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Method development for the quantification of nine nitazene analogs and brorphine in Dried Blood Spots utilizing liquid chromatography – tandem mass spectrometry

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ABSTRACT

The detection of nitazenes in biological fluids is increasingly needed as they are repeatedly reported in intoxication and overdose cases. A simple method for the quantification of low levels of nine nitazene analogs and brorphine in Dried Blood Spots (DBS) was developed and validated. 10 μL of spiked whole blood is deposited on a Capitainer®B card and allowed to dry. The spot is punched out, and extracted with 500 μL methanol:acetonitrile (3:1 v/v) added with 1.5 μL of fentanyl-D5 as the internal standard. After stirring, sonication, and centrifugation of the vial, the solvent is dried under nitrogen, the extract is reconstituted in 30 μL methanol, and 1 μL is injected into a UHPLC-MS/MS instrument. The method validation showed linear calibration in the 1–50 ng/mL range, LOD values ranging between 0.3 ng/mL (isotonitazene) and 0.5 ng/mL (brorphine), average CV% and bias% within 15 % and 10 % for all compounds, respectively. The matrix effect due to blood and filter paper components was within 85–115 % while recovery was between 15–20 %. Stability tests against time and temperature showed no significant variations for storage periods up to 28 days. Room temperature proved to represent the best samples storage conditions. UHPLC-MS/MS proved capable to reliably identify all target analytes at low concentration even in small specimen volumes, as those obtained from DBS cards, which in turn confirmed to be effective and sustainable micro-sampling devices. This procedure improves the efficiency of toxicological testing and provides an innovative approach for the identification of the nitazene class of illicit compounds.

1. Introduction

In the last years, numerous Novel Psychoactive Substances (NPS) with effects similar to traditional drugs of abuse gained an important part of the global illicit substance market. Recently published studies reported in detail the trends of consumption of fentanyl and related compounds, the diffusion throughout the world as their presence combined with other drugs of abuse, alcohol and medicinal drugs leads to acute intoxications and may contribute to the causes of death [1]. Indeed, significant number of deaths have been attributed to the use of opioids, mostly fentanyl and its analogs [2,3]. Based on Brunetti et al., the number of opioids related cases may be underestimated, as they are likely taken in combination with other substances and may be unnoticed, but even low concentration may be harmful. The information about their potency and lethal dosage is yet to be defined, thus it is important to develop novel and sensitive methods to identify and report the new trends of opioids for further investigation on their toxicity and fatal potency.

New synthetic opioids (NSO) appeared recently on the stage and their presence raises concerns due to their adverse effects, equal or higher than the fentanyl ones. Nitazenes represent a new group of potent synthetic opioids with a 2-benzyl-benzimidazole structural core. Remarkably, the first studies on their possible synthesis routes date back to the 1960s, in view of potential analgesic usage, but only in 2019 their abrupt appearance was noticed in the illicit market [4]. Isotonitazene was the first analog identified in illicit drugs samples' and, after full characterization, it revealed a biological activity several times higher than fentanyl [5,6]. Other studies have shown that other analogs, (most prominent, etonitazene), are thousands of times more potent than morphine. This group of NSO has never been approved for

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pharmaceutical or clinical use [7,8].

Explicit data from case reports involve mainly isotonitazene, with metonitazene, N-pyrrolidino etonitazene, flunitazene and/or butonitazene being present in fewer cases [9]. They are prominently centred in the US [10], but their presence was reported also in Canada, Australia, the UK, and northern European countries [3,11–13], together with the structurally different brorphine [14]. Brorphine is a piperidine-based opioid compound [15], mainly mixed with - or used - as an alternative of isotonitazene, and repeatedly detected together with flualprazolam and other synthetic opioids [16,17]. The consumption of these drugs may lead to typical opioid effects, such as respiratory depression, possible dependence, and risk of severe intoxication and death [18], as the first studies have already revealed [19,20]. Studies have also been conducted to post-mortem samples (blood and urine) and nitazenes were detected: in one case metonitazene is deemed the only possible cause of death in the absence of others [21], while most of them do not report nitazenes as the only responsible for death [22]. Further studies are still needed to fully understand the exact role of nitazenes in lethal intoxication cases and their health risks in case of chronic use. Nitazenes currently represent a small portion of the NSO diffusion in the world, especially in the European territory, but the opioid market is growing and poses a challenge to the forensic toxicology laboratories that contribute to the fight against the illicit drug trade. Accordingly, these laboratories are forced to develop and apply expanded NPS/NSO screening analyses to identify and study the exact substances consumed and their adverse effects [23,24]. Based on the current situation, aim of the present study is to provide a novel, easy and effective method for the identification and quantification of the most prominent and potent nitazene analogs in Dried Blood Spots (DBS).

DBS is a micro-sampling technique based on the collection of a capillary blood drop on a filter paper. It is gaining increasing interest both in clinical and forensic contexts [25,26]. The collection of small blood samples is performed preferably from a finger (toe or heel as alternatives) pricked by a conventional lancet. Nowadays, various types of filter papers and DBS collectors are commercially available. After collection, the blood spot is left to dry and either analyzed immediately after drying or it is easily stored or transported to other laboratories for delayed analysis, as the analytes have greater stability when dried [27,28]. The capillary blood collection is fast and minimally invasive as the collected blood volume is small, the pre-analytical treatment is simple, quick, resistant to adulteration, unlike other matrices, and safe for handling [29]. DBS simplify the everyday workflow of routine laboratories and are highly sustainable, since they allow easier sampling and large-scale testing, reduce the required solvent volume, the analysis time, and the energy requirements [30]. Recent research invested on automation of sample preparation and analysis [31].

This study contributes to the definition of expanded NPS/NSO protocols [32] to be used in everyday applications, such as roadside and workplace drug testing, but also aims to help clinicians have a better insight in intoxication cases and differentiate their causes. The analytes panel includes nine nitazene analogs: N-pyrrolidino etonitazene, butonitazene, etodesnitazene, etonitazepipne, flunitazene, isotonitazene, metodesnitazene, metonitazene, protonitazene and brorphine. For the present work, DBS technique was combined with a highly sensitive UHPLC-MS/MS system: the SCIEX Triple Quad™ 7500 LC-MS/MS - QTRAP®.

2. Materials and methods

2.1. Reagents and standards

Methanol and acetonitrile were purchased from Sigma-Aldrich (Milan, Italy), formic acid (purity > 95 %) from Fisher Chemical (Milan, Italy) and Double-distilled water was obtained from a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). Stock standard solutions were prepared in methanol at 1.0 mg/mL and stored at –20 °C until used. All 10 analytes and fentanyl-D5 were purchased from Comedical (Trento, Italy). Working solutions of all analytes were prepared at the final concentration of 1.0 μg/mL and 100 ng/mL by dilution with methanol. The internal standard working solution (ISTD) was prepared at a final concentration of 1.0 μg/mL. Validation of the method was performed using the Capitainer®B cards as micro sampling devices.

2.2. Sample preparation

The sample preparation method was based on a previously published procedure for an expanded NPS screening protocol, with minor modifications [32]. The spiked specimens used in the method development and validation were prepared from blank whole blood obtained by mixing aliquots collected from five volunteers, stored in EDTA blood collection tubes at 4 °C. 50 μL aliquots of blank blood was fortified with a working solution of all ten analytes at six concentration levels (1, 2, 5, 10, 25 and 50 ng/mL), that were processed in three working sessions along five days. 30 μL of fortified blood was deposited onto the blood inlet, to ensure smooth flow into the microfluidic system, but only 10 μL dried blood spots were generated. Capitainer®B cards are haematocrit-independent and designed to ensure that the exact amount of blood is flowed in the microfluidic tube while the excess blood volume is deposited to another collection disc [33]. The spots were allowed to dry for at least 3 h at room temperature (approximately 21 °C), away from direct sunlight and humidity. The 10 μL spot was then punched out, transferred into a glass tube and extracted with 500 μL methanol: acetonitrile (3:1 v/v) added with 1.5 μL fentanyl-D5 as the internal standard (1 μg/mL, resulting in a final concentration of 100 ng/mL). The extraction vial was subjected to intense stirring and ultra-sonication for 30 min at room temperature. The extract was transferred into a fresh tube, where the solvent was evaporated under nitrogen at room temperature. The dry residue was reconstituted with 30 μL methanol and centrifuged for 10 min at 4000 rpm. 1 μL was injected into the UHPLC-MS/MS. Quality Control samples were prepared in the same way of calibrators using blank whole blood from five independent volunteers fortified at three concentration levels (low, medium and high).

2.3. Instrumentation

Sample analysis was performed on a UHPLC SCIEX ExionLC™ (AB SCIEX, Framingham, USA) coupled with a SCIEX 7500 TripleQuad (Darmstadt, Germany), triple quadrupole mass spectrometer, equipped with an electrospray ion source (ESI) operated in the positive ion mode. Data were elaborated by the SCIEX OS software for both qualitative and quantitative purposes.

The UHPLC was equipped with a C18 Kinetex column (100 × 2.1 mm, 1.7 μm) by Phenomenex (Torrance, CA, USA). The mobile phase consisted of (A) 5 mM formic acid in water and (B) 5 mM formic acid in acetonitrile. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following gradient conditions (A:B, v-v): isocratic elution at 95:5 for 0.5 min, linear elution 95:5 to 5:95 in 8.0 min, isocratic elution at 5:95 for 0.5 min, and final equilibration at the initial conditions for 1.5 min resulting in a total run of 10 min.

The targeted analysis was carried out using the selected reaction monitoring (SRM) technique, selecting three MS/MS transitions for each analyte around the expected chromatographic retention time (the details are reported in Table 1). The MS conditions were set as follows: ion source gas 1 and 2 at 30 and 70 psi respectively, curtain gas at 40 psi, source temperature at 600 °C and spray voltage at 1500 V.

3. Method validation

The calibration samples were analyzed in three working sessions along five days, following the protocols previously published for the validation of analytical methods [34]. The performance parameters evaluated with this data set (9 replications × 6 levels) included:

Table 1
Optimized MS parameters for the detection of the 10 analytes. Retention time (min), precursor and fragment ion masses (*m/z*) and collision energy (V) are reported. The underlined fragment ion masses are used for the quantification.

Analyte	Retention time (min)	Precursor ion mass^{Q}1 (*m/z*)	Fragment ion mass^{Q}3 (*m/z*)	CE (V)
Brorphine	3.7	402.0	218.2	35
			104.1	63
Butonitazene			100.1	31
	4.4	425.2	72.0	67
			107.0	75
Etodesnitazene			100.1	26
	3.0	352.1	109.1	57
			72.1	54
Etonitazepipne			112.1	46
	3.5	409.2	135.1	60
			107.1	82
Flunitazene			100.1	33
	3.5	371.1	109.1	65
			72.1	58
Isotonitazene			100.0	20
	3.7	411.2	106.9	52
			72.0	42
Metodesnitazene			100.0	23
	2.6	338.1	72.0	53
			121.0	33
Metonitazene			100.0	26
	3.5	383.0	72.1	58
			121.0	38
N-Pyrrolidino etonitazene	3.7	395.0	98.0	27
			107.0	65
			56.0	82
Protonitazene			100.0	20
	3.8	411.2	106.9	82
			72.0	82

calibration curve, intra- and inter-day accuracy and precision at all concentration levels, limit of detection (LOD), limit of quantification (LOQ). Recovery, matrix effects, and analytes stability were evaluated from independent experiments.

3.1. Calibration

The calibration curve for each analyte was created in the 1–50 ng/mL range by calculating the peak-area ratios between the target analyte and the internal standard for all concentration levels and plotting them on the y-axis against the six concentration levels (x-axis). The homoscedastic vs. heteroscedastic data distribution was evaluated by examining the variance of nine data-points at six concentration levels; once heteroscedasticity was recognized ($p < 0.05$), the relative weighting factor ($1/x$ or $1/x^2$) was assessed by testing the variance increase from low to high concentrations, depending on the linear or quadratic relationship occurring between concentration and variance [35]. The order of the calibration model (linear vs. quadratic) was chosen on the basis of Mandel and lack-of-fit tests results.

3.2. LOD and LOQ

The limit of detection (LOD) for the various analytes was determined using the Hubaux-Vox method [36] applied to the linear portion of the calibration curve (typically the three-four lowest concentration levels). The calculated LOD values were subsequently confirmed in experiments using blank samples spiked at the corresponding concentrations and verifying a $S/N > 3$. The limit of quantification (LOQ) was defined as the minimum analyte concentration that could be determined with an acceptable level of precision and trueness.

3.3. Precision and accuracy

Intra-day accuracy (expressed as bias%) was calculated for each of the three days in which the sequence of 3 repetitions × 6 calibration levels was replicated. Two repetitions were cyclically used to create a calibration model that was then used for back-calculating the six-remaining data-points [34]. The inter-day accuracy was calculated similarly, with the difference that a calibration curve was cyclically constructed with the six data-points at six concentration levels collected in two working days and this calibration curve was subsequently used to back-calculate the concentrations resulting from the data-points collected in the third working day. The outcome of these back-calculations was then averaged to provide a final inter-day accuracy value. In this way, accuracy results were obtained for all six concentration levels. Considering the variability factors affecting the sampling operations and the small blood volume collected, the accuracy was considered optimal if the bias was lower than 15 % and good with bias < 20 %. The method was deemed as validated if the average intra- and inter-day accuracy over all calibration levels remained below 20 % [34].

Intra-day precision was independently assessed for the three days of analysis. The coefficient of variation (CV%) was determined for each concentration level by averaging the precision obtained for the three days. The inter-day precision followed a similar procedure, that makes use of the nine replications collected during all three days. In practice, the protocol used for calculating accuracy and precision is based on the same data collected for preparing the calibration curve, obtained in the three separate days.

3.4. Matrix effect and extraction recovery

The matrix effect (ME) was estimated at three concentration levels: 1 ng/mL, 10 ng/mL, and 50 ng/mL, and was aimed to test whether the blood, as well as the filter paper used, may have an influence on the intensity of the analytes' signal. Three replicates of blank dried blood samples, three spots without blood and three samples without the filter paper (just extraction solvents) were equally spiked (with target analytes and ISTD) and analyzed. The matrix effect was calculated as the percentage ratio between the two measured signals for each concentration level. Then, the ME% value for each analyte was calculated by averaging the three replicates. Acceptable ME% values had to be between 85 % and 115 % [37].

The extraction recovery (RE) was determined at the same concentration levels, 1 ng/mL, 10 ng/mL, and 50 ng/mL, by comparing the experimental results obtained from six whole blood samples three of which spiked before and three after the extraction step. The result was expressed as the mean percentage ratio between the two signals, expressed as extraction repeatability (CV%) and completed with its uncertainty.

3.5. Stability

The stability of the target analytes was examined on purposely spiked blank samples maintained at different temperatures for different time intervals. The stability of the analytes was evaluated on day 1, day 14, and day 28, at storage temperatures of –20 °C (freezer room), 4 °C (cold room), 25 °C (room temperature). When designing the experiments, the most common storage or transportation conditions that are used in a forensic or clinical context were considered. To this purpose, the DBS were stored away from sunlight and important humidity generators at room temperature and no other limitation factor was applied at –20 °C or 4 °C. Thus, the results obtained are interpreted on the basis of an everyday use of DBS. The humidity factor that exists at –20°C and 4 °C may influence the results obtained. Blank whole blood was spiked at 1 ng/mL, 10 ng/mL, and 50 ng/mL and the DBS cards were prepared in triplicate for each condition. All samples were prepared at the same

moment, using the same working solutions, and were stored according to the various condition combinations. An analysis of variance (ANOVA) was performed from the integrated areas for each variable and allowed the construction of boxplots described in the following section.

4. Results

The present analytical method allowed the detection in DBS of all the target analytes at all concentration levels tested (1, 2, 5, 10, 25, 50 ng/mL). The haematocrit effect on the concentrations was not evaluated since all the validation process was performed using volumetric Capitainer®B cards on the same blank blood. For future large studies with possibly other collection cards, the haematocrit range of the calibrators should be determined and matched to the one of the target populations. Moreover, the MS/MS instrument presently used provided high sensitivity, allowing the detection and quantification of the analytes even at very low concentrations. The precursor ion *m/z* value was different for all the analytes, except isotonitazene and protonitazene which however differ in their retention time, making their discrimination easy.

4.1. Calibration

Residues analysis and variance distributions at low, medium and high concentration levels indicated strong heteroscedasticity resulting in the adoption of a $1/x^2$ weighting factor for all analytes. Depending on the linear or quadratic relationship occurring between response and concentration, as verified by Mandel and lack-of-fit tests, a linear or quadratic calibration model was defined, with equal distribution among the analytes (5 linear and 5 quadratic). The regression models obtained are reported in the Supplementary material (Table S1).

4.2. LOD and LOQ

The LOD value for isotonitazene, both calculated and experimentally verified, was as low as 0.3 ng/mL, while for butonitazene, flunitazene, metodesnitazene, metonitazene, N-pyrrolidino etonitazene, and protonitazene was equal to 0.4 ng/mL, and for brorphine and etonitazepipne was 0.5 ng/mL. These values should be considered as optimal since - to the best of our knowledge - no other study reported the detection of nitazenes in DBS at such low levels and with the present high accuracy and starting from a sample volume of only 10 μL. Indeed, the present LOD values are comparable with those obtained by other quantification studies applied on much larger whole blood sample volumes [14], [38]. All LOQ values corresponded to the lowest level of the calibration curves (i.e., 1.0 ng/mL) at which the precision and trueness requirements were positively verified (full values are reported in Table S2 of the Supplementary material). The chromatogram for a blank sample shows that the method is free from interferences. The possible coelution of analytes, is also considered. Even though some analytes do have the same retention time as expected since they are structurally similar, by observing the precursor and fragment ions masses, it can be concluded that the quantification of is not affected as the transitions resulted are different for each analyte. The underlined fragment ion masses are used for the quantification (Table 1). The total ion chromatogram obtained as well as the extracted ion chromatograms for each analyte, including a characteristic negative chromatogram as an example, are reported in the Supplementary material.

4.3. Accuracy and precision

Inter-day and intra-day accuracy and precision data (expressed as bias % and variation coefficient, CV%, respectively) for the six concentration levels tested are reported in the Supplementary material (Table S3). The intra- and inter-day accuracy, as well as intra- and inter-day precision obtained proved optimal for most target analytes and calibration levels with bias% and CV% values lying below 15 %. For few others, accuracy and precision values remained below 20 % or slightly above this cut-off (three values, randomly scattered). In general, intra- and inter-day precision did not depend on absolute concentration in the 1–50 ng/mL investigated range and was homogeneously disseminated among the analytes, with absolute mean values ranging from 7.0 % to 15.5 % intra-day and from 9.3 % to 15.8 % inter-day. Inter-day average precision was only slightly higher than intra-day average precision (13.1 % vs. 10.8 %). Even more homogeneous were the intra- and inter-day accuracy data averaged for the six concentrations, ranging from 5.8 % (intra-day protonitazene and inter-day isotonitazene) up to 14.8 % (intra-day metonitazene). The absence of any systematic shift from suitable accuracy and precision values confirms the overall reliability of quantitative determinations.

4.4. Matrix effect and extraction recovery

The results obtained from the experiments addressed to the estimation of potential matrix effects produced from both blood and collection paper revealed little influence of these two matrices on the analytical results (Table 2). As a matter of fact, all values determined for single concentrations fall within the 78 %–123 % range, while the data averaged for the three concentrations ranged between 86 % (metodesnitazene, corresponding to a 14 % suppression) and 113 % (protonitazene,

Table 2
Matrix effect (%), ion suppression (%) and recovery (%) calculated at three concentration levels (1, 10 and 50 ng/mL), followed by the average of three for each one.

Analyte	Concentration (ng/mL)	Matrix effect (%)	Ion suppression (%)	Recovery (%)
	1	78	–22	22
Brorphine	10	88	12	11
	50	102	2	15
	Average	**89**	**–11**	**16**
	1	116	16	15
Butonitazene	10	103	3	12
	50	112	12	19
	Average	**110**	**10**	**15**
	1	79	–21	22
Etodesnitazene	10	85	–15	11
	50	99	–1	19
	Average	**88**	**–12**	**19**
	1	113	13	27
Etonitazepipne	10	97	–3	11
	50	107	7	15
	Average	**105**	**5**	**18**
	1	107	7	25
Flunitazene	10	101	1	12
	50	105	5	19
	Average	**104**	**4**	**19**
	1	112	12	25
Isotonitazene	10	103	3	12
	50	108	8	20
	Average	**108**	**8**	**19**
	1	79	–21	23
Metodesnitazene	10	84	–16	13
	50	96	–4	22
	Average	**86**	**–14**	**19**
	1	104	4	26
Metonitazene	10	107	7	12
	50	110	10	21
	Average	**108**	**8**	**20**
	1	93	–7	22
N-pyrrolidino etonitazene	10	88	–12	11
	50	106	6	18
	Average	**95**	**–5**	**17**
	1	123	23	26
Protonitazene	10	107	7	11
	50	109	9	17
	Average	**113**	**13**	**18**

corresponding to a 13 % signal enhancement). Even if the limited extension of matrix effects can be partly attributed to the compensating contribution provided by referring the integrated peak areas of the analytes to those of the ISTD, still the matrix components of dried blood did not occur to produce appreciable ion suppression nor ion enhancement effects on the analytical signal. Also, the data obtained from the second evaluation (i.e., ratio between the filter paper without blood and pure extraction solvent) demonstrate that the filter paper does not significantly impact the analytical signal.

The recoveries measured by comparing the analytes' signals arising from adding the spikes either before or after the paper extraction stage confirm the significant influence of the collecting paper in seizing most part of the spiked analytes. Actually, recoveries in the range 15 %–20 % were typically observed (Table 2). Nevertheless, the recovery percentages are rather replicable and independent from concentration. Moreover, they are quite similar for all the targeted analytes and do not affect the obtainment of low detection concentrations limits (LOD) below the first point of the calibration range (LOQ). These relatively low recoveries are expected and is in line with other DBS studies [32], since the DBS procedure involves extraction of a dried substrate from a relatively hydrophilic support, leading to potential interactions with the endogenous components present in the DBS matrix [39].

Further possible experimental factors inherent the analytical process may contribute to the low recovery observed. Bearing in mind that the blood spot volume consists of only 10 μL and should be entirely removed from the card, the conventional tweezers consistently used throughout the sample manipulation may subtract part of the sample. In future studies, alternative ways to efficiently remove the spot should be explored. Moreover, the present study was carried out with the scope of being integrated within a broader NPS screening, so the solvents and the extraction steps were not specifically optimized towards nitazenes. Different solvents, ratios or additional extraction steps should be evaluated for class-specific procedures.

4.5. Stability

The stability of the 10 analytes was assessed against time (day 1, day 14 and day 28) and temperature (–20 °C, 4 °C, 25 °C room temperature). The peak intensity for every analyte in every condition was collected without ISTD correction and its variability was evaluated. Based on ANOVA, the results showed high comparability among the different analytes. The results obtained from isotonitazene are presented as a typical example (Fig. 1): room temperature conditions showed the lowest variability, providing the greatest level of stability, with respect to the other two storage conditions (the difference was statistically significant). In contrast, no significant variability was observed between the different durations of storage. It is concluded that the analytes can be considered as stable over a period of four weeks, at least. Lastly, the variability between the three chosen concentration levels was also compared: quite obviously, it proved statistically significant, with increasing variability observed when the analytes concentration increases. This is in agreement with the heteroskedastic distribution obtained in the calibration.

The experimental results relative to the analytes' stability demonstrate that the nine nitazene analogs and brorphine are stable on DBS for at least 28 days and suggest storing the DBS cards at room temperature, away from humidity.

5. Discussion

The results discussed so far must be related to the objective of this study, that was the development and validation of an effective analytical procedure for realistically monitoring the diffusion in selected populations of nitazenes analogs and brorphine. The screening of this class of potent opioids of recent toxicological interest by using DBS is an easy and fast alternative sampling method for biological matrices, with several advantages. The results obtained from the validation process demonstrate that the method is reliable and suitable for the declared purpose and can now be transferred to real samples application. Actually, the literature reporting on authentic cases recorded variable concentrations for the most common nitazenes analogs, including a few as low as 0.5 ng/mL [6,14,22], namely a concentration still above or equivalent to the experimental LOD values measured in the present study. Nitazenes are still a rare phenomenon in Europe, and especially in Italy where only recently the first "nitazene" case was identified in postmortem samples while as it can be observed the majority of authentic nitazenes casework is observed mostly in the U.S. The lack of authentic samples testing is a limitation of the study and is taken under consideration.

Compared to the conventional blood sampling methods used in the previous studies, the dried blood spots matrix appears to be more advantageous, especially for large-scale epidemiological studies. Firstly, the sample collection is significantly less invasive than with intravenous needle draw. Secondly, the sampling itself, and transportation and conservation requirements of the samples are particularly simple.

The stability tests aimed to evaluate the possible occurrence and

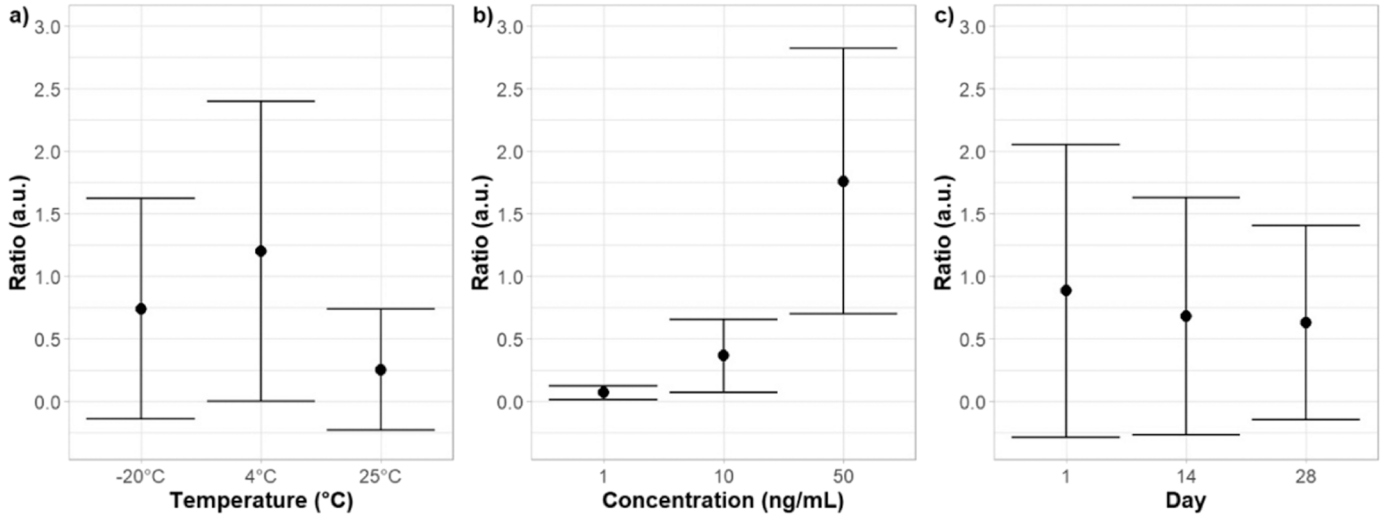

Fig. 1. ANOVA performed to assess the DBS stability against a) different storage temperatures (20 °C, 4 °C, 25 °C) b) low, medium, and high concentration levels (1, 10 and 50 ng/mL) c) time differences (day 1, day 14 and day 28).

extent of analytes degradation after they are deposited onto a DBS card is of particular importance. Indeed, the collected samples could potentially be stored for short or long periods of time, considering the needs of forensic toxicology laboratories either to receive the samples after transportation from the point of collection or to perform confirmation or counter-analysis after the results have been challenged. For example, in previous epidemiological studies conducted in cooperation among international institutions, we tested hair samples for abused drugs and NPS, collected during extended periods of time and after single overseas transportation, relying on the considerable stability of the investigated analytes in properly packaged hair specimen [40–42]. Conceivably, the same could apply with blood samples collected and dried on DBS cards, once the stability at room temperature of the target analytes had been positively verified. This is potentially a crucial asset of the DBS technique.

Our experimental data proved that there is no need to extract the DBS devices and analyze the extracts immediately, since the targeted nitazenes show great stability once the collected blood droplet has been dried. Thus, storage of the DBS cards for several weeks at room temperature do not apparently pose the risk of analytes degradation. On the other hand, the adoption of appropriate UHPLC-MS/MS conditions demonstrated adequate sensitivity and specificity for the unequivocal identification of the target analytes and their detection at low concentration levels despite the small specimen volume and the low recovery yield of the analytes from the DBS cards.

6. Conclusions

A robust and sensitive analytical method for the quantitative determination of nine nitazenes and brorphine in blood has been developed and validated, that combines DBS sampling and UHPLC-MS/MS detection. This method will likely be applied as is on future epidemiological campaigns for the selective detection of nitazenes and/or combined with other existing methods for exploring the diffusion of a wider range of NPS in selected populations.

A clear advantage of DBS sampling is that the sample collection is allowed in non-clinical environments and without the need of special equipment and trained healthcare personnel, so it is suitable for several toxicological and epidemiological contexts of NPS monitoring. It is possibly suitable also for road-side testing, workplace drug testing and crime scenes [43]. The DBS sampling device used in this study proved to guarantee accurate and precise results relying on the microfluidic system that allows constant volumetric sampling.

Further refinement of the present method may possibly address an improvement of the analytes' recovery from the DBS card, especially when the detection of nitazenes has to be combined with that of other NPS classes. This refinement may involve the comparative testing of different spot removal techniques, extraction solvents and incubation times, under design-of-experiment framing and supervision. Beside epidemiological studies, authentic samples analysis will be conducted within expanded NPS screening protocols in combination DBS sampling, that can be managed outside a medical laboratory and feasibly in experimental roadside testing campaigns.

Author statement

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

CRediT authorship contribution statement

Salomone Alberto: Conceptualization, Data curation, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Ververi Christina:** Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Massano Marta:** Conceptualization, Methodology, Validation. **Galletto Martina:** Conceptualization, Data curation, Methodology, Validation. **Vincenti Marco:** Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. **Alladio Eugenio:** Data curation, Validation, Software, Visualization.

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.jpba.2024.115975.

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