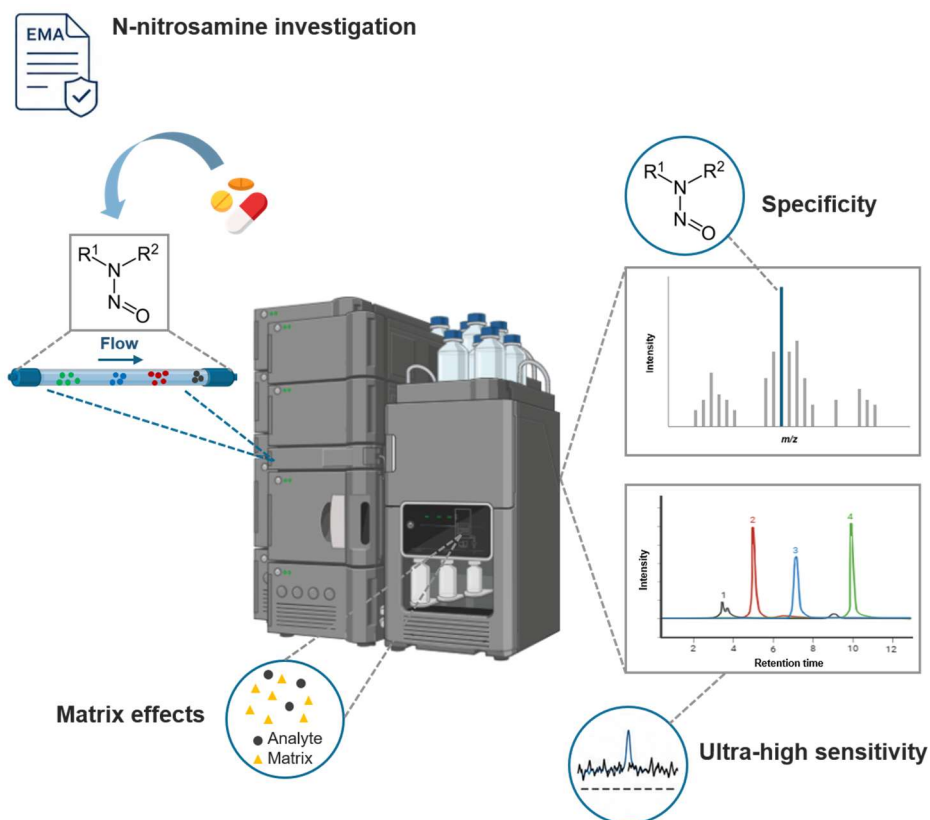


Evaluation of N-nitrosamine impurities in medicines: Analytical challenges and regulatory context



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*To my beloved grandparents,
whose unconditional love and unwavering support mean everything to me.*

献给我挚爱的外公外婆
你们的无私关爱与坚定支持
是我前行的勇气

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Abstract

N-nitrosamines are a class of compounds considered potentially carcinogenic to humans by the International Agency for Research on Cancer. Although the presence of these compounds at trace levels has long been known, particularly in the environment and food, the successive detection in 2018 of certain N-nitrosamines at levels far exceeding safety thresholds in several medicinal products for human use marked the beginning of an N-nitrosamine crisis in the global pharmaceutical industry. To address this public health concern, international regulatory authorities rapidly collaborated to establish guidelines aimed at framing the investigation and control of these impurities posing a potential risk to patient safety.

This doctoral thesis is conducted within the context of this N-nitrosamine crisis in the pharmaceutical industry and follows its evolution. In response to regulatory requirements, this work aims to develop and validate suitable analytical methods based on liquid chromatography hyphenated to tandem mass spectrometry (LC-MS/MS). These methods meet regulatory requirements, particularly during two key stages of the investigation: first, the confirmation and quantification of N-nitrosamines in drugs; and second, the implementation and monitoring of risk mitigation strategies when corrective actions are necessary to restore product compliance, safety, and quality.

Initially, the work focused on the determination of small-molecule N-nitrosamines, which are generic impurities that may originate from multiple sources. An innovative approach based on quantitative structure-retention relationships (QSRR) was proposed to support the development of LC-MS/MS methods enabling ultra-sensitive detection of these compounds at trace levels. These methods, validated according to ICH Q2(R2) guideline, were applied to the confirmatory testing of a marketed medicinal product, thereby illustrating the investigation workflow recommended by regulatory authorities.

As the N-nitrosamine crisis evolved, an unexpected turning point occurred in 2021 with the identification of a new class of N-nitrosamines: N-nitrosamine drug substance-related impurities (NDSRIs). Unlike generic N-nitrosamines, each NDSRI is specific to a given active pharmaceutical ingredient. Following this evolution, the work subsequently focused on a particular NDSRI, N-nitroso-fluoxetine, formed by nitrosation of fluoxetine in the presence of nitrosating agents. Fluoxetine is a widely prescribed medicinal product used in the treatment of major depressive episodes. The detection of N-nitroso-fluoxetine at levels exceeding the acceptable intake limit led to several product recalls and ultimately to the withdrawal of a fluoxetine-based pharmaceutical product from the Belgian market. A robust LC-MS/MS method was developed using the Analytical Quality by Design (AQbD) approach, enabling the

simultaneous quantification of N-nitroso-fluoxetine and the detection of 13 other small-molecule N-nitrosamines at levels below their acceptable limits. This method was subsequently validated according to ICH Q2(R2) guideline to assess the effectiveness of the mitigation strategies implemented to maintain the N-nitroso-fluoxetine content below its safety threshold.

Overall, this work provides analytical tools and scientific approaches contributing to the control of N-nitrosamines in medicines, addressing regulatory requirements and ensuring the safety and quality of pharmaceutical products.

Résumé

Les nitrosamines sont une classe de composés considérés par le centre international de recherche sur le cancer comme potentiellement carcinogènes pour l'Homme. Bien que la présence de ces composés à l'état de traces soit connue depuis longtemps, notamment dans l'environnement et l'alimentation, la détection successive en 2018 de certaines nitrosamines à des niveaux largement supérieurs aux seuils de sécurité dans plusieurs médicaments à usage humain a marqué le début de la crise des nitrosamines dans l'industrie pharmaceutique mondiale. Afin de répondre à ce problème de santé publique, les autorités réglementaires internationales ont rapidement collaboré pour établir des lignes directrices visant à encadrer l'investigation et le contrôle de ces impuretés présentant un risque potentiel pour la santé des patients.

Cette thèse de doctorat s'inscrit dans le contexte de cette crise des nitrosamines dans l'industrie pharmaceutique et en suit l'évolution. Face aux exigences réglementaires, ce travail vise à développer et valider des méthodes analytiques adaptées, basées sur la chromatographie liquide couplée à la spectrométrie de masse en tandem (LC-MS/MS). Ces méthodes satisfont aux exigences réglementaires, en particulier lors de deux étapes clés de l'investigation : d'une part, la confirmation et la quantification des nitrosamines dans le médicament ; d'autre part, le déploiement et le suivi des stratégies d'atténuation du risque lorsque des mesures correctives s'imposent pour garantir à nouveau la conformité, la sécurité et la qualité du produit.

Dans un premier temps, les travaux ont porté sur la détermination de nitrosamines de faible masse moléculaire. Celles-ci représentent des impuretés génériques pouvant provenir de multiples sources. Une approche innovante basée sur les relations quantitatives structure-rétention (QSRR) a été proposée afin de soutenir le développement de méthodes LC-MS/MS permettant leur détection à l'état de traces. Ces méthodes, validées conformément aux recommandations ICH Q2(R2), ont été appliquées au contrôle confirmatoire d'un médicament commercialisé, illustrant ainsi le processus d'investigation recommandé par les autorités compétentes.

Au cours de l'évolution de la crise des nitrosamines, un tournant inattendu est survenu en 2021 avec l'identification d'une nouvelle classe de nitrosamines : les impuretés de nitrosamines spécifiques de substance active (NDSRIs). Contrairement aux nitrosamines génériques, chaque NDSRI est spécifique à une substance active donnée. Suivant cette évolution, les travaux de cette thèse se sont alors concentrés sur une NDSRI particulière, la nitroso-fluoxétine, formée par nitrosation de la fluoxétine en présence d'agents nitrosants. La fluoxétine est un médicament largement prescrit dans le traitement des épisodes de dépression majeure. La détection de nitroso-fluoxétine à des niveaux dépassant la limite d'apport autorisée a conduit à plusieurs rappels de

lots et finalement au retrait d'une spécialité pharmaceutique à base de fluoxétine du marché belge. Une méthode LC-MS/MS robuste a été développée en appliquant l'approche Analytical Quality by Design (AQbD), permettant la quantification simultanée de la nitroso-fluoxétine ainsi que la détection de 13 autres nitrosamines de faible masse moléculaire à des niveaux inférieurs à leurs limites autorisées. Cette méthode a ensuite été validée conformément aux recommandations ICH Q2(R2) afin d'évaluer l'efficacité des stratégies de mitigation mises en œuvre pour maintenir la teneur en nitroso-fluoxétine en dessous de son seuil de sécurité.

L'ensemble de ces travaux propose ainsi des outils analytiques et des approches scientifiques contribuant au contrôle des nitrosamines dans les médicaments, afin de répondre aux exigences réglementaires et de garantir la sécurité et la qualité des produits pharmaceutiques.

List of abbreviations

#	
+	Positive
A	
ADC	Antibody-drug conjugate
AHP	17 α -hydroxyprogesterone
AI	Acceptable intake
AL	Acceptable limit
Al	Aluminum
AmAc	Ammonium acetate
AQbD	Analytical Quality by Design
APCI	Atmospheric pressure chemical ionization
API	Active pharmaceutical ingredient
APPI	Atmospheric pressure photoionization
ARB	Angiotensin II receptor blocker
ATP	Analytical target profile
AUC	Area under the curve
C	
CID	Collision-induced dissociation
CMA	Critical method attribute
CMP	Critical method parameter
CPAP	1-cyclopentyl-4-aminopiperazine
CPCA	Carcinogenic potency categorization approach
CPNP	1-cyclopentyl-4-nitroso-piperazine
D	
DC	Direct current
DoE	Design of experiment
DL	Detection limit
E	
EAT	Enhanced Ames test
EIC	Extracted ion chromatogram
EMA	European Medicines Agency
ESI	Electrospray ionization
F	
FA	Formic acid
FDA	Food and Drug Administration
FT-ICR	Fourier transform ion cyclotron resonance
G	
GC	Gas chromatography

H	
HPLC	High-performance liquid chromatography
I	
IARC	International Agency for Research on Cancer
IC	Ion chromatography
ICH	International Council for Harmonisation
IUPAC	International Union of Pure and Applied Chemistry
L	
LC	Liquid chromatography
LQL	Lower quantification limit
LTL	Less-than-lifetime
M	
MAP	1-methyl-4-aminopiperazine
MAH	Marketing authorization holder
ME	Matrix effect
MeNP	1-methyl-4-nitrosopiperazine
MeOH	Methanol
MODR	Method operable design region
MRM	Multiple reaction monitoring
MS	Mass spectrometry / Simple quadrupole mass detector
MS/MS	Tandem mass spectrometry / Tandem mass spectrometer
<i>m/z</i>	Mass-to-charge
N	
NaN ₃	Sodium azide
NaNO ₂	Sodium nitrite
NAP	Nitrosation assay procedure
NDBA	N-nitrosodi-N-butylamine
NDEA	N-nitroso-diethylamine
NDELA	N-nitroso-diethanolamine
NDIPA	N-nitroso-diisopropylamine
NDLRI	N-nitrosamine drug linker-related impurity
NDMA	N-nitroso-dimethylamine
NDPA	N-nitrosodi-N-propylamine
NDPhA	N-nitrosodiphenylamine
NDSRI	N-nitrosamine drug substance-related impurity
NEIPA	N-nitroso-ethylisopropylamine
NMBA	N-nitroso-N-methyl-4-aminobutyric acid
NMEA	N-nitrosomethylethylamine
NMOR	N-nitrosomorpholine
NMPA	N-nitroso-methylphenylamine

NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NO _x	Nitrogen oxide
NPIP	N-nitrosopiperidine
NPYR	N-nitrosopyrrolidine
NTHP	N-nitroso-1,2,3,6-tetrahydropyridine
O	
OECD	Organisation for Economic Co-operation and Development
OFAT	One-factor-at-a-time
OMCL	Official medicines control laboratory
P	
Ph. Eur.	European Pharmacopoeia
PVC	Polyvinyl chloride
Q	
QbD	Quality by Design
QC	Quality control
QL	Quantification limit
QSRR	Quantitative structure retention relationship
R	
R ²	Coefficient of determination
RF	Radiofrequency
RH	Relative humidity
RSD	Relative standard deviation
S	
SAR	Structure-activity relationship
SIL	Stable isotope-labeled
SIR	Selective ion recording
SRM	Selective reaction monitoring
SST	System suitability test
S/N	Signal-to-noise
T	
TIC	Total ion chromatogram
ToF	Time-of-flight
TOST	Two one-sided t-Test
TTC	Threshold of toxicological concern
U	
UHPLC	Ultra-high-performance liquid chromatography
USP	United States Pharmacopeia
W	
WHO	World Health Organization

Table of contents

Chapter I. Introduction

Preamble	1
1 “N-nitrosamine risk assessment in pharmaceuticals: Where are we from a regulatory point of view in 2025?”	5
1.1 Abstract.....	5
1.2 N-nitrosamines	5
1.3 Carcinogenicity and acceptable intake limit determination.....	10
1.3.1 Carcinogenicity mechanisms.....	10
1.3.2 Acceptable intake limit.....	12
1.3.3 Acceptable intake limit determination.....	15
1.4 A 3-step N-nitrosamine investigation guidance.....	19
1.4.1 Theoretical risk assessment.....	19
1.4.2 Analytical confirmatory testing.....	20
1.4.3 Comprehensive risk mitigation measure implementation	23
1.5 Sources of N-nitrosamine impurity formation.....	23
1.5.1 Chemical synthesis of APIs.....	24
1.5.2 Manufacturing of finished products	25
1.5.3 Storage of drug substances and products.....	29
1.5.4 Combined root causes	29
1.6 N-nitrosamine risk mitigation measures	31
1.7 Risk-based prediction tools for N-nitrosamine formation	34
1.7.1 Nitrite excipient database	34
1.7.2 Nitrite testing.....	34
1.7.3 Nitrosation assay procedure	37
1.7.4 Carcinogenicity prediction – Ames test	37
1.7.5 Forced degradation study	38
1.7.6 Stability testing.....	39
1.7.7 Extractable and leachable assessment	39
1.7.8 In silico purge-based approach.....	40
1.8 Conclusion.....	41
2 Analytical Quality by Design	43
2.1 Regulatory framework.....	44
2.1.1 Quality by design	44

2.1.2	Analytical quality by design.....	45
2.2	Analytical procedure lifecycle.....	47
2.3	Workflow of analytical quality by design.....	48
2.3.1	Analytical target profile	48
2.3.2	Knowledge management	49
2.3.3	Quality risk management.....	49
2.3.4	Design of experiment	50
2.3.5	Method operational design region and robustness	51
2.3.6	Working point selection	51
2.3.7	Analytical procedure validation	52
2.3.8	Analytical control strategy	52
3	Hyphenated technique selection	53
3.1	Liquid chromatography	54
3.2	Mass spectrometry	56
3.2.1	Ion sources	57
3.2.2	Mass analyzers	61
3.2.3	Ion detectors	65
3.3	Hyphenation of liquid chromatography to tandem mass spectrometry.....	67
3.3.1	Selectivity and specificity	67
3.3.2	Sensitivity.....	68
3.3.3	Matrix effects	70
	References.....	72

Chapter II. Objectives 87

Chapter III. Results

Section 1(A). Analytical development of LC-MS/MS methods for the determination of small-molecule N-nitrosamine impurities

	Preamble	91
	Abstract.....	94
1	Introduction	95
2	Materials and methods.....	100
2.1	Chemicals and reagents	100
2.2	LC-MS/MS instrumentation	101

2.3	QSRR dataset description	101
2.4	<i>In silico</i> screening methodology	102
2.5	Analytical method development	103
2.5.1	MS/MS parameter optimization.....	103
2.5.2	LC separation verification.....	104
2.6	Validation protocol.....	105
2.6.1	17 α -hydroxyprogesterone solution	106
2.6.2	Internal standards.....	106
2.6.3	Calibration standards	106
2.6.4	Validation standards.....	106
2.6.5	Quality control solution	107
2.7	Analytical method for validation study	107
2.7.1	Chromatographic conditions.....	107
2.7.2	MS/MS parameters	107
2.8	Stability assessment.....	111
2.9	Software.....	111
3	Results and discussion	111
3.1	Desirability index graph	111
3.1.1	Desirability index graph including 17 N-nitrosamines	111
3.1.2	Desirability index graph for 5 N-nitrosamines.....	112
3.2	Analytical method development	113
3.2.1	MS/MS parameters	113
3.2.2	Chromatographic selectivity – pH 2.7	113
3.2.3	pH impact.....	115
3.2.4	Generic methods	115
3.3	Analytical method validation.....	116
3.3.1	Combined approach for accuracy and precision	118
3.3.2	Linearity.....	123
3.3.3	Working range	123
3.3.4	Detection limit	124
3.3.5	Stability.....	124
3.4	System suitability tests	124
3.4.1	Equipment verification.....	124

3.4.2	Tailing factor.....	126
3.4.3	Ion ratio.....	126
3.4.4	QC recovery.....	126
3.4.5	System sensitivity verification.....	126
4	Conclusion.....	126
	Supplementary material	129
	References.....	134

Section 1(B). Illustration of the practical workflow for N-nitrosamine investigation according to the “Call for Review” regulatory guidance

	Preamble	137
	Abstract.....	139
1	Introduction	141
2	Materials and methods.....	146
2.1	Chemicals and reagents	146
2.2	Instrumentation and software.....	147
2.3	Analytical methods	147
2.3.1	Sample preparation.....	147
2.3.2	Chromatographic conditions	148
2.3.3	Mass spectrometric parameters	150
2.4	Validation protocol	154
2.4.1	Internal standards	154
2.4.2	Calibration standards.....	155
2.4.3	Validation standards.....	158
2.4.4	Unspiked matrices	159
2.5	Equipment performance verification	159
2.6	System suitability test solutions.....	159
2.6.1	System sensitivity verification for trace N-nitrosamine analysis	159
2.6.2	Ion ratio	159
2.6.3	NDBA carry-over verification.....	160
2.7	Confirmatory testing.....	160
3	Results	161
3.1	Validation studies	161
3.1.1	Selectivity.....	161

3.1.2	Detection limit.....	162
3.1.3	Recovery	165
3.1.4	Equipment performance verification.....	171
3.1.5	System suitability tests	171
3.2	Confirmatory testing.....	171
3.2.1	Equipment performance verification and system suitability tests	172
3.2.2	Testing results	172
4	Discussion	174
	Supplementary material	176
	References.....	182

Section 2. Analytical development of an LC-MS/MS method for the determination of an N-nitrosamine drug substance-related impurity

	Preamble	185
	Abstract.....	186
1	Introduction	187
2	Materials and methods.....	189
2.1	Chemicals and reagents	189
2.2	Instrumentation and software.....	190
2.3	Risk assessment	190
2.4	Analytical quality by design	190
2.4.1	Definition of analytical target profile.....	190
2.4.2	Selection of analytical technique.....	190
2.4.3	Optimization experimental design.....	191
2.4.4	Second optimization experimental design.....	193
2.5	Analytical procedure validation.....	193
2.5.1	Method parameters.....	193
2.5.2	Internal standard.....	194
2.5.3	Calibration standards.....	194
2.5.4	Validation standards.....	195
2.5.5	Blank matrix.....	196
2.6	Risk mitigation measures.....	196
2.6.1	Confirmatory testing	196
3	Results and discussion.....	197

3.1	Risk assessment	197
3.2	Analytical quality by design	198
3.2.1	Definition of analytical target profile	198
3.2.2	Selection of analytical technique.....	201
3.2.3	Optimization experimental design.....	201
3.2.4	Second optimization experimental design.....	204
3.3	Analytical procedure validation.....	205
3.3.1	Selectivity and specificity	205
3.3.2	Detection limits	207
3.3.3	Quantitative performance.....	210
3.3.4	System suitability tests	213
3.4	Risk mitigation measures.....	214
3.4.1	System suitability tests	214
3.4.2	Confirmatory testing	214
4	Conclusion.....	217
	Supplementary material	219
	References.....	237

Chapter IV. General discussion

1	Small-molecule N-nitrosamine impurity analysis	241
2	N-nitrosamine drug substance-related impurity analysis.....	245
3	Advantages of combining QSRR and AQbD approaches	248
	References.....	250

Chapter V. Conclusion..... 253

Chapter VI. Perspectives 257

	Reference	258
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Publication report 259

Chapter I

Introduction

Preamble

Certain N-nitrosamines are classified by the International Agency for Research on Cancer as probably or possibly carcinogenic to humans [1]. The carcinogenic potential of N-nitrosamines was first demonstrated in 1956 when Barnes and Magee reported that N-nitrosodimethylamine (NDMA) induced hepatic cancers in rats [2]. This finding prompted extensive investigation into the carcinogenicity of other N-nitrosamines.

Human exposure to N-nitrosamines occurs predominantly through dietary sources and may arise via both exogenous and endogenous pathways. Exogenous exposure results from the ingestion of contaminated food or beverages, whereas endogenous formation originates from nitrosation reactions between dietary nitrites and amines, a process favored by the acidic conditions of the gastric environment [3].

During the 1970s, NDMA and N-nitrosopyrrolidine were detected in several cured meat products [4]. These compounds, together with N-nitrosodiethylamine, were later found in beer [5,6]. Moreover, the extensive use of nitrogen-based fertilizers, pesticides, and herbicides in agriculture has increased the release of nitrosation precursors, leading to the detection of additional N-nitrosamine impurities in cereals, vegetables, and fruits [7]. N-nitrosamines are also present in tobacco products and are referred to as tobacco-specific N-nitrosamines. [8].

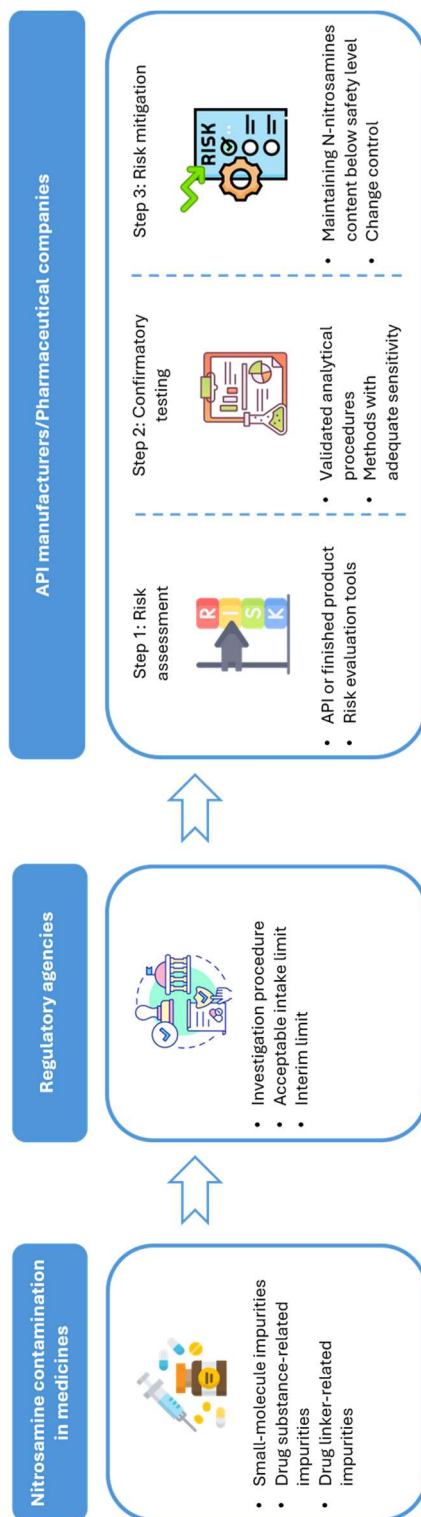
In 2018, a major concern regarding these impurities emerged in the pharmaceutical industry following the detection of significant levels of NDMA in multiple products containing valsartan [9]. In response, international regulatory authorities rapidly established and periodically revised acceptable intake limits for N-nitrosamines in medicinal products [10,11].

In contrast, no specific acceptable intake limits have yet been formally established for N-nitrosamines in food. The establishment of maximum allowable levels in foodstuffs is currently under discussion following the publication of the “Risk assessment of N-nitrosamines in food” by the European Food Safety Authority [12]. Gushgari *et al.* estimated daily N-nitrosamine exposure based on typical American consumption patterns, identifying tobacco as the primary source ($21,800 \pm 4,350$ ng/day), followed by food intake ($1,900 \pm 380$ ng/day) [13]. By comparison, the acceptable intake for NDMA in pharmaceutical products is set at 96 ng/day [10,11]. Based on these data, exposure from medicinal products is likely to represent only a minor contribution to the overall human exposure to N-nitrosamines [3].

The first section of this introduction presents an overview of the regulatory framework that emerged in response to the N-nitrosamine crisis in the pharmaceutical industry, with a focus exclusively on exogenous exposure related to drug intake.

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Graphical abstract. Regulatory measures for N-nitrosamine impurities in medicinal products and structure of the 3-step investigation procedure.

1 “N-nitrosamine risk assessment in pharmaceuticals: Where are we from a regulatory point of view in 2025?”

Y. Zhang, J. Widart, E. Ziemons, Ph. Hubert, C. Hubert, **Journal of Pharmaceutical and Biomedical Analysis Open**, 6 (2025), 100084.

1.1 Abstract

N-nitrosamines have been a concern for decades due to their potential mutagenicity and widespread occurrence across various matrices. While evidence suggests carcinogenicity in animals, their potential carcinogenicity in humans has prompted their initial inclusion in the “cohort of concern” since ICH M7(R1), and the current ICH M7(R2) guideline is now in effect. Intensified control of N-nitrosamines began in 2018 following the detection of N-nitrosodimethylamine in valsartan-containing products. Subsequent investigations revealed N-nitrosamine contamination across multiple drug classes, triggering widespread recalls, withdrawals, and regulatory actions. Recently, the emergence of N-nitrosamine drug substance-related impurities and drug linker-related impurities has drawn additional regulatory attention.

This review presents the methodologies used to determine the acceptable daily intake of N-nitrosamines and traces the evolution of regulatory guidelines, offering a comparative analysis of the 3-step investigation approaches adopted by the European Medicines Agency and Food and Drug Administration. It provides a comprehensive examination of potential root causes for N-nitrosamine contamination, outlines the analytical requirements for confirmatory testing, as well as mitigation strategies to prevent or minimize contamination. Additionally, the review summarizes risk assessment tools used to predict N-nitrosamine formation.

By presenting a comprehensive workflow for impurity investigations, this review aims to assist industrial stakeholders in managing N-nitrosamine risks, ensuring regulatory compliance, and safeguarding public health.

1.2 N-nitrosamines

N-nitrosamines are a class of organic compounds sharing the common chemical structure of a nitroso functional group bonded to an amine, represented by the general structure $R^1N(-R^2)-N=O$, as illustrated in Figure 1 [1]. These compounds can naturally occur in the environment, found in air, drinking water, and soil [2], and also form in various matrices, including food, beverages, tobacco, and cosmetics [3]. N-nitrosamines have raised concerns for decades due to their potential mutagenicity in humans, most are classified by the International Agency for Research on Cancer into groups 2A or 2B [4]. While these classifications suggest evidence of carcinogenicity in animals, further research is necessary to confirm the risk in humans. The impurities

are also included in the “cohort of concern” as specified in the ICH M7(R2) guideline [5].

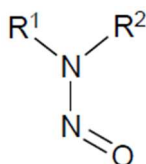


Figure 1. General chemical structure of nitroso functional group.

The pharmaceutical spotlight on N-nitrosamine impurities began in June 2018 with the detection of harmful levels of N-nitrosodimethylamine (NDMA) in batches of valsartan-containing medicinal products. Valsartan is an angiotensin II receptor blocker (ARB) prescribed to treat chronic heart failure and hypertension [6]. The unexpected NDMA contamination was reported to the European Union authorities and the United States Food and Drug Administration (FDA). It was identified as originating from the valsartan active pharmaceutical ingredient (API) manufacturer, Zhejiang Huahai Pharmaceutical Co., Ltd., China [7,8]. The API manufacturer attributed the undesirable NDMA formation to process-related impurities introduced after chemical synthesis modifications during the tetrazole ring-forming step. Subsequent investigations revealed additional N-nitrosamines, such as N-nitrosodiethylamine (NDEA) and N-nitroso-N-methyl-4-aminobutyric acid (NMBA), in ARBs containing a tetrazole moiety, including losartan and irbesartan, as shown in Figure 2 [9]. The scope of contamination expanded to include other drug classes, such as antacids (*e.g.*, ranitidine and nizatidine [10,11]), antidiabetics (*e.g.*, metformin [12] and pioglitazone [13]), antibiotics (*e.g.*, rifampin, rifapentine [14]). Despite their trace presence, these genotoxic impurities often exceeded acceptable limits, posing significant health risks [15–18]. Numerous decisions of recalls, suspensions, and withdrawals of affected pharmaceuticals have been taken to protect patient safety [9,11].

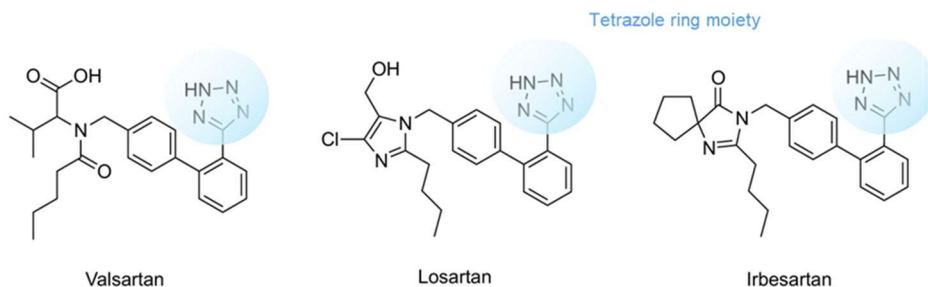


Figure 2. Chemical structures of valsartan, losartan, and irbesartan.

A new class of impurities, N-nitrosamine drug substance-related impurities (NDSRIs), emerged in 2021, beginning with the discovery of N-nitroso-varenicline in varenicline-containing drugs [19]. Unlike small-molecule N-nitrosamines (*e.g.*, NDMA, NDEA, NMBA, etc.), which can be generated from multiple sources, NDSRIs share structural similarities with APIs. In general, they are larger molecules and unique to specific drugs, generally forming through the nitrosation of drug substances in the presence of nitrite [1], as illustrated in Figure 3.

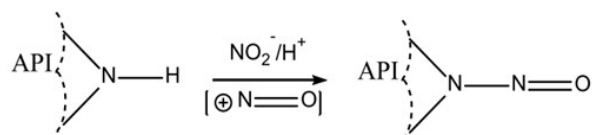


Figure 3. Nitrosation reaction of NDSRI formation.

Recent regulatory attention has also shifted towards biologicals, particularly antibody-drug conjugates (ADCs). ADCs, which combine monoclonal antibodies with synthetic drugs via drug linkers, present a risk for N-nitrosamine drug linker-related impurities (NDLRIs) due to their synthetic components [20,21]. These major events, which have challenged the entire pharmaceutical industry and global health regulators, are illustrated in Figure 4.

Chapter I: Introduction - Section 1

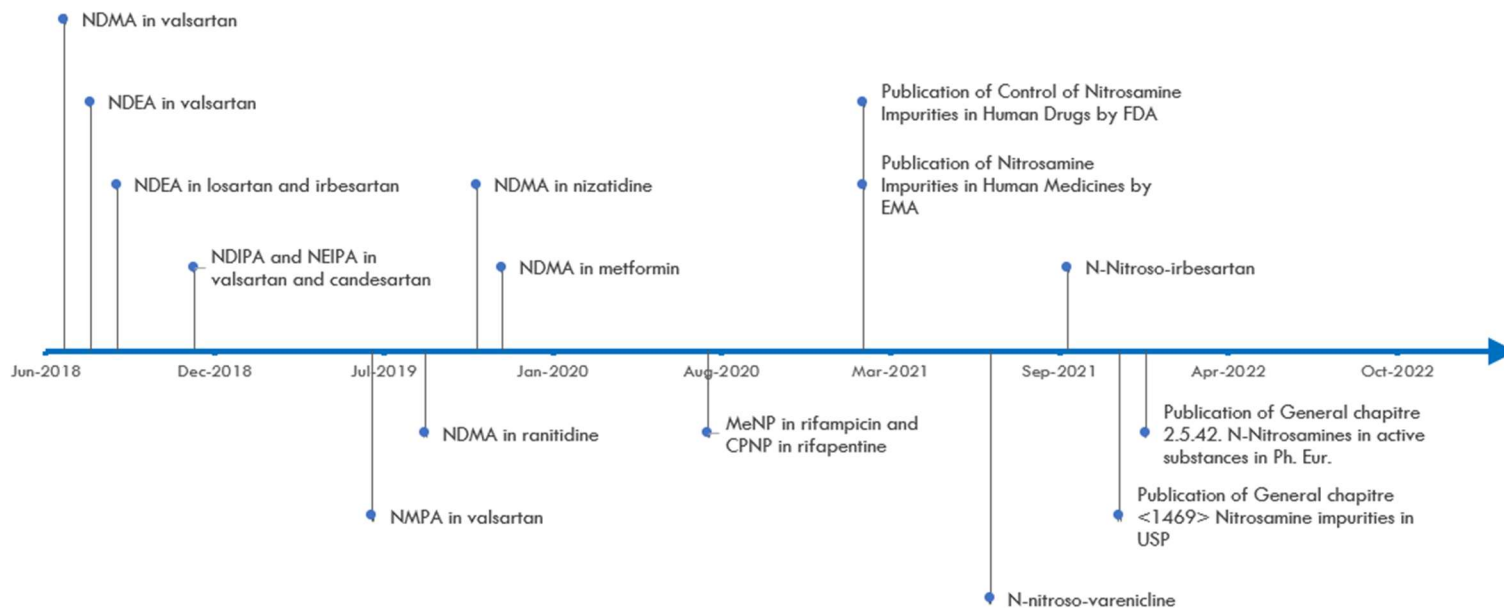


Figure 4. Timeline of key N-nitrosamine events for the pharmaceutical industry.

International regulatory bodies have responded promptly to concerns about N-nitrosamine contamination by forming a global network. Regulatory bodies, industrial stakeholders, and official medicines control laboratories (OMCLs) worldwide have collaborated to centralize information and develop strategies to address and control N-nitrosamine impurities. One key initiative led by the United States Pharmacopeia (USP) is the “Nitrosamine Exchange” platform [22], an interactive, knowledge-based online forum that brings together pharmaceutical companies, API manufacturers, and excipients/packaging suppliers actively engaging, discussing, and sharing best practices related to N-nitrosamines [22,23]. Moreover, this platform hosts the “Nitrosamine Analytical Hub”, a publicly accessible online repository of analytical methods for the detection of N-nitrosamine impurities in pharmaceuticals [23,24].

In February 2021, key regulatory authorities, including the European Medicines Agency (EMA) and the U.S. FDA, released guidance documents Revision 1 [25]. Subsequently, in April 2024, the World Health Organization (WHO) introduced a draft working document titled WHO Good Manufacturing Practices: Considerations for the Prevention and Control of Nitrosamine Contamination in Pharmaceutical Products, soliciting public feedback [26]. These documents outline detailed procedures and mandate API manufacturers, marketing authorization holders (MAHs), and applicants to undertake a systematic 3-step investigation to assess the presence of N-nitrosamine impurities in their products [1,27].

In late 2020, both the USP and the European Pharmacopoeia (Ph. Eur.) introduced general chapters addressing N-nitrosamine impurities. These chapters were officially implemented in December 2021 for the USP and January 2022 for the Ph. Eur. The pharmacopeias provide a wide range of quantitative and limit test analytical procedures [28,29]. Furthermore, a large selection of reference standard materials has been specifically developed to facilitate analytical testing for these impurities. Key publication dates for these documents are summarized in the event timeline shown in Figure 4.

Numerous reviews in the field of N-nitrosamines have been published, focusing on various aspects such as analytical procedures [30,31], formation pathways in pharmaceutical products [32,33], or documented cases of detection [34]. This review first presents the mechanisms underlying the genotoxicity of N-nitrosamines and methodologies used to determine the acceptable intake (AI) limit of N-nitrosamines. It also traces the evolution of regulatory guidelines, offering a comparative analysis of the 3-step investigation approaches adopted by the EMA and FDA, highlighting their similarities and differences. A comprehensive workflow is proposed to guide the investigation process. Finally, the review summarizes risk assessment tools used to predict N-nitrosamine formation.

1.3 Carcinogenicity and acceptable intake limit determination

1.3.1 Carcinogenicity mechanisms

N-nitrosamines can be ingested or form endogenously within the organism via nitrosating reactions in the acidic environment of the stomach. In this reaction, nitric oxide¹, derived from nitrite or nitrate, reacts with amines present in food [3]. Once ingested, N-nitrosamines are metabolically activated *in vivo*, primarily by cytochrome P450 enzymes. While CYP2E1 is known to metabolize smaller N-nitrosamines, a broader range of cytochrome isoforms can contribute to their activation. Regardless of the cytochrome involved, the metabolic activation pathway is generally consistent across different classes of N-nitrosamines: hydroxylation at the α -carbon adjacent to the N-nitroso group. In the case of aliphatic N-nitrosamines, the α -hydroxylation generates an unstable dealkylated N-nitrosamine that decomposes rapidly into a reactive alkyl diazonium ion. Alkyl diazonium ions can alkylate DNA material via either S_N1 or S_N2 mechanisms, forming adducts that lead to DNA damage and potentially contribute to cancer development [2,35]. The metabolic biotransformation pathways and subsequent DNA adduct formation are represented in Figure 5.

In contrast to aliphatic N-nitrosamines, aromatic N-nitrosamines ultimately yield aryl diazonium ions, which are incapable of undergoing S_N1 or S_N2 reactions and instead form diazo adducts. Consequently, aromatic N-nitrosamines tend to exhibit lower carcinogenic potential than their aliphatic counterparts. This distinction is reflected in the AI limits: for instance, N-nitroso-diphenylamine (NDPhA) has a considerably higher AI limit of 78,000 ng/day, compared to 96 ng/day for NDMA. Additionally, cyclic N-nitrosamines may form cyclic diazonium intermediates that can rearrange into oxonium ions, which serve as alternative alkylating agents [35].

¹ *Correction:* The term “nitric oxide” should read “nitrosonium”.

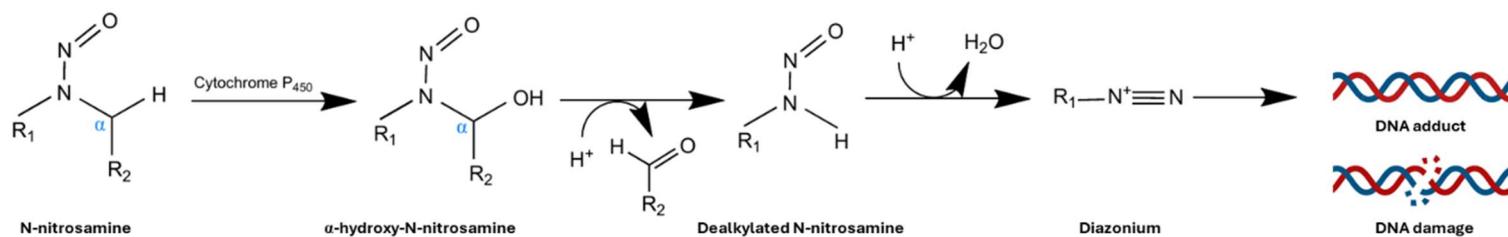


Figure 5. Metabolic biotransformation of N-nitrosamines and formation of DNA adducts.

1.3.2 *Acceptable intake limit*

The EMA and FDA have elaborated, periodically reviewed, and updated comprehensive lists of N-nitrosamines, including both small-molecule N-nitrosamines and NDSRIs, along with their respective AI limit. These AI limits are established based on the carcinogenic risk associated with the impurities. Given the large number of small-molecule impurities and NDSRIs identified to date and the ongoing discovery of new ones, some of these are summarized in Table 1, along with the source and CAS number. Notably, 1-methyl-4-nitrosopiperazine (MeNP) and 1-cyclopentyl-4-nitrosopiperazine (CPNP) represent particular cases: although they have low molecular weights, they are classified as NDSRIs because they are specifically associated with rifampin and rifapentine, respectively [33,36]. These examples will be discussed in detail in Section 1.5.4.2. Furthermore, the EMA and FDA lists provide complementary information, with each including different N-nitrosamines [27,37].

Table 1: CAS number, source, CPCA category, and recommended AI limit of N-nitrosamines according to EMA and FDA procedures.

Small-molecule N-nitrosamines	CAS No.	Source [27]	CPCA category [27,38]	AI limit (ng/day)	
				EMA [27]	FDA [37]
N-nitroso-dimethylamine (NDMA)	62-75-9	Multiple	2	96	96
N-nitroso-diethylamine (NDEA)	55-18-5	Multiple	1	26.5	26.5
N-nitroso-N-methyl-4-aminobutyric acid (NMBA)	61445-55-4	Multiple	4	1500	1500
N-nitroso-ethylisopropylamine (NEIPA)	16339-04-1	Multiple	3	400	400
N-nitroso-diisopropylamine (NDIPA)	601-77-4	Multiple	5	1500	1500
N-nitroso-methylphenylamine (NMPA)	614-00-6	Multiple	2	100	100
N-nitrosodi-N-propylamine (NDPA)	621-64-7	Multiple	1	26.5	UA
N-nitrosodi-N-butylamine (NDBA)	924-16-3	Multiple	1	26.5	UA
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	64091-91-4	Multiple	2	100	UA
N-nitrosomorpholine (NMOR)	59-89-2	Multiple	N/A	127	UA
N-nitrosodiphenylamine (NDPhA)	86-30-6	Multiple	N/A	78000	UA
N-nitrosopyrrolidine (NPYR)	930-55-2	Multiple	N/A	1700	UA
N-nitrosopiperidine (NPIP)	100-75-4	Multiple	N/A	1300	UA
NDSRIs					
1-methyl-4-nitrosopiperazine (MeNP)	16339-07-4	Rifampin	3	400	400 3000 [#]
1-cyclopentyl-4-nitroso-piperazine (CPNP)	61379-66-6	Rifapentine	3	UA	400
N-nitroso-desmethyl-tramadol	UA	Tramadol	1	18	26.5
N-nitroso-STG-19 (NTTP)	2892260-32-9	Sitagliptin	2	100	100
N-nitroso-varenicline	2755871-02-2	Varenicline	3	400	400

Table 1 Continued.

NDSRIs	CAS No.	Source [27]	CPCA category [27,38]	AI limit (ng/day)	
				EMA [27]	FDA [37]
N-nitroso-betaxolol	UA	Betaxolol	4	1500	1500
N-nitroso-enalapril	2994317-06-3	Enalapril	5	1500	1500
N-nitroso-duloxetine	2680527-91-5	Duloxetine	2	100*	100*

*: Recommended AI limit based on read-across analysis from a surrogate N-nitrosamine (NNK); #: Recommended interim AI limit for N-nitrosamines for approved or currently marketed products; CAS No.: Chemical abstracts service number; UA: Unavailable; N/A: Not applicable.

Data from the EMA was last updated in February 2025, and from the FDA in March 2025.

1.3.3 *Acceptable intake limit determination*

The ICH M7(R2) guideline defines the AI limit for mutagenic impurities as a threshold representing an increased cancer risk of one case per 100,000 people, based on lifetime daily exposure (70 years) [5]. For most unstudied substances, this corresponds to a threshold of toxicological concern (TTC) of 1.5 µg/day, which is associated with a negligible cancer risk. However, highly potent mutagenic compounds classified as part of the “cohort of concern”, such as N-nitrosamines, pose a significant cancer risk even at doses below this threshold. Consequently, the TTC approach is inappropriate for determining the AI limit for N-nitrosamines, necessitating a compound-specific assessment instead [5].

1.3.3.1 Carcinogenic potency categorization approach

The EMA and FDA have suggested a novel predictive carcinogenic potency categorization approach (CPCA) for determining AI limits for both small-molecule N-nitrosamines and NDSRIs [27,38]. This approach offers a significant advantage in its applicability even when robust carcinogenicity data are lacking for the identified N-nitrosamines in pharmaceutical products. This is particularly relevant for NDSRIs, as their carcinogenicity data are rarely available due to their recent identification and ongoing detection in drugs [39].

The CPCA is a conservative approach and user-friendly prediction tool that leverages SAR principles and databases [39,40], such as the Carcinogenic Potency Database [41,42] and Lhasa Carcinogenicity Database [43]. This methodology assumes that enzymatic α -hydroxylation by cytochrome P450 is the primary mechanism responsible for the mutagenic and genotoxic effects observed for N-nitrosamines [40].

The structural features of molecules can either enhance or reduce the enzymatic activation of metabolism mechanisms or facilitate alternative biological clearance pathways [40]. Using SAR-assisted predicted carcinogenic potency, the CPCA classified molecules into five potency categories, assigning four discrete AI levels: 18 or 26.5, 100, 400, and 1500 ng/day, as summarized in Table 2.

Notably, the EMA and FDA have adopted different AI values for category 1, using distinct strategies. Specifically, the EMA sets the AI limit for category 1 at 18 ng/day, while the FDA assigns a slightly higher limit of 26.5 ng/day, as detailed in Table 2. This classification system enables AI limits to be derived solely from the evaluation of structural features [27,38,40]. The potency categories for selected N-nitrosamines are listed in Table 1.

Chapter I: Introduction - Section 1

Table 2: Five predicted CPCA categories and associated recommended AI limits for N-nitrosamine impurities.

CPCA category	Recommended AI limit (ng/day)		Comments [27,38]
	EMA [27]	FDA [37]	
1	18	26.5	N-nitrosamines assigned to category 1 are predicted to have high carcinogenic potency but no higher than the class-specific threshold. The class-specific TTC for N-nitrosamine impurities is considered sufficiently protective for patients. EMA: The recommended AI limit of 18 ng/day is set, equivalent to the class-specific TTC for N-nitrosamines, as outlined in the Assessment report Procedure under Article 5(3) of Regulation EC (No) 726/2004 Nitrosamine impurities in human medicinal products Procedure number: EMEA/H/A-5(3)/1490. FDA: The recommended AI limit of 26.5 ng/day is set based on the carcinogenic potency of NDEA, the most potent and robustly studied N-nitrosamine.
2	100	100	N-nitrosamines assigned to category 2 are predicted to have carcinogenic potency no higher than NDMA and NNK, which have recommended AI limits of 96 ng/day and 100 ng/day, respectively. The recommended AI limit of 100 ng/day is set and aligns with the limits for NDMA and NNK.
3	400	400	N-nitrosamines assigned to category 3 are predicted to have lower carcinogenic potency than potency category 2 due to certain structural features. The recommended AI limit of 400 ng/day is set to reflect a 4-fold reduction in carcinogenicity potency compared to category 2.
4	1500	1500	N-nitrosamines assigned to category 4 are predicted to be probably metabolically activated through an enzymatic α-hydroxylation pathway but with low carcinogenic potency. The recommended AI limit of 1500 ng/day (or 1.5 μg/day) is set at the TTC defined in the ICH M7(R2) guideline.

Chapter I: Introduction - Section 1

Table 2 Continued.

CPCA category	Recommended AI limit (ng/day)		Comments [27,38]
	EMA [27]	FDA [37]	
5	1500	1500	N-nitrosamines assigned to category 5 are not predicted to be metabolically activated through an enzymatic α -hydroxylation pathway due to steric hindrance or the absence of α -hydrogens or are predicted to form unstable species that will not react with DNA. The recommended AI limit of 1500 ng/day (or 1.5 μ g/day) is set at the TTC defined in the ICH M7(R2) guideline.

1.3.3.2 Read-across analysis

An alternative to the CPCA is a read-across analysis. This method consists of using a structurally similar N-nitrosamine, referred to as a “surrogate”, which has sufficient and robust carcinogenicity data to predict the carcinogenic potency of the compound of interest [1,27]. The application of this strategy must be scientifically justified and can be used to determine AI limits for both small-molecule N-nitrosamine impurities and NDSRIs [1]. An example of this approach is provided in Table 1. For instance, the recommended AI limit for N-nitroso-duloxetine is set at 100 ng/day based on read-across analysis from NNK, which serves as the surrogate N-nitrosamine [37].

1.3.3.3 Temporary approaches

If drug product batches in distribution contain N-nitrosamine impurities at levels exceeding the recommended AI limits, both the FDA and EMA have proposed interim measures to mitigate supply disruptions [1,27]. The FDA’s approach includes updated recommended interim AI limits for certain N-nitrosamine impurities, applicable for a temporary period under specific conditions. At the end of this period, the FDA intends to reassess these interim limits [1].

In contrast, the EMA has adopted a less-than-lifetime (LTL) approach to calculate interim limits for N-nitrosamine impurities above AI limits. This approach is applied only after consultation with competent authorities and serves as a temporary measure until further steps can be taken to reduce the contaminant levels to or below the established AI limits. The recommended interim limits under the LTL approach depend on the treatment duration of the drug product, with two adjustment factors: 13.3× for treatments lasting up to 12 months and 6.7× for treatments exceeding 12 months [27].

Both the EMA’s and FDA’s temporary approaches represent conservative measures to prevent disruptions in drug supply and ensure patients’ continued access to essential treatments.

1.3.3.4 Other approaches

For any N-nitrosamines with sufficient and robust compound-specific carcinogenicity data from animal studies, the TD₅₀ (the dose giving a 50% tumor incidence) should be determined and used to derive a compound-specific AI limit for lifetime exposure as recommended in the ICH M7(R2) guideline [27].

A negative result in a Good Laboratory Practice-compliant enhanced Ames test (EAT) permits the control of the N-nitrosamine at the TTC of 1.5 µg/day. Furthermore, a negative result in a relevant well-conducted *in vivo* mutagenicity study allows the classification of the N-nitrosamine as a non-mutagenic impurity, enabling its control

under less stringent criteria [27]. For instance, transgenic rodent mutation assays study and measure gene mutations *in vivo* for carcinogenic risk and potential assessment. The mutation assay procedure is outlined in the Organisation for Economic Co-operation and Development's (OECD) test guideline [44].

1.4 A 3-step N-nitrosamine investigation guidance

Regulatory agencies have extended the requirement for N-nitrosamine investigations to all medicines, including herbal and homeopathic medicinal products, as well as those containing chemically synthesized APIs and biologicals containing chemically synthesized fragments. Investigations apply to marketed drugs and drugs under marketing authorization applications [1,27]. The FDA has further recommended extending this investigation to semi-synthetic and fermentation products at risk of N-nitrosamine contamination [1]. The investigation guidance follows a standardized 3-step approach [1,27]:

- Step 1: Risk assessment,
- Step 2: Confirmatory testing,
- Step 3: Implementation of risk mitigation measures.

The EMA refers to this procedure as a “Call for Review”. Deadlines for completing these steps depend on the regulatory agency and the product nature or N-nitrosamine class. While the EMA categorizes deadlines based on the nature of chemically synthesized APIs or biologicals, the FDA differentiates deadlines by N-nitrosamine structure (*e.g.*, small-molecule N-nitrosamines versus NDSRIs) [1,27]. The inclusion of NDSRIs is a new requirement introduced in Revision 2 of the FDA's Control of Nitrosamine Impurities in Human Drugs Guidance for Industry [1]. Notably, the FDA's timeline shows a two-year difference between the deadlines for small-molecule N-nitrosamines and NDSRIs. This gap reflects that the discovery and recognition of NDSRIs as a distinct risk emerged approximately three years after the initial detection of NDMA in valsartan [1].

1.4.1 Theoretical risk assessment

The initial step in investigating N-nitrosamine contamination involves a theoretical, risk-based assessment using prior knowledge and industry experience. This approach evaluates the likelihood of N-nitrosamine formation during the manufacturing of APIs, as well as throughout the drug product manufacturing and storage. Collaboration with API manufacturers is essential to ensure a complete and accurate assessment.

The risk assessments should clearly outline the impact of potential N-nitrosamine formation on the product's benefit-risk balance [1,27]. The evaluation should consider all possible root causes of N-nitrosamine contamination, including any pathways that could contribute to N-nitrosamine formation, as detailed in Section 1.5.

If the risk assessment (Step 1) indicates a potential N-nitrosamine presence, confirmatory testing (Step 2) becomes mandatory to confirm their presence or absence in the product. The analytical methods used for Step 2 must be sufficiently sensitive, capable of accurately detecting trace levels of N-nitrosamines [1,27]. Conversely, if a robust risk assessment concludes that N-nitrosamine formation is not possible, no further action is required.

1.4.2 Analytical confirmatory testing

1.4.2.1 Regulatory requirements for analytical methods

During the development of analytical procedures, several technical aspects must be carefully considered. In the context of trace analysis, it is essential to prevent both contamination and *in situ* formation of N-nitrosamines during sample preparation and analysis, as these can result in false-positive results [27]. Given the relatively large quantities of APIs and excipients compared to the trace levels of N-nitrosamines, sample preparation protocols must be meticulously designed. Effective sample preparation enables the removal of interfering substances, minimizes matrix effects, and lowers the detection limit.

Beside sample preparation, an appropriately sensitive analytical method needs to be developed. Regulatory agencies recommend the use of mass spectrometers due to their high specificity and sensitivity in trace-level, multi-analyte analysis [27]. Tandem mass spectrometry (MS/MS) is considered the gold standard for the quantification of trace impurities, owing to its dual mass-to-charge ratio selection, which enhances specificity and sensitivity. When a method is intended for the simultaneous determination of multiple N-nitrosamines, its selectivity/specificity must be demonstrated. Method sensitivity is typically characterized by lower limits like the detection and quantification limits [45]. The required quantification limit depends on the acceptable limit (or specification limit) of a N-nitrosamine in a given product. The acceptable limit in ppm is calculated by dividing the AI limit of a specific N-nitrosamine in ng/day (see Tables 1 and 2) by the maximum daily dose of the product expressed in mg/day [27]. Certain high-dose products, such as metformin or paracetamol formulations (≥ 3 g/day), may face challenges in meeting these sensitivity requirements due to technical limitations. In such cases, regulators have suggested that scientifically justified alternatives may be considered [27].

To assist pharmaceutical companies, organizations such as the FDA, OMCLs, USP, and Eur. Ph. have developed and published validated analytical procedures for analyzing both small-molecule N-nitrosamines and NDSRIs. Additionally, several highly sensitive LC-MS/MS methods have been published in the literature, with detection limits reported as low as 0.75 pg/mL in a synthetic drug product [46] and 5 pg/mL in a biological medicine [47].

1.4.2.2 Possible future testing scenarios

Before performing confirmatory studies, analytical procedures must undergo rigorous validation to ensure the compliance with regulatory standards [1,27]. The use of validated analytical procedures guarantees the quality and reliability of confirmatory testing results, providing confidence to health authorities and safeguarding public health.

Both the EMA and FDA require that API manufacturers, MAHs and applicants test a representative number of API or finished product batches. The number of batches to be tested should be commensurate with the annual production volume and should include both newly manufactured and retained samples that remain within the expiry dates [1,27].

1.4.2.2.1 EMA investigation procedure

Following confirmatory testing (Step 2), the EMA outlines 3 possible testing strategies depending on the test results, as illustrated in Figure 6 [27]:

- Specification omission: No further testing is required.
- Periodic or skip testing: Future testing is performed at release on pre-selected batches or predetermined intervals. This requires assurance that untested batches still must meet all acceptance criteria [48].
- Routine analysis: Future testing is performed on a batch-to-batch basis [48].

Testing scenarios depend on both the sensitivity of the analytical methods and N-nitrosamine levels detected in the product [27]. Even if theoretical levels of N-nitrosamine remain consistently below 10% of the acceptable limit, inadequate method sensitivity may disqualify or exclude a product from specification omission. Conversely, if N-nitrosamine levels exceed the acceptable limit, regulatory actions such as recalls or withdrawals may be necessary following a benefit-risk assessment. In certain circumstances, interim limits may be temporarily established to prevent supply disruptions while risk mitigation measures are being implemented to reduce impurity levels below the acceptable limit. For products still in development, further formulation or process optimization is required to ensure that N-nitrosamine levels remain within safe limits prior to submission of a marketing authorization or new drug application.

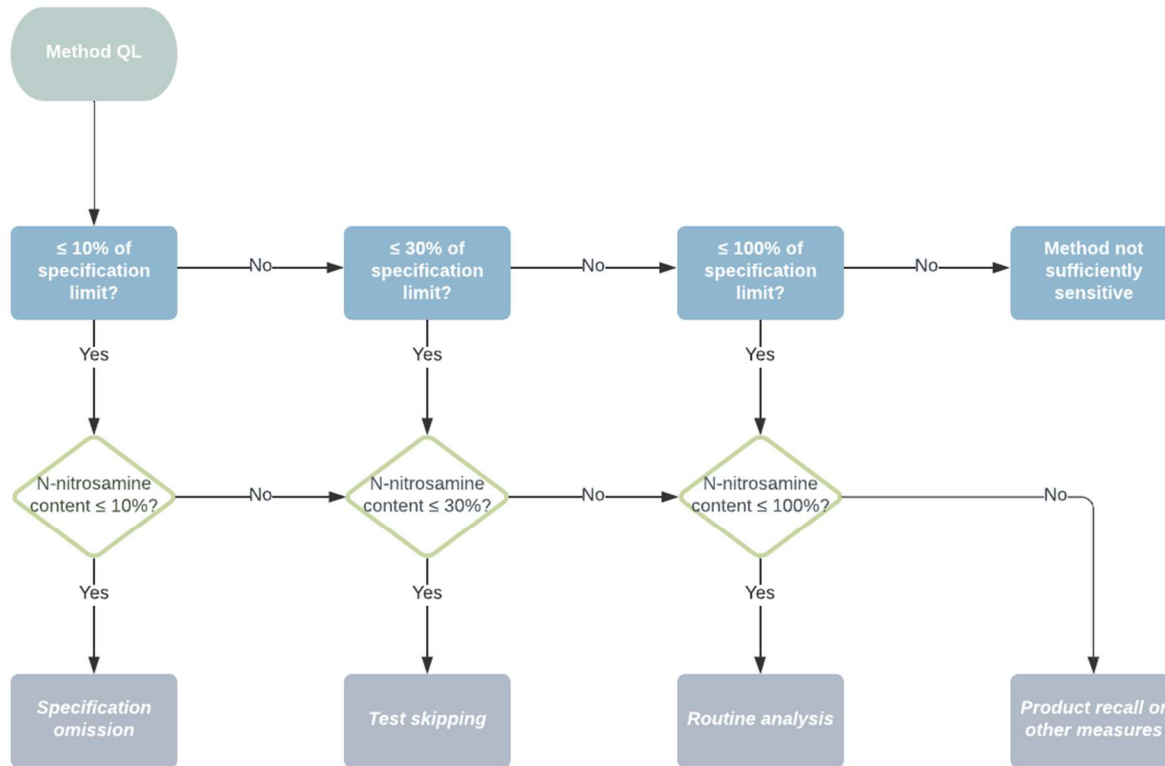


Figure 6. Possible scenarios following analytical method performance and confirmatory testing outcomes according to the EMA investigation approach. QL: Quantification limit.

1.4.2.2.2 *FDA investigation procedure*

In contrast to the EMA's unified approach, the FDA distinguishes between API manufacturers and drug product manufacturers/applicants, with different expectations and testing strategies.

According to FDA guidance, if N-nitrosamines are consistently found above 10% of the acceptable limit in APIs, API manufacturers should test each production batch at release as well as retained stability samples. For drug products, even when N-nitrosamines are detected below 10% of the AI limit, the FDA still recommends testing. As with the EMA procedure, API and drug product batches containing N-nitrosamine levels above the acceptable limit should generally not be released for distribution. However, exceptions may be granted by the regulatory bodies on a case-by-case basis when the API or product is deemed essential to prevent or mitigate a drug shortage [1].

1.4.3 *Comprehensive risk mitigation measure implementation*

As illustrated in Figure 6, under the EMA approach, if N-nitrosamine levels consistently remain below the acceptable limit, indicating compliance with drug safety requirements, no risk mitigation measures are required. However, if the levels exceed the acceptable limit, API manufacturers, MAHs or applicants must implement mitigation strategies based on identified root causes [27].

The FDA takes a more stringent stance, if N-nitrosamines are detected above the quantification limit, API, drug product manufacturers or applicants should develop mitigation strategies to ensure that the level remains within the acceptable limit. Furthermore, the FDA recommends introducing control measures when there is a significant risk of N-nitrosamine formation due to the API structure, the synthetic route, or the manufacturing process [1].

Risk mitigation measures aim to effectively prevent or reduce N-nitrosamine formation in drug substances and products [1,27]. Section 1.6 provides detailed source-specific mitigation strategies proposed by the regulatory agencies to guide pharmaceutical companies in managing contamination risks.

1.5 Sources of N-nitrosamine impurity formation

Pharmaceutical impurity designates any components present in the drug substance or product that are not the API or an excipient [49,50]. Impurities are unwanted compounds that remain in APIs or finished product formulations. N-nitrosamine impurities, including small-molecule N-nitrosamine impurities and NDSRIs, can form through various pathways and at different stages of drug manufacturing, namely i) during the chemical synthesis of APIs, ii) in the manufacturing processes, and iii) during the storage of the drug product formulations.

1.5.1 Chemical synthesis of APIs

During API synthesis, most N-nitrosamines formed are small-molecule N-nitrosamine impurities, which arise from several root causes.

1.5.1.1 Routes of chemical synthesis

The N-nitrosamine crisis in the pharmaceutical industry began with the detection of high levels of NDMA in sartan medications. The root cause was traced to poorly controlled modifications in the synthetic routes of APIs. In particular, the formation of N-nitrosamines was found to be intrinsically linked to the synthetic routes of certain functional groups, such as tetrazole rings in sartans. Azide compounds are commonly employed in the synthesis of nitrogen-containing heterocycles, including tetrazole rings. The tetrazole moiety is typically formed through a reaction between nitrile and azide compounds, often using tributyltin azide as a reagent [51].

The contamination of valsartan API with NDMA was reported by Zhejiang Huahai Pharmaceutical Co. as a consequence of changes to the API manufacturing process, specifically replacing tributyltin azide with sodium azide (NaN_3) in the tetrazole ring-forming step [52,53]. The use of NaN_3 , a more reactive and cost-effective inorganic alternative, resulted in a higher yield of valsartan compared to the original patented method developed by Novartis, which utilized tributyltin azide [53]. However, NaN_3 has poor solubility in organic solvents and is often added in excess to compensate for its limited solubility in dimethylformamide, a preferred solvent employed by the Chinese manufacturer [32]. Sodium nitrite (NaNO_2) is then introduced to quench excess NaN_3 after tetrazole ring formation [52–54]. Under acidic conditions, NaNO_2 can convert to nitrous acid, a strong nitrosating agent [52,55]. Dimethylamine and diethylamine, which are known impurities in dimethylformamide, can undergo nitrosation in the presence of nitrous acid or nitrite under acidic conditions to form NDMA or NDEA, respectively. Such impurities were absent in the originator Novartis product Diovan[®] [53,56].

1.5.1.2 Use of nitrosating agents

Nitrosating agents, such as nitrites and nitrous acid, used in API synthesis can directly contribute to N-nitrosamine formation when secondary, tertiary, or quaternary amines² are present [1]. Moser *et al.* experimentally compared the secondary and tertiary amine reactivity and highlighted that secondary amines are significantly more reactive with nitrite [57]. The nitrosation reaction is most pronounced under acidic conditions, with an optimal pH of approximately 3 to 4 [58]. Nitrite may also come from impurities from solvents, water, and reagents used during API manufacturing.

² The risk of nitrosation of quaternary ammonium compounds is considered negligible.

Even trace amounts of nitrite-contaminated materials can lead to N-nitrosamine formation.

The formation of N-nitrosamines is not limited to the direct use of nitrosating agents, nitrite can also be generated indirectly during various synthetic steps, including via the oxidation of hydroxylamine [32,59]. Notably, Jires *et al.* demonstrated that nitrosation in metformin film-coated tablets can occur even in the absence of nitrosating agents. In this case, the process involves the oxidation of dimethylamine in the presence of strong oxidants such as hydrogen peroxide. The NDMA content formed through this pathway is relatively low, contributing less than 10% of the total impurities [59].

1.5.1.3 Use of low-quality materials

The introduction of low-quality or contaminated materials into the production process inevitably leads to the presence of N-nitrosamine impurities. Sources of contamination include starting materials, intermediates, recycled solvents, reagents, and catalysts. For instance, solvents such as ethyl acetate, ortho-xylene, toluene, and methylene chloride have been reported to contain N-nitrosamines [1,60]. Kosuri *et al.* analyzed commercial solvent samples and found that toluene was contaminated with 550 ppb of NDEA, while ortho-xylene contained 120 ppb of NDEA and 3400 ppb of N-nitrosodi-N-butylamine (NDBA) [60]. Ensuring the quality of raw materials involved in the chemical synthesis is therefore essential to maintaining the quality of the finished product.

1.5.1.4 Reaction parameters

Reaction parameters such as pH and temperature can significantly promote or reduce the yield of API synthesis and influence the formation of N-nitrosamines [1]. Elevated temperatures and humidity exacerbate N-nitrosamine formation during synthesis [32]. Nitrosation reactions are facilitated in the pH range from 3 to 4 [58], while alkaline conditions favor nitrite formation through hydroxylamine oxidation [59]. Additionally, poor control over the sequence of reagent, intermediate, catalyst, or solvent addition may increase the risk of N-nitrosamine formation. Uncontrolled reaction conditions can lead to batch-to-batch inconsistencies in API quality [1].

1.5.2 Manufacturing of finished products

The manufacturing of finished pharmaceutical products includes two critical stages: drug product formulation and packaging. These operations are more commonly associated with the formation of NDSRIs rather than small-molecule N-nitrosamine impurities. NDSRIs predominantly form due to the coexistence of nitrite and nitrosatable drug substances containing amine moieties. Over the past two years, NDSRIs have attracted increasing regulatory attention, their reported numbers have

grown substantially. As of the most recent update in February 2025, the EMA has reported 194 N-nitrosamines, of which 176 are classified as NDSRIs, representing over 90% of the total, while small-molecule N-nitrosamines account for less than 10% [27].

Processes involving nitrite or generating it in the presence of amines should be considered high-risk for N-nitrosamine formation [61]. The reaction between secondary amines and nitrite under acidic conditions is the most likely pathway for N-nitrosamine generation during the manufacturing and storage stages of APIs and drug products [62]. Furthermore, starting materials and intermediates may be susceptible to carry-over or cross-contamination if manufacturing is conducted in shared facilities without sufficient cleaning controls [1,63].

1.5.2.1 Drug product formulation

1.5.2.1.1 Nitrite-contaminated excipients

Excipients play an essential role in the formulation of dosage forms and the selection of delivery routes [64,65]. Although they are pharmacologically inert, excipients contribute various functional roles, such as enhancing the volume or size of dosage forms, promoting disintegration, binding particulates, taste masking, and modifying drug release [64]. However, nitrite contamination in excipients has emerged as a major source of NDSRI formation due to unintended interactions with nitrosatable APIs. Jires *et al.* used ultra-high-performance liquid chromatography coupled with a simple quadrupole mass detector (UHPLC-MS) to evaluate nitrite content in excipients, finding nitrite levels below the QL (0.2 ppm) in most excipients, although some excipients showed nitrite levels up to 3.3 ppm [61]. Schlingemann *et al.* reported nitrite levels of a similar magnitude, with a maximum of 6.1 ppm nitrite detected in excipients [66]. Although small-molecule N-nitrosamines are formed less frequently, they can still occur during manufacturing or storage. For instance, dimethylamine is an impurity in metformin hydrochloride and reacts with nitrite to form NDMA [67].

Interestingly, Moser *et al.* highlighted that secondary amines originating from excipients present a considerably lower risk of N-nitrosamine formation compared to secondary amines that are integrated into the drug substance structures [57]. Nevertheless, higher nitrite levels are generally associated with an increased risk of N-nitrosamine formation in pharmaceuticals [61].

These data highlight the crucial need for rigorous quality control of excipients, particularly those used in solid oral dosage forms, given their prevalence in the pharmaceutical industry, their simplicity and high acceptability by patients [68].

1.5.2.1.2 Manufacturing processes

Purified water, one of the most widely used utilities in the pharmaceutical industry, plays an indispensable role in various steps of API synthesis and manufacturing. It represents a potential source of trace N-nitrosamines and nitrite contamination. The quality of purified water is directly influenced by the quality of the input water [32]. Regarding N-nitrosamine presence, although disinfection with chloramine can generate trace amounts of NDMA and other N-nitrosamines in input water [69], the EMA has stated that water used in pharmaceutical manufacturing is unlikely to be a significant source of N-nitrosamine contamination in APIs. Nevertheless, the potential risk for degradation processes in drug substances due to disinfected water cannot be entirely excluded [70]. Among existing techniques, UV direct photolysis has been identified as the most effective for removing several N-nitrosamines from water [71,72]. In addition, despite ongoing rigorous purification processes, purified water may still contain residual nitrite at very low levels. Suresh Kumar *et al.* developed an ion chromatography (IC) method to quantify nitrite content in manufacturing water. Their study revealed that 21 of 22 purified water samples contained nitrite levels below the quantification limit (QL) of 0.1 ppb, indicating that the risk of N-nitrosamine formation from this source is negligible [73].

Nitrogen oxides (NO_x) in the air may act as nitrosation precursors and react with at-risk APIs [32]. For example, trace levels of nitrite can result from oxidation processes involving NO_x species during drying operations [61,74].

Certain manufacturing processes characterized by excessive energy, temperature, forced air, or high moisture can create favorable conditions for N-nitrosamine formation [1]. Solid oral dosage forms, such as tablets and capsules, remain the most popular pharmaceutical products [68]. Among common manufacturing processes, wet granulation often involves the use of solvents, introducing moisture into the process [75], whereas processes such as jet milling and fluidized bed drying typically operate under high temperatures and pressures [76].

Therefore, manufacturing process parameters must be carefully controlled to ensure batch-to-batch consistency. Poorly optimized process parameters could inadvertently facilitate N-nitrosamine formation. The lack of process control may also lead to significant variability in product quality and inconsistent N-nitrosamine levels across batches [1].

1.5.2.2 Packaging and printing

Packaging materials, integral to pharmaceutical products, are classified as primary, secondary, or tertiary. Primary packaging, which comes into direct contact with the formulation, logically poses the highest risk of interacting with the drug product [77].

Certain polymers, elastomers, and adhesives used in packaging can either contain N-nitrosamines or contribute to their formation under specific conditions, particularly during storage [78,79]. Among these, nitrocellulose-based blisters have been identified as a major concern regarding N-nitrosamine contamination [27]. Blister packaging, widely used for solid dosage forms, often incorporates lidding foils containing nitrocellulose in the print primer and/or lacquer layers [80]. The classical structure of a multi-layer aluminum lidding foil is illustrated in Figure 7 [81].



Figure 7. Structure of a multi-layer lidding foil.

Golob *et al.* demonstrated that nitrocellulose can act as a nitrosating agent, capable of reacting with vulnerable amines in printing inks, *e.g.*, dimethylamine or diethylamine, to form NDMA or NDEA. N-nitrosamines or their precursors may migrate from the packaging materials into the product, particularly when there is direct contact. High-temperature heat-sealing processes can volatilize N-nitrosamines from the lidding foil, allowing their transfer and deposition onto tablets in adjacent blister cavities [81]. The possibility of nitroso-compounds migrating into the blister cavity and subsequently interacting with the bulk product to form NDSRIs cannot be excluded [32].

Additionally, elastomers used as vial stoppers in container closure systems are a well-established potential source of N-nitrosamine contamination in the primary packaging of sterile injectable or parenteral dosage forms [78,82].

While secondary packaging and manufacturing equipment pose a smaller risk, they may still contribute to N-nitrosamine contamination through extractable and leachable substances. However, Regulatory agencies now mandate extractable, leachable, and ink migration studies during Step 1 of the investigation procedure to assess the risks of substance migration from packaging materials to drug formulations [1,77]. For example, the USP has identified N-nitrosamines as “special case” compounds, and 6 N-nitrosamines (NDMA, NDEA, NDBA, N-nitrosomorpholine, N-nitrosopiperidine, and N-nitrosopyrrolidine) are typically investigated as extractables and leachables in orally inhaled and nasal drug products [79]. The assessment of extractables and leachables associated with pharmaceutical packaging and delivery systems will be discussed in Section 1.7.7.

1.5.3 Storage of drug substances and products

Inappropriate storage conditions, such as high temperatures, humidity, or exposure to light, can lead to the degradation of APIs. This degradation compromises the efficacy, quality, and safety of medicines. Under such conditions, N-nitrosamines can form as degradation impurities through chemical reactions such as oxidation, hydrolysis, and photolysis. Additionally, they can arise as byproducts, for instance, following the nitrosation of APIs or API-related substances in the presence of precursors.

For example, Jires *et al.* studied the stability of sitagliptin during storage and identified that the hydrolysis of its amide bond results in the formation of degradation products such as amino acid compounds and 3-(trifluoromethyl)-5,6,7,8-tetrahydro-(1,2,4)triazolo(4,3-a)pyrazine, the latter can react with nitrosating precursors to form N-nitroso-STG-19 (N-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-(1,2,4)triazolo(4,3-a)pyrazine) [83].

Furthermore, chemical reactions or degradation processes occurring within the packaging material itself during storage can also contribute to N-nitrosamine formation.

1.5.4 Combined root causes

Although N-nitrosamine contamination is sometimes attributed to a single source, it is often the result of multiple interacting factors. Understanding these combined root causes is central for implementing effective risk mitigation strategies. This section explores the topic through selected illustrative examples.

1.5.4.1 Metformin

In metformin, dimethylamine, designated as Impurity F in the Ph. Eur. monograph, is limited to a maximum of 0.05% in metformin hydrochloride [84]. Dimethylamine is a well-documented impurity in metformin raw materials and forms NDMA when exposed to nitrite [67]. However, forced degradation studies by Hao *et al.* revealed that NDMA formation is not limited to dimethylamine contamination. These studies demonstrated that metformin degrades into dimethylamine and NDMA under stress conditions such as elevated temperatures, strong alkaline and oxidative conditions. NDMA can also form during the storage of metformin APIs or finished products under inappropriate conditions [85]. It is important to note that NDMA is not the only N-nitrosamine that can form through the nitrosation of synthesis byproducts or degradation impurities.

1.5.4.2 Rifampin and rifapentine

Rifampin and rifapentine are associated with two impurities, MeNP in rifampin, and CPNP in rifapentine [33,36]. Although both MeNP and CPNP have low molecular weights, they are considered NDSRIs due to their structural similarities with rifampin and rifapentine, as illustrated in Figure 8.

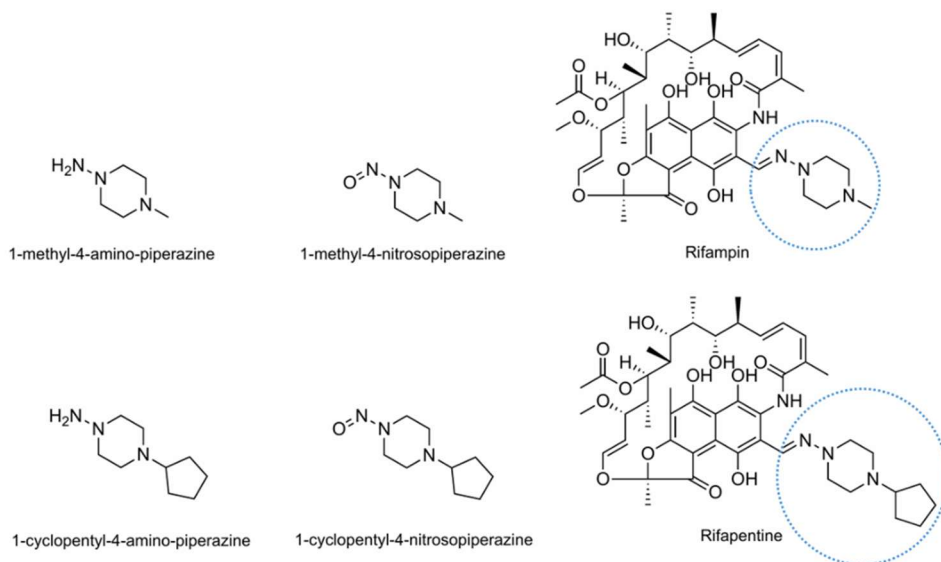


Figure 8. Structures of MeNP, CPNP, MAP, CPAP, rifampin and rifapentine.

MeNP and CPNP can be formed from the synthesis of 1-methyl-4-aminopiperazine (MAP) and 1-cyclopentyl-4-aminopiperazine (CPAP), respectively, using NaNO_2 [86]. These intermediates are key precursors in the synthesis of rifampin and rifapentine [33,36,86]. Tian *et al.* confirmed that MeNP contamination could originate from MAP supplied by manufacturers [86]. Furthermore, higher levels of MeNP and CPNP have been generally observed in drug products compared to their corresponding API batches [36]. This observation may be attributed to the oxidation of MAP or CPAP, or the hydrolysis of APIs during storage, leading to the formation of MeNP and CPNP as degradation products [36,86]. Additionally, the polymorphic form of rifampin API affects the generation of MeNP. Tian *et al.* found that the crystal form I of rifampin is chemically more stable than form II, resulting in significantly lower MeNP content when form I is used [86].

1.5.4.3 Particular case of biopharmaceuticals

This section addresses distinct considerations related to biopharmaceuticals. Although biological drugs generally pose a lower risk of N-nitrosamine formation compared to APIs derived from chemical synthesis, the risk cannot be entirely dismissed. Several studies have identified specific risks associated with biopharmaceuticals, particularly regarding NDLRIs in ADCs. ADCs represent a unique case within the biopharmaceutical category due to their hybrid nature, combining drug components with monoclonal antibodies via chemical drug linkers. The synthetic components within ADCs inherently increase the risk of NDLRIs. Consequently, this therapeutic class is considered higher risk compared to monoclonal antibodies and oligonucleotides, without synthetic components in their structures [20,21,87].

As already mentioned, the quality of water, reagents, and raw materials are critical factors in risk assessment. For instance, the risk of N-nitrosamine contamination in biological products is lower than in synthetic drugs due to the use of water for injection and high-purity reagents or excipients. Furthermore, the presence of nitrosating precursors as impurities in drug linkers is typically negligible, as these precursors can be effectively removed post-conjugation through purification techniques such as ultrafiltration or diafiltration [20,21]. Unlike oral solid dosage forms, biopharmaceuticals are usually packaged in glass containers, which are free from nitrocellulose to minimize the presence of nitrosating precursors. Glass container closure systems are specifically designed to prevent permeation of oxygen, moisture, or microorganism, thereby ensuring sterility and reducing degradation caused by oxidation or hydrolysis. However, when elastomeric materials are used as vial stoppers, the associated risk should be carefully evaluated, as previously discussed [82]. Moreover, biopharmaceuticals are typically stored under controlled conditions and low temperature to maintain product stability and prevent degradation.

1.6 N-nitrosamine risk mitigation measures

When a risk of N-nitrosamine contamination over the acceptable limit is identified, well-established, and confirmed, API manufacturers and (bio)pharmaceutical companies are required to implement mitigation measures. The actions are tailored to address the specific root causes of contamination, aiming to effectively prevent or reduce N-nitrosamine levels in APIs and drug products.

Regulatory agencies have published official documents to guide manufacturers to control N-nitrosamine impurities [1,27]. The mitigation plans focus on specific root causes, including synthesis pathways, manufacturing processes, packaging materials, and storage. Based on the potential sources of N-nitrosamine impurity formation presented in Section 1.5, a summary of possible mitigation strategies is provided in Table 3.

Table 3: Risk mitigation measures.

Stage	Root cause	Mitigation measure
Chemical synthesis of APIs	Routes of chemical synthesis	Use of alternative synthesis routes Use of alternative quenching agents other than NaN_3
	Use of nitrosating agents	Use of alternative reagents Addition of scavengers to remove the residual nitrosating precursors [35]
	Use of low-quality materials	Screening materials for pharmaceutical use [1] Supplier qualification and audits [1]
	Reaction parameters	Control and optimization of reaction parameters to maintain N-nitrosamine contents within acceptable limits and ensure batch-to-batch consistency [1]
Formulation of finished products	Nitrite-contaminated excipients	Screening excipients for nitrite content [61] Supplier qualification and audits [1] Use of low-nitrite excipients [35] Addition of a scavenger [35]
	Uncontrolled manufacturing processed	Control and optimization of process parameters (<i>e.g.</i> , temperature, moisture, energy) to reduce N-nitrosamine formation and ensure batch-to-batch consistency [1]
Packaging and printing	Use of nitrocellulose-based lidding foils	Use of nitrocellulose-free lidding foils or alternative materials [81] Use of alternative packaging such as plastic bottles
	Use of elastomeric vial stoppers	Use of alternative packaging forms
	Inappropriate packaging	Redesigning packaging systems or employing alternative packaging materials/forms to minimize the risk of N-nitrosamine formation or migration

Table 3 Continued.

Stage	Root cause	Mitigation measure
Storage	API degradation - oxidation	Addition of antioxidants to inhibit NDSRI formation in drug products due to oxidation [35] Use of vapor(air)-impermeable packaging
	API degradation - hydrolysis	Addition of desiccant in packaging to prevent or control excessive moisture Use of moisture-impermeable packaging Addition of Na ₂ CO ₃ in tablet formulation to modify the microenvironment to neutral or weak basic pH for inhibition of NDSRI formation in drug products [59,85]
	API degradation - polymorphism	Use of stable crystal forms [86] (without compromising the solubility and processability if possible)
	Inappropriate storage conditions	Avoid exposure to light, and storage under high temperature or humid conditions
	Intrinsic API instability	Shorten the expiration date

1.7 Risk-based prediction tools for N-nitrosamine formation

1.7.1 Nitrite excipient database

As developed in previous sections, N-nitrosamine impurities predominantly originate from nitrite in excipients and amines, whether present as impurities or as functional groups within drug substances. However, the availability of reliable information regarding nitrite levels in excipients remains limited. This lack of data creates challenges for regulators and manufacturers to predict and evaluate the risks of N-nitrosamine formation in medicinal products [65].

To address this challenge, Lhasa Limited, a non-profit organization and educational charity, has established a data-sharing initiative to encourage and motivate pharmaceutical companies to report nitrite concentrations in excipients, measured using validated analytical procedures. Excipients covered in the database are representative of common N-nitrosamine formations. Generally, the nitrite contribution is dominated by major excipients such as diluents (*e.g.*, microcrystalline cellulose, mannitol), which are used in larger quantities in drug products but typically contain low nitrite levels. In contrast, excipients like magnesium stearate (a lubricant) and crospovidone (a disintegrant), although present in smaller amounts, are associated with higher nitrite levels. The assessment also takes into account factors such as the average nitrite content, batch-to-batch variability, and differences between suppliers. The database, which continues to expand, is a valuable tool for supporting the risk assessment of N-nitrosamine presence in drug products [65].

1.7.2 Nitrite testing

The determination of nitrite content in excipients plays a central role in the quality control of excipients and aids indirectly in predicting or assessing the potential N-nitrosamine formation. Commonly employed methods for nitrite determination include IC with conductivity detection and the Griess reaction followed by liquid chromatography coupled with UV detection [65,73]. Both techniques have demonstrated adequate sensitivity and specificity to distinguish nitrite from nitrate [73]. Table 4 summarizes various analytical procedures for nitrite determination in excipients reported in the literature.

However, Yamamoto *et al.* highlighted that other compounds than nitrite, such as NO_x species in excipients, could also contribute to the nitrosation of amines. Focusing solely on nitrite may underestimate the risk of N-nitrosamine formation during manufacturing and storage. To overcome this limitation, a novel approach based on the quantification of total reactive NO_x species in excipients was proposed to evaluate the risk of N-nitrosamine formation [89].

Table 4: Summary of analytical procedures for nitrite determination in excipients.

Technique	Sample	DL	Range	Sample preparation	Column	Mobile phase/gas	Reference
IC-Conductivity suppressor	Purified water	0.033 ppb	0.1 – 0.6 ppb	25-fold concentration	Dionex IonPac RFIC Analytical AS19 column, 4 × 250 mm Dionex IonPac RFIC Guard AG19, 4 × 50 mm	20 mM sodium hydroxide in water	[73]
UHPLC-(ESI+)MS	Excipients and metformin tablets	0.005 ppm	0.2 – 10 ppm	Liquide extraction 2,3-diamino-naphthalene Derivatization	Acquity UPLC HSS T3, 100 × 2.1 mm (1.8 µm)	0.1% formic acid acetonitrile	[61]
GC-MS	Excipients	Not reported	Lower range: 0.05 – 2.5 ppm Upper range: 1.0 – 50.0 ppm	Stearate magnesium: liquid-liquid extraction Carbonates: CO ₂ release Other samples: liquid extraction	DB-624 UI, 30 m × 0.25 mm (1.4 µm)	Helium	[62]

Table 4 Continued.

Technique	Sample	DL	Range	Sample preparation	Column	Mobile phase/gas	Reference
Two dimensional IC-MS	Hydroxypropyl methylcellulose	8.9 ppb	29.6 – 5005.8 ppb	Liquid extraction	¹ D: Dionex IonPac AS-24A, 4 × 250 mm (7.0 µm, 2000 Å) and IonPac AG-24A 4 × 50 mm (11.0 µm, <10 Å) ² D: IonPac AS-19, 2 × 250 mm (7.5 µm, 2000 Å) and IonPac AG-19 2 × 50 mm (11.0 µm, < 1 Å)	Potassium hydroxide in water (concentration not specified)	[88]

IC: Ion chromatography; UHPLC: Ultra-high-performance liquid chromatography; ESI: Electrospray ionization; MS: Simple quadrupole mass spectrometer; GC: Gas chromatography (by analyzing cyclohexene generated from the reaction of cyclamate and nitrite); DL: Detection limit.

1.7.3 Nitrosation assay procedure

In 1978, the WHO Expert Group proposed the nitrosation assay procedure (NAP test) as a general *in vitro* test system for evaluating the nitrosatability of drug substances [90]. The conventional NAP test is conducted under standardized conditions using an API concentration of 10 mM and a nitrite (NaNO_2) concentration of 40 mM. The reaction is carried out at 37°C in an aqueous acidic solution with a pH of 3 – 4 for 1 – 4 hours to facilitate the nitrosation reaction, intentionally leading to the formation of NDSRIs [90].

Regulatory authorities have started recommending NAP tests to assess the risk of NDSRI formation from APIs. It is advised that the NAP test be performed on starting materials, intermediates, and APIs during manufacturing process development [70]. For instance, applicants may include NAP test results as supportive documentation in the N-nitrosamine risk evaluation (Step 1) of the Common Technical Document [27]. The Common Technical Document is a harmonized format designed for the submission of new marketing authorizations or new drug applications across all regions [91].

In 2022, Schmidtsdorff *et al.* applied the conventional NAP test to evaluate the potential formation of NDSRIs via direct nitrosation of drug substances. Among 67 compounds studied, 33 APIs were found to form N-nitroso compounds as a result of API-nitrite interactions [92].

In 2023, Nitish *et al.* introduced a modified version of the NAP test to address certain limitations of the conventional method. These shortcomings include the restricted aqueous solubility of drugs belonging to biopharmaceutical classification system classes II and IV, as well as the degradation of many drugs into unintended by-products rather than NDSRIs. In the modified procedure, the drug is solubilized in an organic solvent and incubated at 37°C with tertiary butyl nitrite as the nitrosating agent in a 1:10 molar ratio. This alternative approach is more reactive and specific, enabling the generation of higher levels of NDSRIs in organic solvents within a shorter time frame [93].

1.7.4 Carcinogenicity prediction – Ames test

If NAP tests yield positive results, regulators recommend further investigations, including the Ames test, in accordance with the ICH M7(R2) guideline. The Ames test, developed by Ames in the 1970s, is an *in vitro* bacterial bioassay designed to evaluate the mutagenic potential of chemical compounds [94]. This test provides direct experimental evidence regarding a compound's ability to induce mutations in bacteria, which correlates with an increased risk of carcinogenicity [95]. Standardized recommendations for conducting the Ames test are outlined in the OECD's test guideline [96].

Ames test data have been incorporated into the Carcinogenic Potency Database and have played an important role in the development of structure-activity relationship (SAR) models [95]. While the Ames test has demonstrated positive results for most N-nitrosamines, a small number of these compounds have shown negative outcomes.

Although the Ames test data serve as highly effective qualitative tools for predicting carcinogenicity in rodent studies, it has limitations in providing a quantitative assessment of carcinogenic potency [97,98]. To establish non-mutagenicity, reliable data from a well-conducted Ames test, as outlined in the ICH M7(R2) guideline, are essential [70]. However, reduced sensitivity has been observed for certain N-nitrosamines under standard Ames test conditions [27,37].

To address this limitation, the FDA recommends using of the EAT, a modified version of the classic Ames test. The EAT is specifically designed to evaluate N-nitrosamine compounds that may exhibit limited sensitivity in the standard Ames test due to constrained metabolic activation [37]. The EAT protocol was developed based on research conducted by the FDA's National Center for Toxicological Research [99].

If the classic Ames test yields positive results, these findings are considered sufficient, and conducting an additional EAT is not required [27,37].

1.7.5 Forced degradation study

While NAP tests are utilized to predict NDSRI formation from drug substances, forced degradation studies serve as a complementary approach to assess the potential formation of N-nitrosamine impurities as degradants [100,101]. These studies involve exposing drug substances and/or products to stress conditions, such as heat, moisture, strong acidic or alkaline environments, oxidation, and photolysis to simulate extreme storage conditions that are more severe than accelerated conditions [102]. Forced degradation studies provide valuable insights, including comprehensive impurity profiling, degradation behavior, structural elucidation of degradation products, and an understanding of degradation pathways and kinetics. These studies are a regulatory requirement for assessing the intrinsic stability of drug substances and products [100,101]. Moreover, they provide insights that guide manufacturing, packaging, and storage optimizations to enhance product stability and minimize impurity formation.

For instance, Hao *et al.* demonstrated that metformin undergoes significant degradation into NDMA under strong oxidative conditions and elevated temperatures, highlighting the importance of controlling such conditions during manufacturing and storage to minimize impurity formation [85]. Tian *et al.* investigated the pathways of MeNP formation in rifampin through forced degradation studies. Their findings revealed that MeNP is not only a by-product of the oxidative degradation of rifampin but also introduced during the synthesis process involving MAP, either through

oxidation or nitrosation reactions [86]. Emery Pharma's stability study revealed that elevated temperatures significantly promote and accelerate ranitidine degradation leading to NDMA formation. The NDMA content in ranitidine API increased 6-fold when stored at 70 °C compared to 25 °C after 12 days. For ranitidine formulations (Zantac®, 150 mg), NDMA levels in a tablet increased from 19 ng at the initial time to 70 ng after 14 days at 70 °C [103].

1.7.6 Stability testing

Unlike forced degradation studies, the purpose of stability testing is to generate evidence on how the quality of an API or finished product changes over time under the influence of temperature and humidity. Another objective is to establish appropriate storage conditions that ensure the quality of the product and to determine the retest period for APIs or the shelf life of finished products. Stability studies should be performed on packaged forms, mimicking real use conditions, to provide supporting data on the suitability of the packaging and potential packaging-product interactions [100].

Stability studies should take into account zone-specific climate factors, particularly temperature and humidity. Several types of stability testing exist. Long-term testing evaluates the stability under recommended storage conditions for extended periods. Intermediate and accelerated testing are conducted under increased temperature and humidity for shorter periods to assess the impact of short-term excursions beyond recommended storage conditions [100]. In line with the guidance, confirmatory testing (Step 2) must include both newly manufactured batches and stability batches stored under long-term conditions and still within their expiry date [27].

1.7.7 Extractable and leachable assessment

Management of extractables and leachables in packaging systems and final drug products has become a critical aspect of pharmaceutical development and regulatory submissions. This is due to their potential safety risks for patients and possible compatibility issues with the drug product. Scientific principles and best practices for the assessment of extractables and leachables are described in the USP general chapters <1663> [104] and <1664> [105], respectively.

Extractables and leachables refer to inorganic and organic chemical entities. Extractables are compounds that can be released from pharmaceutical packaging or delivery system into extraction solvents under controlled laboratory conditions. In contrast, leachables are substances that migrate from the packaging or delivery system into the drug product under normal storage and use conditions, or during accelerated stability studies [104,105].

Extraction studies are conducted to determine the extractable profile of packaging components. It is essential that these studies are carefully designed to ensure the extraction of substances. Key factors include the choice of solvent, extraction time, temperature, and technique. For examples, rubber stoppers from vials may be extracted using buffers at pH 5.2 or 9.5, or a 50%/50% (v/v) mixture of isopropanol and water; the mouthpiece of a dry powder inhaler may be extracted with water or isopropanol; the elastomer seal of a metered-dose inhaler valve may require extraction with organic solvents. Once generated under laboratory conditions, extractables must be characterized to understand their potential impact [104].

After packaging, dosage forms come into direct contact with packaging materials, and interactions can occur. To evaluate the risk of leachables, it is necessary to identify them and determine the level to which they may accumulate in the final product over its shelf life. Among various dosage forms, orally inhaled and nasal drug products are considered the most susceptible to interactions with packaging components [105]. These interactions may lead to the formation of leachables such as N-nitrosamines [79]. A specific case is migration, which involves the transfer of a substance across a physical barrier, for instance, ink migrating through blister packaging into the drug product.

In a recent study, Dalkılıç *et al.* investigated the presence of 15 N-nitrosamines as extractables in various pharmaceutical packaging materials, including polypropylene bags, disposable eye drop containers, low-density polyethylene bottles, and bromobutyl stoppers. Multiple extraction methods such as Soxhlet extraction, reflux, oven heating, and ultrasonic bath were used to extract N-nitrosamines from the packaging components. Obtained results showed that all N-nitrosamines were below the method's detection limits, which ranged from 0.006 to 0.909 ng/mL. In parallel, leachable studies were conducted on commercial drug formulations containing sugammadex, metformin, gliclazide, and paracetamol. These studies also confirmed that none of the formulations contained detectable levels of N-nitrosamines [106].

1.7.8 *In silico* purge-based approach

Potential mutagenic impurities, including N-nitrosamines, can be introduced or formed at any stage during the synthesis of APIs [107]. Relying solely on analytical testing of pharmaceuticals to investigate N-nitrosamine presence poses significant challenges to MAHs and/or applicants. To address this, Burns *et al.* proposed a novel purge-based approach in 2020. This conservative, risk-based strategy maintains regulatory confidence and ensures patient safety [54]. This approach also offers the benefit of minimizing costs and time associated with analytical development and testing, when potential mutagenic impurities are unlikely to be present in the final drug product [108].

In the purge-based approach, purge factors are calculated by evaluating physicochemical properties, such as reactivity, solubility, volatility, and acid-base characteristics (pKa/pKb), in relation to the reactions involved, to estimate the likelihood of impurity removal [54]. Understanding N-nitrosamine reactivity is essential for assessing the risk of their presence in the final product and evaluating their expected removal during existing chemical processes [109].

MirabilisTM is an *in silico* risk assessment tool developed through collaboration between Lhasa Limited and pharmaceutical companies [54,107]. This software standardizes purge factor calculations to enhance the control and management of potential mutagenic impurities, harmonizing industry practices. In 2022, the release of MirabilisTM 4 introduced refinements for improved predictive accuracy [108]. The tool utilizes advanced predictive technologies to evaluate PMI formation risks and identifies potential impurity structures based on the reagents and conditions used during each step of API manufacturing. By integrating the purge-based approach with *in silico* tools, the industry can perform rapid, consistent, and reproducible risk assessments for mutagenic impurities [54].

1.8 Conclusion

Facing the challenges posed by N-nitrosamine impurities, regulatory guidelines have rapidly evolved. Regulatory bodies have required API manufacturers and (bio)pharmaceutical companies to adopt a proactive, systematic 3-step approach for controlling these impurities. The approach involves evaluating contamination risks, performing analytical confirmatory testing, and implementing mitigation strategies.

The establishment of collaborative platforms such as the USP Nitrosamine Exchange, combined with advancements in the analytical chemistry field, has significantly enhanced the industry's ability to respond effectively. While the current understanding of small-molecule N-nitrosamines has progressed significantly, recent concerns focusing on NDSRIs and NDLRIs highlight persistent challenges. Further research is essential to refine risk assessment methodologies and establish robust carcinogenic potency data for these emerging impurities.

The aim of this review is to provide a clear overview of the current situation of N-nitrosamine control in pharmaceutical products. It presents methodologies used to determine acceptable daily intake limits for N-nitrosamines and traces the evolution of regulatory guidelines, offering a comparative analysis of the 3-step investigation approaches adopted by the EMA and FDA. The review examines potential root causes for N-nitrosamine contamination, outlines the analytical requirements for confirmatory testing, and describes mitigation strategies to prevent or minimize contamination. Additionally, it summarizes risk assessment tools used to predict N-nitrosamine formation.

Finally, by providing a comprehensive workflow for N-nitrosamine impurity investigation, this review also aims to support API manufacturers and (bio)pharmaceutical companies in managing N-nitrosamine risks. It emphasizes the need for continued vigilance in light of their carcinogenic potential and highlights the importance of innovation and collaboration among regulatory agencies, pharmaceutical manufacturers, and researchers to minimize the presence of N-nitrosamine in medicines, thereby ensuring drug safety.

Acknowledgements

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2 Analytical Quality by Design

Analytical activities occupy a central position within the pharmaceutical industry and play a fundamental role in ensuring product quality throughout the pharmaceutical lifecycle (Figure 9). Reliable analytical procedures support drug development by enabling the characterization of each critical quality attributes and by ensuring appropriate process monitoring and control during manufacturing. In addition, they are essential for routine quality control activities, including raw material testing, intermediate product control, batch release, impurity profiling, and stability studies, thereby contributing to the safety, efficacy, and consistent quality of drug substances and drug products [110–112].

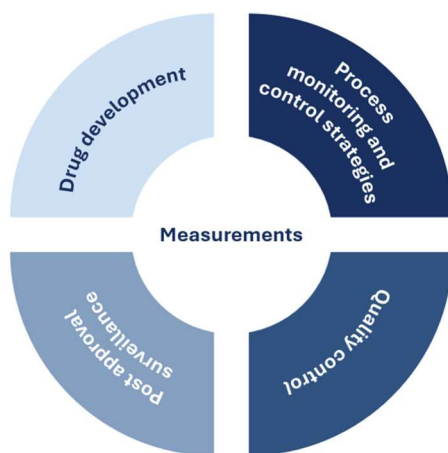


Figure 9. Integration of analytical measurements within the ICH quality framework across the product lifecycle.

Traditionally, analytical procedure development has relied on empirical strategies, often based on trial-and-error experimentation or the one-factor-at-a-time (OFAT) methodology [111]. The trial-and-error approach lacks prior science-based understanding and is often time-consuming and resource-intensive [113]. In the OFAT approach, one experimental factor is varied while all others are kept constant in order to optimize a response [114,115]. Although such approaches may lead to functional analytical procedures, they generally require a large number of experiments and provide little information regarding potential interactions between analytical method parameters [111,116].

Over the past decades, regulatory authorities have encouraged a paradigm shift toward more systematic and science-based development strategies through the implementation of Quality by Design (QbD). The concept was initially developed by Joseph M. Juran and was later introduced into the pharmaceutical field in the early

2000s as a science-driven and risk-based approach to process development and manufacturing and then later introduced to the pharmaceutical industry in early 2000's as new science-based, risk management approach for manufacturing process [117–119].

Initially, QbD principles were mainly applied to drug manufacturing processes. However, analytical procedures are essential components of pharmaceutical quality systems, as they verify compliance with product specifications. Consequently, QbD principles were progressively extended to analytical method development, leading to the concept of Analytical Quality by Design (AQbD). The AQbD approach aims to establish a structured framework for analytical method development that integrates prior knowledge, risk assessment, and design of experiments (DoE) to enhance method understanding, and regulatory flexibility through the method lifecycle [120].

2.1 Regulatory framework

2.1.1 *Quality by design*

Historically, pharmaceutical quality was primarily ensured through extensive end-product testing. However, this approach provides only limited assurance of consistent product performance and does not adequately address product variability originating from manufacturing processes. To overcome these limitations, regulatory authorities introduced the QbD paradigm to pharmaceutical industry, initially promoted by the United States Food and Drug Administration (FDA) [118].

The QbD concept was subsequently incorporated into the regulatory framework through guidelines issued by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). In particular, the guideline ICH Q8 (R2) defines QbD as *“a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management”* [119]. Complementary guidelines, including ICH Q9, ICH Q10, and ICH Q12 further establish the foundations of a comprehensive quality management system across the entire product lifecycle [120–123]. Unlike the traditional quality-by-testing paradigm, in which product quality is mainly assessed through end-product testing, QbD promotes the concept that quality should be built into products and processes by design. As emphasized by Juran, most quality failures originate from inadequate design rather than from manufacturing execution [117].

Over the past two decades, regulatory bodies have increasingly encouraged the pharmaceutical industry to adopt QbD principles in pharmaceutical development. Within this framework, quality is incorporated into pharmaceutical products and processes from the earliest stages of development, rather than being assessed solely

through final product testing. This philosophy promotes enhanced understanding and control of manufacturing processes based on scientific knowledge and quality risk management. Consequently, QbD contributes to reducing product variability and the occurrence of out-of-specification results, while improving overall product quality and manufacturing efficiency. Moreover, by minimizing batch failures and associated financial losses, QbD also supports the production of safe and effective medicines of consistent quality for patients [119,124]. Maintaining consistent pharmaceutical quality is essential to ensure the availability, safety, efficacy, and quality of every dose administered to patients.

Although the initial focus of QbD guidelines was on drug substance and drug product manufacturing processes, analytical procedures represent a critical component of pharmaceutical quality systems. As they ensure compliance with product specifications, the principles of QbD were progressively extended to analytical procedure development, leading to the concept of AQbD.

2.1.2 Analytical quality by design

International regulatory authorities increasingly encourage the implementation of systematic and science-based approaches in analytical procedure development. According to ICH Q14 guideline, the objective of analytical development is to establish analytical procedures that are fit for their intended purpose and capable of reliably measuring a quality attribute with adequate performance [120].

Within the regulatory framework, the principles of QbD have progressively been extended to analytical methods through the concept of AQbD. Analogous to a manufacturing process, an analytical procedure can be considered an analytical process, in which method parameters are equivalent to process parameters. The analogies and equivalences between QbD and AQbD are illustrated in Figure 10 [111,119]. From this perspective, AQbD provides the same benefits as QbD while offering a structured alternative to traditional trial-and-error approaches for analytical method development.

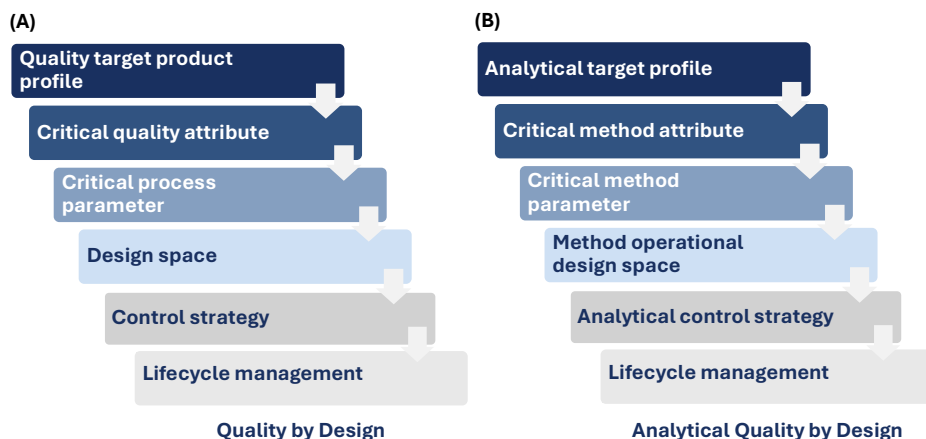


Figure 10. Conceptual parallelism between (A) QbD applied to pharmaceutical manufacturing and (B) AQbD applied to analytical procedure development.

The AQbD framework is formally recognized as an enhanced approach to analytical procedure development in ICH Q14 guideline [120]. In addition, regulatory authorities such as the Medicines and Healthcare products Regulatory Agency and the U.S. FDA have incorporated AQbD principles into the British Pharmacopoeia and the United States Pharmacopeia (USP), respectively [125,126]. In contrast to minimal development concepts, AQbD integrates elements such as prior knowledge, structured risk assessment, DoE, and lifecycle-oriented continuous improvement. This approach facilitates and accelerates analytical method development while improving method knowledge and enabling greater regulatory flexibility throughout the method lifecycle [114,120]. More specifically, AQbD integrates systematic knowledge management and risk management approaches to enhance method reliability, thereby reducing the risk of inadequate analytical performance and erroneous results, while enabling continuous method improvement throughout the product lifecycle [120].

As illustrated in Figure 11, ICH Q14 together with ICH Q2 (R2) define the regulatory framework for science- and risk-based analytical procedure development and validation [45,120]. These guidelines introduce the concept of analytical procedure lifecycle management, encouraging deeper understanding of analytical methods and continuous monitoring of their performance. Furthermore, these guidelines are closely connected with other ICH quality guidelines, including ICH Q8, ICH Q9, and ICH Q10. Together, they establish a comprehensive regulatory framework addressing pharmaceutical development, quality risk management, manufacturing processes, and lifecycle management of pharmaceutical products and analytical procedures [119,121–123,127,128].

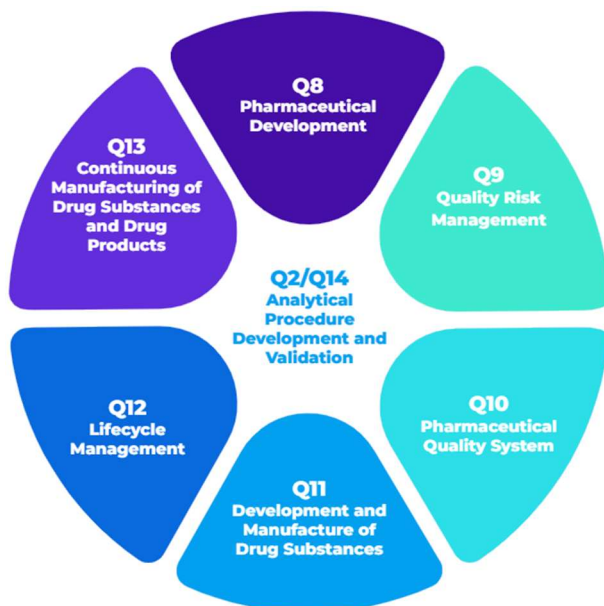


Figure 11. Central role of ICH Q2 (R2) and ICH Q14 within the ICH quality guidelines framework.

2.2 Analytical procedure lifecycle

ICH Q12 guideline focuses on the technical and regulatory aspects of lifecycle management for pharmaceutical products [123]. In ICH Q14 guideline, the concept of lifecycle management has been extended to analytical procedures [120].

Analytical procedure lifecycle management has also been introduced and described in the general chapter <1220> of the USP [126].

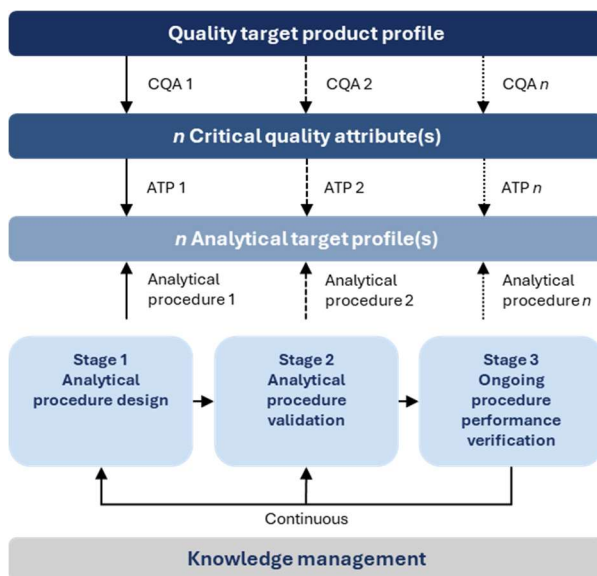


Figure 12. Main stages of the analytical procedure lifecycle. Adapted from references [120,126].

The main stages of analytical procedure lifecycle management are illustrated in Figure 12.

- Stage 1, analytical procedure development, involves the selection of an appropriate analytical technique and the identification of optimal conditions for method parameters in order to achieve the desired method performance as described in the analytical target profile (ATP).
- Stage 2 corresponds to analytical procedure qualification, during which the method undergoes validation to demonstrate that it meets the requirements for its intended application.
- Stage 3, ongoing procedure performance verification, takes place during routine use of the validated method and involves continuous monitoring to ensure that the expected performance is consistently maintained.

If the method fails to meet the predefined performance criteria, appropriate changes must be implemented and managed. When necessary, redevelopment and revalidation of the analytical procedure should be considered.

2.3 Workflow of analytical quality by design

2.3.1 Analytical target profile

The AQbD workflow begins with the definition of the ATP, which describes the purpose of the analytical procedure and the performance characteristics required for

its intended application. The ATP forms the foundation of analytical development and provides a clear description of the desired analytical performance characteristics, together with the associated acceptance criteria necessary to measure a product quality attribute. Consequently, the ATP aligns with a specific product critical quality attribute derived from the quality target product profile (Figure 12) [120,126].

2.3.2 Knowledge management

Knowledge management plays a central role in analytical procedure development and throughout its lifecycle. A key concept of the AQbD approach is prior knowledge, which may originate from scientific literature, previously acquired experimental data, historical data, or accumulated laboratory experience. The integration of prior knowledge facilitates science-based analytical development and may accelerate the method development process. Knowledge is continuously generated, refined, and actively managed throughout the analytical procedure lifecycle [120,129].

2.3.2.1 Technological choice

Prior knowledge of the pharmaceutical product, including its quality target product profile and critical quality attributes, is essential for accurately defining the ATP. In parallel, product-related knowledge and relevant regulatory guidelines contribute to the selection of an appropriate analytical technique and suitable instrumentation capable of fulfilling the requirements defined in the ATP before starting method development [120,129].

2.3.2.2 Identification of critical method attributes

Critical method attributes (CMAs) correspond to the responses of an analytical procedure that ensure the desired quality of analytical measurements within appropriate limits, ranges or distributions [111]. An in-depth understanding and comprehensive identification of CMAs are essential for controlling the performance of an analytical procedure. The identification of CMAs strongly depends on technological prior knowledge, as different analytical techniques may lead to different CMAs [129].

2.3.3 Quality risk management

Quality risk management is encouraged and should be initiated at an early stage of analytical development. Risk assessment, supported by prior knowledge, aims to identify factors that may affect method performance, represented by CMAs [126]. All steps of the analytical procedure should be considered during risk evaluation, including standard preparation, sample preparation, instrumental analysis, and data processing [120,126].

To facilitate risk identification, qualitative risk assessment tools such as process mapping and Ishikawa diagrams (or fishbone diagrams) may be employed to identify analytical procedure parameters that could potentially impact analytical performance. Alternatively, quantitative tools such as failure mode and effects analysis and failure mode, effects, and criticality analysis can be used. The quantitative approaches integrate ranking systems and risk priority numbers to assign numerical values to each method parameter, enabling prioritization based on the degree of risk criticality [115,120,121,126]. High-criticality risks are typically associated with high severity, high probability of occurrence, and low detectability of potential failures.

Based on the results of the risk assessment, the most crucial method parameters are selected from the initially identified set, and are defined as critical method parameters (CMPs). CMPs correspond to experimental factors that influence the CMAs. These parameters include both sample preparation conditions and instrumental settings [129].

The set of CMPs defines the knowledge space to be investigated. Within this space, the relationships between CMPs and CMAs, including the influence of potential interactions between different CMPs on CMAs, are evaluated through experimentation. The integration of quality risk contributes to a better understanding of the method [126,129].

2.3.4 Design of experiment

Experimentation is a direct manner to generate data and information for evaluating the influence of CMPs on CMAs. DoE is an effective statistical tool that enables experiments to be conducted on a scientific basis [126].

In contrast to OFAT experimentation, DoE allows the simultaneous evaluation of multiple method parameters, enabling the identification of main effects, interactions, and potential quadratic effects on responses while reducing the number of experiments, development time, and associated costs [114,130].

2.3.4.1 Screening

At the early development stages, knowledge regarding the influence of CMPs on CMAs is often limited. CMPs identified during risk assessment are included in the screening phase. The primary goal of this phase is to identify the parameters that significantly influence CMAs. Although a large number of CMPs may initially be considered, the application of DoE significantly reduces the number of required experiments and accelerates the screening process. Only the parameters demonstrating a strong impact on CMAs are retained for further optimization studies [131].

2.3.4.2 Optimization and response surface methodology

Experimental domains often contain maxima, minima, or saddle points. For this reason, response surface methodology is often applied during optimization phase for estimating interactions and quadratic effects of CMPs on CMAs. In these designs, CMPs are typically evaluated at three or more levels in order to detect potential curvature in the response surface [111,131]. This phase aims to explore the ranges of CMPs, evaluate their interactions, and assess their effects on CMAs within the knowledge space in order to optimize analytical performance [126]. It is supported by risk assessment or its review when an initial screening phase has already been performed to prioritize and reduce the number of interactions to be investigated.

2.3.5 Method operational design region and robustness

The ultimate objective of the AQbD concept is the establishment of a method operable design region (MODR). The MODR is a multidimensional space comprising ranges of analytical procedure parameters within which the ATP is consistently fulfilled. Within this region, the analytical procedure guarantees the quality of analytical measurements, demonstrating that the method is fit for its intended purpose. Therefore, the MODR is considered a robust operating region for analytical procedure parameters. An analytical procedure is considered robust if it remains unaffected by small but deliberate variations in method parameters while maintaining suitability during normal use [45,120,126]. In contrast to the traditional approach, in which robustness is generally evaluated at the end of method development, the AQbD strategy integrates robustness assessment from the early stages and throughout the entire development process [120,132].

Regulatory authorities encourage applicants to include MODR alongside with analytical procedure validation [120]. The MODR should be investigated using well-designed experiments (*i.e.*, DoE) and consider potential multivariate interactions between parameters. Once the MODR is validated and approved, adjustments to method parameters within this region generally do not require any regulatory notification, thereby providing greater regulatory flexibility while maintaining compliance and robustness throughout the product lifecycle [120,126].

2.3.6 Working point selection

The MODR defines a range of acceptable operating conditions, whereas a working point corresponds to a specific set of conditions within this region. Multiple working points may be considered within the MODR but a single working point is selected for analytical procedure validation. The selection can be based on the greatest desirability index or on the highest probability that CMAs will meet ATP requirements. Practical aspects may also influence this selection. However, robustness should remain a key factor when choosing the final working point for validation studies [111,133].

2.3.7 *Analytical procedure validation*

ICH Q2(R2) guideline describes the methodology for analytical procedure validation, which represents a key stage of the analytical procedure lifecycle [45].

Validation studies aim to demonstrate that an analytical procedure is suitable for its intended purpose. Within an AQbD framework, this involves verifying that the analytical procedure meets the predefined performance criteria described in the ATP, which serve as the basis for validation acceptance criteria. Typical performance characteristics described in ICH Q2(R2) include specificity, range, linearity, accuracy, and precision. For identification methods, demonstration of specificity is generally sufficient. In contrast, quantitative methods require validation of additional characteristics such as range, accuracy, and precision, which can be jointly evaluated using a combined approach [45,120,126].

2.3.8 *Analytical control strategy*

An analytical control strategy consists of a planned set of controls implemented to ensure that the analytical procedure consistently performs as expected. The control strategy should be defined during the method development stage. Effective monitoring of analytical procedures provides continued confidence in their performance during routine use [126]. System suitability test (SSTs) are performed alongside sample analyses to verify the ongoing performance of the analytical system at the time of testing [134]. Both the European Pharmacopoeia and the USP incorporate SST requirements in monographs.

Analytical procedures developed using the AQbD approach benefit from a well-characterized risk profile, facilitating the anticipation of method performance during routine use and enabling identification of potential sources of variability. The ATP and CMAs form the basis of the analytical control strategy. In practice, CMAs may be monitored through SSTs to ensure that the analytical method remains compliant with ATP requirements [111]. SSTs are preliminarily designed during method development based on risk assessment and method understanding, in order to ensure that the procedure meets the ATP in routine applications [120,126]. These tests are subsequently verified during validation studies.

If SST criteria are not met, the analytical sequence cannot be considered valid and the generated data must be discarded. In such cases, the root cause of the deviation should be investigated in order to prevent recurrence. In the absence of SSTs, the performance of the analytical system cannot be effectively monitored.

3 Hyphenated technique selection

As previously mentioned in Section 2 of this chapter, analytical development within an Analytical Quality by Design framework begins with the selection of an appropriate technology based on the analytical target profile and prior knowledge. This section introduces the hyphenated technique employed throughout the thesis. First, the principles of the separation technique are briefly described, followed by those of the detection technique. Finally, the analytical challenges encountered during method development and the strategies implemented to overcome them are discussed.

Hyphenated techniques usually refer to the integration of a separation technique with a detection technique into a single analytical system, commonly designated as a “hyphenated technique”. Hyphenation does not necessarily involve only two techniques. In some cases, more than two techniques may be combined, resulting in so-called multi-hyphenated techniques [135,136].

Among hyphenated techniques, chromatography remains the most widely used separation technique [135]. Chromatographic separation is based on the differential distribution of analytes between a stationary phase and a mobile phase. The mobile phase flows through the stationary phase and transports the analytes. Separation occurs because analytes interact differently with the stationary and mobile phases depending on their physicochemical properties. The mobile phase may be either a gas or a liquid. When the mobile phase is a gas, the technique is referred to as gas chromatography (GC). Conversely, when the mobile phase is a liquid, the technique is known as liquid chromatography (LC) [137].

GC and LC are complementary techniques with distinct application domains determined by analyte physicochemical properties. GC is primarily used for the analysis of volatile and thermally stable compounds, whereas LC is more versatile and applicable to a broader range of compounds, including non-volatile, polar, and thermally labile analytes. A comparison of GC and LC applications and separation principles is presented in Table 5.

Table 5: GC and LC applications and separation principle comparison [137–139].

	LC	GC
Analyte properties	Volatile and non-volatile Low to highly polar Thermally labile and stable Small molecules to large biomolecules	Volatile Non-polar Thermally stable Primarily small molecules
Sample type	Analytes must be dissolved in a suitable solvent prior to injection	Gas, liquid, or solid samples
Stationary phase dimension	Shorter and wider columns Length: 5 – 30 cm Internal diameter: 2 – 5 mm	Longer and narrower columns Length: 15 – 105 m Internal diameter: 0.10 – 0.53 mm
Mobile phase	Aqueous and organic solvents	Helium, hydrogen, or nitrogen
Separation mechanism	Based on analyte polarity and ionic properties	Based on analyte volatility
Operating temperature	Typically 20 – 40 °C (can be higher depending on the method)	Typically 150 – 300 °C

Chromatography is commonly coupled to mass spectrometry (MS) to address complex analytical challenges [135]. Therefore, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was selected in this thesis to overcome the various analytical challenges associated with N-nitrosamine determination.

3.1 Liquid chromatography

Among chromatographic techniques, LC-MS(/MS) is currently the most widely used hyphenated technique due to the broad applicability and versatility of LC. Various LC approaches exist because no single chromatographic mode provides universal applicability for all analytes. Different LC types offer selectivity based on analyte physicochemical properties, charge, and polarity, as illustrated in Figure 13.

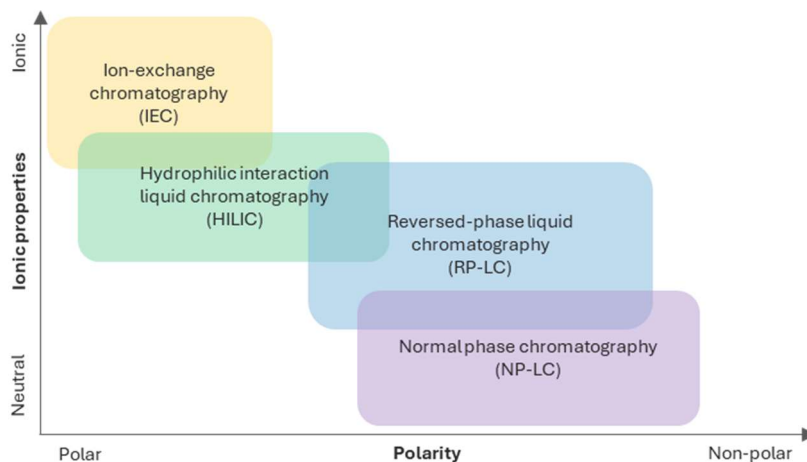


Figure 13. Comparative overview of LC types, illustrating their respective applications as a function of analyte polarity and ionic properties. Adapted from reference [140].

LC can be classified into different generations of instrumentation, namely conventional high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC). In this thesis, an UHPLC instrument was used throughout the experimental work rather than HPLC.

UHPLC was introduced in 2004 by Waters Corporation and represents an advancement over HPLC. UHPLC systems utilize columns packed with sub-2 μm particles, typically based on reversed-phase stationary phases [141]. As shown in Figure 14, smaller particles improve efficiency by decreasing height equivalent of a theoretical plate, resulting in higher resolution. When particle size decreases, mass transfer is improved, and the mobile phase optimal velocity tends to higher values meaning faster separations [142,143]. However, they also generate significantly higher backpressures, requiring instrumentation specifically designed to operate at ultra-high pressures. For this reason, UHPLC is also referred to as ultra-high-pressure liquid chromatography [141,142].

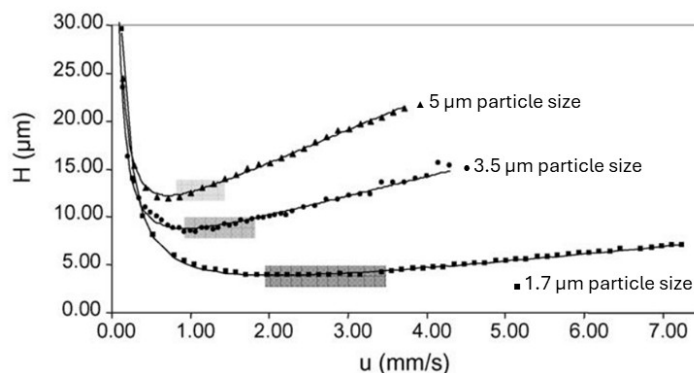


Figure 14. Effects of particle size on height equivalent of a theoretical plate (H) and mobile phase linear velocity (u). From reference [143].

3.2 Mass spectrometry

MS is a highly sophisticated and powerful analytical detection technique that is widely employed across diverse scientific fields for the identification and quantification of a broad range of chemical compounds. Owing to its superior sensitivity and specificity, MS has become an indispensable tool in modern analytical sciences [144,145].

In biomedical and clinical research, MS is extensively used for biomarker discovery and disease diagnosis [146,147]. In environmental analysis, it plays a crucial role in monitoring pollutants in air, soil, and wastewater [146,148,149]. In food safety, MS is applied to the detection and quantification of pesticide residues and other contaminants [145,146]. Furthermore, MS is widely utilized in forensic science to support criminal investigations through the identification of drugs and toxins in biological fluids and tissues, as well as the detection of gunshot residues and explosive materials [150,151]. In pharmaceutical analysis, MS contributes significantly across the entire drug development pipeline, ranging from early-stage drug discovery to routine quality control processes [152].

MS is a destructive analytical technique [153], as analytes are ionized and transferred into the gas phase during analysis. The generated ions, which may carry either positive or negative charges, are characterized by their mass-to-charge (m/z) ratios [144]. These ratios serve as fundamental parameters for compound identification and structural elucidation.

In most analytical applications, MS is coupled with separation techniques such as LC, SFC, GC, and capillary electrophoresis [144,146,154]. This hyphenation enhances analytical performance by integrating high separation efficiency with the superior specificity of MS detection in complex matrices. Thanks to its high specificity and extremely high sensitivity, MS often outperforms conventional detection methods.

Consequently, the use of MS has increased greatly over recent decades, and it is expected to become the detector of choice for most chromatographic applications across many scientific disciplines.

The principal components of a mass spectrometer are illustrated in Figure 15. A typical mass spectrometer consists of an inlet system that enables the introduction of samples from LC or GC systems. After entering the instrument, analytes are transferred to the ion source, where they are vaporized and ionized to form positively or negatively charged molecular ions. Following ionization, the generated ions are guided into the mass analyzer, where they are separated according to their m/z ratios. The separated ions are subsequently detected by the ion detector, which records their m/z values and corresponding ion abundance and transmits this information to the data acquisition system [144]. Both the mass analyzer and the detector operate under ultra-high vacuum conditions, typically in the range of 10^{-6} to 10^{-9} bar [155], which are essential to ensure efficient ion transmission.

The data system collects the acquired signals and generates output in the form of a mass spectrum. In a mass spectrum, the m/z ratio is represented on the x-axis, while the signal intensity is displayed on the y-axis [153]. Each peak corresponds to a unique m/z value present in the sample, and the peak intensity reflects the relative abundance of the corresponding analyte.

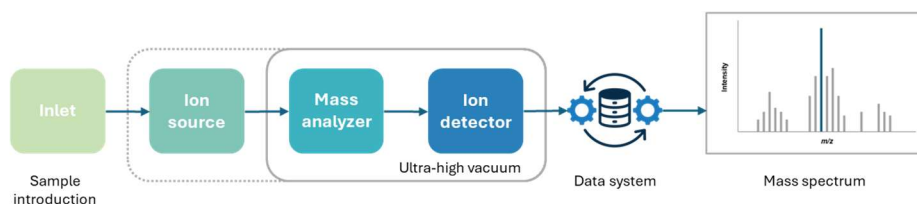


Figure 15. Schematic representation of the different parts of the mass spectrometer. Adapted from reference [153].

3.2.1 Ion sources

The primary role of ion sources is to transfer analytes from samples into gas-phase, generally by adding or removing protons, thereby generating positively or negatively charged species [146,156,157]. As mentioned previously, since MS detection is based on m/z ratios, ionization of compounds is essential for their detection.

A wide variety of ion sources are available for mass spectrometers, each designed to accommodate different inlet techniques and sample characteristics. This section focuses on ion sources compatible with LC systems, namely electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). All three are atmospheric-pressure ionization (API) techniques that generate ions from analytes in solution [156].

The main characteristics and applications of ESI, APCI, and APPI are compared and summarized in Table 6. All three ionization techniques cover a specific range of applications considering analytes physicochemical properties regarding the polarity, molecular weight, thermal stability, and volatility.

Table 6: Comparative characteristics and applications of ESI, APCI, and APPI [144,153,156,158].

	ESI	APCI	APPI
Ionization mechanism	Ionization occurs primarily in the liquid phase through charged droplets	Ionization occurs in the gas phase by corona discharge needle	Ionization occurs in the gas phase by UV irradiation
Ionization softness	Softest	Soft	Softer
Types of ions formed	Monocharged and multicharged ions, enables analysis of high-mass molecules	Monocharged ions, limiting the effective mass range	Monocharged ions, limiting the effective mass range
Matrix effects	Susceptible to matrix effects, particularly ion suppression	Lower sensitivity to matrix effects compared with ESI	Lower sensitivity to matrix effects compared with ESI
Typical analyte mass range	Low to high-mass molecules (<i>i.e.</i> , > 1000 Da)	Low-mass molecules	Low-mass molecules
Analyte polarity	Moderate to high	Low to moderate	Low to moderate
Analyte thermal stability	Not required	Must be thermally stable	Must be thermally stable
Analyte volatility	Not required	Require some degree of volatility	Require some degree of volatility

The following discussion will focus on ESI and APCI, as both ion sources were employed in the work presented in this thesis. Their mechanisms are complementary and provide distinct advantages.

3.2.1.1 Electrospray ionization

ESI is the most widely used ion source and is estimated to be employed in more than 90% of LC-MS(/MS) applications [159]. Operating under atmospheric pressure, ESI is one of the softest ionization techniques, associated with minimal in-source fragmentation. In-source fragmentation often refers to the undesired dissociation of ions during their transfer from an atmospheric pressure ion source to the vacuum region of the mass spectrometer [157]. ESI is particularly suitable for a broad range of analytes, from small molecules to large biomolecules such as proteins, including

compounds with moderate to high polarity and molecules that do not require volatility or thermal stability [158]. These remarkable advantages contribute to the widespread adoption of ESI and make it the primary choice considered during method development.

In LC-MS(/MS) analysis, analytes are transported by the mobile phase and introduced into the mass spectrometer in solution. Ionization process in ESI positive mode is represented in Figure 16. The eluent passes through a stainless-steel capillary and is nebulized at its outlet into a fine spray of charged droplets under the influence of a strong electric field and a nebulizing gas, typically nitrogen. This process results in the formation of electrically charged droplets. Subsequently, solvent evaporation occurs, leading to progressive droplet shrinkage. As the droplets decrease in size, the surface charge density increases until repulsion exceeds surface tension, releasing ions into the gas phase. To enhance solvent evaporation and reduce cluster formation, a drying gas is introduced into the ion source region [144,158]. The proportion of organic solvent in the mobile phase plays a critical role in ESI. Higher organic solvent content facilitates droplet desolvation and evaporation, leading to more efficient ion formation and improved signal intensity [160].

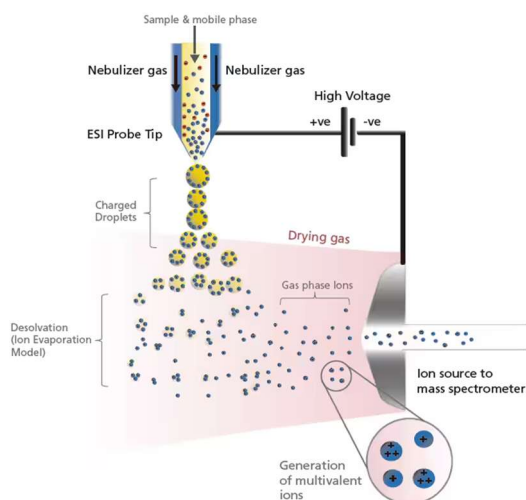


Figure 16. Schematic of the ionization process in ESI positive mode. From reference [158].

ESI is extensively applied in biochemical, pharmaceutical, and medical research, because it generates singly and multiply charged ions, it is particularly advantageous for the analysis of high-molecular-weight molecules [144].

3.2.1.2 Atmospheric pressure chemical ionization

APCI enables the analysis of a wide range of compounds, including pharmaceuticals, environmental contaminants, and petrochemical constituents. In contrast to ESI, APCI is more suitable for nonpolar to moderately polar compounds with a small molecular weight [153,156]. However, due to the elevated operating temperatures, some thermally labile compounds may undergo partial degradation during this ionization process [156].

APCI shares a design concept similar to ESI but employs a heated probe instead of a capillary for sample introduction. In APCI, the LC eluent is first nebulized and vaporized using the heated probe in combination with a nebulizing gas (typically nitrogen), forming an aerosol. Unlike ESI, ionization in APCI occurs predominantly in the gas phase. A high-voltage corona discharge needle generates primary ions by ionizing solvent molecules, which subsequently transfer charge to analytes through ion-molecule reactions to form singly charged species, molecular ions or adducts [144,153,156]. In contrast to the softer ionization process of ESI, APCI involves higher energy and therefore practically does not produce multiply charged ions [158]. The APCI ionization mechanism is illustrated in Figure 17.

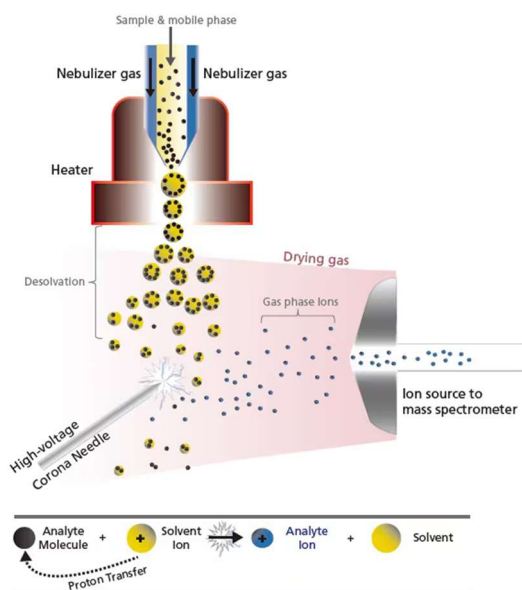


Figure 17. Schematic of the ionization process in APCI positive mode. From reference [158].

3.2.2 Mass analyzers

The primary function of a mass analyzer is to separate ions according to their m/z ratios, whereas ion detection is performed by a dedicated detector, which is discussed in Section 3.2.3. Various types of mass analyzers have been developed, and ion separation is achieved through the application of electric and/or magnetic fields [153]. These analyzers differ in performance, mass accuracy, resolution, sensitivity, acquisition speed, and mass range [153,161].

According to the International Union of Pure and Applied Chemistry (IUPAC), mass accuracy is defined as the difference between the exact (theoretical) mass and the experimentally measured mass, it is typically expressed in parts per million (ppm) [157]. IUPAC defines mass resolution as the ability of a mass spectrometer to distinguish between two adjacent ions with slightly different m/z values. It reflects the “sharpness” of a spectral peak at a given m/z ratio and represents the separation power of the analyzer [157]. In practice, higher mass resolution generally leads to improved mass accuracy.

The mass range corresponds to the range of m/z values that a mass spectrometer is capable of detecting or over which it is operated to record a mass spectrum [157].

Acquisition speed refers to the number of mass spectra acquired per second, whereas the scan rate is the mass range scanned per unit time and is expressed in Da/s [162]. A slower acquisition speed is generally associated with reduced sensitivity. Similarly, increasing the scan rate may compromise sensitivity, as faster scanning across the mass range reduces the time available for the detector to measure ions at a specific m/z value. Conversely, slower scan rates result in better-resolved and more accurate mass spectra [162].

Mass analyzers are commonly classified as either low-resolution or high-resolution instruments [149,161]. High-resolution analyzers are capable of accurate mass measurements, whereas low-resolution instruments are more frequently employed for quantitative applications thanks to their great sensitivity [161]. A comparison of the resolving power of these two categories of mass spectrometers is presented in Figure 18.

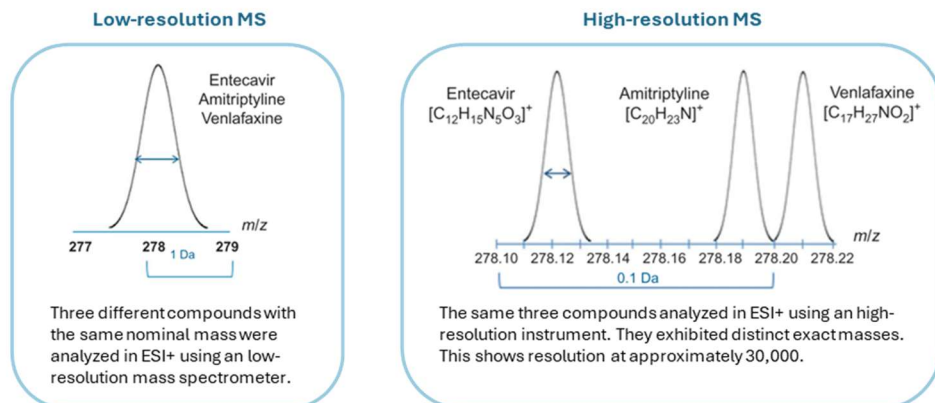


Figure 18. Analysis of entecavir, amitriptyline, and venlafaxine by low- and high-resolution MS in positive ESI mode. Adapted from reference [161].

Low-resolution mass analyzers are typically capable of distinguishing ions that differ by one mass unit (unit-mass resolution). An instrument providing unit-mass resolution generally enables the determination of m/z values for singly charged ions with a mass accuracy of approximately ± 0.1 Da. Quadrupole and ion trap analyzers are commonly classified within this category [163].

High-resolution analyzers include time-of-flight (ToF), Orbitrap, and Fourier transform ion cyclotron resonance (FT-ICR) [161]. These analyzers typically provide mass accuracy in the ppm range and very high resolving power. They are widely used for qualitative and structural analyses, particularly for exact mass determination and structural elucidation. Consequently, ToF and Orbitrap analyzers are especially valuable in metabolomics and proteomics studies. The main characteristics of commonly used mass analyzers are summarized in Table 7.

Table 7: Comparison of mass analyzer characteristics [161,163,164].

Mass analyzer	Quadrupole	Ion trap	ToF	Orbitrap
Mass accuracy	± 0.1 Da	± 0.1 Da	< 3 ppm	< 1 ppm
Resolution	Unit-mass	Unit-mass	$\leq 70,000$	$\leq 140,000$
Acquisition speed	10 Hz	20 Hz	30 Hz	1.5 or 3 Hz
Cost	Low	Moderate	High	High

Hz: hertz, number of complete mass spectra acquired per second.

Mass analyzers may be used individually or in combination. In hybrid configurations, multiple analyzers are integrated within a single instrument to perform tandem mass spectrometry (MS/MS) or higher-order MS^n experiments, thereby enhancing analytical performance [144,156,157,161]. Common examples include triple quadrupole spectrometers and hybrid quadrupole-ToF systems.

3.2.2.1 Quadrupole

Charged species, when subjected to electric or magnetic fields, experience forces that alter their trajectories. In quadrupole analyzers, ion separation is achieved exclusively through the application of electric fields. A quadrupole consists of four parallel hyperbolic metal rods arranged symmetrically around a central axis [144]. Opposite rods are maintained at identical electrical potentials, while adjacent rods carry opposite potentials. One pair of opposing rods is supplied with a potential of $+(U + V \cos \omega t)$, whereas the other pair with $-(U + V \cos \omega t)$, as illustrated in Figure 19. In this expression, U represents the direct current (DC) component, which provides a constant electric potential, while $V \cos \omega t$ represents the radiofrequency (RF) component, characterized by an amplitude (V) and a sinusoidal frequency (ω). The combination of DC and RF potentials generates an oscillating electric field within the quadrupole. Under the influence of this dynamic electric field, ions follow complex oscillatory trajectories between the rods. The stability of these trajectories depends on the ion m/z ratio as well as the applied DC and RF voltages and the RF frequency [144,156].

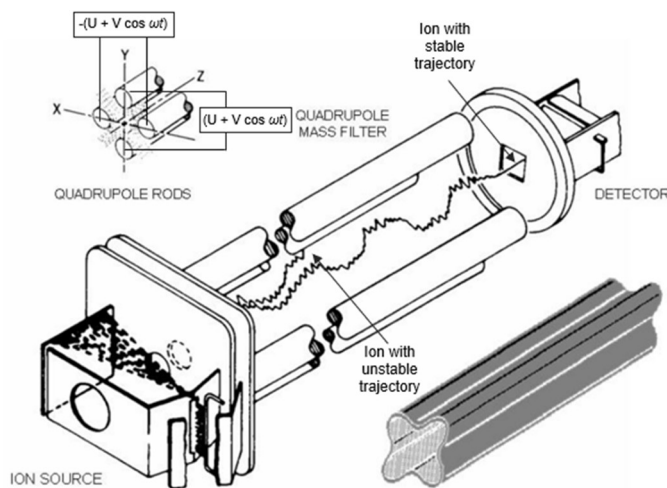


Figure 19. Schematic representation of a quadrupole mass analyzer. Adapted from reference [165].

By carefully adjusting the DC and RF potentials, the quadrupole can function either as a mass filter that selectively transmits ions with a specific m/z ratio or as a scanning analyzer that sequentially transmits ions over a defined m/z range. In selective ion recording (SIR) mode, only ions with specific m/z ratios maintain stable trajectories within the electric field and are successfully transmitted through the rod assembly (Figure 19). All other ions with unstable trajectories are deflected and collide with the rods, preventing their transmission to the detector. This selective transmission

enhances analytical specificity and sensitivity [144,156]. Alternatively, continuous variation of the applied DC and RF voltages enables scanning across a wide m/z range, allowing full mass spectrum acquisition.

3.2.2.2 Tandem mass spectrometer

In MS/MS, two mass analyzers are combined in series, either in-time or in-space, and separated by a collision cell. The fundamental principle of MS/MS relies on collision-induced dissociation (CID), which enables controlled fragmentation of selected precursor ions [163,165]. Instruments such as ion trap and FT-ICR mass spectrometers are classified as tandem-in-time systems, because precursor ion selection, fragmentation, and product ion analysis occur within the same physical device but at different time intervals. In contrast, tandem-in-space instruments perform these processes in physically distinct regions of the instrument. Triple quadrupole and Q-ToF mass spectrometers are the most widely used systems in this category [163,165,166]. CID enables the generation and study of fragment ions and plays a central role in qualitative analysis by providing structural information. As the collision cell allows selective fragmentation and generation of specific product ions, MS/MS provides enhanced structural elucidation, improved specificity, and greater sensitivity compared with single mass analyzer systems [144,156,163].

The following discussion focuses on the triple quadrupole mass spectrometer, which was used throughout this thesis.

Single quadrupole mass spectrometers contain only one mass analyzer and measure ions generated in the ion source. These ions may correspond to intact molecular ions or fragment ions formed through in-source fragmentation. In the absence of CID, these instruments provide limited structural information and exhibit lower specificity and sensitivity compared to triple quadrupole mass spectrometers [156].

Triple quadrupole mass spectrometers are widely regarded as the gold standard for quantitative applications in pharmaceutical and bioanalytical analysis thanks to its higher specificity and sensitivity [147]. This configuration consists of two quadrupole mass analyzers (Q1 and Q3) arranged in series and separated by a collision cell (Q2). The collision cell is typically a quadrupole operated in RF-only mode or another dedicated device, and serves as the region where precursor ions undergo controlled fragmentation via CID [144,156,165].

The most commonly used acquisition mode in quantitative MS/MS applications is multiple reaction monitoring (MRM) [165,167], which is conceptually analogous to the SIR mode in single quadrupole mass spectrometry. MRM was originally referred to as selective reaction monitoring (SRM), and these terms are often used interchangeably. MRM/SRM provides significantly higher selectivity, specificity and

sensitivity than SIR because it employs two sequential mass filtering steps [165,168,169].

The dual mass filtering principle in MRM mode is illustrated in Figure 20. In this mode, precursor ions (also called parent ions) are first selectively isolated by the first quadrupole (Q1) and subsequently transmitted to the collision cell (Q2), where they undergo fragmentation via CID. The resulting fragment ions, known as product ions (or daughter ions), are then filtered by the third quadrupole (Q3) before reaching the detector [165,167]. CID occurs through collisions between precursor ions and an inert collision gas, most commonly argon, although helium and nitrogen may also be used [144,165]. This process generates specific product ions whose formation depends on the chemical structure of the precursor ion, the nature of collision gas, and the applied collision energy. The combination of a precursor ion and one of its product ions is defined as a mass transition (*e.g.*, m/z 200 $\rightarrow$ m/z 50), as illustrated in Figure 20. The selective monitoring of these specific mass transitions enables highly sensitive and specific detection and quantification of analytes.

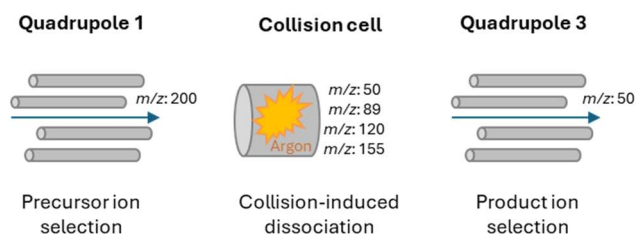


Figure 20. MRM/SRM acquisition mode in quantitative MS/MS applications.

3.2.3 Ion detectors

Similar to ion sources and mass analyzers, several types of ion detectors are available in MS. Although they differ in their physical operating principles, all detectors share the same objective: to convert incident ions into measurable electrical signals. The most commonly employed detectors are the electron multiplier and the photomultiplier. The detector integrated in the tandem mass spectrometer used in the thesis is a photomultiplier. The mechanism of the latter is illustrated in Figure 21 and described below.

In a photomultiplier system, transmitted ions first impact a conversion dynode, producing secondary electrons. These electrons strike a phosphor screen, which emits photons. The emitted photons then enter a photomultiplier tube and strike a photocathode, where they are converted back into electrons. These electrons are subsequently multiplied through a cascade process, generating a measurable current [170].

A major advantage of photomultiplier detectors is that the amplification unit can be maintained in a sealed vacuum environment, which minimizes contamination and significantly extends the operational lifetime of the detector [170].

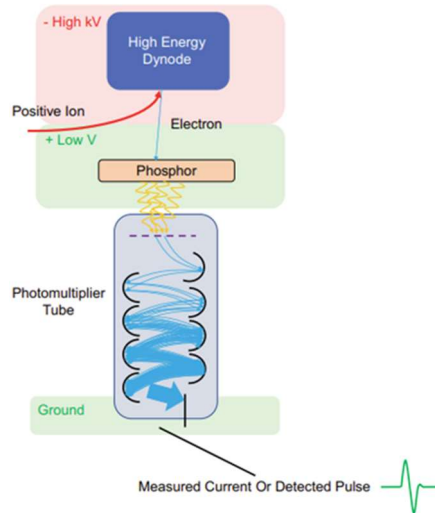


Figure 21. Positive ion detection using a photomultiplier. From reference [170].

3.3 Hyphenation of liquid chromatography to tandem mass spectrometry

Although a wide range of detectors can be coupled with LC, MS remains the most powerful detection technique due to its superior specificity, sensitivity, and structural identification capabilities [171]. A comparative overview of MS and other detectors commonly coupled with LC, in terms of specificity, detection limits, and typical applications, is presented in Table 8.

Table 8: Comparison of specificity, detection limits, and applications of MS *versus* other detectors [168].

Detector	Specificity	Detection limit	Application
Refractive index	Not specific	µg	Universal
UV-visible	Slightly specific	ng	Restrictive: compounds that absorb in the UV–visible region (e.g., 190 – 600 nm)
Fluorescence	Specific	fg	Restrictive: compounds that fluoresce when excited by UV radiation
Amperometric	Specific	pg	Restrictive: compounds that can be oxidized or reduced when an electric potential is applied
ELSD	Not specific	ng	Universal
MS	Specific	fg	Universal

UV: ultraviolet; ELSD: evaporative light scattering detector; MS: mass spectrometry.

As a result, UHPLC-MS/MS, the technique used in the thesis, combines the superior separation efficiency of UHPLC with the advantages of MS. Nevertheless, method development involves several analytical challenges. The main challenges encountered in this thesis, together with the strategies implemented to address them, are presented in the following sections.

3.3.1 Selectivity and specificity

Although MS-based detection is highly specific, additional confidence in analyte identification can be achieved in MS/MS applications by monitoring ion ratios between MRM transitions. Typically, two MRM transitions are selected for each analyte: the most intense transition referred to as the quantifier ion, is used for quantitative purposes, whereas the second most intense transition serves as the qualifier ion [172]. The ratio between the qualifier and quantifier ion intensities corresponds to the ion ratio. Consistent ion ratios observed between standards and samples contribute to confirming analyte identity in samples [173].

MS detection offers an additional dimension of separation based on m/z ratios. Consequently, analytes do not necessarily need to be completely chromatographically separated before entering the detector. However, chromatographic selectivity becomes critical in specific situations, particularly when isobaric compounds are present. According to IUPAC, isobars are molecular species sharing the same nominal mass but differing in exact masses [157]. In the thesis, a particular case involving isomeric compounds was encountered. These species share the same elemental composition but differ in stereochemistry [161]. In such scenarios, MS/MS systems are unable to discriminate between isomers [174], as they exhibit identical MRM transitions. Therefore, differentiation of isomeric species relies on chromatographic selectivity.

3.3.2 Sensitivity

Sensitivity is a critical parameter in method development for the determination of N-nitrosamine impurities. As previously introduced in Section 1 of this chapter, N-nitrosamines are trace-level impurities that may be present in pharmaceutical products at amounts down to the ppb range [175], thereby requiring very high sensitivity. This requirement is also emphasized by the European Medicines Agency, which strongly recommends the use of MS/MS or high-resolution MS [176].

Method sensitivity refers to the ability of an analytical procedure to detect small changes in analyte concentration and is defined by the slope of the calibration curve [171,177,178]. A steeper slope indicates that a small variation in analyte concentration produces a larger change in the measured signal, reflecting higher method sensitivity, as illustrated in Figure 22. Sensitivity may also refer to the magnitude of the signal generated by the analyte and is commonly used to compare detector performance [171].

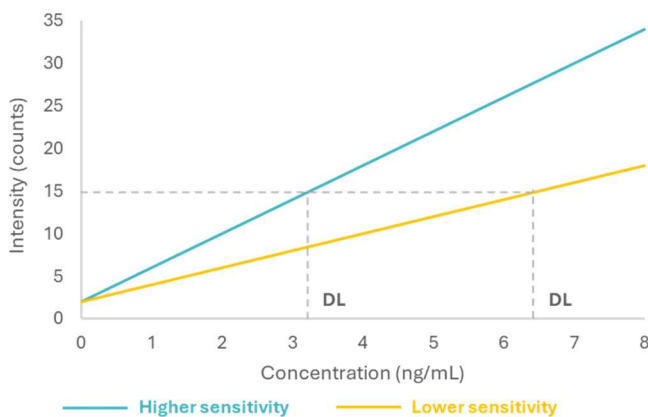


Figure 22. Illustration of sensitivity and detection limit (DL). Adapted from reference [171].

Fundamentally, the ability of an analytical method to generate reliable qualitative and quantitative results depends on the signal-to-noise (S/N) ratio [171]. According to ICH Q2(R2) guideline, the detection limit is defined as the smallest amount of an analyte in a sample that can be reliably detected but not necessarily quantified. An S/N ratio greater than or equal to 3 is generally considered acceptable for estimating the detection limit [45]. Although sensitivity and detection limit are distinct parameters, they are closely related. In practice, an analytical method is often described as “sensitive” when it exhibits a low detection limit [179].

The sensitivity of LC-MS methods can be improved through several optimization strategies. In the following sections, key factors influencing the method detection limit are discussed. These parameters must be carefully optimized to maximize method sensitivity and minimize detection limits.

3.3.2.1 Signal-to-noise ratio

Improvement of the S/N ratio is a key strategy for enhancing sensitivity, particularly in triple quadrupole mass spectrometers [167]. For targeted quantitative analysis, these instruments operated in MRM mode provide superior sensitivity compared to full-scan acquisition or tandem-in-time mass spectrometers, thanks to the selective monitoring of precursor-to-product mass transitions [165,167].

Chemical background noise, originating from unwanted species such as matrix components, solvents, or instrument contamination, can interfere with analyte determination. The dual mass filtering principle of MRM significantly reduces background noise by selectively transmitting only ions corresponding to the chosen mass transitions. This selective transmission improves the S/N ratio, resulting in lower detection limits [169]. The S/N ratio can also be increased by optimizing the dwell time.

3.3.2.2 Ionization and transmission efficiencies

Analyte signal intensity is directly influenced by both ionization and ion transmission efficiencies. In LC-MS application, ionization efficiency is not only governed by the ion source but is also strongly affected by the mobile phase composition, including the nature of the organic modifier, pH, and buffer composition [160,171].

Ion transmission refers to the ion transfer from the atmospheric pressure region of the ion source to the high-vacuum region of the mass analyzer. This process involves passage through an atmospheric pressure-to-vacuum interface, which inherently results in ion losses. Transmission efficiency varies depending on the design of the interface and the ion optics used to guide ions into the mass analyzer. The use of ion-guiding devices can significantly improve ion transmission efficiency [180].

Optimization of ion transmission is particularly important in quadrupole-based mass spectrometers, where improved ion focusing enhances sensitivity [167].

3.3.2.3 Dwell time

Dwell time refers to the duration during which the mass spectrometer monitors a specific MRM transition, typically ranging from 5 to 200 ms. Increasing the dwell time improves sensitivity because the instrument spends more time collecting ion signal for the selected transition, resulting in higher signal intensity and an improved S/N ratio [163].

Longer dwell time can enhance measurement precision and accuracy by increasing the number of data points. However, in multi-analyte analysis where multiple MRM transitions are simultaneously monitored, the dwell time allocated to each transition decreases due to reduced acquisition cycle time [163,174]. Therefore, in these situations, dwell time should be carefully adjusted during method development to achieve an appropriate balance between overall sensitivity and adequate peak definition.

3.3.3 *Matrix effects*

While LC-MS offers numerous advantages and broad applicability in across diverse analytical fields, one of its main limitations is the occurrence of matrix effects.

Matrix effects refer to alterations in the ionization efficiency of analytes caused by the presence of co-eluting substances originating from the sample matrix. These compounds may compete with analytes for charge during the ionization process. As a consequence, matrix effects can lead to either ion suppression or ion enhancement, resulting in a decrease or increase in analyte signal intensity. Therefore, these effects compromise analytical performance by affecting method precision, accuracy, robustness, and lower limits [181,182].

The extent of matrix effects depends on several factors, including the ionization technique used, the effectiveness of sample clean-up procedures, and the chromatographic separation. Appropriate sample preparation and efficient chromatographic separation can prevent or minimize the introduction of matrix components into the mass spectrometer and thereby mitigate matrix effects [181–183].

In quantitative LC-MS analysis, the use of internal standards is a widely adopted strategy to compensate for matrix effects and normalize analyte responses [184]. Ideally, the internal standard should be either a structural analogue or a stable isotope-labeled (SIL) analogue of the target analyte [156]. In MS applications, SIL analogues represent best practice as internal standards, in which one or more atoms are replaced by their stable isotopes, such as ^2H (deuterium), ^{13}C , or ^{15}N [181,184,185]. In practice,

SIL analogues with a mass difference of at least three mass units relative to the unlabeled analyte are generally recommended to ensure clear spectral distinction [156]. Because SIL analogues possess nearly identical physicochemical properties to their corresponding standards, they undergo the same extraction, chromatographic, and ionization processes. As a result, they are affected in a similar manner by ion suppression or enhancement and exhibit the same retention time. However, due to their different molecular masses, they produce distinct m/z values in the mass spectrum [181,185].

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Chapter I: Introduction

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Chapter II

Objectives

Since the onset of the global N-nitrosamine crisis in the pharmaceutical industry in 2018, regulatory agencies have initially focused their attention on generic small-molecule N-nitrosamine impurities. In 2021, the first detection of an N-nitrosamine drug substance-related impurity (NDSRI) marked the emergence of a new class of N-nitrosamine impurities. Following this discovery, regulatory concerns have progressively shifted toward NDSRIs, which currently represent more than 90% of pharmaceutical N-nitrosamine impurities listed by the European Medicines Agency (EMA).

In response to this evolving situation, international regulatory agencies rapidly implemented “Call for Review” guidance to investigate and manage N-nitrosamine risks in medicinal products. Within this regulatory framework, analytical testing plays a central role, particularly during Step 2 (confirmatory testing) and Step 3 (implementation and monitoring of risk-mitigation measures) of the N-nitrosamine investigation workflow.

Against this background, two main scientific questions arise regarding the analytical testing of different categories of N-nitrosamines. These questions constitute the guiding thread of the present thesis and also reflect the evolving landscape of the N-nitrosamine crisis.

1) Can reliable LC-MS/MS methods be developed for the determination of small-molecule N-nitrosamine impurities in the selected pharmaceutical product while ensuring reliable analytical performance?

As mentioned earlier, N-nitrosamines are trace-level impurities that may be present in medicinal products at amounts in the ppm or ppb range. Given these extremely low concentration levels, the primary analytical challenge is to achieve sufficiently low detection limits. Considering both technological capabilities and regulatory requirements, hyphenated techniques combining chromatographic separation with mass spectrometric detection are generally preferred. Furthermore, since some N-nitrosamines are non-volatile, liquid chromatography represents a more appropriate analytical approach than gas chromatography for their determination.

To answer this first question, however, two sub-questions must be addressed.

a) Are the developed methods able to manage matrix effects while maintaining suitable quantitative analytical performance?

The use of mass spectrometric detection does indeed introduce an additional analytical challenge: matrix effects. When high concentrations of matrix components co-elute with the analytes, alterations in analyte ionization efficiency may occur, leading to signal suppression or enhancement. Matrix effects can therefore compromise analytical performance, including specificity, accuracy, precision, and detection limits. Moreover, in the analysis of trace N-nitrosamines in pharmaceutical products, matrix effects mainly arise from the large concentration difference between active pharmaceutical ingredients (APIs) and analytes. Typically, APIs are introduced into the analytical system at concentrations in the mg/mL range, whereas N-nitrosamines are present at ng/mL levels.

Although removing the API during sample preparation might appear to be a straightforward solution, selective clean-up techniques such as solid-phase extraction or liquid-liquid extraction generally fail to isolate N-nitrosamines while completely eliminating the API in the selected formulation. This additional step in the analytical process can also introduce sources of error and potentially compromise the reliability of the results.

Consequently, a more effective strategy consists of achieving adequate chromatographic separation between the API and the target analytes, thereby minimizing matrix effects while maintaining satisfactory analytical performance.

b) Do such validated analytical methods meet current regulatory requirements for the investigation of N-nitrosamines and for supporting risk-mitigation decisions?

Before being applied to confirmatory testing requested by regulatory agencies, the developed methods must undergo rigorous validation to demonstrate adequate analytical performance for their intended use as screening methods. In particular, the methods must provide sufficient sensitivity to determine N-nitrosamine levels at or below the regulatory safety thresholds. Based on the results of confirmatory testing, if an N-nitrosamine concentration exceeds its acceptable intake limit as defined by the EMA, appropriate risk-mitigation measures must be implemented.

2) Can the Analytical Quality by Design (AQbD) approach serve as an appropriate strategy for developing reliable analytical methods to support risk-mitigation decisions for both N-nitrosamine drug substance-related impurities (NDSRIs) and small-molecule N-nitrosamine impurities?

With the evolution of the N-nitrosamine crisis and the increasing regulatory focus on NDSRIs, the present work followed this trend by investigating a pharmaceutical product, Fluoxone® Divule® 20 mg, which was withdrawn from the Belgian market following the detection of the NDSRI, N-nitroso-fluoxetine, at levels significantly exceeding the acceptable safety limit.

Building on the knowledge and experience gained from the analytical development described in Chapter III, Sections 1(A) and 1(B), an AQbD approach was considered to guide the development of a specific analytical method for the quantification of N-nitroso-fluoxetine in Fluoxone® Divule® 20 mg, used as a case study.

Chapter III

Results

Analytical development of LC-MS/MS methods for the
determination of small-molecule N-nitrosamine
impurities

Section 1(A)

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

Preamble

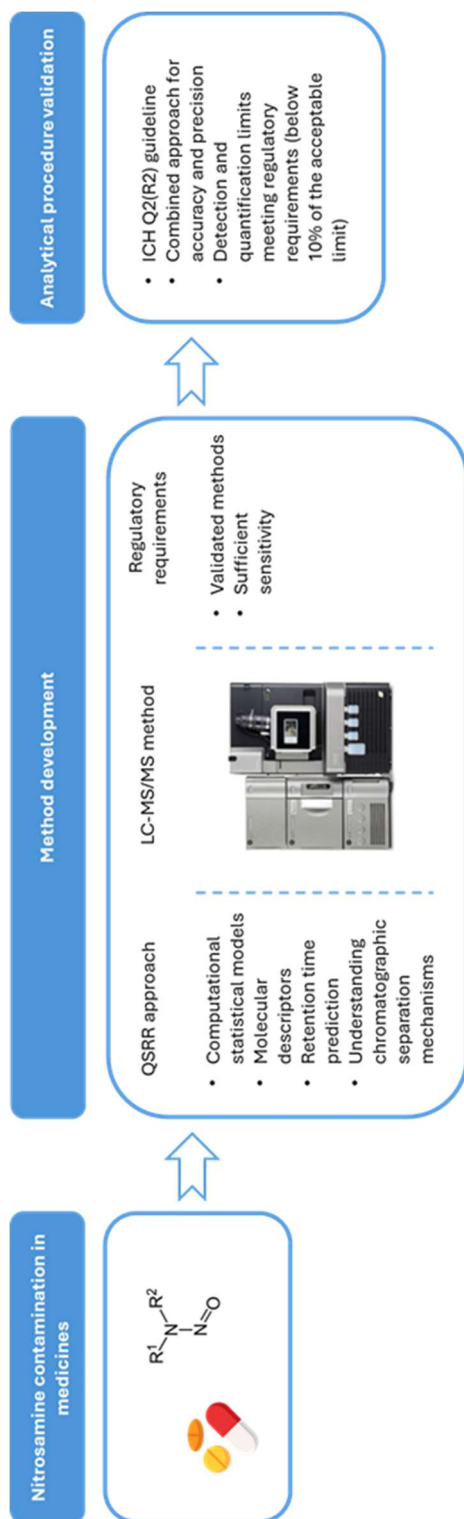
N-nitrosamines are a class of compounds widely monitored as potentially genotoxic to humans [1]. Concerns regarding these impurities intensified following the detection of N-nitrosodimethylamine in valsartan products at levels exceeding safety thresholds in 2018, marking the onset of the N-nitrosamine crisis in the pharmaceutical industry [2]. In response, major international regulatory authorities, including the U.S. FDA and the EMA, released guidance mandating the systematic investigation and control of N-nitrosamine impurities in medicinal products [3,4].

This section exclusively focuses on small-molecule N-nitrosamine impurities, which mostly form during the chemical synthesis of APIs. These impurities may be present in diverse drug substances and drug products [5]. Given the extremely low detection limits required for these trace-level compounds, liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) is the technique of choice, as it represents the gold standard for quantitative analysis [6]. To address the analytical challenges associated with their determination using LC-MS/MS technique, an *in silico* approach based on Quantitative Structure Retention Relationship modeling was employed [7,8]. This computational tool facilitates method development by predicting chromatographic retention behavior, thereby minimizing the risk of N-nitrosamine co-elution with the API. High concentrations of the API or other matrix components introduced into the ion source under co-elution conditions, can alter the ionization efficiency of analytes, leading to ion suppression or enhancement, a phenomenon often observed in mass spectrometry [9]. Therefore, matrix effects may result in poor quantitative performance of analytical methods, including precision, accuracy, and robustness [10,11]. The section describes the analytical development, focusing on the mitigation of matrix effects while maintaining suitable performance. Quantitative performance was demonstrated through the validation of one of the resulting methods for the determination of five N-nitrosamines, both as quantitative impurity assays and limit tests, consistent with EMA sensitivity requirements.

The proposed methodology may be applicable to other pharmaceutical products, and its utility is demonstrated herein through a case study involving a marketed drug product. Due to a non-disclosure agreement with the industrial partner, the pharmaceutical product investigated in the study cannot be disclosed.

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Graphical abstract. Illustration of the work described in Chapter 3 – Section 1(A).

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 Figure 1. Their origin lies in the nitrosation process of secondary, tertiary, or quaternary¹ 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 2A: 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].

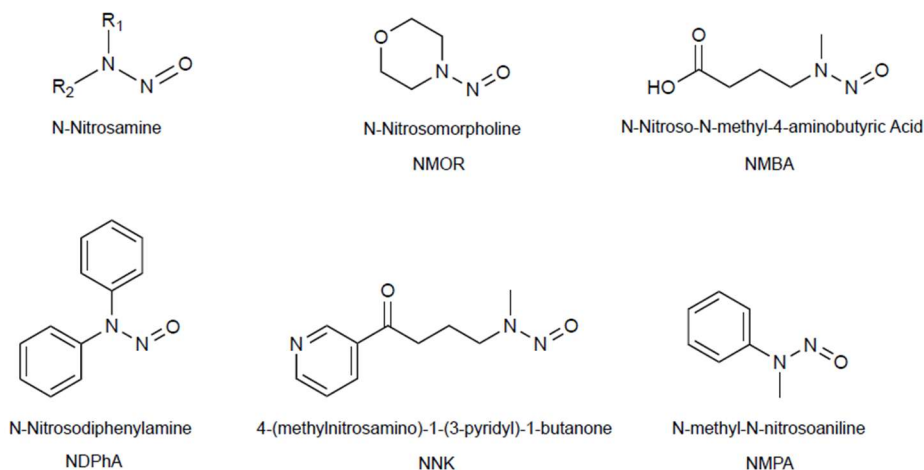


Figure 1. Chemical structures of N-nitrosamines and the N-nitrosamines involved in this study.

¹ The risk of nitrosation of quaternary ammonium compounds is considered negligible.

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 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.

Chapter III: Results - Section 1(A)

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 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.

17 α -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 $\times$ 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 $^{\circ}\text{C}$; autosampler temperature: 6 $^{\circ}\text{C}$. 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 $\times$ 100 mm) was selected over the XSelect HSS T3 column (3.5 μm , 2.1 mm $\times$ 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 $\times$ 100 mm). To verify the chromatographic separation of N-nitrosamines under the predicted optimal conditions indicated on the desirability index graph (Figure 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.

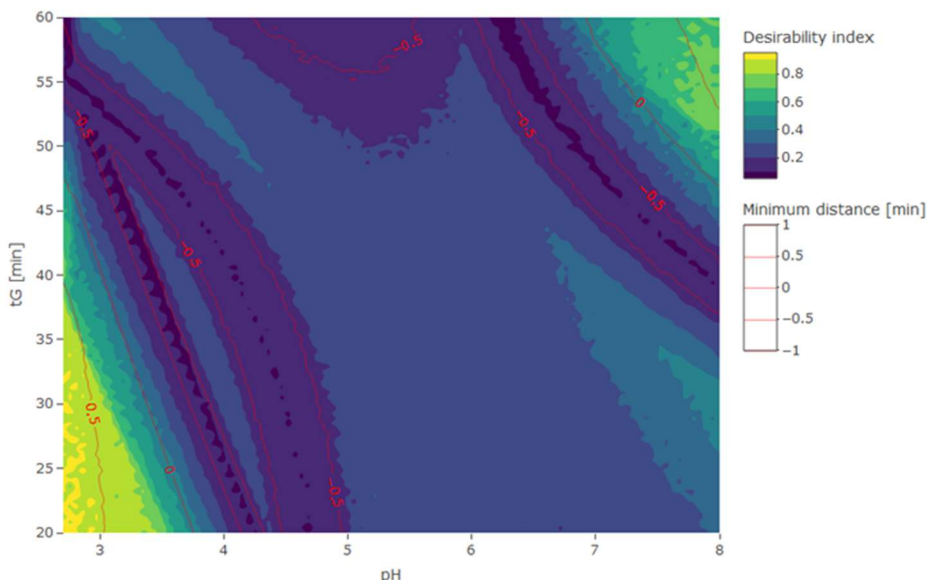


Figure 2. Desirability index graph computed using following weighted criteria for separation (1.0), robustness (0.5), and analysis time (0.1) for 17 N-nitrosamines and API separation. The red lines represent the minimum distance between two consecutive peaks.

Additionally, according to the second desirability index graph (Figure 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.

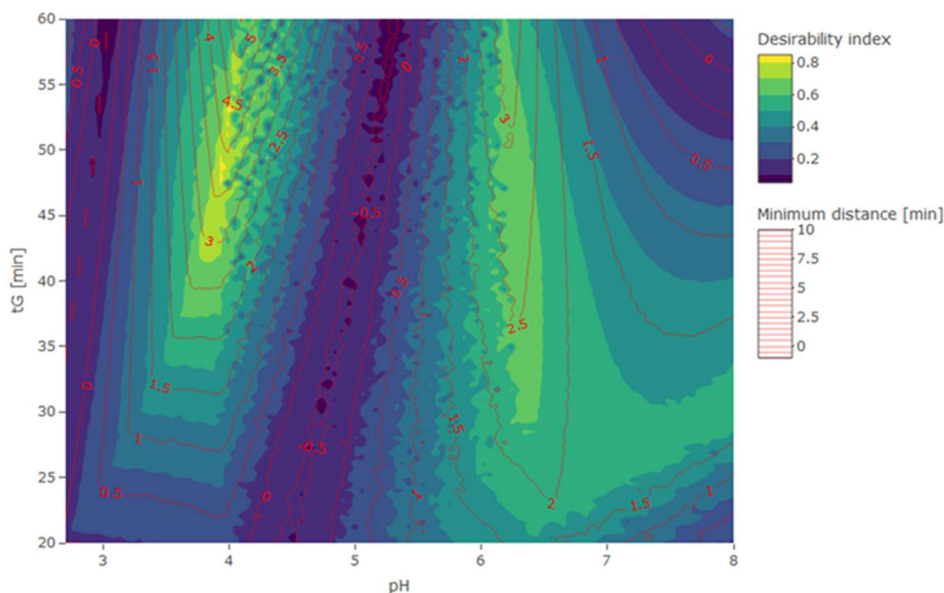


Figure 3. Desirability index graph computed using following weighted criteria for separation (1.0), robustness (0.5), and analysis time (0.1) for 5 N-nitrosamines and API separation. The red lines represent the minimum distance between two consecutive peaks.

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 $\times$ 100 mm) and Acquity® UPLC® HSS T3 column (1.8 μm , 2.1 mm $\times$ 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 17 α -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 μ g/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 μ g/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 daily at a concentration of 1000 μ g/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 μ g/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 μ L 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 to 95% B, 10.9 – 13.5 min: 95% B, 13.5 – 13.6 min: 95 to 0% B, 13.6 – 17.0 min: 0% B. The injection volume was set at 10 μ L. 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 μ A; 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.

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 → 86.93 117.10 → 45.09	2.20 – 3.80	27	13 13	No
NMPA	137.05 → 66.10 137.05 → 107.15	6.20 – 7.50	28	12 11	No
NMBA	147.10 → 117.10 147.10 → 44.10	2.80 – 3.90	17	7 10	Activated
NDPhA	199.10 → 66.10 199.10 → 169.10	8.30 – 10.20	23	22 12	No
NNK	208.10 → 122.10 208.10 → 148.00	4.20 – 5.30	32	14 10	No
NDMA	75.05 → 58.05 75.05 → 43.00	1.65 – 2.90	32	9 13	No
NDBA	159.20 → 57.10 159.20 → 103.23	8.70 – 9.70	30	12 10	No
NMEA	89.11 → 61.00 89.11 → 43.00	3.00 – 4.20	24	9 9	No
NEIPA	117.20 → 74.99 117.20 → 47.10	5.40 – 6.50	25	8 14	Activated

Chapter III: Results - Section 1(A)

Table 2 Continued.

N-nitrosamine	MRM transition (m/z)	Retention window (min)	Cone voltage (V)	Collision energy (eV)	Soft transmission
NDIPA	131.20 → 89.10 131.20 → 43.10	6.30 – 7.50	17	10 9	No
NDPA	131.16 → 89.16 131.16 → 43.14	6.90 – 7.90	30	11 11	Activated
NPYR	101.12 → 55.00 101.12 → 41.00	3.10 – 4.30	22	15 18	No
MeNP	130.00 → 58.00 130.00 → 100.00	2.00 – 3.00	27	13 5	No
NDEA	103.20 → 74.90 103.20 → 46.90	4.30 – 5.30	33	9 12	No
NPIP	115.15 → 69.00 115.15 → 41.00	4.80 – 5.70	13	14 17	No
NTHP	113.10 → 67.10 113.10 → 83.10	4.40 – 5.40	17	10 13	No
NDELA	135.10 → 74.00 135.10 → 104.00	0.70 – 2.20	8	10 6	Activated
NMOR-d8	125.17 → 95.00	2.40 – 3.80	20	15	No

Chapter III: Results - Section 1(A)

Table 2 Continued.

N-nitrosamine	MRM transition (m/z)	Retention window (min)	Cone voltage (V)	Collision energy (eV)	Soft transmission
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.

2.8 Stability assessment

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

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-nitrosamine 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 Figure 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 Figure 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 (Figure 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 Figure 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 Figure 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 Figure 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

Figure 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.

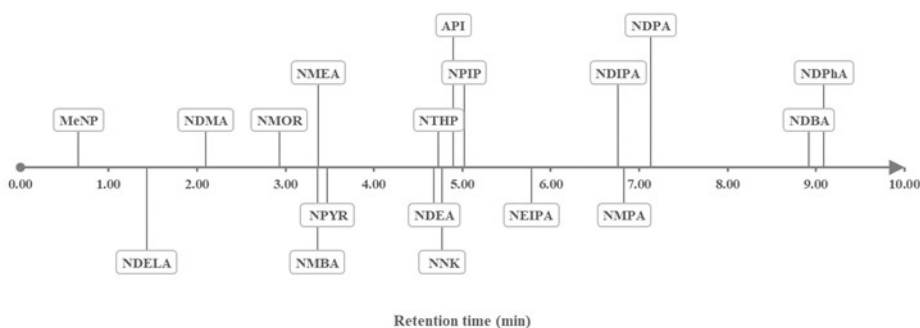


Figure 4. Illustration of LC separation of 17 N-nitrosamines and API under conditions of pH 2.7 and a gradient time of 9.9 minutes.

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 Figure 5 under pH 2.7 and 9.9 minutes of gradient time. The TIC of each N-nitrosamine under the optimal pH of 2.7 and gradient time of 9.9 minutes are illustrated in Figure 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 Figure 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.

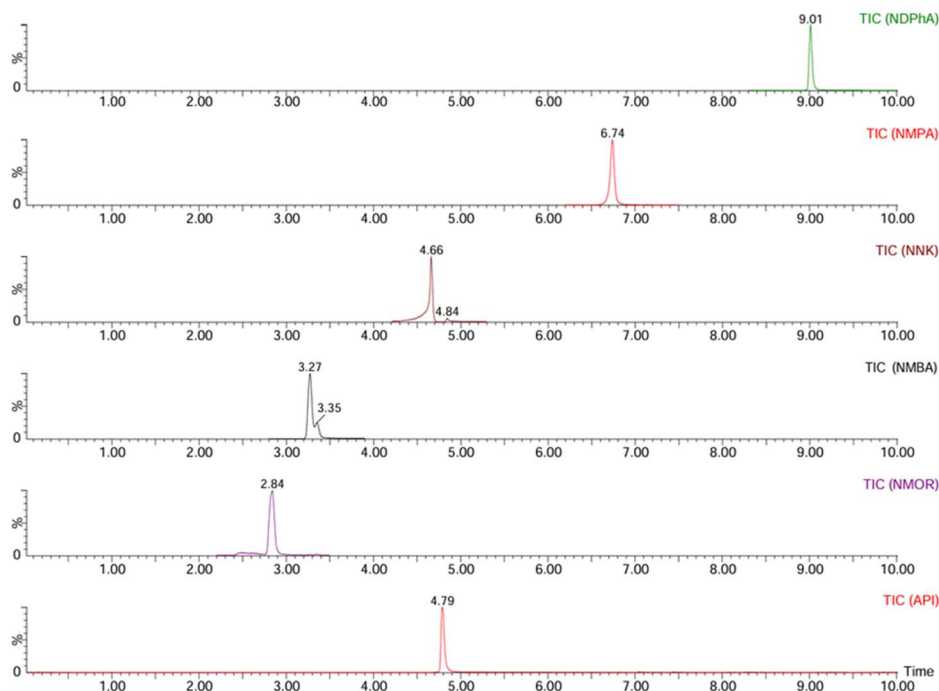


Figure 5. TICs of NMOR, NMBA, NDPhA, NNK, NMPA, and API at a concentration of 5 ng/mL in the matrix under the optimized experimental conditions (pH 2.7, gradient time: 9.9 minutes, APCI probe temperature: 250°C).

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 to 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 both 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 Figure 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 (Figure S3) 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.

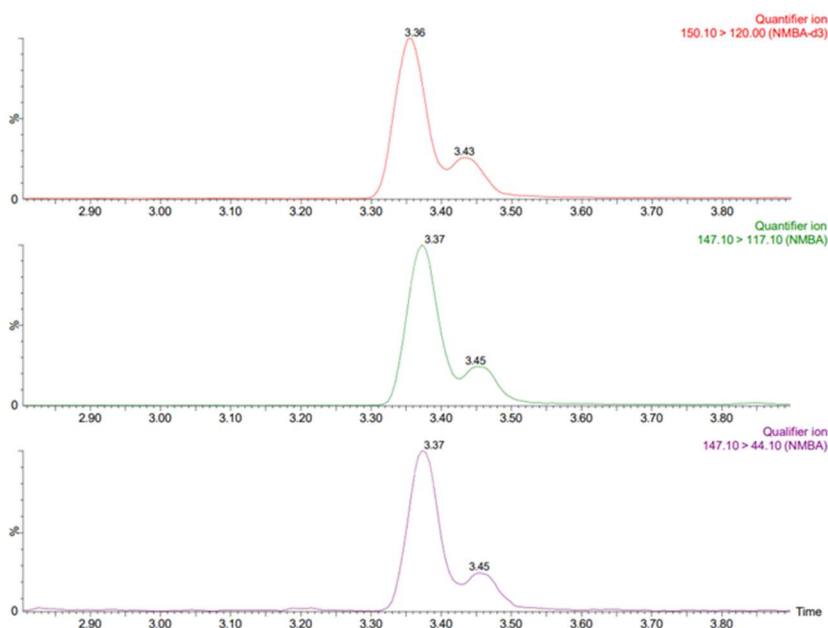


Figure 6. EICs of NMBA and NMBA-d3 at a concentration of 5 ng/mL in matrix under the optimized experimental conditions. (red) NMBA-d3 quantifier ion; (green) NMBA quantifier ion; (purple) NMBA qualifier ion.

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 to 30 ng/mL covers at least 10% to 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.

Chapter III: Results - Section 1(A)

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

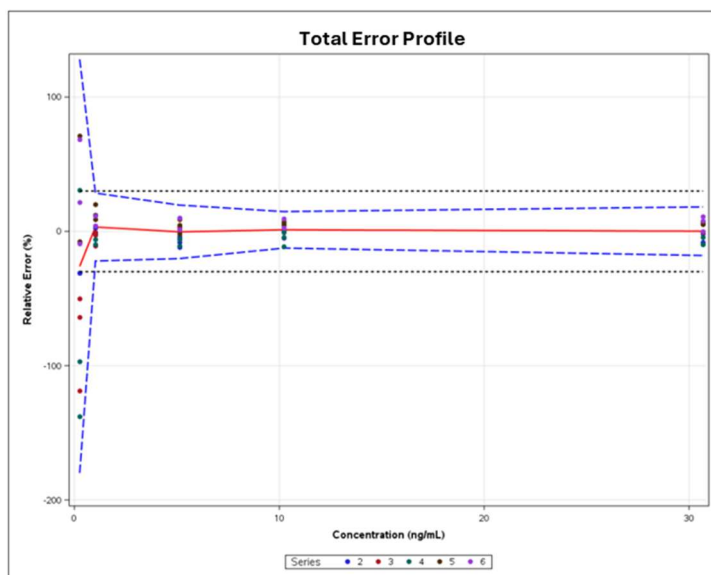
	NMOR ($p = 5; n = 3$)	NMBA ($p = 6; n = 3$)	NDPhA ($p = 6; n = 3$)	NNK ($p = 5; n = 3$)	NMPA ($p = 6; n = 3$)
Response function					
Calibration model	Linear regression through 0 (fitted at level 3)	Linear regression through 0 (fitted at level 3)	Linear regression through 0 (fitted at level 3)	Linear 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.

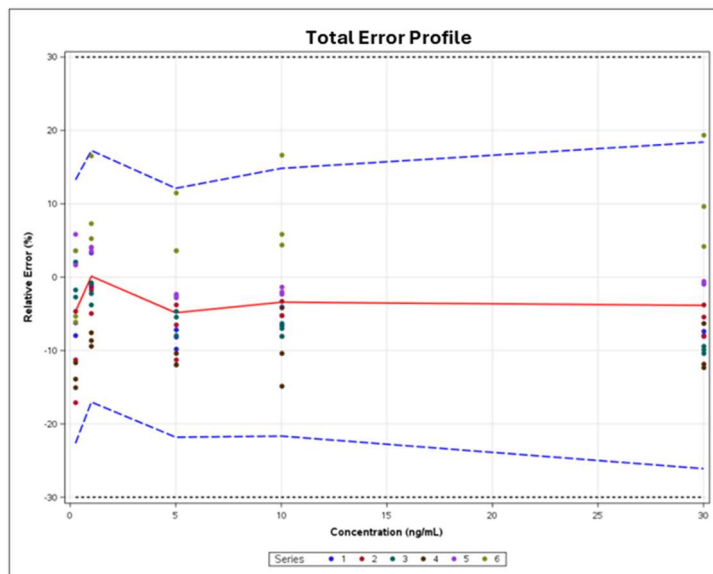
An alternative to the traditional separate evaluation of accuracy and precision is to assess their total impact using a combined approach. This combined methodology can be performed using a prediction interval, a tolerance interval, or a confidence interval [30].

The total error profiles are illustrated in Figure 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 $\pm 30\%$, considering the scope of trace analysis for impurities. The acceptance criterion of $\pm 30\%$ 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.

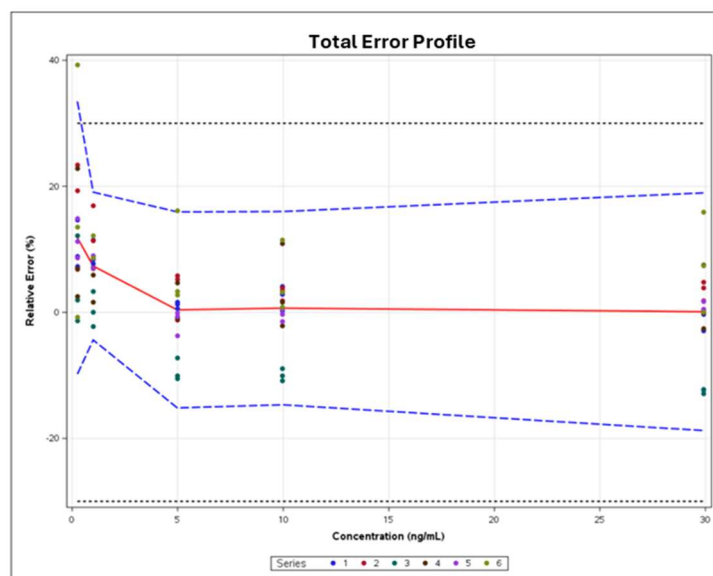
7a)



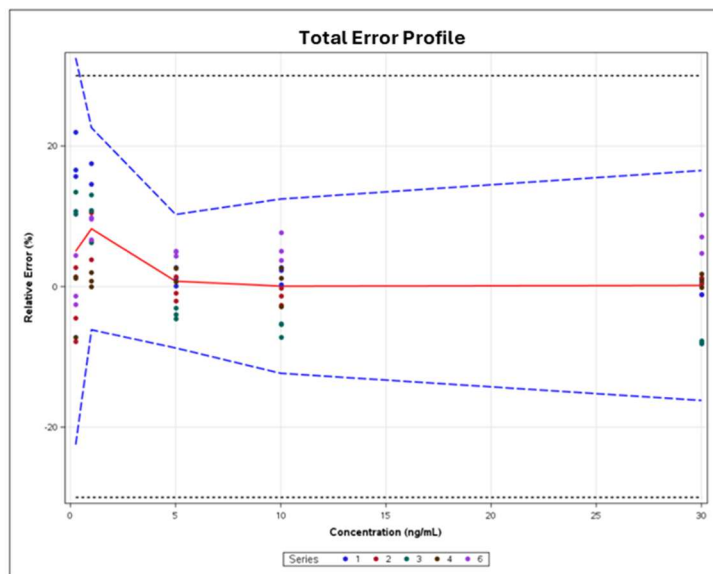
7b)



7c)



7d)



7e)

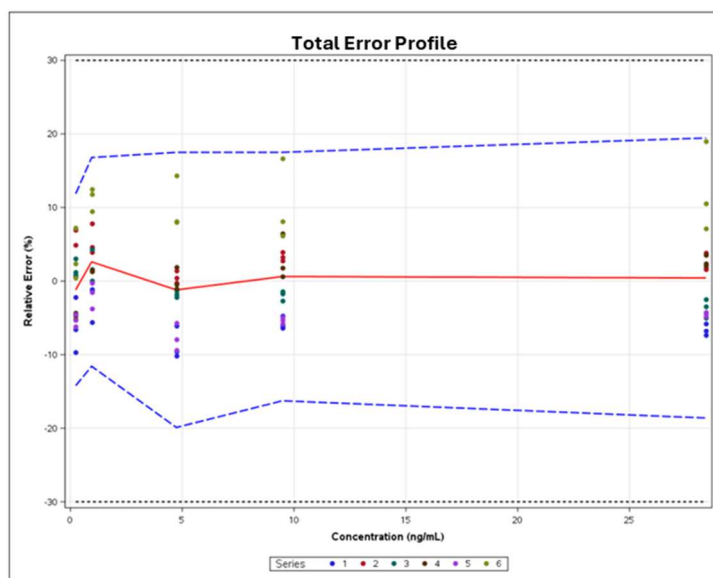


Figure 7. Total error profiles computed by considering linear regression through 0 (fitted only at concentration level 3) to assess the quantitative performance of the LC-MS/MS method for NMOR (a), NMBA (b), NDPhA (c), NNK (d), and NMPA (e) in the tablet formulation. On the figures, the plain red line 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 $\pm 30\%$, the dots represent the relative error of the individual results and are plotted with respect to the targeted concentration.

Illustrated in Figures 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 $\pm 30\%$ at all concentration levels.

In contrast, as shown in Figure 7a, the lower and upper bounds of the β -expectations tolerance intervals for NMOR did not fall within the acceptance limits of $\pm 30\%$ solely at the lowest concentration level. Regarding NDPhA (Figure 7c), and NNK (Figure 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.

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 $\pm 30\%$ 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_{24h} , and T_0 versus T_{72h} , 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_{24h} or T_0/T_{72h} 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_{24h} and T_{72h} . Therefore, the solutions are considered stable after T_{24h} and T_{72h} , and can be re-injected up to T_{72h} 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 4).

Chapter III: Results - Section 1(A)

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

	Mean intensity (counts, n = 3) ≥ 8,000,000 counts		Equipment verification 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.

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 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.

Acknowledgments

The authors would like to express their gratitude for the assistance provided by Laurence Van Oudenhove and the LC-MS application team from Waters (Belgium) for their valuable technical support.

Supplementary material

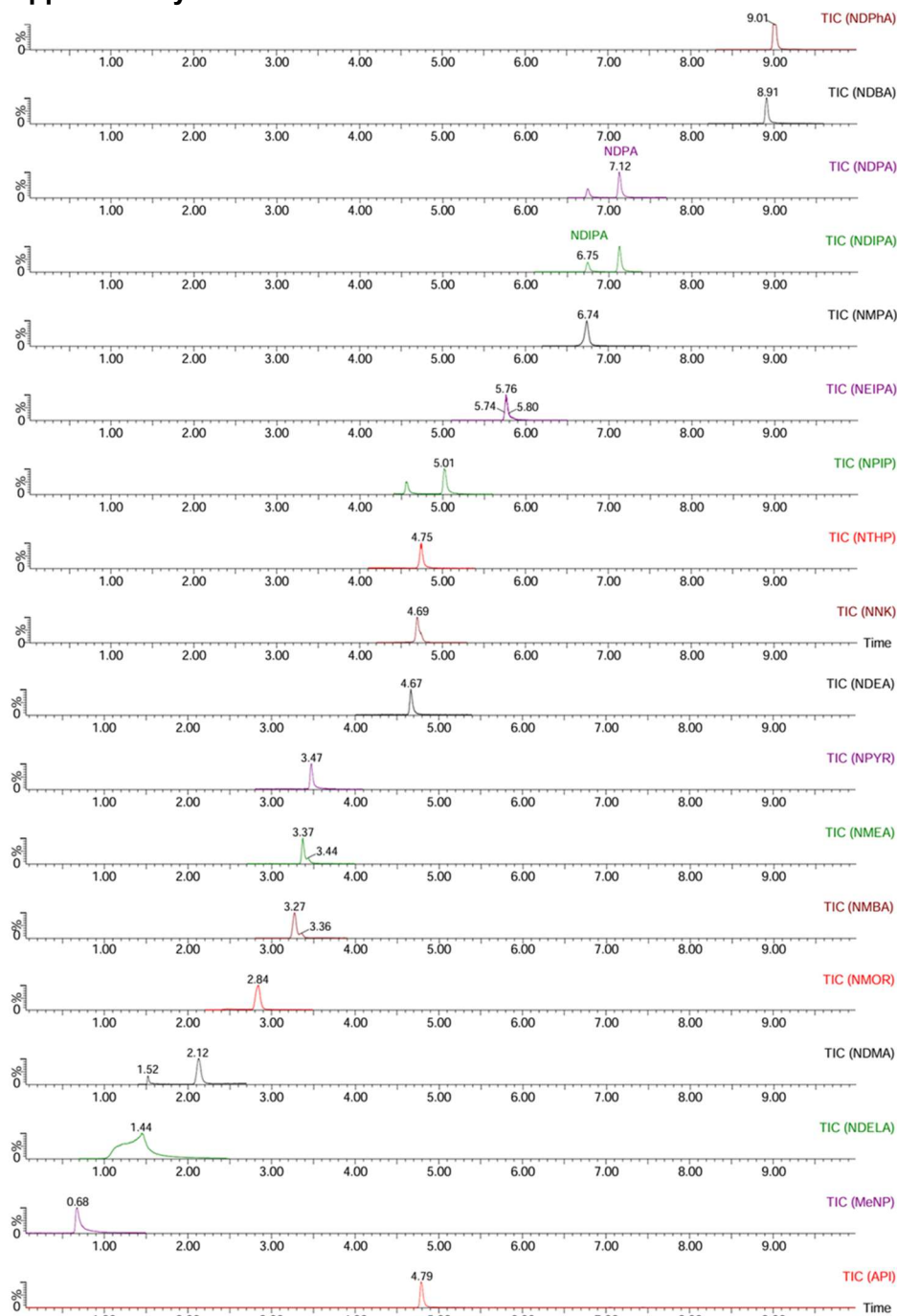


Figure S1. LC separation of 17 N-nitrosamines and API under conditions of pH 2.7 and a gradient time of 9.9 minutes.

Chapter III: Results - Section 1(A)

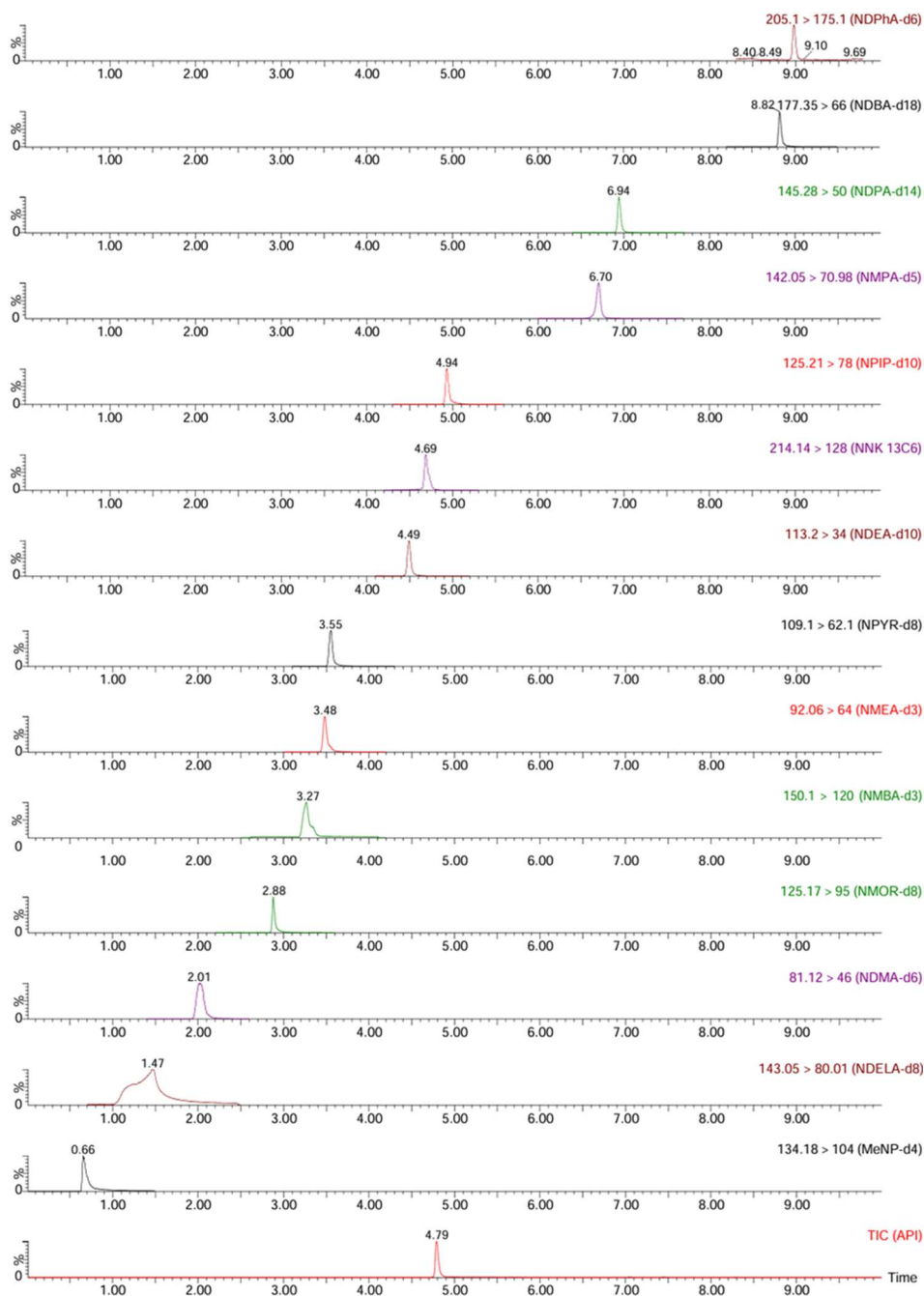


Figure S2. LC separation of internal standards and API under conditions of pH 2.7 and a gradient time of 9.9 minutes.

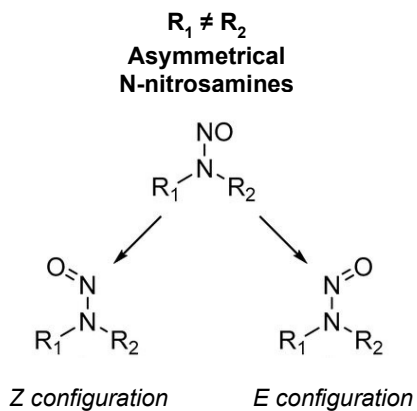


Figure S3. *E/Z* isomerism of asymmetrical N-nitrosamines².

E (entgegen) and *Z* (zusammen) designate the relative positions of the substituents according to the Cahn-Ingold-Prelog priority rules. The group R_1 is assigned the higher priority.

² Adapted from Guan *et al.*, *Molecules*, 27(2022), 4749.

Chapter III: Results - Section 1(A)

Table S1: Total error index and validated range of all calibration models.

Calibration model	NMOR (<i>p</i> = 5)		NMBA (<i>p</i> = 6)		NDPhA (<i>p</i> = 6)		NNK (<i>p</i> = 5)		NMPA (<i>p</i> = 6)	
	Total error index	Validated range (ng/mL)	Total error index	Validated range (ng/mL)	Total error index	Validated range (ng/mL)	Total error index	Validated range (ng/mL)	Total error index	Validated range (ng/mL)
Linear regression through 0, fitted using level 3 only	0.68	1.01 – 30.67	0.71	0.25 – 30.00	0.76	0.43 – 29.89	0.81	0.44 – 30.00	0.74	0.24 – 28.42
Linear regression through 0, fitted using the highest level (level 5) only	0.62	2.02 – 30.67	0.70	0.25 – 30.00	0.78	0.37 – 29.89	0.81	0.25 – 30.00	0.73	0.24 – 28.42
Linear regression	0.44	2.76 – 30.67	0.70	0.53 – 30.00	0	9.03 – 29.89	0.80	0.85 – 30.00	0.73	0.69 – 28.42
Quadratic regression	0.37	3.70 – 30.67	0.71	0.25 – 30.00	0	7.74 – 29.89	0.83	0.41 – 30.00	0.74	0.24 – 28.42
Weighted (1/X) quadratic regression	0.38	3.63 – 30.67	0.71	0.25 – 30.00	0	9.02 – 29.89	0.83	0.25 – 30.00	0.74	0.24 – 28.42
Weighted (1/X ²) quadratic regression	0.39	3.91 – 30.67	0.70	0.25 – 30.00	0	28.24 – 29.89	0.83	0.25 – 30.00	0.74	0.24 – 28.42
Weighted (1/X ²) linear regression	0.40	3.93 – 30.67	0.70	0.25 – 30.00	0	N.D.	0.83	0.25 – 30.00	0.74	0.24 – 28.42
Weighted (1/X) linear Regression	0.39	3.97 – 30.67	0.70	0.25 – 30.00	0	9.07 – 29.89	0.82	0.25 – 30.00	0.75	0.24 – 28.42
Linear regression after square root transformation	0.58	4.72 – 30.67	0.70	0.25 – 30.00	0	16.83 – 29.89	0.82	0.25 – 30.00	0.75	0.24 – 28.42
Linear regression after logarithm transformation	0.48	6.80 – 29.73	0.70	0.25 – 30.00	0	N.D.	0.84	0.25 – 30.00	0.72	0.24 – 28.42

p: number of series of validation; N.D.: Not defined. The total error index accounts for accuracy, precision, and range.

Table S2: TOST interval for solution stability assessment.

NMOR stability evaluation	T ₀ VS T _{24h} TOST interval (ng/mL)	T ₀ VS T _{72h} TOST interval (ng/mL)	Acceptance limits (ng/mL)	Stability
Conc. level 3	[-0.29 , 0.29]	[-0.35 , 0.23]	± 0.77	Stable
Conc. level 4	[-0.56 , 0.55]	[-0.61 , 0.50]	± 1.10	Stable
Conc. level 5	[-2.05 , 1.97]	[-2.36 , 1.66]	± 4.16	Stable
NMBA stability evaluation	T ₀ VS T _{24h} TOST interval (ng/mL)	T ₀ VS T _{72h} TOST interval (ng/mL)	Acceptance limits (ng/mL)	Stability
Conc. level 3	[-0.38 , 0.18]	[-0.40 , 0.16]	± 0.65	Stable
Conc. level 4	[-0.82 , 0.47]	[-0.93 , 0.36]	± 1.45	Stable
Conc. level 5	[-2.70 , 1.66]	[-2.97 , 1.39]	± 5.16	Stable
NDPhA stability evaluation	T ₀ VS T _{24h} TOST interval (ng/mL)	T ₀ VS T _{72h} TOST interval (ng/mL)	Acceptance limits (ng/mL)	Stability
Conc. level 3	[-0.36 , 0.39]	[-0.36 , 0.38]	± 0.64	Stable
Conc. level 4	[-0.69 , 0.56]	[-0.66 , 0.58]	± 1.25	Stable
Conc. level 5	[-2.02 , 2.23]	[-2.01 , 2.24]	± 4.52	Stable
NNK stability evaluation	T ₀ VS T _{24h} TOST interval (ng/mL)	T ₀ VS T _{72h} TOST interval (ng/mL)	Acceptance limits (ng/mL)	Stability
Conc. level 3	[-0.10 , 0.16]	[-0.06 , 0.19]	± 0.41	Stable
Conc. level 4	[-0.28 , 0.19]	[-0.09 , 0.38]	± 1.07	Stable
Conc. level 5	[-0.82 , 0.85]	[-0.67 , 1.00]	± 4.27	Stable
NMPA stability evaluation	T ₀ VS T _{24h} TOST interval (ng/mL)	T ₀ VS T _{72h} TOST interval (ng/mL)	Acceptance limits (ng/mL)	Stability
Conc. level 2	[-0.07 , 0.01]	[-0.08 , 0.00]	± 0.10	Stable
Conc. level 3	[-0.30 , 0.05]	[-0.27 , 0.08]	± 0.66	Stable
Conc. level 4	[-0.66 , 0.39]	[-0.64 , 0.41]	± 1.23	Stable

Supplementary Files S1 – S10 are available in the online supplementary material via the link below.

<https://www.sciencedirect.com/science/article/pii/S2949771X25000155#sec0235>

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Illustration of the practical workflow for N-nitrosamine
investigation according to the “Call for Review”
regulatory guidance

Section 1(B)

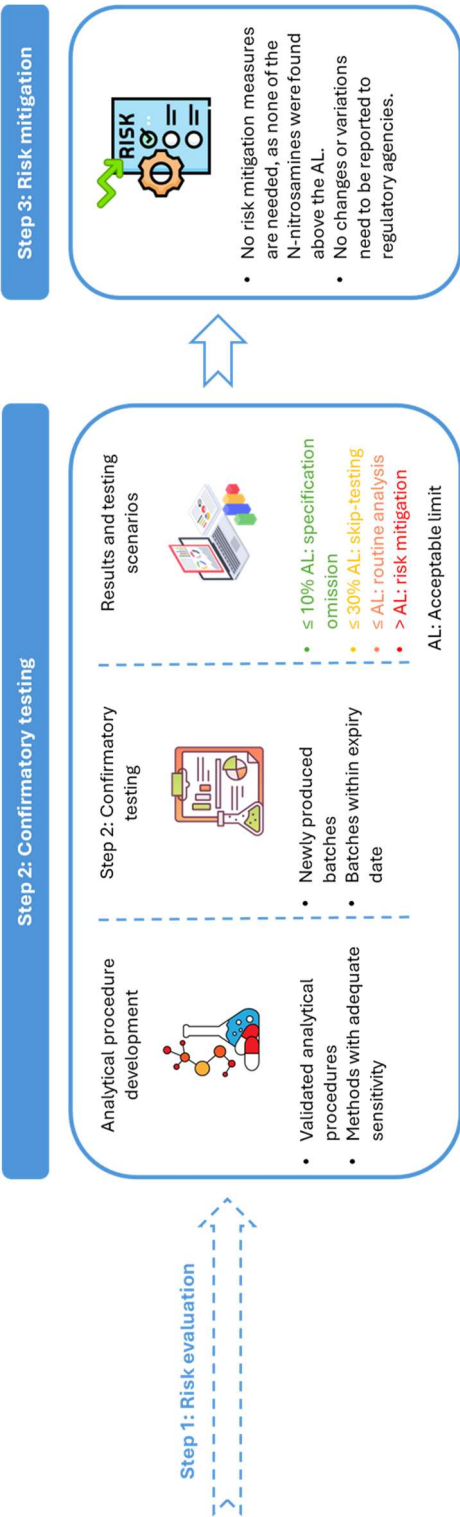
**A case study on N-nitrosamine investigation
in a marketed drug: From analytical
procedure validation to risk mitigation
decision**

Preamble

While the previous section describes the analytical development leading to the establishment of three flexible methods designed to minimize matrix effects, this section demonstrates their performance and presents a case study on the determination and control of small-molecule N-nitrosamine impurities in a marketed drug product, in accordance with the investigation guidance issued by the EMA.

Additionally, this section outlines the N-nitrosamine investigation workflow in the context of the EMA regulatory initiative “Call for Review”. The work aims to provide a practical and illustrative example to support pharmaceutical manufacturers in facilitating the investigation. It details the analytical performance requirements for the preparation and execution of confirmatory testing, and contributes to decision-making regarding risk mitigation measures.

Due to a non-disclosure agreement with the industrial partner, the pharmaceutical product investigated in the study cannot be disclosed. For this reason, the confirmatory testing results are presented using numerical identifiers for the evaluated N-nitrosamine impurities as part of the practical example.



Graphical abstract. Illustration of the 3-step investigation procedure for N-nitrosamine impurities.

Abstract

Introduction: N-nitrosamines, a class of organic compounds recognized for their mutagenic and carcinogenic properties, have been raising major public health concerns following their detection in pharmaceuticals since 2018. These impurities can form during manufacturing processes, particularly through the interaction of amines with nitrosating agents. Regulatory bodies have launched procedures mandating a systematic investigation of medicinal products for N-nitrosamine contamination. This process involves risk evaluation, confirmatory testing, and the implementation of risk mitigation measures. Hyphenated analytical techniques, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), have become the standard for N-nitrosamine detection thanks to their high sensitivity and specificity.

Methods: In this work, based on a previously developed Quantitative Structure Retention Relationship approach for the determination of N-nitrosamines, sensitive LC-MS/MS methods were proposed and validated. These methods were subsequently applied to confirmatory testing of a marketed drug product.

Results: The methods were successfully validated in accordance with the ICH Q2(R2) guidelines as limit tests for trace-level detection of N-nitrosamines. The confirmatory testing demonstrated the absence of most N-nitrosamines throughout the product's shelf life, except for one potentially problematic impurity that nevertheless remained below the established acceptable limit, thus requiring routine quantitative analysis.

Discussion: These findings highlight the need for continued monitoring through routine analysis to ensure the safety and quality of pharmaceutical products.

1 Introduction

N-nitrosamines are a class of organic compounds characterized by the presence of a nitroso functional group. The majority of N-nitrosamines have been identified as mutagenic and carcinogenic in animal studies and are considered potentially carcinogenic to humans. However, Thresher *et al.* reported that approximately 18% of N-nitrosamines are considered non-carcinogenic [1]. These compounds can occur naturally or form in trace amounts in the environment, food, drinking water, and cosmetics [2–4]. Since mid-July 2018, the detection of N-nitrosamines in pharmaceuticals intended for human use has raised substantial public health concerns, prompting action from international regulatory bodies [5]. N-nitrosamine formation may arise from the use of contaminated solvents, reagents, or raw materials, as well as from the application of nitrosating agents in the presence of certain amines during chemical synthesis or manufacturing processes [6,7]. Notably, excipients may also serve as a minor yet significant source of nitrite [8], a well-established nitrosating agent that can contribute to the formation of N-nitrosamines. Therefore, interactions between active pharmaceutical ingredients (APIs) or their related substances and excipients can lead to the formation of a distinct subclass of N-nitrosamines, known as N-nitrosamine drug substance-related impurities (NDSRIs). To date, approximately 90% of reported N-nitrosamines fall into this category [6,9]. Within this subclass, N-nitroso-varenicline was the first NDSRI to be identified, detected in Chantix® or Champix® [6,10] at levels exceeding the established safety threshold, ultimately leading to the product’s recall and market withdrawal in 2021 [11,12]. N-nitroso-varenicline is absent in the varenicline raw material but forms in the finished product due to nitrosation of varenicline in the presence of nitrite originating from excipients. This nitrosation specifically occurs at the secondary amine functional group.

In response to the potential safety risks, the European Medicines Agency (EMA) launched a “Call for Review”. This initiative mandates that marketing authorization holders (MAHs) or applicants review their manufacturing processes, from raw materials through to finished products. The review applies to all medicinal products, encompassing both investigational and marketed drugs, as well as chemically synthesized APIs and biologicals [6,7].

The “Call for Review” involves a systematic investigation procedure to evaluate and detect the presence of N-nitrosamine impurities in pharmaceuticals intended for human use. This process consists of three key steps:

- Step 1: Conduct a comprehensive documentary risk evaluation for each API or finished product to identify or exclude the risk of N-nitrosamine impurities.
- Step 2: If any risk is identified, perform confirmatory testing to confirm the presence or absence of N-nitrosamines.
- Step 3: If the presence of N-nitrosamine(s) is confirmed, implement risk mitigation measures and submit a variation to the marketing authorization, if necessary [6].

The drug product selected for this case study is an oral solid dosage form formulated as uncoated tablets. Based on the documentary risk analysis [8], among excipients, cellulose derivatives, lactose, povidone, and magnesium stearate were identified as potential sources of nitrite. Nitrite is a well-known nitrosating precursor capable of interacting with APIs or related byproducts bearing amine functional groups. Since a risk was identified during Step 1, confirmatory testing (Step 2) was deemed necessary. This work focuses exclusively on small-molecule N-nitrosamines, as the API does not appear to form NDSRIs due to the absence of secondary amine functional groups in its chemical structure. As currently understood, secondary amines are the most reactive and susceptible functional groups for N-nitrosamine formation when exposed to nitrosating agents [13].

For confirmatory testing, analytical methods should be developed with adequate sensitivity to determine trace amounts of N-nitrosamine impurities. According to the EMA, these methods must be validated prior to testing to ensure they provide reliable, high-quality results [6]. The required sensitivity is determined by the calculated acceptable limit for each N-nitrosamine in a specific product. This limit, expressed in ppm, is calculated by dividing the acceptable intake (AI) of the N-nitrosamine (in ng/day) by the maximum daily dose of the product (in mg/day) [6]. AI limits are fixed values established by the EMA, while the maximum daily dose is outlined in the summary of product characteristics. In this case study, the maximum daily dose for the product under investigation is 16 mg/day. It is important to note that (recommended) AI values and relevant N-nitrosamines are listed and periodically reviewed by EMA in Appendix 1 of the document “*Questions and Answers for Marketing Authorization Holders/Applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 Referral on Nitrosamine Impurities in Human Medicinal Products*” [6], as well as on the website of the United States Food and Drug Administration (FDA) through the Center for Drug Evaluation and Research [14]. Both the EMA and the FDA have stated that AI values should be established using a carcinogenic potency categorization approach (CPCA) based on the chemical

structure of N-nitrosamines, which classifies N-nitrosamines into 5 categories, each associated with a specific AI value [6,9,15]. To facilitate this process, Novartis has developed an automated tool that assigns the CPCA category and corresponding AI value for NDSRIs based solely on SMILES-encoded structural information [16]. Exceptionally, the agencies have proposed interim measures to mitigate unnecessary supply disruptions when N-nitrosamine levels exceed the recommended AI limits [6,9,10,14]. The EMA has adopted a less-than-lifetime approach, using conservative adjustment factors to derive interim limits for N-nitrosamine impurities superior to established AI values. This approach is intended as a temporary measure while corrective and preventive actions are implemented to reduce contaminant levels to or below the defined AI limits [6]. In contrast, the FDA's approach consists of updated interim recommended AI limits for only certain NDSRIs, applicable for a limited duration under specific regulatory conditions [14].

Several scenarios can arise depending on the method's lower quantification limit (LQL), which may influence the outcomes of impurity testing [6]. Additionally, the impurity levels detected during confirmatory testing play a crucial role in guiding final decisions regarding the necessity and frequency of further testing, as illustrated in Figure 1.

Chapter III: Results - Section 1(B)

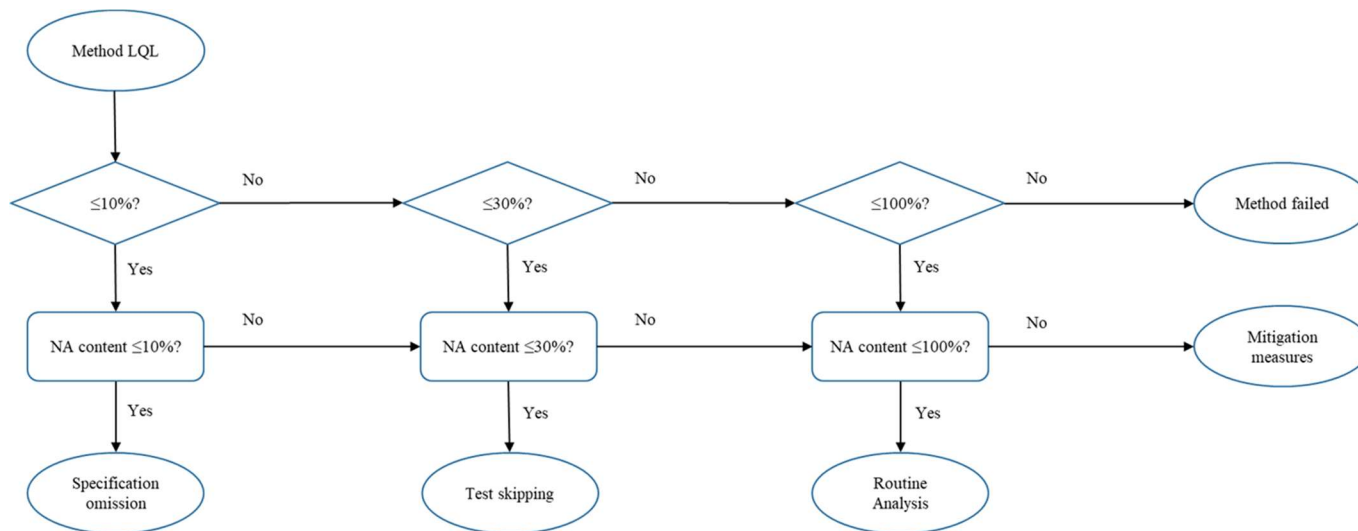


Figure 1. Possible testing scenarios based on the method's LQL and the N-nitrosamine content determined through confirmatory testing. LQL: Lower quantification limit; NA: N-nitrosamine.

In accordance with the EMA's "Call for Review", a representative number of both newly manufactured batches and retained batches that remain within their expiry date must be included in the confirmatory testing to ensure the entire shelf life of the product [6]. The specification of an N-nitrosamine can be omitted if the method's LQL is below 10% of the acceptable limit and the N-nitrosamine content consistently remains below 10% of its acceptable limit across all tested batches throughout the product's shelf life. In such cases, testing for N-nitrosamines is no longer obligatory. The distinction between skip testing and routine analysis lies in the frequency of testing. Skip testing is performed at release on pre-selected batches or predetermined intervals, rather than on every batch, as is the case with routine analysis [17].

Historically, techniques such as thin-layer chromatography, polarography, and spectrophotometry were employed for the detection of N-nitrosamines. However, these methods lacked the sensitivity required to detect trace levels of these genotoxic compounds. In contrast, modern hyphenated techniques, which combine chromatographic separation with mass spectrometric detection, offer superior sensitivity and specificity. Techniques like gas chromatography, liquid chromatography (LC), and supercritical fluid chromatography are commonly paired with mass spectrometers, ranging from simple quadrupole detectors to advanced hybrid systems such as tandem quadrupole (MS/MS) or quadrupole time-of-flight detectors [5]. LC is more versatile and offers a broader analytical scope compared to gas chromatography, as some N-nitrosamines are non-volatile, making gas chromatography unsuitable for their determination. Additionally, determining N-nitrosamines in drug products is more challenging than in drug substances due to the presence of excipients, which usually constitute the major portion of the formulation. In the literature, an LC-MS/MS method capable of simultaneously analyzing up to 13 N-nitrosamines has been reported for biological medicines [18]. Sample preparation protocols vary significantly depending on the dosage form. For instance, protocols designed for biological formulations containing high levels of antibodies, salts, and surfactants are not suitable for oral solid dosage forms, which typically include a high proportion of insoluble excipients such as diluents, disintegrants, lubricants, and colorants.

As outlined in previous work [19], LC-MS/MS was selected as the analytical technique of choice due to its ability to detect analytes based on both LC retention time and structurally specific fragmentation patterns, while also reducing chemical background noise, thus improving sensitivity. This paper builds on previous work and presents three analytical methods validated under the ICH Q2(R2) guidelines, employing a three-level limit test approach for the determination of N-nitrosamine content in a marketed product.

The three methods validated are generic, complementary, and designed to provide flexibility in routine quality control applications. Notably, the methods require no modifications to sample preparation or equipment configuration, enabling the detection of up to 17 small-molecule N-nitrosamines within a single-sequence analysis. This approach ensures i) compliance with regulatory sensitivity requirements for N-nitrosamine control, and ii) effective resolution of co-elution with the API. The case study, centered on a marketed drug product, provides a practical illustration of the structured workflow involved in conducting analytical confirmatory testing for MAHs or applicants.

2 Materials and methods

2.1 Chemicals and reagents

The same chemicals and reagents described previously [19] were purchased and used in this work.

500 µg/mL certified reference stock solutions 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) were obtained from the EDQM (Strasbourg, France). Certified reference stock solutions at 1 mg/mL of 1-methyl-4-nitrosopiperazine (MeNP), and N-nitroso-1,2,3,6-tetrahydropyridine (NTHP) were supplied by the United States Pharmacopeia (Rockville, MD, USA). Reference stock solutions at 1 mg/mL of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosomethylphenylamine (NMPA) were purchased from Sigma-Aldrich/Merck (St. Louis, MO, USA). Similarly, 1000 µg/mL reference stock solutions of N-nitrosomethylethylamine (NMEA), N-nitrosopyrrolidine (NPYR), and N-nitroso-piperidine (NPIP) were purchased from Cambridge Isotopes Laboratories (Tewksbury, MA, USA). Reference standards of N-nitrosomorpholine (NMOR), N-nitrosodiphenylamine (NDPhA), and N-nitroso-diethanolamine (NDELA) were obtained from TCI (Tokyo, Japan).

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

17 α -hydroxyprogesterone was obtained from TCI. ULC-MS grade methanol, water, formic acid, and ammonium acetate were purchased from Biosolve (Dieuze, France). The pharmaceutical formulation used in the validation and case studies was sourced by a pharmaceutical industry partner. Due to a confidentiality agreement, the nature and dosage of the API cannot be disclosed.

Prior to the validation studies and confirmatory testing, individual stock solutions of internal standards NDPA-d14, MeNP-d4, and NDELA-d8 were prepared at a concentration of 1 mg/mL. Both reference stock solutions and prepared stock solutions were aliquoted and stored at -20 °C to prevent instability of the compounds due to repeated freeze-thaw cycles.

2.2 Instrumentation and software

Validation studies and confirmatory testing were conducted using an Acquity® Premier UPLC® system (Waters, Milford, MA, USA), equipped with an Acquity® UPLC® HSS T3 VanGuard pre-column (1.8 μ m, 2.1 mm $\times$ 5 mm) connected to either an Acquity® Premier HSS T3 column (1.8 μ m, 2.1 mm $\times$ 100 mm) or an Acquity® UPLC® HSS T3 column (1.8 μ m, 2.1 mm $\times$ 100 mm) from Waters. The chromatographic system was coupled with a Xevo® TQ-Absolute tandem quadrupole spectrometer (Waters), featuring an atmospheric pressure chemical ionization (APCI) source.

Instrument control, data acquisition, and data processing were managed using MassLynx® Security software (Waters).

2.3 Analytical methods

2.3.1 Sample preparation

An extraction protocol was employed to isolate the analytes from the drug product. Uncoated tablets were crushed into a fine powder. Following the addition of internal standards (see Section 2.4.1 for preparation details) to 425 mg of powdered material, 5.0 mL of methanol was added to solubilize and extract N-nitrosamines while simultaneously removing insoluble excipients such as cellulose derivatives, lactose, and magnesium stearate. The mixture was ultrasonicated for 25 minutes and then centrifuged at 11,000 rpm for 15 minutes. To ensure the removal of residual water-soluble excipients, 1.0 mL of the resulting supernatant was collected and mixed with 4.0 mL of 1% formic acid solution, followed by a second centrifugation under the same conditions. The final supernatant was transferred into an analytical vial for injection.

2.3.2 *Chromatographic conditions*

The chromatographic conditions used for the two validation studies and confirmatory testing are summarized in Table 1. As shown, the only difference between the mobile phase conditions 1 and 2 is their composition. An aqueous mobile phase adjusted to pH 2.7 was used for the validation of Method 2, whereas a pH of 5.0 was applied for the validation of Method 3. As demonstrated in previous work, Method 1 was also validated under pH 2.7 conditions. This decision was made during the development of the three methods to optimize sensitivity and mitigate matrix effects. Working at pH 2.7 yielded greater signal intensities for most N-nitrosamines. However, under these experimental conditions, MeNP, NDEA, NTHP, NPIP, and NDELA co-eluted with the API. Therefore, an alternative pH of 5.0 was selected for the analysis of these compounds to prevent such co-elution observed at pH 2.7. In addition, the N-nitrosamines were divided into two source temperature groups, 150 and 250 °C, to further enhance signal intensities [19]. The effect of source or probe temperature must be carefully considered, as elevated temperatures can cause in-source fragmentation by promoting analyte dissociation. Therefore, thermolabile compounds required a lower probe temperature and were analyzed at 150 °C to prevent undesirable thermal degradation, while the source temperature was consistently maintained at 120 °C.

Chapter III: Results - Section 1(B)

Table 1: Chromatographic conditions.

Stationary phase	Pre-column: - Acquity® UPLC® HSS T3 VanGuard pre-column (1.8 μm, 2.1 mm × 5 mm) Columns: - Acquity® Premier HSS T3 column (1.8 μm, 2.1 mm × 100 mm) - Acquity® UPLC® HSS T3 column (1.8 μm, 2.1 mm × 100 mm)																							
Mobile phase	pH condition 1: - Solvent A: 0.1% FA in water, pH 2.7 - Solvent B: 0.1% FA in methanol	pH condition 2: - Solvent A: 10 mM AcAm, pH 5.0 - Solvent B: Methanol																						
Flow rate	0.4 mL/min																							
Gradient	<table><tr><th>Time (min)</th><th>Solvent A (%)</th><th>Solvent B (%)</th></tr><tr><td>Initial</td><td>100.0</td><td>0.0</td></tr><tr><td>1.00</td><td>100.0</td><td>0.0</td></tr><tr><td>10.90</td><td>5.0</td><td>95.0</td></tr><tr><td>13.50</td><td>5.0</td><td>95.0</td></tr><tr><td>13.60</td><td>100.0</td><td>0.0</td></tr><tr><td>17.00</td><td>100.0</td><td>0.0</td></tr></table>			Time (min)	Solvent A (%)	Solvent B (%)	Initial	100.0	0.0	1.00	100.0	0.0	10.90	5.0	95.0	13.50	5.0	95.0	13.60	100.0	0.0	17.00	100.0	0.0
Time (min)	Solvent A (%)	Solvent B (%)																						
Initial	100.0	0.0																						
1.00	100.0	0.0																						
10.90	5.0	95.0																						
13.50	5.0	95.0																						
13.60	100.0	0.0																						
17.00	100.0	0.0																						
Temperature	Column: 45 °C Autosampler: 10 °C																							
Injection volume	10 μL																							
Needle wash	Water/Methanol (10%/90%, v/v)																							
Purge solvent	Water/Methanol (90%/10%, v/v)																							

FA: formic acid; AcAm: acetate ammonium.

Based on these parameters, the N-nitrosamines were categorized into three groups, each assigned to one of the flexible methods for validation, as follows:

- Method 1 (pH 2.7/250 °C): NMOR, NMBA, NDPhA, NNK, and NMPA,
- Method 2 (pH 2.7/150 °C): NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA,
- Method 3 (pH 5.0/150 °C): MeNP, NDEA, NTHP, NPIP, and NDELA [19].

These methods only differ in the mobile phase composition or the temperature applied to the APCI probe, enabling i) compliance with regulatory sensitivity requirements for N-nitrosamine control, and ii) effective resolution of co-elution with the API. Following successful validation, the same chromatographic conditions were used for confirmatory testing.

2.3.3 *Mass spectrometric parameters*

The ion source was configured in positive mode with a corona current of 1.5 μ A and a source temperature of 120 °C. The APCI probe temperature was set to 150 °C (Methods 2 and 3) or 250 °C (Method 1). Desolvation, nebulization, cone, and collision gas flows were adjusted to 950 L/hr, 250 L/hr, 250 L/hr, and 0.15 mL/min, respectively. Data acquisition was performed in multiple reaction monitoring (MRM) mode, targeting specific precursor ions and product ions. The autodwell option was enabled for all N-nitrosamines, while the soft transmission was activated only for fragile analytes: NMBA, NEIPA, NDPA, NDELA, and their deuterated analogs. The MRM parameters are detailed in Table 2.

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 → 86.93 117.10 → 45.09	2.20 – 3.80	27	13 13	No
NMPA	137.05 → 66.10 137.05 → 107.15	6.20 – 7.50	28	12 11	No
NMBA	147.10 → 117.10 147.10 → 44.10	2.80 – 3.90	17	7 10	Activated
NDPhA	199.10 → 66.10 199.10 → 169.10	8.30 – 10.20	23	22 12	No
NNK	208.10 → 122.10 208.10 → 148.00	4.20 – 5.30	32	14 10	No
NDMA	75.05 → 58.05 75.05 → 43.00	1.65 – 2.90	32	9 13	No
NDBA	159.20 → 57.10 159.20 → 103.23	8.70 – 9.70	30	12 10	No
NMEA	89.11 → 61.00 89.11 → 43.00	3.00 – 4.20	24	9 9	No
NEIPA	117.20 → 74.99 117.20 → 47.10	5.40 – 6.50	25	8 14	Activated

Chapter III: Results - Section 1(B)

Table 2 Continued.

N-nitrosamine	MRM transition (m/z)	Retention window (min)	Cone voltage (V)	Collision energy (eV)	Soft transmission
NDIPA	131.20 → 89.10 131.20 → 43.10	6.30 – 7.50	17	10 9	No
NDPA	131.16 → 89.16 131.16 → 43.14	6.90 – 7.90	30	11 11	Activated
NPYR	101.12 → 55.00 101.12 → 41.00	3.10 – 4.30	22	15 18	No
MeNP	130.00 → 58.00 130.00 → 100.00	2.00 – 3.00	27	13 5	No
NDEA	103.20 → 74.90 103.20 → 46.90	4.30 – 5.30	33	9 12	No
NPIP	115.15 → 69.00 115.15 → 41.00	4.80 – 5.70	13	14 17	No
NTHP	113.10 → 67.10 113.10 → 83.10	4.40 – 5.40	17	10 13	No
NDELA	135.10 → 74.00 135.10 → 104.00	0.70 – 2.20	8	10 6	Activated
NMOR-d8	125.17 → 95.00	2.40 – 3.80	20	15	No

Chapter III: Results - Section 1(B)

Table 2 Continued.

N-nitrosamine	MRM transition (m/z)	Retention window (min)	Cone voltage (V)	Collision energy (eV)	Soft transmission
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

Q: Quantifier ion; q: Qualifier ion.

2.4 Validation protocol

Method 1 (pH 2.7/250 °C) was successfully validated in previous work for the determination of NMOR, NMBA, NDPhA, NNK, and NMPA, both as a quantitative method and as a limit test. This approach was justified by the identification of one of these five N-nitrosamines as being “at risk” of occurring in the finished product due to the nature of the API. Validation results for Method 1 are detailed in the previous publication [19].

The present work focuses on the validation of Methods 2 and 3, which target groups 2 and 3 of N-nitrosamine impurities, respectively. These methods were developed under the experimental conditions outlined in the earlier study. Specifically, Method 2 (pH 2.7/150 °C) was used for the determination of NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA; whereas Method 3 (pH 5.0/150 °C) was applied for the determination of MeNP, NDEA, NPIP, NTHP, and NDELA [19].

Validation of Methods 2 and 3 was conducted in accordance with ICH Q2(R2) guidelines, using a limit test approach. This decision was based on the absence of any N-nitrosamine from groups 2 and 3 being identified as “at risk” for presence in the final product. Four independent validation series were performed, involving two operators to account for variability. The Acquity® Premier HSS T3 column and the Acquity® UPLC® HSS T3 column were used to evaluate the feasibility of employing them interchangeably in routine analyses. Due to the photosensitivity of N-nitroso compounds, all preparations were carried out under light-protected conditions to prevent photodegradation.

2.4.1 Internal standards

On each validation day, aliquots of stock solutions were thawed and used once they reached room temperature. Individual stock solutions of internal standards were diluted to 100 µg/mL with methanol. Two distinct concentrations of a mixture containing NDMA-d6, NDPA-d14, NDBA-d14, NPYR-d8, and NMEA-d3 were prepared: one (IS 1) was used for spiking the validation standards, while the other (IS 2) was used to supplement the calibration standards. In both validation and calibration standards, the injected concentrations of NDMA-d6, NDPA-d14, NDBA-d14, NPYR-d8, and NMEA-d3 were 5, 1.25, 1.25, 50, and 5 ng/mL, respectively.

Similarly, two mixtures of MeNP-d4, NDEA-d10, NPIP-d10, and NDELA-d8 were prepared: one (IS 3) was used to spike the validation standards, while the other (IS 4) was used to supplement the calibration standards. In both validation and calibration standards, the injected concentrations of MeNP-d4, NDEA-d10, NPIP-d10, and NDELA-d8 were 20, 1.5, 50, and 10 ng/mL, respectively.

To normalize the signal, a known quantity of internal standards was added to calibration and validation standards to ensure consistent internal standard concentrations across all standards. This practice is recommended to account for variability in sample preparation and to compensate for variability in the ionization process inherent to the MS(/MS) technique. Although stable isotope-labeled internal standards are expensive, they are generally preferred over structural analogs. These internal standards share identical physicochemical properties and theoretically co-elute with the analytes in LC-MS/MS analyses. Their use can mitigate the enhancement or suppression of ionization in the ion source caused by matrix effects [20].

2.4.2 Calibration standards

In alignment with the three-level limit test approach proposed based on the acceptable limit, calibration standards were prepared at three concentration levels: 10, 30 and 100% of the respective acceptable limit. The calculated three levels of acceptable limits, derived from the AI limits [6] and maximum daily dose for each N-nitrosamine, are presented in Table 3.

Chapter III: Results - Section 1(B)

Table 3: Recommended AI limits published by the EMA and respective acceptable limits for N-nitrosamines in the product under investigation.

Method 1 – pH 2.7/250 °C					
	AI limit (ng/day)	MDD (mg/day)	Acceptable limit (ppm)		
			100%	30%	10%
NMOR	127	16	7.94	2.38	0.79
NMBA	1500		93.75	28.13	9.38
NDPhA	78000		4875.00	1462.50	487.50
NNK	100		6.25	1.88	0.63
NMPA	100		6.25	1.88	0.63
Method 2 – pH 2.7/150 °C					
	AI limit (ng/day)	MDD (mg/day)	Acceptable limit (ppm)		
			100%	30%	10%
NDMA	96	16	6.00	1.80	0.60
NDPA	26.5		1.66	0.50	0.17
NDIPA	1500		93.75	28.13	9.38
NEIPA	400		25.00	7.50	2.50
NDBA	26.5		1.66	0.50	0.17
NPYR	1700		106.25	31.88	10.63
NMEA			AI limit undefined by the EMA		

Table 3 Continued.

Method 3 – pH 5.0/150 °C					
	AI limit (ng/day)	MDD (mg/day)	Acceptable limit (ppm)		
			100%	30%	10%
MeNP	400	16	25.00	7.50	2.50
NDEA	26.5		1.66	0.50	0.17
NTHP	37		2.31	0.69	0.23
NPIP	1300		81.25	24.38	8.13
NDELA	1900		118.75	35.63	11.88

This work was conducted using data published by the EMA, as updated in September 2024.

For each validation series, a 1 mg/mL stock solution of NDELA was freshly prepared in methanol. Aliquots of the stock solutions were thawed and used after reaching ambient temperature. Each stock solution was individually diluted to 10 µg/mL with diluent. The diluent consisted of a mixture of water/methanol (80%/20%, v/v) containing 0.1% formic acid.

Two mixtures of NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA were prepared at concentrations corresponding to the acceptable limits for each N-nitrosamine: one with internal standards (solution IS 2) and one without internal standards. The mixture without internal standards was then diluted and supplemented with solution IS 2 to reach 10 and 30% of the respective acceptable limits.

Similarly, two mixtures of MeNP, NDEA, NPIP, NTHP, and NDELA were prepared at concentrations corresponding to the acceptable limits of each N-nitrosamine: one with internal standards (solution IS 4) and one without. The mixture without internal standards was diluted and supplemented with solution IS 4 to reach 10 and 30% of the respective acceptable limits.

2.4.3 Validation standards

The pharmaceutical product under investigation, in tablet form, was crushed into a powder and used as the drug matrix to prepare validation standards, simulating real samples. Validation standards were freshly prepared in independent triplicate on each validation day, and separately from the calibration standards. For NDELA, a 1 mg/mL stock solution was prepared. Each stock solution was then diluted to the appropriate concentration before being used to prepare mixtures for supplementing validation standards by adding known quantities of N-nitrosamines.

Two N-nitrosamine validation mixtures were prepared: one containing NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA (solution M1), and the other containing MeNP, NDEA, NPIP, NTHP, and NDELA (solution M2).

Solutions M1 and IS 1 were added to the drug matrix, as were solutions M2 and IS 3. Validation standards were prepared to match the concentrations of the calibration standards, corresponding to 10, 30, and 100% of the acceptable limits for each N-nitrosamine. The respective concentrations are presented in Table 3.

Following the addition of N-nitrosamine standards and internal standards, each validation standard was prepared according to the procedure described in Section 2.3.1. For NDELA, a 10-fold dilution was applied prior to injection.

2.4.4 *Unspiked matrices*

Unspiked matrix solutions were prepared independently in triplicate without the addition of N-nitrosamine standards or internal standards. These solutions were processed following the same extraction protocol as the samples detailed in Section 2.3.1 to assess method selectivity.

2.5 Equipment performance verification

17 α -hydroxyprogesterone is recommended by the instrument manufacturer for verifying system performance when operating in APCI positive mode. Accordingly, a 50 ng/mL solution of 17 α -hydroxyprogesterone was freshly prepared each day and injected in triplicate prior to the N-nitrosamine analysis sequence. This verification step was performed independently of the formal N-nitrosamine analyses to confirm instrument sensitivity and repeatability. The equipment performance verification is considered compliant if the mean intensity calculated from the three injections is equal to or greater than 8,000,000 counts, and the relative standard deviation (RSD%) of the intensities obtained is not more than 5%, as established from data obtained during method development.

2.6 System suitability test solutions

System suitability tests (SSTs) are commonly used by laboratories to ensure that the analytical system is suitable for its intended use on the day of analysis. In agreement with the ICH Q14 guidelines, a set of SSTs was validated alongside the validation studies. SSTs are an integral and critical part of an analytical procedure to verify its performance [21].

2.6.1 *System sensitivity verification for trace N-nitrosamine analysis*

Two N-nitrosamine standard solutions at 5% of the acceptable limits were independently prepared: one containing NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA, and the other containing MeNP, NDEA, NPIP, NTHP, and NDELA. The signal-to-noise (S/N) ratio for each analyte was calculated to verify the system's sensitivity. There were no predefined specifications for this SST, instead, specifications were established based on data generated throughout the validation studies.

2.6.2 *Ion ratio*

The ion ratio was calculated by dividing the area under the curve (AUC) of the less intense by that of the most intense ion. The standard solution containing the highest concentration of N-nitrosamines at the acceptable limit was used to establish the reference value. The ion ratio in all calibration and validation standards was then compared to this reference value to determine conformity.

Considering that the ion ratio can vary slightly from series to series, there were no predefined specifications for this SST, instead, specifications were established based on data generated throughout the validation studies.

2.6.3 *NDBA carry-over verification*

An NDBA-related carry-over has been observed since the method's development. To monitor this carry-over, multiple blank injections were performed at the beginning of the analysis sequence. The mean AUC of the carry-over-related peaks observed at the quantifier transition and retention time of NDBA was calculated using the last three blank injections before injecting calibration standards. The SST is considered compliant if the mean AUC of the carry-over-related peaks, corresponding to the retention time of NDBA, does not exceed 10% of the AUC of NDBA at the quantifier transition obtained from the calibration solution at 10% of the acceptable limit.

2.7 Confirmatory testing

Confirmatory testing is part of Step 2 of the "Call for Review" procedure, and it was performed on the finished product in tablet form. According to the investigation procedure, the number of batches to be tested should correspond to the risk and be representative of annual production. Since fewer than 10 batches were manufactured each year, three batches were randomly selected from each production year between 2021 and 2024, resulting in a total of 12 batches. This included three newly produced batches and nine retained samples from batches still within their expiry date. The retained samples were stored under controlled stability conditions of 25 °C and 60% relative humidity (RH). Testing was performed using Method 1, previously validated [19], along with Methods 2 and 3, validated in the present study. All three LC-MS/MS methods were successfully validated in accordance with the ICH Q2(R2) guidelines.

From an experimental perspective, confirmatory testing was carried out using a three-level limit test approach, corresponding to 10, 30, and 100% of the acceptable limit for each N-nitrosamine. To maintain consistency with the validation studies, calibration standards were prepared at these same three concentration levels (refer to Table 3). A known amount of internal standards was added to both the calibration standards and the samples, mirroring the procedure applied during validation.

Sample preparation adhered to the protocol described in Section 2.3.1, employing an extraction procedure to isolate N-nitrosamines from the samples, if present. 12 batches were analyzed over two separate days. On the first day, batches 1 to 6 were tested, while on the second day, batches 7 to 12 were analyzed.

3 Results

3.1 Validation studies

3.1.1 Selectivity

Both methods were validated as limit tests for impurity, in compliance with the ICH Q2(R2) recommendations for screening purposes. At the onset of the validation studies, the selectivity and specificity of the methods were independently demonstrated for each N-nitrosamine by injecting the following solutions: i) blank; ii) N-nitrosamine calibration standard at the lowest concentration (10% of the respective acceptable limit); iii) unspiked matrix solutions in independent triplicate. The selectivity of the methods is illustrated in Figure S1, demonstrating chromatographic separation of all N-nitrosamines from the API.

For the N-nitrosamines and their paired internal standards (where only the quantifier transition was monitored), no interfering peaks were observed at their respective retention times. However, only one exception occurred with NDBA, for which a consistent carry-over was detected across the validation series. This carry-over-related peak appeared at the same retention time as NDBA in both the quantifier and qualifier transitions, even in blank injections or when no injection was performed, as demonstrated in Figure 2.

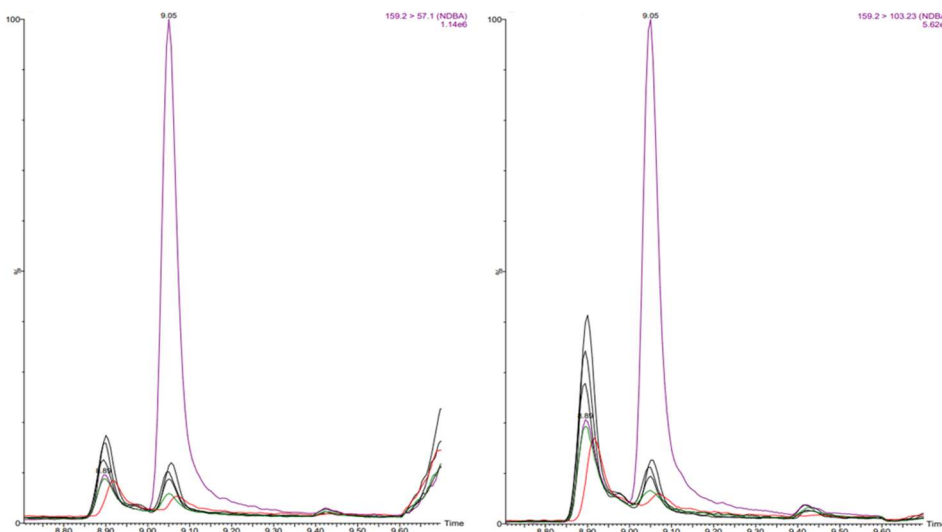


Figure 2. Overlaid extracted ion chromatograms. (A) left: NDBA at quantifier ion transition; (B) right: NDBA at qualifier ion transition. (red) No injection; (green) Blank; (black) Unspiked matrices in triplicate; (purple): Calibration standard at the lowest concentration level (10% of the acceptable limit).

Carry-over refers to the unintended appearance of residual analyte signals, often observed as undesirable small peaks in blank injections. Understanding and controlling carry-over is essential because the residual signals may interfere with analyte detection and potentially compromise the accuracy of the method. Therefore, identifying and eliminating the sources of carry-over during the method development phase is crucial [22].

The source of this unexpected carry-over related to NDBA was investigated during method development. Through testing, which included the assessment of different needle wash solution compositions, use of two different columns, involvement of two operators, and evaluation of various batches of solvents from multiple suppliers, the carry-over was confirmed to be related to the equipment itself, rather than external factors like solvents or isolated contamination events.

To validate the selectivity of NDBA, throughout all validation series, the mean percentage of the NDBA-related carry-over AUC in matrices A, B, and C consistently remained below 10% of the AUC of NDBA at the quantifier transition at the lowest concentration level of the calibration solution (as shown in Table S1). This approach ensures that the AUC of the carry-over-related peak will not exceed 1% of the AUC at the acceptable limit (or the highest concentration level). Therefore, the interference due to carry-over is considered acceptable and does not compromise the method's selectivity for NDBA determination concerning the intended purpose.

3.1.2 *Detection limit*

The detection limit is the lowest amount of an analyte that can be detected in a sample but cannot be precisely and accurately quantified [21,23]. According to the ICH Q2(R2) recommendations, an S/N ratio of 3 is generally considered acceptable for estimating the detection limit. In the studies, the detection limit was estimated by extrapolating the average S/N ratio obtained at the lowest concentration level across all validation series to a threshold of 3. The main advantage of this extrapolation-based approach lies in its ability to reasonably lower the method detection limit based on experimental data. However, its principal limitation is that the estimated detection limit was not experimentally demonstrated. At the extrapolated concentration level, there remains a risk that the analyte may not be detectable. Nevertheless, as the experimentally confirmed detection limits (corresponding to 10% of the acceptable limit) exhibited overall S/N ratios significantly greater than 3, the risk of non-detectability is thus lower than 0.3% and is therefore considered negligible in the context of the present case study.

Chapter III: Results - Section 1(B)

As summarized in Table 4, the detection limit estimated for each N-nitrosamine was found to be below 10% of the acceptable limit in the product, meeting the required method sensitivity criteria for omitting the specification. Since NMEA is not listed in Appendix 1 of the document “*Questions and Answers for Marketing Authorization Holders/Applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 Referral on Nitrosamine Impurities in Human Medicinal Products*”, no AI limit is available for NMEA. Based on the concentration range chosen in the previous validation study, the highest concentration level of 30 ng/mL was arbitrarily and exceptionally selected as the threshold for NMEA.

Table 4: Estimated detection limit for each N-nitrosamine.

Estimated detection limit (<i>p</i> = 4)							
Method 2 pH 2.7/150 °C	NDMA	NDPA	NDIPA	NEIPA	NDBA	NPYR	NMEA
Detection limit (ppb)	40	9	1	2	7	22	3 pg/mL*
Method 3 pH 5.0/150 °C	MeNP	NDEA	NPIP	NTHP	NDELA		
Detection limit (ppb)	52	23	135	23	89		

*The detection limit is expressed in pg/mL, as the EMA has not established an AI limit for NMEA. For the purpose of confirmatory testing, a threshold concentration of 30 ng/mL was exceptionally and arbitrarily selected for NMEA.

p: Number of series of validation.

3.1.3 *Recovery*

Matrix effects may compromise the sensitivity, accuracy, and precision of analytical procedures. They arise from various factors, including the components present in the sample matrix, the sample preparation technique, and the ionization type used in MS analyses [24]. Matrix effects were evaluated by assessing the recovery of responses calculated for both the calibration and validation standards at three levels of acceptable limits: 10, 30, and 100%. Response is defined as the ratio of the analyte AUC to that of its corresponding internal standard. Recovery was individually calculated for each validation standard across the validation series, and determined by comparing the responses obtained in the validation standard to those in the calibration standard at the same concentration level.

To ensure that the matrix effect is acceptable, the individual recovery must fall within the range of 85 to 115% for each validation standard across all validation series. Additionally, the repeatability of recovery was assessed based on three replicates at the same concentration level for each validation series. Except for NMEA, recovery and repeatability were assessed only at an arbitrarily chosen concentration of 30 ng/mL. Within a single validation series, the repeatability, expressed as the RSD% of recovery for the three replicates, must not exceed 15%.

Detailed data for recovery and repeatability are presented in Table 5. Overall, the recovery and RSD% values meet the pre-established specifications, indicating that the matrix effects can be considered acceptable for all N-nitrosamines.

Chapter III: Results - Section 1(B)

Table 5: Response recovery and RSD% for matrix effects evaluation – Method 2 (pH 2.7/150 °C) and Method 3 (pH 5.0/150 °C).

Acceptance limit	NDMA							
	<i>(p = 4; n = 3)</i>							
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	99 – 101	1	102 – 103	1	93 – 99	3	97 – 100	1
Level 30%	99 – 103	2	99 – 103	2	90 – 92	1	95 – 97	1
Level 100%	101 – 106	2	106 – 108	1	93 – 94	1	97 – 99	1
NDPA								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	101 – 104	2	97 – 102	2	98 – 101	1	92 – 98	3
Level 30%	105 – 106	1	99 – 100	1	91 – 95	3	90 – 93	1
Level 100%	105 – 108	1	99 – 101	1	93 – 96	2	94 – 96	1
NDIPA								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	101 – 106	2	98 – 102	2	94 – 101	4	96 – 100	2
Level 30%	101 – 107	3	95 – 97	1	89 – 95	4	90 – 95	2
Level 100%	101 – 105	2	96 – 99	2	90 – 92	1	92 – 96	2

Chapter III: Results - Section 1(B)

Table 5 Continued.

NEIPA								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	92 – 94	1	99 – 103	2	93 – 94	1	90 – 95	3
Level 30%	98	1	98 – 100	1	86 – 89	2	89 – 92	1
Level 100%	99	0	98	0	90 – 93	2	93 – 96	1
NDBA								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	101 – 103	1	101 – 105	2	101 – 102	1	99 – 102	2
Level 30%	103 – 106	1	98 – 101	2	96 – 98	1	94	0
Level 100%	103 – 105	1	102 – 104	1	100 – 102	1	103 – 104	1
NPYR								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	90 – 95	3	95 – 96	1	99 – 102	2	100 – 103	1
Level 30%	96 – 98	1	93 – 94	1	92 – 94	1	95 – 97	1
Level 100%	93 – 95	1	96 – 98	1	97 – 98	1	96 – 97	1

Chapter III: Results - Section 1(B)

Table 5 Continued.

NPYR								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	90 – 95	3	95 – 96	1	99 – 102	2	100 – 103	1
Level 30%	96 – 98	1	93 – 94	1	92 – 94	1	95 – 97	1
Level 100%	93 – 95	1	96 – 98	1	97 – 98	1	96 – 97	1
NMEA								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
30 ng/mL	99 – 102	1	103 – 105	1	99 – 101	1	99 – 101	1
MeNP								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	96 – 100	2	93 – 100	4	101 – 102	1	99 – 105	3
Level 30%	97 – 104	3	98 – 100	2	98 – 100	1	100 – 103	1
Level 100%	102 – 110	4	99 – 102	1	102 – 104	1	104 – 105	1

Chapter III: Results - Section 1(B)

Table 5 Continued.

NDEA								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	98 – 109	5	92 – 96	2	102 – 107	2	96 – 98	1
Level 30%	109 – 112	1	93 – 95	1	101 – 102	1	93 – 100	4
Level 100%	112 – 113	0	95 – 97	1	100 – 102	1	101 – 107	3
NPIP								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	103 – 110	3	95 – 96	0	103	0	100 – 101	1
Level 30%	112 – 114	1	93 – 94	1	95 – 101	3	100 – 102	1
Level 100%	112 – 113	1	95 – 96	1	99 – 101	1	101 – 103	1
NTHP								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	101 – 110	4	92 – 96	2	97 – 100	1	96 – 100	2
Level 30%	111 – 113	1	91 – 92	0	100 – 104	2	101 – 103	1
Level 100%	112 – 113	0	93 – 95	2	102 – 103	1	102 – 104	1

Chapter III: Results - Section 1(B)

Table 5 Continued.

NDELA								
<i>(p = 4; n = 3)</i>								
	Series 1		Series 2		Series 3		Series 4	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
	Min. – Max.		Min. – Max.		Min. – Max.		Min. – Max.	
Level 10%	97 – 100	1	99 – 100	0	93 – 98	3	100 – 105	2
Level 30%	98 – 102	2	97 – 99	1	97 – 99	1	97 – 99	1
Level 100%	95 – 97	1	100 – 101	1	101 – 103	1	101 – 103	1

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

3.1.4 Equipment performance verification

In the first instance, the equipment's sensitivity and repeatability were verified daily before the principal validation analysis sequence. The mean intensity and RSD (%) values obtained from the three injections of 17 α -hydroxyprogesterone solution are reported in Tables S2 and S3. All averaged intensities and RSD (%) values fulfill the specifications pre-established, *i.e.* mean intensity not less than 8,000,000 counts, and RSD (%) not more than 5%.

3.1.5 System suitability tests

The ICH Q14 guidelines emphasize that SSTs are established and implemented to ensure that the measurement system and the associated analytical operations fit the intended applications, thereby enhancing the detectability of undesirable and unacceptable performance [21].

3.1.5.1 System sensitivity verification for trace N-nitrosamine analysis

Analytical system sensitivity is crucial for trace analyses such as the determination of N-nitrosamine impurities. According to the ICH Q2(R2) guidelines, an S/N ratio of 3 is typically considered acceptable for estimating the detection limit. Therefore, the S/N ratio should not be less than 3 for each N-nitrosamine in the standard solution prepared at 5% of the acceptable limit. All values are summarized in Tables S2 and S3, and conform to the specifications.

3.1.5.2 Ion ratio

The ion ratio is commonly used in MS/MS analyses, particularly when using MRM acquisition mode. This SST confirms the identity of analytes and was verified for all calibration and validation standards. All ion ratio values fell within the maximum permitted tolerance for the LC-MS/MS technique [25]. Detailed data are provided in Tables S2 and S3.

3.1.5.3 NDBA carry-over verification

Throughout all validation series, the mean percentage of the NDBA-related carry-over AUC consistently remained below 10% of the AUC of NDBA at the quantifier transition in the calibration solution at 10% of the acceptable limit. Therefore, the verification of the carry-over conforms to the pre-established specifications (refer to Table S2).

3.2 Confirmatory testing

Alongside the validation studies, equipment performance verification and SSTs were also validated to support the routine implementation of the methods. During confirmatory testing, these verifications were carried out to ensure analytical performance on the day of analysis.

3.2.1 *Equipment performance verification and system suitability tests*

Confirmatory testing was conducted over two separate days of analysis. On each day, equipment performance verification and SSTs, based on validation studies, were verified to ensure the equipment's sensitivity and repeatability, system sensitivity (S/N ratio), ion ratio compliance, and to monitor the carry-over related to NDBA. The equipment performance verification and all SSTs met the required specifications, validating the analysis sequences. Table S4 summarizes the results.

3.2.2 *Testing results*

Due to confidentiality considerations, the results of the confirmatory testing are presented in Table 6 without identifying the specific N-nitrosamines detected. This case study is only intended to provide a practical example of the workflow involved in conducting analytical confirmatory testing.

Confirmatory testing was conducted under the "Call for Review" procedure. Appropriate future testing scenarios were determined based on the N-nitrosamine levels detected during the study, as summarized in Figure 1. The results demonstrated that most N-nitrosamines were below the detection limit, indicating that their levels consistently remained under 10% of the respective acceptable limits throughout the product's shelf life. This observation supports the omission of their specifications and eliminates the need for further testing. For N-nitrosamine 12, no intra-year batch-to-batch variation was observed, however, inter-year variation was noted. This impurity was detected in 6 out of 12 batches, with its content consistently remaining below 10% of the acceptable limit. As a result, N-nitrosamine 12 also does not require further testing, and its specification can be omitted. In contrast, one N-nitrosamine impurity, N-nitrosamine 6, was consistently detected across all batches, including both release and ongoing stability samples stored under 25 °C / 60% RH conditions. At release (T_0), its content remained consistently below 10% of the acceptable limit. At 12 months, the amount exceeded 10% but stayed below 30% of the acceptable limit. By 24 months, its content consistently surpassed 30%, though it remained strictly below the acceptable limit. These findings indicate that the product remains compliant with the specification at the end of its shelf life. Risk mitigation measures are therefore not required. However, quantitative routine analysis remains necessary for N-nitrosamine 6, in alignment with the "Call for Review" procedure (Figure 1).

Chapter III: Results - Section 1(B)

Table 6: Summary of confirmatory testing results.

	N-nitrosamine content											
	Batch 1 T0	Batch 2 T0	Batch 3 T0	Batch 4 T12M	Batch 5 T12M	Batch 6 T12M	Batch 7 T24M	Batch 8 T24M	Batch 9 T24M	Batch 10 T30M	Batch 11 T36M	Batch 12 T36M
Manufacturing year	2024	2024	2024	2023	2023	2023	2022	2022	2022	2021	2021	2021
NA 1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 6	≤ 10%	≤ 10%	≤ 10%	≤ 30%	≤ 30%	≤ 30%	≤ 100%	≤ 100%	≤ 100%	≤ 100%	≤ 100%	≤ 100%
NA 7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 12	≤ 10%	≤ 10%	≤ 10%	N.D.	N.D.	N.D.	≤ 10%	≤ 10%	≤ 10%	N.D.	N.D.	N.D.
NA 13	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 14	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 15	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 17	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

The retained samples were stored under controlled stability conditions of 25 °C / 60% RH. NA: N-nitrosamine; N.D.: Not detected.

Given the evolving profile of N-nitrosamine 6 over storage, this impurity is presumed to originate from API degradation. If sufficient financial and human resources are available, efforts may be undertaken to establish conditions enabling skip testing. To achieve this objective, the level of N-nitrosamine 6 would need to be reduced to, and consistently maintained below 30% of its acceptable limit. Potential risk mitigation strategies include the use of low-nitrite excipients, although this approach may increase manufacturing costs. Lowering the storage temperature to 5 °C instead of 25 °C may slow API degradation and, consequently, the kinetics of N-nitrosamine formation. An alternative measure involves redesigning or modifying the packaging, for example by replacing the primary packaging material with a nitrocellulose-free lidding foil for blister packs in direct contact with tablets [9].

4 Discussion

The control of N-nitrosamine impurities in pharmaceuticals has become a critical focus due to their mutagenic and carcinogenic potential, necessitating rigorous regulatory oversight. Based on a previously developed Quantitative Structure Retention Relationship approach to the determination of N-nitrosamines, this study successfully proposed specific and sensitive LC-MS/MS methods for detecting trace levels of small-molecule N-nitrosamines in a marketed pharmaceutical product. These methods have been successfully validated in accordance with the ICH Q2(R2) guidelines, meeting the requirements for limit tests as specified therein. The methods demonstrated high specificity, adequate sensitivity, and acceptable recovery with detection limits below 10% of the acceptable limit, ensuring reliable screening capabilities. The proposed methods provide a solid basis for further development, particularly for the determination of newly identified small-molecule N-nitrosamines, and may also serve as a starting point for the investigation of NDSRIs in both drug substances and drug products. For NDSRI investigations, it is necessary to verify and eliminate potential interference from small-molecule N-nitrosamines and to demonstrate method specificity to ensure method validity for the intended use.

This case study serves as a practical example illustrating the structured workflow of analytical confirmatory testing for MAHs or applicants. The results showed that most N-nitrosamines were undetected, eliminating the need for further testing. However, one impurity was consistently present and increased over time, though remaining below the acceptable limit, necessitating routine quantitative analysis. This finding highlights the importance of ongoing surveillance, particularly for impurities resulting from API degradation. Future efforts could focus on optimizing storage conditions or packaging materials to mitigate impurity formation risk, ensuring continued product safety. The methods proposed in this work provide a useful tool for routine quality control of 17 small-molecule N-nitrosamines in pharmaceutical products, supporting regulatory compliance and safeguarding public health.

From a broader perspective, the development of platform methods for small-molecule N-nitrosamines could help centralize analytical efforts, thereby supporting wider applications and facilitating testing across products with diverse dosage forms, APIs, and excipients. To achieve this goal, the main challenges lie in adapting sample preparation protocols and managing matrix effects in mass spectrometry, which may lead to ion suppression or enhancement.

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Supplementary material

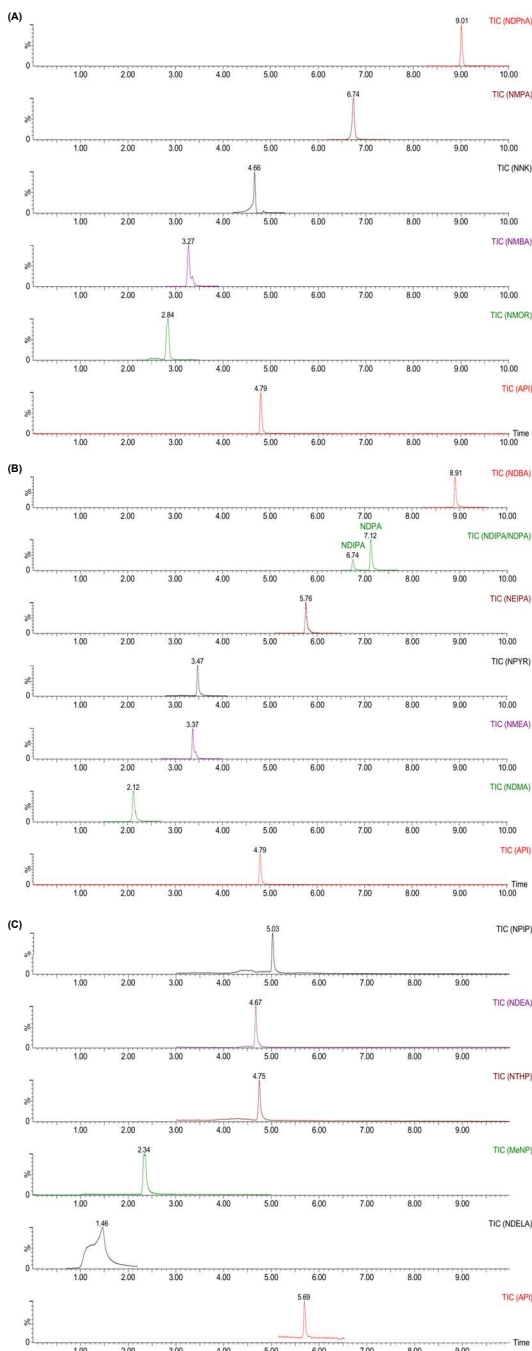


Figure S1. Total ion chromatograms illustrating the selectivity of the analytical methods. (A) Method 1, (B) Method 2, and (C) Method 3.

Table S1: AUC comparison between NDBA-related carry-over in matrices and NDBA peak in calibration standard for selectivity evaluation.

AUC % carry-over versus NDBA peak* (n = 3)	
Series 1	4
Series 2	3
Series 3	3
Series 4	0

*NDBA peak AUC in the calibration standard at 10% of the acceptable limit.

Chapter III: Results - Section 1(B)

Table S2: Summary of SST results for the validation studies of N-nitrosamine impurities – Method 2.

Method 2: pH 2.7/150 °C							
	Equipment performance verification						
	Mean intensity (counts, n = 3)	RSD% (n = 3)		Conformity			
	≥ 8,000,000 counts	≤ 5%					
Series 1	18,280,847	1					
Series 2	20,178,409	1					
Series 3	11,311,710	1		Conforms			
Series 4	10,983,810	0					
<i>p</i> = 4	NDMA	NDPA	NDIPA	NEIPA	NDBA	NPYR	NMEA
Sensitivity							
S/N ratio ≥ 3							
Min. – Max.	18 – 34	18 – 35	138 – 335	68 – 155	46 – 63	882 – 1843	977 – 2435
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Ion ratio							
Within tolerance							
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
NDBA-related carry-over verification							
	Mean AUC % in blank injections (n = 3)					Conformity	
	≤ 10%						
Series 1	4						
Series 2	3						
Series 3	3					Conforms	
Series 4	0						

p: Number of series of validation.

Chapter III: Results - Section 1(B)

Table S3: Summary of SST results for the validation studies of N-nitrosamine impurities – Method 3.

Method 3: pH 5.0/150 °C					
	Equipment performance verification				
	Mean intensity (counts, n = 3)		RSD% (n = 3)		Conformity
	≥ 8,000,000 counts		≤ 5%		
Series 1	19,543,997		1		Conforms
Series 2	9,724,814		1		
Series 3	10,556,798		1		
Series 4	16,1007,22		2		
(p = 4)	MeNP	NDEA	NPIP	NTHP	NDELA
Sensitivity					
S/N ratio ≥ 3					
Min. – Max.	84 – 282	4 – 26	92 – 183	14 – 30	19 – 47
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms
Ion ratio					
Within tolerance					
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms

p: Number of series of validation.

Chapter III: Results - Section 1(B)

Table S4: Summary of equipment performance verification and SST results for the confirmatory study.

Day 1 – Batches 1 to 6									
Day 1	Equipment performance verification							Conformity	
	Mean intensity (counts, n = 3)			RSD% (n = 3)					
	≥ 8,000,000 counts			≤ 5%					
	17,508,013			1%				Conforms	
Sensitivity	NA 1	NA 2	NA 3	NA 4	NA 5	NA 6	NA 7	NA 8	NA 9
S/N ratio ≥ 3									
S/N ratio	526	12	164	294	105	24	33	34	232
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Ion ratio									
Within tolerance									
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
	NA 10	NA 11	NA 12	NA 13	NA 14	NA 15	NA 16	NA 17	
Sensitivity									
S/N ratio ≥ 3									
S/N ratio	98	943	54	2227	273	31	13	16	
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	
Ion ratio									
Within tolerance									
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	
NDBA-related carry-over verification									
	Mean AUC % in blank injections (n = 3)							Conformity	
	≤ 10%								
Day 1	8							Conforms	

Chapter III: Results - Section 1(B)

Table S4 Continued.

Day 2 – Batches 7 to 12									
Day 2	Mean intensity (counts, n = 3)			Equipment performance verification				Conformity	
	≥ 8,000,000 counts			RSD% (n = 3)					
	17,396,959			≤ 5%				Conforms	
	NA 1	NA 2	NA 3	NA 4	NA 5	NA 6	NA 7	NA 8	NA 9
Sensitivity									
S/N ratio ≥ 3									
S/N ratio	591	27	283	360	98	16	43	38	237
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Ion ratio									
Within tolerance									
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
	NA 10	NA 11	NA 12	NA 13	NA 14	NA 15	NA 16	NA 17	
Sensitivity									
S/N ratio ≥ 3									
S/N ratio	181	1362	66	2718	184	47	10	16	
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	
Ion ratio									
Within tolerance									
Conformity	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	
NDBA-related carry-over verification									
Day 2	Mean AUC % in blank injections (n = 3)						Conformity		
	≤ 10%								
	10						Conforms		

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Chapter III: Results - Section 1(B)

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Analytical development of an LC-MS/MS method for the determination of an N-nitrosamine drug substance-related impurity

Section 2

An Analytical Quality by Design-guided method development for the quantification of N-nitroso-fluoxetine to support mitigation strategies for a fluoxetine drug product

Preamble

The N-nitrosamine crisis has continuously evolved and gained increasing momentum in the pharmaceutical industry since 2018. During this period, the regulatory focus has progressively shifted toward a new class of N-nitrosamines known as N-nitrosamine drug-substance-related impurities (NDSRIs). To date, NDSRIs account for more than 90% of the N-nitrosamines identified by the EMA [1].

Unlike small-molecule N-nitrosamine impurities, NDSRIs are specific to the corresponding APIs and may therefore be present in drug products following manufacturing. Moreover, NDSRIs are not primarily formed during API synthesis, rather, their formation is directly associated with the presence of nitrite. Nitrite is mainly introduced through pharmaceutical excipients. When an API contains vulnerable secondary amine functional groups, contact with residual nitrite can lead to nitrosation reactions, resulting in the formation of the corresponding N-nitroso derivative of the API [2]. Consequently, NDSRIs are more likely to be observed in drug products, and their formation may be further promoted by environmental conditions during storage.

This section focuses on a case involving an NDSRI identified on the Belgian market, N-nitroso-fluoxetine. This impurity was detected at excessive levels in Fluoxetine® Divul® 20 mg, leading to its withdrawal from the market [3]. To monitor the effectiveness of implemented risk mitigation strategies, an Analytical Quality by Design approach, a science- and risk-based framework, was employed to accelerate analytical development [4]. The development process was also facilitated by integrating the prior knowledge and experience acquired from the studies presented in the preceding sections.

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Abstract

This work focused on N-nitroso-fluoxetine, a N-nitrosamine drug substance-related impurity (NDSRI) formed in fluoxetine-containing products via nitrosation pathways. N-nitroso-fluoxetine was previously detected above the established safety threshold of 100 ng/day, leading to temporary withdrawal of a marketed product in Belgium.

An Analytical Quality by Design (AQbD) approach supported the development of a reversed-phase LC-(ESI+)-MS/MS method. Design of experiments identified aqueous mobile phase pH and gradient time as critical method parameters affecting detection or quantification limits and chromatographic separation performance, respectively. Adequate separation was achieved using Acquity® HSS T3 guard-column and column with a run time of 22 minutes. The method was validated according to ICH Q2(R2) guideline using a combined approach based on total error. It enables accurate and precise quantification of N-nitroso-fluoxetine from 0.09 to 4.75 ppm (or injected concentration in ng/mL) while simultaneously screening for other small-molecule N-nitrosamines in both drug substance and finished products.

The validated method was applied to assess risk mitigation measures implemented by the manufacturer, including the use of low-nitrite excipients, desiccant-integrated aluminum/aluminum blister packaging, and reduced shelf-life. In the updated formulation, N-nitroso-fluoxetine contents were 0.58 ppm at release and increased to 1.15 ppm after 21 months of storage at 25 °C/60% RH, remaining below the safety threshold of 1.67 ppm calculated according to the EMA “Call for Review” procedure. Together, these modifications have effectively controlled N-nitroso-fluoxetine formation.

Therefore, the present study demonstrates a robust AQbD-guided LC-MS/MS method, contributes to NDSRI analysis, and supports science- and risk-based control strategies for these emerging impurities.

1 Introduction

In medicinal products, N-nitrosamine impurities can be broadly classified into three categories: (i) generic small-molecule N-nitrosamines, which may originate from multiple sources across different drug products; (ii) N-nitrosamine drug substance-related impurities (NDSRIs), which are specific to a given active pharmaceutical ingredient (API) and its related substances; and (iii) N-nitrosamine drug linker-related impurities (NDLRIs), which are encountered in antibody drug conjugates. Since the emergence of the so-called N-nitrosamine crisis in the pharmaceutical industry, NDSRIs have continued to be identified. The first reported case, N-nitroso-varenicline, resulted in voluntary product recalls in 2021 [1]. According to data updated by the European Medicines Agency (EMA) in March 2026, NDSRIs now account for approximately 92% of the N-nitrosamine impurities identified in pharmaceutical products [2]. This observation highlights the progressive shift of regulatory and scientific attention toward NDSRIs as the crisis has evolved.

In line with this trend, the present study investigates a case of an NDSRI detected on the Belgian market: N-nitroso-fluoxetine, which is specifically associated with fluoxetine-containing formulations. Fluoxetine is a widely prescribed antidepressant. Several fluoxetine-based products have been recalled worldwide following the detection of N-nitroso-fluoxetine at levels exceeding the recommended acceptable intake limit [3–5]. Although NDSRIs generally lack carcinogenicity data and their acceptable intake limits are therefore typically assigned using the carcinogenic potency categorization approach [6,7], the acceptable intake for N-nitroso-fluoxetine was established at 100 ng/day based on a structure-activity relationship/read-across approach, with the TD₅₀ of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as the point of departure, given its positive results in *in vivo* mutagenicity studies [8,9]. More recently, due to the limited carcinogenicity data available for N-nitroso-fluoxetine, Vogel *et al.* investigated its mutagenic potential. Their study demonstrated that, following metabolic activation by cytochrome P450 enzymes (CYP2B6, CYP2C19, and CYP3A4), N-nitroso-fluoxetine generates higher levels of DNA methylation adducts and induces more DNA strand breaks than N-nitrosodimethylamine (NDMA), a well-characterized small-molecule N-nitrosamine [10]. In light of the associated mutagenic risk, the Federal Agency for Medicines and Health Products in Belgium mandated marketing authorization holders to assess the levels of N-nitroso-fluoxetine in fluoxetine-containing products. After N-nitroso-fluoxetine amounts exceeding established safety thresholds were detected, Fluoxone[®] Divule[®] 20 mg was temporarily withdrawn from the Belgian market in 2023 [11–13].

The principles of quality by design (QbD) were first introduced to the pharmaceutical industry by the United States Food and Drug Administration (FDA) [14]. QbD is defined in the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q8(R2) as a systematic approach to development that begins with predefined objectives, emphasizing product and process understanding and process control [15]. In combination with ICH Q9(R1) guideline, QbD strengthens science- and risk-based regulatory decision-making [15,16].

Unlike the traditional quality by testing paradigm, in which product quality is assessed primarily through end-product testing, QbD proposes that quality should be built into the process by design [15]. This philosophy promotes enhanced understanding and control of manufacturing processes, thereby reducing the risk of out-of-specification products and improving overall product quality. Consequently, QbD not only minimizes financial risks for manufacturers by limiting batch rejections, but also safeguards patient health by ensuring the consistent production of high-quality medicines.

The concept of analytical quality by design (AQbD) extends QbD principles to analytical procedure development, as outlined in ICH Q14 guideline. AQbD shares the advantages of QbD while providing a structured alternative to minimal method development strategies. Method development phases are often time-consuming due to extensive trial-and-error experimentation. By integrating prior knowledge, risk assessment, design of experiments (DoE), and a lifecycle-oriented continuous improvement mindset, AQbD enhances analytical robustness and regulatory flexibility. Specifically, AQbD incorporates systematic knowledge and risk management to achieve two primary objectives: (i) improving method robustness to reduce the risk of inadequate analytical performance and erroneous results; and (ii) enabling continuous method improvement throughout the product lifecycle [17].

Compared with development approaches based on one-factor-at-a-time (OFAT) experimentation, AQbD represents a more scientific and risk-based strategy that relies on DoE. The OFAT approach varies a single factor while holding all others constant, limiting the ability to identify factor interactions and often requiring a large number of experiments. This limitation may compromise method robustness and increase the risk of analytical failure. In contrast, DoE evaluates multiple factors simultaneously, allowing the assessment of main effects, interactions, and quadratic effects while reducing experimental effort, time, and cost [18].

To date, most published studies have focused on the determination of small-molecule N-nitrosamines [19,20]. Although NDSRIs have attracted increasing regulatory and scientific attention, relatively few analytical methods have been reported for their determination [21,22]. The Taiwan FDA and the German official medicines control

laboratory have published liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for the quantification of N-nitroso-fluoxetine, achieving quantification limits of approximately 0.1 ppm in the drug substance (equivalent to 1 ng/mL) and in immediate-release tablets (equivalent to 0.1 ng/mL), respectively [23,24].

The acceptable limit for N-nitroso-fluoxetine in the studied product is obtained by dividing its acceptable intake limit (100 ng/day) by the maximum daily dose of fluoxetine (60 mg/day), yielding a specification of 1.67 ppm. Applying the AQBd framework, this work developed a robust LC-MS/MS method capable of quantifying N-nitroso-fluoxetine down to 0.1 ppm (or ng/mL) while simultaneously screening for other small-molecule N-nitrosamines in fluoxetine drug substance and drug products. The study provides a valuable contribution to NDSRI determination and supports robust, science-based control strategies for these emerging impurities.

2 Materials and methods

2.1 Chemicals and reagents

Certified reference stock solutions (500 µg/mL) of NDMA, N-nitrosodi-n-propylamine (NDPA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), N-nitrosodi-n-butylamine (NDBA), N-nitrosodiethylamine (NDEA), and N-nitroso-N-methyl-4-aminobutyric acid (NMBA) were obtained from the European Directorate for the Quality of Medicines and HealthCare (Strasbourg, France). A certified reference stock solution (1 mg/mL) of N-nitroso-1,2,3,6-tetrahydropyridine (NTHP) was supplied by the United States Pharmacopeia (Rockville, MD, USA). Reference stock solutions (1 mg/mL) of NNK and N-nitrosomethylphenylamine (NMPA) were purchased from Sigma-Aldrich/Merck (St. Louis, MO, USA). Reference stock solutions (1 mg/mL) of N-nitrosopyrrolidine (NPYR), and N-nitroso-piperidine (NPIP) were purchased from Cambridge Isotopes Laboratories (Tewksbury, MA, USA). Reference standards of N-nitrosomorpholine (NMOR), N-nitrosodiphenylamine (NDPhA), N-nitroso-diethanolamine (NDELA), and fluoxetine hydrochloride were obtained from TCI (Tokyo, Japan). N-nitroso-fluoxetine was purchased from Merck (Kyiv, Ukraine). Deuterated N-nitroso-fluoxetine-d5 (stable isotope-labeled standard) was obtained from Toronto Research Chemicals (Vaughan, Canada).

UPLC-MS grade methanol, water, formic acid, and ammonium acetate were purchased from Biosolve (Dieuze, France). A 25% ammonia solution was obtained from VWR (Rosny-sous-Bois, France). Fluoxetine hydrochloride drug substance, excipients (Table S1), and Fluoxone® Divule® 20 mg used in the work were supplied by a pharmaceutical industry partner.

2.2 Instrumentation and software

LC-MS/MS analyses were performed on an Acquity® Premier® UPLC® hyphenated to a tandem mass spectrometer Xevo TQ-Absolute (Waters, Milford, MA, USA). The UHPLC instrument is equipped with a binary pump, a flow-through needle sample manager, and a pre-heated column manager. The MS/MS detector was equipped with an electrospray ionization (ESI) ion source. Acquity® UPLC® HSS T3 VanGuard pre-column (1.8 µm, 2.1 mm × 5 mm), and Acquity® UPLC® HSS T3 column (1.8 µm, 2.1 mm × 100 mm) were purchased from Waters.

The LC-MS/MS equipment was controlled, data were acquired and processed using MassLynx® Security® 4.2 software. The tailing factor and signal-to-noise (S/N) ratios were automatically calculated using the same software. Experimental designs were computed using JMP® Statistical Discovery Student Edition 19 software (Cary, NC, USA). Validation data were analyzed using E-noval® Version 6.3 software (Cencora Pharmalex, Mont-Saint-Guibert, Belgium).

2.3 Risk assessment

In accordance with the “Call for Review” investigation procedure, an initial documentary risk assessment was performed. This assessment aimed to identify potential N-nitrosamine impurities that may be present in the finished product [8] by evaluating risks associated with two key stages: (i) the manufacturing process of the finished product; and (ii) storage throughout the shelf-life.

2.4 Analytical quality by design

2.4.1 *Definition of analytical target profile*

Within the AQbD framework, the first stage of analytical procedure development consists of defining the analytical target profile (ATP). The success of subsequent analytical development is directly dependent on the accuracy and clarity of the ATP definition. Therefore, the ATP must be established in a precise and unambiguous manner.

2.4.2 *Selection of analytical technique*

Preliminary experiments demonstrated that N-nitroso-fluoxetine generated higher signal intensities with electrospray ionization (ESI) than with atmospheric pressure chemical ionization (APCI). These findings were incorporated into the prior knowledge and guided the selection of the analytical technique.

2.4.3 Optimization experimental design

Based on extensive prior knowledge, an initial screening design was deemed unnecessary, and an optimization experimental design was implemented.

For this study, a D-optimal design was selected to achieve precise estimation of factor effects and to distinguish which effects are active *versus* inactive. Eight factors were included in the design: three chromatographic parameters were investigated at two levels, while five mass spectrometric parameters were studied at three levels, as quadratic effects could not be excluded. Irrelevant quadratic terms and interactions were intentionally excluded from the design. For example, quadratic effects of pH and the interaction between column temperature and capillary voltage were considered improbable. Details and justification for the deselection of these effects are provided in Table S2.

The experimental design results in a total of 28 experiments divided into 4 plots. The experimental design was organized into randomized blocks (split-plot design) due to the inclusion of pH as a technically hard-to-change factor. Fixed factors included a flow rate of 0.4 mL/min and an injection volume of 10 μ L. The studied factors were: pH of the aqueous mobile phase (F1: 2.7 or 5.0), gradient time (F2: 5 or 10 min), column temperature (F3: 40 or 50 $^{\circ}$ C), capillary voltage (F4: 1.5, 2.25 or 3.0 kV), desolvation temperature (F5: 200, 400 or 600 $^{\circ}$ C), desolvation gas flow (F6: 900, 1050 or 1200 L/h), source temperature (F7: 100, 125 or 150 $^{\circ}$ C), and cone gas flow (F8: 150, 225 or 300 L/h). Elution details for both gradient times are provided in Table S3. The mobile phase at pH 2.7 was a 0.1% formic acid solution, while the other was prepared with a 10 mM ammonium acetate solution adjusted to pH 5.0 with formic acid.

The responses (or critical method attributes) were defined as follows: separation criterion between isobaric N-nitrosamines (R1), separation criterion between fluoxetine and its nearest N-nitrosamine (R2), S/N ratio for N-nitroso-fluoxetine (R3), and the number of detectable small-molecule N-nitrosamines with S/N ratio ≥ 3 (R4). The selection criteria and justifications for these responses are summarized in Table 1.

Experimentally, a neat solution containing generic small-molecule N-nitrosamines and N-nitroso-fluoxetine (NDSRI) was prepared at 1 ng/mL, and fluoxetine was added at 2 mg/mL to simulate real sample matrices. This solution was injected into the analytical system under the varying factor settings defined by the D-optimal design (Table S4).

Table 1: Definition of the responses and justifications.

Response	Acceptance criteria	Justification(s)
R1: Separation criterion between isobaric N-nitrosamines	≥ 0.0 min	Separation is calculated between the end of the first peak and the start of the adjacent peak. A separation of 0.0 min indicates baseline separation. NDIPA and NDPA are isobaric and cannot be distinguished by their m/z ratios, requiring complete chromatographic resolution.
R2: Separation criterion between fluoxetine and the nearest N-nitrosamine	≥ 0.0 min	Separation is calculated between the end of the first peak and the start of the adjacent peak. Co-elution with the highly abundant fluoxetine may suppress or enhance N-nitrosamine ionization, reducing precision and accuracy. Adequate separation minimizes matrix effects and allows fluoxetine to be diverted to waste, protecting the mass spectrometer.
R3: S/N ratio for N-nitroso-fluoxetine	≥ 10	A minimum S/N ratio of 10 is required at the lower quantification limit, in accordance with ICH Q2(R2) guideline.
R4: Number of small-molecule N-nitrosamines with $S/N \geq 3$	Maximize	A minimum S/N ratio of 3 is required at the detection limit, in accordance with ICH Q2(R2) guideline.

2.4.4 Second optimization experimental design

A second optimization experimental design was performed to reoptimize method parameters by incorporating the knowledge gained from the first experimental design. As discussed in the Results and Discussion section, the gradient time was increased from 10 to 15 min to improve the NDPA – fluoxetine separation. To account for this modification, the second design was generated with the gradient time fixed at 15 min. This design served two complementary purposes: (i) to evaluate the effect of fixing the gradient time at 15 min, together with the influence of the remaining factors at their predicted optimal settings, on the responses; and (ii) to assess robustness by deliberately introducing small metrological variations in the remaining factors while maintaining the gradient time fixed at 15 min.

To achieve these objectives, a second D-optimal design was computed, including seven factors, each evaluated at three levels. This design comprised 16 experiments, included three replicates at the central point, and was sorted according to aqueous mobile phase pH. The investigated factors were as follows: pH of the aqueous mobile phase (F1: 2.7, 2.8 or 2.9), column temperature (F2: 48, 50 or 52 °C), capillary voltage (F3: 1.50, 1.75 or 2.00 kV), desolvation temperature (F4: 200, 250 or 300 °C), desolvation gas flow (F5: 1100, 1150 or 1200 L/h), source temperature (F6: 100, 110 or 120 °C), and cone gas flow (F7: 250, 275 or 300 L/h). The mobile phase consisted of a 0.1% formic acid solution (pH 2.7), adjusted to a pH of 2.8 or 2.9 using a 25% ammonia solution. The experimental design is summarized in Table S5.

2.5 Analytical procedure validation

Three series were performed to validate the method both as a quantitative assay for N-nitroso-fluoxetine and as a limit test for screening of small-molecule N-nitrosamines. Each validation series included freshly prepared calibration and validation standards. Validation standards were prepared independently in triplicate at each determined concentration level.

Solution stability was not evaluated as a formal validation parameter because all calibration and validation standards were prepared extemporaneously and analyzed immediately after preparation in each series. Under these experimental conditions, this assessment was not necessary.

2.5.1 Method parameters

Chromatographic separations were performed using an Acquity® UPLC® HSS T3 VanGuard pre-column connected to an Acquity® UPLC® HSS T3 column. The mobile phase consisted of (A) 0.1% formic acid in water (pH 2.7) and (B) 0.1% formic acid in methanol. The needle wash and purge solvent were composed of water/methanol (10%/90%, v/v) and water/methanol (90%/10%, v/v), respectively. The final

chromatographic conditions obtained after optimization (Section 3.2.3) were as follows: flow rate, 0.4 mL/min; column temperature, 50°C; injection volume, 10 µL; and linear gradient program, 0.0 to 1.0 min: 100% A, 1.0 to 16.0 min: 100% → 5% A, 16.0 to 19.5 min: 5% A, 19.5 to 19.6 min: 5 → 100% A, 19.6 to 22.0 min: 100% A. The LC flow was diverted to waste to eliminate potential polar matrix components and fluoxetine. The LC effluent was introduced into the mass spectrometer from 0.80 to 9.50 min and from 11.50 to 14.30 min.

Mass spectrometric detection was performed using a tandem mass spectrometer equipped with an ESI source operated in positive polarity. Optimized source parameters (Section 3.2.3) were: capillary voltage, 1.5 kV; desolvation temperature, 200 °C; desolvation gas flow, 1200 L/hr; source temperature, 100 °C; and cone gas flow, 300 L/hr.

Data acquisition was carried out in multiple reaction monitoring (MRM) mode, providing enhanced sensitivity and selectivity through dual mass filtering. The optimized MRM acquisition parameters are summarized in Table S6. The soft transmission option was activated to minimize losses of labile molecular ions during transfer to the first mass analyzer.

2.5.2 *Internal standard*

As a rule of thumb, internal standards are employed to normalize analytical signals and compensate for variability arising from sample preparation and compound ionization.

N-nitroso-fluoxetine-d5 was dissolved in methanol to prepare a stock solution at 1000 µg/mL, which was subsequently diluted to 1 µg/mL. Two independent internal standard solutions were prepared for the entire validation study: one for the preparation of calibration standards (SI1) and one for the preparation of validation standards (SI2). Both solutions were used to obtain a final injected concentration of 1 ng/mL of N-nitroso-fluoxetine-d5. SI1 contained 25 ng/mL of N-nitroso-fluoxetine-d5, whereas SI2 contained 100 ng/mL. All dilutions were performed using water/methanol (80%/20%, v/v). Both internal standard solutions were stored at 5 °C.

2.5.3 *Calibration standards*

Individual stock solutions of NMOR, NDPhA, and NDELA were prepared at 1000 µg/mL, while the N-nitroso-fluoxetine stock solution was prepared at 100 µg/mL. All commercial solutions and stock solutions were stored at -20 °C.

On each validation day, calibration standards were freshly prepared by combining individual stock solutions to obtain a mixture containing 2.5 µg/mL of N-nitroso-fluoxetine and 10 µg/mL of the other N-nitrosamines, maintaining a 4-fold concentration ratio. This intermediate solution was serially diluted, and 200 µL of SI1 solution was added to obtain final concentrations of 0.1, 0.5, 1, 2.5, and 5 ng/mL for N-nitroso-fluoxetine, and 0.4, 2, 4, 10, and 20 ng/mL for the other impurities. The final concentration of N-nitroso-fluoxetine-d5 was 1 ng/mL. All dilutions were performed in water/methanol (80%/20%, v/v).

The range for N-nitroso-fluoxetine was defined in accordance with its acceptable limit in Fluoxone® and ICH Q2(R2) requirements, covering at least 30% to 120% of the acceptable limit, corresponding to 0.50 – 1.67 ppm (or 0.50 – 1.67 ng/mL after sample preparation). For small-molecule N-nitrosamines, NDEA, NDBA, and NDPA were selected as worst-case compounds due to their higher carcinogenicity and lower acceptable intake limit (26.5 ng/day) [8]. As small-molecule impurities must be detectable at least at their respective acceptable limits, the detection limits (DLs) for the three worst-case N-nitrosamines were required to be ≤ 0.44 ppm (≤ 0.44 ng/mL). Detailed acceptable limit calculations are provided in Table S7.

2.5.4 Validation standards

A reconstituted drug matrix was prepared by homogenizing fluoxetine and all excipients according to the quantitative composition of the new formulation of Fluoxone®. The qualitative composition information can be found in the summary of product characteristics [25] and Table S1. The mass corresponding to one capsule was weighed to simulate real sample matrices.

On each validation day, validation standards were freshly prepared. A N-nitrosamine spiking solution was prepared from individual stock solutions at concentrations of 200 ng/mL for N-nitroso-fluoxetine and 800 ng/mL for the other impurities, maintaining a 4-fold concentration ratio.

For validation standard preparation, 200 µL of SI2 solution and the N-nitrosamine spiking solution were added to powdered matrix equivalent to one capsule. Methanol was added to adjust the volume to 4.0 mL. The mixture was subsequently diluted 5 times with water and centrifuged at 14,000 rpm for 15 min prior to injection. Final injected concentrations were 0.1, 0.5, 1, 2.5, and 5 ng/mL for N-nitroso-fluoxetine, and 0.4, 2, 4, 10, and 20 ng/mL for the other impurities. The final concentration of N-nitroso-fluoxetine-d5 was maintained at 1 ng/mL, consistent with that used in the calibration standards.

2.5.5 *Blank matrix*

The blank matrix consisted of an unspiked drug matrix and was used to evaluate the selectivity and specificity of the method. Following the same procedure as for the preparation of validation standards, 4.0 mL of methanol was added to the matrix. The resulting solution was subsequently diluted 5 times with water and centrifuged at 14,000 rpm for 15 min prior to injection.

2.6 Risk mitigation measures

The N-nitroso-fluoxetine content in the former formulation of Fluoxone[®] Divule[®] exceeded the acceptable limit of 1.67 ppm, causing its withdrawal from the Belgian market. Comparison of the former formulation (Table S8) with the updated formulation [25] several modifications, including changes in excipients and primary packaging materials. In addition, the product shelf-life was reduced as part of the risk mitigation strategy.

2.6.1 *Confirmatory testing*

Following these changes, a confirmatory study was conducted to evaluate whether the changes in excipients and blister materials effectively reduced N-nitroso-fluoxetine levels and to verify the effectiveness of the implemented risk mitigation measures. Two batches of fluoxetine hydrochloride raw material were analyzed, along with one batch of the former Fluoxone[®] capsule formulation at the end of its shelf-life, and three independent batches of the updated formulation at release (T0), 3 months (T3M), and 21 months (T21M), respectively, to quantify N-nitroso-fluoxetine content. Stability samples were stored under long-term storage conditions of 25 °C/60% relative humidity (RH). Additionally, small-molecule N-nitrosamines were simultaneously assessed as a conservative approach. The influence of primary packaging material was also investigated using a single production batch packaged in either polyvinyl chloride (PVC)/aluminum (Al) or Al/Al blisters.

Sample preparation followed the protocol used during the validation study. For fluoxetine hydrochloride raw material analysis, 22.36 mg of fluoxetine hydrochloride, corresponding to 20 mg of fluoxetine, was accurately weighed. For finished product analysis, five capsules were crushed into a homogeneous powder, and an amount equivalent to one capsule was accurately weighed and processed as a representative sample. The subsequent sample preparation steps were identical to those described in the validation study.

3 Results and discussion

3.1 Risk assessment

Risk factors potentially contributing to N-nitrosamine formation during the fluoxetine chemical synthesis stage were not evaluated, as this assessment was performed by the API manufacturer. Therefore, the present section focuses on risks associated with finished product manufacturing.

An *in silico* evaluation was first conducted using the N-nitrosamine risk calculator developed by DFE Pharma to estimate the potential formation of small-molecule N-nitrosamines in Fluoxetine[®] based on its quantitative composition [26]. The calculator predicted the possible presence of these impurities but the estimated levels were below the acceptable intake limits. Subsequent assessment identified N-nitroso-fluoxetine as the only relevant at-risk impurity in Fluoxetine[®], resulting from potential interactions between fluoxetine and excipients.

According to the “Call for Review” guidance, further investigation is not required when no risk has been identified. Although the risk associated with the presence of precursor amines forming small-molecule N-nitrosamines is considered low, it cannot be fully excluded. Nevertheless, as a conservative measure, these compounds were included in the present work.

As summarized in Table S8, the risk assessment identified the N-nitroso-fluoxetine as the sole impurity potentially present in the finished product. This impurity may be formed both during the manufacturing process and throughout storage. Fluoxetine contains a secondary amine functional group, which is highly susceptible to nitrosation in the presence of nitrosating agents, conferring an intrinsic potential for the formation of its corresponding NDSRI, N-nitroso-fluoxetine [7]. Furthermore, as fluoxetine is formulated as a hydrochloride salt, acidic conditions may favor and accelerate the nitrosation reaction. The chemical structures of fluoxetine and N-nitroso-fluoxetine, together with the identified nitrosation site, are presented in Figure 1.

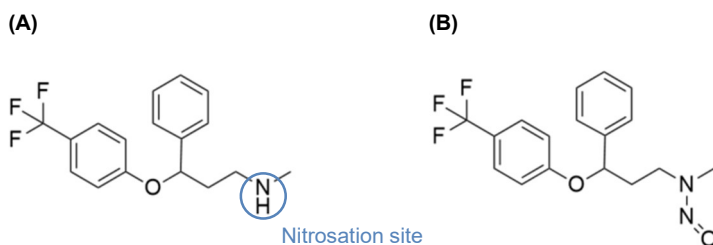


Figure 1. Chemical structures of (A) fluoxetine, and (B) N-nitroso-fluoxetine.

The risk of N-nitroso-fluoxetine formation is associated with the formulation and packaging stages, as excipients may contain nitrites. Based on the nitrite excipient database, microcrystalline cellulose, widely used as a diluent in oral solid dosage forms, shows one of the highest nitrite contents among pharmaceutical excipients [27], and is therefore considered the primary nitrite source in the finished product. Owing to their relatively low proportions, the contribution of other excipients to NDSRI formation is considered negligible. The qualitative composition of the former Fluoxone® formulation (withdrawn from the market) and the function of each component are presented in Table S8. Following formulation, the capsules are packaged in PVC/Al blisters that do not contain nitrocellulose, thereby limiting the risk of N-nitrosamine formation during packaging and subsequent storage. However, PVC is a plastic material with relatively low barrier properties [28]. In particular, its permeability to oxygen, light, and moisture is known to promote degradation and nitrosation processes. Oxygen and light may initiate oxidative reactions that facilitate nitrosation, while moisture has also been identified as a key risk factor for N-nitrosamine formation [29,30]. Furthermore, N-nitroso-fluoxetine may continue to form during storage in the presence of residual nitrosating precursors, potentially leading to an evolving impurity profile over the product shelf life.

3.2 Analytical quality by design

3.2.1 *Definition of analytical target profile*

In the present study, the ATP was defined separately for two distinct purposes: (i) screening of the widest possible range of N-nitrosamines as a secondary analytical objective; and (ii) quantitative determination of N-nitroso-fluoxetine as the primary objective, as it is classified as an at-risk impurity in fluoxetine-containing products, for post-marketing surveillance. The ATPs corresponding to these two intended purposes are detailed in Table 2. Depending on the application, the analytical method was validated either as a limit test or as a quantitative impurity test for both drug substance and finished product, with specific analytical performance criteria defined according to the chosen validation strategy. The analytical method was therefore required to enable the determination of each impurity individually and independently of one another, as well as from potential interferents such as fluoxetine or formulation excipients. Given that the excipients present in the formulation are insoluble, the risk of interference with the analytes was considered negligible.

Table 2: Intended purpose and ATP acceptance criteria.

Screening – Limit test for all N-nitrosamine impurities: Secondary objective		
Selectivity/specificity	The analytical method must be capable of discriminating all targeted N-nitrosamines from potential interferents.	Potential interferences may arise between certain N-nitrosamines and fluoxetine. No interference from excipients in the finished product is anticipated, as excipients are insoluble and can be effectively removed during sample preparation by centrifugation.
Detection limit	≤ 10% of the AL for N-nitroso-fluoxetine ≤ 100% of the AL for small-molecule N-nitrosamines The number of small-molecule N-nitrosamines meeting the ATP acceptance criteria should be maximized.	Given that N-nitroso-fluoxetine has been identified as the only at-risk impurity in Fluoxone® (Section 3.1), a more stringent detection limit is required for this compound than for small-molecule N-nitrosamines, which are not considered relevant risks in the drug product. Accordingly, the analytical method was primarily designed for the determination of N-nitroso-fluoxetine, while also aiming where feasible to enable the screening of small-molecule N-nitrosamines.
Post-marketing surveillance – Quantitative impurity testing for N-nitroso-fluoxetine: Primary objective		
Selectivity/specificity	The analytical method must be capable of discriminating N-nitroso-fluoxetine from potential interferents.	Potential interferences may arise between N-nitroso-fluoxetine and fluoxetine. No interference from excipients in the finished product is anticipated, as excipients are insoluble and can be effectively removed during sample preparation by centrifugation.
Range	30 – 120% of the N-nitroso-fluoxetine AL (0.50 – 2.00 ppm)	According to ICH Q2(R2), the lower end of the reportable range is defined by the reporting threshold, which in this case corresponds to 30% of the AL. The upper end of the reportable range should be at least 120% of the specification acceptance criterion, corresponding here to 120% of the AL.
Combined approach for precision and accuracy	Acceptance limits: maximum ± 15% α-risk level: 5%	<i>Remark: the acceptable limit for N-nitroso-fluoxetine is 1.67 ppm*.</i> As no harmonized acceptance criteria currently exist for quantitative impurity testing at trace levels, acceptance limits of 85 – 115% are generally considered appropriate and acceptable for pharmaceutical impurity analyses. For the combined approach mentioned in ICH Q2(R2) guideline, an α-risk of 5% is commonly applied in the validation of quantitative analytical methods in the pharmaceutical field.

Table 2 Continued.

Post-marketing surveillance – Quantitative impurity testing for N-nitroso-fluoxetine: Primary objective		
Lower limit	Quantification limit: ≤ 30% of the N-nitroso-fluoxetine AL (0.50 ppm)	Based on retrospective analyses performed by the API manufacturer, N-nitroso-fluoxetine levels in fluoxetine drug substance were found to range between 10 and 30% of the AL, supporting the implementation of skip testing for the raw material. As higher levels are generally expected in finished products, demonstration of a quantification limit at or below 30% of the acceptable limit is required to consistently support skip testing for both drug substance and finished product. The method must be capable of detecting N-nitroso-fluoxetine at 10% of the AL.
	Detection limit: ≤ 10% of the N-nitroso-fluoxetine AL (0.17 ppm)	

AL: acceptable limit.

*The acceptable limit of N-nitroso-fluoxetine is calculated by dividing its acceptable intake limit (100 ng/day) by the maximum daily dose of fluoxetine (60 mg/day).

3.2.2 *Selection of analytical technique*

Based on prior knowledge, reversed-phase ultra-high-performance liquid chromatography hyphenated to tandem mass spectrometry (RP-UHPLC-MS/MS) was selected as the most appropriate analytical technique for this study [31]. The rationale for selecting this analytical technique over alternative approaches is summarized in Table S9.

3.2.3 *Optimization experimental design*

Optimization designs aim to evaluate how experimental factors (or critical method parameters) influence the analytical responses of interest, to understand the relationships between factors and responses, and to identify parameter settings that yield the desired outcomes. Like classical experimental designs, custom or optimal designs provide a flexible and efficient approach to method development. Moreover, they enable the experimental plan to be tailored to specific objectives, available resources, and constraints on factors, while minimizing unnecessary interactions and reducing the total number of experiments.

Input factors and obtained output responses are summarized in the D-optimal design presented in Table S4. To fit the specific needs and intended purpose of the method, the importance assigned to the responses was set to 0.3 for both separation criteria (R1 and R2) and for the number of detectable impurities (R4), as these responses exhibit greater variability and are more strongly affected by the studied factors. The importance of N-nitroso-fluoxetine S/N ratio (R3) was set to 0.1, since it was consistently above the minimum requirement of 10 across all optimization experiments and met the acceptance criterion (Table S4). The ATP was not intended to achieve the lowest possible quantification limit but rather to ensure an adequate lower quantification limit (LQL).

The relationships between responses and factors are illustrated using prediction profilers (Figure 2). The profilers show how predicted responses vary with factor settings, allow exploration of potential main, interaction, and quadratic effects, and support identification of optimal conditions.

The gradient time was identified as the most influential factor affecting separation-related responses, namely: (i) the separation of the isobaric analytes NDIPA and NDPA; and (ii) the separation between fluoxetine and NDPA, the N-nitrosamine eluting closest to fluoxetine.

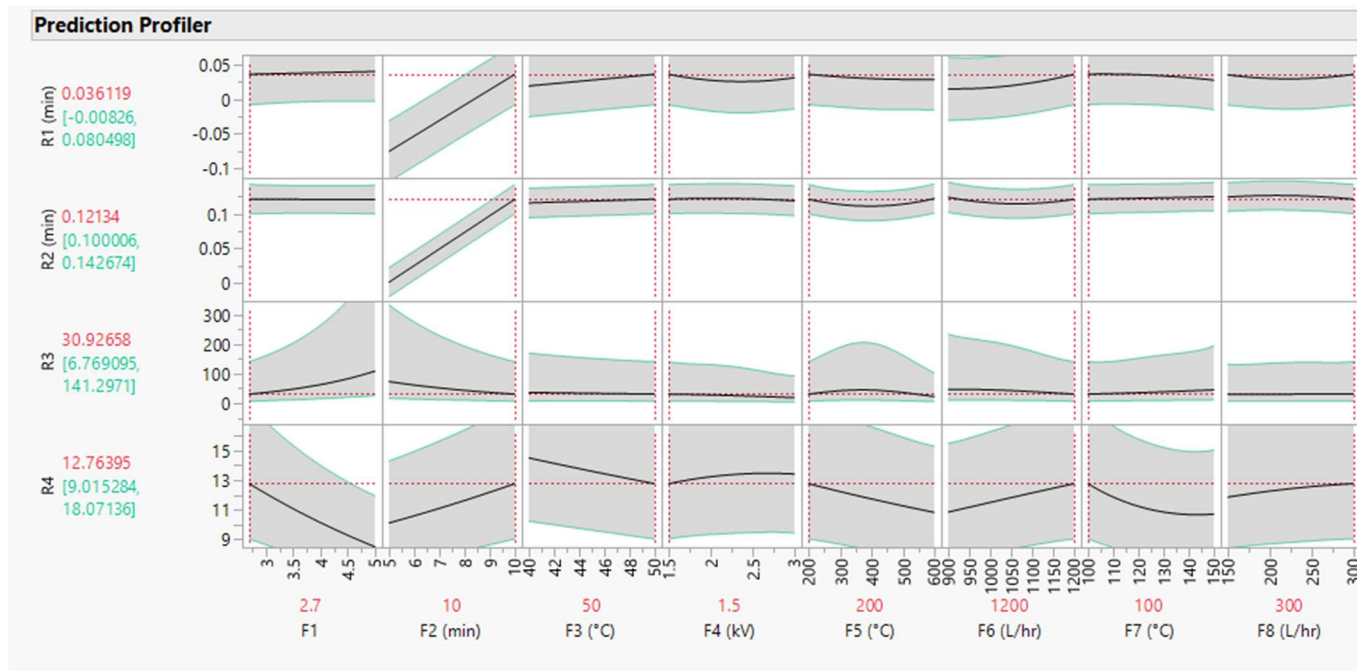


Figure 2. Prediction profiler illustrating influence on responses as a function of evaluated factors. Red: responses predicted with optimal factors; Green: prediction intervals at 95%. F1: pH of the aqueous mobile phase; F2: gradient time; F3: column temperature; F4: capillary voltage; F5: desolvation temperature; F6: desolvation gas flow; F7: source temperature; F8: cone gas flow; R1: separation criterion between isobaric N-nitrosamines; R2: separation criterion between fluoxetine and its nearest N-nitrosamine; R3: S/N ratio for N-nitroso-fluoxetine; R4: number of detectable small-molecule N-nitrosamines with S/N ratio ≥ 3 .

For both gradient times of 5 and 10 min, NDIPA and NDPA were chromatographically separated. However, considering the extremely high fluoxetine concentration (2 mg/mL), its elution extended over more than 1 min. With a gradient time of 5 min, this resulted in co-elution with NDPA, as evidenced by negative separation values reported in Table S4. Increasing the gradient time to 10 min slightly improved the separation, and NDPA no longer co-eluted with fluoxetine, with peak separation being initiated. Experimentally, it was observed that the critical NDPA – fluoxetine pair could not be adequately separated using a gradient time shorter than 10 min. To account for potential retention time shifts and to further improve robustness, increasing the gradient time to 15 min was therefore considered appropriate to enhance the separation between NDPA and fluoxetine, as detailed later in the manuscript.

The pH of the aqueous mobile phase moderately influenced both the number of detectable N-nitrosamines and the S/N ratio of N-nitroso-fluoxetine. More N-nitrosamines were detected under acidic conditions, whereas at pH 5.0, N-nitroso-fluoxetine exhibited higher S/N ratios, corresponding to lower DL and LQL. If the analytical goal were solely to analyze N-nitroso-fluoxetine, a 5-min gradient would be sufficient, or the gradient program could be adjusted to shorten analysis time, with pH 5.0 prioritized to maximize S/N ratios. Among the 15 investigated small-molecule N-nitrosamines, NDMA and NDEA were not detected at 1 ng/mL in any experiment. Their non-detection was independent of the evaluated factors and attributed to insufficient signal intensity in ESI mode, as both compounds produced detectable signals under APCI conditions on the same instrument [32]. Similarly, Herrmann *et al.* demonstrated that APCI provides higher signal intensities and is more suitable than ESI for the analysis of small-molecule N-nitrosamines [31].

Column temperature and mass spectrometric parameters did not significantly affect the evaluated responses. Regardless of factor settings, the S/N ratio for N-nitroso-fluoxetine was always above 10.

The optimal experimental conditions identified from the prediction profiler (Figure 2) were as follows: mobile-phase pH 2.7, gradient time 10 min, column temperature 50 °C, capillary voltage 1.5 kV, desolvation temperature 200 °C, desolvation gas flow 1200 L/h, source temperature 100 °C, and cone gas flow 300 L/h.

Overall, the gradient time was confirmed as the factor exerting the greatest influence on method performance. Consequently, a second experimental design was implemented with the gradient time fixed at 15 min to reassess the performance of the analytical method under the predicted optimal conditions for the remaining factors (Figure 2).

3.2.4 Second optimization experimental design

Compared with the initial design, the lower and upper levels of the factors were narrowed by tightening the experimental ranges around the previously identified optimal conditions. The acceptance criteria for responses R1 to R4 remained unchanged. This approach allowed verification that, with a gradient time of 15 min instead of 10 min, all defined response criteria could still be met within the established acceptance limits despite the presence of deliberate, minor variations in the other experimental factors. The corresponding responses obtained for each experimental condition are summarized in Table S5.

For each output response, the data distribution was evaluated and two-sided prediction intervals were calculated with an α -risk of 5%. The resulting prediction intervals are presented in Table 3.

Table 3: Summary of acceptance criteria and prediction interval for the responses.

	Acceptance criteria	Prediction interval*	Prediction interval#
R1	≥ 0.0 min	0.00 – 0.08 min	0.24 – 0.34 min
R2	≥ 0.0 min	0.10 – 0.14 min	0.16 – 0.35 min
R3	≥ 10	7 – 141	61 – 340
R4	Maximize	9 – 18	13

*: derived from the first D-optimal design at optimal conditions (gradient time of 10 min; Figure 2);

#: derived from the second D-optimal design with a gradient time of 15 min.

The results demonstrate that increasing the gradient time to 15 min improves the separation criteria between NDIPA and NDPA (R1), as well as between NDPA and fluoxetine (R2), with average values close to 0.2 min, thereby confirming the expected influence of gradient time on chromatographic separation. The corresponding prediction intervals indicate that, with 95% confidence, future measurements are expected to fall within 0.24 – 0.34 min for R1 and 0.16 – 0.35 min for R2, thus fully meeting the predefined acceptance criteria.

For R3, the prediction interval of 61 – 340 for the S/N ratio of N-nitroso-fluoxetine complies with the minimum requirement of ≥ 10 . Although this interval is wider than that obtained from the first experimental design, this variability can be attributed to inter-day variability in instrumental response. The prediction interval of the second design for R4 is 13, as 13 small-molecule N-nitrosamines were consistently detected across all experiments (Table S5), and this value falls within the range observed in the initial optimization design.

Overall, extending the gradient time primarily improved separation criterion-related responses (R1 and R2), while R3 and R4 remained stable. As all prediction intervals consistently met the established acceptance criteria, the method was demonstrated to be robust under the selected optimal conditions. The compliance of responses R1 and

R2 confirms that the qualitative ATP requirements related to selectivity and specificity were fulfilled. Consequently, the method was considered suitable for the subsequent validation step.

In parallel, robustness was assessed by fixing the gradient time at 15 min and introducing small metrological variations in the other studied factors around their optimal values obtained from the first experimental design, as illustrated in Figure 2. Robustness refers to the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters and to meet predefined performance criteria during routine use [17].

3.3 Analytical procedure validation

Regardless of whether the method is intended for use as a limit test or for quantitative purposes, selectivity/specificity and DLs must be validated [33]. When quantitative application is intended, additional validation criteria must be evaluated to demonstrate that the method is capable of delivering precise, accurate, and robust results.

3.3.1 *Selectivity and specificity*

The selectivity and specificity of the method were independently demonstrated for each impurity by injecting a blank matrix solution and the calibration standard level 3. Level 3 was selected because it represents the central point of the calibration curve and enables detection of all analytes at this concentration.

Chromatographic selectivity is illustrated in Figure 3. None of the N-nitrosamines co-eluted with fluoxetine. Although partial co-elution was observed between certain N-nitrosamines, these compounds were successfully differentiated based on their unique mass-to-charge ratios, which constitutes the fundamental principle of separation in mass spectrometry. This selectivity was further enhanced by the use of tandem mass spectrometry, enabling dual mass filtering in quadrupoles Q1 and Q3.

Chapter III: Results - Section 2

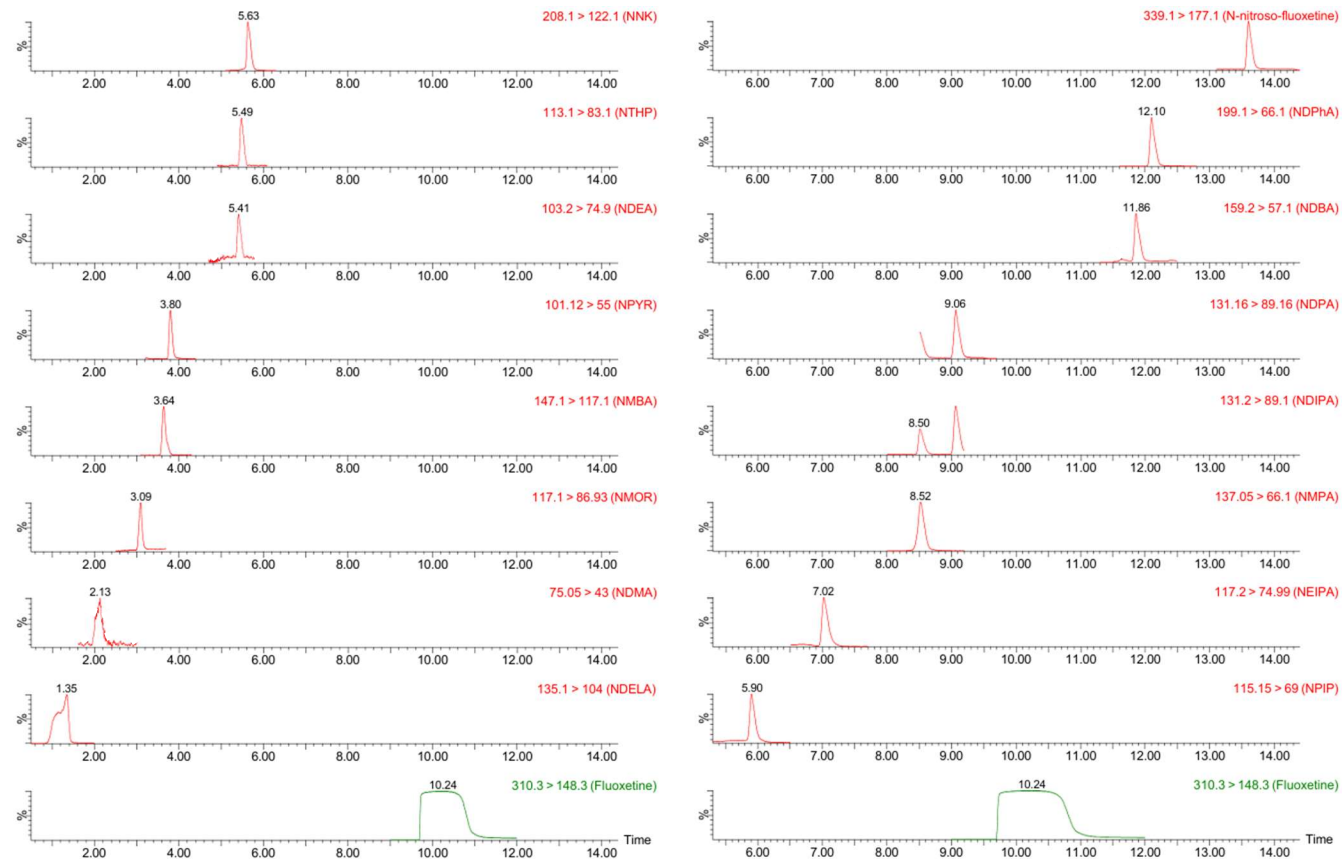


Figure 3. Chromatographic selectivity of N-nitrosamines in calibration standard level 3 (small-molecule N-nitrosamines: 4 ng/mL; N-nitroso-fluoxetine: 1 ng/mL) and fluoxetine (1 mg/mL). The chromatograms were used to support the evaluation of method selectivity (Section 3.4.1).

Specificity was evaluated by comparing extracted ion chromatograms obtained from the calibration standard level 3 with those of the blank matrix. The specificity of N-nitroso-fluoxetine was confirmed by the absence of interfering signals at its mass transition and retention time (Figure 4). The extracted ion chromatograms demonstrating the specificity of all other N-nitrosamines are presented in Figure S1.

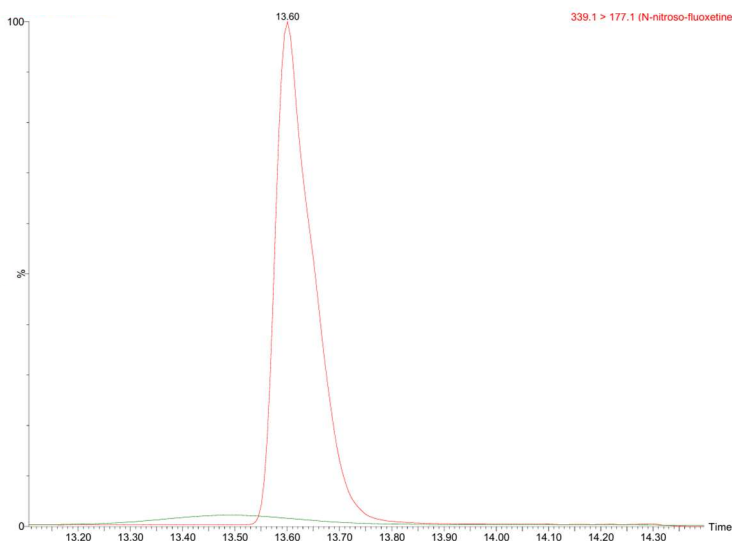


Figure 4. Overlaid extracted ion chromatograms at the mass transition of N-nitroso-fluoxetine. Green: blank matrix; Red: calibration standard level 3 (1 ng/mL).

3.3.2 Detection limits

The DL is defined as the smallest amount of an analyte that can be detected but not necessarily quantified. For screening purposes, a S/N ratio ≥ 3 was considered acceptable, in accordance with ICH Q2(R2) guideline [33].

DLs were experimentally verified using the lowest concentration level. As a conservative approach, the experimentally evaluated concentration levels were reported as DLs. The S/N ratios for all N-nitrosamines are presented in Table S10, while the corresponding DLs are summarized in Table 4, together with their associated detection levels and compliance with the ATP acceptance criteria.

Overall, S/N ratios exceeded 3 at the lowest calibration and validation levels across the three validation series, with the exception of NDEA and NDMA. NDEA was detectable only from concentration level 2 (2 ng/mL), whereas NDMA was detectable from 4 ng/mL. The relatively high DLs are likely related to their low molecular weights, for which APCI is more suitable than ESI. Most N-nitrosamines exhibit significantly lower DLs under APCI conditions on the same instrument [32,34].

Conversely, NNK, NDPhA, and N-nitroso-fluoxetine showed high S/N ratios in ESI mode, which is likely attributable to their higher molecular weights (approximately 200 Da or above), favoring more efficient ionization under ESI conditions.

Compared with the LC-ESI-MS/MS method proposed by the United States FDA for the determination of 8 N-nitrosamines with DLs ranging from 0.1 to 0.5 ng/mL [35], the method developed in this work enables the detection of 13 small-molecule N-nitrosamines at 0.4 ng/mL, below the respective acceptable intake limits. Although the reported DLs appear slightly higher than those of the FDA method, the S/N ratios presented in Table S10 suggest that further lowering of DLs may be achievable. However, this assumption should be experimentally verified at lower concentration levels.

As NDEA is classified among the 3 worst-case impurities, its DL exceeded the acceptable limit of 0.44 ppm (0.44 ng/mL) and therefore did not meet the ATP acceptance criteria defined in Table 2. Similarly, the DL for NDMA of 4 ng/mL resulted in non-compliance with ATP requirements. These findings indicate that the ionization efficiency of NDEA and NDMA under ESI conditions is insufficient to achieve the targeted DLs. Consequently, APCI appears more appropriate for these compounds, under which estimated DLs of at least 0.1 ng/mL were achieved [32].

However, according to the risk assessment, only the impurity identified as relevant for the investigated drug product, N-nitroso-fluoxetine, required evaluation. The inclusion of additional small-molecule N-nitrosamines, such as NDMA and NDEA, was therefore adopted as a conservative but non-mandatory measure. Consequently, although the DL criteria for NDEA and NDMA did not meet the ATP acceptance criteria, the associated residual risk was considered acceptable because the corresponding precursor amines are improbable in the synthetic route of fluoxetine or during the manufacturing of Fluoxone[®].

Chapter III: Results - Section 2

Table 4: DLs and corresponding acceptable limits in Fluoxone®.

N-nitrosamine	DL (ppm or ng/mL)	AL (ppm or ng/mL)	Detection level	ATP acceptance criteria
N-nitroso-fluoxetine	0.09	1.67	< 10 % AL	Compliant
NDEA	2.00	0.44	> 100% AL	Not compliant
NDPA	0.40	0.44	< 100 % AL	Compliant
NDBA	0.40	0.44	< 100 % AL	Compliant
NTHP	0.38	0.62	< 100 % AL	Compliant
NDMA	4.00	1.60	> 100% AL	Not compliant
NMPA	0.38	1.67	< 30 % AL	Compliant
NNK	0.40	1.67	< 30 % AL	Compliant
NMOR	0.40	2.12	< 30 % AL	Compliant
NEIPA	0.40	6.67	< 10 % AL	Compliant
NPIP	0.39	21.67	< 10 % AL	Compliant
NDIPA	0.40	25.00	< 10 % AL	Compliant
NMBA	0.40	25.00	< 10 % AL	Compliant
NPYR	0.40	28.33	< 10 % AL	Compliant
NDELA	0.40	31.67	< 10 % AL	Compliant
NDPhA	0.40	1300.00	< 10 % AL	Compliant

DL: detection limit; AL: acceptable limit; N/A: not applicable.

3.3.3 Quantitative performance

Compliance with the quantitative ATP requirements was assessed through validation of the quantitative method for N-nitroso-fluoxetine. The quantitative performance is summarized in Table 5. Two calibration models were retained owing to their complementary advantages. All evaluated calibration models, together with their corresponding accuracy indices and validated ranges, are presented in Table S11.

Table 5: Summary of quantitative validation criteria for N-nitroso-fluoxetine.

N-nitroso-fluoxetine ($p = 3$; $n = 3$)		
Response function		
Calibration model	Linear regression after logarithmic transformation*	Linear regression through 0 (fitted at level 3)
Level	Relative β -expectation tolerance limits (%)	Relative β -expectation tolerance limits (%)
1	[-1.8 , 7.2]	[-20.0 , 17.9]
2	[-5.4 , 7.9]	[-8.0 , 7.8]
3	[-3.3 , 7.2]	[-1.6 , 5.1]
4	[-4.1 , 3.5]	[-3.5 , 5.2]
5	[-4.7 , 7.9]	[-2.6 , 10.6]
Linearity		
Slope	1.014	1.040
95% confidence interval of slope	[1.004, 1.025]	[1.028, 1.051]
Intercept	-0.005	-0.024
95% confidence interval of intercept	[-0.030 , 0.020]	[-0.051 , 0.004]
R ²	0.999	0.999
Lower limit		
DL (ng/mL or ppm)	0.09	0.09
Range (ng/mL or ppm)	0.09 – 4.75	0.25 – 4.75

*Base-10 logarithmic transformation of both concentration and response.

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

3.3.3.1 Precision and accuracy – combined approach

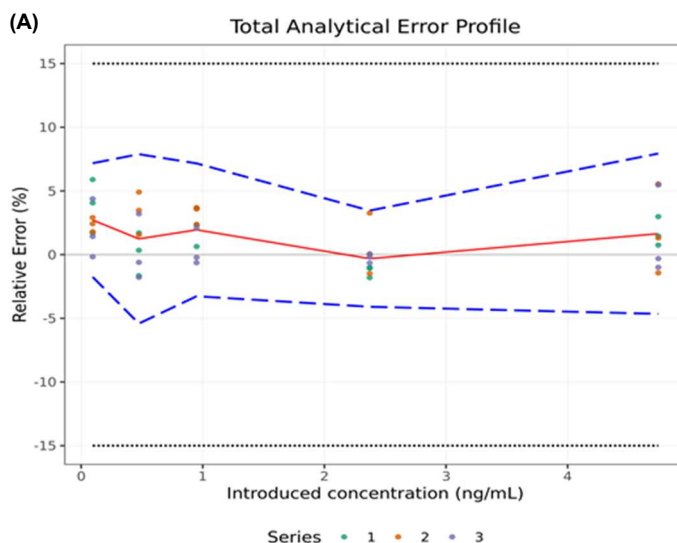
A combined approach was adopted to evaluate total analytical error by simultaneously integrating precision (random error) and accuracy (systematic error). This methodology represents an alternative to conventional separate assessments. Total analytical error was assessed using a β -expectation tolerance interval, which corresponds to a prediction interval for future measurements.

Two total error profiles were generated using the selected calibration models. These profiles provide a comprehensive risk-based visualization of quantitative performance. Acceptance limits were set at $\pm 15\%$, and β -expectation tolerance limits were calculated with a beta-coverage of 95%.

The logarithmically transformed model exhibited a slightly higher total analytical error index (Table S11) with a wider validated range (Table 5) than the linear model. The total analytical error index, formerly known as the accuracy index, is a desirability index used to estimate the overall quality of an analytical procedure [36]. However, the linear model offers the practical advantage of one-point calibration, thereby simplifying preparation. In contrast, application of the logarithmically transformed model requires the construction of a multi-level calibration curve.

A positive bias below 5% was observed in both Figures 5(A) and (B). Figure 5(A) presents a narrower β -expectation tolerance interval than Figure 5(B), particularly at the lowest concentration level. Wider tolerance intervals at low concentrations are typically associated with LQLs. Specifically, the first model yielded tolerance limits of $[-1.8\%, 7.2\%]$ at level 1, compared with $[-20.0\%, 17.9\%]$ for the second model.

In Figure 5(B), a pronounced series effect is observed at the lowest concentration level, with results clustered by validation series. This effect is mitigated using the logarithmically transformed model, as evidenced by the randomly distributed results of the series in Figure 5(A). The first model is more appropriate to address this inter-series variability. Moreover, this model offers a wider validated range, particularly relevant in the framework of the present mitigation study, and was therefore retained for further analysis.



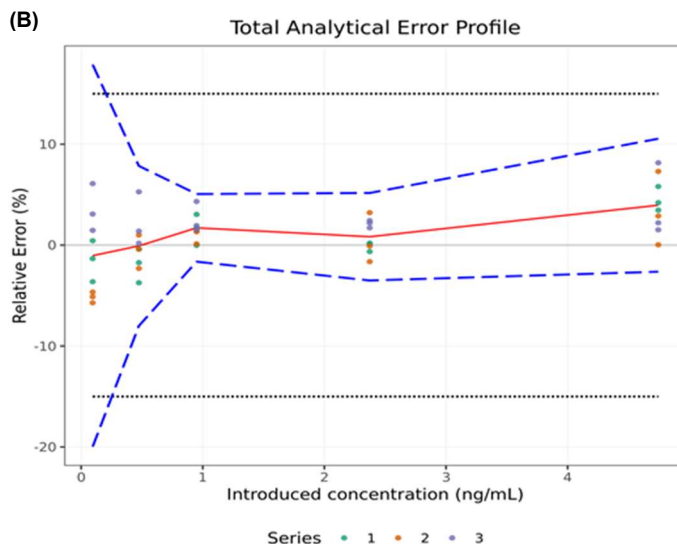


Figure 5. Total analytical error profiles computed by considering (A) linear regression after logarithm transformation of both concentration and response, and (B) linear regression through 0 (fitted only at concentration level 3) to assess the quantitative performance of the method for N-nitroso-fluoxetine in Fluoxone[®] 20 mg. On the figures, the plain red line is the relative bias (%), the dashed blue lines are the lower and upper ends of the β -expectations tolerance limits with a beta-coverage of 95%, the dotted black lines represent the acceptance limits of $\pm 15\%$, the dots represent the relative error of the individual results and are plotted with respect to the targeted concentration.

3.3.3.2 Range

The range is defined as the interval between the lowest and the highest amounts of an analyte, in which the analytical method provides results with suitable precision and accuracy [33].

The validated range is readily visualized in the total error profiles. In Figure 5(A), β -expectation tolerance limits remain within acceptance limits over the entire range, confirming full validation of the pre-selected range using the logarithmically transformed model. In contrast, Figure 5(B) shows partial validation of the evaluated range, as tolerance limits fall outside the acceptance limits at the lowest concentration.

3.3.3.3 Lower limits

The DL for N-nitroso-fluoxetine is reported in Section 3.3.2. Unlike the DL, the LQL is the smallest amount of an analyte that can be quantified in a sample with adequate precision and accuracy. According to ICH Q2(R2), an S/N ratio ≥ 10 is required for LQL [33]. The overall S/N ratios of N-nitroso-fluoxetine at the lowest concentration

level were above 10 (Table S10), indicating that this level can be validated as the LQL, provided that all quantitative performance criteria are met.

In Figure 5(A), the entire concentration range was successfully validated, with the lowest level (0.09 ppm, equivalent to 0.09 ng/mL) confirmed as the LQL. This value is tenfold lower than the quantification limit of 1 ng/mL reported in the analytical procedure reported by the Taiwan FDA [23]. Although the LQL is comparable to that of the method proposed by the German OMCL [24], the present method additionally enables the simultaneous detection of small-molecule N-nitrosamines. In contrast, Figure 5(B) shows that the LQL was validated at 0.25 ppm, as indicated by the intersection between the β -expectation tolerance limits and the acceptance limits.

3.3.3.4 Linearity

The result linearity refers to the ability of an analytical method to provide results directly proportional to the concentration of an analyte in a sample, within a defined range [37].

Both calibration models demonstrate excellent result linearity, with coefficients of determination (R^2) of 0.999. Furthermore, the value of 0 fall within the 95% confidence interval of the intercept. Although the value of 1 is not strictly included within the 95% confidence interval of the slope, it is very close to the interval. These results provide complementary evidence of robust linearity. The corresponding data are presented in Table 5.

3.3.4 System suitability tests

System suitability tests (SSTs) as part of the AQbD approach, verify that the analytical system meets established performance criteria [17,38]. For this purpose, two SSTs were defined and performed in parallel with the validation study to confirm that the analytical system and procedure were fit for purpose on the day of analysis.

3.3.4.1 Tailing factor

The tailing factor assesses peak symmetry and serves as an indicator of column integrity. In this study, the tailing factor was evaluated exclusively for N-nitroso-fluoxetine, for which a quantitative purpose was defined, using calibration standard level 3 (1 ng/mL). According to the European Pharmacopoeia, the recommended tailing factor for quantitative analysis ranges from 0.8 to 1.8 [39]. Values of 1.6, 1.7, and 1.7 were obtained across the three validation series, indicating peak tailing, as illustrated in Figure 4. No peak fronting was observed. The peak tailing was not considered critical, as no isobaric interferences with N-nitroso-fluoxetine were detected, and the validation results demonstrated compliance with the ATP requirements.

Given the validation data, the lower acceptance limit of 0.8 recommended by the European Pharmacopoeia was retained, while the upper limit was broadened to 2.2 to provide an appropriate operational margin (0.8 – 2.2) that better reflects the method performance under routine conditions.

3.3.4.2 System sensitivity

System sensitivity is critical for the trace analysis of impurities and is commonly assessed using S/N ratios. For impurity limit test and quantitative testing, the S/N ratio should be equal to or greater than 3 and 10, respectively [33].

To monitor the system sensitivity, S/N ratios were evaluated for each analyte in the calibration standard at the lowest concentration level. Across the three validation series, S/N ratios were greater than 3 for all small-molecule impurities for which a screening purpose was defined, with the exception of NDEA and NDMA. For N-nitroso-fluoxetine, for which quantitative analysis was intended, S/N ratios were greater than 10. These data are reported in Table S10.

3.4 Risk mitigation measures

3.4.1 System suitability tests

SSTs confirmed adequate system performance during confirmatory analysis. The tailing factor of N-nitroso-fluoxetine was 1.9, complying with the predefined criteria of 0.8 – 2.2. S/N ratios (Table S12) were generally above 3 for small-molecule N-nitrosamines (except for NDEA and NDMA), and above 10 for N-nitroso-fluoxetine.

3.4.2 Confirmatory testing

A confirmatory study was performed to demonstrate the effectiveness of the implemented risk mitigation measures. Fluoxetine hydrochloride raw material, newly produced batches, and retention batches were analyzed. Although the former Fluoxone® formulation had been withdrawn and was no longer manufactured, a sample at the end of its shelf-life was tested. The method specificity and absence of matrix effects were previously verified. The former formulation was analyzed using the same analytical procedure without modification of the sample preparation protocol.

Results obtained using the calibration model based on linear regression after logarithmic transformation are reported in Table 6.

Table 6: Summary of N-nitroso-fluoxetine contents in fluoxetine hydrochloride drug substance and finished products.

	Detected small-molecule N-nitrosamines	N-nitroso-fluoxetine		
		Linear regression after logarithmic transformation		AL: 1.67 ppm
		Content (ppm)	AL level	AL level
Fluoxetine hydrochloride				
Batch 1	None	0.21	13%	10 – 30 % of AL
Batch 2	None	0.25	15%	10 – 30 % of AL
Former formulation of Fluoxone®				
End of shelf-life	None	> UQL (26.59*)	16 x AL	> 100% AL
New formulation of Fluoxone®				
T0 (release sample)	None	0.58	35%	30 – 100 % of AL
T3M	None	0.66	40%	30 – 100 % of AL
T21M – desiccant-integrated Alu/Alu blister	None	1.15	69%	30 – 100 % of AL
– PVC/Alu blister	None	> UQL (4.90*)	3 x AL	> 100% AL

Stability samples were stored at 25 °C/60% RH conditions. AL: acceptable limit; LQL: lower quantification limit; UQL: upper quantification limit.

*Value provided for information purposes only.

Testing results showed that only N-nitroso-fluoxetine was detected in both the drug substance and finished product, while all other small-molecule N-nitrosamines were not detected. As declared by the API supplier, N-nitroso-fluoxetine content in the drug substance was below 30% of the acceptable limit, 0.21 and 0.25 ppm were found in two batches. As expected, higher levels were observed in the finished product, with 0.58 ppm detected in the release sample (T0), suggesting that approximately 0.3 ppm of N-nitroso-fluoxetine formed during manufacturing, likely due to contact with nitrosating agents. The impurity continued to form during storage, reaching 0.66 and 1.15 ppm after 3 and 21 months at 25 °C/60% RH, respectively, remaining below the acceptable limit of 1.67 ppm. These results indicate that replacing microcrystalline cellulose with mannitol did not completely eliminate nitrite but reduced excipient-related nitrite levels and consequently decreased N-nitroso-fluoxetine formation in Fluoxone®.

For the secondary screening objective, 13 out of 15 small-molecule N-nitrosamines were not detected and were below their respective acceptable limits. Although NDEA and NDMA were also not detected in the samples, their DLs are higher than the corresponding acceptable limits. Therefore, the potential presence of these impurities at concentrations below the DLs but exceeding the acceptable limits cannot be completely excluded. However, small-molecule N-nitrosamines were screened using available LC-APCI(+)-MS/MS methods [32,34], which have been reported to achieve DLs of at least 0.1 ng/mL for NDEA and NDMA, demonstrated that both impurities were below their respective regulatory limits.

The T21M batch was packaged using two different blister systems: Al/Al with integrated desiccant and conventional PVC/Al. The product packaged in PVC/Al materials generated approximately 4-fold higher quantities of N-nitroso-fluoxetine compared to the Al/Al system, resulting in amounts largely exceeding the acceptable limit. The Al/Al blister provides superior barrier properties. In addition, the incorporated desiccant particles absorb residual moisture, offering further protection against nitrosation and hydrolysis. Consequently, this packaging system effectively minimizes product deterioration and impurity formation throughout the shelf-life [28,40,41].

In the former formulation, N-nitroso-fluoxetine content exceeded the upper limit of the calibration range and required a 10-fold dilution with water/methanol, 80%/20%, v/v. As this dilution also reduced the concentration of N-nitroso-fluoxetine-d5 by a factor of 10, an appropriate correction factor was applied. At the end of shelf-life, the N-nitroso-fluoxetine content was estimated to be approximately 27 ppm. The EMA authorized interim acceptable limits for N-nitrosamines exceeding the recommended acceptable intake during the implementation of corrective and preventive actions. As fluoxetine is typically prescribed for chronic use, a conservative correction factor of

6.7 x for treatment durations longer than 12 months results in an interim intake limit of 670 ng/day for N-nitroso-fluoxetine, corresponding to 11.17 ppm in Fluoxetine® [8]. However, the measured content exceeded the recommended limit by more than 15-fold, thereby justifying the withdrawal of the former formulation and the need for redevelopment.

In addition to formulation and packaging modifications, the product shelf-life was reduced from 36 to 24 months as a conservative measure within the overall risk mitigation strategy.

4 Conclusion

The AQbD framework streamlines method development through science-based risk management. Using DoE rather than the OFAT strategy, interactions between method parameters can be systematically evaluated with fewer experiments, reducing failure risks, shortening timelines, and optimizing resources.

By applying the AQbD approach, a robust LC-MS/MS method was developed for N-nitrosamine analytics in fluoxetine-containing products, minimizing the risk of inadequate analytical performance. The method was successfully validated according to ICH Q2(R2) guideline, functioning both as a limit test for 13 small-molecule N-nitrosamines and as a quantitative method for N-nitroso-fluoxetine. This method is able of quantifying N-nitroso-fluoxetine down to 0.1 ppm (ng/mL) while simultaneously detecting most N-nitrosamines below their respective acceptable limits in fluoxetine hydrochloride drug substance and fluoxetine-containing products, except for NDMA and NDEA. Although the confirmatory study did not experimentally demonstrate that these two impurities were below their respective acceptable limits, the associated residual risk is considered low, as small-molecule N-nitrosamines are not regarded as at-risk impurities in Fluoxetine®. Complementary confidence was provided by analyzing these impurities under APCI conditions, which confirmed that NDMA and NDEA levels were below their respective limits. This study provides a valuable contribution to NDSRI determination and supports robust, science-based control strategies for these emerging impurities.

Furthermore, the manufacturer's risk mitigation strategies were demonstrated to be effective. The use of low-nitrite excipients successfully reduced nitrite levels, thereby limiting N-nitroso-fluoxetine formation. In addition, modifications to the primary packaging, specifically, the use of desiccant-integrated Al/Al blisters enhanced protection against environmental factors promoting nitrosation by maximizing barrier properties against oxygen, moisture, and light. Together, these mitigation measures maintained N-nitroso-fluoxetine content below the safety threshold of 1.67 ppm in Fluoxetine® Divul® 20 mg through the product shelf-life.

N-nitroso-fluoxetine was experimentally confirmed as the only relevant impurity in Fluoxone®. This acquired product knowledge may support further method refinement within the AQbD framework, with a focus solely on N-nitroso-fluoxetine quantification. Such an approach is fully aligned with the analytical procedure lifecycle management principles described in ICH Q14. For this potential new analytical objective arising from the enhanced understanding of the product, the information generated from the first optimization experimental design can be leveraged and redirected toward N-nitroso-fluoxetine quantification. Through continuous knowledge management, the method can be further refined by exploiting previously acquired knowledge. For example, previously investigated conditions, including the use of a pH 5.0 aqueous mobile phase to potentially lower the DL or LQL if required, as well as a reduced gradient time to shorten analysis time and decrease solvent consumption, could support future method adaptation in line with the principles of white analytical chemistry [42].

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Supplementary material**Table S1:** Qualitative composition of the updated formulation of Fluoxetine® 20 mg.

Qualitative composition of Fluoxetine® 20 mg	
Active substance	Fluoxetine hydrochloride
Excipients	Mannitol
	Croscarmellose sodium
	Magnesium stearate
	Hypromellose
	Diethyl Phthalate

Table S2: Deselection of irrelevant quadratic effects and interactions in the D-optimal design and justification.

	Estimability	Justification(s)
Main effects		
Intercept	Necessary	Main effects are essential in an experimental design.
Aqueous solvent pH	Necessary	Main effects are essential in an experimental design.
Gradient time	Necessary	Main effects are essential in an experimental design.
Column temperature	Necessary	Main effects are essential in an experimental design.
Capillary voltage	Necessary	Main effects are essential in an experimental design.
Desolvation temperature	Necessary	Main effects are essential in an experimental design.
Desolvation gas flow	Necessary	Main effects are essential in an experimental design.
Source temperature	Necessary	Main effects are essential in an experimental design.
Cone gas flow	Necessary	Main effects are essential in an experimental design.
Interactions		
Aqueous solvent pH*Gradient time	Necessary	Interactions may exist.
Aqueous solvent pH*Column temperature	Unnecessary	No interactions expected.
Aqueous solvent pH*Capillary voltage	Necessary	Interactions may exist.
Aqueous solvent pH*Desolvation temperature	Unnecessary	No interactions expected.
Aqueous solvent pH*Desolvation gas flow	Unnecessary	No interactions expected.
Aqueous solvent pH*Source temperature	Unnecessary	No interactions expected.
Aqueous solvent pH*Cone gas flow	Unnecessary	No interactions expected.
Gradient time*Column temperature	Unnecessary	No interactions expected.
Gradient time*Capillary voltage	Unnecessary	No interactions expected.
Gradient time*Desolvation temperature	Unnecessary	No interactions expected.
Gradient time*Desolvation gas flow	Unnecessary	No interactions expected.
Gradient time*Source temperature	Unnecessary	No interactions expected.
Gradient time*Cone gas flow	Unnecessary	No interactions expected.

Chapter III: Results - Section 2

Table S2 Continued.

	Estimability	Justification(s)
Interactions		
Column temperature*Capillary voltage	Unnecessary	No interactions expected.
Column temperature*Desolvation temperature	Unnecessary	No interactions expected.
Column temperature*Desolvation gas flow	Unnecessary	No interactions expected.
Column temperature*Source temperature	Unnecessary	No interactions expected.
Column temperature*Cone gas flow	Unnecessary	No interactions expected.
Capillary voltage*Desolvation temperature	Necessary	Interactions may exist.
Capillary voltage*Desolvation gas flow	Necessary	Interactions may exist.
Capillary voltage*Source temperature	Necessary	Interactions may exist.
Capillary voltage*Cone gas flow	Unnecessary	No interactions expected.
Desolvation temperature*Desolvation gas flow	Necessary	Interactions may exist.
Desolvation temperature*Source temperature	Necessary	Interactions may exist.
Desolvation temperature*Cone gas flow	Unnecessary	No interactions expected.
Desolvation gas flow*Source temperature	Necessary	Interactions may exist.
Desolvation gas flow*Cone gas flow	Unnecessary	No interactions expected.
Source temperature*Cone gas flow	Unnecessary	No interactions expected.
Quadratic effects		
Aqueous solvent pH*Aqueous solvent pH	Unnecessary	Quadratic effects are not anticipated.
Gradient time*Gradient time	Unnecessary	A linear effect is expected.
Column temperature*Column temperature	Unnecessary	A linear effect is expected.
Capillary voltage*Capillary voltage	Necessary	Quadratic effects may exist.
Desolvation temperature*Desolvation temperature	Necessary	Quadratic effects may exist.
Desolvation gas flow*Desolvation gas flow	Necessary	Quadratic effects may exist.
Source temperature*Source temperature	Necessary	Quadratic effects may exist.
Cone gas flow*Cone gas flow	Necessary	Quadratic effects may exist.

Table S3: Elution program for the gradient times of 5, 10, and 15 minutes.

Mobile phase composition	Gradient time of 5 minutes	Gradient time of 10 minutes	Gradient time of 15 minutes
100 % A	0.0 – 1.0 min	0.0 – 1.0 min	0.0 – 1.0 min
100 → 5% A (gradient)	1.0 – 6.0 min	1.0 – 11.0 min	1.0 – 16.0 min
5% A	6.0 – 8.5 min	11.0 – 13.5 min	16.0 – 18.5 min
5 → 100% A	8.5 – 8.6 min	13.5 – 13.6 min	18.5 – 18.6 min
100% A	8.6 – 12.0 min	13.6 – 17.0 min	18.6 – 22.0 min

Chapter III: Results - Section 2

Table S4: D-optimal design for optimization computed with 8 input factors and 4 associated output responses.

Run	Whole plots	Input factors								Output responses			
		F1	F2 (min)	F3 (°C)	F4 (kV)	F5 (°C)	F6 (L/hr)	F7 (°C)	F8 (L/hr)	R1 ≥ 0.00 min	R2 ≥ 0.00 min	R3 ≥ 10	R4 Maximize
1	1	5.0	5	50	2.25	400	900	150	300	0.00	-0.10	366	8
2	1	5.0	5	50	3.00	200	1200	150	150	0.00	-0.10	234	8
3	1	5.0	10	50	3.00	600	1200	125	300	0.13	0.05	101	6
4	1	5.0	10	40	1.50	200	900	150	225	0.12	0.00	261	7
5	1	5.0	5	40	1.50	600	1050	125	225	0.00	-0.10	114	8
6	1	5.0	10	50	1.50	200	1200	100	150	0.13	0.05	115	8
7	1	5.0	10	40	3.00	200	900	100	150	0.14	0.00	149	8
8	2	2.7	5	50	3.00	200	900	150	300	0.00	-0.10	60	10
9	2	2.7	5	40	2.25	600	1050	100	150	0.00	-0.10	56	9
10	2	2.7	5	50	1.50	400	900	100	225	0.00	-0.10	115	8
11	2	2.7	10	50	3.00	600	900	100	225	0.15	0.05	14	10
12	2	2.7	10	40	1.50	200	1050	125	300	0.11	0.00	71	13
13	2	2.7	10	40	3.00	400	1200	150	150	0.10	0.00	57	12
14	2	2.7	5	40	3.00	200	1200	100	225	0.00	-0.10	55	13
15	3	5.0	10	50	2.25	200	1200	125	225	0.12	0.00	102	7
16	3	5.0	5	50	1.50	600	1200	150	300	0.00	-0.10	123	8
17	3	5.0	5	40	3.00	600	1200	100	150	0.00	-0.10	101	8
18	3	5.0	10	50	3.00	400	1050	150	225	0.11	0.00	141	7
19	3	5.0	5	50	1.50	200	900	100	150	0.00	-0.10	160	8
20	3	5.0	10	40	1.50	600	900	100	300	0.12	0.00	79	7
21	3	5.0	5	40	3.00	600	900	150	300	0.00	-0.10	70	8

Chapter III: Results - Section 2

Table S4 Continued.

Run	Whole plots	Input factors								Output responses			
		F1	F2 (min)	F3 (°C)	F4 (kV)	F5 (°C)	F6 (L/hr)	F7 (°C)	F8 (L/hr)	R1 ≥ 0.00 min	R2 ≥ 0.00 min	R3 ≥ 10	R4 Maximize
22	4	2.7	10	50	1.50	600	900	150	150	0.13	0.02	14	8
23	4	2.7	5	40	1.50	200	1200	150	300	0.00	-0.10	112	9
24	4	2.7	5	40	3.00	400	900	125	150	0.00	-0.10	97	8
25	4	2.7	10	40	2.25	600	1200	150	225	0.13	0.00	28	11
26	4	2.7	10	40	1.50	600	1200	100	225	0.12	0.00	33	11
27	4	2.7	5	50	3.00	600	1050	150	225	0.00	-0.10	81	7
28	4	2.7	10	50	3.00	400	1050	100	300	0.11	0.03	31	10

F1: pH of the aqueous mobile phase; F2: gradient time; F3: column temperature; F4: capillary voltage; F5: desolvation temperature; F6: desolvation gas flow; F7: source temperature; F8: cone gas flow; R1: separation criterion between isobaric N-nitrosamines; R2: separation criterion between fluoxetine and its nearest N-nitrosamine; R3: S/N ratio for N-nitroso-fluoxetine; R4: number of detectable small-molecule N-nitrosamines with S/N ratio ≥ 3 .

Chapter III: Results - Section 2

Table S5: Second D-optimal design for reoptimization computed with 7 input factors and 4 associated output responses.

Run	Input factors							Output responses			
	F1	F2 (°C)	F3 (kV)	F4 (°C)	F5 (L/hr)	F6 (°C)	F7 (L/hr)	R1 (min) ≥ 0.00 min	R2 (min) ≥ 0.00 min	R3 ≥ 10	R4 Maximize
1	2.7	52	1.50	200	1200	120	300	0.28	0.23	13	228
2	2.7	48	2.00	300	1200	100	250	0.28	0.20	13	314
3	2.7	52	1.50	300	1100	100	300	0.31	0.20	13	269
4	2.7	48	1.50	200	1200	120	250	0.29	0.21	13	198
5	2.7	48	2.00	200	1100	100	300	0.30	0.20	13	156
6	2.7	48	1.50	300	1100	120	300	0.31	0.21	13	277
7	2.7	52	2.00	200	1100	120	250	0.29	0.21	13	193
8	2.8	50	1.75	250	1150	110	275	0.33	0.30	13	160
9	2.8	50	1.75	250	1150	110	275	0.28	0.29	13	154
10	2.8	50	1.75	250	1150	110	275	0.33	0.29	13	157
11	2.9	48	2.00	300	1200	120	300	0.28	0.30	13	121
12	2.9	52	1.50	300	1200	100	250	0.28	0.29	13	248
13	2.9	52	2.00	200	1200	100	300	0.24	0.24	13	295
14	2.9	48	1.50	200	1100	120	300	0.30	0.30	13	126
15	2.9	52	2.00	300	1100	120	250	0.25	0.27	13	185
16	2.9	48	1.50	200	1100	100	250	0.28	0.32	13	128

F1: pH of the aqueous mobile phase; F2: column temperature; F3: capillary voltage; F4: desolvation temperature; F5: desolvation gas flow; F6: source temperature; F7: cone gas flow; R1: separation criterion between isobaric N-nitrosamines; R2: separation criterion between fluoxetine and its nearest N-nitrosamine; R3: S/N ratio for N-nitroso-fluoxetine; R4: number of detectable small-molecule N-nitrosamines with S/N ratio ≥ 3 .

Chapter III: Results - Section 2

Table S6: Cone voltage and MRM parameters of N-nitrosamines and N-nitroso-fluoxetine-d5.

N-nitrosamine	MRM transition (<i>m/z</i>)	Acquisition window (min)	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)	Soft transmission
N-nitroso-fluoxetine	339.10 → 177.10	13.10 – 14.40	10	10	108	Activated
N-nitroso-fluoxetine-d5	344.10 → 182.00	13.10 – 14.30	10	10	108	Activated
NMOR	117.10 → 86.93	2.50 – 3.70	27	13	19	No
NMPA	137.05 → 66.10	8.00 – 9.20	28	12	38	No
NMBA	147.10 → 117.10	3.10 – 4.30	17	7	19	Activated
NDPhA	199.10 → 66.10	11.60 – 12.80	23	22	52	No
NNK	208.10 → 122.10	5.10 – 6.30	32	14	27	No
NDMA	75.05 → 58.05	1.60 – 3.00	32	9	52	No
NDBA	159.20 → 57.10	11.30 – 12.50	30	12	52	No
NEIPA	117.20 → 74.99	6.50 – 7.70	25	8	163	Activated
NDIPA	131.20 → 89.10	8.00 – 9.20	17	10	38	No
NDPA	131.16 → 89.16	8.50 – 9.70	30	11	38	Activated
NPYR	101.12 → 55.00	3.20 – 4.40	22	15	19	No
NDEA	103.20 → 74.90	4.70 – 5.90	33	9	27	No
NPIP	115.15 → 69.00	5.30 – 6.50	13	14	27	No
NTHP	113.10 → 67.10	4.90 – 6.10	17	10	27	No
NDELA	135.10 → 104.00	0.50 – 2.00	8	10	108	Activated

Chapter III: Results - Section 2

Table S7: Acceptable limit calculations.

N-nitrosamines	AI limit (ng/day)	MDD (mg/day)	100% AL		30% AL	10% AL		
			AL (ppm)	AL (ng/mL)	AL (ppm)	AL (ng/mL)	AL (ppm)	AL (ng/mL)
N-nitroso-fluoxetine	100.0	60	1.67	1.67	0.50	0.50	0.17	0.17
NDEA	26.5	60	0.44	0.44	0.13	0.13	0.04	0.04
NDPA	26.5	60	0.44	0.44	0.13	0.13	0.04	0.04
NDBA	26.5	60	0.44	0.44	0.13	0.13	0.04	0.04
NTHP	37.0	60	0.62	0.62	0.19	0.19	0.06	0.06
NDMA	96.0	60	1.60	1.60	0.48	0.48	0.16	0.16
NMPA	100.0	60	1.67	1.67	0.50	0.50	0.17	0.17
NNK	100.0	60	1.67	1.67	0.50	0.50	0.17	0.17
NMOR	127.0	60	2.12	2.12	0.64	0.64	0.21	0.21
NEIPA	400.0	60	6.67	6.67	2.00	2.00	0.67	0.67
NPIP	1300.0	60	21.67	21.67	6.50	6.50	2.17	2.17
NDIPA	1500.0	60	25.00	25.00	7.50	7.50	2.50	2.50
NMBA	1500.0	60	25.00	25.00	7.50	7.50	2.50	2.50
NPYR	1700.0	60	28.33	28.33	8.50	8.50	2.83	2.83
NDELA	1900.0	60	31.67	31.67	9.50	9.50	3.17	3.17
NDPhA	78000.0	60	1300.00	1300.00	390.00	390.00	130.00	130.00

AI: acceptable intake; MDD: maximum daily dose of Fluoxone®; AL: acceptable limit.

The AL expressed as concentration (ng/mL) depends on the sample preparation protocol. Sample preparation: internal standard added to (reconstituted matrix equivalent to) 1 tablet of Fluoxone® solubilized with methanol to 4.0 ml, then diluted 5 times with water.

Table S8: Identification of hazardous N-nitrosamines in the former formulation (withdrawn from the market) Fluoxone® and related sources.

Source of risk of N-nitrosamine formation	Justification(s)
API chemical synthesis	
Investigation under API manufacturer's responsibility: N-nitroso-fluoxetine detected between 10 – 30% of its acceptable limit in fluoxetine hydrochloride raw material	
Finished product manufacturing – formulation	
API	Vulnerable (nitrosatable) secondary amine in the chemical structure
Fluoxetine hydrochloride	N-nitroso-fluoxetine formation facilitated by the acid environment (HCl) in the presence of precursors
Excipients	Source of nitrite leading to N-nitroso-fluoxetine formation
	Function
Microcrystalline cellulose	Diluent
Calcium hydrogen phosphate dihydrate	Diluent
Crospovidone	Disintegrant
Magnesium stearate	Lubricant
Hypromellose	Film-forming agent
Diethyl phthalate	Film-forming agent
Formulation process	Direct compression at room temperature; low risk of N-nitrosamine formation in solid state and in absence of elevated temperature, energy, and moisture
Utilities	Stainless steel-based equipment unlikely to contain N-nitrosamines; no risk of N-nitrosamine formation
Cleaning and cross-contamination	Validated cleaning procedure; low risk of N-nitrosamine formation
Finished product manufacturing – packaging	
PVC/Alu blister	Low risk of N-nitrosamine formation associated with non-nitrocellulose-based lidding foil; however, PVC is permeable to oxygen, light, and moisture
Storage	
Room temperature ($\leq 25\text{ }^{\circ}\text{C}$), 3-year shelf-life	Evolving nitrosation of the secondary amine functional group leading to N-nitroso-fluoxetine formation in the presence of precursors

Table S9: Comparison and justifications for the selection of the analytical technique.

Comparison	Justification(s)
Reversed-phase vs. normal phase chromatography	Reversed-phase chromatography is more suitable for separating compounds in complex mixtures. Given that N-nitrosamines are polar to moderately polar compounds, reversed-phase chromatography is preferred over normal phase chromatography.
LC vs. GC	Not all N-nitrosamines are sufficiently volatile or thermally stable for GC analysis. LC therefore represents a more versatile approach, enabling the analysis of both volatile and non-volatile N-nitrosamines.
UHPLC vs. HPLC	UHPLC offers enhanced chromatographic efficiency, improved peak shape and resolution, reduced analysis time, increased sample throughput, and lower solvent consumption compared with HPLC.
ESI(+) vs. APCI(+)	ESI(+) provides greater signal intensity and signal-to-noise ratio for N-nitroso-fluoxetine compared with APCI(+), making it more suitable for trace-level analysis.
Tandem mass spectrometer vs. other detectors	Tandem mass spectrometry is considered the gold standard for trace-level quantification due to its high sensitivity and specificity.
RP-UHPLC-ESI(+)-MS/MS	The selected hyphenated technique is ideal for the intended purpose of the method. In addition, the system is compatible with AQbD-driven method development, enabling efficient automated DoE and systematic method optimization.

Chapter III: Results - Section 2

Table S10: S/N ratios obtained in the lowest concentration level of calibration and validation standards across 3 series.

Standard	Series 1				Series 2				Series 3			
	CAL 1	A1	B1	C1	CAL 1	A1	B1	C1	CAL 1	A1	B1	C1
N-nitroso-fluoxetine	19	32	25	26	23	25	25	26	32	28	37	33
NDEA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NDPA	18	23	18	20	16	29	33	23	22	29	14	23
NDBA	40	38	37	41	57	40	52	45	56	41	30	45
NTHP	26	17	27	15	18	26	17	26	16	27	14	16
NDMA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NMPA	78	75	73	56	115	76	48	44	94	38	96	140
NNK	449	343	415	522	350	320	378	347	330	403	291	328
NMOR	7	10	10	8	13	12	7	6	12	10	8	8
NEIPA	16	19	17	21	25	26	18	21	26	15	22	20
NPiP	18	17	20	24	16	22	13	13	7	9	11	12
NDiPA	8	12	17	12	12	16	9	9	13	12	12	15
NMBA	43	15	15	13	35	10	16	11	59	17	15	13
NPYR	20	19	21	17	14	14	15	11	8	8	9	8
NDELA	68	73	87	22	25	42	21	27	25	27	29	26
NDPhA	431	424	499	560	551	425	805	321	597	446	408	446

N-nitroso-fluoxetine concentration: 0.1 ng/mL; small-molecule N-nitrosamine concentration: 0.4 ng/mL. CAL: calibration standard; A, B, C: triplicate; ND: not detected.

NDEA was detectable from the concentration level 2: 2 ng/mL (> acceptable limit or 0.44 ng/mL); NDMA was detectable from the concentration level 3: 4 ng/mL (> acceptable limit or 1.60 ng/mL).

Table S11: Total analytical error index and validated range of all calibration models.

Calibration model	N-nitroso-fluoxetine ($p = 3$)	
	Total error index	Validated range (ng/mL)
Linear regression after logarithm transformation	0.87	0.09 – 4.75
Linear regression through 0, fitted using level 3 only	0.84	0.25 – 4.75
Weighted ($1/X^2$) linear regression	0.88	0.09 – 4.75
Weighted ($1/X^2$) quadratic regression	0.87	0.09 – 4.75
Weighted ($1/X$) quadratic regression	0.86	0.09 – 4.75
Weighted ($1/X$) linear Regression	0.83	0.15 – 4.75
Linear regression after square root transformation	0.81	0.37 – 4.75
Quadratic regression	0.76	0.37 – 4.75
Linear regression through 0, fitted using the highest level (level 5) only	0.66	0.55 – 4.75
Linear regression	0.00	0.46 – 4.75

p : number of series of validation. The total error index accounts for accuracy, precision, and range.

Table S12: S/N ratios obtained in the lowest concentration level of calibration (risk mitigation measures).

Standard	S/N ratio
N-nitroso-fluoxetine	43
NDEA	Not detectable
NDPA	21
NDBA	72
NTHP	25
NDMA	Not detectable
NMPA	127
NNK	402
NMOR	9
NEIPA	37
NPIP	21
NDIPA	24
NMBA	3
NPYR	15
NDELA	69
NDPhA	1055

N-nitroso-fluoxetine concentration: 0.1 ng/mL; small-molecule N-nitrosamine concentration: 0.4 ng/mL.

Chapter III: Results - Section 2

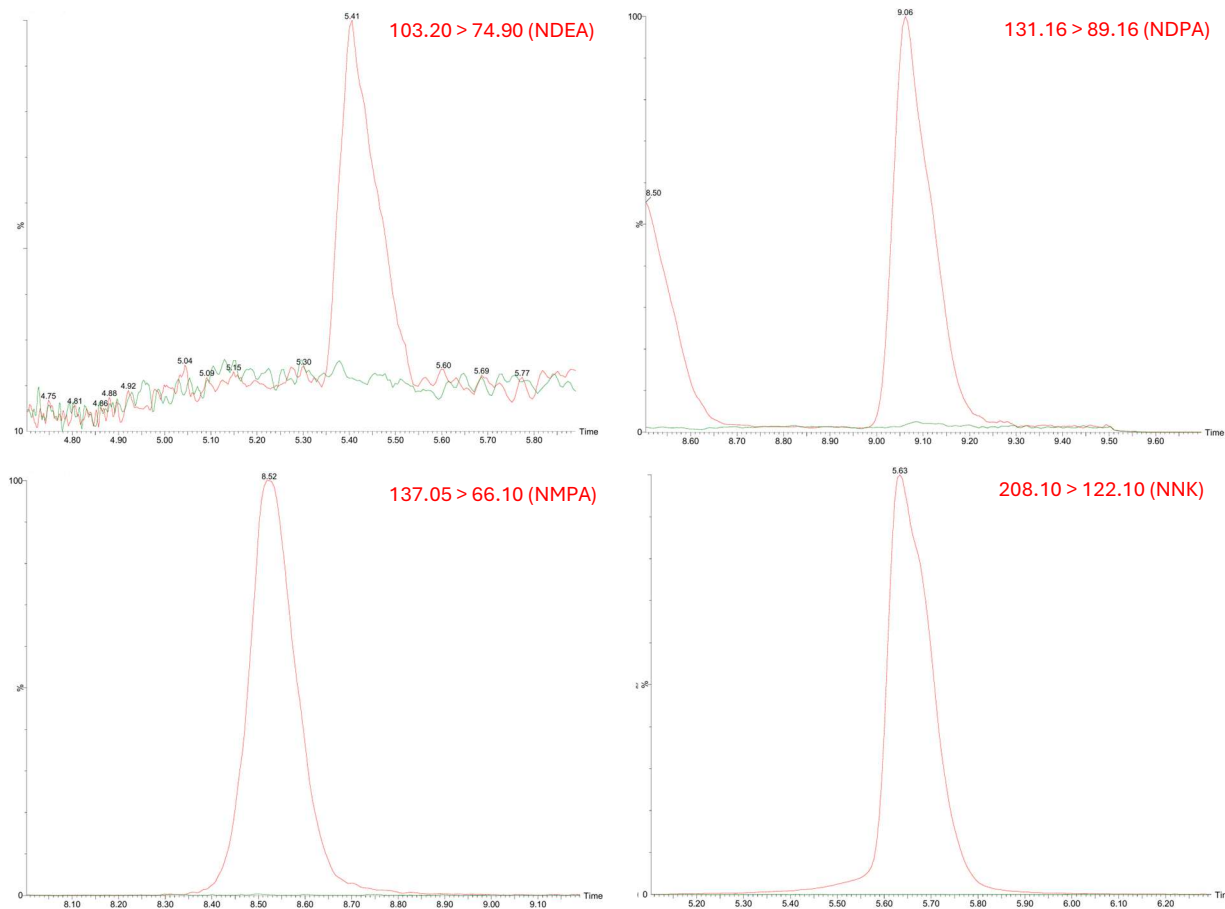


Figure S1. Specificity evaluation for small-molecule N-nitrosamine impurities. Green: blank matrix; Red: calibration standard level 3 (4 ng/mL).

Chapter III: Results - Section 2

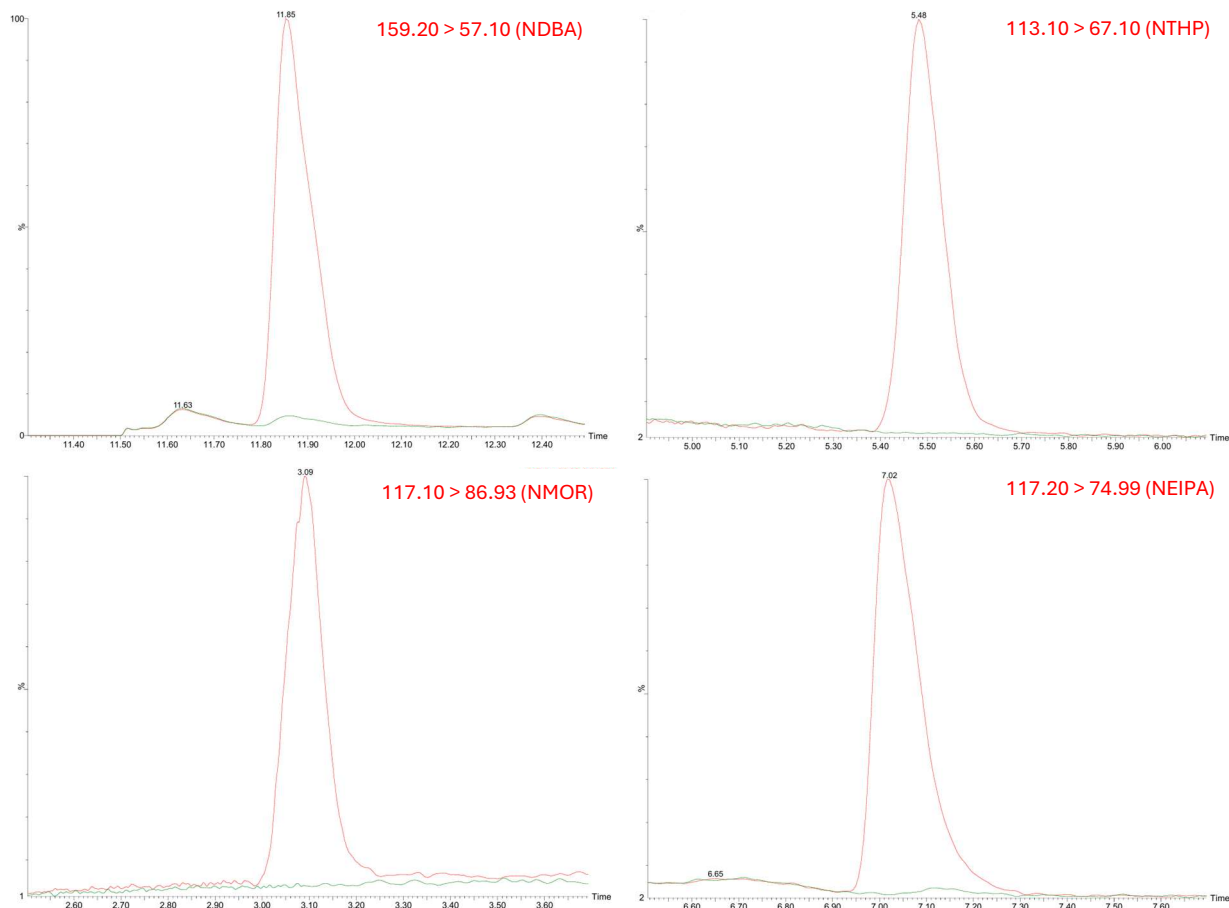


Figure S1. Specificity evaluation for small-molecule N-nitrosamine impurities. Green: blank matrix; Red: calibration standard level 3 (4 ng/mL).

Chapter III: Results - Section 2

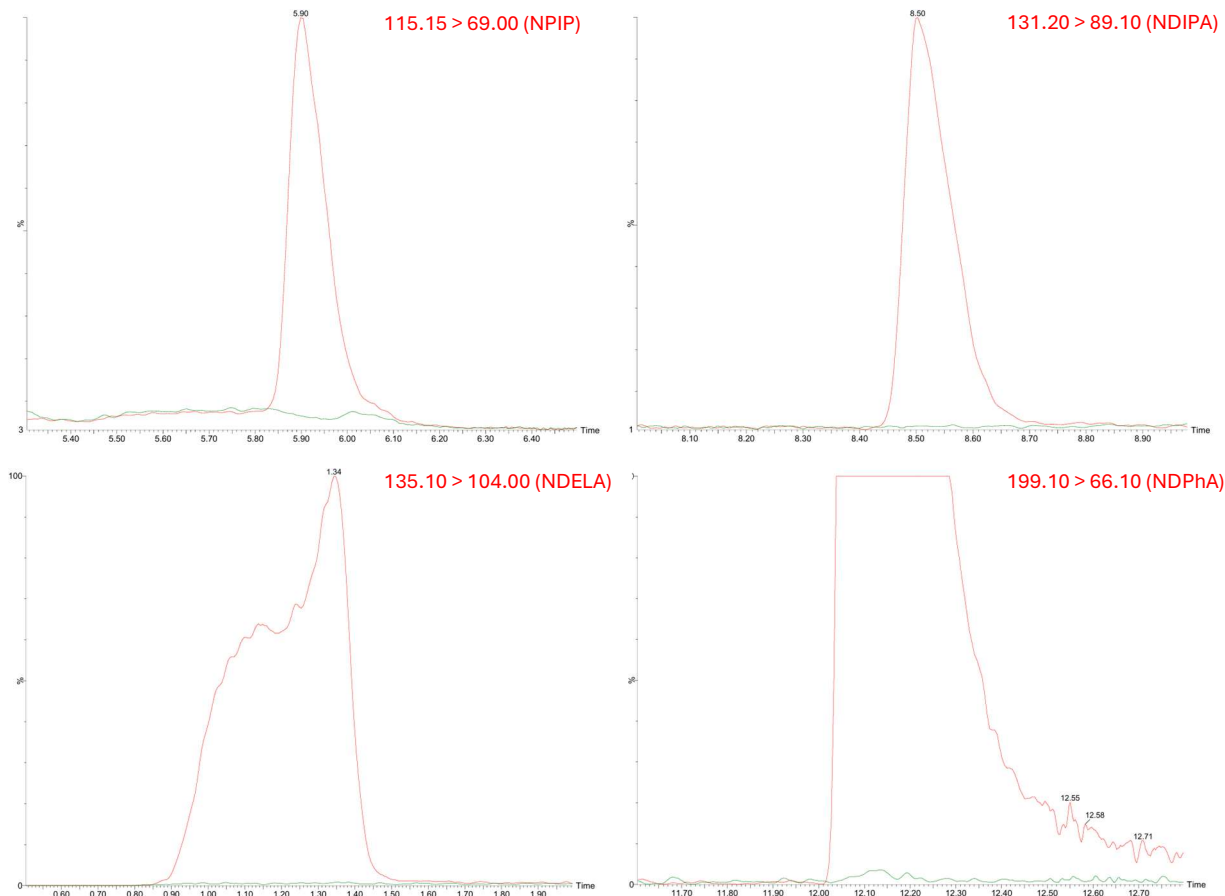


Figure S1. Specificity evaluation for small-molecule N-nitrosamine impurities. Green: blank matrix; Red: calibration standard level 3 (4 ng/mL).

Chapter III: Results - Section 2

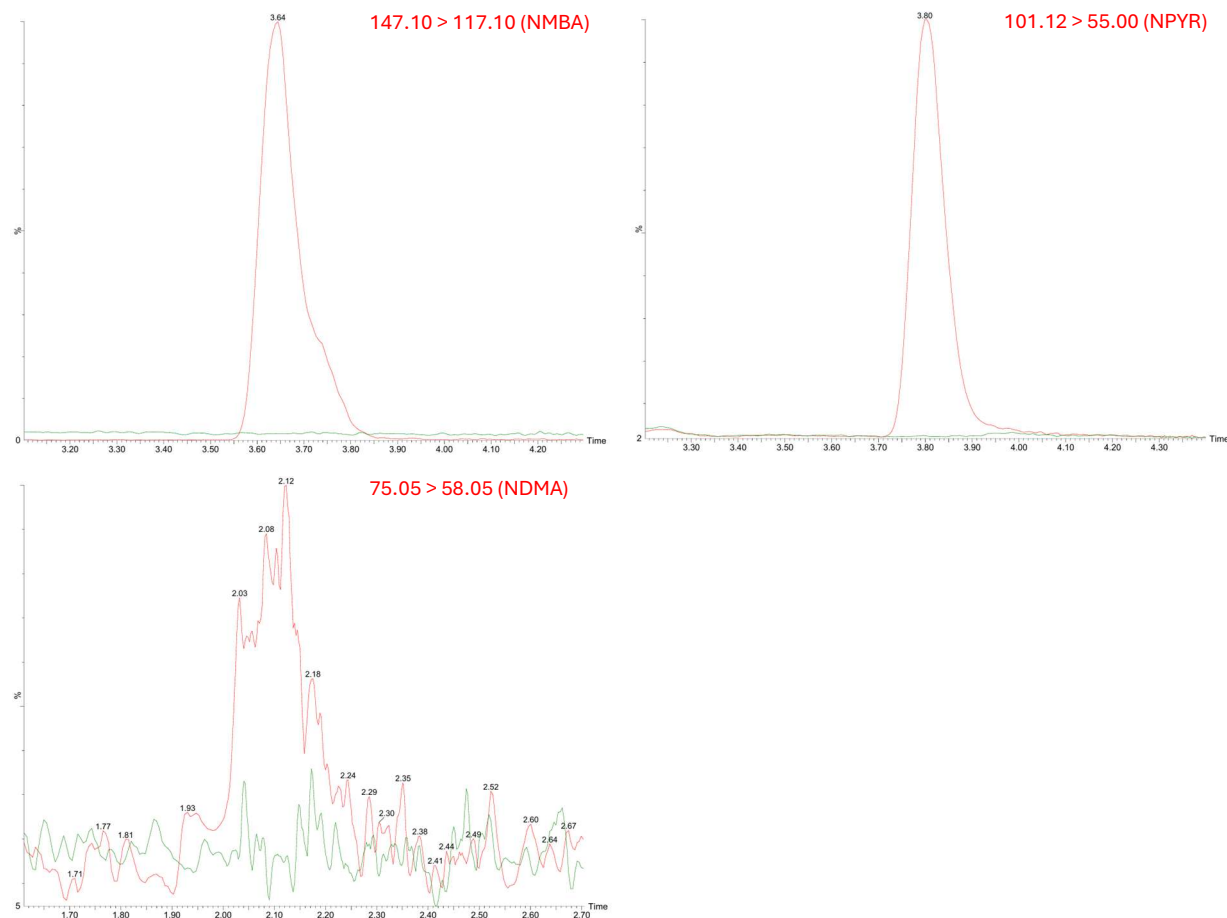


Figure S1. Specificity evaluation for small-molecule N-nitrosamine impurities. Green: blank matrix; Red: calibration standard level 3 (4 ng/mL).

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Chapter IV

General discussion

In the thesis, from the early stages of analytical development, all the LC-MS/MS methods were designed and developed to meet the regulatory requirements of the EMA guidance on N-nitrosamine investigation (“Call for Review”).

In addition, all developed methods underwent rigorous validation according to ICH Q2(R2) guideline. Depending on the analytical objectives, two approaches were adopted to support Step 2: confirmatory testing; and Step 3: implementation of risk mitigation measures of the “Call for Review”, i) a limit test to assess impurity levels, or ii) a quantitative method to determine impurity content.

Quantitative performance was assessed using a combined approach for total error (accuracy and precision) evaluation [1], and successfully demonstrated suitable specificity, sensitivity, range, accuracy, and precision. All the validated range complied with regulatory requirements. The limit tests were also validated by demonstrating, at a minimum, their specificity and suitability with respect to the target concentration levels.

1 Small-molecule N-nitrosamine impurity analysis

The Ph. Eur. updated the general chapter 2.5.42 in January 2026 and extended the application of the methods to medicinal products of solid dosage forms. In this chapter, the Ph. Eur. proposes three procedures for the control of seven small-molecule N-nitrosamine impurities associated with the sartan class of medicines. These procedures rely on three different analytical techniques: HPLC-MS/MS, GC-MS, and GC-MS/MS. All methods were validated as limit tests but only the HPLC-MS/MS and GC-MS/MS methods have been validated for quantitative determination [2]. In parallel, the USP published in monograph <1469> four quantitative methods using HPLC-HRMS, GC-MS, HPLC-MS/MS, and GC-MS/MS for the determination of small-molecule N-nitrosamine impurities in sartans [3].

It is noteworthy that N-nitroso-N-methyl-4-aminobutyric acid (NMBA) is analyzed exclusively using LC and not by GC-based techniques in both the Ph. Eur. and USP [2,3]. This is explained by the fact that NMBA is non-volatile and therefore not amenable to GC analysis [4]. A key advantage of LC is its suitability for a broad range of analytes regardless of their volatility, including NMBA.

Table 1 summarizes the LC methods published in the Ph. Eur. and USP and compares them with those proposed in this thesis. Among detection techniques, triple quadrupole detectors enable quantification at lower concentrations, thus providing higher sensitivity than high-resolution mass spectrometers. Regarding ionization techniques, APCI+ is commonly preferred for small-molecule N-nitrosamine analysis because lower detection limits are typically obtained compared with ESI+ mode.

The HPLC-MS/MS method described in the Ph. Eur. allows the simultaneous quantification of five small-molecule N-nitrosamines. In comparison, the USP HPLC-HRMS and HPLC-MS/MS methods enable the quantification of seven and six impurities, respectively, but at higher concentrations. Compared with these compendial methods, the UHPLC-MS/MS methods proposed in Chapter III, Sections 1(A) and 1(B), together, enable the detection of up to 17 N-nitrosamines. Although some of these methods were validated as limit tests rather than quantitative methods, they nevertheless achieve lower concentration levels than those reported for the compendial procedures. Another advantage of the proposed quantitative approach lies in the use of a single-point calibration strategy covering the entire validated range, in contrast to the multi-level (three- or five-point) calibration curves required by the Ph. Eur. and USP methods. This simplification is particularly advantageous for routine industrial applications, where high analytical throughput is often required. Moreover, the comparison presented in Table 1 also indicates that UHPLC methods generally have shorter analysis times than HPLC methods. Although the run time (17.0 min) of these methods is longer than that of the USP HPLC-MS/MS method (12.1 min), they offer improved separation efficiency while maintaining reasonable analysis times.

The development of these UHPLC-MS/MS methods was supported by an *in silico* QSRR-based approach in order to address matrix effects. This led to the development of three flexible analytical methods enabling the determination of 17 N-nitrosamines within the same analytical sequence with only minor modifications to method parameters. These adjustments include the pH of the mobile phase to resolve matrix effects arising from co-elution with the API, and the APCI probe temperature to prevent thermal degradation while improving sensitivity and lowering detection limits.

Chapter IV: General discussion

Table 1: Comparison of compendial HPLC methods and UHPLC methods proposed in this thesis for the determination of small-molecule N-nitrosamine impurities.

	Ph. Eur. [2]	USP [3]		Methods proposed in this thesis	
Purpose	Quantitative	Quantitative	Quantitative	Quantitative	3-level limit test*
Technique	HPLC-MS/MS	HPLC-HRMS	HPLC-MS/MS	UHPLC-MS/MS	UHPLC-MS/MS
Ionization	APCI+	ESI+	APCI+	APCI+	APCI+
Calibration curve	3 levels	1 level	5 levels	1 level	N/A
Run time	35.0 min	25.0 min	12.1 min	17.0 min	17.0 min
N-nitrosamine	Concentration or range (ng/mL)				
NDMA	0.9 – 3.6	6.0	1.33 – 90.00	N/A	1.54, 4.61, 15.36
NDEA	0.9 – 3.6	6.0	0.66 – 59.40	N/A	0.42, 1.27, 4.24
NMBA	0.9 – 3.6	6.0	1.33 – 90.00	0.25 – 30.00	1.54, 4.61, 15.36
NDPA	N/A	N/A	N/A	N/A	0.42, 1.27, 4.24
NDIPA	0.9 – 3.6	6.0	1.33 – 90.00	N/A	0.42, 1.27, 4.24
NEIPA	0.9 – 3.6	6.0	1.33 – 90.00	N/A	0.42, 1.27, 4.24
NDBA	N/A	6.0	1.33 – 90.00	N/A	0.42, 1.27, 4.24
NMPA	N/A	6.0	N/A	0.24 – 28.42	0.55, 1.65, 5.49
Comment				Range (ng/mL) NMOR: 1.01 – 30.67 NDPhA: 0.43 – 29.89 NNK: 0.44 – 30.00	3 flexible methods capable of analyzing 9 additional N- nitrosamine impurities within the same sequence

*To simplify the comparison, concentrations are expressed in ng/mL instead of ppm as described in Chapter III, Section 1(B), corresponding to 10%, 30%, and 100% of the acceptable limit.

Chapter IV: General discussion

Once validated in accordance with ICH Q2(R2) guideline, the three developed flexible methods were successfully applied within an uninterrupted sequence of analysis. The Step 2 investigation was conducted on a marketed prolonged-release tablet product in full regulatory compliance, including validated methods, sensitivity requirements, and the analysis of representative samples from production to end of shelf-life. The results confirmed consistent detection of one impurity whose concentration increased during storage but remained below the established safety threshold at the end of shelf-life, while other impurities remained below 10% of their respective acceptable limits, eliminating the need for further routine testing. As no impurity exceeded its respective acceptable limit, no risk-mitigation measures were required at Step 3. The N-nitrosamine investigation for the product was therefore concluded at this stage.

2 N-nitrosamine drug substance-related impurity analysis

API-related substances are compounds structurally related to the API and may be formed during chemical synthesis as by-products or during storage as degradation impurities [5]. For example, 1-methyl-4-nitrosopiperazine (MeNP) was initially classified as a small-molecule N-nitrosamine due to its molecular mass of 129.16 Da [6]. However, it was later identified as an NDSRI associated with rifampin because it originates from the nitrosation of a rifampin-related impurity (Chapter 1, Section 1) [7]. For this reason, MeNP was not included in the work presented in Chapter III, Section 2.

As currently understood and also confirmed by the work presented in this part of the thesis, the primary factor responsible for the formation of NDSRIs is the presence of nitrite originating from pharmaceutical excipients. Therefore, improved control of nitrite levels in excipients should be implemented by suppliers to limit the formation of NDSRIs during drug product manufacturing and storage. In this context, the use of low-nitrite excipients, which are already available on the market, should be considered as a potential mitigation strategy.

Fluoxetine® Divule® 20 mg contains 20 mg of fluoxetine base per capsule was used as case study in this thesis. Fluoxetine is a selective serotonin reuptake inhibitor widely prescribed for the treatment of major depressive episodes [8]. This product was withdrawn from the Belgian market after its corresponding NDSRI, N-nitroso-fluoxetine, was found at levels exceeding the acceptable intake limit of 100 ng/day [9,10], considering a maximum daily dose of three capsules [11].

In contrast to small-molecule N-nitrosamines, no compendial analytical methods for the determination of NDSRIs have yet been published in either the Ph. Eur. or the USP. Nevertheless, several analytical methods for NDSRI determination have been reported by OMCLs. While small-molecule N-nitrosamines generally exhibit higher signal intensities when analyzed in APCI+ mode, NDSRIs are typically larger molecules and tend to produce stronger signals in ESI+ mode. For this reason, N-nitroso-fluoxetine was analyzed in ESI+ mode in Chapter III, Section 2, although this required accepting a compromise in sensitivity for some small-molecule N-nitrosamines.

The method developed in this part of thesis demonstrated a detection limit tenfold lower than that of the method published by the Taiwan FDA for the quantification of N-nitroso-fluoxetine. Moreover, the proposed method presents another significant advantage, as it additionally enables the simultaneous detection of 13 small-molecule N-nitrosamine impurities at amounts below their respective acceptable limits within the same analytical run.

Thus, the method successfully contributed to Step 2 of “Call for Review”, enabling the detection of N-nitroso-fluoxetine at approximately 30 ppm, corresponding to about 18 times its acceptable intake limit, in Fluoxone® Divule® 20 mg. The method was subsequently used to effectively monitor the implementation of risk mitigation measures.

At the same time, the results obtained in this thesis demonstrate that N-nitrosamine impurity levels may evolve during product storage. Environmental conditions may contribute to the continuous formation of N-nitrosamines over time. Therefore, controlling environmental factors may help slow the conversion of APIs into their corresponding N-nitroso derivatives. In this context, optimization of primary packaging systems may also contribute to improving product safety.

Not all medicines are exposed to N-nitrosamine risks to the same extent, the risks associated with chemically synthesized APIs are generally greater than those associated with biological medicines. Biological therapeutics are derived from biological sources and are produced through cell culture processes, where the introduction of chemical reagents is limited. These medicines include hormones, vaccines, monoclonal antibodies, and products derived from human blood or plasma [12]. As a result, the risk of N-nitrosamine formation in such products is significantly reduced.

Another factor relates to the route of administration and formulation characteristics. Biotherapeutics are most often administered via intravenous, intramuscular, or other parenteral routes as sterile dosage forms. These formulations typically use higher-purity excipients compared with oral dosage forms, which further reduces the risk of N-nitrosamine formation.

In contrast, solid oral dosage forms are more frequently associated with N-nitrosamine risks. Excipients used in these formulations may contain residual nitrite, which can lead to the formation of NDSRIs when APIs or related impurities contain reactive secondary amine functional groups. The USP has therefore stated in general chapter <2> that N-nitrosamine impurities should be systematically considered as part of the general testing strategy for oral drug products when a potential risk is identified [13]. Additionally, in chapters <4> and <5>, N-nitrosamines are included in impurity considerations for mucosal drug products as well as inhalation and nasal products [14,15].

Packaging materials may also influence N-nitrosamine formation. Sterile formulations are typically packaged in glass containers that provide strong barrier properties and are filled in classified environments, which limits contamination risks. However, elastomers used as vial stoppers have been associated with N-nitrosamine risks in injectable pharmaceutical products [16]. The oral solid dosage forms

Chapter IV: General discussion

investigated in this thesis were packaged in blister systems. Conventional PVC/Alu blister packaging has relatively poor barrier properties due to its permeability to gases, moisture, and light, which may contribute to product alteration during storage. Therefore, the presence of N-nitrosamines with potential safety concerns should also be evaluated in packaging systems [17].

3 Advantages of combining QSRR and AQbD approaches

Based on the above, it is evident that multiple pathways can lead to the formation of N-nitrosamine impurities in pharmaceutical products. Consequently, effective strategies for the development of analytical methods able of detecting these impurities have become essential. As illustrated in this thesis, such a strategy could be based on an AQbD approach, potentially combined with or complemented by *in silico* QSRR models.

To date, numerous new drug applications for medicines developed using a QbD approach have been approved by regulatory authorities such as the FDA and the EMA. These regulatory agencies increasingly encourage the adoption of QbD strategies because they provide greater regulatory flexibility and improved process understanding. Since the early 2010s, the increasing use of QbD-based approaches can largely be attributed to regulatory initiatives promoting science- and risk-based development strategies [18].

Review reports of new drug applications have shown that deficiencies related to drug product control are among the most frequently observed issues in the Common Technical Document. In some cases, regulatory withdrawals have been linked to inappropriate analytical techniques used for impurity testing or to missing validation studies [19]. These observations highlight the critical importance of reliable analytical method development and validation. By adopting a science- and risk-based framework such as AQbD, the likelihood of successful analytical method development can be significantly improved. Furthermore, the integration of well-designed experimental strategies contributes to the development of more robust analytical procedures.

Reliable analytical methods generate reliable results by minimizing uncontrolled variability and reducing the probability of incorrect analytical outcomes. On the one hand, inaccurate results may lead to unnecessary batch rejection, or product recalls due to false positive out-of-specification or out-of-trend results caused by unexpected method failures, representing a significant financial risk for manufacturers. On the other hand, and more critically, incorrect results may result in the release of non-compliant or potentially unsafe products that fail to meet specification requirements, thereby posing a potential risk to patients and public health.

The AQbD approach applied in Chapter III, Section 2, for N-nitrosamine determination is particularly valuable for small-molecule N-nitrosamines. Because these impurities may occur across a wide range of pharmaceutical products, a broad method operable design region (MODR) facilitates the identification of suitable operating conditions for future applications involving new matrices and products. This

flexibility can help mitigate matrix effects by allowing experimental conditions to be adjusted when matrix components interfere with analyte determination.

In addition, the value of the *in silico* QSRR approach lies in its ability to anticipate potential co-elution between analytes and matrix components solely from structural information (molecular descriptors). This computational tool supports the early selection of chromatographic conditions, such as pH and gradient time, that may reduce matrix interferences. Combined with the AQbD framework, this knowledge facilitates navigation within the modeled design space through desirability functions, enabling the identification of operating conditions that achieve the desired analytical performance for new small-molecule N-nitrosamines, NDSRIs, and pharmaceutical products using only molecular descriptors.

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Chapter V

Conclusion

The EMA has recommended the use of mass spectrometric detection to meet the sensitivity requirements associated with the investigation of N-nitrosamine impurities in medicines. Although gas chromatography-based methods are documented in the literature and pharmacopoeias for the determination of these impurities, this thesis adopts liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) because not all N-nitrosamines are volatile. This choice enables the analysis of all N-nitrosamines (small-molecule and NDSRIs) using a single analytical technique.

Consequently, this thesis focuses on the development and validation of LC-MS/MS methods and the demonstration of their compliance with regulatory requirements, thereby supporting Steps 2 (confirmatory testing for N-nitrosamines) and 3 (implementation and monitoring of risk mitigation measures) of the EMA investigation guidance, known as the “Call for Review”. Indeed, analytical testing plays a central role in these steps by controlling pharmaceutical product quality throughout its lifecycle to ensure the safety, efficacy, and quality of medicines for patients.

The following sections revisit the two main scientific questions defined in the objectives and provides concluding answers based on the investigations conducted in this thesis.

1) Can reliable LC-MS/MS methods be developed for the determination of small-molecule N-nitrosamine impurities in the selected pharmaceutical product while ensuring reliable analytical performance?

The case study presented in Chapter III, Sections 1(A) and 1(B) demonstrated that LC-MS/MS is an appropriate analytical technique for the determination of small-molecule N-nitrosamines using a single analytical platform. The developed methods exhibited suitable performance through rigorous validation studies performed according to the ICH Q2(R2) guideline. The methods achieved detection limits in the pg/mL or ppb range, enabling the reliable determination of trace N-nitrosamine impurities in the selected pharmaceutical product.

a) Are the developed methods able to manage matrix effects while maintaining suitable quantitative analytical performance?

Chapter III, Section 1(A), also describes the application of an innovative QSRR approach as an *in silico* tool to facilitate analytical method development, particularly to address co-elution between certain N-nitrosamines and the API. Having chosen a rather simple sample preparation protocol to minimize sources of error, the API is typically present at concentrations in the mg/mL range, whereas N-nitrosamines are present at ng/mL levels. This large concentration disparity can lead to matrix effects, *i.e.*, alterations in analyte ionization efficiency caused by co-eluting matrix components, which are frequently encountered in MS. The QSRR-assisted strategy successfully demonstrated its applicability for the analysis of small-molecule N-nitrosamine impurities in a marketed drug product by preventing N-nitrosamine-API co-elution and thereby mitigating matrix effects.

Matrix effects were effectively managed through optimization of chromatographic conditions. In particular, modification of the aqueous mobile phase pH influenced the retention behavior of the API. At pH 2.7, co-elution between the API and several N-nitrosamines resulted in matrix effects characterized by ion suppression. Increasing the mobile phase pH to 5.0 enhanced the retention of the API on the chromatographic column, delaying its elution and thereby eliminating the co-elution responsible for the observed matrix effects.

Once matrix effects were controlled, analytical sensitivity was further optimized. Most N-nitrosamines exhibited higher signal-to-noise ratios at pH 2.7 compared with pH 5.0. Additionally, the APCI probe temperature significantly influenced analyte signal intensities. Consequently, two APCI probe temperatures (150 °C and 250 °C) were employed to maximize sensitivity, with lower temperatures being more suitable for thermolabile compounds. The analytical development resulted in three flexible LC-MS/MS methods that addressed the encountered challenges. Matrix effects were mitigated by avoiding co-elution between N-nitrosamines and the API through appropriate pH selection, while overall sensitivity was further optimized by adjusting the APCI probe temperature.

A key advantage of these methods is their ability to determine 17 N-nitrosamines using a single analytical technique within the same analysis sequence, without requiring modifications to the instrument configuration. To demonstrate compliance with EMA requirements, one of the developed methods successfully underwent a comprehensive validation study in accordance with ICH Q2(R2) guideline, using a combined validation approach for total error (accuracy and precision) evaluation. This method demonstrated suitable quantitative performance in terms of accuracy and precision within the validated concentration range.

b) Do such validated analytical methods meet current regulatory requirements for the investigation of N-nitrosamines and for supporting risk-mitigation decisions?

The validated methods demonstrated adequate analytical performance and successfully supported the N-nitrosamine investigation workflow, including Steps 2 and 3 of the “Call for Review” regulatory guidance.

During confirmatory testing, most N-nitrosamines were not detected above 10% of their respective acceptable intake limits, confirming the absence of a significant risk and eliminating the need for further control of these impurities. However, the analytical testing consistently revealed the presence of one N-nitrosamine impurity across all analyzed samples. The amounts of this impurity increased over time, suggesting that it originates from product degradation during storage. Despite this progressive formation, its content remained below the regulatory safety threshold at the end of the product shelf life.

These findings demonstrate that the product remains safe and compliant with quality requirements, therefore, no immediate risk-mitigation measures were required. Nevertheless, routine quantitative monitoring of the detected impurity is necessary to ensure continued product quality and patient safety. The strategy presented in this work, applied to a marketed drug product, enabled full regulatory compliance with the EMA “Call for Review” procedure.

2) Can the Analytical Quality by Design (AQbD) approach serve as an appropriate strategy for developing reliable analytical methods to support risk-mitigation decisions for both N-nitrosamine drug substance-related impurities (NDSRIs) and small-molecule N-nitrosamine impurities?

The AQbD approach was successfully applied to guide analytical method development by integrating the scientific knowledge and experience gained from the earlier investigations presented in this thesis. The incorporation of prior knowledge facilitated a systematic risk assessment, allowing the identification of critical method parameters requiring evaluation and enabling a better understanding of their influence on analytical responses.

In addition, the AQbD framework incorporates design of experiments (DoE), which allows the investigation of potential interactions between method parameters through a limited number of well-designed experiments. The combination of risk analysis, prior knowledge, and DoE enabled relatively straightforward development of a specific analytical method for the simultaneous quantification of N-nitroso-fluoxetine for post-marketing surveillance and the detection of 13 small-molecule N-nitrosamine impurities below their respective acceptable limits. The approach used in this case study integrated prior knowledge to enhance method understanding and support more effective control of analytical performance.

The resulting method was also successfully validated according to the ICH Q2(R2) guideline and demonstrated suitable analytical performance. Importantly, this method effectively supported the implementation and monitoring of risk-mitigation strategies. Major changes implemented by the manufacturer included the redesign of the formulation and packaging system. The formulation was updated through excipient modifications to reduce nitrite content and thereby limit the formation of N-nitroso-fluoxetine in the presence of precursors. In parallel, the packaging was improved to enhance barrier properties and protect the product against environmental conditions during long-term storage at 25°C / 60% RH. The implementation of all mitigation measures, coupled with a reliable method for determining N-nitroso-fluoxetine, helped restore the quality and safety of Fluoxone® Divule® 20 mg, ultimately allowing its reintroduction to the Belgian market.

Chapter VI

Perspectives

Chapter VI: Perspectives

The present thesis presents the development and rigorous validation of LC-MS/MS methods to detect and control N-nitrosamine impurities in pharmaceuticals. All the work supports EMA “Call for Review” confirmatory testing (Step 2) and risk mitigation (Step 3), directly addressing the global industry crisis.

Chapter III, Sections 1(A) and 1(B) demonstrate the value of an innovative QSRR-assisted strategy for analytical development. This approach enabled the anticipation and mitigation of matrix effects, which can significantly compromise analytical method performance and should therefore be investigated at early stages of development. This proof of concept now allows us to envisage the use of the proposed QSRR strategy to other APIs in both drug substance and drug product forms in order to anticipate and mitigate matrix effects. Furthermore, extending the workflow to the emerging class of NDSRIs represents a possible direction for future research.

In addition, as the method for the determination of both N-nitroso-fluoxetine and small-molecule impurities was validated at a single working point (Chapter III, Section 2), further work could focus on method operable design region (MODR) validation after its verification. In this context, the innovative bracketing strategy recently proposed by the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) could represent a particularly appropriate approach [1].

Another observation made in Chapter III, Section 2 concerns the ionization behavior of different classes of N-nitrosamines. Small-molecule N-nitrosamines exhibited high signal intensities and improved S/N ratios in APCI+, whereas larger N-nitrosamines (typically NDSRIs) showed higher sensitivity when analyzed using ESI+. To benefit from the advantages of both ionization modes, the instrument used in this work is equipped with a combined ion source, enabling the possible use of the ESCI+ ionization. Future studies could therefore investigate the performance of ESCI+ for the simultaneous analysis of N-nitrosamines covering a wide range of molecular masses.

Building on the knowledge gained throughout the thesis, novel LC-MS/MS methods could be developed by integrating this knowledge within an AQbD framework. This is particularly relevant for the evaluation of other NDSRIs, for which only a limited number of methods are currently available. In parallel, the determination of small-molecule N-nitrosamines could benefit from the implementation of platform analytical methods. Such methods offer a major advantage because they can be applied to multiple pharmaceutical products while allowing minor modifications of method parameters within regulatory tolerance. An approach fully aligned with AQbD strategy and the determination of the MODR. Consequently, such dedicated platform method could be implemented for specific NDSRIs when risks are identified for a given API formulated in different dosage forms or strengths. Ultimately, an extend of

this approach allowing simultaneously addressing both small-molecule N-nitrosamines and NDSRIs may represent an attractive future perspective.

As a more ambitious perspective, the platform strategy could be further extended by integrating QSRR models within the AQbD framework, in combination with MODR. In this context, molecular descriptors (derived from QSRR approaches) of newly identified N-nitrosamines or previously uninvestigated APIs could be incorporated to predict potential co-elution risks and identify suitable operating conditions within the modeled design space. Such a strategy may support the progressive refinement of the MODR, facilitate the selection of new working points, and reduce experimental effort during future method development through a combination of *in silico* predictions and targeted experiments.

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