despite these unusual findings regarding the ME, criteria for bias and precision were still met (≤20%) according to validation guidelines, especially at LOD and LOQ values (Tables III and IV). Furthermore, in terms of RE, values varied from 27.4% to 60.0% and from 13.0% to 55.2% for whole blood and urine, respectively; the PE varied from 45.0% to 192.0% and 17.9% to 58.4% for whole blood and urine, respectively (Tables IV and V). The overall analyte recovery was low compared to another DLLME reported in the literature (8, 21–23); nonetheless, the LODs and LOQs achieved with the present method are fit for purpose.

Interference studies performed by extracting 10 blank samples, a blank sample spiked only with ISs or analytes and direct analysis of a pool containing commonly used substances (prescription medications and drugs of abuse) showed no

Table IV. LOD, LOQ, Bias and Precision Obtained during Method Validation for Urine Samples

Analyte	LOD (ng/mL)	LOQ (ng/mL)	QC (ng/mL)	Bias (%)	Precision (%)	
					Within-run	Between-run
Cathinone	1	10	20	7.8	11.7	11.0
			400	8.3	5.5	8.0
			800	−2.6	6.1	10.8
Methcathinone	1	10	20	0.7	6.3	5.7
			400	8.9	7.6	8.4
			800	4.2	6.2	9.9
Methylone	1	10	20	−4.9	10.6	9.8
			400	8.0	7.9	10.4
			800	7.5	6.5	8.7
Dimethylone	1	10	20	−0.5	6.5	18.2
			400	−2.7	2.8	3.7
			800	−1.6	5.0	5.1
Methedrone	1	10	20	−2.3	6.0	7.7
			400	4.9	5.3	7.7
			800	2.3	5.4	7.5
Butylone	1	10	20	−2.5	8.9	9.9
			400	9.0	9.3	11.5
			800	6.7	7.9	8.6
Mephedrone	1	10	20	−3.5	8.8	8.1
			400	2.9	8.5	10.2
			800	−3.3	5.2	8.3
Eutylone	1	10	20	−2.7	5.5	4.9
			400	1.5	6.0	7.3
			800	−3.4	5.4	5.8
4-CEC	1	10	20	10.7	6.9	6.4
			400	−1.6	7.0	7.1
			800	−7.1	9.0	11.6
Pentedrone	1	10	20	1.4	6.0	7.8
			400	−7.2	8.9	11.7
			800	−11.4	9.8	12.5
Dibutylone	1	10	20	−0.7	8.5	8.8
			400	4.0	4.9	7.3
			800	−1.2	4.9	4.8
N-ethylpentedrone	1	10	20	3.6	10.7	10.6
			400	−14.4	9.4	12.2
			800	−15.4	10.4	13.3
Dipentylone	1	10	20	0.5	6.1	5.4
			400	−0.2	6.7	9.2
			800	0.3	6.6	7.1
N-ethylpentylone	1	10	20	−2.0	5.4	5.5
			400	2.5	7.3	9.7
			800	0.4	7.2	6.5
N-ethylheptedrone	1	10	20	9.9	10.9	11.6
			400	−5.4	8.4	9.9
			800	−9.0	12.5	11.3

4-CEC: 4-chloroethcathinone.

interference by these endogenous or exogenous compounds in the analyte's retention times. Thus, the absence of interfering peaks proves the selectivity of the method for the aimed SC.

Finally, the applicability of the method was tested in nine authentic samples collected at the Laboratory of Analytical Toxicology (Table VI). In blood samples, *N*-ethylpentylone and dibutylone were found at 8.91 and 3.57 ng/mL, respectively. As for urine samples, it was only possible to quantitate *N*-ethylpentedrone in one of the samples (34.40 ng/mL), while SCs were found below LOQ on the remaining samples (Table VI). Although some reports in the literature show that these substances can be found at concentrations >1 μg/mL, this was not the case for the authentic samples analyzed in this study (4, 6, 7, 15). In fact, the opposite was observed as most urine samples were below LOQ and were only able to be identified in the specimens. This finding could be explained by the late intake of the drug by the users before sample collection, thus resulting in low concentrations of the substances in the urine. In contrast, it could also mean recent intake of the drugs with the low levels representing early excretion of the substances, provided that these specimens were collected at a toxicological center. In addition, SCs are known not to be stable in aqueous samples, hence degradation could have occurred, although all samples were stored at −20°C prior to analysis (29, 30). Drug concentration in urine samples may fluctuate depending on the time of sample collection, level of patient hydration, renal disorders, etc., resulting in dilution or concentration of metabolites. Different factors could explain the low levels of SCs found in the urine samples analyzed by the method. In any case, our findings are in agreement with concentrations of SCs found

Table V. ME, RE and PE Values for All SCs in Whole Blood (Top) and Urine (Bottom) Samples

	ME (%)			RE (%)			PE (%)		
Analyte	Low	Med	High	Low	Med	High	Low	Med	High
Cathinone	64.0	128.6	178.0	27.4	38.3	32.4	45.0	87.6	90.1
Methcathinone	84.0	148.9	203.3	32.9	39.7	34.5	60.6	98.8	104.5
Methylone	41.9	40.8	29.2	32.9	41.0	35.8	46.7	57.7	46.3
Dimethylone	56.5	53.2	37.3	46.5	58.9	51.3	72.7	90.2	70.4
Methedrone	48.4	72.6	59.1	32.3	40.5	34.8	47.9	69.9	55.3
Butylone	34.2	29.3	17.6	40.3	51.6	44.2	54.1	66.7	52.0
Mephedrone	80.0	168.7	215.5	35.6	48.0	40.1	64.1	128.9	126.5
Eutylone	42.2	41.1	23.2	39.8	55.6	48.5	56.7	78.5	59.7
4-CEC	108.6	189.0	219.3	36.9	54.2	44.7	76.9	156.6	142.8
Pentedrone	86.9	201.7	250.6	42.2	57.6	49.3	78.9	173.7	172.9
Dibutylone	27.8	16.4	1.9	38.8	57.8	50.2	49.6	67.2	51.1
N-ethylpentedrone	108.6	201.5	260.2	42.9	60.0	53.3	89.4	181.0	192.0
Dipentylone	41.1	26.3	14.3	42.8	59.1	50.2	60.4	74.7	57.4
N-ethylpentylone	24.8	15.3	4.5	40.5	56.7	51.3	50.5	65.3	53.6
N-ethylheptedrone	114.3	118.5	86.4	35.2	54.5	48.1	75.5	119.1	89.7
Cathinone	28.3	5.1	70.5	32.1	25.6	21.3	41.2	26.9	36.3
Methcathinone	19.1	8.6	112.2	27.7	16.5	13.0	33.0	17.9	27.6
Methylone	−6.5	−12.3	−3.4	40.8	35.6	33.8	38.2	31.2	32.7
Dimethylone	6.4	−1.6	10.7	50.1	35.6	30.0	53.3	35.1	33.3
Methedrone	0.1	−7.5	4.8	38.6	32.1	30.2	38.6	29.7	31.7
Butylone	−3.2	−8.8	−3.5	49.0	42.4	39.5	47.5	38.7	38.1
Mephedrone	18.2	2.8	71.2	40.9	28.1	22.9	48.3	28.9	39.2
Eutylone	18.2	2.8	71.2	52.7	43.5	40.5	55.2	42.6	42.8
4-CEC	14.3	2.1	54.6	39.1	24.2	20.6	44.7	24.7	31.8
Pentedrone	38.7	20.3	137.7	39.4	23.3	18.4	54.6	28.0	43.8
Dibutylone	4.3	−1.7	0.0	55.1	48.3	49.1	57.5	47.5	49.1
N-ethylpentedrone	35.2	20.0	139.6	36.8	21.4	17.4	49.7	25.6	41.8
Dipentylone	5.7	−0.1	6.6	55.2	43.2	40.7	58.4	43.2	43.4
N-ethylpentylone	−2.4	−4.8	0.5	54.8	45.7	45.2	53.5	43.5	45.4
N-ethylheptedrone	22.4	4.5	25.6	44.2	28.9	26.8	54.2	30.2	33.7

4-CEC: 4-chloroethcathinone.

Table VI. Results of the Authentic Case Samples Analyzed by the Proposed DLLME

Samples	Analyte	Concentration (ng/mL)
Postmortem blood		
01	*N*-ethylpentylone	8.91
02	Dibutylone	3.57
Urine		
01	Methylone	<LOQ
02	*N*-ethylpentedrone	<LOQ
03	*N*-ethylpentedrone	34.40
04	*N*-ethylpentedrone	<LOQ
05	Dimethylone	<LOQ
06	*N*-ethylpentylone	<LOQ
07	Methylone	<LOQ

in urine samples reported by previous works. In addition, considering no authentic specimens analyzed were above linear range, validation of dilution integrity was not necessary (2, 6, 7, 16).

The proposed DLLME was fully optimized and validated for SC in whole blood and urine samples. Although intensive ME and medium-to-low analyte RE were achieved when compared to previous works, this method proved to be fit for its purpose. Moreover, a reasonable number of SCs were included, of which *N*-ethylheptedrone was reported for the first time. Thus, the focus of the technique was SC analysis, meaning other compounds of this NPS group can be easily included, if necessary. Finally, this study serves the purpose to showcase the development of a green microextraction technique that can be a valuable tool for routine analyses.

Conclusions

In the present study, we described the development, optimization and validation of a DLLME for SC using greener extraction solvents than the commonly employed with this technique. Acceptable LODs and LOQs for all 15 SCs in both biological samples were achieved, and the applicability of the method was proven with the analysis of real case samples. In addition, a practical illustration of exploring greener alternatives during method development in toxicological analyses, according to GAT, was provided. Thus, a simple and inexpensive miniaturized extraction technique that uses low volumes of samples and less toxic solvents was proposed.

Data availability

Data are available on request.

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6. ARTIGO III

A new application of the Switchable Hydrophilicity Solvent-Based Homogenous Liquid–Liquid Microextraction to analyze synthetic cannabinoids in plasma by LC-MS/MS

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Short communication

A new application of the switchable hydrophilicity solvent-based homogenous liquid–liquid microextraction to analyze synthetic cannabinoids in plasma by LC-MS/MS

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ABSTRACT

Synthetic cannabinoids are still a growing trend among drug users and consist of a group of hundreds of highly potent compounds. To investigate the use of such substances, sample preparation of biological matrices is a crucial step prior to instrumental analysis. Although different efficient extraction techniques have been proposed for that aim, they usually do not fit eco-friendly guidelines that have been gaining popularity in recent years, such as Green Analytical Toxicology. This work uses describes for the first time the use of switchable hydrophilicity solvent-based homogenous liquid–liquid microextraction (SHS-HLLME) for synthetic cannabinoids. This is a green technique that replaces highly toxic organic reagents for switchable hydrophilicity solvents (SHS), substances that can be either water-miscible or immiscible depending on their protonation. Thus, by simply adjusting the pH of the system, these SHS can be used as extraction solvents. A full optimization study including type of SHS, volume of protonated SHS, volume of NaOH, salting-out effect, and extraction time was performed. The optimized procedure consisted of precipitating the proteins of 300 µL of plasma with 300 µL of acetonitrile followed by centrifugation; evaporation of the organic solvent under N_2 stream; addition of 500 µL of the protonated DPA, DPA-HCl (6 M) (1:1, v/v); addition of 500 µL of NaOH (10 M); and finally centrifugation and evaporation. Validation results showed determination coefficients $\geq$ 0.99 for the 0.1–10 ng/mL linear range; 0.01–0.08 ng/mL as limit of detection; 0.1 ng/mL as limit of quantitation; accuracy and imprecision were within acceptable ranges; matrix effect, recovery, and process efficiency ranged from −55.6 to 185.9%, 36–56.7%, and 18.5–148.4%, respectively. The SHS-HLLME herein described was fully optimized providing satisfactory recoveries of 31 synthetic cannabinoids at low concentrations requiring only 300 µL of plasma. In addition, the validation results showed that the technique is a reliable eco-friendly alternative for clinical and toxicological analysis.

1. Introduction

Synthetic cannabinoid agonist receptors (SCRA), or simply synthetic cannabinoids, were first synthesized in the 1970 s with the goal of understanding the endocannabinoid system [1,2]. In the early 2000 s, however, these synthetic compounds were found in the illicit drug market with the first reports dating to 2008 with JWH-018 in Austria and Germany [3]. This was the beginning of an unprecedented phenomenon that eventually flooded the illicit drug market with hundreds of novel chemicals, which were soon named New Psychoactive Substances (NPS). A total of 1182 have been reported so far and are grouped according to their pharmacological effects or chemical structure [4].

These synthetic substances that mimic the effects of Δ-9-tetrahydrocannabinol (THC) are artificially added to herbs and sold to drug users as alternatives to cannabis, although this has not been the only presentation reported [5,6]. In addition, synthetic cannabinoids became one of the most important groups of NPS due to a large number of substances and chemical variety. Up to December 2022, 224 SCRA were being monitored by the European Union Early Warning System and this

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group has been among the most detected NPS in the last decade [4]. As new compounds continue to appear in the drug market, slight to more drastic changes in their chemical structures have been observed, as shown in Fig. 1. Due to such differences, NPS tend to circumvent the existing legislation and can be legally commercialized – reason why they are also referred to as "legal highs". This chemical complexity makes it difficult for professionals and analytical methodologies to keep up-to-date to deal with this threat. Thus, with the increasing number of

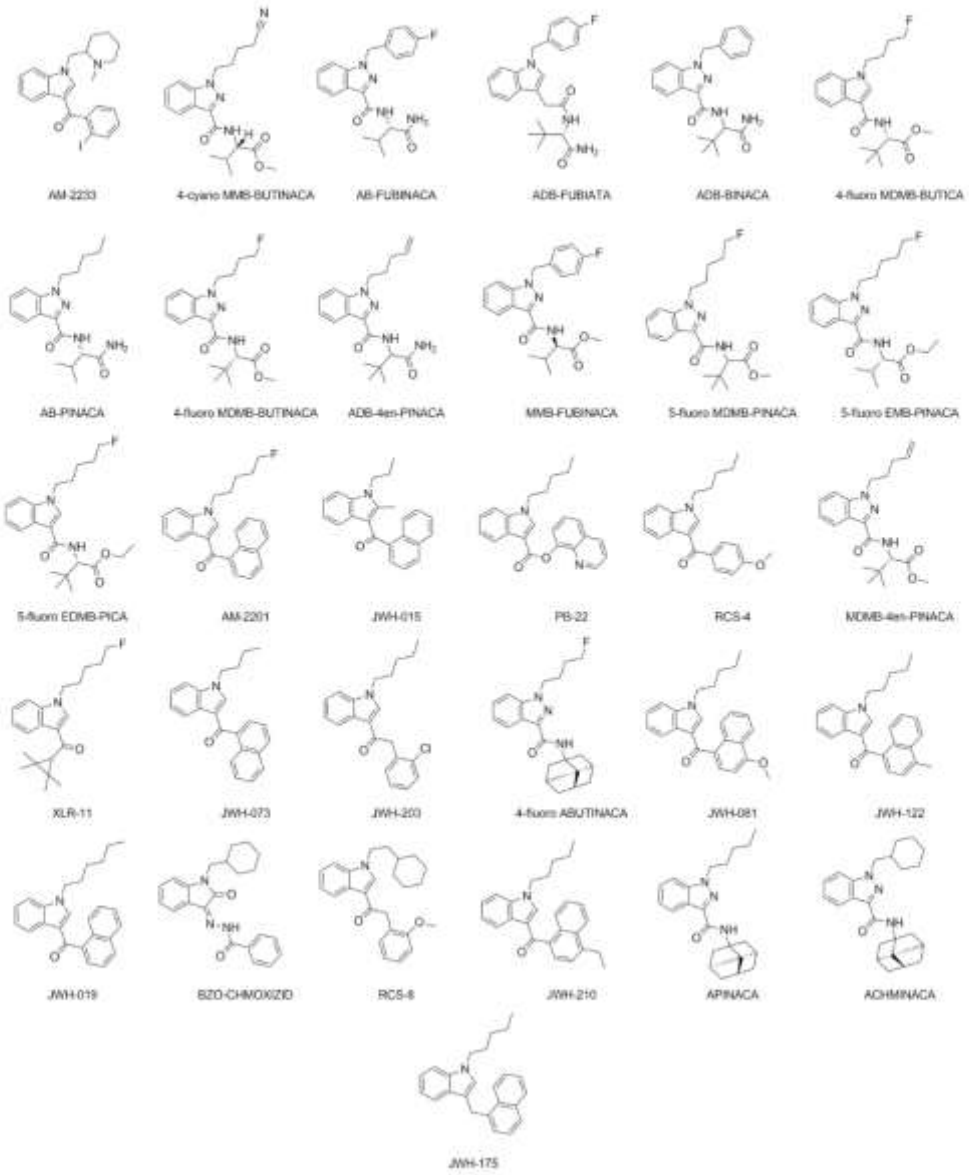

Fig. 1. Chemical structures of all synthetic cannabinoids covered by the method.

novel chemically diverse compounds flooding the illicit drug market, the development of new analytical techniques suited to analyze such substances is warranted.

The development of techniques aiming to analyze SCRA on biological specimens, such as blood [7,8], serum [9], plasma [10,11], urine [11–15], oral fluid [16–18], and hair [19,20], have been reported. To that end, sample preparation is mandatory for analyte extraction and preconcentration prior to analysis. In fact, this is the main challenge during the development of any analytical method addressed to drugs of abuse on biological samples, especially SCRA due to the low concentration that they are found associated with matrix complexity. Thus, solid-phase extraction [11,15] and liquid-liquid extraction (LLE) [9,17,18] have been the traditional sample preparation procedures for drug analysis in decades, which have also been applied for SCRA. However, these techniques have many disadvantages and the use of alternatives have been explored and encouraged [21]. For instance, methods using magnetic solid-phase extraction [10], micro-solid-phase extraction with molecularly imprinted polymers [14], dispersive liquid–liquid microextraction (DLLME) [7,16], or protein precipitation [8,12,13] to the analysis of SCRA on biological specimen have been reported. Nevertheless, although such alternatives are an improvement, they still have shortcomings, such as using hazardous organic solvents (chloroform, toluene, etc.), large sample volumes (> 500 µL), and requiring expensive reagents or devices. These practices are discouraged by the growing trend of finding more environmentally friendly alternatives during method development, such as it is postulated by Green Analytical Toxicology (GAT) [21].

The use of switchable hydrophilicity solvents (SHS) has been proposed in 2010 for the extraction of soybean oil [22]. These SHS are molecules with poor polarity but can become completely water-miscible by simply switching the pH of the system. This change in polarity occurs due to the protonation or deprotonation of specific functional groups in their chemical structures, e.g. amidines and amines. The switchable hydrophilicity solvent-based homogenous liquid–liquid microextraction (SHS-HLLME) makes use of that chemical feature to extract non-polar substances from aqueous samples, such as drugs of abuse and pharmaceuticals from biological specimens [23–27]. Briefly, the SHS is mixed with a concentrated acid solution so the excess of protons in the system protonates the amino group of the SHS making it positively charged, thus allowing polar interactions, such as hydrogen bonds. In this state, the SHS is hydrophilic and forms a miscible solution with aqueous samples, such as urine or plasma. Thereafter, to separate an organic layer and extract substances with low polarity, a base is then added in excess enough for the hydroxyl groups to neutralize the protons, increase the pH, and consequently deprotonate the SHS. In its neutral state, non-polar interactions are favored instead of hydrogen bonds, thus the SHS has a hydrophobic behavior and is immiscible in the aqueous sample, forming a two-layered system (Fig. 2). Although the SHS-HLLME technique is somewhat recent, few applications have been reported and it is already considered a promising green alternative for sample preparation [28]. Therefore, considering SHS-HLLME merits further studies, we applied this technique to the analysis of SCRA for the first time and describe in this article the full optimization and validation process covering 31 compounds.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical or HPLC grade. Methanol, acetonitrile, chloroform, toluene, hexane, ethyl acetate, dipropylamine (DPA), N,N-dimethylcyclohexylamine (DMCHA), sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), ammonium formate, and formic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using a Milli-Q system (Millipore, Billerica, Massachusetts).

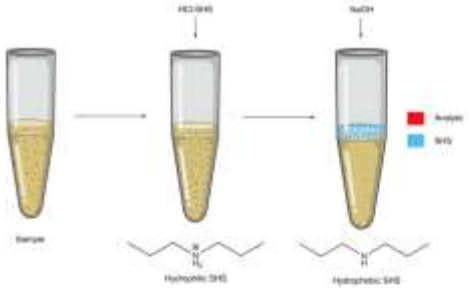

Fig. 2. Principle of SHS-HLLME. The SHS is mixed with a proton donor substance (HCl) and then added to an aqueous sample, e.g. human plasma. The protonation of the SHS increases its hydrophilicity, making it miscible with the plasma sample. Finally, addition of a base (NaOH) deprotonates the SHS making it once again hydrophobic and immiscible to the sample; as a consequence, it is possible to collect the organic layer from the vial. SHS-HCl: mixture of the switchable hydrophilicity solvent with HCl (i.e. protonated SHS).

All analytes and internal standards (IS) were acquired from Cerilliant Corporation (Round Rock, Texas, EUA). The stock solutions were available at 1.0 mg/mL, while for JWH-210-OH-*d*5 was at 100 µg/mL. For validation, the standard solutions were diluted and a pool containing all analytes at 1 µg/mL was prepared. Nine different concentrations, 0.6, 1.2, 1.5, 3, 4.8, 6, 30, 48, and 60 ng/mL, were then prepared from this pool and used to fortify blank plasma samples to obtain the calibration curve and quality controls (QC). All solutions were diluted in methanol and stored at − 20 °C.

2.2. Liquid chromatography-tandem mass spectrometry

A Waters ultra-performance liquid chromatography (UPLC) Acquity System equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm) was used for all analyses. This system was coupled to a Quattro Premier XE mass spectrometer (MS) (Waters Corporation, Milford, MA). Mobile phase A was ammonium formate (pH 3.1, 1 mM)-formic acid (999:1, v/v) and mobile phase B was methanol-formic acid (999:1, v/v). The method was kept at a constant flow rate of 0.3 mL/min with column oven temperature at 40 °C. The chromatographic gradient was as follows: initially, 10% of B for 0.5 min, ramp to 70% within 3.5 min, which was kept for 2.5 min; second ramp to 100% of B within 2 min, which was kept for another 2 min. Initial conditions were restored in 1 min and kept for 1.5 min to re-equilibrate the system. The method's run time was 13 min and 7.5 µL of the extracts were injected.

The multiple reaction monitoring (MRM) approach was used for all analytes with three transitions for each compound and the MS was operated in positive ionization mode. The electrospray capillary voltage was set to 2.5 kV, desolvation gas flow rate to 1100 L/h, cone gas flow rate to 200 L/h, desolvation temperature to 450 °C, and source temperature to 120 °C. The capillary voltage, collision energy, and *m/z* transitions used as quantifiers and qualifiers for each analyte, and retention times are displayed in Table S1.

2.3. Sample preparation

The extraction procedure consisted of two steps: i) protein precipitation and ii) SHS-HLLME technique.

2.3.1. Protein precipitation

Three hundred microliters of acetonitrile were added to a 300 µL aliquot of human plasma to obtain protein precipitation. In addition,

40 µL of the pool of analytes and 20 µL of the IS were added to each vial to obtain the intended concentrations. Then, vials were vortexed for 10 s, centrifuged (5 min at 6000 rpm), and the supernatant was transferred to a thin glass vial in which the acetonitrile was evaporated under a gentle N_2 stream at room temperature.

2.3.2. SHS-HLLME technique

Once the acetonitrile used for protein precipitation was evaporated, 500 µL of a DPA-HCl (6 M) (1:1, v/v) mixture were added to the samples and briefly vortexed until complete miscibility of the SHS (≈ 2 s). Next, 500 µL of NaOH (10 M) were added, vortex for 5 s, and 2 min later centrifuged for 5 min at 6000 rpm. Finally, the organic layer was transferred to autosampler vials, evaporated to dryness under N_2 stream at room temperature, and resuspended with 50 µl. of mobile phase A.

2.4. Method optimization

Two-way Analysis of Variance (ANOVA) was used for univariate analysis and a factorial screening 2^k followed by a central composite design (CCD) were used for multivariate statistical analyses. In both statistical approaches, significance was considered when $p < 0.05$, as for multivariate analyses, the adjusted coefficient of determination (r^2) obtained by ANOVA served to determine the percent of data variance explained by the model. All data were processed using GraphPad Prism® 8 and Statistica® 10 software.

2.5. Method validation

The method was validated for the quantitation of 31 SCRA in plasma samples according to the ANSI/ASB standard 036, 1st Edition 2019 guide for method validation in Forensic Toxicology, and supporting literature [29,30]. Therefore, linearity, limit of detection (LOD), limit of quantitation (LOQ), bias, precision, selectivity, carryover, matrix effect (ME), recovery (RE), and process efficiency (PE) were the parameters evaluated.

2.5.1. Linearity

The linear range encompasses the plasmatic concentrations in which SCRA are usually found [9,31]. To determine the linearity, five calibration curves each composed of six concentrations were freshly prepared and analyzed on the same day. Each concentration was prepared using fortified blank plasma samples and the linear regression was accepted when $r^2 \geq 0.99$. In addition, heteroscedasticity weighting was applied whenever necessary.

2.5.2. Limit of detection and limit of quantitation

To establish the LOD values, fortified blank samples in decreasing concentrations were experimentally tested. Plasma from three different donors was employed to ensure reproducibility. The LOD for each analyte was then determined as the concentration capable of providing a signal-to-noise ratio of, at least, 3:1 with variations in the retention times not exceeding ± 0.05 min. In addition, the ratio of daughter ions for each analyte should be consistent with higher concentrations.

The LOQ established for all analytes was the first concentration of the calibration curve. To establish this value as LOQ, three samples from different donors were analyzed on three different days with freshly prepared calibration curves. The identification and quantitation evaluated were: bias (± 20%), precision (≥ 80%), signal-to-noise ratio (≥ 3:1), and retention times (± 0.05 min).

2.5.3. Bias and precision

The evaluation of bias and precision was performed by preparing three QC levels using blank plasma samples. The specimens were fortified to achieve the intended concentrations and analyzed on five different days with a freshly prepared calibration curve for each run. Bias should not exceed ± 20% and precision should be ≥ 80%. Both within-run and between-run precisions were assessed and are expressed as coefficient of variation (CV).

2.5.4. Matrix effect, recovery, and process efficiency

The ME, RE, and PE were examined according to Matuszewski et al. [29]. To that end, the three QC were used in three different experiments. In set 1, neat standards prepared in mobile phase A were analyzed. In set 2, blank plasma samples were extracted and the analytes were added to the organic supernatant, evaporated under N_2 stream, and injected into the instrument. In set 3, blank plasma samples were fortified before the extraction procedure. For all sets, five replicates of each QC level were used, while each blank samples used for sets 2 and 3 were from different donors. The ME was calculated by dividing set 2 by set 1, RE by dividing set 3 by set 2, and PE by set 3 by set 1.

2.5.5. Selectivity

The selectivity of the method was evaluated with three different approaches. First, ten blank plasma samples from different subjects were extracted and analyzed by the method to assess the presence of common endogenous interferents. Next, neat standards of exogenous substances were prepared in mobile phase A at a 100 ng/mL concentration and injected into the instrument. The common drugs analyzed were amphetamine-type stimulants (amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, cathinone, methcathinone, methylone, dimethylone, methedrone, butylone, mephedrone, eutylone, 4-chloroethcathinone, pentedrone, dibutylone, N-ethylpentedrone, dipentylone, N-ethylpentylone, and N-ethylheptedrone), cannabinoids (THC, cannabidiol, and cannabinol), cocainics (cocaine, benzoylecgonine, cocaethylene, and anhydroecgonine methyl ester), opioids (morphine, 6-acetylmorphine, and codeine), antidepressants (paroxetine, fluoxetine, norfluoxetine, sertraline, amitriptyline, nortriptyline, imipramine, and desipramine), benzodiazepines (midazolam, nitrazepam, nordazepam, oxazepam, flunitrazepam, bromazepam, alprazolam, clonazepam, and diazepam), barbiturates (phenobarbital, secobarbital, and pentobarbital), and others (caffeine, nicotine, and cotinine). Finally, as JWH-210-OH-d5 was used as IS, the presence of non-labeled molecules (i.e. JWH-210-OH) as impurity or product of degradation was assessed. To that end, a blank plasma sample was fortified only with the labeled IS (JWH-210-OH-d5) and another only with the SCRA aimed by the method. Samples were then extracted and analyzed by the method. For the three interference studies described above, no interfering peaks should be visualized in the analyte's detection window.

2.5.6. Carryover

The carryover effect was investigated by analyzing three consecutive blank injections after a 10-fold higher concentration (100 ng/mL) was analyzed. There should not be any analyte signal in the blank injections.

3. Results and discussion

3.1. Preliminary experiments

The first experiment performed in this study sought to find a microextraction technique suited to analyze SCRA in plasma that would also fit GAT guidelines [21]. With that in mind, DLLME seemed a good candidate, for it is simple, fast, and efficient. Thus, a screening of common organic solvents used for cannabinoid extraction was performed and as expected, the mixture of chloroform-methanol (1:3, v/v) yielded the best recovery for most analytes (Fig. 3). In the same experiment, however, the SHS-HLLME was also included in the comparison. The technique was performed using two SHS with basic properties (DPA and DMCHA) and simultaneously compared with DLLME in terms of analyte recovery. The only DLLME mixture that provided better recoveries than that of chloroform-methanol was toluene-acetonitrile (1:3, v/v) for APINACA (24%) and ACHMINACA (49%). In contrast, when

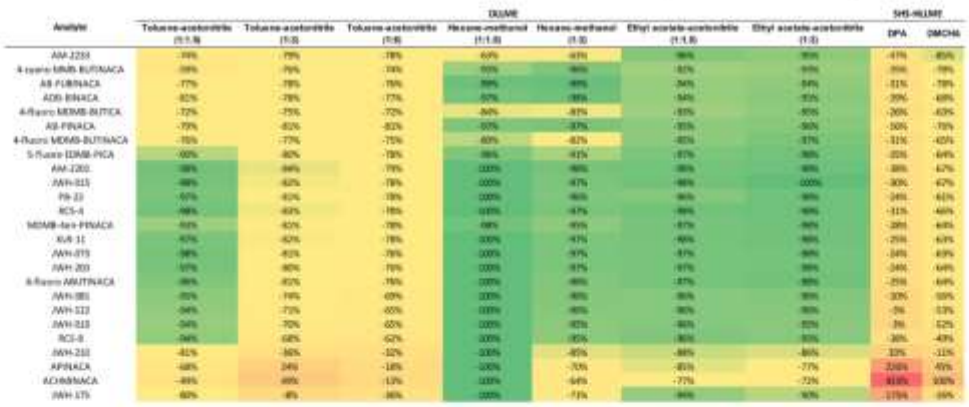

Fig. 3. Heat map showing the recoveries achieved with DLLME and SHS-HLLME. Each value represents the relative recovery of each mixture compared to chloroform-methanol (1:3, v/v) in DLLME. Negative values indicate lower recoveries, while positive values indicate higher recoveries than those of chloroform-methanol were achieved. SHS-HLLME for was performed with a SHS-HCl (6 M) (1:1, v/v) solution for both SHS. DPA: dipropylamine; DMCHA: N,N-dimethylcicloheylamine.

SHS-HLLME was employed with DPA, extraction of JWH-210, APINACA, ACHMINACA, and JWH-175 was 20%, 226%, 423%, and 175% better than DLLME, respectively. Better recoveries were also observed with DMCHA for APINACA (45%) and ACHMINACA (100%) (Fig. 3). This preliminary finding suggested that SHS-HLLME could be a good candidate for the aimed purpose. Interestingly, data published by Xu and colleagues agree with these findings as they compared DLLME with SHS-HLLME, and showed that both yielded similar analyte recovery [25]. However, although these miniaturized techniques are fast, cheap, and simple to perform, DLLME has more disadvantages, e.g. more steps are involved and higher volumes of more toxic solvents are commonly used. It is also worth pointing out that chloroform is highly hazardous and its use is strongly discouraged by eco-friendly guidelines [21]. With that in mind, we decided to investigate the use of SHS-HLLME as a sample preparation for analyzing SCRA in plasma samples.

A following experiment was carried out to confirm which SHS would work better for the target analytes. As shown in Fig. 4a, the extraction using DMCHA yielded statistically significant higher recoveries for 5-fluoro EDMB-PICA, PB-22, MDMB-4en-PINACA, APINACA, and ACHMINACA, while DPA was only better for 4-cyano MMB-BUTINACA. This could be explained by the higher hydrophobicity of DMCHA compared to DPA (LogP = 1.9 and 1.6, respectively), which represents it has a higher affinity for non-polar substances, such as the synthetic cannabinoids herein studied. However, except for the aforementioned SCRA, both SHS yielded similar recoveries for most of the aimed analytes ($p > 0.05$). Hence, DPA was chosen to favor analytes with lower intensities, such as 4-cyano MMB-BUTINACA (Fig. 4a). As a result, this experiment confirms that SHS-HLLME can efficiently extract the aimed analytes from plasma samples at low concentrations (1 ng/mL), thus the technique was further optimized to improve its efficiency.

3.2. SHS-HLLME optimization

For the optimization study, four conditions that could significantly impair analyte recovery were identified and screened in a full factorial screening 2^4. As the type of SHS was studied in previous experiments, it was not included in the present screening (Fig. 4a). Thus, the volumes of the DPA-HCl (6 M) (1:1, v/v) mixture and NaOH (10 M) (200–500 μL for both solutions), addition of NaCl (60 mg), and extraction time (0–5 min) were examined. The only statistically significant variables were the volumes of DPA-HCl and NaOH (Fig. 4b and c). Both these factors were then studied in a CCD to find the optimum volumes that would yield the best response, i.e. analyte recovery. For all SCRA analyzed, the most important factor was the volume of NaOH and optimum responses were achieved with 400–600 μL (Fig. 4d and e). In addition, for SCRA that the volume of DPA-HCl was also relevant, the highest analyte recovery was achieved with volumes ranging from 400 μ to 600 μL as well (Fig. 4d and e).

The addition of salts, such as NaCl, also known as salting-out effect, can improve the extraction of drugs from biological samples by reducing their solubility in the aqueous phase. However, previously published works have studied the salting-out effect in SHS-HLLME and showed no positive impact on analyte recovery [25–27,32]. This was also observed in the present work, as adding NaCl did not improve the extraction of SCRA from plasma samples (Fig. 4b and c). After NaOH is added to trigger SHS deprotonation, HCl is neutralized forming H_2O and NaCl. It is plausible to assume that the NaCl formed during this step is enough to produce the salting-out effect or that it indeed has no impact on SHS-HLLME. Another factor that was not relevant for SCRA recovery was the extraction time (Fig. 4b and c). After NaOH is added, different times have been considered for analyte extraction and phase separation (0–10 min) [23–25,27,33]. Based on such reports, we evaluated up to 5 min of extraction time but no statistical difference was observed in overall SCRA recovery (Fig. 4b and c). This could be explained by the infinitely large surface area between SHS and sample, making the transfer of analytes to the organic layer very fast. Altogether, both these parameters make the technique simpler and faster, as no addition of salt is required and analyte extraction happens quickly.

In contrast to the previous parameters, the volumes of DPA-HCl and NaOH solutions were statistically significant for the technique, of which higher volumes improved analyte recovery (Fig. 4d and e). As more volumes of DPA-HCl and NaOH were added to the samples, more organic supernatant was formed and capable of being recovered. That could explain the higher analyte recovery achieved when a combination of 400–600 μL of both DPA-HCl and NaOH was employed. On the other hand, high volumes of DPA-HCl (> 300 μL) combined with low volumes of NaOH (< 300 μL) do not suffice for triggering the pH switch and SHS deprotonation, therefore was incompatible with the technique. Thus, 500 μL of each solution was chosen. It is worth mentioning that such volume is not an inconvenience as it corresponds to only 250 μL of DPA

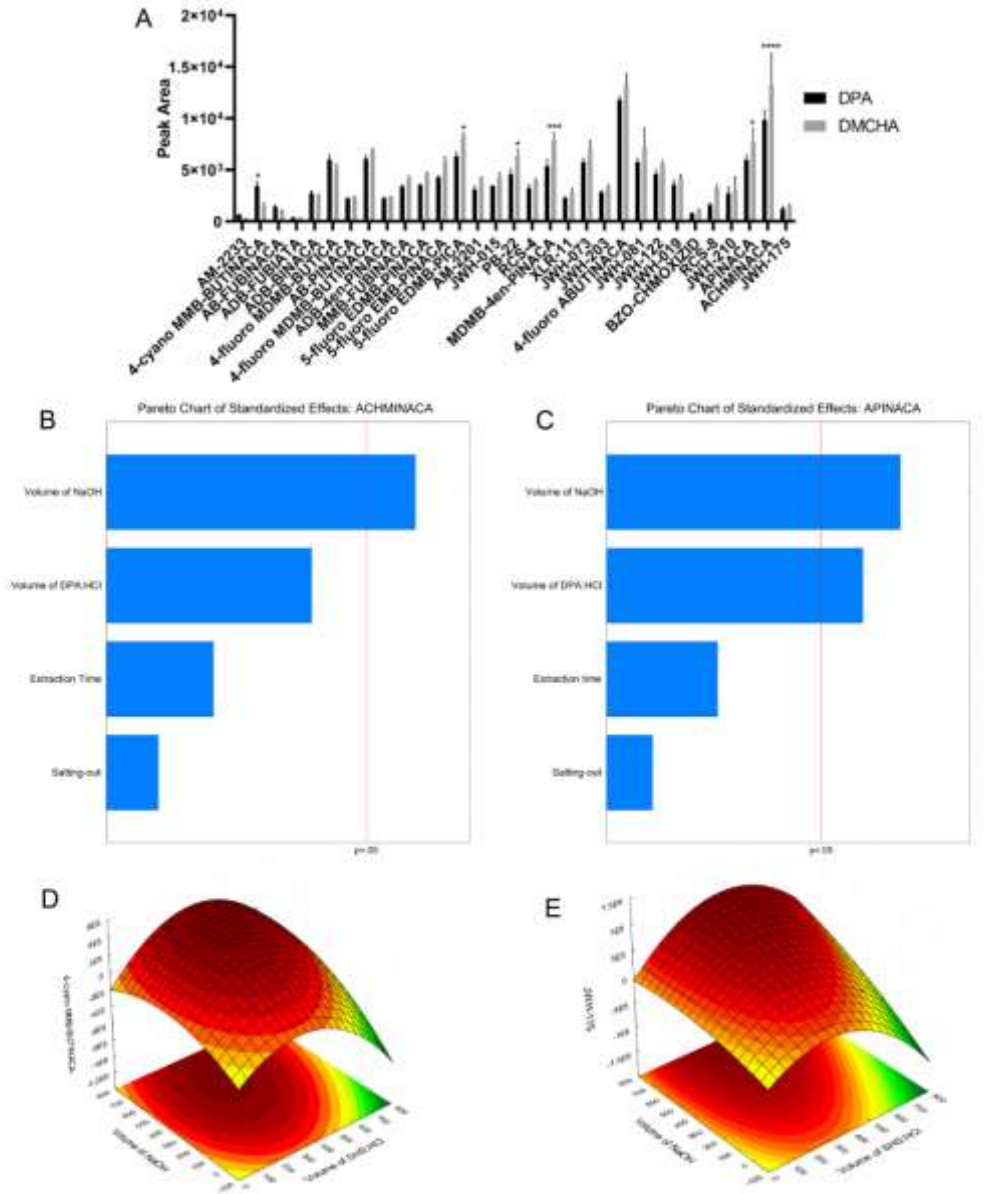

Fig. 4. Optimization of the SHS-HLLME. Comparison between analyte recoveries yielded by two different SHS (A). Pareto charts showing the significant variables among those screened in the Factorial Screening (B and C). Surface response graphs of the significant variables that were identified in the previous experiment, volumes of DPA-HCl and NaOH, indicating the optimum conditions for SCRA extraction (D and E). Two-way ANOVA followed by post-hoc Tukey Test was used for graph A (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.001$), while bars crossing the red line indicate $p < 0.05$ in graphs B and C. SHS: switchable hydrophilicity solvent; DPA: dipropylamine; DMCHA: dimethylhexylamine.

Table 1
Validation results for all analytes covered by the proposed SHS-HLLME.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)	QC (ng/mL)	Bias (%)	Imprecision (%)	
						Within-run	Between-run
AM-2233	0.08	0.1	0.1–10	0.2	2.4	13.0	14.7
				0.8	3.6	8.6	11.8
				8.0	3.9	9.4	10.5
4-cyano MMB-BUTINACA	0.03	0.1	0.1–10	0.2	4.1	14.2	18.8
				0.8	1.6	12.9	17.4
				8.0	3.4	7.2	10.8
AB-FUBINACA	0.03	0.1	0.1–10	0.2	2.1	5.1	9.9
				0.8	2.7	5.9	7.0
				8.0	4.5	10.6	11.5
ADB-FUBIATA	0.08	0.1	0.1–10	0.2	0.8	8.6	11.4
				0.8	2.3	5.2	7.3
				8.0	4.4	9.4	9.8
ADB-BINACA	0.03	0.1	0.1–10	0.2	1.4	9.3	11.4
				0.8	2.1	3.2	5.8
				8.0	3.9	5.7	7.3
4-fluoro MDMB-BUTICA	0.03	0.1	0.1–10	0.2	4.3	10.7	11.2
				0.8	6.7	10.5	11.6
				8.0	4.2	8.2	9.8
AB-PINACA	0.05	0.1	0.1–10	0.2	7.7	11.9	14.9
				0.8	5.6	10.2	11.1
				8.0	5.6	6.8	7.8
4-fluoro MDMB-BUTINACA	0.03	0.1	0.1–10	0.2	4.0	8.4	10.0
				0.8	5.0	8.6	9.2
				8.0	4.5	4.6	6.5
ADB-4en-PINACA	0.03	0.1	0.1–10	0.2	-0.6	2.4	5.9
				0.8	9.1	8.9	9.7
				8.0	6.5	9.5	10.2
MMB-FUBINACA	0.05	0.1	0.1–10	0.2	-2.7	10.7	12.0
				0.8	3.4	7.1	8.0
				8.0	1.0	2.6	5.7
5-fluoro MDMB-PINACA	0.05	0.1	0.1–10	0.2	6.5	6.0	7.0
				0.8	3.4	3.0	5.6
				8.0	1.5	4.3	5.3
5-fluoro EMB-PINACA	0.03	0.1	0.1–10	0.2	-1.1	8.9	11.5
				0.8	2.7	2.5	4.7
				8.0	1.7	3.9	5.5
5-fluoro EDMB-PICA	0.03	0.1	0.1–10	0.2	6.6	8.0	8.6
				0.8	3.9	6.2	7.1
				8.0	3.3	6.7	7.4
AM-2201	0.03	0.1	0.1–10	0.2	1.4	4.9	7.2
				0.8	1.9	6.8	7.7
				8.0	3.4	4.1	5.1
JWH-015	0.01	0.1	0.1–10	0.2	3.5	6.5	13.4
				0.8	4.6	9.8	10.9
				8.0	5.3	13.5	14.5
PB-22	0.01	0.1	0.1–10	0.2	5.3	4.0	8.6
				0.8	6.4	9.3	11.0
				8.0	1.5	9.5	10.8
RCS-4	0.01	0.1	0.1–10	0.2	0.7	5.4	7.6
				0.8	5.3	4.1	5.5
				8.0	0.8	12.3	12.6
MDMB-4en-PINACA	0.01	0.1	0.1–10	0.2	2.2	6.0	8.3
				0.8	3.5	8.2	10.3
				8.0	1.3	7.7	9.2
XLR-11	0.03	0.1	0.1–10	0.2	7.0	9.8	12.0
				0.8	5.7	11.7	13.2
				8.0	4.0	4.8	7.7
JWH-073	0.01	0.1	0.1–10	0.2	7.7	12.1	14.3
				0.8	1.0	9.0	12.3
				8.0	1.3	6.5	9.5
JWH-203	0.03	0.1	0.1–10	0.2	-0.7	11.2	13.3
				0.8	1.0	7.9	11.3
				8.0	-0.6	9.2	11.4
4-fluoro ABUTINACA	0.01	0.1	0.1–10	0.2	-0.7	8.7	9.4
				0.8	4.3	10.2	11.9
				8.0	-1.4	8.9	12.1
JWH-081	0.03	0.1	0.1–10	0.2	5.0	14.8	17.4
				0.8	6.4	10.9	12.4
				8.0	8.9	14.3	15.3
JWH-122	0.01	0.1	0.1–10	0.2	8.7	12.5	13.7
				0.8	4.6	5.5	8.5
				8.0	11.8	9.2	10.9

(continued on next page)

Table 1 (continued)

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)	QC (ng/mL)	Bias (%)	Imprecision (%)	
						Within-run	Between-run
JWH-019	0.01	0.1	0.1–10	0.2	3.4	7.5	10.1
				0.8	4.7	10.4	12.0
				8.0	-0.5	9.5	12.6
BZO-CHMOXZID	0.03	0.1	0.1–10	0.2	-0.6	13.0	14.4
				0.8	4.5	2.7	3.7
				8.0	4.5	7.2	9.1
RCS-8	0.08	0.1	0.1–10	0.2	2.1	13.5	14.4
				0.8	1.3	11.4	14.1
				8.0	4.8	5.8	7.8
JWH-210	0.05	0.1	0.1–10	0.2	10.6	11.9	13.1
				0.8	3.2	5.9	7.0
				8.0	4.0	3.7	5.6
APINACA	0.08	0.1	0.1–10	0.2	12.4	7.8	10.9
				0.8	4.3	2.5	3.5
				8.0	5.0	4.3	6.0
ACHMINACA	0.08	0.1	0.1–10	0.2	6.3	9.9	12.1
				0.8	-1.3	7.8	10.1
				8.0	3.9	6.7	9.7
JWH-175	0.08	0.1	0.1–10	0.2	7.0	11.2	14.1
				0.8	7.7	8.1	10.5
				8.0	5.7	6.1	11.0

LOD: limit of detection; LOQ: limit of quantitation; QC: quality controls.

that is considerably less hazardous than other solvents commonly used in microextraction techniques, e.g. chloroform and toluene [28]. In addition, the concentrated acid and base solutions neutralize each other producing H_2O and NaCl, also contributing to the greenness of the technique.

3.3. Method validation

Linearity, LOD, and LOQ were established according to relevant plasmatic concentrations of SCRA [9,31]. Therefore, the linear range and LOQ were defined as 0.1–10 ng/mL and 0.1 ng/mL, respectively (Table 1). In addition, a statistical analysis showed a lack of homogeneity in the calibration model, thus the 1/x weighting was applied for all analytes providing $r^2 \geq 0.99$. As for LOD, the lowest concentration was aimed, thus the values achieved varied among analytes (0.01–0.08 ng/mL) (Table 1). Based on available literature, these values should suffice for detecting SCRA in plasma when not possible to quantitate [9,31].

Table 2
ME, RE, and PE values for the synthetic cannabinoids at each QC level. ME: matrix effect; RE: recovery; PE: process efficiency.

Analyte	ME (%)			RE (%)			PE (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
AM-2233	43.9	31.2	36.2	46.4	47.7	46.9	66.7	62.5	63.9
4-cyano MMB-BUTINACA	-6.2	-12.5	-3.8	42.9	40.8	41.3	40.2	35.7	39.8
AB-FUBINACA	23.9	12.9	13.2	43.3	43.9	41.3	53.6	49.6	46.7
ADB-FUBIATA	143.3	142.1	153.7	49.7	47.2	39.3	120.9	114.3	99.7
ADB-BINACA	54.8	43.9	49.1	46.0	44.6	39.3	71.2	64.2	58.6
4-fluoro MDMB-BUTICA	54.3	47.9	53.4	45.3	47.1	41.7	69.9	69.6	63.9
AB-PINACA	61.6	63.6	61.8	44.2	41.5	40.9	71.4	67.9	66.2
4-fluoro MDMB-BUTINACA	77.1	67.5	62.3	42.3	41.9	41.4	75.0	70.1	67.3
ADB-4en-PINACA	62.5	52.5	54.9	43.6	45.8	42.5	70.8	69.9	65.8
MMB-FUBINACA	29.5	27.0	24.4	40.1	38.0	36.3	51.9	48.2	45.2
5-fluoro MDMB-PINACA	6.0	0.4	-2.1	44.4	40.7	40.1	47.1	40.9	39.3
5-fluoro EMB-PINACA	141.3	129.4	126.5	41.3	42.4	40.5	99.7	97.2	91.8
5-fluoro EDMB-PICA	84.0	78.1	72.4	47.3	43.7	42.0	87.0	77.8	72.3
AM-2201	59.1	51.3	46.0	47.6	44.9	41.2	75.6	67.9	60.2
JWH-015	85.7	73.5	66.1	43.9	40.9	40.1	81.4	70.9	66.7
PB-22	71.2	68.9	68.5	46.2	41.7	41.4	79.0	70.4	61.5
RCS-4	17.5	9.1	0.5	51.0	44.0	43.8	59.9	48.0	44.0
MDMB-4en-PINACA	14.5	12.7	0.9	50.5	41.0	42.6	57.8	46.2	43.0
XLR-11	11.1	4.6	4.9	45.3	43.6	41.0	50.4	45.6	43.0
JWH-073	109.9	101.5	107.3	44.4	40.5	40.6	93.3	81.7	84.2
JWH-203	52.8	57.6	56.2	44.0	43.2	41.4	67.3	68.0	64.6
4-fluoro ABUTINACA	60.9	62.2	58.1	51.0	45.8	41.6	82.0	74.2	65.7
JWH-081	90.6	75.8	68.6	43.7	40.9	39.3	83.3	87.3	66.3
JWH-122	65.6	55.8	49.4	43.9	41.8	40.9	72.6	65.1	61.1
JWH-019	72.6	68.7	64.0	46.8	43.1	42.1	80.8	72.8	60.7
BZO-CHMOXZID	47.0	66.2	61.7	56.7	43.1	36.5	83.4	71.6	59.0
RCS-8	-5.4	-16.8	-14.1	51.2	39.7	40.7	45.9	33.1	35.0
JWH-210	-45.3	-55.6	-51.9	47.9	41.5	39.6	26.1	18.5	19.1
APINACA	-46.2	-50.5	-49.3	38.9	40.9	40.9	20.9	20.3	20.8
ACHMINACA	185.9	121.0	111.0	51.9	42.6	37.5	148.4	94.2	79.2
JWH-175	72.3	51.0	52.3	36.0	38.6	37.9	62.0	58.3	57.6

The concentrations for the low, medium, and high QC levels are: 0.2, 0.8, and 8 ng/mL, respectively. ME: matrix effect; RE: recovery; PE: process efficiency.

The accuracy and imprecision for all SCRA were assessed on five different days by analyzing the three QC levels. The within-run precision was higher than 85% for all analytes, which is represented by CV < 15% (Table 1). As for between-run precision, there were only 3 analytes with CV values > 15%, 4-cyano MMB-BUTINACA, JWH-081, and JWH-175 (18.8%, 17.4%, and 11%, respectively). In addition, the accuracy of the method was also high for all analytes (> 90%), with the exceptions being JWH-122, JWH-210, and APINACA with biases of 11.8%, 10.6%, and 12.4%, respectively (Table 1). Thus, even though the between-run imprecision and bias were > 15% and ± 10%, respectively, for three of the analytes covered by the method, these are still within acceptable limits, indicating the technique has satisfactory accuracy and precision for the intended purpose.

The ME study showed that most SCRA were affected by intense ion enhancement (> 50%), especially ADB-FUBIATA, 5-fluoro EMB-PINACA, JWH-073, and ACHMINACA with values > 100% (Table 2). Curiously, analytes with high chemical similarity and eluting very closely were affected with considerably different ME, such as 5-fluoro MDMB-PINACA (≈ 0.4%), 5-fluoro EMB-PINACA (≈ 129.4%), and 5-fluoro EDMB-PICA (≈ 78.1%) or also APINACA (≈ −50.5%) and ACHMINACA (≈ 121.0) (Tables S1 and 2). On the other hand, RE was between 36% and 56.7%, and PE ranged from 18.5–148.4% (Table 2). Although the ME observed in the present study was intense for some of the aimed SCRA, it did not impact bias and precision, hence is in agreement with validation guidelines [30]. In addition, intense ME for SCRA has been reported elsewhere with other techniques [9]. In the last step of interferents assessment, no relevant signal in the analytes' detection windows and carryover effect was observed. Thus, the method proved to be selective for the aimed SCRA suffering no relevant interference from common endogenous and exogenous substances.

As proof of applicability, the method was employed to analyze one authentic sample from a known user of marijuana. Analysis showed the presence of MDMB-4en-PINACA at 0.81 ng/mL, although the subject reported having no intention of using synthetic cannabinoids. A representative chromatogram from the authentic sample is presented in Fig. S1 and this confirms that the method can be used for real sample analysis.

We presented a full optimization followed by validation of the proposed SHS-HLLME resulting in a technique suited to efficiently detect and quantitate 31 synthetic cannabinoids at low concentrations. The present method requires only 300 µL of plasma samples, which can be considered a low volume if compare with other works. For example, Hwang and co-workers described a micro-solid-phase extraction to analyze SCRA using only 180 µL of plasma [10]. However, the method can be complex and time-consuming for it has more steps than the SHS-HLLME reported herein and requires over 7 min of only vortexing and sonication. In addition, 300 µL of toluene were used to extract each sample and only LODs above 2 ng/mL were achieved. Although the work focuses on 13 SCRA and other drugs of abuse, such drawbacks as using highly toxic extraction solvents, expensive devices, and having a complex analytical pipeline are not in agreement with GAT guidelines [10]. In contrast, Kneisel and Auwärter proposed a highly sensitive analytical method in which an LLE was developed and validated to analyze 27 SCRA on serum samples [9]. Briefly, 1 mL of samples were extracted with 1.5 mL of a hexane-ethyl acetate (99:1, v/v) mixture, mixed for 5 min, and centrifuged for another 20 min. The reported LODs ranged from 0.01 to 2 ng/mL and were similar to the values achieved with the SHS-HLLME reported in the present work, especially for the SCRA included in both techniques: JWH-210 (0.07 ng/mL); JWH-203 (0.05 ng/mL); JWH-019 and RCS-8 (0.03 ng/mL); JWH-073, JWH-081, JWH-122, RCS-4, AM-2201, and AM-2233 (0.02 ng/mL); and JWH-015 (0.01 ng/mL) (Table 1) [9]. Adamowicz and Tokarczyk have published a method for the screening of 143 drugs of abuse, which included 4 SCRA. The sample preparation consisted of precipitation of 200 µL of blood with 600 µL of acetonitrile added 50 µL at a time with a 10 s-vortexing after each addition. Although the approach of using only protein precipitation is simple, the procedure described could be time-consuming when analyzing multiple samples simultaneously. In comparison, the SHS-HLLME proposed herein takes similar time and involves fewer pipetting steps. In addition, the LODs reported for the SCRA in their work ranged from 0.07 to 0.46 ng/mL, of which AM-2201 was the only analyte included in both works (0.09 ng/mL against 0.03 ng/mL achieved by the SHS-HLLME) (Table 1) [8].

As for DLLME in SCRA analysis, we have found only two works. In the first one, Odoardi et al. used 500 µL of whole blood and 350 µL of chloroform-methanol (1:2.5, v/v) as extraction and dispersive solvents, respectively [7]. In this screening method, 70 NPS from different groups were included, but the LODs achieved for synthetic cannabinoids were inferior (≥ 0.2 ng/mL) to those obtained by the SHS-HLLME herein described (Table 1). On the other hand, Tomai et al. reported a DLLME for the analysis of 6 SCRA in oral fluid using 500 µL of sample followed by protein precipitation with 1 mL of acetonitrile [16]. Although the technique was highly sensitive yielding LODs of 0.002–0.021 ng/mL, chloroform was used as extraction solvent. In addition, when taking the analytes included in both methods into comparison, the LODs achieved in the present work were over 2-fold higher than the DLLME reported by Tomai et al., JWH-073 (0.021 ng/mL) and JWH-019 (0.018 ng/mL) (Table 1), indicating that the SHS-HLLME could also be a valuable technique to analyze oral fluid [16]. In terms of simplicity of handling, DLLME and SHS-HLLME can be somewhat equivalent. For example, previously published articles show the use of dry ice in SHS-HLLME, which can be inconvenient and prone to analytical variability [27,32,33]. This can be easily overcome by using basic solutions, such as NaOH, to change the pH of the system, thus improving reproducibility [23–26,34]. In contrast, DLLME by default requires more steps, e.g. the addition of the extraction and dispersive solvents, buffer solutions, NaCl, and even water and protein precipitation in some cases [7,16]. Hence, these techniques could be used as alternatives to each other allowing the simplest and most effective to be chosen.

To our knowledge, SHS-HLLME was mostly used in the analysis of food and urine, while only one other work has applied it to plasma samples [23]. As a result, the use of a protein precipitation step was necessary and this was achieved by the addition of an organic solvent. Although efficient, acetonitrile needed to be removed through evaporation prior to the extraction itself (Section 2.3) and this additional step is laborious and time-consuming. In that regard, Scheid et al. have reported an interesting alternative by using zinc sulfate for protein precipitation instead of organic solvents [23]. This approach dismisses this evaporation step and the zinc sulfate is not incompatible with the extraction as organic solvents are. This should be taken into consideration for future applications of SHS-HLLME, especially for samples that require protein precipitation.

The number of published methods for SCRA analysis in blood-related matrices are scarce. These specimens, such as plasma, are particularly relevant in cases of acute toxicity or pharmacokinetic studies, for example. In addition, synthetic cannabinoids have been shown to be extensively metabolized producing a great number of metabolites that have to be included in urine screenings [12]. However, new substances are constantly introduced into the drug market and the process to identify such biotransformation products is complex and time-consuming. As a result, many SCRA remain undetected until comprehensively studied, hence analyzing the parent compounds in blood or plasma can circumvent that limitation until more information on metabolites is available. Overall, the SHS-HLLME described in the present work can be a valuable tool in that regard, especially if considered that a compound belonging to the new emerging SCRA group called OXIZIDs was included in the method [5,35] (Tables S1, 1, and 2). This shows that the technique reported herein can be used for synthetic cannabinoids in general and has a potential application for those that appear in the illicit drug market in the future.

4. Conclusions

The SHS-HLLME technique is somewhat recent and only few applications have been reported. This work described the first application of this SHS-based extraction for the analysis of synthetic cannabinoids on plasma samples. Method optimization showed that only 250 μL of DPA were required for 300 μL of plasma to achieve satisfactory sensitivity. Moreover, the salting-out effect is dismissed and analyte extraction is achieved in extremely short times, making SHS-HLLME simpler and faster than DLLME. Although some limitations still need to be addressed, such as solvent evaporation, this SHS-based technique is a promising eco-friendly alternative for drug analysis, including the major NPS group of synthetic cannabinoids.

CRediT authorship contribution statement

André Luis Fabris: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. Aline Franco Martins: Writing – review. Jose Luiz Costa: Resources, Authentic samples, Writing – review, Funding acquisition. Maurício Yonamine: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115588.

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7. ARTIGO IV

Solvent-free parallel artificial liquid membrane extraction of drugs of abuse

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Solvent-free parallel artificial liquid membrane extraction for drugs of abuse in plasma samples using LC-MS/MS

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HIGHLIGHTS

- 96-well liquid-phase microextraction of drugs of abuse from plasma.
- Synthetic organic solvents replaced by microliter volumes of essential oil.
- Simple workflow, aqueous extracts injected directly in LC-MS.
- Performance data in compliance with forensic toxicology guideline requirements.
- A simple, fast, and efficient eco-friendly technique for routine analysis.

ARTICLE INFO

Keywords:
Parallel artificial liquid membrane extraction
PALME
New psychoactive substances
Drugs of abuse
Essential oil
LC-MS/MS

ABSTRACT

Background: Parallel artificial liquid membrane extraction (PALME) is a 96-well plate setup variant of liquid-phase microextraction. Basic or acidic analytes are extracted in neutral form from the sample, through a supported liquid membrane (SLM), and into aqueous acceptor. PALME is already considered a green extraction technique, but in the current conceptual work, we sought to make it even greener by replacing the use of organic solvents with essential oils (EO). PALME was combined with LC-MS/MS for analysis of plasma samples and multiple drugs of abuse with toxicological relevance (amphetamines, phenethylamines, synthetic cathinones, designer benzodiazepines, ayahuasca alkaloids, lysergic acid diethylamide, and ketamine).

Results: Fourteen EO were compared to organic solvents frequently used in PALME. The EO termed smart & sassy yielded the best analyte recovery for all drugs studied and was thus selected as SLM. Then, factorial screening and Box-Behnken were employed to optimize the technique. The extraction time, concentration of base, sample volume, and percentage of trioctylamine significantly impacted analyte recovery. The optimum values were defined as 120 min, 10 mmol/L of NaOH, 150 μL, and 0%, respectively. Once optimized, validation parameters were 1–100 ng mL-1 as linear range, accuracy ±16.4%, precision >83%, 1 ng mL-1 as limit of quantitation, 0.1–0.75 ng mL-1 as limit of detection, matrix effect <20%, and recovery 20–106%. Additionally, EO purchased from different production batches were tested and achieved acceptable reproducibility. Data were in compliance with requirements set by internationally accepted validation guidelines and the applicability of the technique was proven using authentic samples.

Significance: In this study, the use of an EO provided a solvent-free sample preparation technique suited to extract different classes of drugs of abuse from plasma samples, dismissing the use of hazardous organic solvents. The

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method also provided excellent sample clean-up, thus being a simple and efficient tool for toxicological applications that is in agreement with the principles of sustainable chemistry.

1. Introduction

In the early 2000s, the New Psychoactive Substances (NPS) began to be commercialized as legal alternatives to conventional drugs of abuse and thus drastically changed the landscape of the illicit drug market. These novel chemicals are designed to mimic the effect of classic psychoactive substances, such as amphetamines and lysergic acid diethylamide (LSD). However, as a result of chemical modifications, the potency of these new compounds might be considerably higher [1–3]. In addition to that, NPS are commonly sold mixed with other drugs and might be inadvertently consumed by drug users. Unwittingly taking substances of unknown potency can result in severe intoxication or even death [3–5]. Over 15 years after the beginning of this phenomenon, law enforcement, toxicologists, and other professionals are still being challenged with the ever-growing number of heterogeneous chemically diverse molecules that are introduced into the illicit drug market. For example, by November 2023, the total number of NPS repoted to The United Nations Office on Drugs and Crime worldwide accounted for 1230 [1,2].

To face the NPS threat, the analysis of biological specimens is crucial in clinical and forensic toxicology. However, some NPS are found at low concentrations in biological samples due to their high potency and thus require the use of efficient extraction techniques combined with highly sensitive analytical instruments [3,5,6]. In fact, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been one of the instruments of choice for that purpose. In addition, although several extraction techniques have been proposed to analyze NPS in blood, urine, and other biological specimens, as new compounds are constantly appearing in the drug market, adapting these existing methodologies or developing new ones is often required [7–10]. These techniques must be also compatible with conventional drugs of abuse, which are still relevant [1].

Among the large number of works proposing approaches to analyze NPS and classic drugs of abuse in biological samples, some also attempt to reduce the environmental impact of such practices [10–13]. This can be accomplished, for instance, by choosing less hazardous organic solvents, reducing sample volume, processing multiple samples simultaneously, and improving overall cost-effectiveness. This goal of developing more eco-friendly methodologies in analytical chemistry and toxicology has been a trend in recent years that can rely on guidelines established specifically to that end [14,15]. In that regard, liquid-phase microextraction (LPME) is a fine example of a green alternative approach. Proposed by Pedersen-Bjergaard and Rasmussen, in 1999, LPME is a miniaturized version of classic liquid-liquid extraction that is able to avert some of its limitations, such as the high volumes of sample and organic solvents that are frequently employed [13,16,17].

In 2013, an adaptation of LPME to a 96-well plate setup was proposed by Gjelstad et al. named parallel artificial liquid membrane extraction (PALME) [18]. In this format, two 96-well plates are placed in parallel but the principle of LPME remains (Fig. 1). Basic or acidic analytes are extracted from aqueous sample, through a thin liquid membrane of a few microliters of organic solvent, and into aqueous acceptor. Essentially, PALME allows the simultaneous extraction of almost a hundred samples, making the technique simpler, more efficient, and of high-throughput. In addition, this setup is amenable to automation, and both sample and organic solvent volumes are substantially reduced, making PALME a remarkable improvement to conventional hollow-fibre LPME, which has been applied in different contexts as its predecessor [19–27].

PALME is already considered a green sample preparation approach

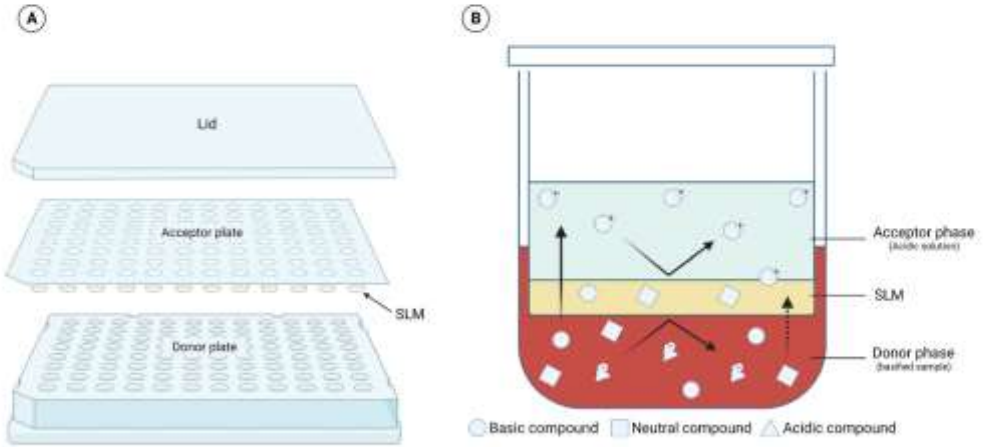

Fig. 1. Illustration of PALME setup and the principle of extracting basic analytes. First, the donor solution is added to the wells of the donor plate and the SLM is pipetted onto the membrane in the acceptor plate. Then, the two individual plates are sandwiched together and the acceptor solution is added to the wells of the acceptor plate. Finally, a lid is placed to prevent evaporation of the SLM (A). Once this setup is assembled, compounds are transferred from the donor to the acceptor phase under agitation, i.e. analyte extraction. For the extraction of basic substances, the donor phase is comprised of a base added to an aqueous sample, such as blood or plasma, to keep basic analytes in their neutral state. A hydrophobic organic solvent is used as SLM to serve as a barrier to charged compounds. The acidic solution protonates basic compounds once they reach this compartment, thus avoiding their back-extraction to the SLM or donor phase (B). SLM: supported liquid membrane.

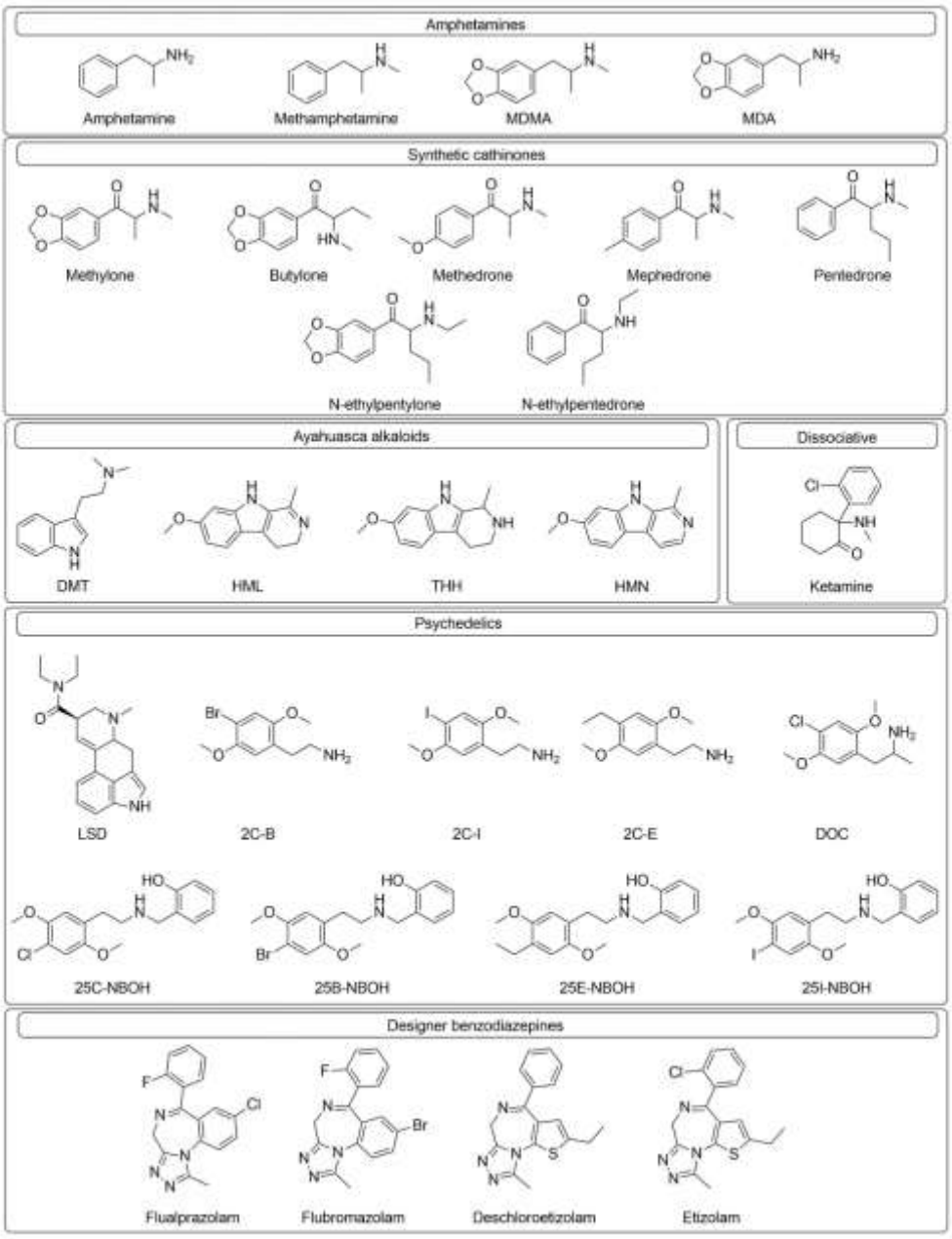

Fig. 2. Chemical structures of the analytes included in the present work.

but we sought to make it even greener by replacing the organic solvents used as supported liquid membrane (SLM) with more eco-friendly alternatives without sacrificing overall efficiency. Essential oils (EO) are hydrophobic concentrated plant extracts that have been previously used in extraction techniques, including LPME [11,13]. The main advantage of using EO is the reduced hazard for both the operator and the environment. With that in mind, we explored the efficiency of using these natural extracts as SLM in PALME in a method aimed at different classes of drugs of abuse, including NPS (Fig. 2). This conceptual article describes the development of this idea.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, formic acid, and ammonium formate of HPLC grade were purchased from Merck (Darmstadt, Germany). Citric acid, sodium hydroxide, sodium bicarbonate, 2-nitrophenyl octyl ether (NPOE), dihexyl ether, dodecyl acetate, and trioctylamine (TOA) were purchased from Merck (Darmstadt, Germany). The EO were purchased from different brands: cedar wood, clove, *Eucalyptus radiata*, lavender, lemon, lemon-grass, lime, peppermint, rosemary, and smart & sassy were purchased from dōTERRA International, LLC (Pleasant Grove, UT, USA); clove, *Eucalyptus globulus*, and peppermint were purchased from Bio-Essência® (São Paulo, Brazil); and a second brand of *Eucalyptus globulus* was purchased from LASZLO© (Minas Gerais, Brazil). Ultrapure water (resistivity 18.2 MΩ cm, total organic carbon (TOC) ≤ 5 ppb) was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

All analytes and internal standards were acquired from Cerilliant Corporation (Round Rock, TX, USA) and were available at 1.0 mg mL^{-1} and 100 μg mL^{-1}, respectively. Analytes were grouped accordingly to psychoactive effect, i.e. classic stimulants: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxyamphetamine (MDA); synthetic cathinones: methylone, methedrone, butylone, mephedrone, pentedrone, *N*-ethylpentedrone, and *N*-ethylpentylone; dissociative: ketamine; psychedelics: LSD, 2,5-dimethoxy-4-chloroamphetamine (DOC), 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-Dimethoxy-4-ethylphenethylamine (2C-E), 2-(((2-(4-chloco-2,5-dimethoxyphenyl)ethyl)amino)methyl)phenol (25C-NBOH), 2-(((4-Bromo-2,5-dimethoxyphenethyl)amino)methyl) phenol (25B-NBOH), 2-((2-(4-Iodo-2,5-dimethoxyphenyl)ethylamino) methyl)phenol (25I-NBOH), and 2-(((4-Ethyl-2,5-dimethoxyphenethyl) amino)methyl)phenol (25E-NBOH); ayahuasca alkaloids: N, N-dimethyltryptamine (DMT), harmaline (HML), harmine (HMN), and tetrahydroharmine (THH); and designer benzodiazepines: deschloroetizolam, flubromazolam, flualprazolam, and etizolam. Dilutions of these stock solutions were prepared for optimization and validation studies. All solutions were in methanol or acetonitrile and stored at −20 °C.

2.2. Samples

Both blank and authentic human plasma samples used in this work were provided by the Laboratory of Analytical Toxicology from the Toxicological Assistance and Information Centre located at the University of Campinas. Samples were collected in blood collection tubes containing EDTA and subsequently centrifuged (5 min at 3500×*g*) to allow plasma separation. Plasma samples were then stored at −20 °C before analysis. The use of human specimens in the present study was approved by the Ethics Committees of both the University of São Paulo and the University of Campinas (CAAE: 46404121.8.3001.0067).

2.3. Sample preparation procedure

A 96-well donor plate of polypropylene with 0.33 mL wells from Agilent (Santa Clara, CA, USA) and a 96-well acceptor plate from Millipore (Billerica, MA, USA) with polyvinylidene fluoride (PVDF) as support for the SLM were used. The PVDF material has 0.45 μm and 6.0 mm of pore size and internal diameter, respectively. A lid provided by the manufacturer was used to reduce or prevent evaporation of the solutions during the procedure. This same setup was described previously (Fig. 1) [21–23,28]. Once assembled, the PALME setup was placed on a ThermoMixer® C (Eppendorf® EP5382000023, Hamburg, Germany) for agitation.

For the extraction procedure, 100 μL of NaOH 25 mM were pipetted into the donor wells in the donor plate. Then, 150 μL of plasma aliquots previously spiked with the analytes were added to each well. Next, 3 μL of the smart & sassy EO were added to each of the PVDF filters to create the SLM, and the two plates were then sandwiched together. Finally, 50 μL of citric acid 50 mM was pipetted into the acceptor wells in the acceptor plate, a lid was placed, and the system was kept for 120 min under 500 rpm of agitation. Once the extraction was finished, the acceptor solutions were simply transferred to autosampler vials, and 7.5 μL were injected into the LC-MS/MS system.

2.4. LC-MS/MS

All analyses were performed using an ultra-performance LC-MS/MS instrument. The LC was an Acquity System equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) and the MS was a Quattro Premier XE model, both from Waters Corporation (Milford, MA, USA). Ammonium formate (pH 3.1, 1 mM) was used as mobile phase A and acetonitrile as mobile phase B, both containing 0.1% of formic acid (v/v). The method was kept at a constant flow rate of 0.3 mL/min with column oven temperature at 40 °C. The chromatographic gradient was as follows: initially, 10% of B was kept for 2 min, followed by a ramp to 20% within 5 min, and a second ramp to 100% of B within 3 min. Then, the initial conditions were restored in 0.5 min and kept for 1.5 min to re-equilibrate the system with a total run time of 12 min. The MS was operated in positive ionization mode with electrospray capillary voltage set to 2.5 kV, desolvation gas flow rate to 1100 L/h, cone gas flow rate to 200 L/h, desolvation temperature to 450 °C, and source temperature to 120 °C. The multiple reaction monitoring approach was used and the specific conditions to each analyte are displayed in Table S1.

2.5. Method optimization

The Design of Experiment statistical approach was used for both univariate and multivariate analysis of the extraction procedure where a fractional factorial screening 2^{k-1} and a central composite design (CCD) were used. Significance was considered when $p < 0.05$ and the adjusted coefficient of determination obtained by Analysis of Variance (ANOVA) served to determine the percentage of data variance explained by the model. All data were processed using GraphPad Prism® 8 and Statistica® 10 software.

2.6. Method validation

The ANSI/ASB standard 036, 1st Edition 2019 guide for method validation in Forensic Toxicology was used with the following parameters assessed: linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, imprecision, selectivity, and carryover. The matrix effect (ME), recovery (RE), and process efficiency (PE) study was performed according to Matuszewski et al. [29,30].

2.6.1. Calibration model

The linear range was assessed from six calibrators with five replicates. Heteroscedasticity weighting (1/x) was applied and the linear regression was acceptable when the coefficient of correlation (r^2) ≥ 0.99.

2.6.2. LOD and LOQ

LOD was experimentally determined as the lowest concentration

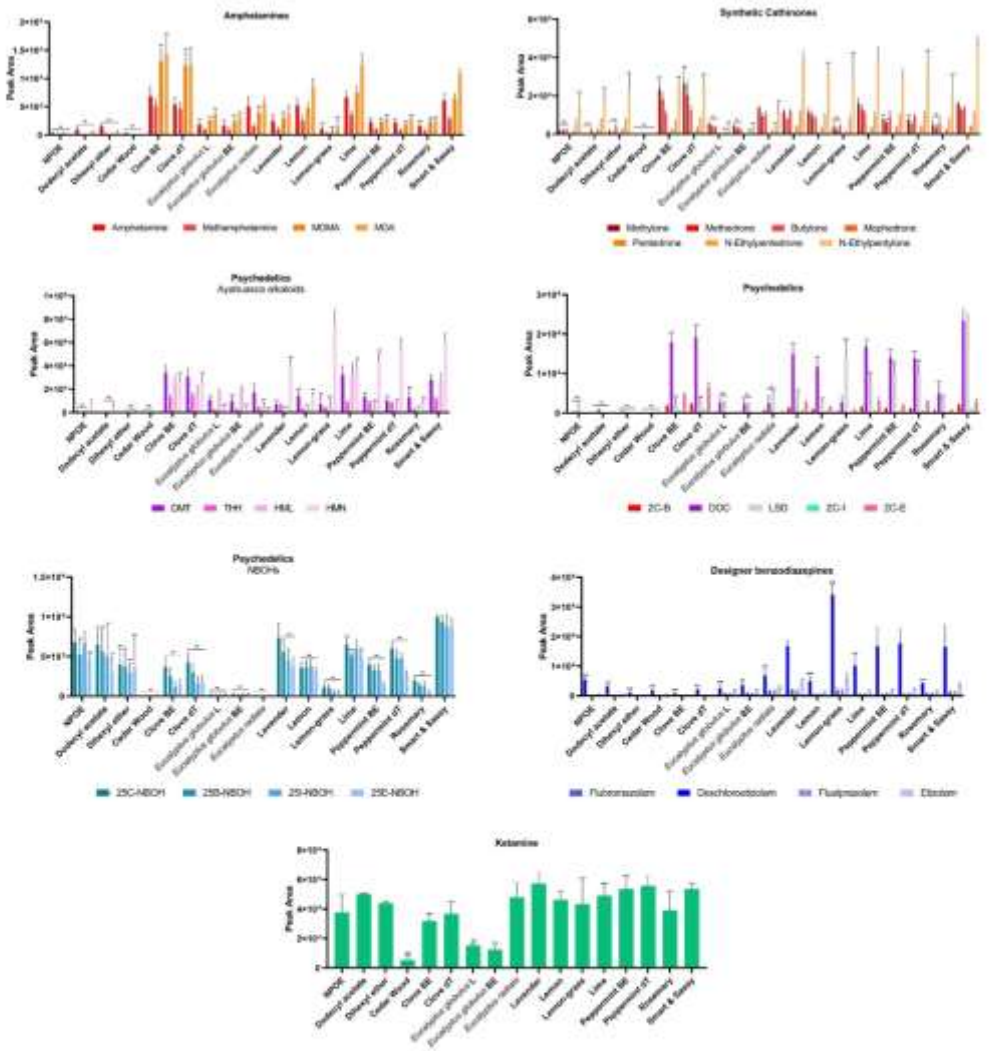

Fig. 3. Comparison between EO and other organic solvents as SLM. The mean of the absolute peak areas is shown (n = 4). Two-way ANOVA with post-hoc Tukey Test was used to determine the difference among groups. *p < 0.05 vs smart & sassy; **p < 0.01 vs smart & sassy. Clove BE: from BioEssência®; clove dT: from doTERRA International; Eucalyptus globulus L: from LANZLOO; Eucalyptus globulus BE: from BioEssência®; peppermint BE: from BioEssência®; peppermint dT: from doTERRA International; SLM: supported liquid membrane.

capable of providing a signal-to-noise ratio above three with retention times ±0.05 min. LOQ was set as the first concentration of the calibration curve by analyzing three samples from different donors on three different days with freshly prepared calibration curves. To determine LOQ, all identification and quantitation criteria should be met: accuracy (±20%), imprecision (≤20%), signal-to-noise ratio (≥10:1), and retention times (±0.05 min).

2.6.3. *Accuracy and imprecision*

Three QC levels were analyzed with a freshly prepared calibration curve on five independent days. The accuracy and imprecision of the method were then considered acceptable when ±20% and ≤20%, respectively. Accuracy was reported as bias and imprecision was expressed as coefficient of variation (CV) for both within-run and between-run imprecisions.

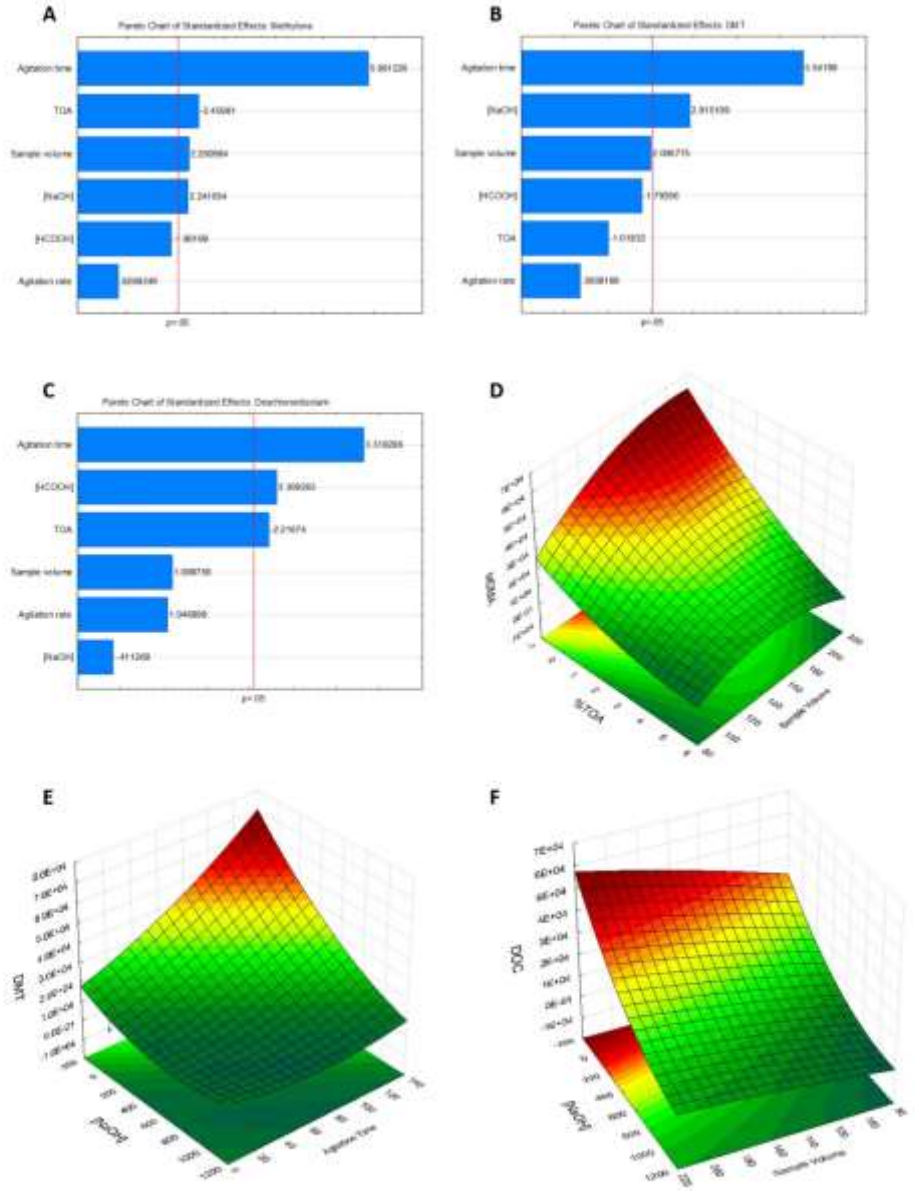

Fig. 4. Optimization study of the EO-based PALME herein proposed. A, B, and C are Pareto charts representing the statistically significant variables in the fractional factorial screening (bars crossing the red line: $p < 0.05$). D, E, and F are surface response graphs showing the optimum combination (red areas) of the statistically significant variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Linearity, LOD, LOQ, accuracy, and imprecision results.

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Calibration range (ng mL^{-1})	Linearity (r^2)	QC (ng mL^{-1})	Bias (%)	Imprecision (%)	
							Within-run	Between-run
Amphetamine	0.2	1	1–100	0.9928	2	10.9	8.9	11.9
					40	−4.3	8.7	12.8
					80	1.4	6.2	7.7
Methamphetamine	0.1	1	1–100	0.9903	2	6.4	12.5	10.4
					40	−9.4	5.6	9.5
					80	−4.7	6.6	6.6
MDMA	0.1	1	1–100	0.9978	2	14.2	5.1	5.4
					40	−8.5	4.4	8.7
					80	−1.2	4.8	4.9
MDA	0.2	1	1–100	0.9966	2	16.1	7.7	7.16
					40	−9.1	6.1	12.3
					80	−6.3	5.9	7.2
Methylone	0.1	1	1–100	0.9966	2	6.9	13.6	14.8
					40	5.0	7.0	9.5
					80	5.8	11.7	9.9
Methedrone	0.1	1	1–100	0.9959	2	3.0	10.7	11.7
					40	−0.5	9.1	9.2
					80	2.2	6.8	5.9
Butylone	0.1	1	1–100	0.9953	2	11.1	9.7	9.4
					40	−0.5	6.1	6.2
					80	2.9	4.2	5.2
Mephedrone	0.5	1	1–100	0.9955	2	3.2	9.0	14.8
					40	0.5	6.7	8.8
					80	5.0	4.6	4.5
Pentedrone	0.2	1	1–100	0.9981	2	4.4	5.8	11.8
					40	−0.5	8.5	10.5
					80	2.9	3.3	8.3
N-ethylpentedrone	0.5	1	1–100	0.9998	2	4.5	11.8	13.4
					40	−1.4	7.5	9.6
					80	0.8	3.9	5.3
N-ethylpentylone	0.1	1	1–100	0.9984	2	14.8	4.7	6.7
					40	−8.3	2.6	3.7
					80	−3.2	2.0	6.7
DMT	0.1	1	1–100	0.9962	2	10.8	10.8	12.0
					40	2.2	6.4	9.8
					80	2.1	6.4	9.3
THH	0.2	1	1–100	0.9961	2	12.3	11.0	14.8
					40	−2.8	9.8	14.7
					80	−2.3	12.2	14.0
HML	0.75	1	1–100	0.9970	2	−0.6	13.8	16.4
					40	2.3	12.9	12.5
					80	−1.9	8.5	7.0
HMN	0.1	1	1–100	0.9974	2	5.0	7.7	13.4
					40	0.7	10.2	13.2
					80	−9.0	8.2	11.3
Ketamine	0.1	1	1–100	0.9965	2	14.8	5.6	5.2
					40	−6.5	3.9	3.7
					80	−5.4	2.2	4.4
2C-B	0.75	1	1–100	0.9950	2	0.8	11.7	12.4
					40	−1.1	10.6	12.3
					80	0.9	7.9	9.9
DOC	0.2	1	1–100	0.9977	2	16.4	5.7	5.2
					40	1.2	6.9	12.5
					80	−1.5	4.1	8.9
LSD	0.1	1	1–100	0.9984	2	12.1	11.4	10.9
					40	−4.9	5.3	6.6
					80	0.5	3.1	4.6
2C-I	0.5	1	1–100	0.9995	2	8.0	10.5	13.3
					40	−5.3	11.5	12.3
					80	−1.4	8.4	11.0
2C-E	0.2	1	1–100	0.9980	2	3.0	7.6	13.0
					40	7.2	11.6	11.7
					80	−5.5	9.4	11.0
25C-NBOH	0.5	1	1–100	0.9981	2	1.1	12.2	14.3
					40	−3.9	13.8	13.7
					80	−4.4	10.3	10.2
25B-NBOH	0.5	1	1–100	0.9913	2	−0.4	14.3	13.3
					40	−4.7	0.7	0.7
					80	−2.3	0.4	0.3
25I-NBOH	0.2	1	1–100	0.9975	2	−3.3	10.7	12.9
					40	−1.5	13.6	14.9
					80	−6.1	12.5	11.7
25E NBOH	0.2	1	1–100	0.9974	2	−2.3	15.0	12.9

(continued on next page)

Table 1 (continued)

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Calibration range (ng mL^{-1})	Linearity (r^2)	QC (ng mL^{-1})	Bias (%)	Imprecision (%)	
							Within-run	Between-run
					40	4.9	12.0	13.0
					80	−2.5	7.0	9.3
Deschloroetizolam	0.2	1	1–100	0.9927	2	8.0	13.4	14.5
					40	−0.5	5.4	12.6
					80	−6.6	6.9	7.1
Flualprazolam	0.75	1	1–100	0.9949	2	−0.2	14.3	14.0
					40	−1.8	8.8	9.3
					80	−2.3	5.5	6.9
Flubromazolam	0.75	1	1–100	0.9935	2	0.6	10.6	10.9
					40	−1.1	6.8	11.8
					80	−2.2	5.2	5.1
Etizolam	0.2	1	1–100	0.9974	2	−8.4	15.9	15.5
					40	−4.5	7.8	12.9
					80	−10.9	14.8	12.6

LOD: limit of detection; LOQ: limit of quantitation; QC: quality control.

2.6.4. ME, RE, and PE

The ME was the calculated ratio between post-spiked extracts with neat analyte injections; RE was calculated as the ratio between spiked plasma samples with post-spiked extracts; and PE was obtained considering the ratio between spiked plasma samples with neat analyte injections [30].

2.6.5. Intermediate imprecision for EO

Five blank plasma samples were spiked at each of the three QC levels and extracted simultaneously with three different batches of the smart & sassy EO. These samples were analyzed in terms of within- and between-batch imprecisions. Values were considered acceptable when ≤20% [11, 29].

2.6.6. Selectivity

The presence of common endogenous substances and exogenous compounds was studied as potential interferents. To that end, ten blank plasma samples from different subjects were extracted, and neat standards of common substances were directly injected into the instrument and analyzed by the method (Table S3). In both studies, no interfering peaks should be visualized in the same retention times as the target analytes.

3. Results and discussion

3.1. Selection of EO as liquid membrane

In a first set of experiments, fourteen different EO were tested as liquid membranes. The model analytes were amphetamines (four compounds), synthetic cathinones (seven compounds), ayahuasca alkaloids (four compounds), psychedelics (nine compounds), designer benzodiazepines (four compounds), and ketamine. These were selected to represent a wide range of compounds in terms of molecular weight (135.2–413.3 g/mol), polarity ($1.23 \leq \log P \leq 4.06$), and basicity ($4.02 \leq pKa \leq 10.26$) (Table S2). In addition to the EO, extractions were also conducted with NPOE, dodecyl acetate, and dihexyl ether for comparison, as these are pure synthetic solvents frequently used for LPME (Fig. 3). Interestingly, for most of the substance classes, the EO were more efficient than the synthetic solvents but none were highly efficient for all the model analytes. The reason behind that could be plenty fold. For example, the composition of the SLM is known to play a major role on extraction efficiency, as it affects mass transfer from the donor to acceptor compartment. Thus, the presence of chemicals in the SLM that allow formation of ionic, hydrogen bond, and π-π interactions, are likely to impact analyte recovery [31]. EO are comprised of multiple natural compounds that have hydrogen-bond donor or acceptor groups and

Table 2
ME, RE, and PE values.

Analyte	ME (%)			RE (%)			PE (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Amphetamine	97.2	95.4	105.7	70.4	70.2	69.1	68.5	67.0	73.1
Methamphetamine	102.4	93.3	106.2	67.5	77.2	68.4	69.1	72.0	72.6
MDMA	91.0	92.0	102.3	75.1	73.6	79.3	68.4	67.7	81.1
Methylone	86.4	83.5	86.6	69.6	72.0	76.3	52.4	60.1	66.1
Mephedrone	91.1	98.0	103.6	72.1	80.7	81.8	65.6	79.1	84.8
Pentedrone	85.6	86.0	109.1	95.3	90.9	91.8	81.5	72.7	100.2
N-ethylpentedrone	91.2	94.8	91.2	89.6	99.4	94.9	81.7	94.2	86.6
DMT	101.0	100.6	99.7	66.0	70.2	79.8	66.6	70.6	79.6
HML	101.9	102.6	109.8	43.7	38.9	42.0	44.5	39.9	46.1
HMN	105.9	96.9	109.2	76.4	78.5	82.8	80.9	76.1	90.4
Ketamine	101.7	93.7	105.6	95.4	88.5	106.1	97.1	82.9	112.0
DOC	98.4	108.7	107.2	53.4	58.2	63.6	52.6	63.3	68.2
LSD	109.6	115.4	119.1	67.8	82.6	82.3	74.3	95.3	98.0
2C-I	102.9	101.2	107.1	49.3	59.0	55.5	50.7	59.7	59.4
2C-B	104.7	118.5	112.2	64.7	55.8	62.3	67.7	66.1	69.9
25C-NBOH	88.3	94.9	103.1	78.3	76.4	77.6	69.2	72.5	80.0
25I-NBOH	96.0	118.0	117.0	55.8	63.6	60.2	53.6	75.1	70.4
25E-NBOH	109.4	103.8	113.7	77.5	88.3	86.3	84.7	91.6	98.1
Flualprazolam	111.4	104.6	110.4	28.4	33.4	34.2	31.6	34.9	37.7
Flubromazolam	110.8	116.0	110.8	20.6	25.3	27.3	22.8	29.4	30.2
Etizolam	119.9	118.8	116.9	30.6	31.3	26.0	36.6	37.2	30.4

ME: matrix effect; RE: recovery; PE: process efficiency.

Table 3
Imprecision assessment of DO from different production batches.

Analyte, QC (ng mL^{-1})	Imprecision (%)	
	Within batch	Between batch
Amphetamine		
2	7.4	6.3
40	7.9	9.7
80	3.5	3.3
Methamphetamine		
2	6.8	6.7
40	7.9	12.8
80	2.9	4.8
MDMA		
2	4.8	4.4
40	3.1	7.8
80	4.1	3.7
MDA		
2	10.8	8.9
40	8.3	12.6
80	6.3	7.2
Methylone		
2	12.6	17.2
40	5.5	8.4
80	12.9	10.6
Methedrone		
2	13.6	11.4
40	10.9	9.3
80	7.0	6.3
Butylone		
2	8.0	8.0
40	8.0	6.5
80	2.7	4.4
Mephedrone		
2	7.5	6.4
40	4.5	11.8
80	2.6	2.1
Pentedrone		
2	3.3	16.4
40	6.3	5.1
80	4.0	11.3
N-ethylpentedrone		
2	3.2	10.3
40	8.3	6.8
80	2.3	7.5
N-ethylpentylone		
2	3.4	3.5
40	2.7	3.2
80	2.1	4.2
DMT		
2	14.5	15.1
40	9.5	11.1
80	6.6	7.7
THH		
2	8.4	17.4
40	13.2	16.8
80	14.0	16.3
HML		
2	9.3	17.3
40	7.1	8.5
80	6.1	5.3
HMN		
2	8.8	12.0
40	11.0	9.1
80	5.7	5.8
Ketamine		
2	3.5	2.9
40	2.0	2.4
80	2.3	2.5
2C-B		
2	4.8	4.2
40	10.4	8.6
80	4.9	9.9
DOC		
2	4.0	3.4
40	7.2	11.1
80	2.3	3.8
LSD		
2	11.2	10.7
40	2.8	2.6
80	3.2	5.3
2C-I		
2	12.1	13.2
40	12.4	13.0
80	5.1	5.4
2C-E		
2	10.2	14.9
40	11.6	10.8
80	13.0	12.9
25C-NBOH		
2	14.2	17.1
40	7.9	10.3
80	7.7	6.3
25B-NBOH		
2	15.3	12.6
40	8.9	7.5
80	13.5	11.0
25I-NBOH		
2	13.9	12.3
40	11.0	12.1
80	11.9	12.5
25E-NBOH		
2	14.1	14.7
40	13.9	16.2
80	4.5	7.8
Deschloroetizolam		
2	4.8	5.4
40	4.4	7.7
80	2.2	3.0
Flualprazolam		
2	12.4	12.7
40	5.6	4.7
80	6.6	6.5
Flubromazolam		
2	9.3	10.7
40	5.6	13.7
80	3.1	3.2
Etizolam		
2	8.9	10.3
40	6.8	7.7
80	5.7	5.6

QC: quality control.

Table 4
Real case samples analyzed by the proposed technique.

Samples	Analyte	Concentration (ng mL^{-1})
01	Butylone	8.9
02	N-ethylpentedrone	19.2
03	N-ethylpentylone	1.3
04	DMT	3.3
	THH	51.3
	HML	26.3
	HMN	26.3
05	DMT	< LOQ
	THH	26.2
	HML	17.6
	HMN	10.6
06	DMT	6.6
	THH	38.4
	HML	12.4
	HMN	9.9
07	25B-NBOH	< LOQ

LOQ: limit of quantitation.

aromatic rings, hence directly affecting the interaction between SLM and target analytes [32–34]. In addition to those chemical interactions, different classes of drugs of abuse were used as model analytes (Fig. 2 and Table S2). Thus, the result of combining a complex SLM composition

Fig. 5. AGREEprep assessment of the EO-based PALME proposed in the present work. The value in the inner circle represents the overall score of the technique (0–1.0). Each of the ten parameters evaluated are placed around the inner circle and the length of each criterion represents the weight on the final score. The darker the green tones in each of the ten criteria, closer to the desired sustainable performance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to extract different groups of drugs with chemically diverse properties seemed to have been the cause for the differences in extraction efficiency observed in Fig. 3. To pinpoint how each EO constituent might be influencing analyte extraction, these EO molecules would have to be studied in isolated or in defined mixtures [35]. For the present work, the EO termed smart & sassy was selected as the best compromise for simultaneous extraction of all compounds and, therefore, was optimized as described below.

3.2. Optimization of operational parameters

Based on previous works, the sample pH, acceptor pH, sample volume, agitation rate, and extraction time were considered the major operational parameters. In addition, TOA was added to the liquid membrane to suppress secondary interactions with the solid support membrane [18,19,22–24]. Therefore, the concentration of TOA was considered an important operational parameter as well. In total, six parameters were included in this first optimization step. The fractional factorial screening 2^{6-1} was performed considering the concentration of base in the sample (10–100 mM), the concentration of acid in the acceptor (10–100 mM), sample volume (100–200 μL), percentage of TOA added to the liquid membrane (0–5%), agitation rate (300–700 rpm), and time (5–120 min). The outcome of the optimization experiments is summarized in Fig. 4.

The agitation time was the most important operational parameter, followed by the concentration of base in the sample, sample volume, and percentage of TOA (Fig. 4A–C). The concentration of acid in the acceptor was critical only for the designer benzodiazepines (Fig. 4C). These compounds are extremely weak bases and, therefore, required strongly acidic conditions in the acceptor (Table S2).

After this screening step, a CCD was performed to find the optimum extraction conditions. Extraction recoveries increased with increasing extraction time up to 120 min. Extractions for 180 min were also tested but these failed due to stability issues with the EO liquid membrane in the final stage. At 120 min, equilibrium was still not obtained. Thus, compared with LPME using synthetic organic solvents as the liquid membrane [18,20–24], the extraction kinetics in the current EO-based system was relatively slow. This was likely attributed to the higher viscosity of the smart & sassy EO.

The extraction recoveries were also affected by the concentration of base in the sample. As seen from Fig. 4E and F, the extraction efficiency decreased upon increasing the concentration of NaOH in the sample. This is not typical for LPME with pure synthetic liquid membranes and indicates that high levels of NaOH affected the chemical stability of the EO-based liquid membrane. Most probably, basic hydrolysis may explain this observation and may also explain why the EO liquid membrane cannot be used for very long extractions. Therefore, 10 mM NaOH was selected for pH adjustment of the sample.

Extraction recoveries increased with increasing sample volume up to 150 μL. Above this level, the convection in the sample was reduced despite strong agitation and the extraction efficiency decreased (Fig. 4D and F). This observation was in accordance with previous studies [18,19,23]. In addition, the performance was not affected by the addition of TOA to the liquid membrane. Most probably, natural constituents of the EO suppressed secondary interactions and, therefore, no additional effect was obtained with TOA [19–22,24,32–34].

In a separate set of experiments, NaOH and formic acid were replaced with sodium bicarbonate and citric acid. The two latter substances are less hazardous and greener [36]. The results are summarized in Fig. S1 in supporting information. Generally, recoveries with sodium bicarbonate were lower than with NaOH, due to pH. For most model analytes, extractions with citric acid and formic acid were comparable, but for the four designer benzodiazepines, the green alternative provided improved extraction recovery. Therefore, in the final method, NaOH and citric acid were selected.

3.3. Method validation

The calibration curves used for all the analytes ranged from 1 to 100 ng mL^{-1} with $r^2 \geq 0.99$ (Table 1). This range was chosen as a compromise based on the typical plasma concentrations of the drugs covered by the method [4,5,10,11]. The established LOD for most analytes varied between 0.1 and 0.5 ng mL^{-1}, while the LOQ was set to 1 ng mL^{-1} for all compounds (Table 1). Although the model analytes were not extracted exhaustively, the LOD were comparable to those obtained in similar PALME setups for methylone, pentedrone, 2C-E, deschloroetizolam, etizolam, and flubromazolam [21,23]. Thus, although the current work used an EO-based liquid membrane and slower kinetics were observed, detection and quantification limits were not seriously affected.

The accuracy and imprecision of the method were also within acceptable limits established by validation guidelines, that is ±20% and ≤20%, respectively (Table 1 and Section 2.5.3) [29]. The ME study showed no ion suppression nor enhancement ±20%, which is in agreement with previous studies that demonstrate PALME provides excellent sample clean-up (Table 2) [19,22,26,37]. In addition, despite the slow kinetics observed, the RE values were high for most model analytes. As for the poorly extracted compounds (RE < 60%), the LOD achieved suffices for the application to real case samples (Table 2). Thus, the technique showed to be efficient even for the analytes with poor recoveries.

In a final validation step, extraction with EO from different production batches was tested. This was based on the fact that EO are plant extracts, and their composition may vary depending on several factors including the harvest, season, and extraction conditions [32–34]. In a new set of experiments, smart & sassy EO of different batches were acquired and evaluated in terms of intermediate precision [11]. As shown in Table 3, the imprecision for most of the analytes was ≤15% at the three QC levels. A closer investigation of these results revealed that the older EO bottle included in this experiment was responsible for producing the higher values observed in the between-batch imprecision.

Table 5
Comparison of the proposed PALME method with previous applications.

Donor phase		SLM	Acceptor phase*	Extraction time (min)	Analytes	RE (%)	ME (%)	Ref.
pH adjustment	Sample							
200 μL of NaOH 20 mM	400 μL of plasma	2 μL of dihexyl ether	Formic acid 20 mM	30	Pethidine, haloperidol, methadone, and nortriptyline	55–89	–	[18]
125 μL of HCl 250 mM	125 μL of plasma	2 μL of dihexyl ether	NH_4 25 mM	60	Ketoprofen, fenoprofen, diclofenac, flurbiprofen, ibuprofen, and gemfibrozil	93–108	–	[20]
115 μL of NaOH 40 mM 115 μL of HCl 250 mM	125 μL of plasma	4 μL of hexadecane 4 μL of isopentyllecanol	Formic acid 20 mM NH_4 25 mM	120	Fluoxetine, fluvoxamine, and quetiapine	91–96	86–109	[37]
125 μL of phosphate buffer 50 mM	125 μL of plasma	2.5 μL of 2-nonanone + 15% DEHP	Trifluoro acetic acid 150 mM	45	Hydralazine, ephedrine, metaraminol, salbutamol, and cimetidine	2–89	–	[19]
115 μL of NaOH 40 mM	125 μL of plasma	5 μL of dodecyl acetate + 1% TOA	Formic acid 20 mM	120	Methylone, 4-Fluoroamphetamine, pentedrone, 3,4-Methylenedioxypyrovalerone, meta	25–117	–	[21]
75 μL of NaOH 80 mM	150 μL of whole blood				Chlorpheniylpiperazine, 6-(2-aminopropyl) benzofuran, methantarsine, ethylphenidate, methylenedioxy 2 aminoindane 2C-B, bromo-dragonfly, and AH-7921	3–86		
250 μL of NaOH 10 mM 250 μL of formic acid 20 mM	DBS	4 μL of dodecyl acetate + 1% TOA	Formic acid 20 mM NH_4 25 mM	60	Ketoprofen, fenoprofen, diclofenac, and flurbiprofen Quetiapine and nortriptyline	58–74 74–88	– 88–102	[22]
115 μL of NaOH 40 mM	125 μL of plasma	5 μL of dodecyl acetate + 1% TOA	Formic acid 20 mM	120	Citalopram, fluoxetine, venlafaxine, o-desmethylvenlafaxine, sertraline, norfluoxetine, and paroxetine	72–111	86–112	[24]
130 of phosphate buffer 50 mM	100 μL of whole blood	2-undecanone and dihexyl ether (1:1) + 1% TOA	150 μL of DMSO + formic acid 200 mM (75:25)	60	Alprazolam, bromazepam, desalkylflurazepam, diazepam, diclazepam, estazolam, phenazepam, flubromazepam, flualprazolam, flunitrazepam, clonazepam, clonazolam, lormazepam, meclonazepam, midazolam, N-desmethylflunitrazepam, nitrazepam, oxazepam, zolpidem, and zopiclone	52–104	87–107	[23]
115 μL of NaOH 40 mM	125 μL of plasma	4 μL of dodecyl acetate + 1% TOA	Formic acid 20 mM	60	Amitriptyline, nortriptyline, quetiapine, venlafaxine, O-desmethyl-venlafaxine, and fluoxetine	67–89	–	[25]
125 μL of NaOH 50 mM or $NaHCO_3$ 500 mM	125 μL of plasma	3 μL of dihexyl ether or sesame oil	Formic acid 20 mM or citric acid 10 mM	120	90 basic compounds (drugs and endogenous metabolites)	0–103	–	[26]
125 μL of NaOH 40 mM	125 μL of breast milk	3 μL of dihexyl ether + 1% TOA	100 of μL formic acid 20 mM	50	Amphetamine, methamphetamine, and MDMA	40–89	88–105	[29]
HCl	Water samples	4 μL of 1 Octanol	NaOH	120	Soman acid, ethyl methylphosphonic acid, sarin acid, cyclohexyl sarin acid, and isobutyl methylphosphonic acid	0–100	–	[44]
Drops of HCl (32%)	350 μL of urine	4 μL of n Octanol	NaOH 1 M	120	Soman acid, ethyl methylphosphonic acid, sarin acid, cyclohexyl sarin acid, and isobutyl methylphosphonic acid	–	–	[45]
140 μL of phosphate buffer 50 mM	100 μL of plasma	5 μL of dodecyl acetate + 1% TOA	DMSO and formic acid 200 mM (50:50)	30	Repaglinide	97–101	98–102	[46]
100 μL of NaOH 25 mM	150 μL of plasma	3 μL of EO	Citric acid 10 mM	120	29 basic drugs of abuse	20–106	80–119	This work.

*Acceptor volume was 50 μL if not specified. EO: essential oil; SLM: supported liquid membrane; RE: recovery; ME: matrix effect; TOA: trioctylamine; DEHP: 2-di(ethylhexyl) phosphate; DBS: dried blood spot; DMSO: Dimethyl sulfoxide.

Nevertheless, all values were ≤20% and, therefore, within accepted limits by validation guidelines [39]. In conclusion from this experiment, the extraction performance with different batches of EO varied to some extent but is avoidable by using the same batch for series of extractions. This could be attributed to loss of stability of the compounds present in the EO.

As a final proof of the applicability of the proposed technique, seven real case samples were analyzed and the results are displayed in Table 4.

3.4. Greenness score

As the sole purpose for studying the use of EO as liquid membrane was environmental consciousness, the greenness of the method was evaluated. The AGREEprep approach was chosen for it allows a thorough evaluation of different aspects of the sample preparation procedure [38,39].

In this work, the focus was on replacing pure synthetic solvents used in PALME with greener alternatives. The use of hazardous solvents in a sample preparation procedure is evaluated by criterion 2 in AGREEprep. As shown in Fig. 5, this parameter was considered highly green. In combination with other features, such as using only 150 μL of plasma and replacing formic acid with citric acid, the overall score was slightly improved in comparison to previous works (Fig. 5) [36]. In contrast, the lowest scores obtained in this evaluation were related to sample preparation placement (1), reusability of the materials (3), lack of automation (7), and type of analytical instrumentation (9). Some of these four limitations are easier to address than others. For example, the liquid membrane is not reusable (3), but the technique performed in this study

is amenable to automation [27,40,41] (7), and other instrument can be used for analysis [20,31] (9). This illustrates that PALME is indeed a green alternative and improvements can still be explored to further reduce the environmental impact of this technique.

3.5. Feasibility of PALME for routine analysis

In this work, LPME in the 96-well plate format was studied as a tool for investigating the consumption of drugs of abuse by patients. With the increasing number of NPS, this has been a troublesome task in routine laboratories dealing with intoxicated patients. To address such cases, a reliable sample preparation technique that is fit to both traditional drugs of abuse and NPS is warranted. Moreover, this technique should also be fast and simple allowing the rapid analysis of urgent cases. The overall extraction of basic drugs of abuse from plasma samples using the herein proposed PALME proved to meet those requirements as it is considered a single-step procedure [19]. In addition to that, this setup allows the simultaneous processing of up to 96 samples, which is excellent for laboratories with massive routine casework.

Another important feature that has a major impact on the implementation of a technique in routine analysis is automation because it minimizes human handling further simplifying the analytical pipeline. In this regard, LPME automation has been reported, although such approaches tend to require sophisticated systems [42,43]. In contrast, PALME is simpler by default and so are the automated systems [27,40, 41]. Thus, even though the PALME setup used in the present work was entirely manual, the advantage of being amenable to automation brings this technique closer to implementation in routine analysis. Of note, PALME automation would meet even closer today's standards of environmental consciousness (Fig. 5) [14].

In combination, the fast and simultaneous analysis of almost a hundred samples makes PALME a valuable tool not only for cases of drug consumption. Table 5 summarizes previously published articles using PALME for different applications. In terms of donor phase composition (NaOH) and extraction time (120 min), the technique described herein uses parameters similar to other works. In contrast, this study stands out in terms of acceptor phase and SLM by replacing formic acid with citric acid and completely dismissing the use of organic solvents as extractors, respectively. Moreover, most studies have focussed on medications, such as antidepressants and anti-inflammatory drugs, while only few have investigated PALME for the analysis of drugs of abuse and included different classes or even NPS (Table 5).

4. Conclusion

In this conceptual work, an EO-based PALME to extract model analytes from plasma samples was explored. A total of 29 illicit substances were chosen due to their toxicological relevance and heterogeneous chemical properties.

Among all SLM tested, the smart & sassy EO was the best candidate for all compounds covered by the method. However, compared to pure synthetic solvents, this EO had higher viscosity resulting in slower extraction rates. Nevertheless, the sensitivity required for the applicability of this method to real cases was still achieved for all analytes, even the ones that were poorly extracted.

Importantly, the natural variabilities of the smart & sassy EO were also considered during this study and were found not to impact the analytical robustness of the method. However, results suggest that EO bottles should be used for series of experiments, as extraction efficiency might decrease over time likely due to stability issues.

From an eco-friendly perspective, using only 3 μL of EO,150 μL of sample, citric acid as acceptor, and being able to simultaneously extract up to 96 samples makes PALME a valuable tool for toxicological analyses – especially considering that the technique is fit for different classes of compounds. This feature is particularly relevant in the current NPS scenario in which novel drugs are constantly appearing in the illicit drug market.

CRediT authorship contribution statement

André Luis Fabris: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Stig Pedersen-Bjergaard:** Writing – review & editing, Visualization, Formal analysis, Conceptualization. **Elisabeth Leere Oiestad:** Writing – review & editing, Resources. **Giordano Novak Rossi:** Resources. **Jaime E.Cecílio Hallak:** Resources. **Rafael Guimarães dos Santos:** Resources. **Jose Luiz Costa:** Writing – review & editing, Resources, Funding acquisition. **Mauricio Yonamine:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.342387.

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8. ARTIGO V

An efficient and green method for the analysis of synthetic cathinones in whole blood using 96-well electromembrane extraction and LC-MS/MS

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An efficient and green method for the analysis of synthetic cathinones in whole blood using 96-well electromembrane extraction and LC-MS/MS

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ABSTRACT

Electromembrane extraction (EME) is a miniaturized technique in which charged molecules are extracted from biological samples, through a porous membrane, into an acceptor solution. This process occurs in the presence of an electric field that enables increased analyte recovery in short extraction times. Studies are being carried out to establish recommended extraction conditions for different groups of compounds based on their physicochemical properties. In this work, pre-established generic liquid membranes for the extraction of basic compounds were tested using synthetic cathinones within a wide log P range as basic model analytes. First, recommended organic solvents for the chosen analytes were evaluated in terms of recovery. The best candidates were included in an optimization study with other relevant EME conditions (voltage, extraction time, and agitation rate). All analyses were performed using a UPLC-MS/MS with the multiple reaction monitoring mode. 2-Undecanone was chosen as the liquid membrane providing the best extraction efficiency in combination with 40 V, 35 min, and 725 rpm. Validation showed $r^2 > 0.99$ in the 1–500 ng/mL range, accuracy of ±12%, imprecision of ≤19%, 1 ng/mL as limit of quantitation, 0.1–0.5 ng/mL as limit of detection, matrix effect 97–118%, and recovery 63–110%. Among the generic liquid membranes studied in this work, 2-Undecanone was the best choice for the target basic analytes. High recoveries and low matrix effects were achieved, thus demonstrating the applicability of recommended generic liquid membranes in the 96-well plate setup and with whole blood samples. Our findings contribute to further establishing EME as an actual sample preparation technique for routine analyses that could be further explored in a commercial format.

1. Introduction

Synthetic cathinones (SC) are a group of substances structurally related to cathinone, a natural compound found in the Khat plant (*Catha edulis*). The first SC was found on the illicit drug market in 2005 but new cathinone derivatives are still emerging. Recent data from the United Nations Office on Drugs and Crime show that more than 70 S C have been reported yearly since 2014. In 2022, the

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seizure of large quantities of SC in Europe has raised the concern of these compounds playing a major role in Europe's stimulant market (European Monitoring Centre for Drugs and Drug Addiction, 2023).

SC have become well-established in some European drug markets due to their psychoactive properties (European Monitoring Centre for Drugs and Drug Addiction, 2023). With similar effects to known drugs of abuse, such as amphetamine, they are frequently sold as replacements for these stimulants. However, different substitution patterns in the core structure of cathinone result in numerous SC of unknown toxicological properties (Fig. 1) (Soares et al., 2021). In addition, SC are also often used as adulterants of stimulant products resulting in the inadvertent use of these mixtures of drugs (European Monitoring Centre for Drugs and Drug Addiction, 2023). In combination, these factors increase the risk of overdoses and deaths, especially in groups with a high prevalence of SC abuse, such as clubbers, high-risk drug users, and treatment entrants (European Monitoring Centre for Drugs and Drug Addiction, 2015). Interestingly, some SC have more adhesion from users and continue on the drug market even after being listed in current legislation (European Monitoring Centre for Drugs and Drug Addiction, 2023). On the other hand, new compounds with novel chemical structures are constantly created and introduced into the drug market to be sold as legal replacements for controlled substances. In addition to this dynamic flow, these drugs are typically not included in routine screenings, thus the development of analytical methods suited to analyze these substances in biological matrices is of growing relevance.

Several extraction techniques aiming at analyzing SC in different biological specimens have been proposed (Lau et al., 2020; Niebel et al., 2020; Aldubayyan et al., 2022). Some works have also attempted to fit the Green Analytical Toxicology or Green Sample Preparation perspective (de Paula Meirelles et al., 2023; López-Lorente et al., 2022). Liquid-phase microextraction (LPME), microextraction by packed sorbent, and dispersive liquid-liquid microextraction are examples of miniaturized techniques that have been proposed in that regard (Fabris et al., 2023; Bavlovic Piskackova et al., 2022; Vardal et al., 2017; da Cunha et al., 2020; Lesne et al.,

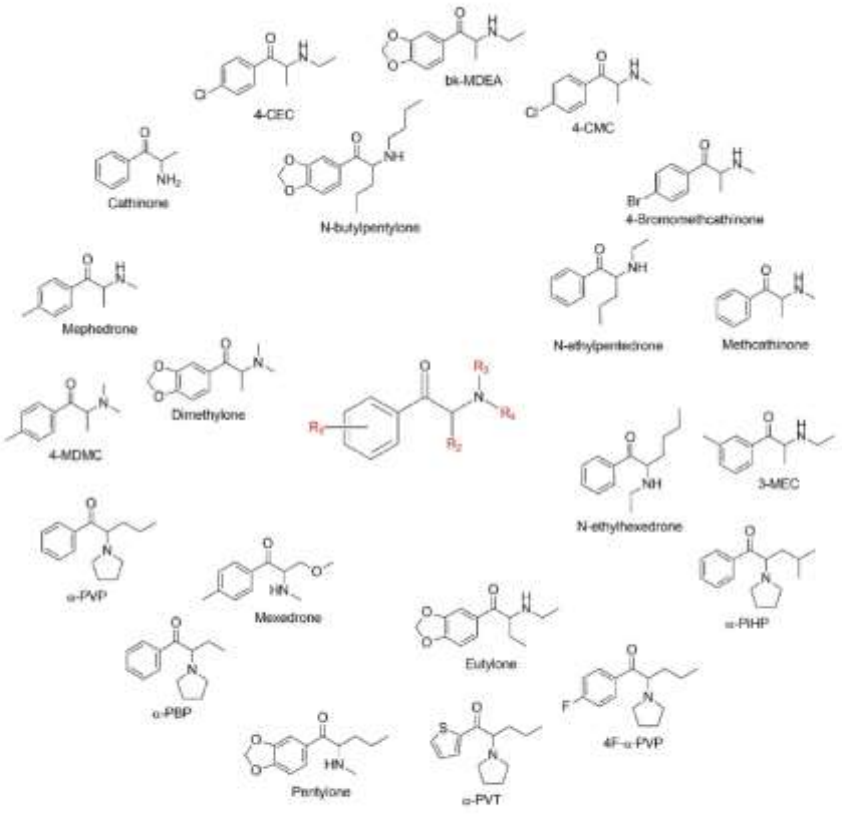

Fig. 1. Chemical structure of the cathinones studied in the present work. In the centre, the core structure of cathinone with the positions where substitutions can be introduced (in red) to obtain new derivatives, i.e. SC. At the borders are the cathinone derivatives included in this study illustrating the diversity of this group of NPS. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2023). The main advantages of using these formats are reducing the volume of samples and organic solvents consumed while improving simplicity and cost-effectiveness. However, a major setback with these new techniques is the lack of commercial equipment for their actual implementation in routine laboratories.

Electromembrane extraction (EME) is a form of LPME that makes use of an electric field to improve mass transfer. Since it was introduced in 2006, EME has been explored in different applications and formats (Gjelstad et al., 2007). For instance, setups using hollow fibers, flat membranes, micro-chip technology, and 96-well plates were described to extract medications, drugs of abuse, etc., from various samples (Eibak et al., 2012; Vardal et al., 2016; Gjelstad et al., 2015; Cristale et al., 2013; Ramos Payan et al., 2016; Hong et al., 2022). These approaches were also summarized in some interesting reviews (Alexovic et al., 2016, 2018). Recently, a commercial device for EME based on conductive vials was made available (Skaalvik et al., 2021, 2023a, 2023b; Schuller et al., 2023a). This is a milestone for this sample preparation technique and brings it one step closer to routine implementation. Nevertheless, efforts to make EME simpler and more practical are still being made.

The 96-well plate format is a promising candidate because it offers higher throughput and less solvent consumption can be achieved compared to the conductive vial-based setup. The single-step three-phase extraction principle of EME is maintained in this configuration (Eibak et al., 2014). Briefly, charged analytes are transferred from an aqueous sample solution, through a hydrophobic liquid membrane, into an aqueous acceptor solution. The application of an electric field facilitates the transfer of charged molecules towards the acceptor solution. For example, extracting basic compounds, such as the SC, is achievable by acidifying the sample and using an acidic acceptor solution (Fig. 2) (Skaalvik et al., 2021, 2023a, 2023b; Schuller et al., 2023a; Schüller et al., 2023b). Finally, clean aqueous extracts can be directly injected into an LC-MS instrument. In that regard, EME is highly selective to small ionized compounds, thus it offers great sample clean-up of matrix components, such as salts, proteins, phospholipids, etc. (Bavlovic Piskackova et al., 2022; Skaalvik et al., 2021).

In theory, EME is applicable to any ionizable compound but this task is challenging when multiple analytes with a range of chemical properties are to be extracted simultaneously. As EME has recently become commercially available and is now an actual alternative for green and sustainable routine analysis, pre-established generic extraction conditions for specific groups of substances are being proposed (Zhou et al., 2023, 2024). These recommendations are mainly based on the log P of the target compounds. In this work, pre-established conditions to extract basic compounds were tested in the prototype 96-well plate format. SC were chosen due to their increasing relevance in toxicology. This class of drugs of abuse has basic properties and compounds within a wide lipophilicity range were considered. This study aimed to evaluate the applicability of generic liquid membrane recommendations in this prototype 96-well EME setup. Additionally, recommended extraction conditions were previously determined for plasma samples, thus this study also evaluates the compatibility and performance in whole blood.

2. Methodology

2.1. Chemicals and solvents

Ammonium formate, 2-Nitrophenyl octyl ether (NPOE), 2-Undecanone, and bis(2-Ethylhexyl) phosphate (DEHP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thymol (Thy) and 6-Methylcoumarin (6 MC) were from Merck (Darmstadt, Germany). Formic acid (LC-MS grade) was purchased from VWR (Radnor, PA, USA). Methanol (MeOH, Chromasolv™ ≥ 99.9%) was obtained from Honeywell/Riedelde Haën™ (Seelze, Germany). Cathinone was obtained from USP, 4-Bromomethcathinone, 4-Methyl *N*,*N*-dimethylcathinone (4-MDMC), bk-MDEA, dimethylone, *N*-butylpentylone, and α-Pyrrolidinobutiophenone (α-PBP) were obtained from Cayman Chemical Company. Eutylone was purchased from LGC Standards, methcathinone from Sigma, and mexedrone, 4-Chloroethcathinone (4-CEC), mephedrone, 4-Chloromethcathinone (4-CMC), 3-Methylmethcathinone (3-MEC), *N*-ethylpentedrone, α-Pyrrolidinopentiothiophenone (α-PVT), α-Pyrrolidinopentiophenone (α-PVP), *N*-ethylhexedrone, 4-Fluoro-α-pyrrolidinopentiophenone (4 F-α-PVP), and α-Pyrrolidinoisohexanophenone (α-PiHP), methcathinone-d3 and α-pyrrolidinopentiophenone-d8 (α-PVP-d8) were purchased from Chiron AS.

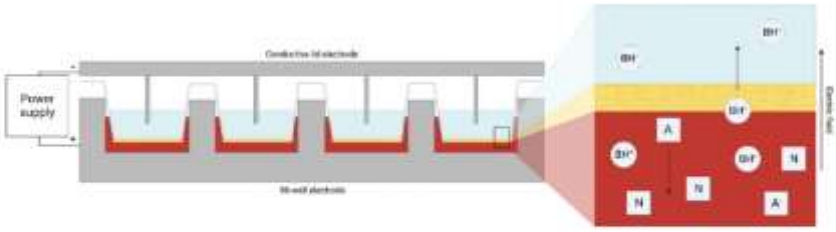

Fig. 2. Principle of EME in the 96-well plate setup. First, samples are placed in the bottom conductive plate. The organic solvent is then pipetted onto the porous membranes and the acceptor solution is added to the wells of the top plate. Finally, the conductive lid with the rods is placed on top of the acceptor plate and the system is connected to a power supply so the electric field can be created. For the extraction of basic compounds, cations migrate towards the acceptor solution where the cath-ode is placed. BH^+: protonated basic compounds; A^-: deprotonated acidic compounds; N: neutral compounds.

2.2. Samples and working solutions

Human blood samples were supplied by the Blood Bank of Oslo (Oslo University Hospital) and stored at −20 °C before use. All stock solutions were diluted into working solutions using the donor phase as diluent (250 mM formic acid).

2.3. EME procedure

The EME setup was performed as previously described (Eibak et al., 2014; Schuller et al., 2023a; Schüller et al., 2023b). In a laboratory-built 96-well format, extractions were performed using a conductive stainless-steel 96-well plate and a MultiScreen-IP filter 96-well plate from Merck Millipore (Carrigtwohill, Ireland) as donor and acceptor, respectively. A laboratory-built conductive stainless-steel lid with 96 rods was placed on top of the acceptor plate and served as the cathode (Fig. 2). This setup was agitated during extractions with a Vibramax 100 agitation system from Heidolph (Kellheim, Germany).

The specific extraction conditions were as follows. The donor phase consisted of a 1:2 dilution of whole blood:buffer (v/v); that is 120 μL of whole blood, 100 μL of 250 mM formic acid, 10 μL of a pool of analytes, and 10 μL of the pool of internal standards, with a final volume of 240 μL. Both analytes and internal standards were prepared in solutions of 250 mM formic acid. The supported liquid membrane (SLM) was created by pipetting 3 μL of 2-Undecanone directly onto the filter and the acceptor phase consisted of 50 μL of 100 mM formic acid. Once the plates were put together, the conductive lid was placed on top and the setup was connected to a power supply set to 40 V (model ES 0300–0.45, Delta Elektronika BV, Zierikzee, the Netherlands). The system remained under 725 rpm of agitation for 35 min.

2.4. Instrumentation

All analyses were performed with an Acquity UPLC coupled to a Xevo-TQ triple quadrupole (Waters, Milford, MA, USA). The chromatographic separation was achieved on a Kinetex Biphenyl column (2.1 × 100 mm, 1.7 μm; Phenomenex, Torrance, CA, USA). Mobile phases A and B consisted of ammonium formate (10 mM, pH 3.1) and MeOH, respectively, at a flow rate of 0.5 mL/min. The gradient was as follows: 0.2–0.3 min, 10–25% B; 0.3–2.8 min, 25–30% B; 2.8–3.5 min, 30–45% B; 3.5–4.0 min, 45–100% B and kept for another 0.5 min; 4.5–4.6 min, 100-10% B and kept for another 0.9 min. The injection volume was 0.8 μL and the column was kept at 60 °C for the entire 5.5-min run.

The mass spectrometer operated with positive electrospray ionization and multiple reaction monitoring was used. A capillary voltage of 0.5 kV, ion source temperature of 150 °C, cone gas flow of 300 L/h, and desolvation gas flow of 1000 L/h at 500 °C were used. The MassLynx 4.2 software from Waters was used to process all data. The specific MS conditions of the analytes studied herein are summarized in Table S1.

2.5. EME optimization

A Box-Behnken design was performed considering the relevant variables for extraction efficiency on EME. The SLM, voltage, extraction time, and agitation rate were chosen based on initial tests and previous studies (Skaalvik et al., 2021, 2023a, 2023b; Schuller et al., 2023a; Schuller et al., 2023b, 2023c; Schüller et al., 2023a; Zhou et al., 2023; Hansen et al., 2021a; Rye et al., 2021). In total, the combination of these four conditions resulted in 34 experiments that were performed in triplicate. From the obtained results, predictive models were constructed using multiple linear regression, assisted by analysis-of-variance (ANOVA), and applied for the determination of optimal EME parameters settings. The analysis was performed using Design-Expert 13 (Stat-Ease Inc., Minneapolis, MN, USA).

2.6. Method validation

Validation was performed according to the ANSI/ASB Standard 036 for method validation in forensic toxicology (AAFS Standards Board, 2019) and supporting literature (Matuszewski et al., 2003).

2.6.1. Limit of detection, limit of quantitation, and linearity

The linearity range was based on relevant concentrations commonly found for SC (Lau et al., 2020; Fabris et al., 2023; Adamowicz, 2021). The calibration model for all analytes was considered acceptable when the coefficient of correlation (r^2) ≥ 0.99. For heteroscedasticity correction, 1/x weighing was applied. The limit of quantitation (LOQ) was then selected as the lowest concentration of the linearity range and was defined when both accuracy and imprecision criteria were met (± 20% and ≤20%, respectively). The limit of detection (LOD) was estimated from scalar dilutions starting at the LOQ and was considered acceptable when signal-to-noise ratio ≥3:1.

2.6.2. Accuracy and imprecision

For the evaluation of accuracy and imprecision, four sets of quality controls (QC) were assessed with freshly prepared calibration curves on five different days. Within-run and between-run imprecisions were then calculated by ANOVA considering all experiments and are expressed as coefficient of variation (CV). The minimum requirements for accuracy and imprecision were variations of ± 20% and ≤20%, respectively.

2.6.3. Analyte recovery and matrix effect

In this study, three different groups were considered: i) neat analyte injection, ii) samples spiked pre-extraction, and iii) samples spiked post-extraction. This experiment was performed in quintuplicate, whereas each replicate was a sample from a different donor

(Matuszewski et al., 2003). Internal standards were added post-extraction in groups ii and ii, so the relative area could be considered for all analyses.

The estimation of extraction recovery (RE) was calculated at the QC levels as the ratio of groups ii by ii. Similarly, the matrix effect (ME) was calculated as the ratio of groups iii by i. Results for RE and ME are expressed as percentages.

2.6.4. Selectivity and carryover

Ten blank samples from different donors were simultaneously extracted and analyzed by the method to evaluate the presence of endogenous interfering compounds. Additionally, a pool containing common exogenous substances at the concentration of 1–24 μM was injected and analyzed by the proposed method. This pool contained buprenorphine, clonazepam, alprazolam, cocaine, benzoylecgonine, morphine, codeine, oxycodone, methadone, tramadol, nitrazepam, diazepam, *N*-desmethyl diazepam, oxazepam, zopiclone, zolpidem, amphetamine, methamphetamine, 3,4-Methylenedioxymethamphetamine, delta-9-tetrahydrocannabinol, and phosphatidylethanol 16:0/18:1.

Carryover between injections was investigated by monitoring chromatograms of blank injections after the highest calibration standards.

2.6.5. Stability of processed samples

The response from twelve extracted samples run at the start and end of a sequence of 96 samples were compared to controls to verify if the time difference would influence the results.

3. Results and discussion

3.1. Selection of the liquid membrane

The cathinone analogs included in this study have low to moderate polarity ($1.18 \leq \log P \leq 3.65$) (Table S2). Extracting these compounds simultaneously can be complicated due to this wide polarity range.

In a first set of experiments, NPOE and 2-Undecanone were tested as the first choices of liquid membranes. These are recommended to extract analytes with polarities within the log P range of 2.2–6.4 and 1.0 to 5.8, respectively (Zhou et al., 2023). In addition, some modifications to NPOE have previously been described to increase the extraction efficiency of more polar analytes. For example, the addition of the ionic carrier DEHP and the mixture of 6 MC and Thy have widened the extraction window of NPOE (Skaalvik et al., 2023b; Schüller et al., 2023c; Hansen et al., 2021a). These additives improve the mass transfer of highly polar compounds from the sample to the acceptor for increasing their affinity to the SLM (Hansen et al., 2021a; Hansen et al., 2021b). Thus, the π-type interactions and hydrogen bond acceptor properties of these additives have been demonstrated beneficial for the extraction of polar basic compounds. Therefore, these alternatives were also included in the first assessment of SLM candidates.

Extractions were carried out for 30 min but the voltages had to be adjusted based on the properties of each liquid membrane. NPOE and NPOE +1% DEHP were more stable and thus 100 V could be applied. In contrast, 2-Undecanone and a 1:2 (v/v) mixture of 6 MC:Thy (1:2, molar ratio) and NPOE (NPOE + 6 MC:Thy) produced higher currents (>50 μA per sample), thus lower voltage was used (60 V) (Skaalvik et al., 2023b; Schüller et al., 2023a). This was based on previous studies that established 50 μA per sample as the recommended limit in EME (Hansen et al., 2020). Higher currents negatively affect extraction performance due to electrolysis that cause bubble formation and drifts the pH in both the sample and acceptor (Zhou et al., 2023, 2024; Hansen et al., 2020).

Interestingly, SC in the lower log P range were not efficiently extracted with NPOE alone, and adding DEHP did not improve this outcome. On the other hand, adding 6 MC:Thy to NPOE significantly improved the recoveries, reaching similar values to those achieved with 2-Undecanone alone (>55%) (Fig. 3). These findings are in agreement with previous data showing that NPOE is not very efficient for basic compounds of moderate polarities ($1.0 \leq \log P \leq 2.0$) but can be improved with additives in the SLM (Skaalvik et al., 2023b; Schüller et al., 2023a; Zhou et al., 2023; Hansen et al., 2021a). In contrast, 2-Undecanone alone efficiently extracted all the SC from whole blood samples. This also corroborates previous data that recommends using this liquid membrane instead of NPOE for compounds with log P ranging from 1.0 to 5.8 (Zhou et al., 2023).

3.2. Optimizing EME conditions

A Box-Behnken design was performed to optimize operational parameters with both NPOE + 6 MC:Thy and 2-Undecanone. Typically, exhaustive extractions in EME are achieved in under 60 min (Skaalvik et al., 2021, 2023a, 2023b; Schuller et al., 2023a; Schüller et al., 2023b; Rye et al., 2021). The extractions in the previous experiment (Section 3.1) were carried out for 30 min and moderate recoveries were achieved, thus similar extraction durations were considered for optimization (20–40 min). After a few exploratory experiments, high extraction efficiencies were found to be sustained at lower voltages than previously used. The voltage range of the optimization study was therefore set to 30–50 V. The fourth parameter included was the agitation rate (400–1000 rpm), which is important for facilitating mass transfer in the sample and acceptor solution.

After performing the experimental plan, the initial data analysis indicated that individual analyte extraction recoveries were highly correlated to the average of all SC. This indicated that all analytes responded in the same manner to changes in EME parameter settings. For subsequent modelling, the response variable was thus defined as the average recovery of all SC, and model terms were taken as significant for ANOVA p-values <0.05. Details on the model statistics are provided in Supplementary information (Tables S3 and S4). Fig. 4 shows the effect of the four parameters on average SC recovery.

For both SLMs (NPOE + 6 MC:Thy and 2-Undecanone), the agitation rate was the most important factor for extraction recovery followed by the voltage, and extraction time to a lesser extent (Fig. 4).

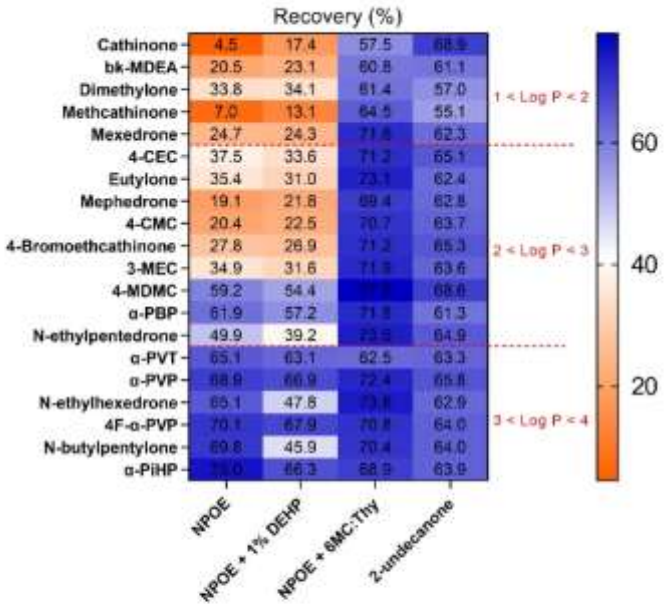

Fig. 3. Recoveries achieved with different liquid membranes. Four liquid membranes (NPOE, NPOE + 1% DEHP, NPOE + 6 MC:Thy, and 2-Undecanone) were studied as first choices for the target basic analytes. Comparisons were based on analyte recovery.

With 2-Undecanone as SLM, the optimal agitation rate was reached at around 800 rpm and increasing the agitation rate beyond this value caused no major gain in analyte recovery (Fig. 4). Interestingly, agitation rate impacted the extraction more drastically with NPOE + 6 MC:Thy as SLM, as recoveries close to zero were achieved at 400 rpm compared to exhaustive extractions achieved at 1000 rpm (>90%) (Fig. 4). In contrast, 2-Undecanone at 400 rpm provided recoveries of about 60%, and increasing this value only slightly improved the extraction efficiency (≈80 %) (Fig. 4). These differences in extraction efficiency between SLM may be associated with the higher viscosity of NPOE + 6 MC:Thy compared to 2-Undecanone.

The voltage and extraction time also played a relevant role in extraction efficiency, although these effects were secondary to the agitation rate. The effects were also independent of agitation rate and type of liquid membrane, however, an interaction occurred between voltage and extraction time (Fig. 4). At shorter extraction durations (for example 20 min) the optimal voltage was 45 V, while at 40 min the optimal voltage was around 35 V. This interaction may be explained by the effects of electrolysis and pH changes in the acceptor solution, which are expected to be more prominent for both increasing voltage and time. The highest efficiency was obtained at 30–40 V for 35 min.

Both liquid membranes studied in this optimization step were able to provide high predicted recoveries (>80%) for all SC aimed to include in the method (Table 1). This could be achieved with different combinations of the variables included in this optimization study. From the model, NPOE + 6 MC:Thy was predicted to be slightly more efficient, however, preparing the mixture of NPOE + 6 MC:Thy requires mixing three components and may introduce variability into the method. Consequently, 2-Undecanone was preferred for the sake of simplicity. The optimum extraction conditions defined for validation were 2-Undecanone as SLM and 40 V for 35 min. Agitation was set to 725 rpm, which is the nearest instrument setting to the predicted optimum of 800 rpm.

3.3. Method validation

The EME technique was validated after optimizing relevant extraction conditions. The validation results are summarized in Table 2, and the corresponding chromatograms of the compounds at the LOQ concentration can be found in Fig. 5.

The calibration range was defined as 1–500 ng/mL to fit all cathinone derivatives covered by the method. The response was linear to all analytes with $r^2 \geq 0.99$. The 1 ng/mL concentration in blood suffices to analyze quantitatively most cases involving SC and was thus established as LOQ. On the other hand, LOD was established as 0.1 ng/mL for all analytes, except for 4-CMC with a LOD of 0.5 ng/mL (Table 2).

The accuracy and imprecision study showed bias was ±12%, within-run CV ≤ 18%, and between-run CV ≤ 19%. Overall values were considered acceptable according to validation guidelines. RE for all analytes were within 79%–117%, except for methcathinone (63–73%), and ME ranged from 97 to 117% (Table 2). Finally, no peaks of interfering compounds were observed in the retention

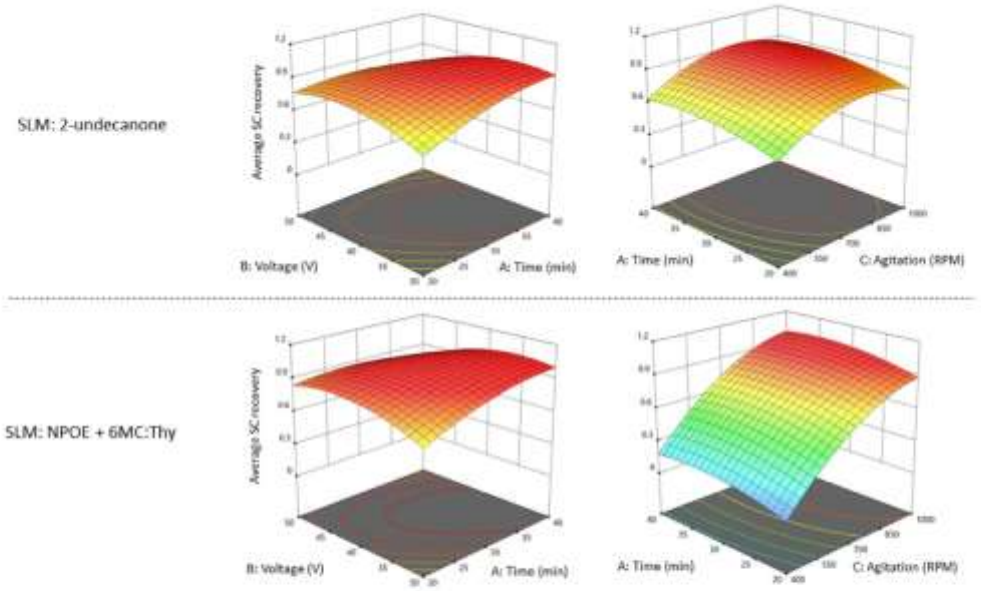

Fig. 4. Surface plots of the average SC recovery (z-axis) as a function of A: Time, B: Voltage, C: Agitation rate, and D: SLM composition (2-Undecanone or NPOE + 6 MC:Thy). For each plot, the factor that is not displayed was set to its optimum value.

Table 1
Prediction of exhaustive recoveries based on optimization data.

Analytes	Predicted RE (%)	
	NPOE+6 MC:Thy	2-Undecanone
Cathinone	83	80
bk-MDEA	102	97
Dimethylone	97	95
Methcathinone	88	87
Mexedrone	99	98
4-CEC	103	99
Eutylone	102	97
Mephedrone	101	97
4-CMC	98	97
4-bromomethcathinone	104	99
3-MEC	101	98
4-MDMC	103	101
α-PBP	105	98
N-ethylpentedrone	103	100
α-PVT	106	99
α-PVP	105	97
N-ethylhexedrone	106	98
4-F-α-PVP	105	98
N-butylpentylone	103	95
α-PiHP	105	96

RE: recovery.

times of the SC included in the method indicating acceptable selectivity. Carryover was not observed in blank samples after high concentration standards were injected.

The proposed method was successfully validated for all target SC, although high CV (≥15%) was observed for some analytes (Table 2). Nevertheless, these values are still within acceptable limits (AAFS Standards Board, 2019). Additionally, LOD and LOQ values achieved with the 96-well plate setup described herein are in agreement with other works that propose methods to analyze SC in

Table 2
Summary of validation results.

Analyte, QC conc. (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Calibration range* (ng/mL)	Accuracy (%)	Imprecision (%)		RE** (%)	ME** (%)
					Within run	Between run		
Methcathinone								
2	0.1	1	1–500	−3.5	6.9	7.9		
15				6.3	11.7	9.0	70	112
200				7.0	8.8	14.9	63	101
400				0.5	9.2	9.8	73	98
Mexedrone								
2	0.1	1	1–500	−2.5	9.2	10.6		
15				−4.6	6.2	12.4	113	115
200				3.7	11.2	12.2	79	105
400				1.4	13.6	14.2	88	104
4-CEC								
2	0.1	1	1–500	−7.8	15.1	16.0		
15				−11.3	5.9	13.0	94	98
200				5.3	10.7	11.4	85	107
400				0	11.5	11.7	96	106
Eutylone								
2	0.1	1	1–500	−7.8	12.3	14.2		
15				−10.9	10.5	14.7	100	102
200				7.0	7.4	7.8	85	102
400				0.3	11.5	11.9	93	103
Mephedrone								
2	0.1	1	1–500	−6.9	11.3	12.1		
15				−10.2	16.2	18.7	98	114
200				6.2	5.3	5.9	79	103
400				0.8	11.7	12.1	90	101
4-CMC								
2	0.5	1	1–500	−5.5	13.0	14.6		
15				−9.0	8.3	13.1	117	109
200				5.5	7.2	7.5	82	102
400				0.4	12.4	12.7	92	102
3-MPC								
2	0.1	1	1–500	−8.1	10.6	12.3		
15				13.3	−11.3	11.9	102	115
200				5.3	8.4	9.0	85	104
400				1.0	11.1	11.3	95	106
N-ethylpentedrone								
2	0.1	1	1–500	−9.5	12.5	13.8		
15				−8.9	11.7	16.7	110	117
200				3.3	6.7	7.3	88	104
400				0	11.4	11.6	101	101
α-PVP								
2	0.1	1	1–500	−4.3	16.0	17.0		
15				15.3	2.1	11.3	104	103
200				2.5	6.6	7.1	86	99
400				−0.2	15.2	15.6	94	97
N-ethylhexedrone								
2	0.1	1	1–500	−11.8	18.4	19.2		
15				−8.2	12.4	17.8	112	118
200				1.3	11.1	11.7	96	110
400				−1.4	14.7	15.5	102	110
α-PiHP								
2	0.1	1	1–500	−4.6	15.7	16.3		
15				−2.6	11.5	14.5	90	112
200				−0.7	12.1	12.5	86	111
400				−2.0	17.3	18.1	100	103

* Weighting of 1/x was applied to the calibration model. ** RE and ME experiments were carried out only for the 2, 200, and 400 ng/mL QC. QC: quality control; LOD: limit of detection; LOQ: limit of quantitation; RE: recovery; ME: matrix effect.

blood samples (Lau et al., 2020; Fabris et al., 2023; Hong et al., 2022; Odoardi et al., 2015). These validation results demonstrate that the EME described herein fits the intended purpose.

The recoveries achieved were >75% for all SC herein studied, except for methcathinone. Thus, exhaustive recoveries could be achieved for most compounds (Table 2). In addition, ME values were <20% for all target SC, indicating excellent sample clean-up. This finding is in agreement with previous works that reported LPME-base techniques such as EME providing high selectivity for ion-

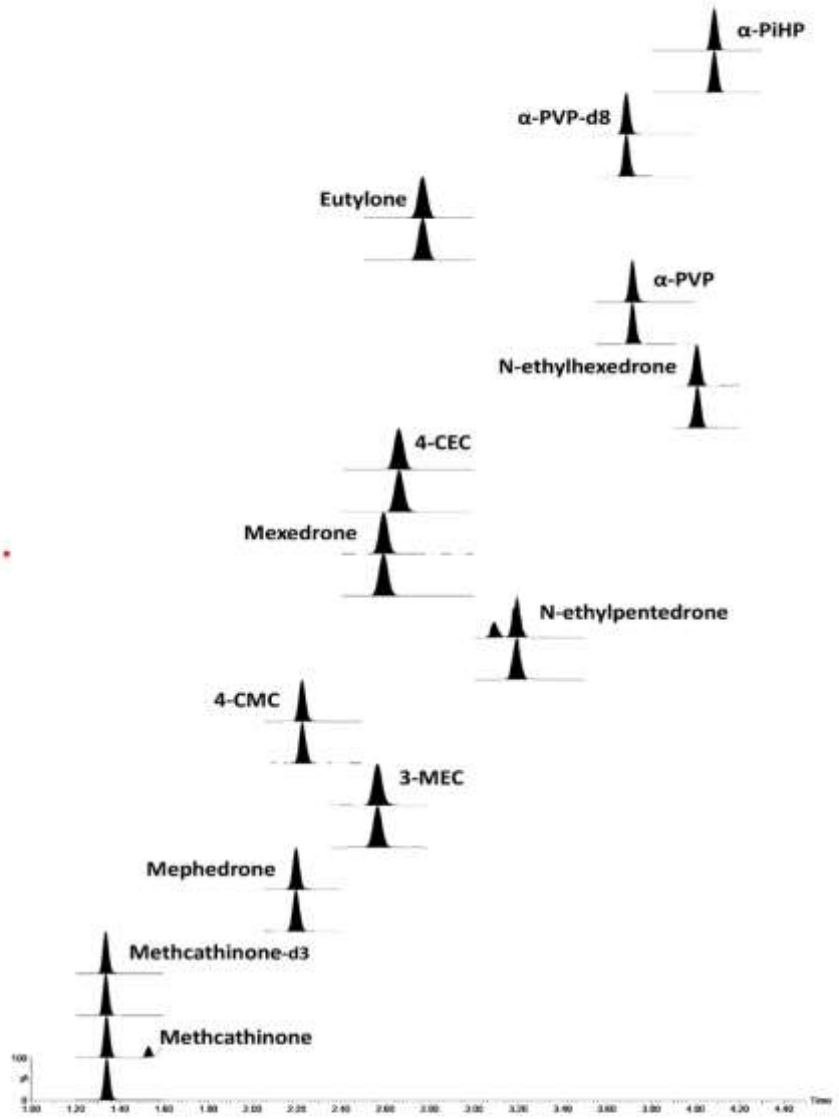

Fig. 5. Chromatograms of the SC included in the method at LOQ (1 ng/mL).

izable compounds while avoiding the extraction of interferents that cause intense ME, such as phospholipids (Bavlovic Piskackova et al., 2022; Skaalvik et al., 2021).

No evidence of sample degradation was observed during a series with 96 samples. The difference in response at the end of the assay was on average within 16% of the response at the start. This experiment shows that the 96-well plate setup proposed in this work can be used at its maximum capacity with no significant impact on the results over the time required to inject all extracted samples.

3.4. Practical application of the proposed EME method

Comparing the EME presented in this work with other methods aimed at analyzing SC in biological samples highlights the advantages of the technique. For example, Hu et al. described an electromembrane microextraction-assisted fluorescent molecularly imprinted polymer to detect SC in urine samples (Hu et al., 2022). In this work, analyte recovery was lower and the setup was not high-throughput, thus limiting the application of this technique in its current state. Similarly, Hong et al. presented an EME system to analyze SC in whole blood and urine samples but using a different setup (Hong et al., 2022). Although decent recovery values were achieved for the analytes included in the study, the EME system was not high-throughput, which faces the same limitation of applicability in laboratories with large routine casework.

For simplicity purposes, protein precipitation is frequently used to clean up biological samples prior to instrument injection. Comparing our EME to a method analysing a range of NPS, including SC (Giorgetti et al., 2022), EME requires less volume of samples and hazardous organic solvents than protein precipitation. Additionally, the workflow with EME is simple and involves the extraction of the analytes into aqueous solutions, which are injected directly into LC-MS. The protein precipitation method, on the other hand, involved multiple operations, including solvent evaporation and reconstitution to make samples compatible with LC-MS. Finally, while protein precipitation mainly removes proteins, EME removes many more endogenous components and provides a very clean sample for LC-MS analysis. A detailed comparison between the EME presented in this work with the mentioned protein precipitation procedure for SC (Giorgetti et al., 2022) is provided in Table 3.

3.5. Greenness score

Estimating the environmental impact of an analytical technique has become relevant in recent years. Wojnowski et al. have proposed an evaluation tool to assess various steps of the sample preparation procedure: AGREEprep (Wojnowski et al., 2022). With this approach, it is possible to attribute a score to the proposed method providing a clearer indication of its environmental impact. The greenness score of the EME performed with the prototype setup herein described is depicted in Fig. 6.

The greenness score ranges from 0 to 1, where the extremes represent the worst and best performances, respectively. The score of the EME developed in the present work was of 0.77 and is thus considered very good (Fig. 6) (Wojnowski et al., 2022). The lowest scores were attributed to sample preparation placement (1), reusability of the materials (3), lack of automation (7), and type of analytical instrumentation (9), which should be addressed in future works. Overall, the score obtained with the proposed EME is similar to those obtained in other studies with the same technique, demonstrating this prototype setup meets the demands of future analytical laboratories in terms of environmental impact even though some steps can still be improved (Skaalvik et al., 2023a; Schuller et al., 2023a).

4. Conclusions

In this work, a prototype 96-well plate setup for EME was used to study the application of generic liquid membranes to basic compounds. Cathinone analogs were chosen as model monobasic substances due to their increasing relevance in toxicology and whole blood was the sample of choice as it is the preferred matrix in forensic toxicological analysis.

Based on the log P of the target compounds, NPOE and 2-Undecanone are the first choices of SLM. However, using NPOE alone was not efficient for extracting SC with moderate polarity. This limitation was overcome by the addition of additives that improved

Table 3
Comparison of EME and protein precipitation methods for cathinones in whole blood.

EME	Protein precipitation
Sample volume	**Sample volume**
120 µL of whole blood	500 µL of whole blood
Solvent, chemicals, and gas per sample	**Solvent, chemicals, and gas per sample**
Undecanone, 3 µL	Acetonitrile, 1650 µL
Formic acid, 1 µL	Formic acid, 1 µL
	Nitrogen gas
Pictograms and signal words x 3	**Pictograms and signal words** x 4
Warning x 1, danger x 1	Danger x 2
Pipetting x 5	**Pipetting** x 5
Operations	**Operations**
Extraction	Vortexing
LC-MS	Centrifugation
	Evaporation
	Reconstitution
	LC-MS
Cleanup	**Cleanup**
Phospholipids	Proteins
Proteins	
Lipids	
Salts	
Neutral substances	
Acidic substances	

Fig. 6. AGREEprep greenness score of the EME developed in this study. The overall score is at the circle in the centre. Around the circle are the 10 criteria evaluated by this assessment (sample preparation placement; hazardous materials; sustainability, renewability, and reusability of materials; waste; size economy of the sample; sample throughput; integration and automation; energy consumption; post-sample preparation configuration for analysis; operator's safety, respectively).

the applicability of NPOE to compounds with log P ≤ 2. In contrast, 2-Undecanone alone sufficed to achieve high recoveries, dismissing the need for any modifications to this liquid membrane. This is in agreement with the recommended polarity range for this SLM ($1.0 \leq \log P \leq 5.0$). After optimizing other relevant EME conditions, the technique was successfully validated in this prototype setup.

This work served to illustrate the practical application of recommended EME conditions to new compounds. Hence, generic liquid membranes can be used for novel applications with slight adjustments or modifications. Additionally, these recommended extraction conditions were previously determined for plasma samples, thus this study also demonstrated the compatibility and performance in whole blood (Zhou et al., 2023, 2024; Hansen et al., 2021a; Hansen et al., 2021a, 2021b). Although a small group of SC were included in the present work when compared to all SC that have at one point been a part of the international drug market, 2-Undecanone proved to be efficient in extracting these compounds within a wide polarity range, indicating other cathinone derivatives can be easily included. Thus, the successful application of this single-step extraction in a simpler setup using pre-established recommended extraction conditions brings EME one step closer to being implemented in routine laboratories, providing the possibility of greener and more sustainable sample preparation in forensic toxicology. In following works, it would be advisable to perform comparison studies between this 96-well plate setup and the commercially available conductive vial format to closely evaluate the performance of both systems with generic membranes. Such studies should contribute not only to the analysis of drugs of abuse in biological samples but also to paving the way to make EME an established sample preparation alternative for routine applications.

CRediT authorship contribution statement

André L. Fabris: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Conceptualization. **Frederik A. Hansen:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Mauricio Yonamine:** Writing – review & editing, Funding acquisition. **Stig Pedersen-Bjergaard:** Writing – original draft, Supervision, Resources, Methodology, Conceptualization. **Elisabeth L. Øiestad:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

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.

Data availability

Data will be made available on request.

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The graphical abstract was made using BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2024.101494.

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9. CONCLUSÕES

Quatro técnicas de extração destinadas a analisar as NPS em amostras biológicas foram desenvolvidas, otimizadas e validadas no presente projeto de pesquisa. As classes das catinonas sintéticas, canabinoides sintéticos e fenetilaminas foram focadas devido sua relevância em território nacional. Entretanto, outras drogas de abuso também foram incluídas em alguns destes estudos, por exemplo a cetamina, anfetaminas, LSD, dentre outros. Durante a execução do presente trabalho, os princípios da GAT foram levados em consideração e aplicados sempre que possível. Assim, alternativas mais sustentáveis foram apresentadas, como substituição dos solventes clorados em DLLME por misturas menos nocivas, o uso de técnicas miniaturizadas, que são mais econômicas e usam menos de 300 µL de amostra, a completa substituição de solventes orgânicos por óleos essenciais, a implementação de uma técnica de microextração pela primeira vez no Brasil (PALME), dentre outros.

O projeto desenvolvido contribuiu com a difusão dos conceitos da GAT, tanto proporcionando técnicas para serem utilizadas e adaptadas bem como exemplificando a aplicação destes conceitos no desenvolvimento de métodos. Além disso, as metodologias aqui apresentadas podem ser ferramentas úteis no controle das NPS no Brasil e em outros países, visto que os trabalhos estão disponíveis em revistas científicas internacionais.

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11. ANEXOS

11.1 Aprovação do uso de amostras biológicas de humanos pelo Comitê de Ética em Pesquisa

UNICAMP - CAMPUS CAMPINAS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PROJETO INSPEQT: INVESTIGAÇÃO DE NOVAS SUBSTÂNCIAS PSICOATIVAS EM AMOSTRAS BIOLÓGICAS APLICADAS EM CASOS DE TOXICOLOGIA

Pesquisador: Jose Luiz da Costa

Área Temática:

Versão: 2

CAAE: 46404121.8.0000.5404

Instituição Proponente: Faculdade de Ciências Farmacêuticas

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.768.570

Apresentação do Projeto:

As informações contidas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa", "Avaliação dos Riscos e Benefícios" e "Comentários e Considerações sobre a Pesquisa" foram obtidas dos documentos apresentados para apreciação ética pelo CEP e das informações inseridas pelo(a) pesquisador(a) responsável pelo estudo, na Plataforma Brasil.

O uso de substâncias psicoativas faz parte da história da humanidade. Existem evidências arqueológicas de que há milhares de anos, nossos ancestrais já buscavam na natureza plantas que alterassem o estado mental e a percepção dos sentidos (Guerra-Doce, 2015). Ao mesmo tempo, nada indica que o Homem um dia abandonará esse hábito, como dito uma vez por Aldous Huxley, em sua obra "As portas da percepção" de 1954: "Parece improvável que a humanidade em geral seja algum dia capaz de dispensar os 'paraísos artificiais', isto é... a busca da autotranscedência através das drogas ou... umas férias químicas de si mesmo..."Similarmente a outros fenômenos sociais, o que ocorre é que o padrão de uso de substâncias psicoativas continua a se alterar com o passar do tempo, sendo moldado por novidades e 'modismos', disponibilidade de novas drogas e produtos, novas formas de uso, formação de novos grupos sociais de usuários, globalização e efeitos das políticas de saúde e segurança públicas implementadas no país (EvansBrown e Sederov, 2018). Contudo, o fenômeno do surgimento das novas substâncias psicoativas (NSP), que emergiu na última década, pode ser considerado sem precedentes em termos do número de substâncias que vem sendo

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Biorepositório / Biobanco	regulamento_de_biorrepositorio_INSPEQT.pdf	14/04/2021 14:11:06	Jose Luiz da Costa	Aceito
Outros	matricula_JLC_CEP.pdf	14/04/2021 13:27:52	Jose Luiz da Costa	Aceito
Declaração de Instituição e Infraestrutura	anuencia_IAPF.pdf	14/04/2021 13:26:43	Jose Luiz da Costa	Aceito
Declaração de concordância	anuencia_SPTC.pdf	14/04/2021 13:24:33	Jose Luiz da Costa	Aceito
Folha de Rosto	folhaDeRosto_INSPEQT_JLC_1269734.pdf	14/04/2021 13:11:45	Jose Luiz da Costa	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 11 de Junho de 2021

Assinado por:
Renata Maria dos Santos Celeghini
(Coordenador(a))

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PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PROJETO INSPEQT: INVESTIGAÇÃO DE NOVAS SUBSTÂNCIAS PSICOATIVAS EM AMOSTRAS BIOLÓGICAS APLICADAS EM CASOS DE TOXICOLOGIA

Pesquisador: Jose Luiz da Costa

Área Temática:

Versão: 1

CAAE: 46404121.8.3001.0067

Instituição Proponente: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.831.168

Apresentação do Projeto:

De acordo com a United Nations Office on Drugs and Crime (UNODC), as novas substâncias psicoativas (NSP) são definidas como "novos narcóticos ou drogas psicotrópicas, apresentadas na forma pura ou preparadas, não controladas pela Convenção Única das Nações Unidas sobre Drogas Narcóticas de 1961 e a Convenção das Nações Unidas sobre Substâncias Psicotrópicas de 1971, mas que podem constituir ameaça à saúde pública, comparáveis a aquelas substâncias listadas nessas convenções". É um fenômeno global e emergente, com 111 países tendo reportado a apreensão e a identificação de mais de 800 compostos, dentre os quais estão canabinoides sintéticos, estimulantes (incluindo anfetaminas, catinonas e piperazinas), alucinógenos (triptaminas e derivados da fenciclidina), opioides sintéticos (principalmente substâncias derivadas do fentanil), benzodiazepínicos e drogas baseadas em plantas. As NSP têm desafiado as abordagens tradicionais de monitoramento, vigilância, controle e medidas de proteção à saúde pública com relação ao tráfico e abuso de drogas. Dificuldades específicas incluem a grande quantidade e diversidade de substâncias classificadas como NSP, a velocidade com que elas entram e saem do mercado ilícito, as diferentes formas de apresentação das drogas, a dificuldade de identificação química das NSP e o desconhecimento da potência e gama de seus efeitos tóxicos em usuários.

Projeto apresentado pela UNICAMP, com financiamento do Programa de Cooperação Acadêmica em

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Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 06 de Julho de 2021

Assinado por:
Neuza Mariko Aymoto Hassimotto
(Coordenador(a))