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Innovative QSRR modeling approach for the development of an ultra-sensitive LC-MS/MS method for trace analysis of N-nitrosamines

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ABSTRACT

To address regulatory concerns regarding N-nitrosamine contamination in pharmaceutical products, generic LC-MS/MS methods for determining N-nitrosamines were developed using an innovative *in silico* approach based on Quantitative Structure Retention Relationship modeling (QSRR). The development process included screening and optimization phases, offering flexibility in targeting N-nitrosamines and addressing the challenges related to the matrix effect. This methodology represents a significant advancement in method development. Among the developed methods, a highly sensitive and accurate LC-MS/MS method was successfully validated to simultaneously determine 5 small-molecule N-nitrosamine impurities in tablets, which was used in the present proof-of-concept study. The validation followed the ICH Q2 (R2) guidelines, employing a combined approach for accuracy and precision based on total error risk-based methodology. The method was validated to function as both an impurity limit test and a quantitative method. Validation results demonstrated adequate quantitative performance of the method, establishing a validated dosing range from 1 to 30 ng/mL for all N-nitrosamines. The estimated detection limit ranged from 0.75 pg/mL to 0.02 ng/mL. The detection and quantification limits for each N-nitrosamine met the EMA N-nitrosamine investigation approach requirements. Moreover, both are always below 10 % of their respective acceptable limit in the studied finished product formulation. This proposed method is suitable for investigating small-molecule N-nitrosamines in pharmaceutical products and also provides a starting point for further method development, particularly for the determination of newly identified small-molecule N-nitrosamines and drug-substance-related N-nitrosamines.

1. Introduction

N-nitrosamines are a class of chemical compounds bearing a common functional $R^1N(-R^2)-N=O$ group, illustrated in Fig. 1. Their origin lies in the nitrosation process of secondary, tertiary, or quaternary amines in the presence of nitrosating agents under acidic conditions [1,2]. Typically present in trace quantities as impurities across diverse matrices, they are notably found in the environment, drinking water, food, tobacco, cosmetics, etc. Designated as potentially mutagenic substances for humans and identified as a "cohort of concern" by the ICH M7 (R2) guidelines [3], certain N-nitrosamines are classified in group 2 A: probably carcinogenic; or in group 2B: possibly carcinogenic to humans, according to the International Agency for Research on Cancer (IARC) [4]. After ingestion, N-nitrosamine first undergoes an enzymatic α-hydroxylation facilitated by cytochrome P450, resulting in the formation of an intermediate dealkylated primary N-nitrosamine. Due to its inherent instability, this primary N-nitrosamine undergoes further decomposition, giving rise to diazonium, which acts as a DNA alkylating agent. The subsequent formation of DNA adducts poses a risk of DNA damage, potentially leading to cancer development [5].

The first N-nitrosamine contamination reported to EU authorities was the detection of N-nitrosodimethylamine (NDMA) in the active pharmaceutical ingredient (API) of valsartan in June 2018. The NDMA-contaminated API was manufactured by Zhejiang Huahai, one of the Chinese API manufacturers authorized in the EU [6]. Later, N-nitrosamine contaminations were detected in drug substances and products within the pharmacological class of sartans, commonly known as angiotensin II receptor antagonists, widely prescribed for the chronic treatment of hypertension and heart failure. Following numerous antihypertensive cases, regulatory agencies initiated comprehensive investigations to other therapeutic categories, including antidiabetics, antacids, antibiotics, and more. These medications are generally

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intended for chronic use, which could significantly increase the risk of exposure to N-nitrosamines over extended periods. The detection of N-nitrosamines in pharmaceuticals prompted recalls and the withdrawal of the drugs from the market, resulting in shortages in several countries. These regulatory actions have heightened awareness among healthcare professionals about the potential risks associated with exposure to these genotoxic impurities.

Regulatory agencies worldwide have intensified their oversight of pharmaceutical manufacturing processes, with a particular focus on those involving chemically related compounds prone to N-nitrosamine formation. N-nitrosamines can arise as byproducts at various stages of drug manufacturing, particularly during the chemical synthesis of APIs by using sodium nitrite as a nitrosating reagent, or due to contaminated raw materials and solvents. Improper storage conditions may also be a source of N-nitrosamines, such as degradation impurities. Moreover, the use of nitrocellulose-containing packaging materials may contribute to the formation of N-nitrosamines. Most of these compounds are classified as small-molecule N-nitrosamine impurities, with NDMA and N-nitrosodiethylamine (NDEA) being the most commonly detected, which do not share structural similarities with APIs. In parallel, N-nitrosamine drug substance-related impurities (NDSRIs) are being reported, which can be found in certain drug substances or products. These impurities arise from a specific nitrosation of APIs and share structural similarities. The detection of N-nitroso-varenicline, N-nitroso-nortriptyline, N-nitroso-dabigatran, N-nitroso-duloxetine, etc., has been reported [7].

Given the comprehensive examination of these cases and the acknowledged risk of N-nitrosamine contamination, both the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) have recommended that marketing authorization holders (MAHs) play a proactive role and extend the lessons learned to all medications intended for human use. The control of N-nitrosamine impurities has now become a priority for both manufacturers and regulators.

Considering the carcinogenic potential of N-nitrosamines, their presence is only tolerated in very small amounts. To address this, international regulatory agencies have established recommended acceptable intake (AI) limits for some small-molecule N-nitrosamines in relation to their toxicological profile (refer to Table 1) [1,7]. Concurrently, MAHs are required to investigate the presence of N-nitrosamine impurities in chemically synthesized and biologically derived active ingredients, as well as finished products [1,7]. All decisions and actions taken by MAHs should be geared towards mitigating or preventing contamination risk, thereby ensuring the safety and quality of pharmaceutical products.

Table 1
Recommended acceptable intake (AI) limit of N-nitrosamine impurities established by the EMA and FDA.

N-nitrosamine	AI (ng/day)	N-nitrosamine	AI (ng/day)
NDMA	96	NDBA*	26.5
NDEA	26.5	NMOR*	127
NEIPA	400	MeNP*	400
NDIPA	1500	NNK*	100
NMPA	100	NMPEA*	8
NMBA	1500	NTHP*	37
NDPA*	26.5	NPIP*	1300
NPYR*	1700	NDELA*	1900
NDPhA*	78000	NDIPLA*	400

* Additional information from the EMA procedure. Data from the EMA was last updated in September 2024, and from the FDA in October 2024.

In trace analysis, the development of analytical methods poses considerable challenges as they must achieve sufficient sensitivity to reliably detect and quantify trace levels of analytes. The choice of an appropriate analytical technique is pivotal and should align with the required sensitivity. Hyphenated techniques, particularly liquid chromatography or gas chromatography hyphenated to mass spectrometry (LC-MS/MS or GC-MS(/MS)), stand out as the most frequently reported methods in the literature. Renowned for their high sensitivity and specificity, these techniques are preferred for their ability to detect N-nitrosamines in the order of 0.1 ng/mL [8]. Regulatory agencies and official medicines control laboratories have released validated LC-MS/MS and GC-MS(/MS) methods for medicinal products derived from chemical synthesis, serving as valuable starting points to aid MAHs in their method development [8–12]. Both the United States Pharmacopeia and the European Pharmacopoeia have established general chapters addressing concerns related to N-nitrosamine impurities and have proposed corresponding analytical procedures [13,14]. Some validated methods for biopharmaceuticals have also been reported [15,16].

Despite the great sensitivity and specificity of spectrometric techniques, their analytical strength can sometimes be restricted, especially when attempting to identify an analyte solely based on its mass-to-charge (m/z) ratio. This limitation becomes more apparent in the presence of isobaric or isomeric compounds. In such scenarios, an orthogonal identification method providing chromatographic retention time proves useful for separating compounds before spectrometric detection [17]. However, chromatographic development can be a laborious and

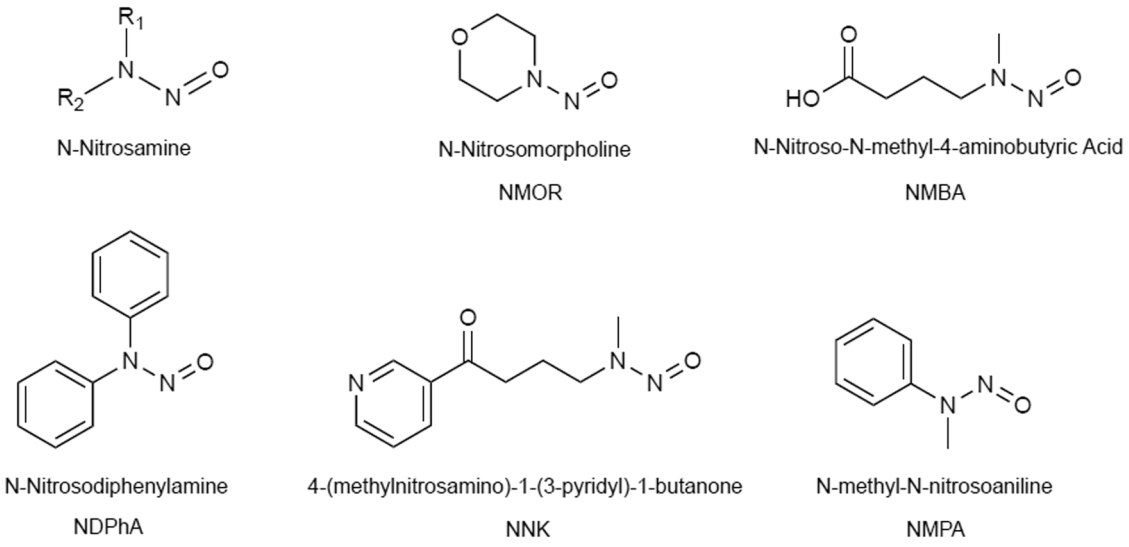

Fig. 1. Chemical structures of the N-nitrosamines and the N-nitrosamines involved in this study.

time-consuming step. Quantitative structure retention relationship (QSRR) modeling emerges as a potent tool for predicting retention time and understanding separation mechanisms in chromatographic analysis. QSRR models are computational statistical models that establish mathematical relationships between molecular descriptors and chromatographic retention parameters [18–20]. Molecular descriptors (or features) are quantitative values representing a compound's physicochemical properties [20]. First, a diverse set of compounds is experimentally tested under various chromatographic conditions to build a dataset encompassing a broad range of molecular descriptors to train QSRR models. After training, these models can then be applied to new compounds with similar features to predict their retention behavior [17, 19]. While the development phase of QSRR modeling is complex and time-consuming, the methodology offers numerous advantages once the models are established. It is user- and environmentally friendly, straightforward to interpret, time-efficient, and capable of predicting compound retention times without the need for additional experimental work [19]. However, the application of QSRR modeling has limitations, as the models are developed using specific columns, eluents, flow rates, etc, making them specific to the stationary and the mobile phases within a range of given chromatographic conditions [17]. Additionally, model error should also be considered. QSRR modeling was proposed as an alternative approach to assist and optimize the development phase of chromatographic methods [18]. This development strategy offers the advantage of integrating prior knowledge and quality risk management, aligning with the ICH Q14 guidelines. On the one hand, this practice enables continuous lifecycle management of analytical procedures using accumulated knowledge. On the other hand, it reduces the risk of poor analytical performance and enhances the reliability of the results [21].

This work aimed to establish an operational space where chromatographic conditions could be wisely selected to determine a set of highly specific and sensitive LC-MS/MS methods for the simultaneous screening and quantification of up to 17 trace impurities of N-nitrosamines. The method was developed using an innovative *in silico* approach combining the screening and optimization phases. This approach provides flexibility in targeting specific N-nitrosamines and addressing the challenges related to matrix effect (ME). ME, a common drawback in mass spectrometry, occurs when compounds co-elute. This phenomenon is characterized by an enhancement or suppression of analyte ionization and signal intensity in the presence of interfering compounds. Subsequently, ME can negatively impact the method's sensitivity and accuracy, thereby influencing the reliability of the results [22].

Proof of this concept was demonstrated in the present study using 5 N-nitrosamines, selected deliberately to align with the requirements of Quality Control for a commercialized pharmaceutical formulation. The developed method was successfully validated under the recommendations outlined in ICH Q2(R2) using the combined approach and effectively serves both as a limit test for impurities and as an assay for the five N-nitrosamines.

2. Materials and methods

2.1. Chemicals and reagents

The chemical reference standards were purchased from various suppliers. 500 μg/mL commercial stock solution of N-nitrosodimethylamine (NDMA), N-nitrosodi-n-propylamine (NDPA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), N-nitrosodi-n-butylamine (NDBA), N-nitroso-diethylamine (NDEA), and N-nitroso-N-methyl-4-aminobutyric acid (NMBA) was purchased from EDQM (Strasbourg, France). 1000 μg/mL commercial stock solutions of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-Nitrosomethylphenylamine (NMPA) were purchased from Sigma-Aldrich/Merck (St. Louis, MO, USA). N-Nitrosomorpholine (NMOR), N-Nitrosodiphenylamine (NDPhA), and N-nitroso-diethanolamine (NDELA) were obtained from TCI (Tokyo, Japan). 1000 μg/mL commercial stock solutions of N-nitrosomethylethylamine (NMEA), N-nitrosopyrrolidine (NPYR), and N-nitroso-piperidine (NPIP) were purchased from Cambridge Isotopes Laboratories (Tewksbury, MA, USA). 1000 μg/mL commercial stock solutions of 1-methyl-4-nitrosopiperazine (MeNP), and N-nitroso-1,2,3,6-tetrahydropyridine (NTHP) were purchased from United States Pharmacopeia (Rockville, MD, USA).

Internal standards (stable isotope-labeled analogs) were obtained from several suppliers. Deuterated N-nitrosamine standards NMBA-d3, NMPA-d5, NDPhA-d6, NDPA-d14, and MeNP-d4 were purchased from BOC Sciences (New York, NY, USA). NMOR-d8 was provided by J.H. Ritmeester B.V. (Nieuwegein, Netherlands). 10 μg/mL commercial stock solution of 13C6-NNK, and 1000 μg/mL commercial stock solutions of NDMA-d6, NDBA-d18, NPYR-d8, NMEA-d3, NDEA-d10, and NPIP-d10 were purchased from Cambridge Isotope Laboratories. NDELA-d8 was obtained from Sigma-Aldrich/Merck.

17α-hydroxyprogesterone was obtained from TCI. ULC-MS grade solvents and additives such as methanol (MeOH), water, formic acid (FA), and ammonium acetate (AmAc) were purchased from Biosolve (Dieuze, France). The pharmaceutical formulation used in this proof-of-concept study was an oral solid dosage form (prolonged-release tablets), sourced by a pharmaceutical industry partner. Due to a confidentiality agreement, the nature and dosage of the API cannot be disclosed.

2.2. LC-MS/MS instrumentation

An Acquity® Premier UPLC® system (Waters, Milford, MA, USA) was used to carry out this study. This system is composed of a binary solvent manager, a sample manager equipped with a 10 μL injection loop, working in the partial loop and the flow through needle mode, and a column heater with an active pre-heater. The system was coupled with a Xevo® TQ-Absolute detector (Waters) equipped with an atmospheric pressure chemical ionization (APCI) source.

Acquity® UPLC® HSS T3 VanGuard pre-column (1.8 μm, 2.1 mm × 5 mm), Acquity® Premier HSS T3 column (1.8 μm, 2.1 mm × 100 mm), and Acquity® UPLC® HSS T3 column (1.8 μm, 2.1 mm × 100 mm) were purchased from Waters.

2.3. QSRR dataset description

The development phase included a test set of compounds with distinct physicochemical properties under various experimental conditions. This methodology was designed to build a dataset that covers a broad range of molecular descriptors [17]. The development of the QSRR model integrates findings from our previous publications [18,23].

The model was initially designed for reverse-phase high-performance liquid chromatography (HPLC). Experiments were carried out using an Alliance® system (Waters) equipped with a photodiode array detector. XSelect HSS T3 columns, 3.5 μm, 2.1 mm × 100 mm (Waters) were used without a pre-column throughout the model development phase. HPLC parameters were maintained at the following settings: flow rate: 0.3 mL/min; injection volume: 5 μL; column temperature: 25 °C; autosampler temperature: 6 °C. A linear binary gradient with variations in gradient time (from 20 to 60 minutes), with a transition from 100 % to 5 % of the aqueous fraction, was applied along with five levels of pH. In total, 10 distinct experimental conditions were tested by combining variations in pH and gradient time, resulting in the generation of a robust and comprehensive dataset. The molecular descriptors for the API and the N-nitrosamines involved in this study were calculated for each condition and are available in Files S1 to S10.

Given the intended routine use, an ultra-high-performance liquid chromatography (UHPLC) method is more suitable than HPLC due to its ability to reduce analysis time, lower solvent consumption, and enhance peak efficiency. For the method development, the Acquity® Premier HSS T3 column (1.8 μm, 2.1 mm × 100 mm) was selected over the XSelect HSS T3 column (3.5 μm, 2.1 mm × 100 mm). Although both column types share similar chemistry (C18), the Acquity® Premier HSS

T3 columns possess a smaller particle size and are compatible with UHPLC instrumentation.

Any modifications to the initial conditions used for model development must be carefully evaluated. Chromatographic parameters should be adjusted as needed to maintain equivalent separation. The transition to a longer and UHPLC-compatible column necessitated a geometric transfer, which was performed using HPLC Calculator v3.1 (University of Geneva) [24,25]. The optimal gradient time post-transfer was determined to be 9.9 minutes, equivalent to 20.0 minutes predicted from the original HPLC method.

2.4. In silico screening methodology

QSRR models initially establish mathematical relationships between molecular descriptors and the chromatographic retention behavior of a set of compounds previously acquired. Once these relationships are defined, following a published workflow [18], the molecular descriptors of new compounds can be provided as input to the models to predict their retention times under specific experimental conditions covered in the training dataset. The subsequent steps, detailed in a second publication [23], include predicting retention times under intermediate conditions and a multi-criteria decision process to select optimal conditions for further investigation. In this context, the separation criterion is tailored to address specific analytical challenges. Notably, the high concentration of the API in tablet formulations may lead to ME if co-elution occurs with N-nitrosamines. To mitigate potential ME, the separation criterion was adjusted to ensure adequate resolution between the studied N-nitrosamines and the API. Additionally, another criterion was established to separate isobaric compounds NDPA and NDIPA. Isobaric compounds share identical quantifier and qualifier mass transitions, making them indistinguishable on the MS/MS detector. The adapted criterion aligns with the defined approach that generated a desirability index for each condition, which can then be graphically represented to illustrate the operational space most likely to fulfill the pre-set separation criterion.

Moreover, weights can be assigned to factors like separation, robustness, and analysis time in the models. Separation refers to the distance (or retention time) between peaks of critical pairs at the baseline. Robustness indicates the sensitivity to small variations in experimental conditions. Analysis time represents the minimum duration needed to analyze all analytes in a sample. Details and calculations related to these factors are provided in the associated publication [23]. To define the separation, the peaks are assumed to be symmetrical with two half-widths of 0.1 minutes (*i.e.* a total peak width of 0.2 minutes), except for the API, which is considered to have two half-widths of 0.5 minutes, due to its predominant presence compared to impurities. The desirability index was computed using multiple criteria to automate the selection of the best experimental condition [23]. The separation, robustness, and analysis time were each assigned weighted values of 1, 0.5, and 0.1, respectively.

2.5. Analytical method development

2.5.1. MS/MS parameter optimization

MS/MS detection was performed in APCI positive (+) mode. Due to the small structure of N-nitrosamines, which lack soft ionization sites, APCI was deemed more suitable than electrospray ionization (ESI). Our previous observations indicated that the MS signal obtained in APCI+ mode surpassed that of ESI+ by at least 10-fold.

Multiple reaction monitoring (MRM) is a highly specific and sensitive acquisition mode in mass spectrometry, widely used for MS/MS quantitative analysis. This mode effectively isolates the analyte signal from the chemical background noise.

To independently define the MRM transitions for each compound, individual standard solutions of N-nitrosamines and internal standards were prepared at a concentration of 50 ng/mL and directly infused into the MS/MS detector in combination with the UHPLC instrument, for the identification of the m/z ratios for both the precursor and product ions. This configuration effectively mimics the analyte's ionization and fragmentation behavior, and maximizes method sensitivity under real experimental conditions.

2.5.2. LC separation verification

The method development phase used an Acquity® Premier HSS T3 column (1.8 µm, 2.1 mm × 100 mm). To verify the chromatographic separation of N-nitrosamines under the predicted optimal conditions indicated on the desirability index graph (Fig. 2), a matrix solution spiked with all the studied N-nitrosamines was prepared at a concentration of 5 ng/mL. This solution was injected under the most favorable separation conditions, including an aqueous mobile phase at pH 2.7 and a gradient of 9.9 minutes based on geometric transfer.

Additionally, according to the second desirability index graph (Fig. 3), which focused on 5 of the 17 N-nitrosamines (NMOR, NMBA, NDPhA, NNK, and NMPA), LC separation under pH 5.0 conditions (10 mM AmAc, pH adjusted to 5.0 with FA) was also tested using a matrix solution containing these 5 N-nitrosamines at the same concentration of 5 ng/mL.

2.6. Validation protocol

The validation study focused on 5 N-nitrosamine impurities (NMOR, NMBA, NDPhA, NNK, and NMPA), studied under the first set of experimental conditions, with an aqueous mobile phase at pH 2.7, as part of the proof-of-concept.

All the stock solutions were prepared in MeOH while being protected from light to prevent photo-degradation of the N-nitrosamines. A total of six validation series were conducted, with each series consisting of three independent replicates. Each validation series considered various sources of variation. The validation study was carried out with the participation of two operators. Additionally, variability associated with the analytical column and day-to-day effects was assessed.

Both Acquity® Premier HSS T3 column (1.8 µm, 2.1 mm × 100 mm) and Acquity® UPLC® HSS T3 column (1.8 µm, 2.1 mm × 100 mm) were used in the validation study. Although the two columns share identical dimensions, particle size, and stationary phase (C18), they differ in the coating technology applied to the column hardware. The Premier® column features a specialized coating designed to minimize analyte-surface interactions, making it particularly suitable for extending applications to biological drugs.

2.6.1. 17α-hydroxyprogesterone solution

A standard solution of AHP at a concentration of 50 ng/mL was freshly prepared each day and injected three times prior to the validation analysis sequence to verify the equipment readiness.

2.6.2. Internal standards

Apart from the commercially available ready-to-use stock solutions, individual stock solutions of internal standards NMOR-d8, NMBA-d3, NDPhA-d6, and NMPA-d5 at a concentration of 1000 µg/mL were prepared once for the entire validation period. These solutions were aliquoted, and stored at −20 °C. For each validation series, an aliquot was thawed and used after reaching room temperature.

Two mixtures of working internal standard solutions were freshly prepared for each validation series: one at 100 ng/mL to be added to the calibration standards and QC solution, and another at 1250 ng/mL (only NDPhA-d6 at 625 µg/mL) for use in the validation standards. Both mixtures were prepared with the aim of achieving a final injected concentration of 5 ng/mL of internal standards in the solutions.

2.6.3. Calibration standards

Except for the commercially available ready-to-use stock solutions, individual stock solutions of NMOR and NDPhA were freshly prepared

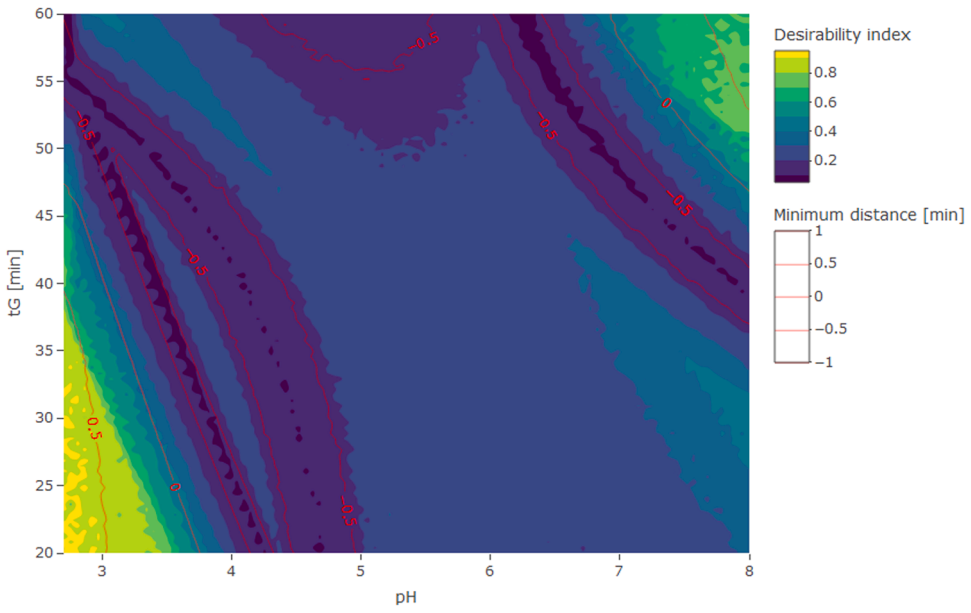

Fig. 2. Desirability index graph computed using following weighted criteria for separation (1.0), robustness (0.5), and analysis time (0.1) for 17 N-nitrosamines and API separation. The red lines represent the minimum distance between two consecutive peaks.

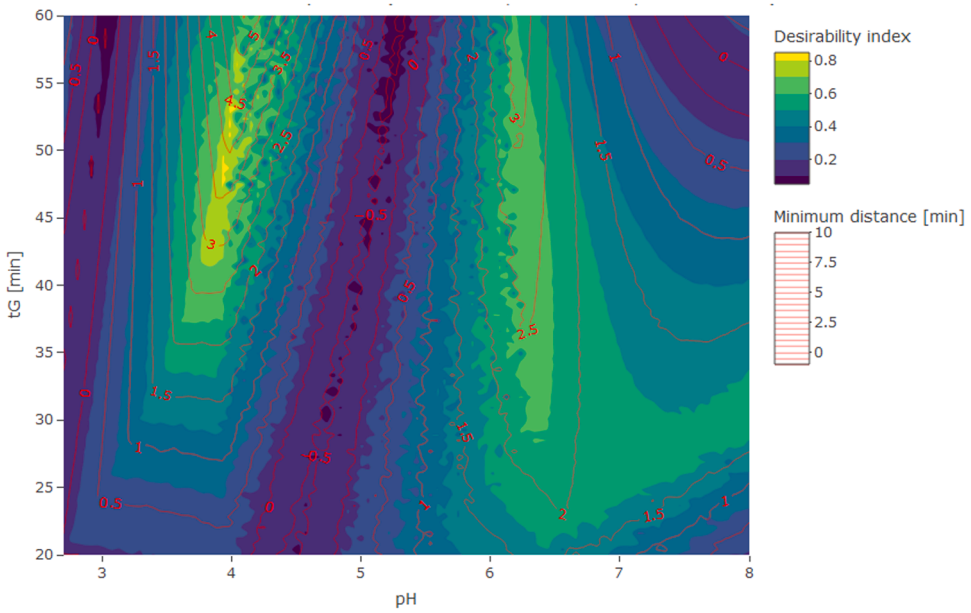

Fig. 3. Desirability index graph omputed using following weighted criteria for separation (1.0), robustness (0.5), and analysis time (0.1) for 5 N-nitrosamines and API separation. The red lines represent the minimum distance between two consecutive peaks.

daily at a concentration of 1000 μg/mL. 20.0 mg of NMOR and NDPhA were accurately weighed and dissolved in 20.0 mL of MeOH using volumetric flasks. A mixture containing all N-nitrosamine standards was then prepared from the stock solutions and diluted in a diluent (composed of water/MeOH, 80 %/20 %, v/v, with 0.1 % FA) to an intermediate concentration at 1000 ng/mL. Subsequently, the intermediate N-nitrosamine mixture underwent successive dilutions to obtain five concentration levels for calibration standards as follows: 0.25 – 1 – 5 – 10 – 30 ng/mL. Internal standards were spiked at a concentration of 5 ng/mL in the calibration standards.

2.6.4. Validation standards

The tablet formulation was crushed into a fine powdered material to serve as the matrix, which was then spiked with precisely measured amounts of N-nitrosamines to prepare validation standards.

Except for the commercially available ready-to-use stock solutions, individual stock solutions of NMOR and NDPhA were freshly prepared daily at concentrations of 1000 and 4165 μg/mL, respectively. 20.0 mg of NMOR and 83.8 mg NDPhA were accurately weighed and dissolved in 20.0 mL of MeOH using volumetric flasks. A mixture containing all N-nitrosamine standards was then prepared from the stock solutions and diluted in MeOH to an intermediate concentration. This solution was successively diluted to obtain two solutions at different concentrations. Finally, these two diluted solutions were used to spike the validation standards at 5-level final concentrations: 0.25 – 1 – 5 – 10 – 30 ng/mL, in 5.0 mL of MeOH. Internal standards were spiked at the concentration of 5 ng/mL in the validation standards. The preparation for validation standards is confidential and not disclosed in detail. The validation

standards underwent liquid extraction to isolate N-nitrosamines, followed by centrifugation. A portion of the supernatant was injected to analyze NMOR, NMBA, NNK, and NMPA. To analyze NDPhA, 100 μL of the supernatant was further diluted 500-fold.

2.6.5. Quality control solution

Like the calibration standards, an independent quality control (QC) solution containing all N-nitrosamines and internal standards was prepared at a concentration of 5 ng/mL.

2.7. Analytical method for validation study

The experimental conditions identified through the *in silico* screening strategy, which demonstrated acceptable separation during method development, were further applied to establish proof-of-concept and subsequently used for validation studies.

2.7.1. Chromatographic conditions

The mobile phase was composed of solvents A (0.1 % FA in water at pH 2.7) and B (0.1 % FA in MeOH). A linear binary solvent gradient was performed at a flow rate of 0.400 mL/min as follows: 0.0 – 1.0 min: 0 % B, 1.0 – 10.9 min: 0–95 % B, 10.9 – 13.5 min: 95 % B, 13.5 – 13.6 min: 95–0 % B, 13.6 – 17.0 min: 0 % B. The injection volume was set at 10 μL. The autosampler and column temperature were at 10 °C and 45 °C, respectively.

2.7.2. MS/MS parameters

The optimized parameters were then applied to configure the ion source as follows: corona current, 1.5 μA; source temperature, 120 °C; APCI probe temperature, 250 °C. Desolvation, nebulization, cone, and collision gas flows were set at 950, 250, 250 L/hr, and 0.15 mL/min, respectively. Data acquisition was carried out in MRM mode to monitor specific precursor and product ions. The auto dwell option was activated for all N–nitrosamines, while the soft transmission was only activated for fragile analytes. Cone voltage and MRM parameters are given in Table 2.

2.8. Stability assessment

A stability study was conducted independently of the validation study. The investigation involved reinjecting five calibration standard

Table 2
Cone voltage and MRM parameters of N-nitrosamines and internal standards.

N-nitrosamine	MRM transition (*m/z*)	Retention window (min)	Cone voltage (V)	Collision energy (eV)	Soft transmission
NMOR	**117.10 → 86.93**	2.20 – 3.80	27	13	No
	117.10 → 45.09			13	
NMPA	**137.05 → 66.10**	6.20 – 7.50	28	12	No
	137.05 → 107.15			11	
NMBA	**147.10 → 117.10**	2.80 – 3.90	17	7	Activated
	147.10 → 44.10			10	
NDPhA	**199.10 → 66.10**	8.30 – 10.20	23	22	No
	199.10 → 169.10			12	
NNK	**208.10 → 122.10**	4.20 – 5.30	32	14	No
	208.10 → 148.00			10	
NDMA	**75.05 → 58.05**	1.65 – 2.90	32	9	No
	75.05 → 43.00			13	
NDBA	**159.20 → 57.10**	8.70 – 9.70	30	12	No
	159.20 → 103.23			10	
NMEA	**89.11 → 61.00**	3.00 – 4.20	24	9	No
	89.11 → 43.00			9	
NEIPA	**117.20 → 74.99**	5.40 – 6.50	25	8	Activated
	117.20 → 47.10			14	
NDIPA	**131.20 → 89.10**	6.30 – 7.50	17	10	No
	131.20 → 43.10			9	
NDPA	**131.16 → 89.16**	6.90 – 7.90	30	11	Activated
	131.16 → 43.14			11	
NPYR	**101.12 → 55.00**	3.10 – 4.30	22	15	No
	101.12 → 41.00			18	
MeNP	**130.00 → 58.00**	2.00 – 3.00	27	13	No
	130.00 → 100.00			5	
NDEA	**103.20 → 74.90**	4.30 – 5.30	33	9	No
	103.20 → 46.90			12	
NPIP	**115.15 → 69.00**	4.80 – 5.70	13	14	No
	115.15 → 41.00			17	
NTHP	**113.10 → 67.10**	4.40 – 5.40	17	10	No
	113.10 → 83.10			13	
NDELA	**135.10 → 74.00**	0.70 – 2.20	8	10	Activated
	135.10 → 104.00			6	
NMOR-d8	**125.17 → 95.00**	2.40 – 3.80	20	15	No
NMPA-d5	**142.05 → 112.01**	6.20 – 7.50	20	12	No
NMBA-d3	**150.10 → 120.00**	2.80 – 3.90	20	5	Activated
NDPhA-d6	**205.10 → 175.10**	8.30 – 9.80	20	11	No
NNK-^{13}C6	**214.14 → 128.00**	4.20 – 5.30	32	12	No
NDMA-d6	**81.12 → 46.00**	1.60 – 2.80	32	13	No
NDBA-d18	**177.35 → 66.00**	8.60 – 9.60	25	13	No
NDPA-d14	**145.28 → 50.00**	6.80 – 7.80	9	12	Activated
NPYR-d8	**109.10 → 62.10**	3.10 – 4.30	22	15	No
NMEA-d3	**92.06 → 64.00**	3.00 – 4.20	25	10	No
MeNP-d4	**134.18 → 104.00**	2.00 – 3.00	17	7	No
NDEA-d10	**113.20 → 34.00**	4.30 – 5.30	15	11	No
NPIP-d10	**125.21 → 78.00**	4.60 – 5.60	22	16	No
NDELA-d8	**143.05 → 80.01**	0.70 – 2.20	8	10	Activated

Bold: Quantifier ion; Thin: Qualifier ion.

levels and the three highest concentration levels of validation standards (5, 10, and 30 ng/mL) from the two selected series after 24, and 72 hours. All solutions were stored at 10 °C in the autosampler during the stability assessment period.

2.9. Software

The LC-MS/MS instrument, along with data acquisition and processing was managed using MassLynx® Security Software (Waters). Analytical method validation and total error profile computations were operated using E-Noval® (Cencora-Pharmalex, Mont-Saint-Guibert, Belgium).

The solution stability evaluation was conducted using a comparison test, which was performed with JMP® (JMP Statistical Discovery LLC) statistical software.

3. Results and discussions

Given the continuous emergence and discovery of new N-nitrosamines, all medicines, whether in the discovery phase or already commercialized, must undergo N-nitrosamine investigation. In this work, an *in silico* QSRR strategy was deliberately chosen to develop a generic methodology that supports and facilitates analytical method development for N-nitrosamines analysis in pharmaceutical products. This approach aimed to explore flexible experimental conditions within a broad modeled operational space, including parameters such as pH and gradient time, to optimize the LC separation of N-nitrosamines based on formulation compositions. At this stage, the strategy deals with 17 small-molecule N-nitrosamines and was applied to a commercialized drug product where a focus on the determination of 5 of these N-nitrosamine impurities was considered as a proof of concept study.

3.1. Desirability index graph

3.1.1. Desirability index graph including 17 N-nitrosamines

The desirability index graph, as illustrated in Fig. 2, visually represents the predicted chromatographic separation conditions. A higher desirability index correlates with an improved separation of the 17 individual N-nitrosamines from one another. The pre-set separation criterion includes two key constraints: the separation between the isobaric compounds NDPA and NDIPA, and the separation of N-nitrosamines from the API, which is predominant in the matrix.

Differences in minimum separation distance are observed. This distance is represented by red lines in Fig. 2 and expressed in minutes, indicating the distance between the end of the first peak and the start of the second peak in the critical pair. Therefore, a minimum distance equal to or greater than 0 minutes is necessary for separation to be considered acceptable and exclude any co-elution with the API. A separation exceeding 1 minute between two peaks is deemed unnecessary and increases analysis times subsequently. Conversely, a negative minimum distance indicates incomplete separation, posing a risk of co-elution. Regions identified with a negative distance were not investigated.

Regarding the desirability index graph illustrating the chromatographic separation conditions for the 17 N-nitrosamines (Fig. 2), the most favorable predicted conditions for optimal separation were found using an aqueous mobile phase at pH 2.7 and a gradient time of 20.0 minutes under HPLC conditions (or 9.9 minutes under UHPLC conditions).

3.1.2. Desirability index graph for 5 N-nitrosamines

The desirability index graph in Fig. 3 was generated based on 5 targeted N-nitrosamines: NMOR, NMBA, NDPhA, NNK, and NMPA, after sorting the N-nitrosamines according to probe temperature (150 or 250 °C) to maximize MS/MS sensitivity. The figure indicates that the most optimal conditions for API separation involve using a mobile phase with an aqueous fraction with a pH around 4, and a linear gradient time exceeding 40 minutes.

The graph additionally shows that the N-nitrosamines may be separated from the API over a considerable portion of the modeled experimental domain. This spans a pH range from 2.7 to 8.0, and a gradient time from 20 to 60 minutes under HPLC conditions. Gradient time needs to be carefully optimized to strike a balance between effective separation and reasonable analysis time. Based on our previous experience, following the geometric transfer, a gradient time of 9.9 minutes proved to be sufficient for this purpose.

Since N-nitrosamines become positively charged in positive ionization mode, using an acidic mobile phase with volatile additives is preferred. Consequently, the pH range from 6.0 to 8.0 in Fig. 2 was considered irrelevant and excluded from further investigation.

In the modeled experimental domain, we prioritized exploring the region characterized by a minimum distance of 0.5 minutes or more. Two distinct regions marked by red lines were identified in Fig. 3. The first region corresponds to a pH around 2.7, while the second region is approximately at pH 5.0.

All the data presented in this section were generated entirely *in silico*. This predictive approach enables the identification of multiple suitable generic methods without performing any experiments. Based on a preference for shorter analysis times, experiments were further performed at these two pH levels, pH 2.7 and pH 5.0, using a gradient time of 9.9 minutes based on geometric transfer considerations.

3.2. Analytical method development

3.2.1. MS/MS parameters

Table 2 summarizes the MRM transitions and acquisition parameters for all N-nitrosamines and their associated internal standards, including the specific cone voltage, collision energy, and retention window for each compound.

3.2.2. Chromatographic selectivity – pH 2.7

Fig. 4 illustrates the LC separation of 17 N-nitrosamines and API under conditions of pH 2.7 and a gradient time of 9.9 minutes, considering their retention times at the peak apex. The total ion chromatograms (TICs) are shown in Figure S1. Given the similar retention behavior between the N-nitrosamines and their internal standards, the latter are separately shown in Figure S2. As illustrated, most N-nitrosamines were successfully separated from the API. While some N-nitrosamine groups such as NMEA/NMBA/NPYR, NDIPA/NMPA, and NDBA/NDPhA co-eluted, this did not affect their separation from a mass spectrometry perspective. The applied *in silico* strategy of QSRR did not consider the additional separation dimension provided by mass spectrometry, which offers a complementary separation based on the m/z ratio when coupled with LC. Despite some co-elution among N-nitrosamines, the separation was deemed acceptable overall.

Importantly, isobaric molecules NDPA and NDIPA were fully resolved under the selected chromatographic conditions. However, NDEA, NTHP, NNK, and NPIP co-eluted with the API, creating an ion suppression zone with no detectable signal. This significantly impacted the method's sensitivity and global performance, highlighting the need for alternative conditions to improve separation from the API. Additionally, MeNP was not retained by the column, indicating that modified conditions are necessary to enhance its retention.

The LC separation evaluation was combined with varying APCI probe temperature conditions. The 17 N-nitrosamines were divided into two temperature groups, 150 and 250 °C, to achieve optimal sensitivity. It is crucial to consider the impact of temperature, as elevated source or probe temperature can induce significant in-source fragmentation by accelerating analyte dissociation [26].

Focused on the 5 N-nitrosamines under investigation to demonstrate the proof-of-concept of the QSRR approach, their LC separation is represented in Fig. 5 under pH 2.7 and 9.9 minutes of gradient time. The

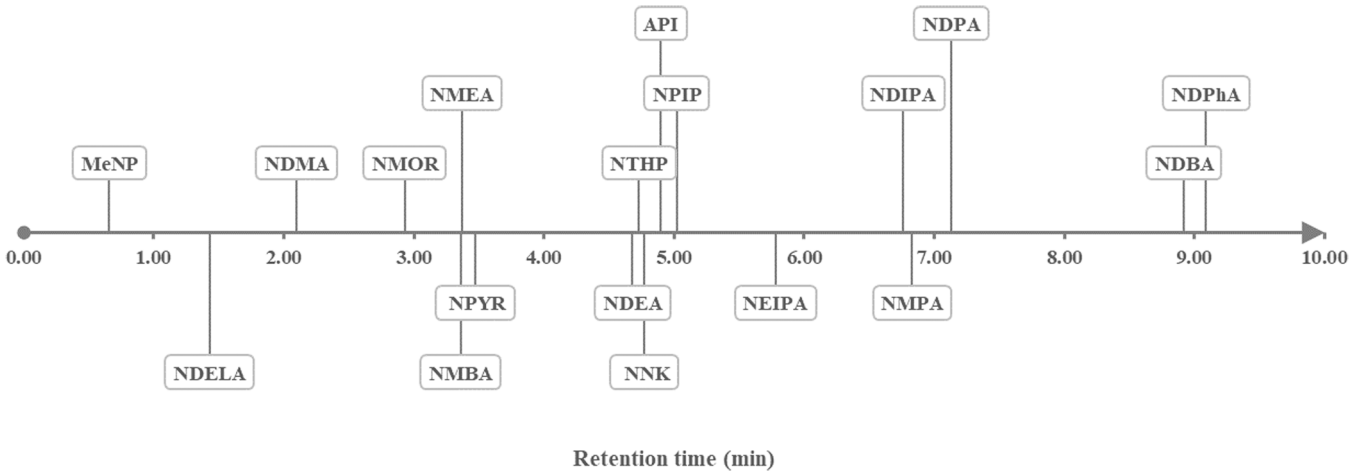

Fig. 4. Illustration of LC separation of 17 N-nitrosamines and API under conditions of pH 2.7 and a gradient time of 9.9 minutes.

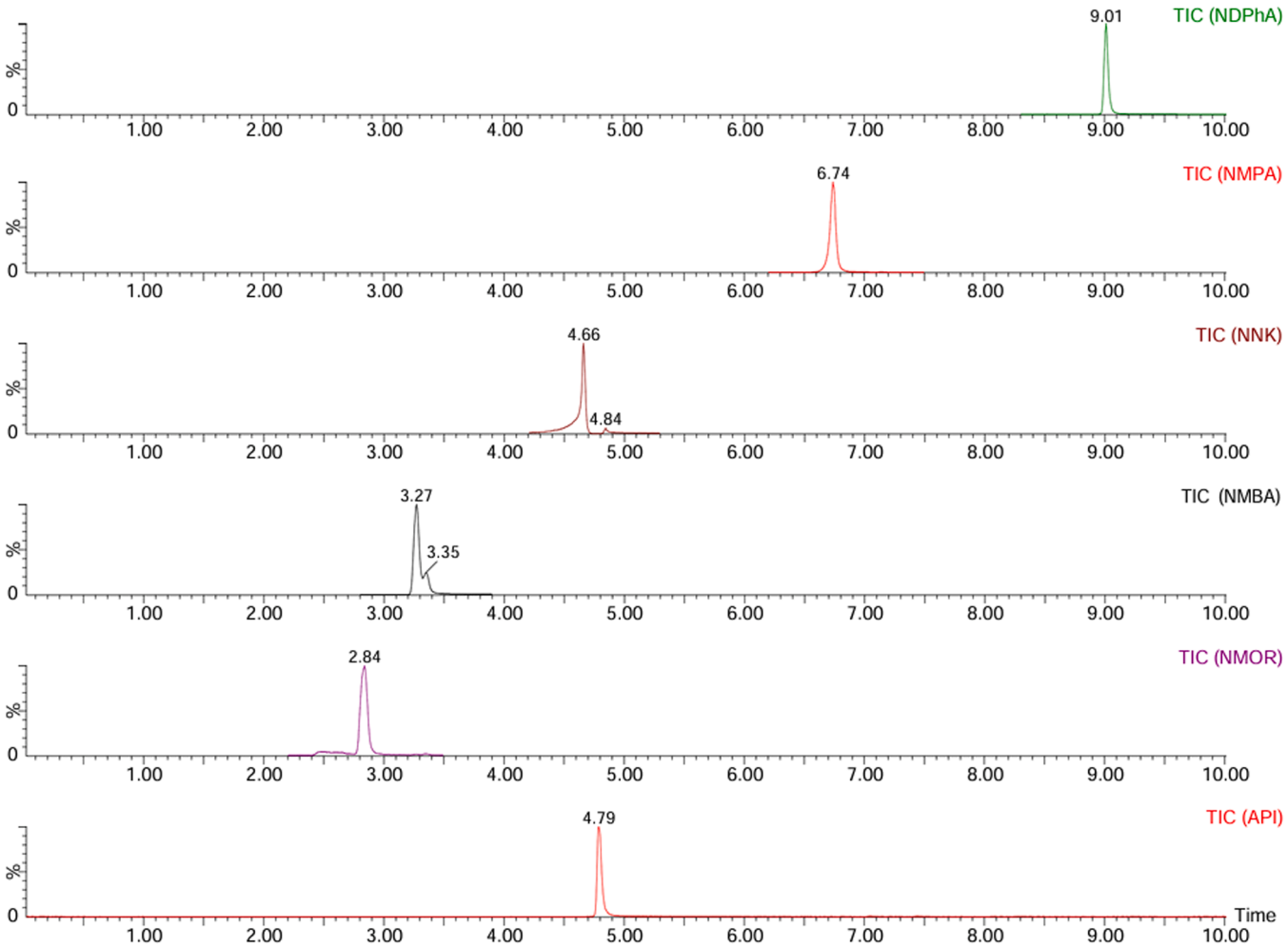

Fig. 5. TICs of NMOR, NMBA, NDPhA, NNK, NMPA, and API at a concentration of 5 ng/mL in the matrix under the optimized experimental conditions (pH 2.7, gradient time: 9.9 minutes, APCI probe temperature: 250°C).

TIC of each N-nitrosamine under the optimal pH of 2.7 and gradient time of 9.9 minutes are illustrated in Fig. 5, along with their respective retention time. NMOR, NMBA, NDPhA, and NMPA exhibit sufficient separation from the API, although complete separation of analytes is not mandatory when a mass spectrometer is used. The MS/MS detector ensures high specificity and provides a complementary dimension of separation based on the *m/z* ratio. Furthermore, a tandem mass spectrometer offers greater specificity than a simple quadrupole detector due to its dual selection of the *m/z* ratio for both ion precursor and ion product. However, in Fig. 5, the peak corresponding to NNK exhibits a region with no signal. This phenomenon was caused by its co-elution with the API, which is predominantly present in the tablets or matrix.

This inevitably resulted in ME, inhibiting the subsequent ionization of NNK.

3.2.3. pH impact

pH variations have a minimal impact on separation but can significantly influence the MS ionization behavior of N-nitrosamines. Observations demonstrated a higher method's sensitivity at pH 2.7 compared to pH 5.0 for most molecules, except NDELA, which showed a more intense signal at this pH than at pH 2.7. This enhancement can be explained by the improved ionization of N-nitrosamines under acidic conditions, leading to increased signal, sensitivity, and potentially a lower detection limit (DL).

3.2.4. Generic methods

Compared to pH 2.7, operating at pH 5.0 showed no impact on the retention mechanisms of most N-nitrosamines, with retention times remaining unchanged. However, MeNP showed increased retention at pH 5.0, with its retention time rising from 0.6 to 2.5 minutes. Using both pH conditions, 16 out of 17 N-nitrosamines were adequately separated from the API, except for NNK. Despite modifications in mobile phase pH, NNK still showed slight co-elution with the API, although the API's retention time was delayed from 4.8 minutes to 5.7 minutes. Extending the gradient times did not improve this separation. NNK showed greater signal intensity and peak symmetry at pH 2.7 than at pH 5.0. In contrast, the delay in API retention time at pH 5.0 improved separation for NDEA, NTHP, and NPIP, resolving their co-elution with the API at pH 2.7. Therefore, pH 5.0 is more suitable for analyzing these molecules.

Consequently, three generic methods were developed to determine the 17 N-nitrosamines in a drug product formulation of oral solid dosage forms (tablets) to address ME and sensitivity challenges. The methods slightly vary in terms of the aqueous mobile phase pH and APCI probe temperature:

- pH 2.7/250 °C: NMOR, NMBA, NDPhA, NNK, and NMPA,
- pH 2.7/150 °C: NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA,
- pH 5.0/150 °C: MeNP, NDEA, NTHP, NPIP, and NDELA.

Instrumentally, all three methods can be programmed into the same analysis sequence without altering the equipment, its configuration, or the sample pretreatment of tablets. However, adequate equilibration time is required before switching methods due to mobile phase pH modification. Compared to the methods proposed by the European Pharmacopoeia [14] and United States Pharmacopeia [13], our strategy offers the advantage of using a single technique to determine up to 17 N-nitrosamines in a single-run analysis. Although S. Schmidtsdorff et al. proposed a supercritical fluid chromatography method to target 16 N-nitrosamines in various drug products, the LC remains the most commonly used technique in the pharmaceutical industry [27].

In accordance with the EMA investigation guidance [7], a documentary risk assessment was performed at Step 1. One of studied N-nitrosamine impurities was identified as "at-risk" of being present in the finished product due the nature of the API. Considering this N-nitrosamine belongs to the first set of experimental conditions, this set was selected and validated to demonstrate the relevance and applicability of the QSRR strategy used in this proof-of-concept study. Although the limit test approach can be used at Step 2 of analytical confirmatory testing, a quantitative method for impurity is preferred to anticipate its potential application in future post-marketing surveillance (Step 3) of the "at-risk" N-nitrosamine in the product.

3.3. Analytical method validation

Among the six validation series conducted, data from one series were excluded due to preparation errors affecting NMOR and NNK. Consequently, five series of data were available for these two N-nitrosamines, which still provided sufficient statistical robustness. For NMBA, NDPhA, and NMPA, data from all six validation series were retained.

For an impurity limit test, the selectivity and DL were validated regarding the ICH Q2(R2) recommendations. At the onset of the validation study, the method's selectivity and specificity for each N-nitrosamine were independently demonstrated, by injecting i) blank solution containing the diluent, ii) N-nitrosamines calibration standard at a concentration of 5 ng/mL (concentration level 3), iii) unspiked matrix solutions. No interfering peaks were observed at both quantifier and qualifier transitions of N-nitrosamines and internal standards (only quantifier transition monitored) at the respective retention time.

In Fig. 6, it is noteworthy that the peaks related to NMBA and NMBA-d3 on the extracted ion chromatograms (EICs) consistently split into two peaks under the experimental conditions in both calibration and validation standards. This uncommon phenomenon, referred to as peak splitting, is potentially attributable to the presence of *E/Z* isomerism and has been previously reported for N-nitrosamine compounds in the literature [28,29]. The split peaks were taken into consideration in their entirety for the quantitative assessment of NMBA.

Quantitative performances of the optimized analytical method were independently assessed for NMOR, NMBA, NDPhA, NNK, and NMPA. A deuterated or ^{13}C-labeled standard was paired with the respective unlabeled N-nitrosamine standard to compensate for variations in sample preparation and the ionization process. The quantitative assessment utilized a combined approach based on the total error profile and β-expectation tolerance intervals. These intervals, computed by considering both bias and the estimated variability of the results, establish an interval where a pre-defined proportion β of future results is expected to fall (*e.g.* 95 %). For each concentration level, these tolerance intervals are calculated to provide control and evaluate the risk associated with acceptance of unsuitable results (*e.g.* 5 %). In practice, if the β-expectation falls within the acceptance limits, it guarantees that future results will meet the acceptance criteria with a pre-defined probability. Thus, the analytical method is suitable for its intended purpose and is considered valid. This total error approach aligns with the ICH Q2(R2) guidelines, ensuring that the quantitative validation criteria, including range, accuracy, and precision are fulfilled. Considering the preparation of samples or validation standards, the proposed validation dosing range of 1–30 ng/mL covers at least 10–120 % of the acceptable limit (or specification limit) for each N-nitrosamine.

3.3.1. Combined approach for accuracy and precision

The mathematical model employing a linear regression through the origin, fitted using only concentration level 3, was a priori selected and is presented in Table 3. This model offers the advantage of requiring only a single-point calibration curve, making it highly convenient for routine applications. Additional mathematical models were also evaluated, and the results are summarized in Table S1.

An alternative to the traditional separate evaluation of accuracy and precision is to assess their total impact using a combined approach. This combined methodology can be performed using a prediction interval, a tolerance interval, or a confidence interval [30].

The total error profiles are illustrated in Fig. 7. The relative β-expectations tolerance limits are presented in Table 3. Given the trace levels of impurities and the absence of specific regulatory specifications for impurity quantification, total error profiles were generated with acceptance limits set at ± 30 %, considering the scope of trace analysis for impurities. The acceptance criterion of ± 30 % was also used in Parfant *et al.*'s work [31]. A risk level was set at 5 %, indicating that at most 5 % of the results may be expected to fall outside the acceptance limits.

Illustrated in Figs. 7b and 7e, the lower and upper bounds of the relative β-expectations tolerance intervals for NMBA, and NMPA consistently fell within the acceptance limits set at ± 30 % at all concentration levels.

In contrast, as shown in Fig. 7a, the lower and upper bounds of the

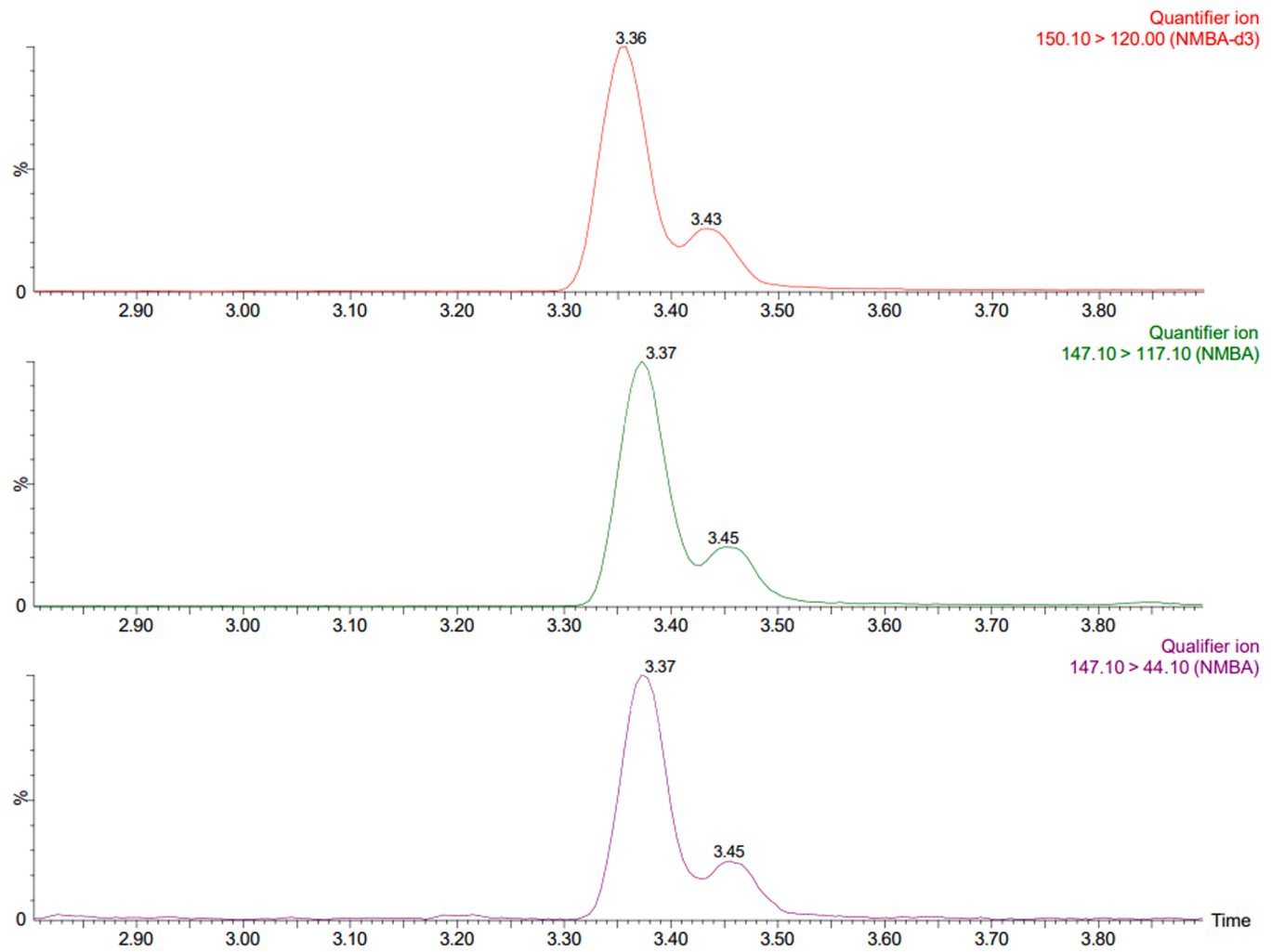

Fig. 6. EICs of NMBA and NMBA-d3 at a concentration of 5 ng/mL in matrix under the optimized experimental conditions. (red) NMBA-d3 quantifier ion; (green) NMBA quantifier ion; (purple) NMBA qualifier ion.

Table 3
Summary of quantitative validation criteria for studied N-nitrosamine impurities.

	NMOR *(p = 5; n = 3)*	**NMBA** *(p = 6; n = 3)*	**NDPhA** *(p = 6; n = 3)*	**NNK** *(p = 5; n = 3)*	**NMPA** *(p = 6; n = 3)*
Response function					
Calibration model	Linear regression through 0 (fitted at level 3)	Linear regression through 0 (fitted at level 3)	Linear regression through 0 (fitted at level 3)	Linear regression through 0 (fitted at level 3)	Linear regression through 0 (fitted at level 3)
Combined approach for accuracy and precision					
Level	Relative β-expectation tolerance limits (%)	Relative β-expectation tolerance limits (%)	Relative β-expectation tolerance limits (%)	Relative β-expectation tolerance limits (%)	Relative β-expectation tolerance limits (%)
1	[−178.0, 128.1]	[−22.6, 13.3]	[−9.8, 33.5]	[−22.5, 32.5]	[−14.2, 11.9]
2	[−22.1, 28.5]	[−17.0, 17.3]	[−4.4, 19.1]	[−6.2, 22.6]	[−11.6, 16.8]
3	[−20.3, 19.6]	[−21.8, 12.1]	[−15.1, 16.0]	[−8.7, 10.3]	[−19.9, 17.5]
4	[−12.4, 14.7]	[−21.7, 14.8]	[−14.7, 16.0]	[−12.4, 12.5]	[−16.3, 17.5]
5	[−17.9, 18.2]	[−26.1, 18.4]	[−18.8, 19.0]	[−16.2, 16.5]	[−18.6, 19.5]
Linearity					
Slope	1.002	0.961	1.000	1.000	1.005
Intercept	0.006	0.007	0.048	0.037	−0.011
R^2	0.994	0.989	0.992	0.996	0.993
Lower limit					
DL	0.02 ng/mL	0.02 ng/mL	0.75 pg/mL	3.91 pg/mL	1.81 pg/mL
Range (ng/mL)	1.01 – 30.67	0.25 – 30.00	0.43 – 29.89	0.44 – 30.00	0.24 – 28.42

p: number of series of validation; *n*: number of replicates per series and per level.

β-expectations tolerance intervals for NMOR did not fall within the acceptance limits of ± 30 % solely at the lowest concentration level. Regarding NDPhA (Fig. 7c), and NNK (Fig. 7d), only the upper bound of the β-expectations interval slightly exceeded the acceptance limits at the lowest concentration level, measuring at 33.5 and 32.5 %, respectively. The method is, therefore, considered accurate.

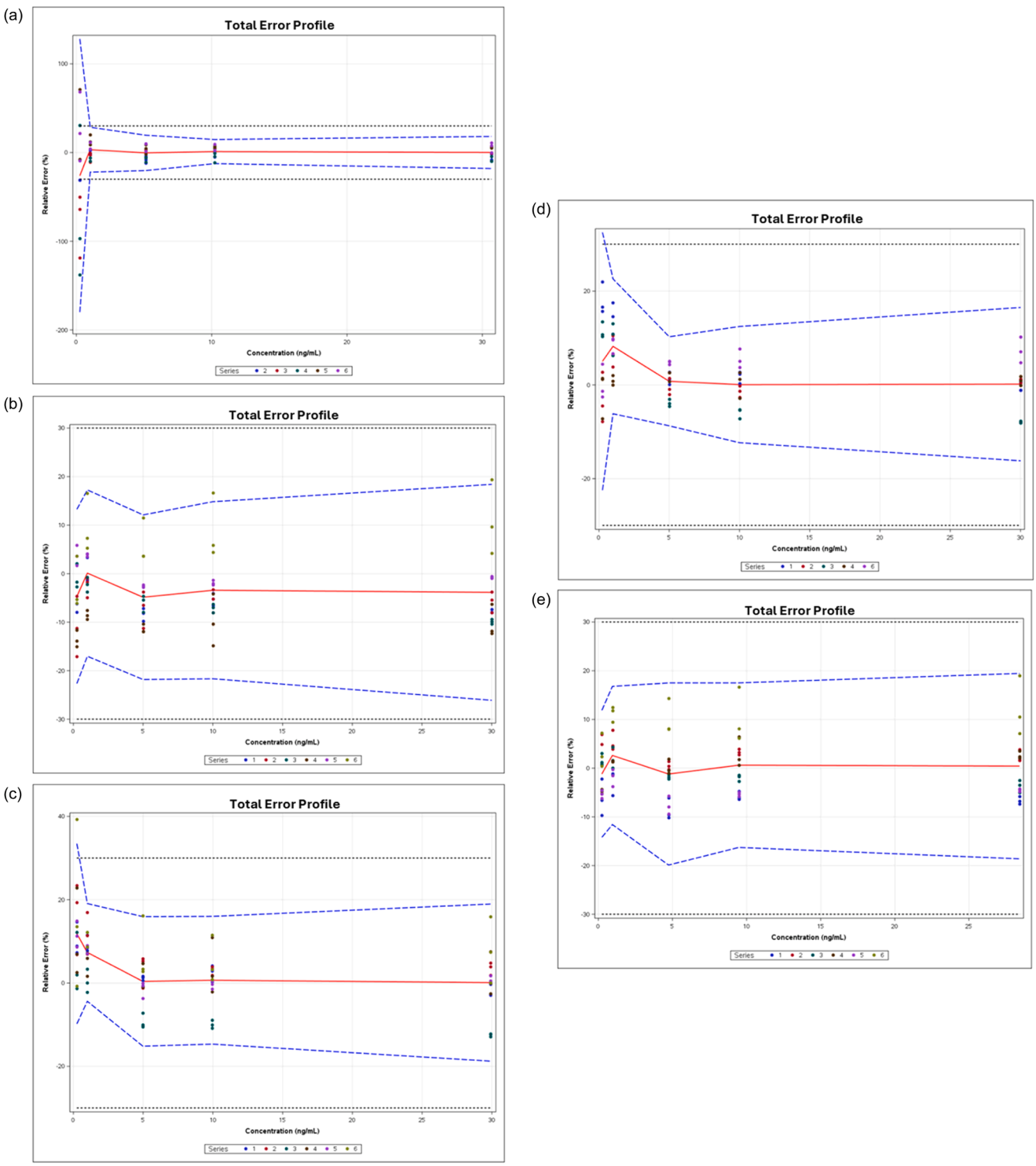

Fig. 7. Total error profiles computed by considering linear regression through 0 (fitted only at concentration level 3) to assess the quantitative performance of the LC-MS/MS method for NMOR (a), NMBA (b), NDPhA (c), NNK (d), and NMPA (e) in the tablet formulation. On the figures, the plain red line is the relative bias (%), the dashed blue lines are the lower and upper bounds of the β-expectations tolerance limits considering 5 % of risk, the dotted black lines represent the acceptance limits of ± 30 %, the dots represent the relative error of the individual results and are plotted with respect to the targeted concentration.

3.3.2. *Linearity*

The linearity of results of an analytical method is its ability within a definite working range, to provide results directly proportional to the concentration of the analyte in the sample [32].

The results indicated that each N-nitrosamine embraced good linearity, with a coefficient of determination (R^2) equal to or greater than 0.989 across the five concentration levels. This demonstrated the consistency of the selected calibration model. Furthermore, the values of slope and intercept, closely approximating 1 and 0, respectively, indicated the robust linearity of results as well. The results are summarized in Table 3.

3.3.3. *Working range*

The range is the interval between the lowest and the highest

quantities of an analyte in which the analytical procedure demonstrates suitable precision, accuracy, and response [30]. It is defined by the limits where the β-expectations tolerance intervals fall outside of the acceptance limits, which can be visually identified on the total error profiles.

For NMBA and NMPA, since all relative β-expectations tolerance limits fell within the acceptance limits of ± 30 % at all concentration levels, their respective ranges are 0.25 – 30.00 ng/mL and 0.24 – 28.42 ng/mL. Conversely, for NMOR, NDPhA, and NNK, the lower and/or upper limits of the β-expectations tolerance limit did not fall within the acceptance limits at the lowest concentration level, this concentration cannot be validated as the lower quantification limit. Therefore, the ranges for NMOR, NDPhA, and NNK were determined to be 1.01 – 30.67 ng/mL, 0.43 – 29.89 ng/mL, and 0.44 – 30.00 ng/mL, respectively.

3.3.4. Detection limit

The DL is the smallest quantity of an analyte that can be detected in the sample but cannot be accurately quantified. DL was estimated by extrapolating the average S/N ratio obtained over all validation series to the reporting threshold (≥ 3), adhering to the ICH Q2(R2) guidelines [30]. The obtained DL values, ranging from 0.75 pg/mL to 0.02 ng/mL, are summarized in Table 3. The DL for all 5 N-nitrosamines met the EMA N-nitrosamine investigation approach requirements and are below 10 % of their respective acceptable limit.

3.3.5. Stability

A stability study was conducted to assess the possibility of re-injecting solutions during the routine use of the method over a defined period. Stability was independently assessed from the validation study for each N-nitrosamine by comparing the results expressed in concentration obtained at T_0 versus $T_{24\,h}$, and T_0 versus $T_{72\,h}$, across two validation series. A two one-sided t-Test (TOST) at α level of 5 % was performed. The results were compared to the acceptance limits defined as two times the intermediate precision standard deviation observed in the validation study for the corresponding concentration level.

The TOST interval falls inside the acceptance limits (see Table S2). This means that the variability of the results between $T_0/T_{24\,h}$ or $T_0/T_{72\,h}$ is less than the variability (intermediate precision) of the method, which was defined using validation data obtained at T_0 from 6 (or 5) validation series, without considering the stability data generated after $T_{24\,h}$ and $T_{72\,h}$. Therefore, the solutions are considered stable after $T_{24\,h}$ and $T_{72\,h}$, and can be re-injected up to $T_{72\,h}$ after preparation when stored at 10 °C.

3.4. System suitability tests

Various system suitability tests (SSTs) were performed independently of the validation study. This is a control strategy designed to verify that an analytical method or a system was fit for purpose on the day the analysis was carried out.

3.4.1. Equipment verification

This SST ensures the equipment's readiness by verifying its sensitivity and repeatability, both of which are critical for the accurate analysis of trace impurities.

The mean signal intensity from the three injections (n = 3) of the 50 ng/mL AHP solution must be no less than 8,000,000 counts, with an RSD (%) not exceeding 5 %, as determined during method development. These criteria ensure that the system is performing within acceptable performance before proceeding with the analysis (Table).

3.4.2. Tailing factor

The tailing factor was independently calculated using the first injection of the calibration standard at level 3, utilizing the quantifier ion of each N-nitrosamine. This calculation was automated by MassLynx Security® in accordance with the European Pharmacopoeia. The obtained minimal and maximal values of the tailing factor are summarized in Table 4.

For NMOR, NDPhA, and NMPA, the specifications of 0.8 – 1.8 from the European Pharmacopoeia can be applied. However, due to the peak splitting phenomenon observed for NMBA, its tailing factor is greater than 1.8. Consequently, specifications should be adjusted based on the results, for example, setting them in the range between 1.5 and 2.5. The column effect highlighted for NNK also had an impact on the peak shape. Taking into account the observed peak fronting, specifications of 0.5 – 1.5 can be considered.

3.4.3. Ion ratio

The ion ratio serves to confirm the identity of the compound of interest. This SST was verified for each N-nitrosamine in the calibration and validation standards, as well as the QC solution. All Ion ratio values met the specifications in accordance with the maximum permitted tolerance for the LC-MS/MS technique [33]. The results are reported in Table 4.

3.4.4. QC recovery

The recovery between the calibration standard at concentration level 3 and the QC solution was calculated based on the selected calibration model. This SST is used to verify the calibration standard preparation and ensure the system stability during the analysis. The minimal and maximal recovery values are presented in Table 4, all of them ranging from 92 % to 106 %. A specification of 90 % – 110 % can be set for the routine use of the method.

3.4.5. System sensitivity verification

System sensitivity is crucial in trace analysis of impurities. For a limit test or quantitative testing, the S/N ratio must be equal to or greater than 3 or 10, respectively. To monitor this SST, the S/N ratio was calculated

Table 4
Summary of SST results for the validation study of N-nitrosamine impurities.

	Equipment verification		
	Mean intensity (counts, n = 3) ≥ 8000,000 counts	**RSD% (n = 3)** ≤ 5 %	**Conformity**
Series 1	14,505,414	1 %	Conforms
Series 2	17,478,304	1 %	Conforms
Series 3	16,559,172	0 %	Conforms
Series 4	17,283,209	4 %	Conforms
Series 5	18,918,641	2 %	Conforms
Series 6	17,518,591	1 %	Conforms

	NMOR *(p = 5)*	**NMBA** *(p = 6)*	**NDPhA** *(p = 6)*	**NNK** *(p = 5)*	**NMPA** *(p = 6)*
Tailing factor Specifications	0.8 – 1.8	1.5 – 2.5	0.8 – 1.8	0.5 – 1.5	0.8 – 1.8
Min. – Max.	1.0 – 1.2	1.7 – 1.9	1.1 – 1.3	0.7 – 1.2	0.9 – 1.0
Ion ratio Within tolerance					
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms
QC Recovery 90 % – 110 %					
Min. – Max.	92 – 104 %	95 – 104 %	92 – 105 %	98 – 103 %	99 – 106 %
Sensitivity (S/N ratio) ≥ 10					
Min. – Max.	27 – 61	19 – 53	297 – 5051	32 – 387	213 – 850

p: number of series of validation.

at the lowest concentration level of the calibration standard. The S/N ratios are summarized in Table 4, and all values are superior to 10.

4. Conclusion

The current study highlighted the feasibility of employing an innovative *in silico* strategy of QSRR modeling to support the development of chromatographic methods in screening of LC parameters for effective chromatographic separation of compounds. Utilizing QSRR methodology clearly facilitated the establishment of robust experimental conditions, such as pH and gradient time, to achieve optimal separation of N-nitrosamines by UHPLC before MS/MS detection. Remarkably, this was achieved solely using molecular descriptors from literature, without the need for experiments. This strategic approach helps address challenges associated with N-nitrosamine targeting and ME.

Following experimental verification of the model's proposed optimal conditions, a single set of conditions (pH 2.7/250°C APCI probe temperature) was successfully validated for 5 N-nitrosamines used as examples in this proof-of-concept study. The method's quantitative performance was rigorously validated in accordance with the ICH Q2 (R2) recommendations, employing the combined approach for accuracy and precision based on the total error profile methodology.

This LC-MS/MS method serves as a proof-of-concept for the QSRR-based approach applied to a real case involving a finished product, where the significant presence of a component led to a pronounced ME. The method demonstrated its suitability for both limit testing and quantitative analysis of 5 N-nitrosamine impurities: NMOR, NMBA, NDPhA, NNK, and NMPA. All required quantitative validation criteria were fulfilled.

The validated LC-MS/MS method is specific, sensitive, and accurate, making it particularly valuable for determining N-nitrosamine impurities in pharmaceutical products. The *in silico* QSRR-based strategy provides a reliable and robust analytical approach within an industrial framework. The approach employed in this work proposes a generic methodology that streamlines the development of methods for analyzing N-nitrosamines in oral solid dosage forms (tablets), especially when a new N-nitrosamine is discovered, or a product requires investigation. This approach aims to explore flexible and generic experimental conditions to address challenges related to ME and sensitivity. The validated DL and QL for all 5 N-nitrosamines met the EMA N-nitrosamine investigation approach requirements and are below 10 % of their respective acceptable limit in the selected finished product formulation.

Furthermore, the proposed *in silico* strategy integrates prior knowledge and quality risk management into the development process, in alignment with the ICH Q14 guidelines and enabling the continuous lifecycle management of analytical procedures. This approach aids marketing authorization holders (MAHs) in method development to comply with the evolving requirements of international regulatory agencies, thereby playing a crucial role in controlling N-nitrosamines and contributing to public health.

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CRediT authorship contribution statement

Houari Sabah: Formal analysis. **Zhang Yue:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Conceptualization. **Hubert Cédric:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Hubert Philippe:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Ziemons Eric:** Writing – review & editing. **Dispas Amandine:** Writing – review & editing, Methodology, Conceptualization. **Van Laethem Thomas:** Writing – review & editing, Software.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly to verify spelling and grammar. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the published article.

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.jpbao.2025.100064.

Data availability

Data will be made available on request.

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