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Desenvolvimento de um Método Analítico Inovador para Determinação Forense de Cocaína, Antidepressivos e Substâncias Correlatas em Sangue *Post Mortem* Utilizando Nanopartículas Magnéticas

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Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção de grau de Mestre.

Orientador: Prof. Dr. Tiago Franco de Oliveira

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RESUMO

O consumo generalizado e a subsequente dependência de substâncias psicoativas (SPAs) têm um impacto significativo na saúde pública, resultando em mudanças nos padrões de mortalidade da população. Substâncias como a cocaína e os antidepressivos destacam-se nesse cenário, sendo frequentemente associadas a fatalidades e suicídios. A determinação precisa desses compostos e de seus produtos de biotransformação em amostras biológicas torna-se crucial para compreender os seus efeitos e para determinar sua contribuição em casos de intoxicação. Portanto, técnicas de extração miniaturizadas estão sendo desenvolvidas para atender às demandas deixadas pelos métodos de extração tradicionais, relacionadas ao elevado volume de solventes orgânicos utilizado e à complexidade dos processos de extração. A exploração do potencial das nanopartículas magnéticas (MNPs) como sorventes pode abrir novas perspectivas na extração de SPAs de matrizes biológicas. Assim, este estudo está focado no desenvolvimento de uma metodologia analítica inovadora para a quantificação de amitriptilina, benzoilecgonina, citalopram, cocaetileno, cocaína, desipramina, desvenlafaxina, éster metílico de ecgonina, fluoxetina, imipramina, norfluoxetina, nortriptilina, sertralina, e venlafaxina, em amostras de sangue *post mortem*, utilizando nanopartículas magnéticas de Fe_3O_4 como sorvente para a e extração em fase sólida dispersiva magnética (m-dSPE), em conjunto com a análise por cromatografia líquida acoplada a espectrometria de massas em tandem. Metodologias de superfície de resposta foram realizadas para otimizar os parâmetros que influenciam a atividade das nanopartículas, utilizando amostras de sangue fortificadas com os analitos. O menor limite de quantificação obtido foi de 10 ng/ml para todos os 15 analitos. O método desenvolvido mostrou-se linear, com uma curva de calibração variando de 10 a 1000 ng/ml. Experimentos de precisão e exatidão intra e inter-dia, bem como efeito matriz se mostraram adequados aos parâmetros do guia de validação. Após a completa validação o método foi aplicado com sucesso a 263 amostras de casos reais, possibilitando a quantificação de diversas substâncias. Esses resultados confirmam a primeira aplicação deste sorvente na análise de sangue *post mortem*, caracterizando-o como uma alternativa eficaz e sustentável às técnicas convencionais para trabalhos forenses.

Palavras-Chave: Cocaína, antidepressivos, nanopartículas magnéticas, extração miniaturizada, sangue *post mortem*.

ABSTRACT

The widespread consumption and subsequent dependence on psychoactive substances (PAS) have a significant impact on public health, resulting in changes in population mortality patterns. Substances such as cocaine and antidepressants stand out in this scenario, often associated with fatalities and suicides. The precise determination of these compounds and their biotransformation products in biological samples becomes crucial for understanding the effects of these substances and for determining their contribution in cases of intoxication. Therefore, miniaturized extraction techniques are being developed to meet the demands left by traditional extraction methods and to quantify various categories of PAS used in toxicological analysis. Exploring the potential of magnetic nanoparticles (MNPs) as sorbents may open new perspectives in the extraction of PAS from biological matrices. Thus, this study focuses on the development of an innovative analytical methodology for the quantification of amitriptyline, benzoylecgonine, bupropion, citalopram, cocaethylene, cocaine, desipramine, desvenlafaxine, EME, fluoxetine, imipramine, norfluoxetine, nortriptyline, sertraline, and venlafaxine, in post-mortem blood samples, using Fe_3O_4 magnetic nanoparticles as sorbent for magnetic dispersive solid-phase extraction (m-dSPE), coupled with liquid chromatography tandem mass spectrometry analysis. Response surface methodologies were performed to optimize the parameters influencing the activity of nanoparticles, using blood samples fortified with analytes. The lowest quantification limit obtained was 10 ng/ml for all 15 analytes. The developed method proved to be linear, with a calibration curve ranging from 10 to 1000 ng/ml. Intra-day and inter-day precision and accuracy experiments, as well as matrix effect assessments, were found to be adequate according to the validation guideline parameters. Following complete validation, the method was successfully applied to 263 samples from real cases, enabling the quantification of various substances. These results confirm the first application of this sorbent in *post mortem* blood analysis, characterizing it as an effective and sustainable alternative to conventional techniques for forensic work.

Keywords: Cocaine, antidepressants, magnetic nanoparticles, miniaturized extraction, *post mortem* blood.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

5-HT	Serotonina
AAFS	Academia Americana de Ciências Forenses, do inglês "American Academy of Forensic Sciences"
ANVISA	Agência Nacional de Vigilância Sanitária
ANSI	Instituto Nacional Americano de Padrões, do inglês "American National Standards Institute"
CYP450	Citocromo P450
DA	Dopamina
dSPE	Extração em Fase Sólida Dispersiva, do inglês "Dispersive Solid Phase Extraction"
d-μSPE	Microextração em Fase Sólida Dispersiva, do inglês "Dispersive Solid Phase Microextraction
EME	Éster Metílico de Ecgonina ou, do inglês "Ecgonine Methyl Ester"
GC	Cromatografia gasosa, do inglês "Gas Chromatography"
GC-MS/MS	Cromatografia Gasosa Acoplada à Espectrometria de Massas em tandem do inglês "Gas Chromatography-tandem Mass Spectrometry"
ICSN	Inibidores da Captação de Serotonina e Norepinefrina
IMAO	Inibidores da Monoamino Oxidase
ISRS	Inibidores Seletivos da Recaptação de Serotonina
kg	Quilograma
LC	Cromatografia Líquida, do inglês "Liquid Chromatography"
LC-MS/MS	Cromatografia Líquida acoplada à Espectrometria de Massas em tandem, do inglês "Liquid Chromatography-tandem Mass Spectrometry"
LLE	Extração líquido-líquido, do inglês "Liquid-Liquid Extraction"
MAO	Monoamino oxidase
m-dSPE	Extração em Fase Sólida Dispersiva Magnética, do inglês "Magnetic Dispersive Solid Phase Extraction"
MIP	Polímero de impressão molecular, do inglês "Molecular Imprinted Polymer"
Mg	Miligrama
mL	Mililitro
MNP	Nanopartícula magnética, do inglês "Magnetic Nanoparticle"
MRM	Monitoramento de reações múltiplas, do inglês "Multiple Reaction Monitoring"

MS	Espectrometria de massas, do inglês “Mass Spectrometry”
m/z	Razão massa/carga
NHSBSA	National Health Service Business Services Authority
ng	Nanograma
OMS	Organização Mundial da Saúde
QuEChERS	Rápido, Fácil, Barato, Efetivo, Robusto e Seguro, do inglês “Quick, Easy, Cheap, Effective, Robust and Safe”
pH	Potencial hidrogeniônico
PANI	Polianilina
RDC	Resolução da Diretoria Colegiada
SNC	Sistema Nervoso Central
SPA	Substâncias Psicoativas
SPE	Extração em fase sólida, do inglês “solid phase extraction”
SPME	Microextração em fase sólida, do inglês “solid phase microextraction”
$t^{1/2}$	Tempo de meia-vida
µg	Micrograma
µL	Microlitro
UNODC	Escritório das Nações Unidas sobre Drogas e Crime, do inglês “United Nations Office on Drugs and Crime”

SUMÁRIO

1. REFERENCIAL TEÓRICO

Nas análises forenses, a cocaína e os antidepressivos estão entre as principais classes de substâncias psicoativas encontradas. De acordo com os dados do Escritório das Nações Unidas sobre Drogas e Crime (UNODC) em 2022 houve uma estimativa global de 21 milhões de usuários de cocaína com idade entre 15 e 64 anos no ano de 2020 (UNODC, 2022). No Brasil, segundo o último Levantamento Nacional sobre o Uso de Drogas pela população Brasileira, estima-se que mais de 1,3 milhões de pessoas tenham utilizado cocaína em 2017 (BASTOS *et al.,* 2017). O estudo do Ônus Global de Doenças para 2019, conduzido pelo Instituto de Métricas de Saúde, destacou a gravidade dos distúrbios atribuídos ao uso de cocaína, com uma taxa de mortalidade de 0,32 por 100.000 indivíduos. Essa taxa sofreu um aumento de 256% no período de 1990 a 2019 (RITCHIE; ROSER, 2022).

Concomitantemente, na Inglaterra, estima-se que 83,4 milhões de medicamentos antidepressivos foram prescritos entre os anos de 2021 e 2022 (NHSBSA, 2022). Em outros países da Europa, o consumo médio diário de antidepressivos chega a 161 por 1000 pessoas, como na Islândia. No Brasil, 4 em cada 100 brasileiros fazem uso de antidepressivos (TIGUMAN *et al*., 2023). Essas prevalências destacam o impacto significativo da cocaína e dos antidepressivos como algumas das substâncias mais utilizadas globalmente, superadas apenas pela *Cannabis* em muitas regiões (UNODC, 2022). Os antidepressivos desempenham um papel significativo em inúmeros casos de overdose, seja intencional ou não. Sua presença, somada a outras substâncias psicoativas, é comumente identificada em relatórios toxicológicos *post mortem* devido à sua prescrição generalizada e às toxicidades relativamente altas associadas a situações de overdose (CHEETA *et al.*, 2004; BERNARDES et al., 2012; MANDOUR, 2012; WIEGAND *et al.*, 2012; METHLING *et al.*, 2019).

1.1. Cocaína e produtos de biotransformação

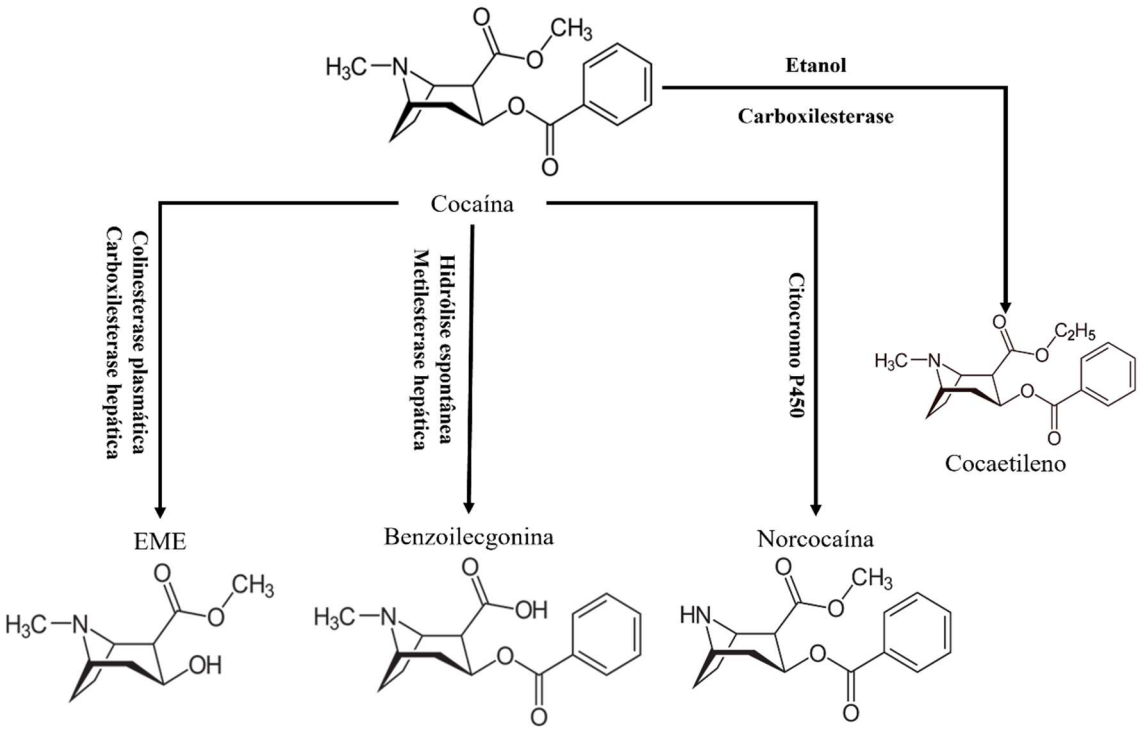
A cocaína é uma droga obtida da ecgonina, um produto dos alcaloides da planta *Erythroxylum coca* e está entre as drogas de abuso mais populares do país, sendo proscrita no Brasil, conforme a resolução RDC nº 344/1998, da Agência Nacional de Vigilância

Sanitária (ANVISA) (ANVISA, 1998). A cocaína em forma de base livre, conhecida popularmente como *crack,* é encontrada na forma de pedras, enquanto a cocaína hidroclorídrica é encontrada na forma de cristais sólidos brancos. Comumente, a droga encontrada nas ruas contém uma alta quantidade de adulterantes e diluentes, podendo ser misturada com anfetaminas, anti-histamínicos, opioides e anestésicos, dentre outros (SOLIMINI *et al.*, 2017).

A absorção da cocaína é efetiva pelas vias intravenosa, intranasal e pulmonar (*crack*), mas o curso temporal e a extensão da absorção diferem entre elas. As rotas que envolvem a via pulmonar permitem que o estimulante alcance a circulação do cérebro de 6 a 8 segundos após a administração (POMARA, *et al.* 2012; CUNHA-OLIVEIRA *et al.*, 2013). A via intranasal permite biodisponibilidade próxima a 90%, e seu efeito dura de 15 a 30 minutos, enquanto o volume de distribuição varia entre 1 e 3 L/kg. A cocaína se liga à albumina e à alfa1-glicoproteína ácida em uma taxa de cerca de 90% e pode ser encontrada nas concentrações mais altas no cérebro, baço, rins e pulmões, seguido pelo sangue, coração e tecido muscular. A meia-vida média da cocaína é entre 40 e 90 minutos, podendo variar dependendo da via de administração (CUNHA-OLIVEIRA *et al.*, 2013; ISENSCHMID, 2020).

Dois ésteres da cocaína são hidrolisados *in vitro* e *in vivo*. O éster alquil da cocaína é hidrolisado em benzoilecgonina via hidrólise espontânea, bem como por metilesterases no fígado, e o éster fenílico é hidrolisado em éster metílico de ecgonina (EME) pelas colinesterases plasmáticas e carboxilesterases no fígado (**Figura 1**) (ISENSCHMID, 2020). O EME representa de 30 a 50% da cocaína biotransformada, enquanto a benzoilecgonina cerca de 40%, sendo ambos produtos inativos. Outras rotas de transformação podem ocorrer, gerando norcocaína a partir de cocaína ou norbenzoilecgonina/ éster metílico de norecgonina, a partir de seus produtos de biotransformação (JAGERDEO; ABDEL-REHIM, 2011). De forma relevante, o uso concomitante de etanol e cocaína leva à transesterificação das duas substâncias no composto cocaetileno (**Figura 1**) (HERBST *et al.*, 2011; JONES, 2019).

Figura 1 - Cocaína e substâncias correlacionadas



Fonte: Adaptado de ISENSCHMID, 2020.

Quanto à eliminação, de 1 a 9% da cocaína é excretada inalterada na urina, com uma proporção maior em urina ácida (ROQUE, *et al.,* 2022). Os produtos EME e benzoilecgonina constituem a maior parte de produtos excretados. Estudos controlados, após a administração intranasal de 20 mg, evidenciaram a detecção de cocaína no sangue em um intervalo de 4 a 6 horas (VERSTRAETE, 2004). Em uma dose de 100 mg, o tempo de detecção aumenta para 12 horas. No caso da benzoilecgonina, após a administração de 1,5 mg/kg, pela via intranasal, é possível detectá-la por 2-3 dias. Os tempos de detecção variam para usuários crônicos, onde é possível realizar a deteção por um período mais longo (ROQUE, *et al.,* 2022; CIUCĂ ANGHEL, *et al.*, 2023). A cocaína inalterada é excretada nas fezes e na saliva. A cocaína e a benzoilecgonina podem ser detectadas no leite materno até 36 horas após a administração e na urina de neonatos por até 5 dias. A cocaína na forma de base livre atravessa a placenta, e a norcocaína persiste por 4 a 5 dias no líquido amniótico, mesmo quando não é mais detectável no sangue materno. Considerando a letalidade do uso de cocaína, já foram retratadas na literatura fatalidades após aplicação na membrana mucosa de 25 mg ou uso intranasal de 400 mg da droga (DART, 2004; CIUCĂ ANGHEL, *et al.*, 2023).

Os efeitos psicoativos da cocaína estão relacionados com o bloqueio da recaptação dos neurotransmissores dopamina (DA), norepinefrina e serotonina (5-HT) (ISENSCHMID, 2020). Em doses baixas, a intoxicação aguda causa euforia e agitação. Doses maiores causam diversos sintomas relacionados ao sistema nervoso central (SNC) e sistema cardiovascular, como hipertermia, náuseas, vômitos, dor abdominal, dor no peito, taquicardia, arritmia ventricular, hipertensão, ansiedade extrema, agitação, alucinações e midríase (SCHWARTZ, 2010; CIUCĂ ANGHEL, *et al.*, 2023). Esses sintomas podem ser seguidos por depressão do SNC com respiração irregular, convulsões, coma, distúrbios cardíacos, colapso e morte. A intoxicação crônica produz euforia, agitação psicomotora, ideação suicida, anorexia, perda de peso, alucinações e deterioração mental (DART, 2004; CIUCĂ ANGHEL, *et al.*, 2023). A cocaína também pode ser responsável por mortes por meio de diversos mecanismos, incluindo delírio, arritmias cardíacas e super estímulo do sistema nervoso central (ISENSCHMID, 2020). Adicionalmente, a liberação de neurotransmissores está associada ao maior desenvolvimento de neurotoxicidade e susceptibilidade à dependência (ISENSCHMID, 2020).

1.2. Antidepressivos

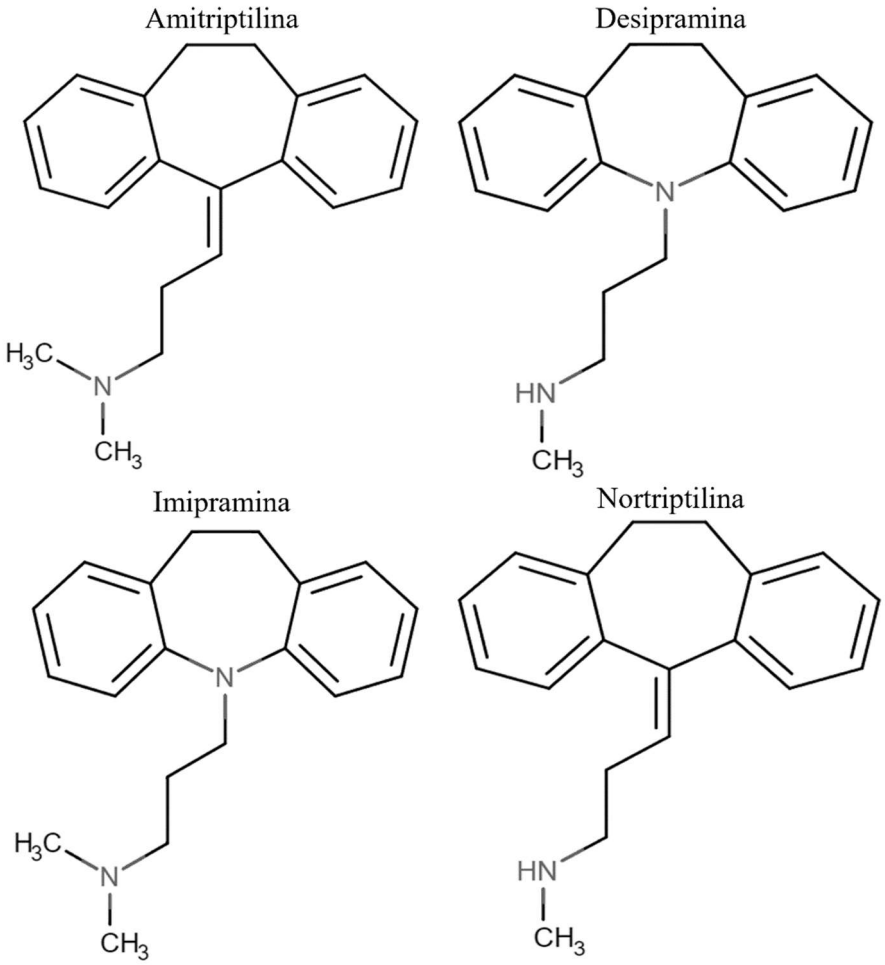
Os antidepressivos atualmente são classificados em antidepressivos tricíclicos, inibidores seletivos da recaptação de serotonina (ISRS), inibidores da captação de serotonina e norepinefrina (ICSN), antidepressivos atípicos e inibidores da monoamino oxidase (IMAO). São fármacos facilmente absorvidos pela via oral, e, em alguns casos, podem ser administrados por outras vias conforme a necessidade do paciente. Por serem medicamentos de amplo acesso, são facilmente adquiridos de forma ilícita e estão presentes em grande parte dos casos de intoxicação, incluindo overdoses e óbito, desempenhando um papel importante na toxicologia no Brasil e no mundo (MATHIAS; GUIDONI; GIROTTO, 2019).

1.2.1. Antidepressivos tricíclicos

A classe dos tricíclicos é uma das classes antidepressivos presentes há mais tempo no mercado e possuem alta taxa de prescrição no país, embora seu uso tenha diminuído drasticamente, considerando os efeitos adversos que possuem. Exemplos dessa classe são a amitriptilina, desipramina, imipramina e nortriptilina (**Figura 2**). Esses fármacos promovem seus efeitos por meio do bloqueio da recaptação de epinefrina, norepinefrina e serotonina no neurônio pós-sináptico, possuem biodisponibilidade variável, em geral, e elevado índice de ligação às proteínas plasmáticas (WHISKEY, 2013). A amitriptilina, sofre desmetilação e hidroxilação durante o metabolismo, transformando-se em nortriptilina, seu maior produto de biotransformação ativo, também utilizado como um fármaco independente. Alguns outros produtos são biotransformados, embora sua ação seja significativamente inferior. A eliminação das substâncias ocorre principalmente pela urina, sendo excretadas conjugadas ao glicuronídeo ou sulfato, com aproximadamente 2% da substância inalterada eliminada na urina (DEAR; BATEMAN, 2016). A amitriptilina inalterada é 50% eliminada 16 horas após a administração, enquanto o tempo de meia vida da nortriptilina é de 30 horas. A concentração plasmática encontrada após dose terapêutica típica é de 100 – 250 ng/mL para amitriptilina e 60 – 150 ng/mL para nortriptilina. Os dados de toxicidade indicam que reações graves podem surgir em concentrações superiores a 500 ng/mL (GHAMBARIAN; YAMINI; ESRAFILI, 2012; WEBER; WEHR; DUCHEMIN, 2014). Os efeitos na overdose são aumentados com a ingestão concomitante de álcool e outras substâncias psicotrópicas. Alguns dos sintomas incluem convulsões, hipotensão, tonturas, alucinações, prejuízo das funções cardíacas e coma (GAO *et al*., 2023; DOS SANTOS PEREIRA *et al.*, 2024).

A desipramina atinge o pico de concentração plasmática entre 4 e 6 horas após administração oral, é extensivamente biotransformada pelo fígado, em maior parte pela CYP2D6 e em menor parte pela CYP1A2, no principal produto ativo, a 2-hidroxi-desipramina. O tempo de meia vida desse fármaco é de em média 30 horas, com concentração plasmática de 125 a 300 ng/mL após administração oral de doses clínicas típicas. Ensaios de toxicidade em ratos indicam que efeitos de sedação e hipotensão podem ser observados com doses de 290 mg/kg em fêmeas e 230 mg/kg em machos. Relatos de caso descrevem intoxicações após a ingestão oral de 1150 a 2500 mg, gerando ataxia, convulsões, coma e em um dos casos, óbito (BENOWITZ, 2018; THANACOODY, 2020).

Figura 2 - Estruturas químicas dos antidepressivos tricíclicos



Fonte: National Center for Biotechnology Information, 2024.

Outro fármaco da classe dos antidepressivos tricíclicos é a imipramina, utilizada no tratamento da depressão e da enurese noturna em crianças. Bem absorvida oralmente, a imipramina, atinge a concentração plasmática máxima de 2 a 6 horas após a administração oral. A imipramina é convertida em desipramina, citada anteriormente, pelas enzimas do fígado. O tempo de meia vida da imipramina é de 12 horas e a concentração plasmática encontrada é entre 175 e 300 ng/mL subsequente à administração oral de doses típicas (BRUNTON, 2022; VOLZ; LAUX, 2022; UL HAQ; TAZNEEM; MARYAM, 2023).

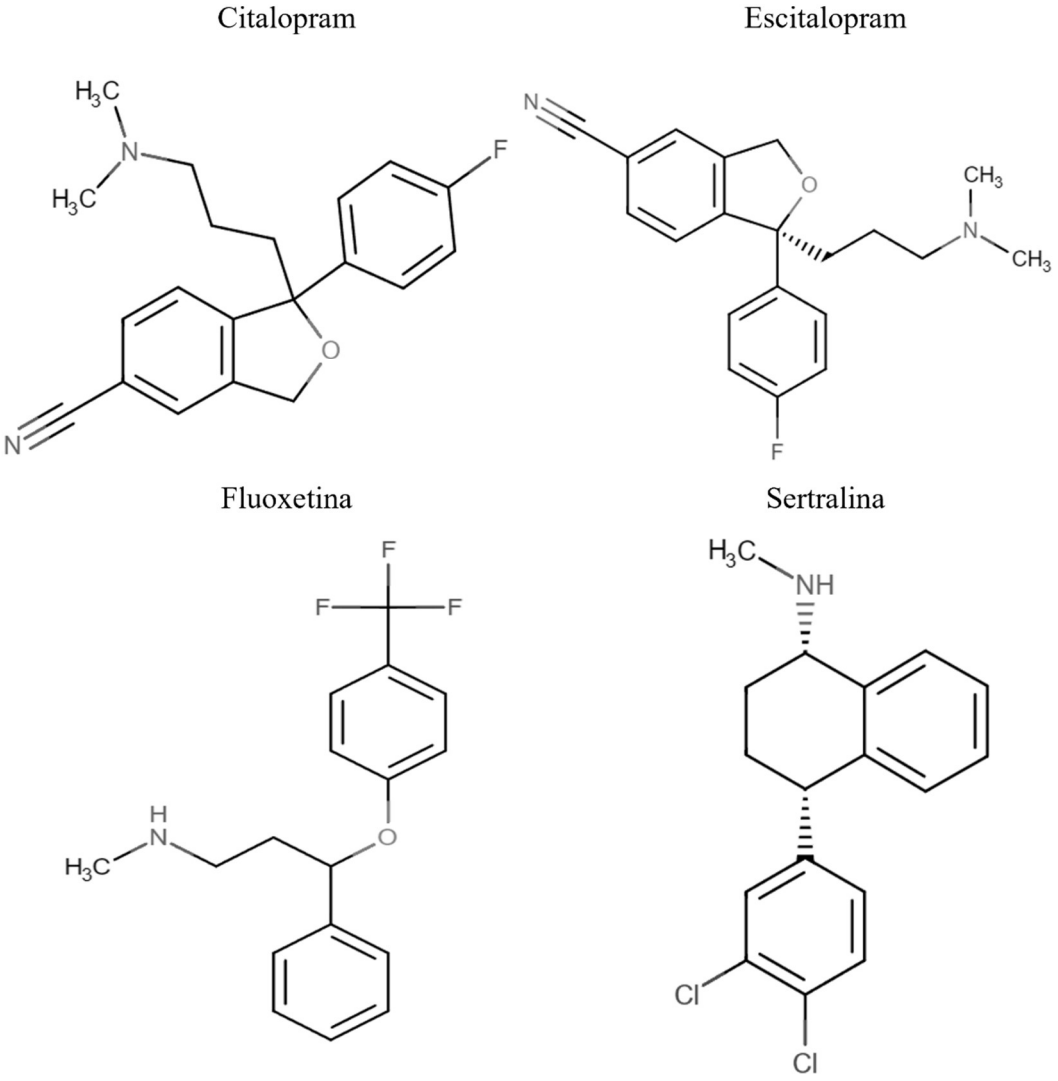
1.2.2. Inibidores Seletivos da Recaptação de Serotonina

Atualmente, os ISRS são os antidepressivos considerados de primeira linha, comumente prescritos devido à alta seletividade e, consequentemente, menos efeitos adversos em relação a outras classes. A ação desses fármacos é eficaz para o tratamento da depressão e outras condições (HIRSCH; BIRNBAUM, 2020). Seu mecanismo de ação envolve a inibição da recaptação específica de serotonina na fenda sináptica, como o nome da classe sugere. Exemplos de ISRS são citalopram, escitalopram, fluoxetina e sertralina (**Figura 3**). O tempo ótimo estimado para o efeito terapêutico desejado varia entre 4 e 8 semanas de uso diário. A maioria dos fármacos da classe é biotransformadas no fígado pelo complexo citocromo P450. A fluoxetina é considerada o ISRS mais potente, bem absorvida após administração oral, com picos de concentração plasmáticas entre 4 e 8 horas. Após a administração oral de 40 mg, por adultos saudáveis, são obtidas concentrações plasmáticas entre 100 e 500 ng/mL. No caso de pessoas em tratamento farmacológico entre duas e quatro semanas, poderá ser observado um estado de equilíbrio entre fluoxetina e norfluoxetina (produto de biotransformação ativo). A meia-vida da fluoxetina é de 53 horas, enquanto a da norfluoxetina é de 240 horas. Essa longa duração permite a administração semanal nos casos em que esse protocolo seja de interesse para a prática clínica. A cinética da fluoxetina não é linear, visto que apresenta um aumento desproporcional nas concentrações plasmáticas após o aumento de dose (EDINOFF *et al.*, 2021). Doses múltiplas proporcionam tempo de meia vida maior quando comparado a doses únicas. A excreção ocorre pela urina, fezes e leite materno. Casos de intoxicação com fluoxetina são relatados a partir de 1500 mg em adultos e 3,6 mg/kg em crianças (POPE; ZARAA, 2016). No entanto, efeitos graves são descritos, como toxicidade serotoninérgica, rabdomiólise, convulsões, atraso na condução cardíaca e arritmia (LEE-KELLAND; ZEHRA; MAPPA, 2018). Casos de overdose foram relatados na ingestão de doses entre de 600 a 1200 mg do fármaco por adolescentes (KOLBECK; SCHULT; NACCA, 2024).

O segundo fármaco mais potente e seletivo na classe dos ISRS é a sertralina, de absorção relativamente lenta, levando de 4 a 10 horas para atingir a concentração plasmática máxima. Os usos de sertralina incluem o tratamento de depressão generalizada, síndrome do pânico, transtorno obsessivo compulsivo e transtorno de estresse pós-traumático. O tempo de meia vida é similar em doses únicas ou uso crônico,

variando em torno de 24 a 32 horas. Seu principal produto de biotransformação é a desmetilsertralina, com atividade 20 a 60 vezes menor, mas tempo de meia vida maior (66 - 120 horas) (HUDDART *et al.*, 2020).

Figura 3 - Estruturas químicas dos principais antidepressivos da classe ISRS



Fonte: National Center for Biotechnology Information, 2024.

Outra substância pertencente à classe dos ISRS é o citalopram, também utilizado para o tratamento de depressão e transtorno de ansiedade. O citalopram e seus produtos N-desmetilados existem como misturas racêmicas, ou seja, uma mistura entre S-citalopram e R-citalopram. Testes *in vitro* e *in vivo* mostraram que os efeitos do citalopram dependem principalmente dos enântiomeros S-citalopram (escitalopram) e S-desmetil-citalopram, que possuem ação mais potente em relação aos R-enântiomeros. O pico de concentração plasmática ocorre no intervalo entre 1 e 4 horas após administração,

e o tempo de meia vida da substância é de aproximadamente 35 horas (SANGKUHL; KLEIN; ALTMAN, 2011). A concentração plasmática conseguinte a administração oral varia de 40 a 80 ng/mL. Geralmente é bem tolerado, com prognósticos positivos mesmo em casos de overdose, mas pode causar taquicardia, hipotensão, confusão mental (COOKE; WARING, 2013).

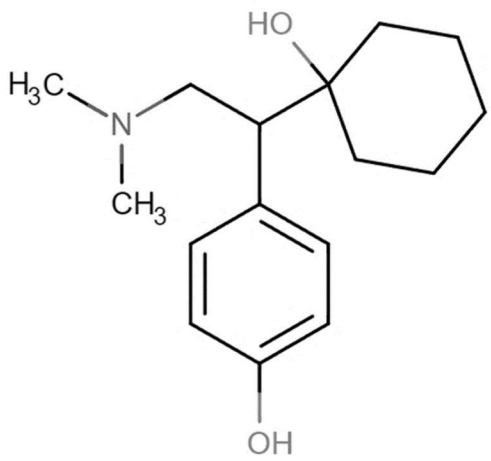
1.2.3. Inibidores da Recaptação de Serotonina e Norepinefrina

A classe dos ICSN não é tão seletiva quanto os ISRS, porém, não possui efeitos adversos exacerbados como os antidepressivos tricíclicos. São fármacos que inibem a captação tanto de serotonina quanto norepinefrina, sendo exemplos a duloxetina, desvenlafaxina e venlafaxina (SANSONE; SANSONE, 2014). A venlafaxina foi o primeiro fármaco da classe a ser desenvolvido e autorizado para uso, tendo inicialmente liberação rápida e administração duas vezes ao dia (**Figura 4**). Posteriormente foi desenvolvida a venlafaxina de liberação prolongada que seria administrada apenas uma vez ao dia. São indicados para, além da depressão, os casos de ansiedade, síndrome do pânico e fobia social. A venlafaxina é biotransformada em desvenlafaxina, ambas possuem tempo de meia vida de 5 horas e 11 horas respectivamente (PAULZEN *et al.,* 2014). Aproximadamente 4,7% do fármaco não biotransformado é eliminado na urina, e 29,4% transformado em desvenlafaxina (**Figura 4**) (MAGALHÃES *et al*., 2013). Um estudo clínico testou as concentrações plasmáticas após administração de variadas doses de venlafaxina em pacientes internados e a concentração encontrada foi de 125 a 400 µg/L de venlafaxina e seu produto de biotransformação, após a administração de doses de 75 a 300 mg (CHARLIER *et al.*, 2002).

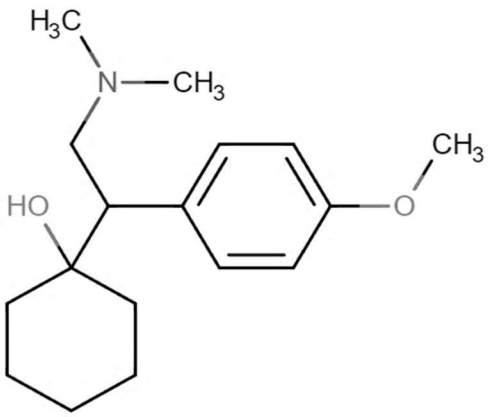
Além de ser o principal produto de biotransformação da venlafaxina, a desvenlafaxina também é utilizada como fármaco, tendo um perfil farmacológico, de eficácia e segurança muito parecido com a venlafaxina. Aproximadamente 45% da substância é excretada inalterada 72 horas após ingestão oral (LIEBOWITZ; TOURIAN, 2010).

Figura 4 - Estruturas químicas dos fármacos ICSN, desvenlafaxina e venlafaxina.

Desvenlafaxina



Venlafaxina



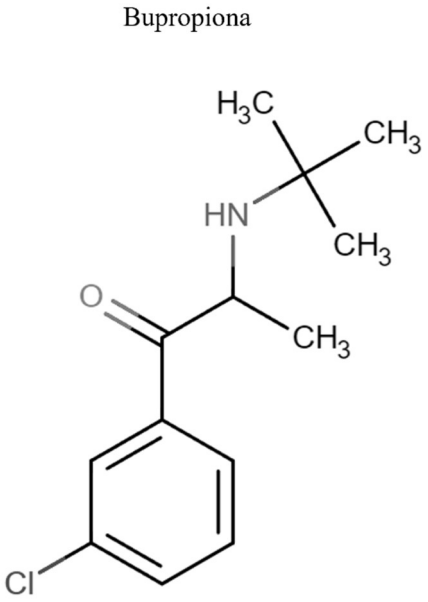
Fonte: National Center for Biotechnology Information, 2024.

1.2.4. Antidepressivos atípicos

Os antidepressivos incluídos nessa classe são aqueles distintos das substâncias presentes nas demais classes. Frequentemente, são utilizados em pacientes com respostas inadequadas ou para evitar efeitos adversos que se manifestaram com o uso de ISRS. Um exemplo de medicamentos dessa classe é a bupropiona, uma opção de interesse por não proporcionar efeitos serotoninérgicos (**Figura 5**). Além do tratamento da depressão, a bupropiona também é prescrita como coadjuvante no tratamento da dependência de nicotina. O mecanismo de ação da bupropiona ainda é incerto, mas estima-se que ocorra através da inibição pré-sináptica dos transportadores da dopamina e norepinefrina (COSTA; OLIVEIRA; DINIS-OLIVEIRA, 2019). Esse fármaco é biotransformado em sua maior parte pela CYP2B6, no produto hidroxi-bupropiona, 50% menos ativo que a forma inalterada, no entanto, encontrado em concentrações plasmáticas 10x maiores. A concentração plasmática máxima é encontrada em torno de 1,5 hora após ingestão oral da forma de liberação imediata e 5 horas para a forma de liberação lenta (ALBERTER; CHAMBERS; WILLS, 2022). O tempo de meia-vida do fármaco e do produto de biotransformação é de 21 horas, e as concentrações plasmáticas encontradas variam de 75 a 100 ng/mL (BRUNTON, 2022). Nos casos de overdose de bupropiona, são

observados os sintomas de convulsões, taquicardia, arritmia e choque. Relatos indicam intoxicações após a administração oral de 75 – 1500 mg (SHEPHERD, 2012).

Figura 5 - Estrutura química do antidepressivo atípico, bupropiona.



Fonte: National Center for Biotechnology Information, 2024.

1.3. Extração e análise de substâncias psicoativas de matrizes biológicas

Considerando a relevância dessas substâncias no campo da toxicologia forense, métodos de determinação precisos e confiáveis são essenciais para sua análise, fornecendo *insights* sobre as circunstâncias de intoxicações ou óbitos suspeitos. As principais técnicas utilizadas na detecção e quantificação são as hifenizadas, como a cromatografia gasosa acoplada à espectrometria de massas (GC-MS) ou a cromatografia líquida acoplada à espectrometria de massas em tandem (LC-MS/MS). Métodos de LC-MS/MS são conhecidos por sua sensibilidade e precisão, tornando-se o sistema analítico de escolha para a maioria das pesquisas forenses (DEEB *et al*., 2014; FRENI *et al*., 2019). No entanto, é fundamental para matrizes complexas, que o material a ser analisado passe pela etapa de preparo de amostra, a qual possibilita a redução de interferências e incompatibilidades das

matrizes biológicas, bem como a capacidade de concentração do analito (SOUSA *et al*., 2011).

Métodos de extração clássicos são consolidados por sua capacidade de isolar os alvos de matrizes complexas, como a extração líquido-líquido (LLE) e a extração em fase sólida (SPE). Embora esses métodos ofereçam diversas vantagens, também apresentam algumas dificuldades associadas à sua implementação e execução na rotina laboratorial. Quanto à LLE, é um processo intensivo, que exige mão de obra qualificada e requer múltiplas etapas, consequentemente prolongando o tempo gasto no processo. Sua principal desvantagem é o impacto ambiental, pois utiliza quantidades significativas de solventes orgânicos (KOESUKWIWAT, *et al.* 2010). Mesmo havendo um tratamento de resíduos adequado, essa etapa é de alto custo para os laboratórios.

A SPE é amplamente utilizada para o preparo de amostras, e utiliza um sorvente para isolar os analitos de uma matriz específica. Uma grande variedade de sorventes e *manifolds* está disponível para fornecer diversos tipos de interações potenciais com os analitos (PLOTKA-WASYLKA, 2016; CALDERILLA *et al.*, 2018). No entanto, quando o sorvente é empacotado em um cartucho, a eficiência da interação sorvente-analito é limitada pela taxa de fluxo selecionada para percolar a amostra. Esse aspecto torna-se crítico quando partículas de tamanho nanométrico são usadas, uma vez que a contrapressão gerada dificulta a passagem da amostra (CHISVERT; CÁRDENAS; LUCENA, 2019). Além disso, a competitividade de interferências da matriz com os sítios ativos do sólido afeta negativamente a recuperação do analito. Considerando os aspectos financeiros, a técnica tem um alto custo devido a cartuchos e sorventes que podem ser relativamente onerosos. Além disso, essa técnica exige uso de diferentes solventes para lavagem, etapas de evaporação e secagem, e ajuste do sistema de vácuo e da vazão dos solventes (SÓCAS-RODRÍGUEZ *et al*., 2015). Todos esses fatores afetam a precisão e reprodutibilidade do método, e mesmo automatizadas, exigem manutenção frequente e investimento financeiro significativo (OJEDA; ROJAS, 2018).

Essas características podem aumentar a complexidade e o tempo necessários para realizar as extrações, tornando importante considerar alternativas mais eficientes e sustentáveis.

1.3.1. Extração em fase sólida dispersiva

A necessidade de maior eficiência, seletividade e sustentabilidade ambiental impulsionou o desenvolvimento de métodos de extração inovadores com técnicas de microextração, que reduzem o volume da amostra e simplificam os procedimentos de extração, utilizando materiais inteligentes e nanotecnologia a seu favor. Um exemplo disso é a extração dispersiva em fase sólida (dSPE) (SÓCAS-RODRÍGUEZ *et al*., 2015).

Sugerida por Anastassiades et al. (2003) a técnica envolve a adição do material adsorvente em alíquota da amostra, seguida de etapas de agitação, centrifugação, e subsequente dessorção, que é realizada com solvente ou solução apropriados. Algumas vantagens a serem citadas são o aumento significativo da capacidade de extração, visto o contato direto entre o material sorvente e analito, diminuição no tempo de processamento, baixo consumo de solvente, e operação simplificada, quando comparado ao método tradicional de SPE (ANASTASSIADES *et al.*, 2003; CHISVERT; CÁRDENAS; LUCENA, 2019). Nesse caso, o sorvente não é empacotado em uma coluna, mas é colocado diretamente em contato com a amostra, evitando a etapa de condicionamento. Enquanto na SPE convencional a taxa de fluxo da amostra é um parâmetro crítico que deve ser constante e controlado para permitir a interação correta entre o sorvente empacotado e as moléculas-alvo, na dSPE o contato entre as duas fases é imediato e mais eficaz. Assim, vale mencionar que a dSPE é adequada para a análise direta de amostras complexas que podem obstruir os cartuchos e levar a falhas na extração na SPE convencional (SÓCAS-RODRÍGUEZ et al., 2015).

O emprego mais conhecido da dSPE é para fins de *clean-up*, como ocorre na etapa de limpeza do método chamado QuEChERS, desenvolvido para ser rápido, fácil, efetivo, robusto e seguro, palavras que em inglês formam o acrônimo do nome (WILKOWSKA; BIZIUK, 2011). De fato, o termo dSPE foi proposto e utilizado quando o método QuEChERS foi introduzido (ANASTASSIADES *et al.*, 2003). No *clean-up* por dSPE, as interferências são retidas no sorvente, que é finalmente descartado, enquanto o sobrenadante é submetido ao restante do processo analítico. No entanto, os sorventes também podem ser usados de modo dispersivo com o objetivo de reter os analitos. Nesse caso, ocorre uma posterior etapa de dessorção com o uso de um solvente apropriado, após a eliminação do sobrenadante (SÓCAS-RODRÍGUEZ *et al*., 2015; CHISVERT; CÁRDENAS; LUCENA, 2019).

Em geral, existem quatro características principais de um composto químico que podem ser usadas para alcançar a extração/retenção em ambas SPE e dSPE; polaridade,

carga elétrica, reconhecimento molecular e tamanho molecular (ISLAS *et al.*, 2017). Os modos de interação que se baseiam na polaridade são chamados de extração em fase normal ou extração em fase reversa. Na primeira, o sorvente é mais polar do que a amostra não aquosa e é usado para isolar compostos polares, através de ligações de hidrogênio ou interações dipolo-dipolo, principalmente, enquanto na segunda ocorre o oposto, um sorvente não polar é usado com uma amostra aquosa polar para isolar analitos relativamente não polares (ISLAS *et al.*, 2017). A extração em fase reversa é baseada em interações de van der Waals ou dispersão entre o sorvente e as moléculas-alvo, normalmente realizada com sorventes de sílica, como octadecil (C18) ou octil (C8), ou ainda sorventes poliméricos orgânicos (CASADO *et al*., 2019; SI, *et al.*, 2020). A extração em fase normal também pode ocorrer em sorventes à base de sílica, ou em materiais inorgânicos (ŚCIGALSKI; KOSOBUCKI, 2020).

A segunda característica é a carga elétrica e o modo é chamado de extração por troca iônica. Neste caso, os analitos são primeiro ionizados e retidos nos sítios iônicos contidos no sorvente, sendo então eluídos com um solvente orgânico, uma vez que passam a ser convertidos de volta para sua forma molecular (NASRIN; MOUSSAVI; GIANNAKIS 2004). A troca de ânions é usada para íons negativamente carregados em um sorvente positivamente carregado, e o contrário para a troca de cátions. Nesse caso, o controle de pH é extremamente importante, já que sua alteração pode neutralizar íons ou até mesmo a carga do sorvente. Além disso, a introdução de íons capazes de deslocar os compostos retidos é necessária para a eluição. Para esse sistema de interação, tanto sorventes de sílica quanto sorventes poliméricos podem ser utilizados (SÓCAS-RODRÍGUEZ *et al.,* 2015).

A terceira característica é o reconhecimento molecular, que é baseado em uma interação específica e altamente seletiva entre uma molécula ou grupos de moléculas e um ligante imobilizado no sorvente. O reconhecimento molecular inclui diferentes tipos de interações, como dipolo-dipolo, interações eletrostáticas e/ou forças hidrofóbicas. Nesse modo, imunossorventes altamente seletivos ou os chamados polímeros de impressão molecular (MIPs) são frequentemente usados, surgindo como alternativas muito interessantes aos sorventes tradicionais (TURIEL; ESTEBAN, 2020; ZHAO et. al., 2021).

A quarta, embora não tão amplamente usada na dSPE, é o tamanho molecular. Esse tipo de sorvente permite a separação de compostos orgânicos com base em um

processo de peneiramento mecânico. Moléculas pequenas penetram nos poros dos sorventes, enquanto moléculas grandes podem ser completamente excluídas ou penetrar poros de tamanhos específicos. Como resultado, eles são eluídos primeiro. Esse tipo de separação pode ser realizado com sorventes em gel (SOCAS-RODRIGUEZ *et al.*, 2015). Além dos mecanismos informados, existem sorventes que combinam diferentes mecanismos de retenção.

Nos processos de dSPE, o volume de amostra a ser utilizado é muito importante, assim como o tipo de solvente utilizado na fase de dessorção. Solventes ou misturas de solventes de dessorção devem ter polaridade aproximadamente igual à polaridade dos compostos (CHISVERT; CÁRDENAS; LUCENA, 2019). Recentemente, o uso de diversos nanomateriais funcionalizados, tais como nanotubos de carbono, grafeno, dióxido de titânio e óxido de zinco têm se mostrado eficientes na extração de diferentes compostos em amostras de solo e água (KHALILIAN; HANZAKI; YOUSEFI, 2015; SONG; CHEN; SHI, 2017; ALOTHMAN; WABAIDUR, 2019; ABDELRAHMAN; HGAZEY; AHMED, 2020). As propriedades físicas, químicas, elétricas e ópticas dos nanomateriais funcionalizados diferem dependendo de seu tamanho e forma, em comparação com partículas maiores. Devido ao seu tamanho diminuto, os nanomateriais possuem área de superfície e razão superfície/volume elevadas, ou seja, quanto menor a nanopartícula, mais significativas serão essas características. Essa relação é uma das razões pelas quais os nanomateriais têm propriedades químicas e físicas superiores, como energia de superfície, reatividade, solubilidade e ponto de fusão baixo. Isso se dá pelo aumento de átomos na superfície, capazes de reagir com o meio e permitir ligações a moléculas, átomos ou íons. A capacidade de adsorção é, portanto, diretamente proporcional ao aumento no número de átomos na superfície (ASHA; NARAIN, 2020). O uso de nanomateriais para o preparo de amostras, confecção de materiais de separação e enriquecimento proporciona alta capacidade de adsorção e fatores de pré-concentração, além de facilitar a funcionalização e reutilização dos nanomateriais, sendo também um fator importante para a eficácia dessas aplicações (BÜYÜKTIRYAKI; KEÇILI; HUSSAIN, 2020; BAIG; KAMMAKAKAM; FALATH, 2021).

A necessidade de um alto nível de sensibilidade e seletividade, ou até mesmo a compatibilidade entre a matriz da amostra e a técnica instrumental, surgem como as principais dificuldades para procedimentos analíticos simples e rápidos. O tratamento da amostra surge como a resposta a essas deficiências. Nesse contexto, a miniaturização e a

automação desses procedimentos são tendências claras para permitir sua implementação final em laboratórios de rotina. Essa abordagem, que faz uso de uma pequena quantidade de sorvente, pode ser chamada de extração em fase sólida microdispersiva (D-μSPE). Pode ser ainda referida como microextração em fase sólida dispersiva, ou mesmo como extração em fase sólida magnética se sorventes magnéticos forem empregados.

1.3.2. Extração em fase sólida dispersiva magnética

Ao longo das últimas duas décadas, partículas magnéticas têm recebido crescente atenção devido às suas propriedades físicas e químicas únicas que permitiram sua aplicação em diversos campos, como microbiologia, biologia celular, biologia molecular e bioquímica, química analítica, bem como em biotecnologia e tecnologia ambiental (AGUILAR-ARTEAGA; RODRIGUEZ; BARRADO, 2010; STARK, 2011; XIE *et al.*, 2014)

Em particular, a aplicação de sorventes magnéticos para a extração em fase sólida dispersiva magnética (m-dSPE) não foi desenvolvida até 1996, quando Towler e colaboradores relataram a recuperação de rádio, chumbo e polônio de amostras de água do mar usando magnetita (Fe_3O_4) revestida com dióxido de manganês como o sorvente magnético (TOWLER; SMITH; DIXON, 1996). No entanto, o termo m-dSPE só foi introduzido três anos depois por Safariková & Šafařík, que extraíram e pré-concentraram um grupo de hidrocarbonetos aromáticos policíclicos e corantes de trifenilmetano de grandes volumes de amostra usando sorventes magnéticos ou magnetizáveis e um campo magnético externo (ŠAFAŘÍKOVÁ; ŠAFAŘÍK, 1999).

A enorme aplicação dessa abordagem é justificada principalmente devido à sua rapidez e simplicidade em comparação com a SPE ou dSPE convencionais. Na abordagem m-dSPE, uma manipulação fácil do sorvente pode ser alcançada com um campo magnético externo fornecido por um ímã. Esses ímãs são frequentemente compostos por uma mistura de três metais diferentes (neodímio, ferro e boro). Dessa forma, os sorventes magnéticos são misturados com a amostra enriquecida e, após o processo de extração, a matriz é separada do sorvente com os analitos usando o ímã (HAGAROVÁ, 2020). Em seguida, a fase magnética é dispersa no solvente de dessorção adequado, podendo ser assistida por ultrassom ou agitação por vórtice, e o sobrenadante posteriormente coletado para análise (VÁLLEZ-GOMIS *et al*., 2022).

Geralmente, os sorventes mais usados em m-dSPE são óxidos de ferro como magnetita (Fe_3O_4) ou maghemita (γ-Fe_2O_3), embora outros materiais magnéticos como, por exemplo, ferrita de cobalto ($CoFe_2O_4$), também tenham sido utilizados. O tamanho dos sorventes geralmente se encontra em escala nanométrica, e pode ser intencionalmente modificado. As alterações de superfície são frequentemente realizadas com um revestimento orgânico ou inorgânico, para direcionar as propriedades das nanopartículas magnéticas (MNPs) a um analito específico ou grupo restrito de moléculas, além de evitar sua aglomeração, que pode influenciar na capacidade de adsorção (VÁLLEZ-GOMIS *et al*., 2022).

A maioria dos estudos recentes sobre sorventes à base de MNPs relata que seus métodos de extração possuem ótima capacidade de recuperação quando aplicados a amostras ambientais e alimentícias (AGHAIE; HADJMOHAMMADI, 2016; VAKH *et al.*, 2018). Uma grande variedade de MNPs revestidas podem ser encontradas na literatura, sendo que essa funcionalização gera uma seletividade elevada para métodos que geralmente possuem um alvo único ou classes de moléculas restritas. Um exemplo dessas aplicações é o trabalho desenvolvido por Socas-Rodríguez *et al*. (2018), onde MNPs de Fe_3O_4 revestidas com polidopamina clássica foram utilizadas na determinação de 21 compostos com atividade estrogênica, em diferentes amostras de leite (SOCAS-RODRÍGUEZ, *et al.*, 2018). Por sua vez, Ge *et al*. (2012), prepararam nanopartículas magnéticas modificadas com polímero para remoção eficiente de íons metálicos pesados como Cd^{2+}, Zn^{2+}, Pb^{2+} e Cu^{2+} de soluções aquosas. Estudos de reutilização mostraram que o nano-adsorvente preparado pode ser usado repetidamente como um adsorvente eficaz no tratamento de água (GE *et al.*, 2012).

Já no trabalho de Rastbood, Hadjmohammadi & Majidi (2020), foram utilizadas MNPs de Fe_3O_4 revestidas de sílica (SiO_2) para isolar e pré-concentrar o fármaco meloxicam de amostras de plasma e urina por m-dSPE (RASTBOOD; HADJMOHAMMADI; MAJIDI, 2020). No caso de substâncias psicoativas, embora não haja muitos relatos, nanocompósitos de Fe_3O_4/Polipirrol e Fe_3O_4/Polianilina (PANI) foram aplicados na extração por dSPE de citalopram e sertralina, e dos benzodiazepínicos nitrazepam e lorazepam, respectivamente (ASGHARINEZHAD *et al*., 2014; ASGHARINEZHAD *et al*., 2015). Esses estudos demonstram a importância e a eficácia do uso de MNPs na simplificação e melhoria dos procedimentos de extração em análises toxicológicas, proporcionando resultados mais precisos e eficientes.

Uma vez que a etapa de preparo de amostra é crucial para a exatidão e precisão da análise, de forma a contornar as dificuldades acerca de cada matriz biológica, a busca pela facilitação e padronização desta etapa se faz constante. O uso de MNPs é vantajoso no desenvolvimento de sistemas de adsorção devido a sua elevada área de superfície, reatividade superficial, além das suas propriedades magnéticas que permitem a simplificação do método de extração a ser desenvolvido. Assim, sugerimos que esses materiais possam ser utilizados como uma alternativa viável na m-dSPE para a extração e subsequente análise de substâncias psicoativas de sangue post-mortem. Essa abordagem pode oferecer uma metodologia eficiente e sensível para a detecção e quantificação de xenobióticos em amostras biológicas, auxiliando na determinação da causa da morte e contribuindo para investigações toxicológicas mais precisas e confiáveis.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo deste trabalho foi desenvolver e validar um método analítico para a determinação de antidepressivos, cocaína e seus produtos de biotransformação em amostras de sangue *post mortem*, empregando nanopartículas magnéticas de Fe_3O_4 como sorvente para micro extração em fase sólida combinada com análise por cromatografia líquida acoplada à espectrometria de massas.

2.2. Objetivos específicos

i. Desenvolver e otimizar o método de cromatografia líquida acoplada a espectrometria de massas para a determinação, em amostras de sangue *post mortem*, de um conjunto específico de substâncias, incluindo amitriptilina, benzoilecgonina, citalopram, cocaetileno, cocaína, desipramina, desvenlafaxina, éster metílico de ecgonina, fluoxetina, imipramina, norfluoxetina, nortriptilina, sertralina e venlafaxina;
ii. Otimizar os parâmetros da m-dSPE utilizando técnicas de planejamento multivariado;
iii. Validar o método conforme o guia de validação 036 da ANSI/ASB "*Standard Practices for Method Validation in Forensic Toxicology*" desenvolvido pela *American Academy of Forensic Science* (AAFS);
iv. Aplicar o método na análise de amostras de sangue *post mortem* fornecidas pelo Departamento de Perícias Laboratoriais do Instituto Geral de Perícias do Rio Grande do Sul (DPL-IGP/RS).

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4. MANUSCRITO

O manuscrito "**Development of an innovative analytical method for forensic detection of cocaine, antidepressants, and metabolites in postmortem blood using magnetic nanoparticles**", que descreve a parte experimental desenvolvida nesta dissertação, foi submetido para publicação na revista **Analytical and Bioanalytical Chemistry** (ISSN 1618-2650, fator de impacto 4.3) e está apresentado a seguir. As normas de publicação estão disponíveis no **Anexo II**.

Development of an innovative analytical method for forensic detection of cocaine, antidepressants, and metabolites in postmortem blood using magnetic nanoparticles

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Abstract

Cocaine and antidepressants rank high globally in substance consumption, emphasizing their impact in public health. The determination of these compounds and related substances in biological samples is crucial for forensic toxicology. This study focuses on developing an innovative analytical method for the determination of cocaine, antidepressants, and their related metabolites in postmortem blood samples, using magnetic Fe_3O_4 nanoparticles as a sorbent for m-dSPE, coupled with LC-MS/MS analysis. An aliquot of 100 µL of the whole blood and 5 µL of the internal standard pool are added to 30 mg of nanoparticles. The nanoparticles are separated from the sample using a neodymium magnet inserted into a 3D-printed microtube rack. The supernatant is discarded, and desorption is 300 µL of solvent added to the nanoparticles. The sample is vortexed and separated, and 1.5 µL of the organic supernatant injected into the LC-MS/MS. After full validation, achieving the established criteria for sensitivity, linearity precision, and bias, the method was successfully applied to 263 postmortem blood samples. All samples evaluated in this study were positive for at least one substance. The most frequent analyte was benzoylecgonine, followed by cocaine and cocaethylene. The most common antidepressant encountered in the analyzed samples were citalopram and fluoxetine, followed by fluoxetine's metabolite norfluoxetine. These results confirm the first use of this sorbent in postmortem blood analysis, demonstrating satisfactory results in linearity, precision, accuracy, and selectivity for all compounds. The method's applicability was confirmed, establishing it as an efficient and sustainable alternative to traditional techniques for forensic casework.

Keywords: magnetic nanoparticles, dispersive extraction, LC-MS/MS, postmortem blood.

Statements and declarations

Ethics approval

This study was approved by the Ethical Committee for Human Studies of the Federal University of Health Sciences of Porto Alegre, Brazil (CAAE 17996819.7.0000.5345).

Competing interests

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

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Introduction

In forensic analysis, the identification of psychoactive substances in biological samples is a crucial aspect. Among the main compounds commonly detected, cocaine and antidepressants hold prominence. Data extracted from the World Drug Report 2022 revealed a staggering global estimate of 21 million cocaine users aged 15 to 64 in the year 2020 [1]. The Global Burden of Disease, conducted by the Institute for Health Metrics, underscored the severity of disorders attributed to cocaine use, with a mortality rate of 0.32 per 100,000 individuals [2]. This rate witnessed a remarkable 256% increase from 1990 to 2019 [3]. Concomitantly, in England, an estimated 83,4 million antidepressant drugs were prescribed in 2021/2022 [4]. In other countries of Europe, the average daily intake of antidepressants is up to 161 per 1000 people in Iceland. The number goes to 130,4 per million people in Canada and North America [5]. This alarming prevalence emphasizes the significant impact of cocaine and antidepressants as a few of the most consumed substances globally, surpassed only by *Cannabis* in many regions [6]. Regrettably, antidepressants play a significant role in numerous overdose cases, whether intentional or unintentional. Their presence, alongside other psychoactive substances, is commonly identified in postmortem toxicology reports due to their widespread prescription and the high toxicities associated with overdose situations [6-10]. Considering the peculiarities of these substances, accurate and reliable detection methods are essential in forensic analysis, providing insights into the circumstances surrounding cases of fatalities or suspicious deaths.

The main techniques used to determine psychoactive substances are hyphenated ones, such as gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). LC-MS/MS is a well-established technique due to its sensitivity and accuracy, becoming the analytical system of choice for most toxicological analyses [11-13]. Nevertheless, when working with biological matrices, it is fundamental that the sample is submitted to an extraction process, which intends to eliminate compounds that can damage the equipment, interfere with the analytical signal, and concentrate the substances of interest [14]. Exhaustive extraction methods such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are consolidated for their capacity to isolate targets from complex matrices

thoroughly. While those methods offer various advantages, they also have some associated difficulties. SPE process has an inflated cost due to the need for expensive commercial cartridges and sorbents. As for LLE, it is a labor-intensive process that requires multiple steps and has an environmental impact, generating significant amounts of organic solvent waste [15]. The need to overcome these limitations and the possibility of improved efficiency, selectivity, and environmental sustainability drives the urge to develop innovative extraction methods. These new methods are mainly based on microextraction techniques, which reduce sample volume, simplify extraction procedures, and can use new materials and nanotechnology in their favor [16].

Dispersive solid-phase extraction (dSPE) is an example of microextraction, which involves the addition of a sorbent material directly into the matrix, enabling an extended interaction with the analytes and selectively absorbing them from the sample. The sorbent is posteriorly separated from the sample, and the desorption of the analytes from the sorbent is performed using an organic solvent [17]. It is possible to use nanomaterials as sorbents due to their high surface area-to-volume ratio, providing ample active adsorption interaction sites with target molecules. Also, nanoparticles have rapid adsorption kinetics due to their small size, which enables fast equilibration between the adsorbent and the solution [18]. A variation of dSPE is Dispersive Magnetic Solid-Phase Extraction (m-dSPE), which uses a sorbent with magnetic properties [19]. This technique applies a magnetic field after the extraction for easy phase separation, eliminating centrifugation and filtration steps. This reduces overall operation time and makes the procedure easily portable and feasible for on-site extractions. The most common magnetic sorbents, like Fe_3O_4 nanoparticles, are usually synthesized from ferrous and ferric salts by simultaneous precipitation in alkaline conditions.

Additionally, magnetic nanoparticles' surfaces can be modified with polymers, biomolecules, or specific ligands. There is also the possibility of reusing the nanoparticles by recovery, making them cost-effective. Different magnetic sorbents have been employed in m-dSPE to extract metals, mycotoxins, and surfactants from various aqueous samples [20-21]. However, only a few applied the technique for the analysis of specific psychoactive substances in biological samples [22-23]. In this work, we aimed to develop and validate an analytical method for the determination of cocaine, several antidepressants, and their related metabolites in postmortem blood samples, using

magnetic Fe_3O_4 nanoparticles as a sorbent for m-dSPE, coupled with LC-MS/MS analysis.

Materials and Methods

Standards and reagents

LC-MS grade acetonitrile, ethyl acetate and methanol, and formic acid 98% were bought from Merck® (Darmstadt, Germany). The analytical standards for the following substances: amitriptyline hydrochloride, desipramine hydrochloride, fluoxetine hydrochloride, imipramine hydrochloride, nortriptyline hydrochloride, sertraline hydrochloride, and venlafaxine hydrochloride (1 mg/mL) were bought from Cerilliant® (Round Rock, TX, USA). Benzoylecgonine, bupropion, cocaethylene, cocaine, desvenlafaxine, ester methyl ecgonine (EME), citalopram oxalate, and norfluoxetine hydrochloride standards were purchased from LGC Standards® (Manchester, NH, USA). The internal standards (IS) cocaine-d_3 (1 mg/mL), paroxetine-d_6, and nortriptyline-d_3 (100 µg/mL) were purchased from Cerilliant® (Round Rock, TX, USA). Ultrapure water was supplied through a Milli-Q water system, with a resistivity of 18.18 MΩ * cm and conductivity of 0.05 µS/cm and, both at 25 °C (Millipore, Billerica, MA, USA). The iron oxide (II, III) nanopowder was commercially obtained from Merck® (Darmstadt, Germany) and has a particle size of 50-100 nm, with 97% purity based on trace metal analysis. It consists of a black powder with magnetic properties when in contact with an external magnetic field.

Solution and blank samples

Working solutions were prepared in acetonitrile, with a concentration of 100 µg/mL. Solutions were stored at -20 °C when not in use. The internal standards pool solution was prepared in acetonitrile at 1 µg/mL, containing cocaine-d_3, nortriptyline-d_3, and paroxetine-d_6. Optimization and validation experiments were performed with blank postmortem samples previously tested with the method developed by Franco de Oliveira and colleagues (2019), fortified with the reference standards working solutions [24].

Instrumentation and analysis

All samples were analyzed using a liquid chromatographic system coupled to a triple quadrupole mass spectrometer model LCMS-8045 (Shimadzu, Kyoto, Japan). The chromatographic separation was performed with a Raptor^{TM} Biphenyl HPLC column (1.8 µm particle size, 90Å, 30 x 2.1 mm) (Restek Corporation), heated at 60 ºC, with a 0.3 mL/min flow rate. The mobile phase used was ultra-pure water (A) and acetonitrile (B), both fortified with formic acid (0.1%, v/v). Chromatography was performed using a gradient, where the concentration of mobile phase B started at 50%, increased to 100% at 3 min, and stabilized at 100% up to 4.5 min. Mobile phase B returned to 2%, remaining stable until the end of the run (8 min). The acquisition in the mass spectrometer for all compounds was performed using electrospray ionization in positive mode. The instrumental parameters were set as follows: heat block temperature, 400 °C; capillary voltage, 1.5 kV; nebulizer gas (N_2) flow, 2.3 L/min; desolvation line temperature, 526 °C; drying gas (N_2) flow, 10 L/min; and collision-induced dissociation gas pressure (Ar), 230 kPa. The analyses were performed in multiple reaction monitoring (MRM) mode. Two MRM transitions were selected, one for the quantification and another for the qualification of each analyte, except for fluoxetine, where only one transition was used. The transitions of each compound, their respective collision energies, and retention time are presented in **Table 1**. Data was extracted using LabSolutions® software (Shimadzu, Kyoto, Japan).

Optimization of sample preparation

The Fe_3O_4 magnetic nanoparticles were weighed in 1.5 mL polypropylene conical microtubes, respecting a standard deviation of ± 0.5 mg. An aliquot of 100 µL of the whole blood sample and 5 µL of the internal standard pool (1 µg/mL) were added to the nanopowder. The mixture was shaken in a vortex mixer. Subsequently, the nanoparticles were separated from the sample using a neodymium magnet inserted into a 3D-printed microtube rack (**Figure 1**). The supernatant was discarded, and a desorption solvent was added to the nanoparticles. The sample was again vortexed and separated, with the resulting organic supernatant being transferred to glass vials. Afterward, 1.5 µL was injected into the analytical system.

A multivariate factorial experiment was conducted to optimize the mass of nanoparticles, time of extraction and desorption, and desorption solvent volume. All variables were assessed at two levels each and simultaneously tested to observe their

combined effect. The solvent volume was evaluated at 100 - 300 µL, the extraction time at 30 - 60 seconds, and the nanoparticle mass at 10 - 30 mg. We also assessed the necessity of protein precipitation as a clean-up step and the influence on sample pH alteration. The desorption solvent was optimized using a centroid-simplex design with a three-component mixture system and a special cubic model. The evaluated solvents were acetonitrile, ethyl acetate, and methanol. The mixtures evaluated were pure solvent, binary mixtures 1:1 (v/v), one ternary mixture 1:1:1 (v/v/v), and mixtures with 2/3 of one of the components and 1/6 of the other two components, totaling ten experiments. All optimization studies were performed using blank postmortem blood samples spiked with all analytes, with a 500 ng/mL concentration. The experimental data obtained from the optimization was evaluated using the Statistica®10.0 software (Statsoft, Tulsa, OK, USA) and Minitab®19.1 (LLC, US).

Validation

The method validation involved the assessment of various parameters, including the lower limit of quantitation (LLOQ), selectivity, linearity, carryover, precision, bias, dilution integrity, and matrix effect. The experiments were conducted based on the ANSI/ASB Standard 036 guideline from the American Academy of Forensic Science [25].

The LLOQ was measured using three different blank postmortem blood samples fortified with decreasing concentrations of each analyte in three replicates. The lowest concentration with a coefficient of variation (CV%) inferior to 20% was determined as the substance's LLOQ. Endogenous interference was conducted with the analysis of ten different blank samples. In contrast, interference from stable isotopes IS was performed in two batches, three replicates each, one constituted by samples with the addition of IS, without the analytes, and the other by adding analytes without IS.

Furthermore, the linearity was calculated using calibration curves with five replicates of blank samples spiked at six concentration levels: 10, 200, 400, 600, 800, and 1000 ng/mL. The presence of homoscedasticity was evaluated using the Fisher's F-test, and in cases where heteroscedasticity was present, a weighted linear regression was employed. The chosen weight was the one that presented the highest coefficient of determination (r^2) and the lower sum of residues. The carryover essay was performed,

with the injection of six replicates of a blank sample, each after injecting the highest concentration of the calibration curve.

Precision and bias assessments were conducted through the analysis of blood samples spiked at three distinct quality control (QC) concentrations: 30 ng/mL for low QC, equivalent to three times the LLOQ; 430 ng/mL for medium QC; and 860 ng/mL for high QC, which is 80-90% the upper limit of the calibration curve. Each QC level was analyzed in five runs, with each run consisting of three replicates. Precision was evaluated by the analysis of within-run and between-run CV%, with the acceptable limit set at 20%. In terms of bias, the verification involved assessing the percentage of the average measured concentration and comparing it to the nominal QC concentration, aiming for tolerance within ±20% at each QC point. Samples spiked at a concentration of 5,000 ng/mL, underwent the evaluation of dilution integrity, using a 1:5 dilution factor, with blank whole blood serving as the diluent and analyzed in triplicates over five different runs. The matrix effect (ME) was determined as in Matuszewski et al. (2003), using six replicates of postmortem blood from different individuals spiked with low and high QC concentrations in comparison to neat standards at the same concentration in purified water [26]. The ME is represented by the average concentration of replicates from six different samples and is expressed as a percentage as follows.

$$ME\% = \frac{\text{peak area in matrix}}{\text{peak area in neat standard solution}} * 100$$

A ME% exceeding 100% shows ionization enhancement, while a ME% below 100% suggests ionization suppression. The normalized matrix effect (NME%) was computed by considering the analyte-to-internal standard ratio instead of the absolute area in the matrix or neat standard solution.

Application to authentic samples

To demonstrate the method's applicability, 263 postmortem blood samples from the General Institute of Expertise of Rio Grande do Sul, Brazil, underwent analysis. This study was approved by the Ethical Committee for Human Studies of the Federal University of Health Sciences of Porto Alegre, Brazil (CAAE 17996819.7.0000.5345).

Results and discussion

Optimization

The responses obtained in the multivariate factorial experiment were evaluated by the geometric mean chromatographic areas of all analytes. The influence of the tested parameters in the analytical response is illustrated in the Pareto Chart shown in **Figure 2**. The solvent volume negative value indicates that volumes closer to the low level represent the best response for this parameter. The fact that it passed the red line suggests that the reduction in solvent volume is significant. The positive value on mass passing the red line implies that the increase in weight is noteworthy and contributes positively to the outcome. The time of extraction had a negative value and did not pass the red line, which means that the reduction of time did not significantly impact the overall result; therefore, a lower time of extraction/desorption was chosen to achieve a faster technique. In summary, the Pareto chart illustrates that the most impactful factor is the reduction in solvent volume, followed by the increase in nanoparticle mass, while the decrease in time has an insignificant effect. However, the reduced solvent volume implied a poor dispersion of the nanoparticles and turbid supernatant, leading to the choice of a higher volume. Following those directions, the selected solvent volume was the higher level, 300 µL, while the nanoparticle mass was 30 mg, and the time of extraction was 30 seconds.

Altering the sample pH was not a practical option since changes in the viscosity of blood end up impairing the dispersion of the sorbent material. Postmortem blood samples can sometimes be more viscous than *in-vivo* samples or show signs of coagulation; further compromising the sample could implicate a bad extraction efficiency. The necessity of protein precipitation with $ZnSO_4$ and NaOH as a clean-up step was also evaluated, yet the ionic charge impaired the adsorption properties of the nanoparticles. The best condition was achieved by adding the matrix directly to the nanoparticle. For the desorption step, the desorption solvent type was optimized, using the geometric mean of all analytes for each experiment as the evaluated response. The surface plot, presented in **Figure 3**, showed a higher intensity in the region with 33.3% methanol, 33.3% acetonitrile, and 33.3% acetyl acetate. The r^2 of the statistical model was 0.94. Thus, the final mixture used for sample preparation was ethyl acetate: methanol: acetonitrile (1:1:1, v/v). The final extraction method was settled as illustrated in **Figure 1**.

Validation

The LLOQ was evaluated considering the guideline that defined the required minimum analytical scope and sensitivity for testing of blood in the suspected toxicological cause of death determination [26]. The guide established cut-off values over 20 ng/mL for the analytes present in this study. In this method, the LLOQ was defined at 10 ng/mL, with a CV% inferior to 20%, ranging from 7.36% to 17.91% as shown in **Table 2.** In **Figure 4** the chromatograms obtained for each analyte at the LLOQ are displayed. The method's selectivity was determined with no interferences detected in the interference assays; all responses were lower than the LLOQ signal.

The achieved linearity results are present in **Table 2**. Heteroscedasticity was present in all analytes, probably due to the extended concentration ranges that were evaluated for linearity. For all analytes, the r^2 was above 0.99. The chosen weights with the best r^2 and residual sum are available in **Table 2.** Analytes in the carryover experiments were not detected.

Within and between-run precision and bias results are reported in **Table 3**. The coefficient of variation in precision was inferior to 20% for all analytes. The range of the CV% for intra-day precision in the low QC was between 2.69% and 8.59% for EME and sertraline, respectively. In the high QC, the range was between 2.56% for sertraline and 12.82% for benzoylecgonine. As for the inter-day precision, the range of CV% was between 4.51% and 9.04% for desvenlafaxine and venlafaxine in the low QC, respectively, and 4.86% for sertraline to 10.9% for EME in the high QC. The bias obtained was below ±20% for all analytes, indicating the accuracy of the method.

The matrix effect study is a critical step in method validation since the presence of compounds capable of suppressing or enhancing ionization will result in a reduced or heightened detector response, respectively [27]. Results are presented in **Table 4**. The analytes amitriptyline, benzoylecgonine, citalopram, EME, nortriptyline, sertraline, and venlafaxine, presented ion suppression in the lowest concentration since the ME (%) values were inferior to 75%. However, the use of internal standard corrected the ME for all analytes except for benzoylecgonine, in which the NME was 61.81%, indicating ionization suppression of this analyte. This can be explained using cocaine-d_3 as the internal standard for benzoylecgonine. Due to their distinct molecular characteristics, achieving accurate correction of matrix effects may prove challenging. This limitation can be overcome by using a deuterated benzoylecgonine standard [28]. None of the analytes presented ionization enhancement over 25% in either concentration, before or

after normalization with IS. Matrix effects are common in biological samples due to the presence of several endogenous compounds, such as lipids, vitamins, and proteins, and exogenous substances, such as other therapeutical or recreational drugs [29]. This effect is even higher for postmortem blood, considering the distinct stages of collection. The quality of the sample varies from well-preserved blood to samples in the decomposition process, as well as other physiological modifications caused by the cause of death [30].

Considering the broad therapeutic levels and the possible toxic concentrations of the analytes, it was important to determine that samples with concentrations above the upper limit of the calibration curve could be diluted to fit the linear model. The dilution integrity was supported within the 1:5 dilution evaluated, for all analytes, with CV% inferior to 20%. The range of the CV% was between 11.6% and 19.63% for benzoylecgonine and bupropion, respectively.

Proof of applicability

The method was successfully applied to 263 postmortem samples. All samples evaluated in this study were positive for at least one substance. The most frequent analyte was benzoylecgonine, which can be explained by the formation of this cocaine metabolite by the plasma cholinesterase [31]. Regarding these samples, six were above dilution integrity, ranging from 5,054 ng/mL to 8,103 ng/mL. Cocaethylene was detected in 102 samples (39%), cocaine in 93 samples (35%), and EME in 42 samples (16%). The high frequency of exposure to these substances is coherent with the high prevalence of cocaine use and overdose reported in Brazil [32-34]. Also, the concomitant use of cocaine and alcohol produces the metabolite cocaethylene and is associated with intoxication accidents [34-35].

The most common antidepressant encountered in the analyzed samples were citalopram and fluoxetine, followed by fluoxetine's metabolite norfluoxetine. Both of those drugs are commonly prescribed in Brazil. Seven samples were positive for venlafaxine in concentrations above the linear range of the method; however, four were within the dilution integrity, and the remaining were above 5,000 ng/mL. **Table 5** presents the concentration range found for each analyte.

Concomitant use of at least one antidepressant and cocaine was found in 44 samples. This association may be related to the fact that antidepressants have a potential effect on remission from cocaine disorder and are constantly prescribed in the treatment

of cocaine dependence [36-38]. However, some relate show that antidepressant drugs may enhance cocaine-induced toxicity, contributing to intoxication cases with this association of substances [39].

Comparison of the proposed method

For the analysis of antidepressants, cocaine, and its metabolites in postmortem blood, SPE is one of the traditional extraction methods. The sample volume varies but usually stays in the range of 1-2 mL, which is 10, 20 times the sample volume used in the method developed in this work. Surrounding organic solvents, the SPE method usually consumes around 6-10 mL, considering the conditioning step, clean-up, and elution, for each sample [40-42], while with m-dSPE we used 300 µL of solvent.

The use of different analytical systems, samples, adsorbents, and analytes can certainly influence the comparison between methods. Very few works that used magnetic nanoparticles as sorbents for psychoactive substances in biological samples are available in the literature. As far as we know, none of those employed these sorbents for the analysis of postmortem blood. In 2014, Asgharinezhad et al. (2014) developed a dSPE method with polyaniline/magnetic nanoparticles for the extraction of lorazepam and nitrazepam from plasma and urine samples. The method uses 5 mg of nanocomposite, 150 µL of elution solvent and the total sample preparation time is 7 minutes [43]. In Asgharinezhad and colleagues (2015) work, a nanocomposite composed of polypyrrole and magnetic nanoparticles was used to extract citalopram and sertraline from urine and plasma samples. The LLOQ was 200 ng/mL, and the applicability was tested in spiked samples rather than real case ones. In summary, although they used fewer nanoparticles and solvents, the extraction time was longer and for only two analytes, using an elevated sample volume compared to the present method [44]. Yang et al. (2017), evaluated m-dSPE with divinyl benzene and vinyl pyrrolidone functionalized Fe_3O_4 nanoparticles for the detection of cocaine and its metabolites in urine. The extraction time was over 20 minutes, using 10 mg of nanoparticles, 1 mL of sample, and 1 mL of elution solvent. The elution step took 10 minutes, and the sample was analyzed in a LC-MS system [45]. A common characteristic of the developed works using nanoparticle adsorption properties is the functionalization of nanoparticles for only one analyte or very few. Little to no paper evaluated several substances with variable structures and properties like the ones in this work.

Conclusion

It was possible to successfully develop, validate, and apply an LC-MS/MS method for the determination of antidepressants, cocaine, and metabolites in postmortem blood samples. The use of magnetic Fe_3O_4 nanoparticles as sorbents to m-dSPE was an easy, cheap, practical, and efficient alternative. As far as we are concerned, this is the first report of using this sorbent to determine these substances in postmortem blood. The method results were satisfactory for linearity, precision, accuracy, and selectivity for all compounds. Proof of applicability was confirmed with the analysis of 263 postmortem blood samples from forensic cases, which were all positive for at least one of the analytes present in this method, often found in toxicology routine. Therefore, the developed methodology can be applied to forensic casework as an efficient, sustainable alternative to classical techniques.

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detection of cocaine and cocaine metabolites in human urine by high-performance liquid chromatography–mass spectrometry. Journal of separation science. 2017; https://doi.org/10.1002/jssc.201700457

Table 1: Analytes and their respective identification parameters for LC-MS/MS detection.

Analytes	Target and internal standards (IS)	MRM transitions (*m/z*)	Collision Energy (V)	Retention time (min)	Internal Standard	MRM Mode
Cocaine and metabolites						
Benzoylecgonine	Target	289.9500>168.2000 289.9500>77.0500	-20 -55	2.907	Cocaine- d_3	Positive
Cocaethylene	Target	318.0500>196.1500 318.0500>82.2000	-20 -35	3.231	Cocaine- d_3	Positive
Cocaine	Target	304.0500>182.1500 304.0500>82.1500	-20 -32	3.064	Cocaine- d_3	Positive
Cocaine-d_3	IS	307.0500>185.1500 307.0500>85.1500	-20 -32	3.058		Positive
EME	Target	200.0500>182.1500 200.0500>82.2500	-20 -26	0.519	Cocaine-d_3	Positive
Antidepressants						
Amitriptyline	Target	278.2000>91.0500 278.2000>105.0500	-27 -25	3.655	nortriptyline- d_3	Positive
Bupropion	Target	240.0000>184.0000 240.0000>166.0000	-13 -16	3,163	Paroxetine-d_6	Positive
Citalopram	Target	325.1000>109.1000 325.1000>234.1000	-30 -31	3.413	Nortriptyline- d_3	Positive
Desipramine	Target	267.2500>72.0500 267.2500>44.0500	-16 -40	3.564	Nortriptyline- d_3	Positive
Desvenlafaxine	Target	264.1000>58.1000 264.1000>246.1000	-20 -13	2.841	Paroxetine- d_6	Positive
Fluoxetine	Target	310.1500>44.1500	-15	3.710	Nortriptyline- d_3	Positive
Imipramine	Target	281.2000>86.3000 281.2000>58.1000	-19 -46	3.600	Nortriptyline- d_3	Positive
Norfluoxetine	Target	296.3000>134.1500 296.3000>30.1500	-7 -24	3.664	Nortriptyline- d_3	Positive
Nortriptyline	Target	264.1500>233.1000 264.1500>91.1000	-15 -25	3.615	Nortriptyline- d_3	Positive
Nortriptiline-d_3	IS	267.1500>233.0500 267.1500>105.0500	-15 -24	3.611		Positive
Paroxetine-d_6	IS	336.1500>198.0500 336.1500>48.1500	-23 -25	3.515		Positive
Sertraline	Target	306.0500>275.0500 306.0500>159.0000	-15 -26	3.723	Paroxetine-d_6	Positive
Venlafaxine	Target	278.1000>58.1500 278.1000>260.1500	-20 -14	3.212	Paroxetine-d_6	Positive

Table 2: Linearity, regression diagnostics, and Lower Limit of Quantitation values for the evaluated analytes.

Compound	LLOQ* (10 ng/mL)	Linearity range (ng/ml)	Weight	Slope	Intercept	r^2
	CV%					
Amitriptyline	17.9	10-1000	$1/X^{1/2}$	0.0059	0.0657	0.9964
Bupropion	10.9	10 - 1000	1/Y	0.0185	0.1562	0.9901
Citalopram	15.3	10 - 1000	$1/X^{1/2}$	0.0022	0.0330	0.9924
Desipramine	13.3	10 - 1000	$1/X^{1/2}$	0.0274	0.3191	0.9960
Desvenlafaxine	12.3	10 - 1000	$1/Y^{1/2}$	0.0275	0.1778	0.9983
Fluoxetine	16.9	10-1000	$1/X^{1/2}$	0.0083	0.0484	0.9986
Imipramine	17.3	10-1000	$1/X^{1/2}$	0.0299	0.2976	0.9970
Norfluoxetine	13.7	10-1000	$1/Y^{1/2}$	0.0023	0.0302	0.9944
Nortriptyline	9.8	10-1000	$1/X^{1/2}$	0.0145	0.0930	0.9986
Sertraline	7.4	10-1000	$1/Y^{1/2}$	0.0008	0.0097	0.9953
Venlafaxine	16.5	10 - 1000	1/X	0.0455	0.0018	0.9984
Benzoylecgonine	18.2	10 - 1000	1/X	0.0010	0.0069	0.9907
Cocaethylene	10.0	10 - 1000	$1/X^{1/2}$	0.0131	0.1291	0.9942
Cocaine	16.1	10 - 1000	1/X	0.0110	0.0180	0.9975
EME	13.6	10 - 1000	1/X	0.0004	0.0029	0.9905

*LLOQ: Lower Limit of Quantitation

Table 3: The intra- and inter-day precision and accuracy of the method at quality control (QC) points: Low (30 ng/mL), Medium (430 ng/mL), and high (860 ng/ml).

Compound	Intra-day						Inter-day					
	Low		Medium		High		Low		Medium		High	
	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Amitriptyline	8.11	13.71	6.36	-14.41	7.26	-5.85	6.46	13.98	6.12	-14.01	7.08	-6.29
Bupropion	4.86	1.47	7.00	-13.24	3.94	-0.76	5.39	5.71	6.99	-14.45	5.05	-1.99
Citalopram	8.57	15.13	0.92	-18.78	3.78	-13.84	7.62	13.52	5.11	-19.08	6.76	-12.88
Desipramine	5.12	16.77	7.64	-15.44	5.13	-9.06	4.94	15.43	6.54	-14.16	6.14	-7.15
Desvenlafaxine	4.30	-2.35	9.62	-10.39	4.74	-5.29	4.51	-2.49	8.06	-9.07	5.83	-4.07
Fluoxetine	7.49	14.24	6.83	-12.90	5.88	-7.19	6.67	13.51	6.22	-12.29	7.27	-4.91
Imipramine	5.10	13.67	5.57	-13.27	6.01	-8.54	5.16	13.77	4.46	-12.16	6.60	-6.69
Norfluoxetine	5.45	15.95	9.90	-14.94	8.50	-2.16	4.89	16.11	8.29	-14.21	7.42	-1.94
Nortriptyline	7.35	16.07	6.76	-7.18	7.35	-4.86	5.93	16.54	6.34	-7.48	7.34	-4.44
Sertraline	8.59	5.32	5.33	1.11	2.56	3.10	8.28	3.18	6.40	1.32	4.86	2.12
Venlafaxine	6.90	14.31	4.73	4.25	8.20	-4.57	9.04	11.52	7.38	1.51	8.93	-2.12
Benzoylecgonine	4.52	4.87	13.23	-12.73	6.91	-2.56	5.59	3.33	10.86	-9.83	8.72	-5.19
Cocaine	8.01	4.47	8.76	-10.83	9.69	-10.78	7.14	4.67	7.08	-10.73	8.48	-8.49
Cocaethylene	5.76	16.00	8.63	-12.14	11.54	-8.14	4.83	16.45	8.39	-11.14	9.61	-6.43
EME	2.69	-2.33	7.46	-11.35	12.82	-3.58	9.00	-3.8	10.5	-12.2	10.9	-4.4

Table 4: Matrix effect in low Quality Control level (30 ng/mL) and high Quality Control level (860 ng/mL) for the evaluated analytes.

Compound	Low QC*		High QC*	
	ME (%)	NME (%)	ME (%)	NME (%)
Amitriptyline	61.04	93.34	83.85	87.84
Bupropion	91.18	115.03	119.77	97.07
Citalopram	68.08	88.29	90.52	92.37
Desipramine	75.22	95.40	105.75	92.98
Desvenlafaxine	75.40	94.08	94.50	93.80
Fluoxetine	76.47	90.95	93.01	90.87
Imipramine	75.28	90.06	91.22	87.14
Norfluoxetine	78.54	101.15	93.12	94.89
Nortriptyline	73.28	95.92	95.36	94.39
Sertraline	74.29	94.03	92.54	94.63
Venlafaxine	69.00	91.73	75.69	88.72
Benzoylecgonine	52.81	61.81	111.01	93.86
Cocaethylene	80.41	82.17	102.52	83.48
Cocaine	86.17	88.15	97.93	84.43
EME	74.07	90.54	112.43	82.96

*QC: Quality Control

Table 5: Positivity frequencies of the analytes (n), the percentage of the results (%) and the range of concentration detected.

Cocaine and metabolites	**n**	**%**	**Range (ng/mL)**
Benzoylecgonine	166	63.1	10.2 - > 5000.0
Cocaethylene	102	38.8	10.0 - 152.5
Cocaine	93	35.4	10.6 - 1249.2
EME	42	16.0	10.0 - 84.4
Antidepressants			
Citalopram	45	17.1	7.2 - > 5000.0
Fluoxetine	45	17.1	76.0 - > 5000.0
Norfluoxetine	32	12.2	17.0 - 1091.4
Nortriptyline	27	10.3	10.1 - 975.9
Amitriptyline	24	9.1	24.0 - 4342.5
Sertraline	18	6.8	11.3 - 2252.6
Desvenlafaxine	17	6.5	162.1 - 4852.8
Venlafaxine	12	4.6	12.0 - > 5000.0
Desipramine	6	2.3	12.1 - > 5000.0
Imipramine	4	1.5	344.4 - 2001.0
Bupropion	4	1.5	36.4 - 856.5

Figure 1: Final developed extraction protocol illustration (A). 3D-printed microtube rack (B)

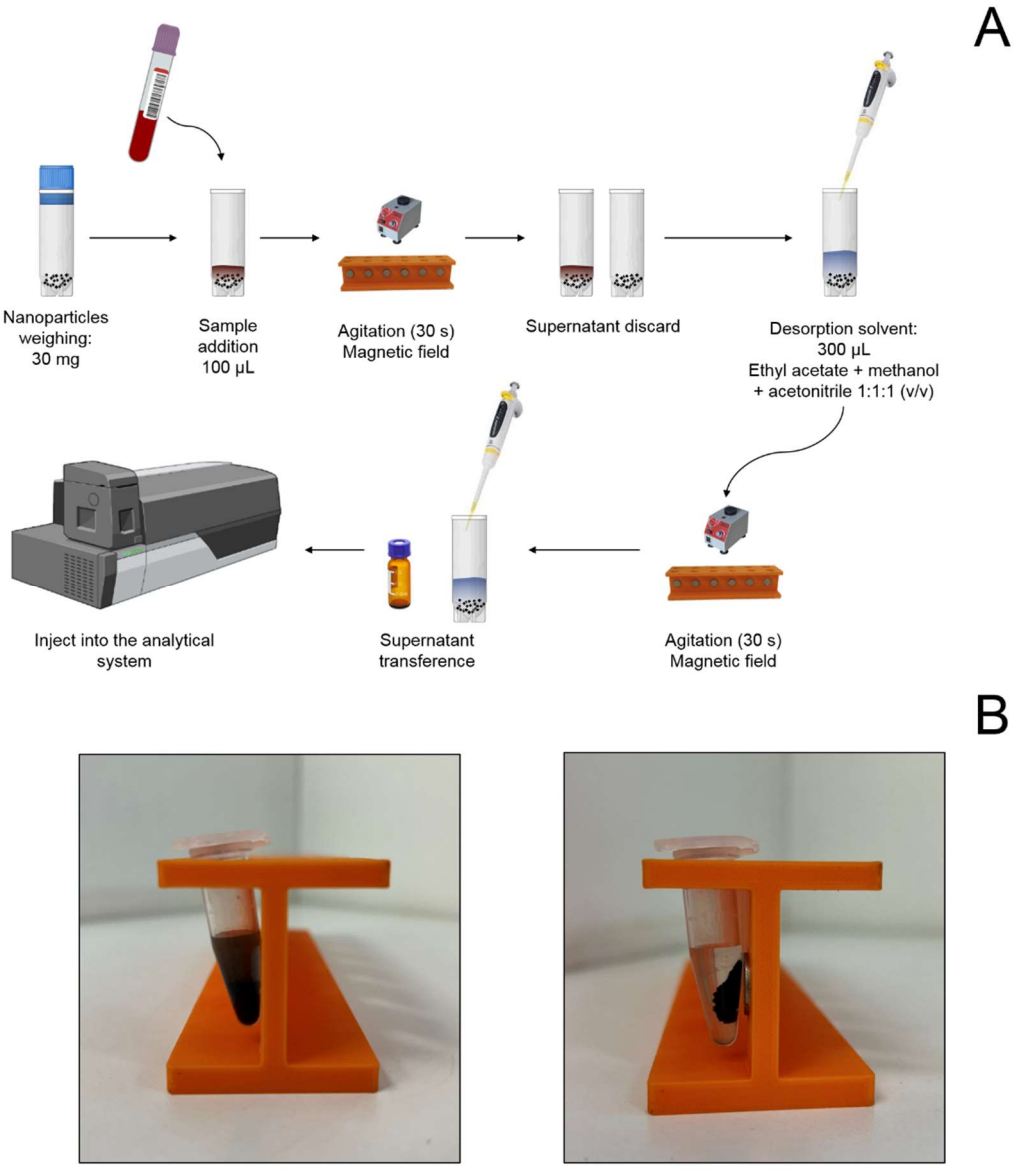


Figure 2: Pareto Chart of standardized effects for the optimization of solvent volume, nanoparticles mass and extraction/desorption time

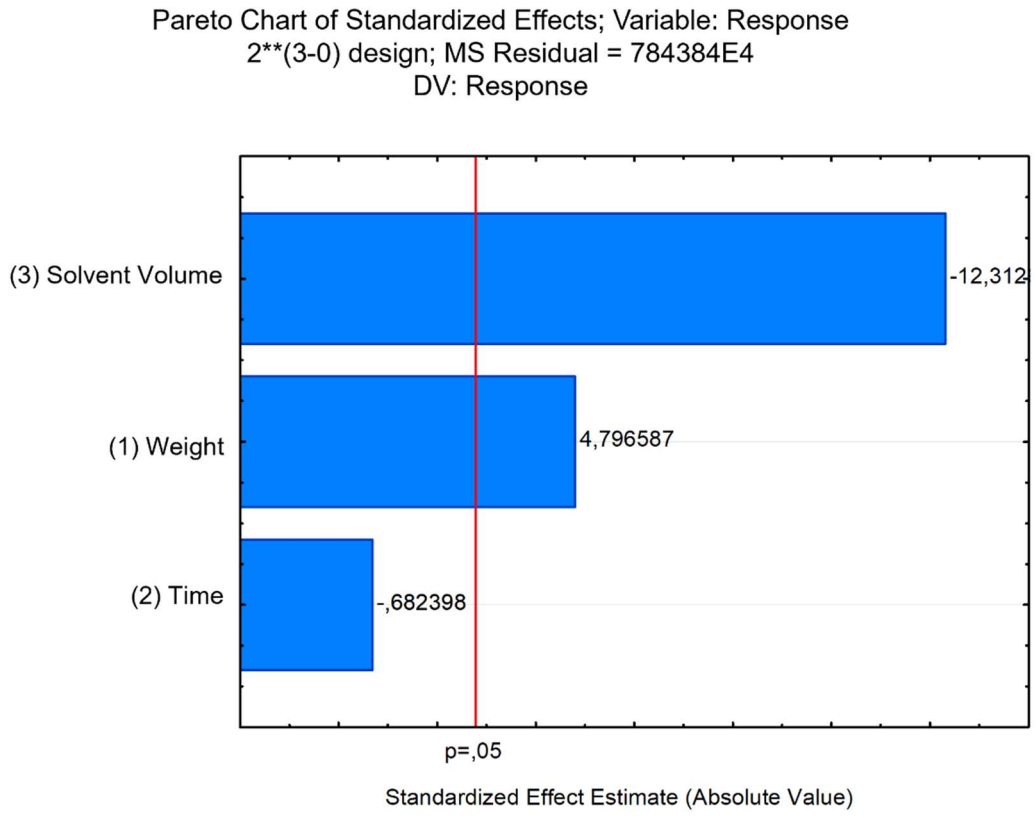


Figure 3: Response mixture surface plot for solvent type optimization

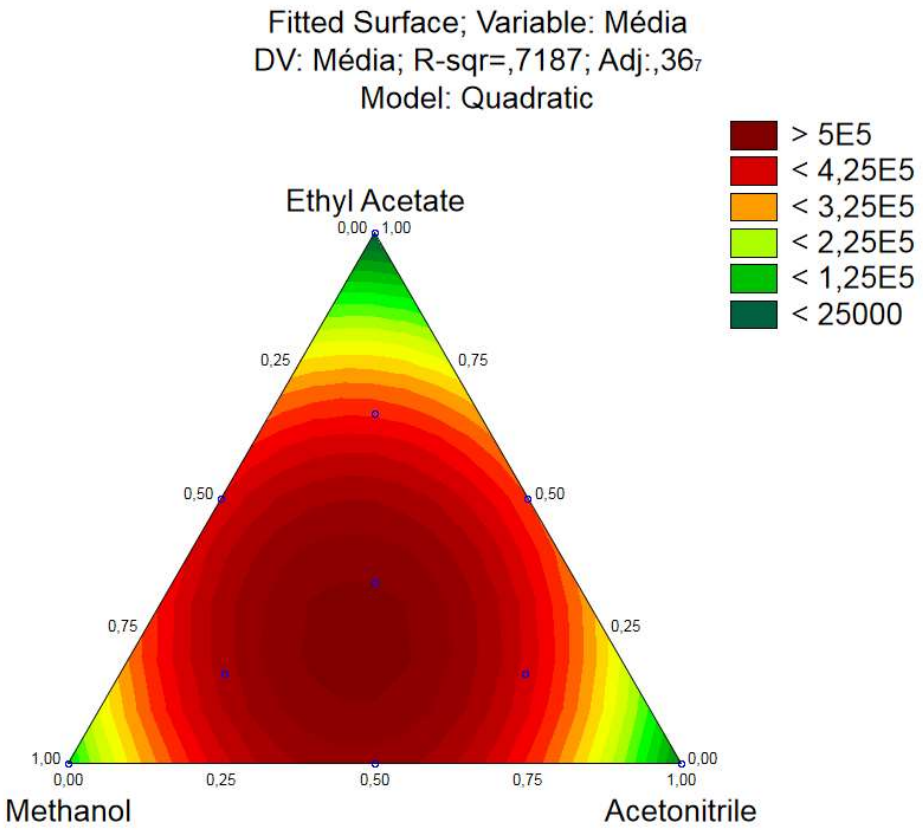
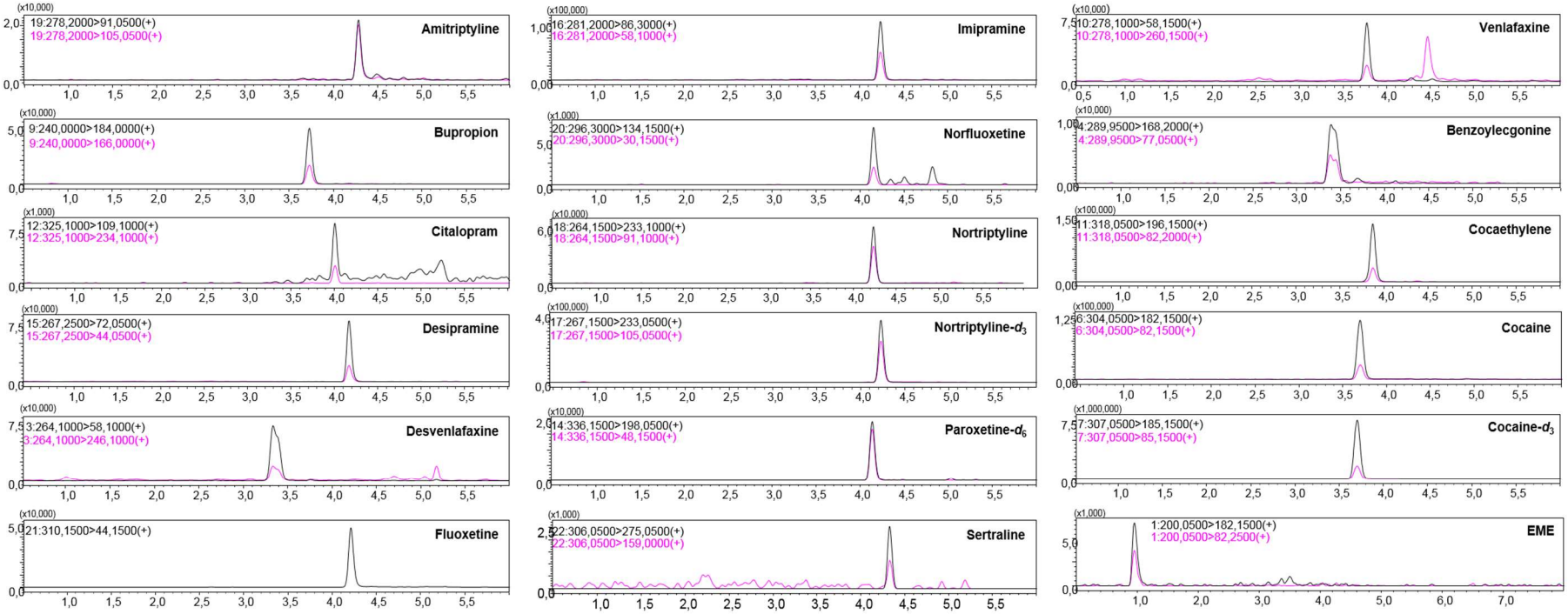


Figure 4: Chromatogram obtained by LC-MS/MS from a sample spiked with all analytes in the LLOQ concentration (10 ng/mL)



5. CONCLUSÕES

Nesse estudo, foi possível desenvolver, validar e aplicar com sucesso um método LC-MS/MS para a determinação de antidepressivos, cocaína e produtos de biotransformação em amostras de sangue post-mortem. O uso de nanopartículas magnéticas de Fe_3O_4 como sorventes para extração sólida em fase dispersiva magnética (m-dSPE) se mostrou uma alternativa de fácil execução, rápida, de baixo custo e eficiente. Pelo que sabemos, este é o primeiro relato do uso deste sorvente para determinar essas substâncias em sangue *post mortem*. Os resultados do método foram satisfatórios quanto à linearidade, precisão, exatidão e seletividade para todos os compostos. A prova de aplicabilidade foi confirmada com a análise de 263 amostras de sangue *post mortem* de casos forenses, todas positivas para pelo menos um dos analitos presentes neste método, frequentemente encontrados na rotina toxicológica. Portanto, a metodologia desenvolvida pode ser aplicada em trabalhos forenses como uma alternativa eficiente e sustentável às técnicas clássicas.

6. ANEXO I – PARECER CONSUBSTANCIADO DO COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Desenvolvimento de métodos analíticos para identificação de novas substâncias psicoativas de interesse forense por espectrometria de massas de alta resolução

Pesquisador: TIAGO FRANCO DE OLIVEIRA
Área Temática:
Versão: 2
CAAE: 17996819.7.0000.5345
Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.784.184

Apresentação do Projeto:
As drogas naturais, como maconha e cocaína, foram gradativamente substituídas pelas sintéticas. O recente relatório intitulado "Global Synthetic Drugs Assessment", publicado pelo United Nations Office on Drugs and Crime - UNODC, revelou que a produção e o consumo de drogas sintéticas têm alcançado números alarmantes, superando os da heroína e
cocaína em muitos lugares do mundo. Segundo o relatório global, foram registradas 348 Novas Substâncias Psicoativas (NPS), de 2008 a 2013, mas o número real de NPS disponível no mundo pode ser significativamente superior, dado que esses números refletem apenas relatos de fontes oficiais e não leva em conta fontes não oficiais. As assim chamadas drogas sintéticas são substâncias ou misturas de substâncias psicoativas produzidas por síntese química a partir de substâncias precursoras encontradas ou não na natureza. A dimensão e os padrões de uso dessas substâncias ainda não são claros e, provavelmente, estão sendo subestimados. Diante do exposto, o objetivo desse projeto é o desenvolvimento de metodologias analíticas para a correta identificação de NPSs (etilona, 2,5-dimetoxi-4-bromoanfetamina, 2,5-dimetoxi-4-metilanfetamina, 25CNBOMe, 25B-NBOMe) e outras substâncias psicoativas em matrizes biológicas por espectrometria de massas de alta resolução. As metodologias aqui desenvolvidas serão disponibilizadas para o Departamento de Perícias Laboratoriais do Instituto Geral de Perícias (DPL-IGP) para a posterior

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implementação na rotina laboratorial, objetivando a elucidação e o mapeamento de casos de envolvendo o consumo de NPSs e outras substâncias psicoativas.

Objetivo da Pesquisa:

1) Objetivo Primário:

Desenvolvimento de metodologias analíticas para a correta identificação de NPSs e substâncias psicoativas convencionais em matrizes biológicas por espectrometria de massas de alta resolução, fornecendo assim subsídios para elucidação de casos com suspeita de ocorrência dessa classe de substâncias.

2)Objetivo Secundário:

a)desenvolvimento de métodos multi-analiticos que permitam a análise simultânea em um intervalo curto de tempo, propiciando uma identificação rápida e precisa das substâncias envolvidas em casos suspeitos de NPS;

b)desenvolvimento e validação de estratégias analíticas de screening em cabelo para as classes de substâncias anfetaminas, antidepressivos, antipsicóticos, barbitúricos, benzodiazepínicos e cocaína;

c)desenvolvimento tecnológico adquirido poderá ser facilmente aplicado a outros projetos de pesquisa, contribuindo assim para avanços importantes no que concerne às implicações da toxicologia analítica à ciências forenses;

d)após a validação metodológica, os procedimentos desenvolvidos estarão acessíveis para Departamento de Perícias Laboratoriais do Instituto Geral de Perícias (DPLIGP);

e) para realizar a transferência do know-how as metodologias serão prioritariamente construídas considerando os métodos de rotina e o parque instrumental do DPL-IGP;

f) os dados gerados no trabalho serão tabulados para confecção de artigos científicos construídos em parceria com a equipe do DPL-IGP.

Avaliação dos Riscos e Benefícios:

Riscos:

Os pesquisadores descrevem que não há riscos para os envolvidos na pesquisa pois as amostras biológicas utilizadas serão oriundas do descarte do laboratório do DPL-IGP. As

amostras terão seu uso liberado pelo responsável do laboratório somente após estarem em posição de descarte. Referem ainda que o possível risco é referente a identificação dos indivíduos, no entanto, os pesquisadores garantem que a identificação dos indivíduos será mantida em anonimato, através da utilização de códigos previamente estabelecidos.

Benefícios:
Não haverá benefícios diretos ao participante da pesquisa, mas o estudo será importante pois será desenvolvido uma metodologia capaz de identificar diferentes substâncias psicoativas em diversas matrizes biológicas que possa ser disponibilizada para os principais serviços de avaliação de intoxicações por drogas de abuso no estado do Rio Grande do Sul. Além disto, espera-se a identificação destes compostos em casos suspeitos possibilitando o mapeamento da distribuição das substâncias elencadas.

Comentários e Considerações sobre a Pesquisa:
Amostra: amostras biológicas de rotina (sangue, urina, vísceras) ou de oportunidade (humor vítreo e cabelo) disponibilizadas pelo DPL-IGP de casos com suspeita de ocorrência de NPS, passíveis de análises toxicológicas, atendidos pelo Instituto durante o período de vigência do projeto. Atualmente, estas amostras são analisadas na rotina Departamento de Perícias Laboratoriais e descartadas, conforme a legislação vigente (ANVISA, RDC 306/04, CONAMA, RDC 358/05), que por tratar de resíduos do Grupo A1 são submetidos a processos de tratamento em equipamentos que promova a redução de carga microbiana e encaminhados para aterro sanitário licenciado para disposição final destes resíduos.

Considerações sobre os Termos de apresentação obrigatória:
- Solicitam a utilização apenas do TCUD, tendo em vista a impossibilidade de obtenção do TCLE, uma vez que se trata de amostras biológicas oriundas do descarte do laboratório do DPL-IGP e que terão seu uso liberado pelo responsável do laboratório somente após estarem em posição de descarte.
- Não encontra-se anexado o termo de compromisso para entrega dos relatórios parciais e final.

Recomendações:
- O projeto somente poderá ter início após sua aprovação na integralidade pelos CEP's envolvidos.
- Solicita-se encaminhar por notificação o termo de compromisso para entrega dos relatórios parciais e final. Como trata-se e um projeto com período de realização abrangente, os relatórios parciais devem ser anuais além do relatório final. Destaca-se a importância da entrega destes relatórios para acompanhamento do CEP, além de possibilitar ao pesquisador, dentro da vigência do mesmo, o envio de qualquer emenda/notificação.
- Data Final de Vigência do Projeto: 01/09/2023.

Conclusões ou Pendências e Lista de Inadequações:

- Os pesquisadores atenderam às solicitações contidas em parecer emitido anteriormente por este CEP.

Considerações Finais a critério do CEP:

De acordo com o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1401883.pdf	06/11/2019 15:31:08		Aceito
Outros	TCUD.pdf	06/11/2019 15:30:43	TIAGO FRANCO DE OLIVEIRA	Aceito
Declaração de Instituição e Infraestrutura	TemoAnuenciaGerLab.pdf	06/11/2019 15:30:30	TIAGO FRANCO DE OLIVEIRA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_parceria_UFCSPA_IGP.docx	23/07/2019 16:09:50	TIAGO FRANCO DE OLIVEIRA	Aceito
Outros	Termo_liberacao_amostras.pdf	23/07/2019 16:09:32	TIAGO FRANCO DE OLIVEIRA	Aceito
Outros	Termo_anuencia_instituicao_coparticipante.pdf	23/07/2019 16:09:11	TIAGO FRANCO DE OLIVEIRA	Aceito
Folha de Rosto	folhaDeRosto_Tiago_Oliveira.pdf	23/07/2019 16:06:23	TIAGO FRANCO DE OLIVEIRA	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

PORTO ALEGRE, 19 de Dezembro de 2019

Assinado por:
Luciane Dalcanale Moussalle
(Coordenador(a))

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7. ANEXO II – NORMAS DE PUBLICAÇÃO DO PERIÓDICO

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Analytical and Bioanalytical Chemistry

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Submission guidelines

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Instructions for Authors

Peer Review Terminology

Analytical and Bioanalytical Chemistry and Springer Nature are participating in a pilot of NISO/STM's Working Group on Peer Review Terminology.

The National Information Standards Organization (NISO) and STM, the International Association of Scientific, Technical and Medical Publishers, has recognized a need to identify and standardize definitions and terminology in peer review practices in order to help align nomenclature as more publishers use open peer review models.

A peer review taxonomy that is used across publishers will help make the peer review process for articles and journals more transparent, and will enable the community to better assess and compare peer review practices between different journals.

The following summary describes the peer review process for this journal:

Identity transparency: Single anonymized

Reviewer interacts with: Editor

Review information published: None

The full terminology is detailed here.

We would welcome your feedback on the Peer Review Terminology Pilot – please can you take the time to fill this short survey:

Survey

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General Information

The central Editorial Office of Analytical and Bioanalytical Chemistry is located at Springer Heidelberg.

Analytical and Bioanalytical Chemistry

Springer

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Manuscripts must be written in English.

The journal publishes Research Papers, Papers in Forefront, Communications, Critical Reviews, Trends, Feature Articles and Letters to the Editor. For more details about the paper types, please go to the hyperlink 'What type is my paper' that you find under "Journal updates" on the journal homepage.

Only new, previously unpublished results given in concise form will be accepted. Known facts should only be mentioned briefly, with appropriate literature citations. Authors are asked to also submit copies of any related papers currently under consideration by another journal.

The manuscript must be accompanied by a Cover Letter. The Cover Letter should have 400 to 600 words and contain the following items:

- Significance of the work

- Novelty of the work

- Contribution to the field

With their submission, authors are encouraged to suggest potential reviewers for their work. They may also prepare a graphical abstract figure which concentrates on the innovative aspects of the submitted work.

Analytical and Bioanalytical Chemistry is a 'color for free' journal with no extra charges for color figures to the author in both the print and online versions. Upon publication the corresponding author will receive a free pdf of the article.

Authors are requested to submit their articles online. Please log directly onto the site http://mc.manuscriptcentral.com/abc or go to the hyperlink 'Submit manuscript' on the right and upload your manuscript following the instructions given on the screen. The Editorial Office is automatically alerted by the system immediately after your manuscript has been received.

If you face any problems using the electronic submission procedures or have any questions regarding the submission of your manuscript, please contact the Editorial Office at abc@springer.com.

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Editorial procedure

Single-blind peer review

This journal follows a single-blind reviewing procedure.

Additional information

This journal is guided by the Springer Portfolio editorial peer review policy.

The following types of contribution to this journal are peer-reviewed: Research Papers and Papers in Forefront, Communications, Critical Reviews, Trends and Feature Articles. Correspondence and all forms of published correction may also be peer-reviewed at the discretion of the editors. Other contributed articles are not usually peer-reviewed. Nevertheless, articles published in these sections, particularly if they present technical information, may be peer-reviewed at the discretion of the editors.

Every paper submitted to ABC is first assessed by the Editors with respect to its scientific content and in particular in terms of its analytical novelty. The corresponding author is notified by e-mail when the editor decides not to send a paper for review. Manuscripts judged to be of potential interest to our readership are sent for formal review, typically to two or three reviewers. Reviewer selection is critical to the publication process, and we base our choice

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The editors then make a decision based on the reviewers' advice, from among several possibilities: (a) Accept, with or without editorial revisions (b) Invite the authors to revise their manuscript to address specific concerns before a final decision is reached (c) Reject, but indicate to the authors if further work might justify a resubmission (d) Reject outright, typically on grounds of specialist interest, lack of novelty, insufficient conceptual advance or major technical and/or interpretational problems.

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Manuscript Submission

Manuscript Submission

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Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

The name(s) of the author(s)

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A clear indication and an active e-mail address of the corresponding author

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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

Trial registration number and date of registration for prospectively registered trials

Trial registration number and date of registration, followed by "retrospectively registered", for retrospectively registered trials

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

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The following statements should be included under the heading "Statements and Declarations" for inclusion in the published paper. Please note that submissions that do not include relevant declarations will be returned as incomplete.

Competing Interests: Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Please refer to "Competing Interests and Funding" below for more information on how to complete this section.

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

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Text

Text Formatting

Manuscripts should be submitted in Word.

The text of a research paper should be divided into Introduction, Materials and Methods, Results, Discussion, Acknowledgements, Conflict of Interest, and References.

Materials and Methods must include statement of Human and Animal Rights.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

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Always use footnotes instead of endnotes.

Acknowledgments and Funding Information

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full. In addition, please provide the funding information in a separate step of the submission process in the peer review system. Funder names should preferably be selected from the standardized list you will see during submission.

If the funding institution you need is not listed, it can be entered as free text. Funding information will be published as searchable metadata for the accepted article, whereas acknowledgements are published within the paper.

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Scientific style

Please always use internationally accepted signs and symbols for units (SI units).

Nomenclature: Insofar as possible, authors should use systematic names similar to those used by IUPAC.

Genus and species names should be in italics.

Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

Please use the standard mathematical notation for formulae, symbols, etc.: Italic for single letters that denote mathematical constants, variables, and unknown quantities; Roman/upright for numerals, operators, and punctuation, and commonly defined functions or abbreviations, e.g., cos, det, e or exp, lim, log, max, min, sin, tan, d (for derivative); Bold for vectors, tensors, and matrices.

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References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1–3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The entries in the list should be numbered consecutively.

If available, please always include DOIs as full DOI links in your reference list (e.g. "https://doi.org/abc").

Journal article

Smith JJ. The world of science. Am J Sci. 1999;36:234–5.

Article by DOI

Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. J Mol Med. 2000; https://doi.org/10.1007/s001090000086

Book

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

Book chapter

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology.

London: Academic; 1980. pp. 251–306.

Online document

Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

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Customised EndNote-Style

Please make sure that you use a term list with abbreviated journal names.

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Analytical and Bioanalytical Chemistry

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Tables

All tables are to be numbered using Arabic numerals.

Tables should always be cited in text in consecutive numerical order.

For each table, please supply a table caption (title) explaining the components of the table.

Definition: Black and white graphic with no shading.

Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

All lines should be at least 0.1 mm (0.3 pt) wide.

Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.

Vector graphics containing fonts must have the fonts embedded in the files.

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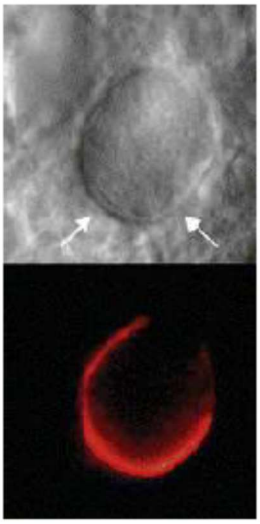
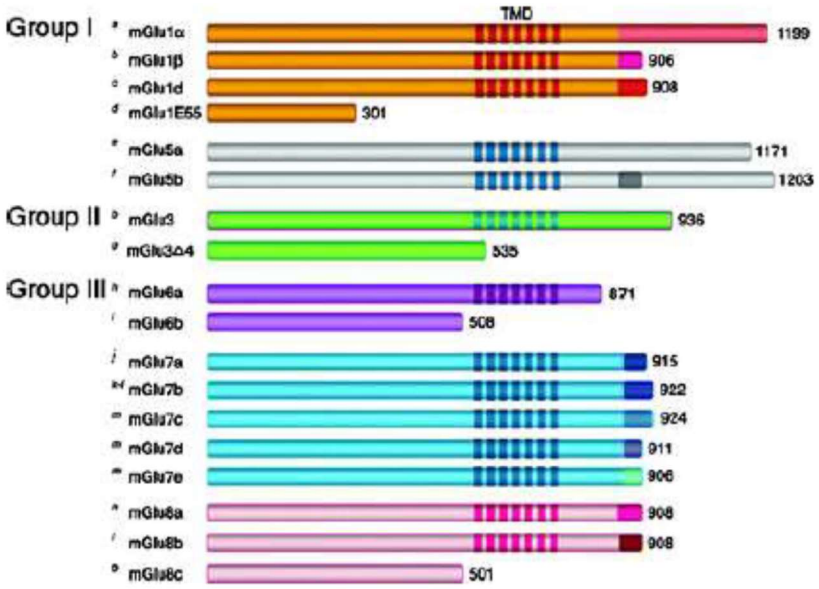


Definition: Photographs, drawings, or paintings with fine shading, etc.

If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.

Halftones should have a minimum resolution of 300 dpi.

Combination Art



Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.

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Color art is free of charge for online publication.

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If the figures will be printed in black and white, do not refer to color in the captions.

Color illustrations should be submitted as RGB (8 bits per channel).

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To add lettering, it is best to use Helvetica or Arial (sans serif fonts).

Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).

Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.

Avoid effects such as shading, outline letters, etc.

Do not include titles or captions within your illustrations.

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All figures are to be numbered using Arabic numerals.

Figures should always be cited in text in consecutive numerical order.

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If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,"A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

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Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.

Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.

No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.

Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.

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The manuscript contains a descriptive caption for each supplementary material

Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

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A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006

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Informed consent

Please note that standards could vary slightly per journal dependent on their peer review policies (i.e. single or double blind peer review) as well as per journal subject discipline. Before submitting your article check the instructions following this section carefully.

The corresponding author should be prepared to collect documentation of compliance with ethical standards and send if requested during peer review or

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