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Analytical approaches for the identification and quantitation of nitazenes: a review

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ABSTRACT

Nitazenes, a class of high-potency novel synthetic opioids containing a benzimidazole moiety, are currently being reported across seized materials and drug-checking submissions, human biological matrices (postmortem and clinical/forensic) and community-level samples including wastewater. The analytical literature converges on a layered, matrix-oriented toolkit. Rapid presumptive strategies (vibrational fingerprints and ambient-ionization techniques) support triage in solids and field-adjacent contexts, while chromatographic separations coupled to mass spectrometry (HPLC-MS/MS/HRMS and less frequently GC–MS/MS/HRMS) deliver selectivity and structure-aware confirmation for closely related analogues. In human biofluids, both targeted triple-quadrupole panels and HRMS suspect screening workflows are present for identification; validated quantitative methods consistently address calibration model and range, sensitivity (LOD/LLOQ), precision and accuracy, selectivity, carry-over and matrix effects, often with explicit attention to isomer resolution. Cross-cutting issues recurring in the field include reference standard availability, isomeric/isobaric interferences, immunoassay cross-reactivity and the promise of miniaturised sampling formats. Organised by matrix and analytical objective, this review provides enough information to enable efficient extraction of details at the method level, while ensuring a comprehensive coverage of the most important topics.

1. Introduction

Nitazenes are potent synthetic opioids originally developed in the mid-20th century as alternative analgesics to morphine [1]. Although none of them progressed to clinical use, their recent appearance on the illicit drug market has renewed interest in this chemical class. Structurally, nitazenes are 2-benzylbenzimidazole derivatives whose core scaffold allows extensive substitution, generating a series of analogues with distinct pharmacological and physicochemical properties [2] (Fig. 1). Several compounds display μ-opioid receptor agonism with potencies that can exceed that of fentanyl by one or more orders of magnitude [3,4], resulting in a high risk of rapid respiratory depression, low effective doses and limited safety margins in recreational use [5], with reports of reduced responsiveness to naloxone treatments [6].

From an analytical perspective, nitazenes present notable challenges. Their expanding structural diversity and the continuous emergence of new analogues limit the availability of certified reference materials and comprehensive spectral libraries [7]. In addition, many analogues exhibit close structural similarity or isomerism, which complicates their discrimination within routine toxicological and forensic workflows [8].

Analytical laboratories must detect, identify and, when needed, quantify nitazenes across several matrices. The available approaches reflect practical requirements: rapid screening of seized materials and drug-checking submissions, selective confirmation and validated procedures for biological samples [9]. At the same time, the evolving repertoire of analogues means that suitable reference materials or spectral data may not yet exist for every candidate [10]. As a result, most workflows combine presumptive screening with chromatographic and mass-spectrometric confirmation [11].

In solid samples and drug-checking submissions, methods are usually based on high-throughput workflows and reliable confirmation. Diagnostic fragments or available reference spectra are used where possible,

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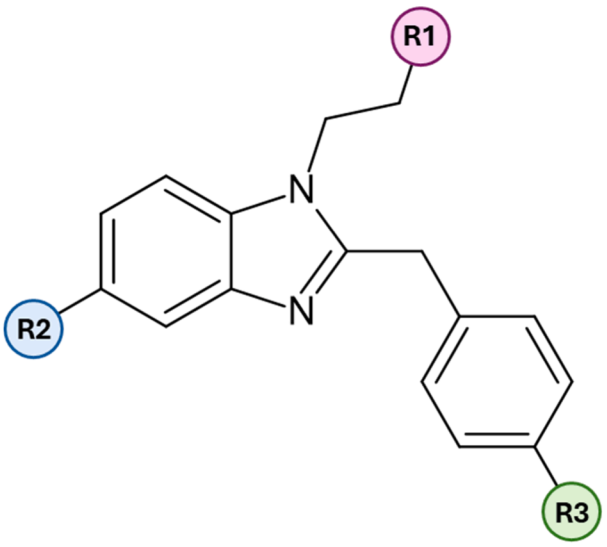


		R1	R2	R3	MW
1.	Etazene	*N*,*N*-diethyl	–	–O–CH_2CH_3	351.5
2.	Nitazene	*N*,*N*-diethyl	NO_2	–	352.5
3.	Isotodesnitazene	*N*,*N*-diethyl	–	–O–CH(–CH_3)CH_3	365.5
4.	Protodesnitazene	*N*,*N*-diethyl	–	–O–$CH_2CH_2CH_3$	365.5
5.	5-methyl-etodesnitazene	*N*,*N*-diethyl	Me	–O–CH_2CH_3	365.5
6.	Flunitazene	*N*,*N*-diethyl	NO_2	F	370.4
7.	Metonitazene	*N*,*N*-diethyl	NO_2	–O–CH_3	382.5
8.	Clonitazene	*N*,*N*-diethyl	NO_2	Cl	386.9
9.	Ethyleneoxynitazene	*N*,*N*-diethyl	NO_2	Tetrahydrofuran	394.5
10.	Etonitazene	*N*,*N*-diethyl	NO_2	–O–CH_2CH_3	396.5
11.	Isotonitazene	*N*,*N*-diethyl	NO_2	–O–CH(–CH_3)CH_3	410.5
12.	Protonitazene	*N*,*N*-diethyl	NO_2	–O–$CH_2CH_2CH_3$	410.5
13.	Butonitazene	*N*,*N*-diethyl	NO_2	–O–$CH_2CH_2CH_2CH_3$	424.5
14.	iso-butonitazene	*N*,*N*-diethyl	NO_2	–O–CH_2CH(–CH_3)CH_3	424.5
15.	sec-butonitazene	*N*,*N*-diethyl	NO_2	–O–CH(–CH_3)CH_2CH_3	424.5
16.	Metonitazepipne	Piperidine	NO_2	–O–CH_3	394.5
17.	Etonitazepipne	Piperidine	NO_2	–O–CH_2CH_3	408.5
18.	Isotonitazepipne	Piperidine	NO_2	–O–CH(–CH_3)CH_3	422.5
19.	Protonitazepipne	Piperidine	NO_2	–O–$CH_2CH_2CH_3$	422.5
20.	Metonitazepyne	Pyrrolidine	NO_2	–O–CH_3	380.4
21.	Etonitazepyne	Pyrrolidine	NO_2	–O–CH_2CH_3	394.5
22.	Isotonitazepyne	Pyrrolidine	NO_2	–O–CH(–CH_3)CH_3	408.5
23.	Protonitazepyne	Pyrrolidine	NO_2	–O–$CH_2CH_2CH_3$	408.5
	Metabolites:				
24.	N-pyrrolidino-4'-OH-nitazene	Pyrrolidine	NO_2	OH	366.4
25.	N-desethyl-etonitazene	*N*,*N*-diethyl	NO_2	–O–CH_2CH_3	368.4
26.	4'-OH-nitazene	*N*-ethyl	NO_2	OH	368.4
27.	5-aminoisotonitazene	*N*-ethyl	NO_2	–O–CH(–CH_3)CH_3	380.5
28.	N-desethyl-isotonitazene	*N*,*N*-diethyl	NO_2	–O–CH(–CH_3)CH_3	382.5

Fig. 1. Representative chemical structures of nitazene analogues and selected metabolites. Compound numbers are referenced in the text where individual analogues are discussed. Adapted from [55].

while high-resolution mass spectrometry or fragment-interpretation approaches support identification when standards are absent [12]. These workflows aim to minimise false positives and false negatives, while remaining compatible with real-world operational requirements [13].

Community-level monitoring offers complementary information. Wastewater-based studies can highlight changes in the illicit market and help prioritise which analogues should be included in screening or confirmation methods [14].

For biological samples, analytical goals typically fall into two categories. Identification relies on targeted multiple-reaction-monitoring or high-resolution MS suspect screening, with matrix-appropriate sample preparation and explicit control of isomeric interferences via chromatographic resolution or diagnostic fragmentation. Quantitative determinations follow current regulatory validation requirements, and in postmortem contexts they support interpretation through consideration of distribution-related variables [15]. Mechanistic studies, both in vitro and in vivo, map metabolic pathways and identify diagnostic fragments and neutral-loss patterns that support suspect screening and confirmation [16]., Several leitmotifs recur across applications: challenges due to extensive isomerism and limited availability of reference standards, problems related to low specificity of screening immunoassays, and growing interest in miniaturised or dried microsampling formats [17].

In this review, analytical approaches to nitazenes are summarised by matrix type and purpose, with method-level detail and emphasis on widely adopted and operationally effective strategies.

2. Analytical workflows for the identification and quantitation of nitazenes

2.1. Analytical workflows for non-biological samples

2.1.1. Screening and identification of nitazenes in solid samples, counterfeit formulations, drug-checking samples, illicit material seizures

Methods in this area converge on a two-tier logic: rapid, low-preparation presumptive indication followed by orthogonal, laboratory confirmation. The presumptive tier is designed to be portable, non- or minimally-instrumental and to operate on heterogeneous solids such as mixed powders, counterfeit dosage forms or paraphernalia residues. Vibrational fingerprints and ambient-ionisation approaches are highlighted for their ability to indicate the presence of benzimidazole-type opioids without exhaustive sample preparation, providing triage value when the composition is unknown or complex.

The confirmatory tier ensures selectivity and legal defensibility. Titles and abstracts emphasise chromatographic separation coupled to MS (triple quadrupole and/or high-resolution) to resolve closely related analogues and to verify identity through retention-time agreement and product-ion patterns. Where reference materials exist, libraries and transition lists are leveraged; where coverage is incomplete, fragment-interpretation strategies and HRMS exact-mass criteria are described to mitigate risks of isomeric or isobaric interference.

Operationally, authors report workflows compatible with rapid turnarounds: screening results guide whether a full confirmatory sequence is warranted, and minimal handling reduces exposure and contamination risks. Co-occurrence with other substances and the presence of excipients/adulterants are recurring considerations that influence both the choice of presumptive signal and the design of confirmatory separation. Overall, these workflows balance speed for triage with the selectivity required for final identification [18–21].

A common pattern is the consideration that unequivocal identification normally requires association of MS spectra with NMR and/or other spectroscopic techniques.

For example, *N*-desethyletonitazene (Fig. 1, **25**) has been identified at high purity (>98 %) by NMR, HPLC-HRMS and capillary zone electrophoresis (CZE - for the identification of anions) in a solid sample provided by a user for drug-checking purposes and sold as isotonitazene (Fig. 1, **11**) [22]. No traces of isotonitazene or etonitazene (Fig. 1, **10**) were found.

In practice, nitazenes are often found as the real active components of illicit formulations sold as containing oxycodone. Indeed, this has been confirmed by solid state techniques such as Raman scattering (RS), surface-enhanced RS (SERS) and Fourier transform infrared spectrometry (FT-IR), which have identified three different nitazenes (*N*-pyrrolidinoetonitazene - Fig. 1, **21**, isotonitazene, etodesnitazene - Fig. 1, **1**) in suspect counterfeit tablets [23]. These methodologies have the advantage of being applicable on-site with handheld/portable instruments; however, complete structure elucidation was not possible even with the application of direct analysis in real-time (DART) coupled to thermal desorption and MS (TD-MS). HPLC-MS was used for identification and HPLC-UV for quantitation. In other instances, the fake oxycodone tablets contained *N*-desethylisotonitazene (Fig. 1, **28**), as assessed by GC–MS, HPLC-HRMS (QTOF), NMR and attenuated total reflectance – FT-IR (ATR-FT-IR) [24]. To compensate the low sensitivity of the last technique, the tablets were extracted by using a MeOH/$CHCl_3$ (1/1, V/V) mixture, and the filtrate was brought to dryness to be analysed as such.

N-Pyrrolidinoisotonitazene (Fig. 1, **22**) was identified for the first time in a tablet supposed to contain oxycodone, by combining the results of UHPLC-ESI-MS/MS (orbitrap), GC-EI-HRMS (QTOF), FT-IR and NMR (after an initial screening with UHPLC-UV and test strips, which simply alerted to the presence of a nitazene analogue) [25]. Other studies have found *N*-pyrrolidino protonitazene (Fig. 1, **23**) [26,27] or metonitazene (Fig. 1, **7**) and protonitazene (Fig. 1, **12**) [28] surreptitiously sold as oxycodone. In this last case, the UHPLC-HRMS method was adapted from a previous analytical study for the general, untargeted identification of NPSs [29].

Another approach to nitazene identification is the laboratory synthesis of analogues, followed by the analytical study of their separation and quantitation. This has been done for 14 different nitazenes [30], and it was possible to separate and analyse most of them simultaneously by HPLC-DAD. Moreover, LC coupled to quadrupole time-of-flight (Q-TOF) MS identified a fragment at *m/z* 100.11 (1,1-diethylaziridinium), which is common to all 14 synthesised compounds and could be a candidate to the role of diagnostic marker for nitazene screening in unknown samples. Similarly, in another study four nitazenes (metonitazene, etonitazene, protonitazene and isotonitazene) and their four nitro group positional isomers (isonitazenes) were synthesised and then characterised by FT-IR, GC–MS and HPLC-MS/MS [31]. Although in GC-EI-MS nitazenes were not unequivocally distinguished from the corresponding isonitazenes, in HPLC-MS/MS characteristic fragment ions (*m/z* 86, 215) were observed in the product ion spectra of isonitazenes, enabling them to be distinguished from nitazenes.

A summary of the main characteristics of the most significant papers published in this area is reported in Table 1.

2.1.2. Environmental and community analysis (wastewater etc.)

Environmental/community studies position wastewater-based epidemiology as a complementary tool to casework. Sampling strategies (composite or time-specific) are paired with enrichment to achieve low detection limits for target analytes and relevant metabolites. Methods based on LC and MS are the norm, with suspect lists updated to reflect shifting availability of analogues. The goal is not individual attribution but population-level signals that can inform early warning and prioritisation.

Analytical narratives emphasise matrix effects and interferences typical of wastewater, necessitating robust clean-up and selective detection. Where HRMS is used, non-targeted or suspect screening enables retrospective mining as new analogues emerge. Interpretation is usually subjected to caveats, such as possible mistakes due to transformation during sewer transport, episodic loading and normalisation choices, keeping the focus on trend indication rather than quantitative prevalence. These outputs can feed back into human-matrix panels, supporting decisions on which nitazenes warrant inclusion in routine

Table 1
Main characteristics of analytical workflows for the identification of nitazenes in non-biological samples.

Identified nitazene(s)	Analytical technique	Sample preparation technique	Sample amount	Notes	Ref.
N-desethyl etonitazene hydrochloride	RP-HPLC-HRMS, NMR, CZE	HPLC: MeOH dissolution NMR: DMSO-D_6 dissolution CZE: H_2O dissolution	HPLC: 1 mg NMR: 2 mg CZE: not declared	NMR: ^{1}H NMR, ^{13}C NMR, COSY, NOESY, HSQC, HMBC CZE: anion analysis	[22]
4-Hydroxynitazene, metonitazene, etonitazene, protonitazene, butonitazene, isotonitazene, clonitazene, flunitazene, *N*-desethyletonitazene, *N*-desethylisotonitazene, metodesnitazene, etodesnitazene, isotodesnitazene, 5-aminoisotonitazene, *N*-pyrrolidinoetonitazene, *N*-piperidinyletonitazene	RS, SERS, FT-IR, DART-MS, HPLC-MS, HPLC-UV	μLSE, MeOH/H_2O 10/90	Half-tablet	DART-MS: SQ	[23]
Etodesnitazene, metodesnitazene, isotodesnitazene, flunitazene, clonitazene, butonitazene, protonitazene, *N*-desethyletonitazene, etonitazene, metonitazene, 5-aminoisotonitazene, 4′-OH-nitazene, *N*-desethylisotonitazene, isotonitazene	HPLC-DAD, GC–MS, HPLC-HRMS	None	Not declared		[30]
N-pyrrolidino isotonitazene	UHPLC-MS/MS, GC-HRMS, NMR, FT-IR	MeOH dissolution	10 mg	UHPLC-MS/MS: orbitrap GC-HRMS: QTOF	[25]
N-desethyl isotonitazene	GC–MS, HPLC-HRMS, NMR, ATR-FT-IR	MeOH/$CHCl_3$ (1/1, V/V) extraction, concentration/drying	20 tablets	GC–MS: EI-SQ HPLC-HRMS: ESI-QTOF	[24]
5-nitro nitazenes (metonitazene, etonitazene, protonitazene, isotonitazene); corresponding 6-nitro nitazenes (isometonitazene, isoetonitazene, isoprotonitazene, isoisotonitazene	FT-IR, GC–MS/MS, HPLC-MS/MS	Dissolution in MeOH	1 mg/mL	GC–MS/MS: TQ HPLC-MS/MS: QTRAP	[31]
Metonitazene, protonitazene	LC-DAD, GC–MS screening; UHPLC-HRMS, UHPLC-MS/MS	Dissolution in MeOH	20 mg	UHPLC-HRMS: orbitrap UHPLC-MS/MS: TQ	[28]
N-pyrrolidino protonitazene	RS, ATR-FT-IR, DART-MS, HPLC-HRMS	Dissolution in ACN/H_2O (1/1, V/V)	50 mg	HPLC-HRMS: Linear ion trap	[26, 27]

screening [32].

For example, eight nitazenes were monitored in wastewater in 22 countries between 2022 and 2024 (Fig. 2), using a validated HPLC-MS/MS (triple quadrupole, TQ) method. Positive samples were found exclusively in USA and Australia, and the only nitazenes found were protonitazene and etonitazepyne [33]. However, the analysis of nitazenes in wastewater remains particularly challenging due to the extremely low expected analyte concentrations and to matrix complexity, requiring effective clean-up strategies and careful consideration of sample storage and transportation conditions, which may significantly affect analyte stability and analytical response.

2.1.3. *Synthetic materials*

These studies focus mainly on the possibility of establishing common rules or workflows, which could help laboratories in identifying and confirming at first the general presence of nitazenes and then the specific

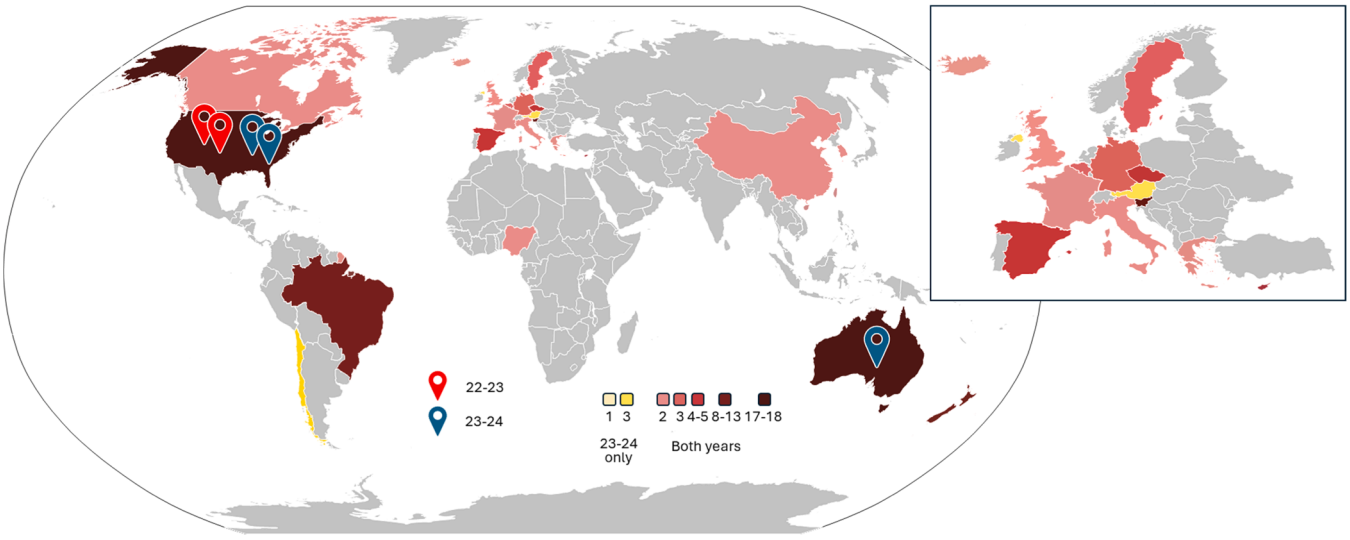


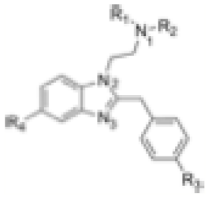
Fig. 2. Map of the areas where nitazene monitoring was carried out. Pins show sites where nitazenes were actually found. Adapted from [33].

presence of some nitazene analogues. Focus is also on the applicability of advanced techniques, which could simplify identification work and in particular the task of discriminating isomers [34].

As usual, most quantitative methods use HPLC or UHPLC coupled to TQ for maximum sensitivity. However, in some cases other separation techniques can be applied, for example GC: Phelps et al. quantitated 20 nitazenes in simulated illicit formulation samples by GC-EI-MS (SQ, SIM scan) [35]. Sensitivity (5–10 μg/mL), however, is far from that of TQ. The fragmentation behaviour of thirty-eight different nitazenes was studied by direct ESI-HRMS (QTOF) infusion, finding diagnostic fragments for most R_1 and R_2, R_3 and R_4 substituents and proposing a standardised workflow for the identification of unknown analogues (see Fig. 4 and Table 2) [36].

Hollerback et al. [37] have confirmed that several nitazenes, like fentanyls, exhibit two ion mobility spectrometry (IMS) collision cross section (CCS) distributions due to the presence of protomers, and that the addition of water in the ESI source significantly changes these distributions, increasing the intensity of the high-mobility distributions and decreasing the intensity of the low-mobility ones. Combined with orbitrap MS fragmentation studies, structures for lossless ion manipulations (SLIM) IMS (+solvent) experiments lead to the hypothesis that the studied nitazenes can produce three protomers in gas phase. Finally, ESI-IMS-HRMS was employed to partially resolve four sets of structurally similar nitazene isomers (i.e., protodesnitazene/isotodesnitazene/5-methyletodesnitazene - Fig. 1, **3–5**); ethyleneoxynitazene/*N*-piperidinyl metonitazene/*N*-pyrrolidinoetonitazene Fig. 1, **9,16,21**; protonitazene/isotonitazene - Fig.1, **11–12**; and butonitazene/isobutonitazene/*sec*-butonitazene - Fig.1, **13–15**), showing that high-resolution IMS separations in MS-based workflows could be suitable for the reference-free identification of emerging nitazenes.

Table 2
Diagnostic ions and fragmentation trends for the most common nitazene substitution groups. Adapted from [42].



Substitution	Diagnostic ions/trends	Common losses (Da)
R_1 and R_2		
Diethyl (R_1 and R_2 = CH_2CH_3)	*m/z* 100, 72, 44	73
Desethyl (R_1 = CH_2CH_3, R_2 = *H*)	No *m/z* 100, base peak = *m/z* 72	71
Piperidine (R_1/R_2 = C_5H_{10})	*m/z* 112, 84, 69, 56	85
Pyrrolidine (R_1/R_2 = C_4H_8)	*m/z* 98, 69, 56	71
R_3		
Methoxy (OCH_3)	*m/z* 121	/
Ethoxy (OCH_2CH_3)	*m/z* 135	/
Propoxy/isopropoxy ($O(CH_2)_2CH_3$)	*m/z* 149	/
Butoxy ($O(CH_2)_3CH_3$)	*m/z* 163	/
R_4		
Desnitazenes (H, CH_3, or NH_2)*	No product ions > *m/z* 200, doubly charged ion in full scan	/

* $[M + H]^+ - R_1/R_2 - R_3$ = benzimidazole core.

2.2. Analytical workflows for biological samples

2.2.1. In vitro and animal studies

Mechanistic investigations using in vitro systems and in vivo animal models can provide the basis for nitazene identification across matrices. By characterising biotransformation routes and fragmentation behaviours, these studies aim to identify diagnostic ions and neutral losses that could later support suspect screening and confirmation in human and environmental workflows. They also help rationalise inter-analogue differences and anticipate metabolites that might be more abundant or stable in certain matrices.

From a practical standpoint, these mechanistic data are reused to populate transition lists, refine chromatographic programs to separate isomers and provide preliminary data for matrix effects or ionisation efficiency. Because reference standard availability may lag behind market emergence, these efforts are highly valuable to reduce false negative risk while standards and libraries are catching up [5,16,38–41].

In this field, an in vitro study has tested the pooled human microsome metabolism of etonitazepipne (Fig.1, **17**), identifying a total of 23 metabolites (13 of them in urine, Fig. 3) by HPLC-HRMS (orbitrap) [42].

Walton et al. determined the main pharmacokinetic and pharmacodynamic characteristics of isotonitazene in rat plasma after LLE with *N*-butyl chloride / ethyl acetate (70:30, V/V) [43] and the *N*-desethyl, 4′-hydroxy and 5-amino metabolites were also quantitated by LC-MS/MS. In this study, isotonitazene reached low-ng/mL peak plasma concentrations and displayed a short elimination half-life in the order of tens of minutes.

Overall, currently available metabolic studies show that nitazenes undergo rapid phase I biotransformation, most consistently through *N*-dealkylation and aromatic hydroxylation, while nitro reduction has also been reported for selected analogues. Across the published in vitro and in vivo studies, the parent compound typically remains the predominant species in plasma, whereas the corresponding *N*-desethyl and hydroxylated metabolites are observed at lower concentrations. PK/PD data indicate a fast systemic clearance and a close temporal association between parent drug exposure and μ-opioid-mediated effects. Taken together, the available evidence outlines a general pattern of rapid metabolism accompanied by the formation of diagnostic metabolites, while also highlighting that detailed PK/PD characterisation is presently available only for a limited number of nitazenes. These metabolomic- and metabolism-driven HRMS approaches are particularly valuable when analytical reference standards are unavailable, as they allow indirect identification through characteristic metabolites and fragmentation patterns.

2.2.2. Nitazene identification in human biological fluids

Analytical approaches for nitazene identification in human biological matrices typically rely on targeted MRM panels or HRMS-based screening applied to blood, plasma/serum, urine, hair and oral fluid. Sample preparation is generally tailored to matrix complexity and expected concentration ranges, balancing throughput with clean-up. Confirmation procedures make use of retention time agreement and diagnostic product ion ratios, with isomeric interferences addressed through chromatographic resolution or fragment-specific criteria.

Postmortem scenarios introduce interpretive nuances (distribution and stability), while clinical/forensic contexts prioritise turnaround and unambiguous calls. Reporting criteria typically emphasise agreement of multiple identifiers, appropriate controls and the handling of potential cross-reactivity when immunoassay screening is carried out before MS [44]. Hair and oral fluid items highlight collection-specific issues (washing procedures, device effects) that can impact identification confidence [45–48]. Despite the growing use of oral fluid for drug testing, no bioanalytical methods specifically developed and validated for nitazenes as a class in this matrix have been reported so far. Only isolated multi-analyte drug of abuse methods, in which nitazene

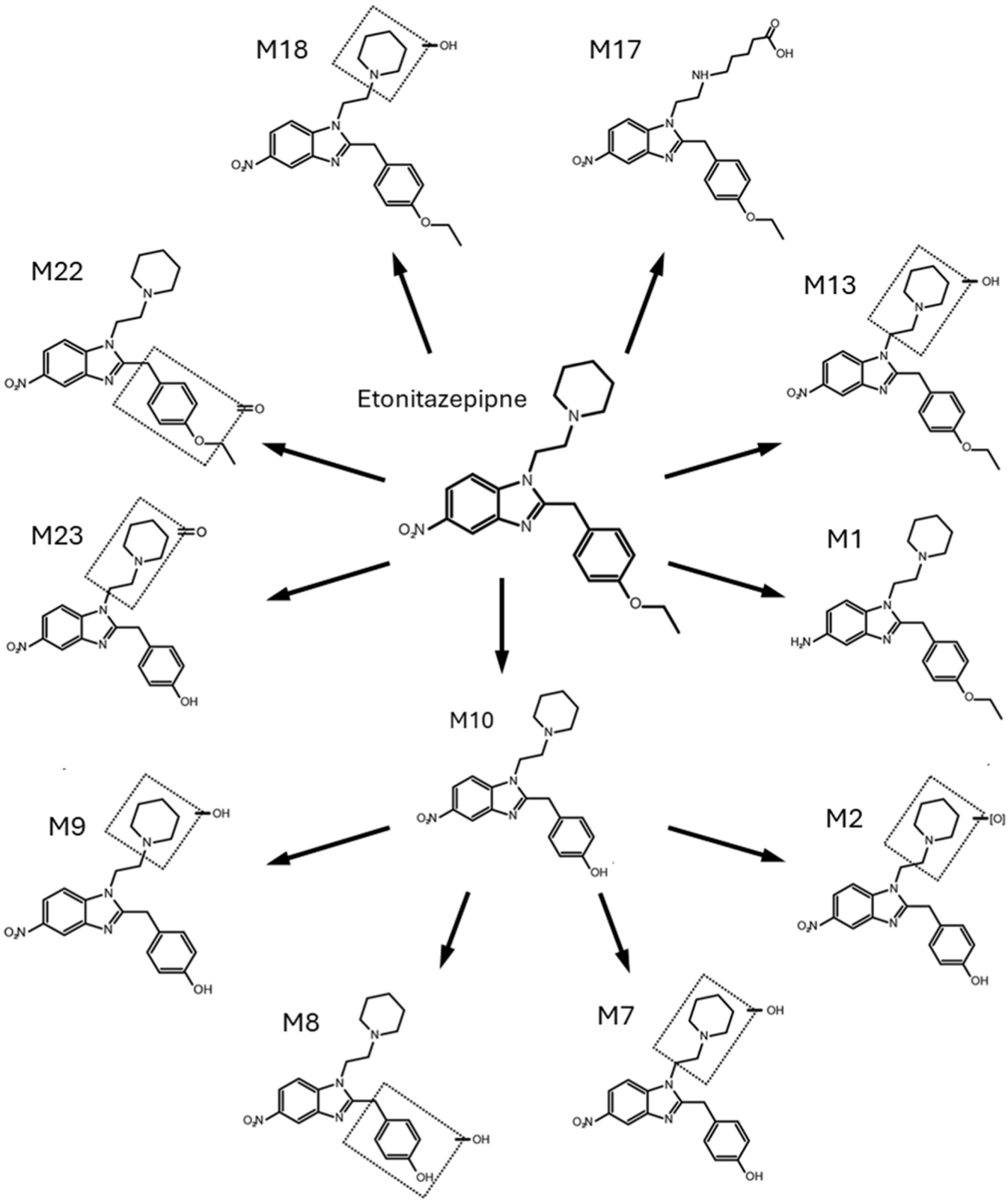


Fig. 3. Map of etonitazepipne metabolites. Adapted from [42].

analogues are occasionally included among the targets, are available [49,50].

In many instances, nitazenes can be identified in biological fluids during forensic scrutiny in cases of poisoning or drug abuse. Untargeted HRMS is currently one of the most potent weapons in the analyst's arsenal for this purpose. Using an approach derived from metabolomics [51], a molecular network is generated (possibly with data from different biological fluids), which allows the identification of parent drug(s) and one or more metabolites (Fig. 5). This workflow was applied to two severe poisoning cases, using an orbitrap MS and resulting in the

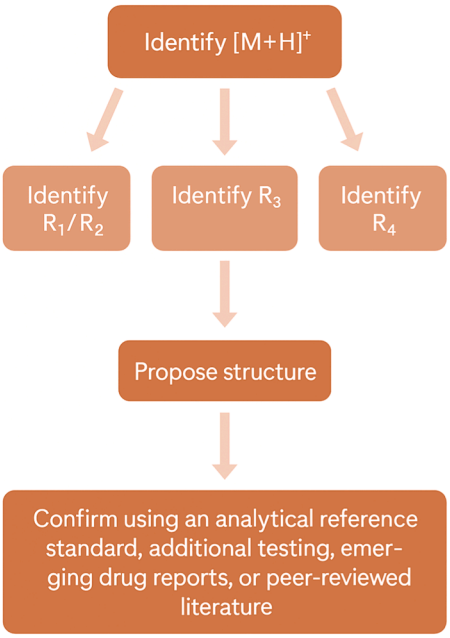


Fig. 4. Possible workflow for the tentative identification of nitazene analogues from their MS spectra. Adapted from [36].

identification in both plasma and urine of protonitazene in the first and protonitazepyne in the second case, together with several phase I (hydroxylated, *N*-desethylate, *O*-despropylated) and phase II (glucuronide) metabolites of the former, and just one of the latter, protonitazepyne acid [52].

Based on analogies with nitrobenzodiazepines, it has been hypothesised that bacterial degradation could lead to the formation of amino- and acetamido- derivatives from nitro-nitazenes. Tentative identification of these metabolites was carried out in non-preserved postmortem blood from a real case of metonitazene poisoning. Indeed, UHPLC-HRMS (QTOF) allowed the authors to detect fragments compatible with the formation of 5-aminometonitazene and 5-acetamidometonitazene. Early addition of stabilising agents (oxalate, fluoride) prevented the detection of these fragments [53].

The main characteristics of these methods are summarised in Table 3.

2.2.3. *Nitazene quantitation in human biological fluids*

Quantitative methods are largely LC-MS/MS (TQ), occasionally HRMS-based, and are framed by validation elements: calibration model and range aligned to clinically/forensically relevant levels; sensitivity expressed as LOD/LLOQ; precision and accuracy across QC levels; selectivity and carry-over controls; and matrix-effect appraisal. Chromatographic programs are tuned to separate close analogues where necessary, or transitions are chosen to maintain specificity when co-elution is unavoidable.

Stability studies (benchtop, freeze-thaw, processed autosampler) and robustness considerations recur, reflecting the intent to support routine deployment. Authors also often highlight the importance of IS strategies and acceptance criteria that are commensurate with the intended use (surveillance, clinical support, or postmortem interpretation) without overreach beyond what the data support [54–56]. Since quantitative application is sought, TQ is by far the most widely used kind of MS, due to its superior sensitivity coupled to good selectivity.

An overview of the variety of approaches and available methods is reported in Table 4.

2.2.3.1. *Blood and other haematic matrices, urine.*

Sample management and analytical challenges differ across whole blood, blood-derived matrices (plasma/serum) and urine. Whole blood is typically more demanding due to its cellular component and higher matrix effects, while plasma/serum generally offers a cleaner background but still requires attention to protein-related interferences; urine, in turn, is influenced by variable dilution and the frequent presence of conjugated metabolites, often motivating hydrolysis and careful calibration strategies.

LC-MS/MS was applied for the determination of 21 nitazenes and 5 metabolites in whole blood after LLE with basic ethyl acetate, reaching outstanding sensitivity (10 pg/mL for 25 of the 26 analytes) also thanks to the 10-fold concentration ratio achieved during sample treatment [57]. The same sample preparation technique (LLE) was also used to extract nine nitazene analogues and/or metabolites using a basic mixture of ethyl acetate and *N*-butyl chloride, and HPLC-MS/MS (TQ) analysis [58].

Nine nitazenes were analysed in whole blood after applying supported membrane - liquid phase microextraction (SM-LPME) in the 96-well plate format (Fig. 6a), making high-throughput possible despite a single extraction taking 45 min (as well as an AGREEprep score of 0.71 on a maximum of 1.0 for sample preparation greenness). Comparison with corresponding procedures by electromembrane extraction (EME, Fig. 6b) highlighted the better performance of the former technique [9].

Unified sample preparation procedures are often adopted for both urine, blood and derived matrices (plasma, serum) in forensic analysis. For example, protein precipitation with ice-cold ACN, coupled to enzymatic hydrolysis for urine, was used by Berardinelli et al. [42] to quantitate etonitazepipne (*N*-piperidinyl etonitazene) by HPLC-MS/MS (QTRAP). The same chromatographic setup and the same sample preparation procedure was later used by Wrbas et al. [59] to quantitate *N*-pyrrolidino protonitazene, again in both urine and blood-derived matrices. In this latter case, the presence of *N*-pyrrolidino protonitazene was hypothesised due to the results of a screening test, which was not detailed in any way. In turn, the same sample preparation and analytical methods were adapted from previously published papers dealing with the quantitation of non-nitazene synthetic opioids brorphine [60] and AP-238 (a cinnamylpiperazine) [61].

2.2.3.2. *Miniaturised samples.*

Given the limited number of published papers on nitazene analysis in biological fluids, it is not surprising that just a few of them include the use of microsampling. However, this approach promises important advantages, such as lower invasiveness, better stability, lower expenses, more practical shipping and storage, easier automation [62,63].

Among them, Ververi et al. [64] applied volumetric dried blood spotting (vDBS) to the analysis of 9 nitazenes and brorphine. vDBS was carried out through Capitainer B cards, which allow the accurate sampling of one 10-μL DBS from a finger prick (up to two DBS can be obtained from a single card, by depositing two blood drops). Sample preparation was carried out by ultrasound-assisted extraction (UAE) with solvent (MeOH/ACN, 3/1, V/V), followed by drying, redissolution (30 μL of MeOH) and injection into UHPLC-MS/MS. Since the IS (fentanyl-D_5) was added with the extraction solvent, it was not extracted together with the analytes, thus limiting its usefulness.

Vitrano et al. [65] have tested the stability of several nitazenes in DBS stored at room temperature or 4 °C by an original HPLC-HRMS method, finding that low-concentration analytes are less stable than high-concentration ones and that temperature has different effects on different analyte concentrations.

2.2.3.3. *Hair analysis.*

After the first report by Kintz et al. [66], retrospective information on nitazene use was obtained from hair analysis by UHPLC-MS/MS (TQ) [67]. This second method allowed the monitoring of 17 different nitazenes (divided into 6 isomer groups, Fig. 7) in the

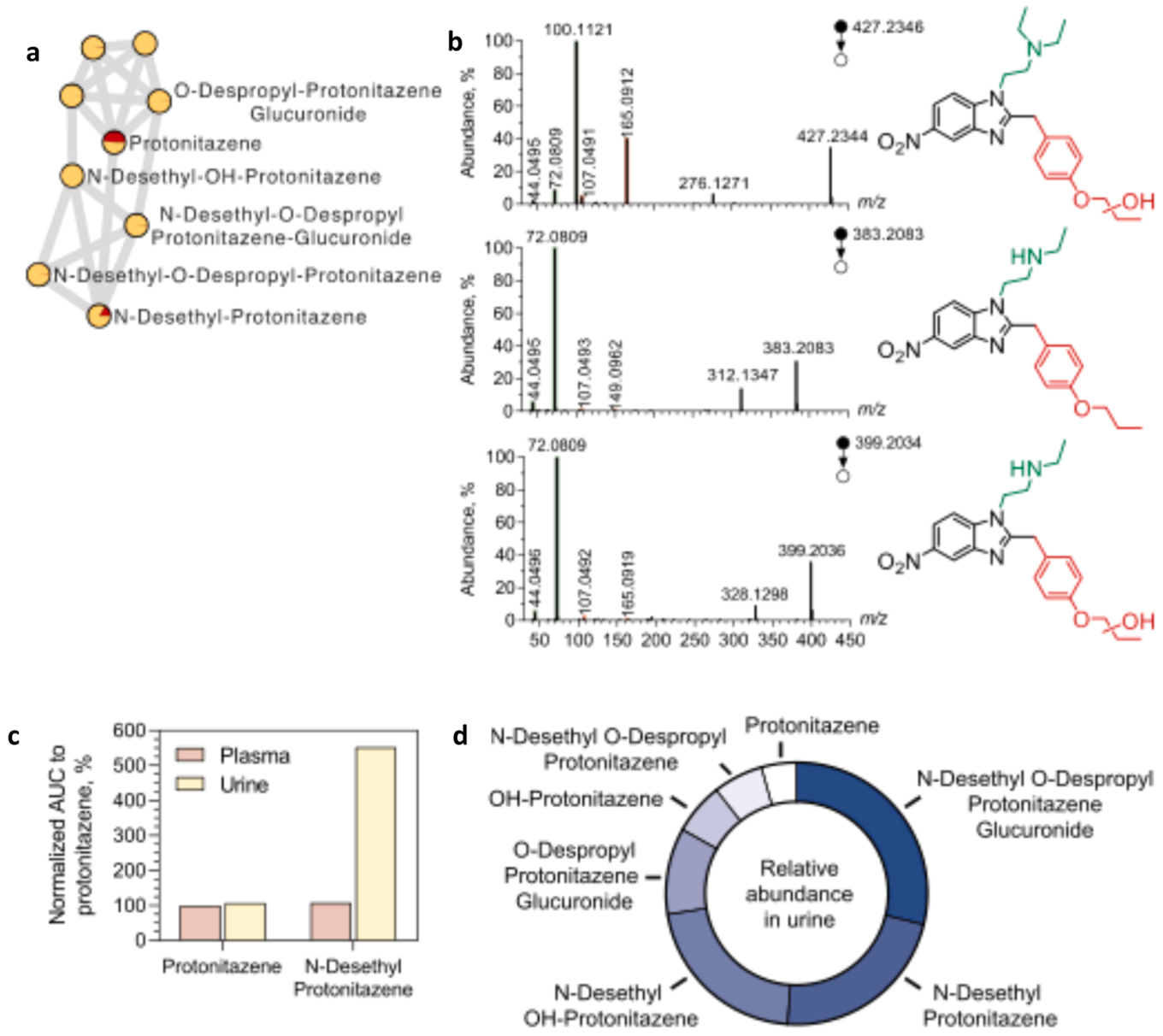


Fig. 5. (a) Molecular network of protonitazene metabolites; (b) Examples of spectra used to build the network. (c) Relative abundance of metabolites in urine and plasma and (d) abundance in urine (based on areas under the curve). Adapted from [51].

Table 3
Main characteristics of analytical workflows for the identification of nitazenes in human biological samples.

Biological matrix	Analytical technique	Sample preparation technique	Sample amount	Identified nitazene(s)	Notes	Ref.
Plasma, urine	HPLC-ESI-HRMS	Protein precipitation, drying, redissolution	100 µL	Protonitazene, protonitazepyne, metabolites	HRMS: orbitrap Molecular network setup	[52]
PM blood, urine	UHPLC-MS/MS, UHPLC-HRMS	Dilution, SPE	200 µL	5-aminometonitazene, 5-acetamidometonitazene	MS/MS: TQ HRMS: QTOF Presumable degradation products	[53]

picograms per milligram concentration range, with chromatographic runs lasting just 6 min. Another retrospective evaluation was carried out by Ballotari et al. [68].

3. CONCLUSION

The analytical investigation of nitazenes reflects a broader evolution in the response to novel synthetic opioids, with laboratory workflows constantly adapting to new analogues and changing market trends. Despite the variety of techniques described in the literature, a common requirement emerges: analytical flexibility grounded in rigorous validation, in which rapid screening and confirmatory analysis are integrated in a way that preserves scientific defensibility.

Nitazenes illustrate many of the difficulties associated with potent

Table 4
Main characteristics of analytical workflows for the quantitative analysis of nitazenes in human biological samples.

Analyte(s)	Matrix	Analytical technique	Key chromatographic conditions	Sample preparation technique	Sample amount	Performance indicators				Notes	Ref.
						Linearity	Precision (RSD %)	Matrix effect (RE %)	Accuracy (bias %)		
N-pyrrolidino etonitazene, butonitazene, etodesnitazene, etonitazepipne, flunitazene, isotonitazene, metodesnitazene, metonitazene, protonitazene	vDBS	UHPLC-ESI-MS/MS (TQ-SRM)	UHPLC, C18 column, gradient elution	Solvent extraction (MeOH/ACN), UAE	10 μL	1–50 ng/mL	<15 %	<15 %	<10 %	28-day stability at RT	[64]
4′-hydroxy-nitazene, 5-aminoisotonitazene, 5-methyletodesnitazene (5-methyletazene), butonitazene, clonitazene, etazene (etodesnitazene), ethyleneoxynitazene, etonitazene, flunitazene, isobutonitazene, isotodesnitazene, etonidazene, metonitazene, *N*-desethyl-etonitazene, *N*-desethyl-isotonitazene, nitazene, *N*-piperidinyl-etonitazene (etonitazepipne), *N*-piperidinyl-isotonitazene (izotonitazepipne), *N*-piperidinyl-metonitazene (metonitazepipne), *N*-piperidinyl-protonitazene (protonitazepipne), *N*-pyrrolidino-etonitazene (etonitazepyne), *N*-pyrrolidino-metonitazene (metonitazepyne), *N*-pyrrolidino-4′-OH-nitazene, protodesnitazene, protonitazene/sec-butonitazene	Whole blood	UHPLC-ESI-MS/MS (TQ-MRM)	UHPLC, PFP, C18 columns, gradient elution, 40 °C	LLE (ethyl acetate, pH 9.0)	500 μL	0.01–15 ng/mL (0.01–15 ng/mL for flunitazene)	<14.9	≤±20.4	−14.1 to +7.1		[57]
Clonitazene, etodesnitazene, etonitazene, etonitazepyne, flunitazene, isotonitazene, metodesnitazene, metonitazene, protonitazene	Whole blood	UHPLC-ESI-MS/MS (TQ-MRM)	UHPLC, biphenyl column, gradient elution, 60 °C	LPME (EME also tested)	120 μL	0.5–50 nM	≤16	233 for 5/9 analytes	−3 to +6		[9]
Metodesnitazene, etodesnitazene, 4′-hydroxy nitazene, *N*-desethyl etonitazene, *N,N*- dimethylamino etonitazene, flunitazene, 5-amino isotonitazene, metonitazene, *N*-desethyl isotonitazene, *N*-pyrrolidino	Hair	UHPLC-MS/MS	UHPLC, biphenyl column, gradient elution	Cryogenic grinding with extraction solution, UAE 10 min	20 mg	5–1000 pg/mg	<15 %	40–99	<±15 %		[67]

(*continued on next page*)

Table 4 (*continued*)

Analyte(s)	Matrix	Analytical technique	Key chromatographic conditions	Sample preparation technique	Sample amount	Performance indicators				Notes	Ref.
						Linearity	Precision (RSD %)	Matrix effect (RE %)	Accuracy (bias %)		
etonitazene, ethyleneoxynitazene, *N*-pyrrolidino protonitazene, *N*-piperidinyl etonitazene, isotonitazene, protonitazene, butonitazene, *sec*-butonitazene											
Etonitazepipne + metabolites	Blood, urine	HPLC-MS/MS (QTRAP)	F5, biphenyl columns, gradient elution, 37 °C	PPT (ice-cold ACN)	100 µL	0.5–10 ng/mL	≤5.6	130	n.d.		[38]
Isotonitazene, metonitazene, protonitazene, etonitazene, clonitazene, flunitazene, *N*-desethyl isotonitazene, 5-amino isotonitazene, 4-hydroxy nitazene	Whole blood, urine, tissue	HPLC-MS/MS (TQ)	C18 column, gradient elution, 30 °C	LLE (N-butyl chloride, ethyl acetate, 70/30, V/V)	500 µL	0.5–50 ng/mL	<14.3	≤234	−7.5 to +8.6		[58]

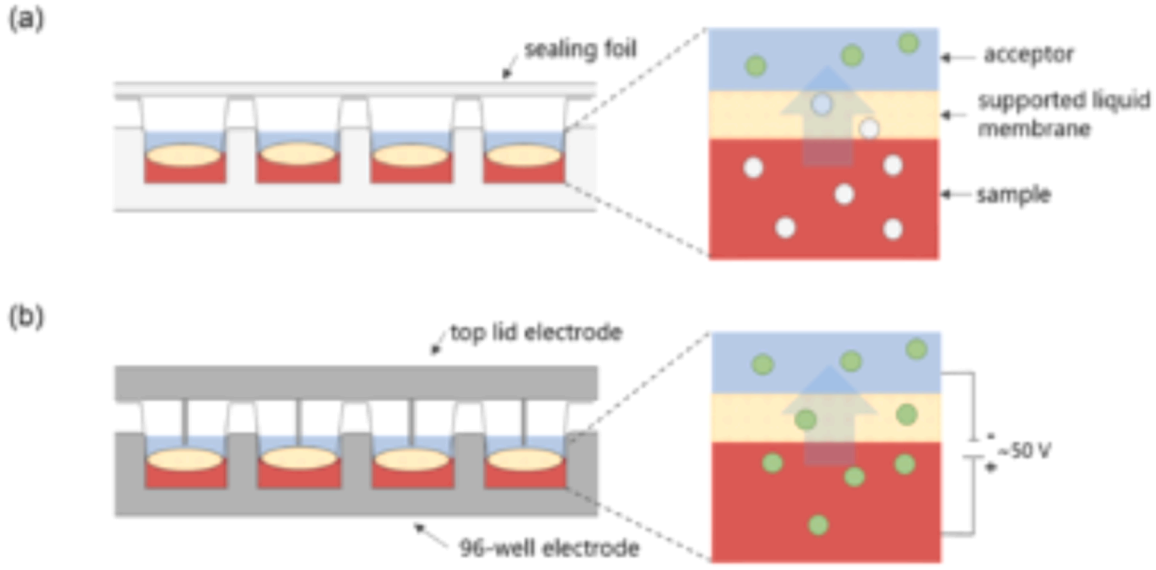


Fig. 6. Visualisation of (a) LPME, (b) EME in a 96-well plate format. Adapted from [9].

and structurally different synthetic opioids, including limited availability of reference standards, isomeric complexity and the unpredictable appearance of new derivatives. In response, laboratories have combined high-resolution mass spectrometry, targeted quantitation and mechanistic information on metabolism and fragmentation. This integrated approach strengthens compound identification and supports more consistent comparison of results across forensic, clinical and monitoring settings.

Another recurring theme is methodological interoperability. The convergence of chromatographic conditions, recurrent diagnostic fragments and shared spectral databases points towards greater harmonisation of analytical practice. Such convergence is essential to ensure that results produced in different laboratories remain comparable and robust, particularly where they may be used in regulatory or legal contexts.

Overall, the framework developed for nitazenes can be viewed as a model for addressing future generations of designer drugs: dynamic and data-driven, but at the same time anchored to clear validation criteria and standardised reporting. Maintaining this balance between innovation and harmonisation will be crucial for ensuring that advanced analytical approaches applied to toxicology, forensics and healthcare scenarios continues to support both immediate and longer-term public health objectives.

CRediT authorship contribution statement

Roberto Mandrioli: Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Michele Protti:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Roberta Di Lecce:** Writing – review & editing, Visualization, Methodology. **Laura Mercolini:** Writing – review & editing, Writing – original draft,

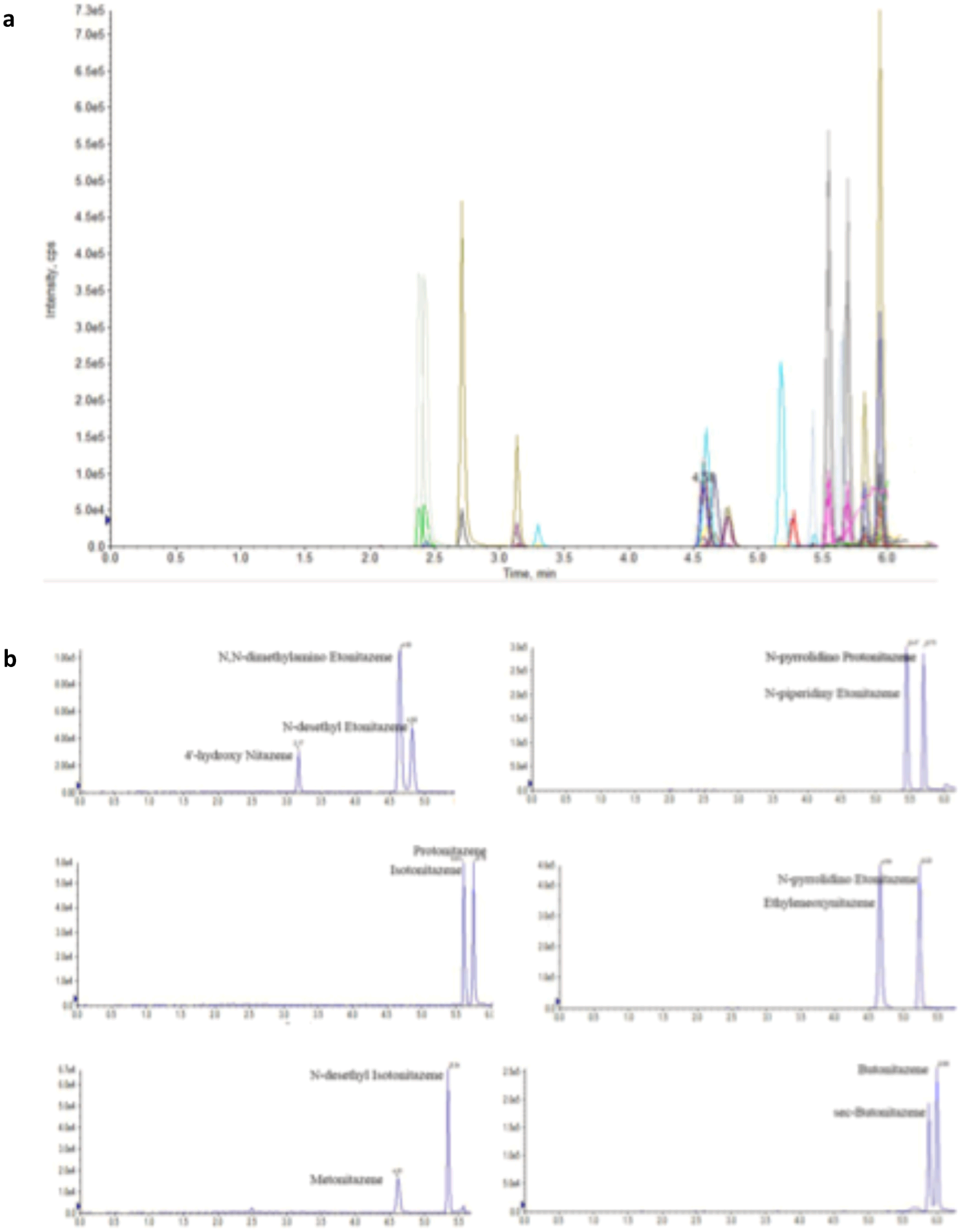


Fig. 7. (a) Chromatograms from the analysis of 17 nitazenes in hair, with (b) their division into six groups of isomers. Adapted from [67].

Visualization, Supervision, Methodology, 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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Data availability

No data was used for the research described in the article.

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