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Development and validation of a simple and fast method for routine analysis of new synthetic opioids and hallucinogens in whole blood using protein precipitation and UHPLC-MS/MS

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ABSTRACT

In forensic toxicology, the rapid and reliable detection of emerging synthetic opioids and hallucinogens is crucial for case investigations and public health monitoring. This work describes the development, optimization and validation of a simple, fast and sensitive methodology for the simultaneous analysis of 6 new synthetic opioids (carfentanil, fentanyl, isotonitazene, metonitazene, norfentanyl, and sufentanil) and 2 hallucinogens (lysergide [LSD] and mescaline), together with the main LSD metabolite 2-oxo-3-hydroxy-lysergide [LSD-OH], in whole blood samples by liquid chromatography coupled to tandem mass spectrometry. Under optimized experimental conditions, linearity was verified between 0.1 and 20 ng/mL for all analytes except mescaline (2.5–500 ng/mL), with $r^2 > 0.99$ for 1/x weighting, and no significant carryover or matrix effects were observed. Good precision (% RSD < 13 %) and trueness (% Bias within ± 20 %) values were achieved. The estimated limit of quantification (LOQ) was 0.1 ng/mL for all compounds except mescaline (2.5 ng/mL). Authentic forensic samples were also analyzed, and positive samples for fentanyl, norfentanyl, and sufentanil were identified. The proposed methodology allows the simultaneous analysis of compounds from different families of psychoactive substances, in both postmortem and *in vivo* samples, using only 50 μL of whole blood. The demonstrated speed, simplicity, and effectiveness make it particularly advantageous for routine implementation in forensic toxicology laboratories.

1. Introduction

Nowadays, drug abuse is a public health issue. The quantity and diversity of substances available in drug markets worldwide have been increasing, mainly due to synthetic drugs, and essentially because of the phenomena of New Psychoactive Substances (NPS), also known as "legal highs" or "designer drugs". NPS are compounds that are not controlled under the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which pose a threat to public health, not being necessarily new inventions, but substances that have recently become available. The NPS phenomena works as a way to circumvent the prohibitive laws applied to classic drugs, providing (legal) substances, easily produced, cheaper, and capable of mimicking the psychoactive effects of these controlled drugs. However, due to the

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lack of knowledge on their pharmacology, the risks associated with the consumption of these drugs are very high. Moreover, users may even not know that they are buying and consuming these substances, as traffickers mix them with other drugs or sell them as their substitution [1–3].

The fastest growing NPS group in recent years are synthetic opioids, increasing their overall percentage from 3 % (in 2015) to 15 % (in 2022) [1]. Opioids are known for their analgesia potential, but when consumed improperly, they may cause several adverse effects, the most serious one being respiratory depression, which, in extreme cases, can trigger acute cerebral hypoxia, coma, and even death. Among new synthetic opioids, the most notorious group is fentanyl and its analogs, particularly relevant, since 2013, in the United States of America Opioid Crisis, being responsible for thousands of fatal intoxications. The implementation of fentanyl-related laws led to a reduction in the number of fentanyls available, however new families of synthetic opioids were quickly introduced into the illicit drug market, being benzimidazole opioids ("nitazenes") the family that stands out and concerns the most nowadays. Currently, dozens of nitazenes have already been identified, mainly sold as heroin and cocaine powders and in fake oxycodone and benzodiazepine tablets [4–7]. Like fentanyls, nitazenes have been responsible for numerous overdoses worldwide, many of which were fatal. These emerging compounds can be dozens to thousands of times more potent than morphine or even fentanyl, being extremely toxic. Thus, in cases of intoxications, they are found in very low concentrations (ng/mL to pg/mL levels) in blood [8–13].

Nevertheless, it is important to remain aware of other more "traditional" psychoactive drugs, such as classic hallucinogens, namely lysergic acid diethylamide (LSD) or mescaline. The consumption of hallucinogens affects the mind, increasing brain neuroplasticity and triggering profound changes in perception, cognition, emotion, mood and consciousness. Due to the global increase in mental health problems, there has been a renewed interest in the therapeutic use of hallucinogens in the treatment of mental/psychological disorders [14]. Although most deaths from hallucinogen use are associated with accidental or self-inflicted traumatic injuries (*e.g.*, defenestration or self-mutilation) that occur as a consequence of psychotic states, there are also records of deaths or near-deaths directly attributed to the toxicity of these compounds, namely mescaline and LSD [15,16].

As a result, it is imperative for forensic toxicology laboratories to implement highly sensitive methodologies for the monitoring of these compounds in biological matrices. In postmortem cases, the need for sensitive methodologies with low sample consumption is particular important, because the amount or volume of available samples is usually very limited. Additionally, considering the enormous number of samples (sometimes thousands) that forensic toxicology laboratories have to analyze monthly, it is also important to develop fast and effective methodologies.

Thus, the present work reports the development, optimization, and validation of a sensitive, fast, simple, and effective methodology for routine analysis in forensic toxicology laboratories of 6 synthetic opioids (fentanyl, norfentanyl, sufentanil, carfentanil, isotonitazene, and metonitazene) and 2 classic hallucinogens (LSD and mescaline), and the primary LSD metabolite, 2-oxo-3-hydroxy-lysergide (LSD-OH), in whole blood samples (postmortem and *in vivo*), by protein precipitation followed by Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). LC-MS/MS is the method of choice for the analysis of new synthetic opioids and hallucinogens in biological matrices, namely blood. Protein precipitation is a sample preparation technique widely used in toxicology, especially in blood samples [17–21]. The chemical structures of the target analytes are present in Fig. 1.

2. Materials and methods

2.1. Chemicals, reagents, and preparation of working solutions

LC-MS grade (≥ 99.9 %) methanol (MeOH), water and acetonitrile (ACN) were purchased from Honeywell - Riedel-de Haën™ (Germany). LC-MS grade formic acid (98 – 100 %) and ammonium formate (≥ 99.9 %) were obtained from Merck (Germany). Certified analytical standards (> 99 %) of fentanyl, fentanyl-D_5 (used as internal standard (IS)), LSD, LSD-OH, mescaline, norfentanyl, and sufentanil were purchased from Cerilliant (USA). Isotonitazene and metonitazene (≥ 98 %) were obtained from Cayman Chemical (USA). Carfentanil (≥ 98 %) was purchased from Toronto Research Chemicals (Canada). Stock solutions of each of the target analytes were prepared by dissolving the respective standard in MeOH or ACN, to a final concentration of 10 µg/mL. A fentanyl-D_5 stock solution at 1 µg/mL was also prepared. Analytes and IS working solutions were prepared weekly in ACN by dilution of the stock solutions at desired concentrations. All certified analytical standards,

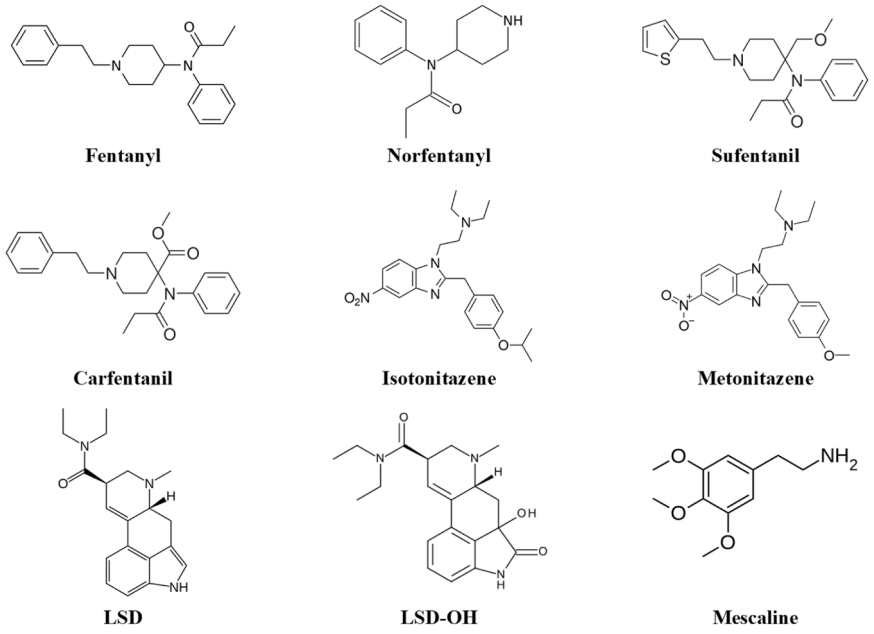

Fig. 1. Chemical structures of the target analytes of the present work.

stock solutions, and working solutions were stored at −20 ºC in amber borosilicate glass vials, until use.

2.2. Blood samples

In the present work, *in vivo* and postmortem human whole blood samples were used. All samples were provided by the Serviço de Química e Toxicologia Forenses da Delegação Sul do Instituto Nacional de Medicina Legal e Ciências Forenses, I.P. (INMLCF, Lisbon - Portugal), having been collected within the INMLCF legal attributions, or supplied from a local blood bank, the Portuguese Blood Institute. These samples were handled according to the INMLCF protocols and ethical regulations concerning data privacy and sample handling. The *in vivo* blood samples were collected into plastic tubes containing ethylenediaminetetraacetic acid disodium salt (EDTA), and the postmortem blood samples were collected into plastic tubes containing 1 % of potassium fluoride. The samples were stored at −20 ºC and, prior to analysis, were placed in a roller mixer in order to thaw and reach room temperature. The blank blood samples were previously screened to verify that they were drug-free. Blank pooled whole blood samples used for validation assays were prepared in the laboratory.

The applicability to real context was tested through the analysis of authentic forensic whole blood samples. In total, 19 *in vivo* and post-mortem samples suspected of containing hallucinogens and/or synthetic opioids were analyzed. Additionally, a serum sample from an inter-laboratory proficiency test organized by ARVECON GmbH (Walldorf, Germany) was also analyzed.

2.3. Protein precipitation

2.3.1. Design of experiments

To obtain the ideal conditions for the analysis of the target compounds, the protein precipitation procedure was optimized using a design of experiments (DoE) approach, specifically a full factorial experimental design. The application of DoE allows systematic investigation of multiple factors and their interactions, providing statistically robust results for identifying the most influential parameters affecting analytical performance. For these assays, blank samples were fortified with the target analytes at a concentration of 10 ng/mL, and the resulting data were evaluated with JMP® Statistical Discovery LLC software (version 17.2.0). The DoE approach consists in performing a series of tests/experiments designed to model and investigate the relationship between factors and selected responses for the identification of experimental conditions that maximize analytical response.

Three experimental factors were selected based on their relevance to protein precipitation efficiency and analyte recovery:

- precipitation solvent volume (X1: 100, 150 and 200 µL), influencing both protein denaturation and the dilution of the supernatant;
- percentage of formic acid to be added to the ACN (X2: 0.1, 0.3 and 0.5 %), which affects the solvent's polarity and pH, thereby modulating both protein aggregation and analyte ionization efficiency; and
- centrifugation time (X3: 2, 5 and 10 min), which determines the completeness of phase separation and removal of residual particulate matter.

All optimization experiments were performed in duplicate, in randomized order, for two days. The response evaluated was the ratio of compound's quantification transition chromatographic peak area and IS chromatographic peak area (relative area), together with the presence of well-defined and identifiable peaks in the two transitions, according to the positivity criteria defined in the World Anti-Doping Agency (WADA) guideline [22].

2.3.2. Sample treatment

The optimized procedure of the developed methodology consists in adding, to a 1.5 mL Eppendorf conical tube, 5 µL of IS at 100 ng/mL followed by 5 µL of spiking solution (for positive quality controls and calibrators) or 5 µL of LC-MS grade water (for samples and negative quality controls). Then, transfer 50 µL of the blood sample to the tube and add, drop by drop, at constant speed and under vortex agitation, 100 µL of the ACN + 0.1 % formic acid solution (stored at - 20 °C). After that, centrifuge for 5 min at 5 °C and 14.000 rpm, transfer part of the supernatant to a polypropylene autosampler vial and inject directly into the UHPLC-MS/MS.

2.4. UHPLC-MS/MS parameters

UHPLC-MS/MS analysis were performed in an ExionLC™ AD Series ultra-high pressure liquid chromatograph (Sciex, USA) coupled to a QTRAP® 6500 + mass spectrometer (Sciex, USA). Extracts were injected onto a C_{18} reverse phase Acquity UPLC HSS T3 column (2.1 mm×100 mm; 1.8 µm; 100 Å) from Waters (USA). The injection volume was 2 µL, with an injection speed of 5 µL/s. Column oven and autosampler temperatures were maintained at 45 ºC and 15 ºC, respectively. The elution method is adapted as described by Matos *et al.* [23] and Antunes *et al.* [24], commonly employed at the Serviço de Química e Toxicologia Forenses da Delegação Sul do INMLCF. The mobile phase consisted of an aqueous solution with 2 mM ammonium formate and 0.1 % formic acid, to which 1 % of organic mobile phase is added (A) and a methanolic solution with 2 mM ammonium formate and 0.1 % formic acid (O). The flow rate was set at 0.4 mL/min. The elution gradient started at 90 % A for the first 0.5 min, transitioning to 5 % A at minute 3 and held for 1 min before reverting to the initial conditions at 4.1 min and held for 0.9 min, resulting in a total run time of 5 min per sample.

Multiple reaction monitoring (MRM) mode was used, monitoring two transitions for each analyte (confirmation transition and quantification transition) and one for the IS. Compounds ionization was performed on positive electrospray ionization (ESI +) with an ion spray voltage of 4500 V and ionization source temperature maintained at 250 ºC. Nitrogen was used as the nebulizer gas (ion source gas 1 and 2 at 60 psi), as the curtain gas (with pressure of 35 psi), and as the collision gas. MRM transitions, retention times and specific UHPLC–MS/MS

Table 1
MRM transitions (ESI +), retention time and optimized LC–MS/MS parameters for the analysis of the target analytes and IS.

Analyte	Q1 (Da)	Q3 (Da)	DP (V)	EP (V)	CE (V)	CXP (V)	RT (min.)
Carfentanil	**395.1**	**335.1**	88	10	26	30	**3.15**
	395.1	246.1	88	10	30	22	3.15
Fentanyl	**337.1**	**188.2**	61	10	33	20	**3.09**
	337.1	105.0	61	10	51	18	3.09
Fentanyl-D_5	342.1	193.2	61	10	33	20	3.09
Isotonitazene	**411.2**	**100.1**	46	10	21	35	**3.30**
	411.2	72.1	46	10	49	35	3.30
LSD	**324.3**	**223.2**	86	10	31	14	**2.94**
	324.1	208.1	66	10	39	24	2.94
LSD-OH	**356.1**	**237.2**	66	10	33	24	**2.50**
	356.1	222.1	66	10	41	24	2.50
Mescaline	**212.2**	**165.1**	31	10	32	4	**2.40**
	212.2	133.0	21	10	35	14	2.40
Metonitazene	**383.2**	**100.1**	48	10	21	25	**3.04**
	383.2	72.1	48	10	45	25	3.04
Norfentanyl	**233.1**	**84.1**	41	10	23	10	**2.75**
	233.1	150.2	41	10	25	18	2.75
Sufentanil	**387.1**	**238.1**	81	10	27	22	**3.27**
	387.1	140.2	81	10	35	16	3.27

Q1: precursor ion; Q3: product ion; DP: decluster potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential; RT: retention time. Quantification transitions in bold.

parameters for each compound are presented in Table 1. Analyst Instrumental Control and Data Processing Software (version 1.7.3, 2022) and Sciex OS Software (version 2.1.6.59781, 2021) (Sciex, USA) were used for data acquisition and data processing/analysis, respectively.

2.5. Method validation

The optimized methodology was validated according to the ANSI/ASB Standard 036 – "Standard Practices for Method Validation in Forensic Toxicology" [25], and the following parameters were evaluated: interference studies, ionization suppression/enhancement, limit of detection (LOD), limit of quantification (LOQ), calibration model, carryover, precision, accuracy, dilution integrity, and processed sample autosampler stability. Positive criteria for detection and identification of chromatographic peaks were established according to the WADA guideline previously mentioned [22]. More information regarding the validation procedure will be presented later on Section 3.2.

3. Results and discussion

3.1. Protein precipitation optimization assays

Several parameters may affect the analytes extraction from the matrix and their subsequent analysis. In that sense and based on the methodology used in the laboratory [23], it was important to optimize the protein precipitation procedure, to maximize the sensitivity of the methodology. The protein precipitation parameters selected for optimization were precipitation solvent volume, percentage of formic acid to be added to the ACN, and centrifugation time.

The precipitation solvent plays the main role in this sample preparation technique, so it was imperative to optimize their constitution and volume. ACN is generally employed in the protein precipitation process; consequently, it was utilized as the precipitation solvent in the present study. Formic acid was added to the ACN solution due to its theoretical capacity to promote protein precipitation and facilitate the extraction of acidic compounds, such as LSD [26,27]. However, it was important to determine the percentage of formic acid that could be added to the ACN without compromising the extractive process of the other non-acidic analytes. Increasing the volume of precipitation solvent added to the blood leads to more easily occurring protein precipitation but also results in greater dilution of the sample, thus affecting the detection of compounds present in the blood at very low concentrations. Consequently, it was imperative to ascertain the optimal precipitation solvent volume to minimize sample dilution while preserving protein precipitation and pellet formation. Finally, the centrifugation time was evaluated to identify an ideal time that was reduced enough to facilitate and speed up the experimental procedure, but which ensured a complete precipitation of the proteins and pellet formation. This type of optimization is commonly performed using the One-variable-at-a-time (OVAT) method. However, OVAT is time consuming and does not allow for the evaluation of possible interactions between variables, which may significantly affect the analytical response. An alternative that has been increasingly used in forensic analytical method development optimization is Design of Experiments (DoE). DoE applies statistical principles to systematically investigate how individual factors and their interactions influence the analytical outcome, extracting maximum information from a minimal number of experiments. Additionally, this approach enables the simultaneous evaluation and control of several parameters that affect method sensitivity, selectivity, precision, and accuracy. Such comprehensive optimization is essential to enhance the reliability and credibility of analytical results, particularly within forensic and legal contexts [28–31].

In this study, a full factorial design (FFD) was employed to evaluate the influence of all factors on the final response, as well as their interactions. This type of DoE (FFD) was selected, because it is simple, systematically examines/investigates all possible combinations of high and low factors' defined levels, and allows the individual and interaction effects of each factor to be estimated independently. The biggest disadvantage of FFD is the fact that, as the number of factors increases, the number of required experiments grows exponentially, making FFD impractical for studies involving more than four parameters. Notwithstanding, as we did not studied more than four factors in the present work optimization, this was not relevant [31].

The influence of the various factors in the relative area was evaluated through the desirability function, which varies between 0 and 1, the latter corresponding to optimal value. Since there were several answers under study, in this case, the results for each analyte, the global desirability response was calculated from the geometric mean of the individual desirable responses [29]. Given the numerous analytes under study and their varied chemical properties, it was inevitable that the conditions deemed optimal for the different variables could not be identical for all compounds. This led to the necessity to compromise, to ensure that the selected values would yield favorable responses for all analytes. Experiments where inadequate pellet formation was observed were excluded, as the presence of protein fragments in the supernatant could potentially impair the performance and functionality of the UHPLC-MS/MS instrument.

The experiment that yielded the highest global desirability (0.545), along with an excellent pellet formation, consisted of protein precipitation with 100 μL of an ACN solution containing 0.1 % of formic acid and subsequent centrifugation for 5 min. Thus, these were considered the optimized experimental conditions.

3.2. Validation results

3.2.1. Interference studies

The interference studies consisted of evaluating matrix interferents, interferences from the IS, interferences on the IS, and interferences from other analytes frequently encountered. To evaluate matrix interferents, 10 different blank samples were analyzed, without IS addition or target analytes spiking, and no significant matrix interferents were observed at the retention times and monitored transitions of the compounds under study. To evaluate whether the IS interfered in the identification of the target analytes, 3 aliquots were extracted with the addition of fentanyl-D_5 at 100 ng/mL, but without the target analytes. The analysis did not cause a false positive identification of any of the analytes, thus there is no interference from the internal standard. Furthermore, three blood aliquots were fortified with the target analytes at 0.2, 3, and 18 ng/mL (5, 75, and 450 ng/mL for mescaline), respectively, but without IS. After extraction, these samples did not show any interference in the IS detection. Additionally, 3 aliquots, without IS addition, were spiked with a 100 ng/mL solution containing over 200 substances commonly found and routinely analyzed in forensic toxicology laboratories, including: amphetamines/methamphetamines, cocaine and metabolites, cannabinoids, opioids, pesticides, antidepressants, benzodiazepines, antipsychotics, antihypertensives, anesthetics, analgesics, anti-inflammatories, among others. In the presence of these substances, no interferences were observed at the retention times and monitored transitions of the compounds under study, as seen in Fig. 2. These results demonstrate the selectivity of the methodology, thereby ensuring the correct detection of target analytes and the absence of false positives or false negatives, which is of paramount importance to prevent any potential impairment to the toxicological interpretation and the judicial process.

3.2.2. Matrix effects (ionization suppression/enhancement)

To evaluate ionization suppression/enhancement, two sets of samples were prepared, each one at two concentrations levels (0.2 and 18 ng/mL or 5 and 450 ng/mL for mescaline). Set 1 consisted of unextracted neat standards, injected six times for each concentration. Set 2 consisted of 10 different blank samples that were extracted and fortified

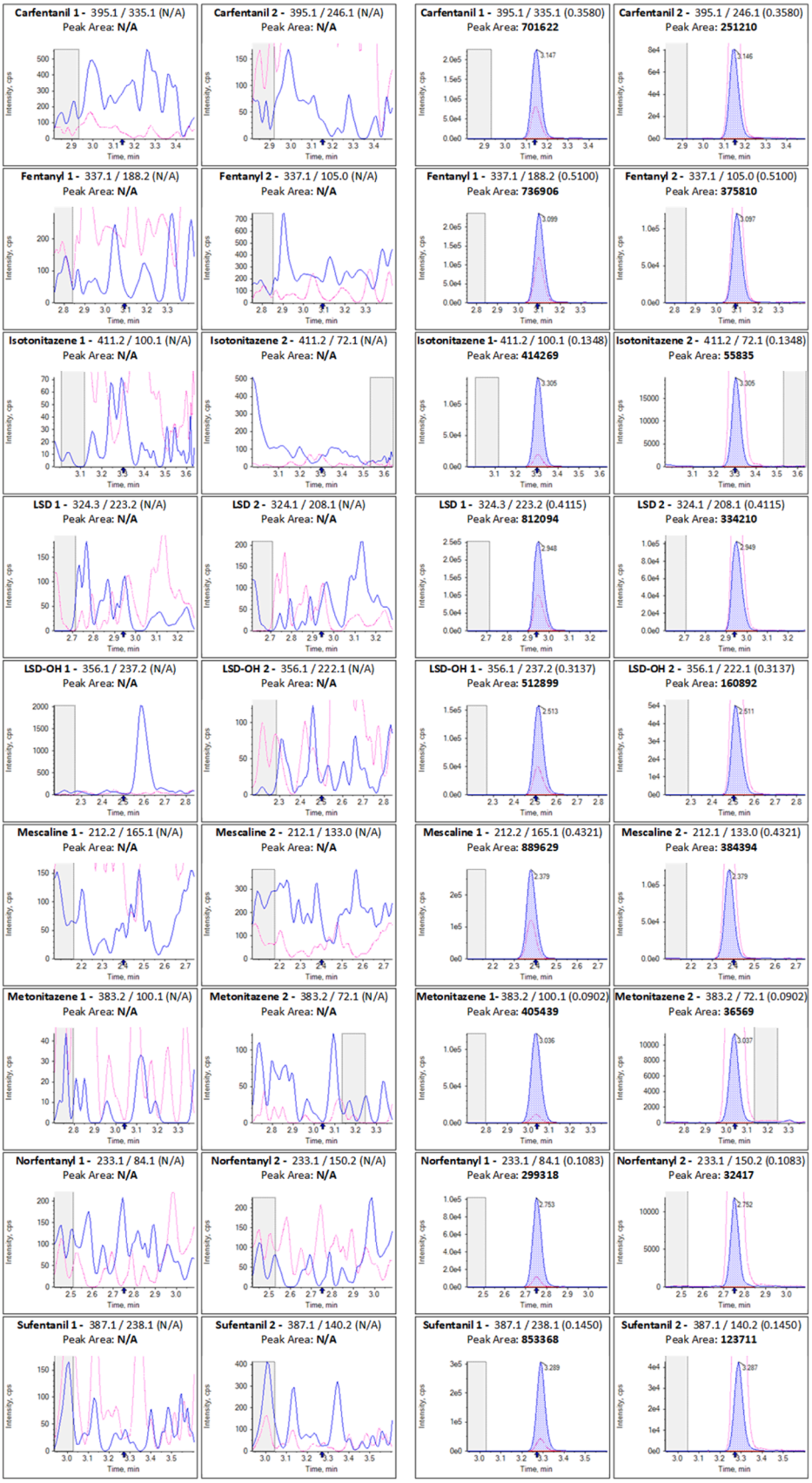

Fig. 2. Extracted ion chromatograms obtained from a sample spiked at 100 ng/mL with over 200 other routinely found and potentially interfering substances (left) and from a positive control sample spiked at 3 ng/mL (75 ng/mL for mescaline) (right). The ion ratio for each analyte is parenthesized in each chromatogram.

(at high and low concentration) after extraction, before injection in the instrument. The average analyte peak areas of each set were used to assess the extent of suppression or enhancement for each concentration. As seen in Table S1, all analytes except mescaline met the matrix effects acceptance criteria, namely % CV under 20 % and matrix effect within ± 25 %. For mescaline, it was verified ion enhancement at lower concentrations, with values higher than 69 %. Although this value exceeds 25 %, it was neither found to be significant nor compromising for mescaline analysis, because CV is under 20 % and all other validation parameters were met, including LOD and LOQ.

3.2.3. Linearity and carryover

The linearity of the methodology was assessed between 0.1 and 20 ng/mL for all compounds excepting mescaline. For mescaline, the linear range was 2.5–500 ng/mL. These values were selected based on typical concentrations encountered in routine cases and *as per* concentrations reported in the literature [21,32–40]. Linearity assays were performed daily for 5 days, using eight calibrators. Data homoscedasticity was evaluated through variance graphs, residuals graphs, and F-test for variances at a 99 % confidence level (data not shown). Since data homoscedasticity was not confirmed, weighting linear regression factors were tested and applied, according to Almeida *et al.* [41]. The best calibration model was selected through several factors, namely: visual evaluation; higher coefficient of determination (r^2); random distribution and smaller amplitude of relative error percentage around the concentration axis; and lower sum of % RE absolute values. Acceptance criteria consisted of $r^2 \geq 0.99$, % RE in between ± 20 %, and replicates % CV ≤ 20 %. For all analytes, the chosen weighting factor was 1/x. The calibration data is summarized in Table 2.

In the context of forensic analysis, particularly in postmortem cases, there is an absence of information regarding the expected concentrations of analytes to be detected in the samples. When a significant interval exists between the consumption of the substance and the collection and subsequent analysis of the sample, the compounds may have already undergone metabolization or elimination processes. Consequently, the concentrations found in the blood will be reduced. Conversely, in cases of lethal overdoses, high concentrations in the blood are likely to be observed. The extensive calibration range employed in this work accounts for these variations, ensuring its suitability for a broad spectrum of sample concentrations.

Carryover was evaluated simultaneously with linearity assays, by analyzing LC-MS grade methanol samples immediately after analysis of the highest calibrator. No carryover phenomena were observed for the majority of monitored ionic transitions. In cases where chromatographic peaks were present, they had areas below 10 % of LOQ signals, thus the carryover was considered absent or insignificant. As a result, it can be concluded that the developed methodology is free of carryover at the established linear range.

3.2.4. LOD and LOQ

To determine the LOD and LOQ, three different pools of blood were fortified at the intended concentrations for each analyte and analyzed in triplicate for three days (n = 27). For LOQ, the samples were fortified at 2.5 ng/mL for mescaline and 0.1 ng/mL for the other analytes. In these studies, WADA positivity peak criteria and precision and accuracy acceptance criteria were met for all the compounds, with % CV values lower than 20 % and % bias falling within the range of ± 20 % (Table 2). The LODs were determined as the minimum concentration in which the substances peaks could be clearly distinguished from a blank sample according to the positivity criteria employed, as shown in Figure S1. LOD was 0.05 ng/mL for all compounds except mescaline, metonitazene and norfentanyl. The established LOD was 1 ng/mL for mescaline, and 0.1 ng/mL for metonitazene and norfentanyl.

Usually, the LOD corresponds to a lower concentration than the LLOQ. However, in this work, for 4-MMC and metonitazene, the values defined as LOD and LLOQ were the same, as lower concentrations did not meet the LOD acceptance criteria. This situation is foreseen in the ANSI/ASB validation guideline adopted [25], which states that these parameters should be defined according to the purpose of the methodology and the needs of the laboratory. Furthermore, the LOD and LLOQ values determined in this study are equivalent with those reported in other works discussed in Section 3.3. It is also important to note that the LLOQ criteria are more stringent than those for the LOD, since it is required that the S/N (signal-to-noise ratio) be equal to or greater than 10, and compliance with established criteria for precision and accuracy.

3.2.5. Precision and accuracy

Precision and accuracy were analyzed simultaneously. Within-run precision (repeatability) and accuracy were estimated daily by extracting aliquots from a fortified blood mixture at three concentration levels (0.2, 3 and 18 ng/mL or 5, 75 and 450 ng/mL in the case of mescaline). These assays were performed in triplicate for each concentration level (n = 3). To estimate between-run precision and accuracy, this process was repeated for five days (n = 15 for each concentration level), always with fresh calibration curves. Accuracy was assessed using the % bias. Within - and between-run precision was assessed using the coefficient of variation (% CV), determined using the one-way ANOVA statistical analysis.

The results for within-run and between-run precision and accuracy studies were in accordance with acceptance criteria (% CV ≤ 20 % and % bias in between ± 20 %). Precision and accuracy data are summarized in Table 3.

3.2.6. Dilution integrity

Sample dilution integrity assessment is of paramount importance in the forensic context, because the expected concentrations to be found in the samples are unknown and may be above the validated linear range. In such cases, it will be necessary to dilute the samples to allow quantification of the compounds. Thus, to validate the sample dilution integrity, a pool sample was spiked at 18 ng/mL (450 ng/mL for mescaline) and subjected to dilutions of 1/2, 1/5 and 1/10, with LC-MS grade water, prior to extraction. Each dilution was performed in triplicate for 5 days, and within-run % CV and % bias were calculated.

As shown in Table S2, for the majority of the target analytes, the

Table 2
Summarized linearity, LOD and LOQ data, obtained for all the target analytes.

Analyte	Linearity (weighting factor 1/x)		LOD (n = 27)	LOQ (n = 27)		
	Linear Range (ng/mL)	r^2	Concentration (ng/mL)	Concentration (ng/mL)	% CV	% Bias
Carfentanil	0.1–20	0.995	0.05	0.1	8.06	7.68
Fentanyl	0.1–20	0.996	0.05	0.1	8.19	11.61
Isotonitazene	0.1–20	0.996	0.05	0.1	12.41	8.25
LSD	0.1–20	0.995	0.05	0.1	7.52	14.48
LSD-OH	0.1–20	0.996	0.05	0.1	10.57	9.69
Mescaline	2.5–500	0.993	1	2.5	11.09	−12.43
Metonitazene	0.1–20	0.996	0.1	0.1	6.82	12.63
Norfentanyl	0.1–20	0.995	0.1	0.1	4.17	19.42
Sufentanil	0.1–20	0.996	0.05	0.1	7.58	14.88

Table 3
Within- and between-run precision and accuracy results (n - number of replicates) obtained for the target analytes at three different concentrations in whole blood samples, under the optimized experimental conditions of the developed methodology.

Analyte	Concentration (ng/mL)	Measured[a] (ng/mL)	Precision (% CV)		Accuracy (% Bias)	
			Within-run (n = 3)	Between-run (n = 15)	Within-run (n = 3)	Between-run (n = 15)
Carfentanil	0.2	0.21 ± 0.01	5.50	5.45	9.09	5.68
	3	2.95 ± 0.19	4.89	6.42	−7.17	−1.53
	18	17.72 ± 1.42	4.84	8.01	−7.79	−1.54
Fentanyl	0.2	0.21 ± 0.02	7.23	7.89	9.78	2.56
	3	2.98 ± 0.26	7.63	8.70	8.47	−0.61
	18	17.22 ± 1.31	6.85	7.71	−12.61	−4.33
Isotonitazene	0.2	0.21 ± 0.02	8.35	10.21	11.64	3.28
	3	2.99 ± 0.27	8.41	9.08	−13.09	−0.24
	18	17.42 ± 1.77	7.39	10.38	−10.60	−3.23
LSD	0.2	0.21 ± 0.02	7.52	8.04	10.29	2.64
	3	2.87 ± 0.18	6.70	6.24	−9.08	−4.26
	18	16.41 ± 1.59	8.34	9.72	−15.05	−8.84
LSD-OH	0.2	0.20 ± 0.02	7.99	11.01	13.67	−1.19
	3	2.90 ± 0.35	7.06	12.12	−18.45	−3.42
	18	16.76 ± 1.64	5.39	9.77	−14.33	−6.91
Mescaline	5	4.90 ± 0.52	9.42	10.70	11.98	−1.95
	75	79.16 ± 7.05	7.68	9.00	10.71	5.55
	450	408.48 ± 31.88	7.98	7.79	−15.87	−9.23
Metonitazene	0.2	0.22 ± 0.02	9.09	8.89	15.60	9.45
	3	3.01 ± 0.26	7.24	8.57	8.62	0.38
	18	17.94 ± 1.34	4.64	7.47	−11.51	−0.34
Norfentanyl	0.2	0.22 ± 0.02	7.40	7.98	19.74	11.22
	3	3.03 ± 0.23	8.25	7.49	5.19	1.10
	18	18.16 ± 1.13	7.00	6.15	4.17	0.90
Sufentanil	0.2	0.22 ± 0.02	8.96	8.64	17.57	9.58
	3	3.08 ± 0.28	8.04	9.22	9.84	2.74
	18	17.62 ± 1.78	6.39	10.11	10.62	−2.13

[a] mean values ± standard deviation

acceptance criteria (same as accuracy and precision) for dilution integrity were met for dilution factor 2, 5, and 10, maintaining confidence in the final results. The exceptions were solely norfentanyl, whose dilution integrity results were only in line with the acceptance criteria for accuracy in the 1/2 ratio, and mescaline, which does not meet accuracy acceptance criteria for none of the dilutions tested.

3.2.7. *Processed sample stability*

When samples are extracted, it is assumed that they will be subjected to chromatographic analysis shortly thereafter. However, due to the high throughput of routine analysis in a forensic toxicology laboratory, this is usually not the case and, consequently, some delay may occur before all the sample extracts are injected into the equipment, ranging from hours to days. It is therefore important to understand whether the extracts remain stable during these waiting times.

Autosampler stability was assessed by reanalyzing the extracts of samples fortified at low (0.2 and 5 ng/mL) and high (18 and 450 ng/mL) concentrations after being kept in the autosampler (at 15 ºC) for one week. Analytes were considered stable while the % CV and % bias were within ± 20 % of the results obtained for the sample prepared at time zero.

All analytes remained stable in the autosampler for one week, at both concentrations, except isotonitazene, metonitazene and norfentanyl that remain stable in the autosampler for 4 days (96 h).

3.3. *Literature comparison and greenness evaluation*

In the present work, the developed methodology was also compared with other methodologies already reported in the literature [21,32–40] for the analysis of the target opioids and hallucinogens in whole blood by LC-MS/MS (triple quadrupole and/or ion trap), but with different sample preparation approaches. Table 4 summarizes the LOD, LOQ, linear range, and sample volume required by the proposed methodology and other approaches.

As seen in Table 4, the proposed methodology requires a very small amount of sample (50 µL), much lower than most of the reported methodologies, which is of upmost importance in forensic laboratories, where the quantity of samples collected/available is usually limited.

Regarding LOD and LOQ, the estimated values are comparable or even better than those present in literature, especially considering the low sample volume (50 µL). These very low detection and quantification limits are particularly important when analyzing extremely potent drugs (such as the target analytes) in a forensic context, where concentrations in biological matrices are likely to be vestigial. Also, the linear ranges are similar to other works. In cases where the target analytes are present in the samples at a concentration higher than the established linear range, it is possible to dilute these samples according to the dilution integrity assays validated for each compound.

Moreover, the proposed methodology was evaluated according to the Analytical greenness (AGREE) and AGREEprep calculator, in which all its steps are individually evaluated concerning their greenness [42,43]. The results obtained are shown in Fig. 3. The overall score is displayed at the center of the pictogram, with values near 1 and a dark green color signifying a greener procedure. The performance for each assessment criterion is represented by the color of the segment associated with its corresponding number.

Considering this classification, the main advantages of this methodology are the number of analytes of different families that can be analyzed simultaneously, the small volume of sample and solvents used, and the quick sample preparation and chromatographic analysis. All these parameters are very important from a forensic point of view, since the flow of analysis in a toxicology laboratory is very high. With the proposed methodology, it is possible to speed up the analysis of samples, contributing to laboratory efficiency, especially in a routine context. Another advantage is the fact that, due to the low use of samples and solvents, the amount of waste produced is also quite small, enhancing the greenness of the methodology. The main limitations of the overall method are associated with the UHPLC-MS/MS equipment, namely energy consumption and reagents sources.

For all these reasons, this methodology presents itself as an

Table 4
Comparison of the proposed methodology with other LC-MS/MS methodologies reported in literature for the determination of the target analytes in whole blood samples.

Ref.	Sample Preparation	Sample Volume (µL)	Compounds	LOD (ng/mL)	LOQ (ng/mL)	Linear Range (ng/mL)
[32]	SPE[a]	1000	Fentanyl	0.1	0.1	0.1–100
			Norfentanyl	0.1	0.1	0.1–100
[33]	LPME[b]	120	Isotonitazene	≈ 0.01 (0.02 nM)	≈ 0.04 (0.1 nM)	≈ 0.04 – 21 (0.1–50 nM)
			Metonitazene	≈ 0.02 (0.05 nM)	≈ 0.19 (0.5 nM)	≈ 0.19 – 19 (0.5–50 nM)
[34]	PP[c]	50	Isotonitazene	0.1	0.1	0.1–100
			Metonitazene	0.1	0.1	0.1–100
[21]	PP	200	LSD	0.01	0.0375	0.0375 – 10
			LSD-OH	0.005	0.0187	0.0187 – 10
	LLE[d]	200	LSD	0.005	0.025	0.025 – 10
			LSD-OH	0.0025	0.0125	0.0125–10
[35]	Mini-QuEChERS	200	Fentanyl	1.22	3.66	5 – 6000
			Mescaline	1.61	4.83	5 – 6000
			Norfentanyl	1.65	4.95	5 – 6000
[36]	LLE	500	Metonitazene	0.1	0.5	0.5–50
[37]	PP	500	Fentanyl	0.07	0.23	0.25–25
			Norfentanyl	0.08	0.27	0.25–25
			Carfentanil	0.07	0.25	0.25–25
[38]	LLE	200	Fentanyl	0.05	0.1	0.1–100
			Norfentanyl	0.06	0.1	0.1–100
			Sufentanil	0.2	0.2	0.2–100
[39]	PP + SPE	300	Carfentanil	0.05	0.05	0.05–20
			Fentanyl			
			Norfentanyl			
			Sufentanil			
[40]	LLE	100	Carfentanil	0.02	0.05	0.05–40
			Fentanyl	0.03	0.2	0.2–40
			Norfentanyl	0.1	0.2	0.2–40
			Sufentanil	0.02	0.05	0.05–40
This work	PP	50	Carfentanil	0.05	0.1	0.1 – 20
			Fentanyl	0.05	0.1	0.1 – 20
			Isotonitazene	0.05	0.1	0.1 – 20
			LSD	0.05	0.1	0.1 – 20
			LSD-OH	0.05	0.1	0.1 – 20
			Mescaline	1	2.5	2.5–500
			Metonitazene	0.1	0.1	0.1 – 20
			Norfentanyl	0.1	0.1	0.1 – 20
			Sufentanil	0.05	0.1	0.1–20

advantageous alternative to other LC-MS/MS methodologies described in the literature.

3.4. Application to authentic samples and interlaboratory assay

The developed methodology was applied to the analysis of 19 whole blood samples from postmortem or *in vivo* cases, having been identified 10 positive samples for fentanyl, norfentanyl and/or sufentanil. A chromatogram of a positive sample with 1.4 ng/mL of fentanyl is presented in Fig. 4. In total, fentanyl was found in 8 samples, in concentrations ranging from 0.6 to 15 ng/mL. Norfentanyl was present in 4 samples at concentrations varying between 0.1 and 1.5 ng/mL. Additionally, one sample contained sufentanil at 5.2 ng/mL. These values are in line with the expected concentrations, according to literature [32,35,37–40] and prove the feasibility of applying the present methodology to routine forensic work cases.

Additionally, the proposed methodology was used to analyze an interlaboratory Proficiency Test serum sample (TAB of Arvecon GmbH). The interlaboratory assay results were evaluated by a z-score (z = [observed value – target value]/standard deviation), where results within ± 2.0 were accepted. The analysis of this serum sample proved the presence of fentanyl at 4.6 ng/mL and sufentanil at 1.1 ng/mL, fulfilling the quantification and confirmation criteria. The z-score calculated was 0.71 and 0.75, respectively, well below the acceptance limit of 2, which demonstrates the accuracy and adequacy of the proposed methodology, even when applied to serum samples.

4. Conclusion

The present study developed, optimized, and fully validated an LC-MS/MS methodology for the routine analysis of 6 new synthetic opioids and 3 hallucinogens in postmortem and *in vivo* whole blood matrices. The method was successfully applied to authentic forensic samples, demonstrating its applicability/suitability to professional forensic context. Under optimized experimental conditions, the proposed methodology exhibited remarkable analytical performance, including high selectivity, minimal matrix effect, accuracy, precision, suitable detection and quantification limits, broad linear ranges and determination coefficients, as well as sample dilution integrity and autosampler stability. Moreover, the results show that the proposed approach compares favorably with other analytical strategies already reported in the literature. This approach is simple, fast, user-friendly, requiring only 50 µL of whole blood samples *per* assay while minimizing organic solvent use, aligning with green chemistry principles and enhancing laboratory throughput. Thus, it represents a viable alternative for routine implementation in forensic toxicology laboratories.

CRediT authorship contribution statement

Suzana Fonseca: Writing – review & editing, Validation, Supervision, Project administration, Formal analysis, Conceptualization. **João Franco:** Resources, Funding acquisition, Formal analysis. **Carla Mustra:** Methodology. **Neng Nuno R:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization. **Mónica Antunes:** Writing – review & editing, Methodology. **Joana R.P. Pereira:** Writing – original draft, Validation, Methodology,

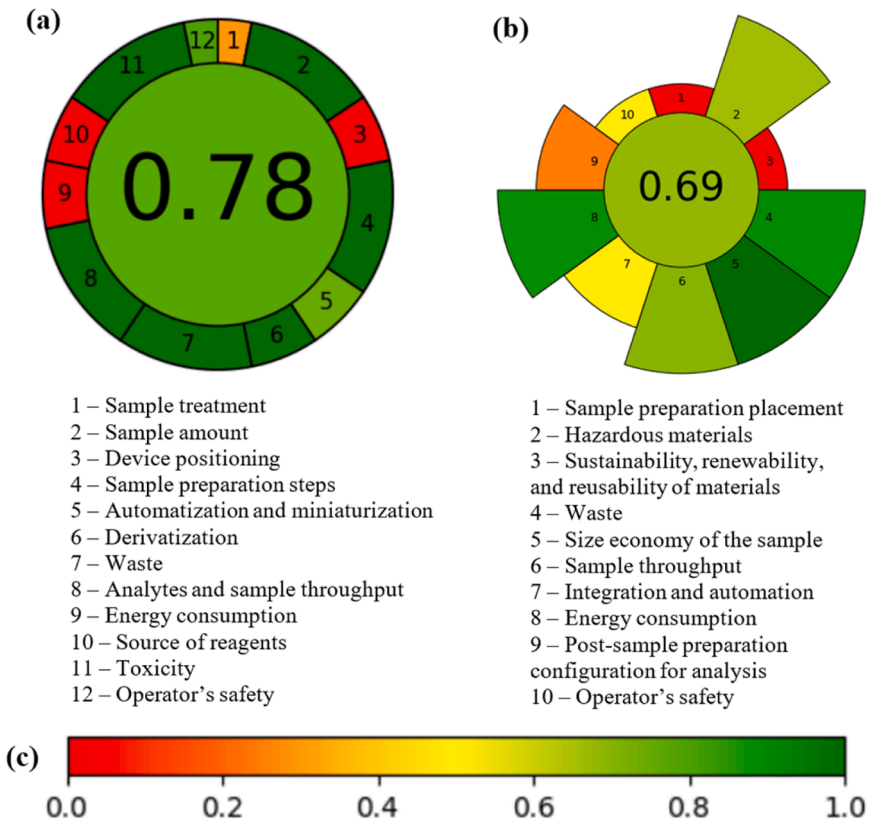

Fig. 3. Method and sample preparation evaluation according to the analytical greenness calculator (a), AGREEprep calculator (b) and the corresponding color scale for reference (c).

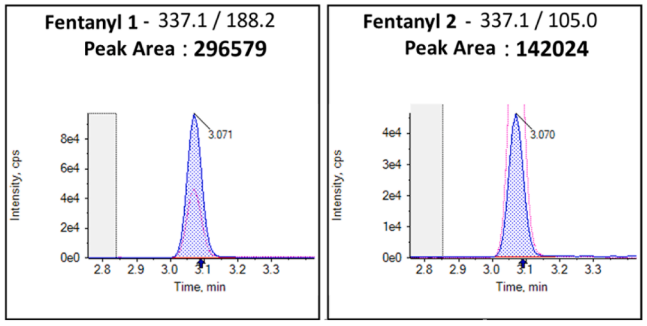

Fig. 4. MRM chromatogram of a sample considered positive for fentanyl (1.4 ng/mL).

Investigation, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2025.112713.

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