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Review Article

Analytical Method Validation Framework for Nitrosamine Impurity Qualification at Trace Levels: A Review

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ABSTRACT

In 2018, the discovery of N-nitrosamine impurities in valsartan uncovered a significant gap between standard pharmaceutical impurity testing and the sensitivity required to fully control potential human carcinogens at nanogram-per-day exposure levels. Since then, regulators and manufacturers have come together on a risk-based framework that connects the identification of sources and their mitigation to highly sensitive and strictly validated analytical methods. This review brings together the current state of analytical method validation for qualifying nitrosamine impurities, drawing from regulatory guidance from the U. S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the International Council for Harmonisation (ICH), in addition to recently published analytical strategies and case studies. It explores the development of acceptable intake (AI) limits under ICH M7(R2) and the Carcinogenic Potency Categorisation Approach (CPCA), compares the performance of liquid chromatography-tandem mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS/MS), and high-resolution mass spectrometry (LC-HRMS) platforms, and outlines sample preparation techniques used to extract trace analytes from complex drug matrices. The review also covers the core validation parameters outlined under ICH Q2(R2) and USP , including specificity, linearity, accuracy, precision, limit of quantitation, and robustness, along with the acceptance criteria typically applied at sub-part-per-billion levels. Ongoing challenges, such as matrix-induced ion suppression, the lack of isotopically labeled internal standards for newly identified nitrosamine drug substance-related impurities (NDSRIs), and the incomplete harmonization of regulations across different regions, are discussed along with emerging solutions like HILIC-based solid-phase extraction and automated online extraction. The review concludes that a sustainable validation framework must remain adaptable, as the list of regulated nitrosamines and their corresponding AI limits continue to grow.

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INTRODUCTION

N-nitrosamines are a class of compounds carrying an N-N=O functional group that are classified by international regulators as probable or possible human carcinogens on the basis of rodent bioassay and mutagenicity data. Their appearance in widely prescribed medicines-first in angiotensin II receptor blockers (the "sartans"), and subsequently in metformin, ranitidine, rifampicin, and a growing list of other drug substances-has driven one of the most consequential impurity-control episodes in modern pharmaceutical quality assurance [1,2]. Because nitrosamines are mutagenic at very low exposure, regulators place them in the ICH M7(R2) "cohort of concern," which requires that daily exposure be controlled to levels associated with a theoretical lifetime cancer risk of roughly one additional case per 100,000 people, rather than the more permissive thresholds applied to non-mutagenic impurities [3,4].

These exposure limits, expressed as acceptable intake (AI) values, are frequently in the tens-to-hundreds of nanograms per day, orders of magnitude below the microgram-level impurity thresholds that conventional HPLC-UV or GC-FID methods are equipped to resolve [2,4]. Meeting such limits within a complex drug product matrix, alongside the parent API and excipients, is as much an analytical chemistry challenge as a toxicological one. The initial failure to detect N-nitrosodimethylamine (NDMA) in valsartan is frequently cited as an illustration of how inadequate method sensitivity, rather than an undetected chemical hazard, can allow a genotoxic impurity to reach patients undetected.[2]

This review consolidates recent literature and regulatory guidance on the analytical method validation framework used to qualify nitrosamine impurities at trace levels. It is organized around four themes: (i) the regulatory and risk-assessment context that defines what "acceptable" detection and quantitation mean for a given nitrosamine-drug product combination; (ii) the analytical platforms capable of the required sensitivity and selectivity; (iii) the sample preparation strategies needed to isolate nanogram-level analytes from milligram-to-gram quantities of API and excipient; and (iv) the specific validation parameters, acceptance criteria, and documented case studies that define current best practice.

Regulatory and Risk-Assessment Framework

1. ICH M7(R2) and the Cohort of Concern

ICH M7(R2), the harmonized guideline on assessment and control of DNA-reactive (mutagenic) impurities, designates nitrosamines as members of a "cohort of concern" alongside aflatoxin-like and alkyl-azoxy compounds, reflecting their disproportionately high mutagenic potency relative to the generic threshold of toxicological concern used for most unstudied impurities [4]. Rather than applying the default 1.5 microgram-per-day threshold used for most mutagenic impurities, regulators derive compound-specific AI limits for individual nitrosamines using available carcinogenicity data, read-across from structurally related compounds, or the newer Carcinogenic Potency Categorisation Approach (CPCA), introduced in 2023 to place a given nitrosamine into one of five potency categories, each carrying its own AI limit ranging from about 18 to 1500 nanograms per day [5].

2. FDA, EMA, and Regional Guidance

Because ICH M7(R2) originally offered limited nitrosamine-specific direction, individual regulatory members developed their own supplementary guidance: the FDA's "Control of Nitrosamine Impurities in Human Drugs" (most recently revised in September 2024), the EMA's

series of nitrosamine review procedures beginning with the 2018 valsartan referral, and equivalent guidance from Health Canada [5]. The FDA guidance recommends an interim AI limit of 26.5 nanograms per day for several small-molecule nitrosamines and directs sponsors of fixed-dose combination products to demonstrate, in aggregate, that total nitrosamine exposure remains below this limit even when more than one API is nitrosamines formed from the API's own amine structure rather than a generic small molecule-the FDA separately maintains a resource of CPCA-derived AI limits and its 2023 NDSRI-specific guidance [6].

A recurring theme across FDA and EMA materials is that nitrosamine control is risk-based rather than universally mandated: manufacturers are expected to conduct a structured risk assessment covering API structure, synthetic route, reagents, recovered solvents, excipients, and storage/degradation pathways, and to test only where that assessment indicates a credible risk of nitrosamine formation [7]. Where testing is warranted, however, the expectation is unambiguous: analytical methods must be validated with sensitivity commensurate with the AI limit and the product's maximum daily dose [7,8].

3.Phase-Appropriate and Lifecycle Validation Expectations

Recent reviews emphasize that nitrosamine method validation should be phase-appropriate: a screening or confirmatory method used early in a risk assessment may justify a less exhaustive validation package than the routine release/stability method used for a marketed product [1]. As new nitrosamines are identified, as AI limits are revised (the FDA's Revision 2 guidance itself updated limits previously set in 2021), and as CPCA categorizations evolve, validated methods may need requalification or re-validation, making the analytical framework inherently a lifecycle exercise rather than a one-time qualification event [6,9].

Table no. 1: Summary of Key Regulatory Reference Points

Reference	Scope	Key Provision
ICH M7(R2)	Mutagenic impurity assessment/control	Defines cohort of concern; AI derivation methodology; CPCA (2023) potency categories
ICH Q2(R2)	Analytical procedure validation	Validation parameters: specificity, linearity, accuracy, precision, LOD/LOQ, robustness
USP <1469>	Nitrosamine impurities in drug products	Method-specific acceptance criteria for nitrosamine assays
FDA Guidance (Rev. 2, 2024)	Nitrosamine control in human drugs	Interim AI limits (e.g., 26.5 ng/day); combination-product aggregation; links to RAIL/NDSRI guidance
EMA Nitrosamine Referrals	Marketing authorization review	Product-specific risk evaluations following the 2018 valsartan referral

Analytical Technologies for Trace-Level Detection

Traditional pharmacopeial techniques such as HPLC-UV and standard GC are generally unable to reach the sub-part-per-billion detection limits that nitrosamine AI values demand, a limitation widely cited as a contributing factor in the initial escape of NDMA from routine valsartan testing [2]. Consequently, the analytical literature has converged on hyphenated mass spectrometric techniques as the practical standard.

1. LC-MS/MS

Triple-quadrupole LC-MS/MS operating in multiple reaction monitoring mode is described across multiple reviews as the reference technique for trace nitrosamine quantitation because its dual mass selection step provides the specificity and sensitivity needed to distinguish nitrosamines from co-eluting matrix components [10]. Reported detection limits in the literature extend down to the low picogram-per-milliliter range for some synthetic and biologic drug products [11]. Representative validated LC-MS/MS methods include a seven-analyte method for mexiletine formulations using a C18 column with formic-acid-modified aqueous/methanol gradients [10], and an eight-analyte UPLC-TQ-MS/MS method for losartan-hydrochlorothiazide fixed-dose tablets using a diphenyl-phase column [12].

2. GC-MS/MS and Derivatization Approaches

For more volatile, low-molecular-weight nitrosamines (e.g., NDMA, N-nitrosodiethylamine), GC-MS/MS remains widely used, sometimes paired with derivatization reagents such as pentafluorobenzyl bromide to improve chromatographic behavior and detection [13]. Related derivatization chemistry is also applied to precursor amines themselves-for example, pre-column derivatization of dimethylamine and diethylamine for HPLC-fluorescence detection, since these small polar amines are poorly retained on reversed-phase columns and weakly UV-absorbing [14]. Monitoring precursor amine levels, in addition to the nitrosamines themselves, supports root-cause investigations when nitrosamine formation is suspected to occur post-manufacture.

3. LC-HRMS

High-resolution mass spectrometry (typically quadrupole time-of-flight or Orbitrap platforms) has gained adoption where multiple nitrosamines, including newly identified NDSRIs without dedicated MRM methods, must be screened simultaneously. A HILIC-based solid-phase extraction coupled to LC-HRMS method developed for a broad panel of regulated nitrosamines was demonstrated across 26 marketed drug products and reported the ability to quantify 15 nitrosamines simultaneously, with the extraction protocol adaptable to newly emerging NDSRIs by adjusting the elution program [15]. The non-targeted acquisition mode of HRMS is particularly valuable for retrospective screening, since data can be reprocessed for newly identified nitrosamines without re-injecting samples [4,15].

Table no. 2: Comparative Considerations

Technique	Typical Sensitivity	Strengths	Limitations
HPLC-UV/GC-FID	µg/mL range	Simple, widely available	Insufficient sensitivity/ selectivity for AI-level limits
GC-MS/MS	Low ng/mL, sub-ppb achievable	Well suited to volatile nitrosamines	Volatility/ derivatization needs; limited for polar/ high-MW NDSRIs
LC-MS/MS (triple quad)	pg/mL–ng/mL	Gold-standard specificity/ sensitivity; routine QC compatible	Requires matrix-matched calibration; matrix effects need active mitigation
LC-HRMS	pg/mL–ng/mL	Multi-analyte/ non-targeted capability; retrospective data mining	Higher capital cost; specialized expertise

Sample Preparation Strategies

Sample preparation is consistently identified as the step most likely to determine whether a

nitrosamine method achieves its required sensitivity, since it governs how effectively trace analytes are isolated from a matrix that may outweigh them by a factor of 10^6-10^9 [1]. Co-extracted excipients and API can suppress or enhance electrospray ionization efficiency, producing biased quantitation if not controlled, and certain excipients (e.g., povidone, crospovidone, croscarmellose sodium, starches, lactose) are themselves recognized sources of nitrite or amine precursors that complicate both the chemistry and the analysis [2,13].

1. Extraction Approaches

Common extraction strategies include liquid-liquid extraction (LLE), solid-phase extraction (SPE), and QuEChERS-style dispersive extraction, each offering a different balance of cleanup efficiency, throughput, and solvent consumption [13]. A methanol-based direct extraction approach, consistent with the FDA's LC-HRMS methodology, has been widely adopted as a starting point for drug substance and drug product samples, though several groups have found that this generic protocol requires product-specific optimization to control matrix effects [16].

2. HILIC-Based SPE for Multi-Analyte Panels

A notable methodological advance combines hydrophilic interaction chromatography (HILIC) principles with solid-phase extraction to selectively retain the API and polar excipients while allowing nitrosamines-generally less polar under HILIC conditions-to pass through in the load/wash fractions. This approach was designed to address the fact that most previously published methods were optimized for a single API, whereas pharmaceutical portfolios require a cleanup strategy flexible enough to accommodate structurally diverse drug substances without redeveloping the extraction from scratch for every product.

3. Matrix-Effect Mitigation

Even after extraction, residual co-eluting material can materially bias LC-MS/MS accuracy. A published case study on rifampin and rifapentine drug products found that a sample preparation protocol modeled on the FDA's generic methanol-extraction LC-HRMS method, while adequate for the original application, introduced significant matrix effects when applied to these specific matrices, and required product-specific re-optimization before acceptable validation data could be generated [16]. This illustrates a broader theme in the literature: matrix effects are not adequately addressed by simply adopting a generic or previously published protocol without confirming its performance for the specific drug product under test [7,13].

Where no isotopically labeled (deuterated) internal standard is commercially available for a given nitrosamine-a common situation for newly identified NDSRIs-matrix effect compensation becomes substantially harder, sometimes requiring matrix-matched calibration, standard addition, or multi-dimensional chromatography to achieve adequate accuracy at sub-nanogram-per-milliliter concentrations [18]. Complex excipients that swell or gel in aqueous media (e.g., croscarmellose sodium) can also physically obstruct conventional offline SPE, motivating a shift toward online SPE and multi-dimensional chromatography for particularly difficult formulations [17].

4. Automation and Emerging Preparation Formats

To improve reproducibility and reduce solvent use, recent methodology reviews describe increasing use of robotic SPE platforms,

microextraction by packed sorbent (MEPS), and headspace solid-phase microextraction (SPME) for volatile nitrosamines, alongside direct coupling of automated extraction to LC-MS/MS or GC-HRMS instrumentation [13]. These approaches are presented as complementary to, rather than a replacement for, careful product-specific method development, since automation improves consistency but does not by itself resolve matrix-specific ionization or recovery issues [13].

Method Validation Framework

Once an extraction and detection strategy has been selected, the method must be formally validated. The literature converges on ICH Q2(R1)/Q2(R2) as the primary validation framework, supplemented by USP <1469> acceptance criteria specific to nitrosamine assays [3]. Because AI limits sit far below the concentrations addressed by typical impurity validation guidance, nitrosamine methods place unusual emphasis on LOQ justification, accuracy/precision at the LOQ, and demonstrated freedom from matrix interference.

1. Specificity and Selectivity

The method must resolve each target nitrosamine from the API, degradation products, excipients, and other nitrosamines that may co-occur, using diagnostic MRM transitions (LC-MS/MS) or accurate mass and isotope pattern matching (LC-HRMS). Specificity is typically demonstrated using blank matrix, placebo, spiked samples, and forced-degradation samples.

2. Linearity and Range

Calibration curves are generally constructed to bracket the AI-derived specification limit, commonly spanning roughly 50-150% of that limit, with correlation coefficients (r^2) of 0.99 or better expected, supported by acceptable back-calculated calibration standard accuracy [3].

3. Limit of Detection and Limit of Quantitation

LOQ is the parameter most directly tied to regulatory acceptability: ICH's nitrosamine-specific FDA guidance explicitly states that the LOQ must be commensurate with, and scientifically justified against, the level at which the impurity is controlled, generally requiring the LOQ to sit at or below a defined fraction of the AI-based specification [18,3]. This differs from generic impurity validation, where LOQ is often set relative to the reporting threshold rather than to a toxicologically derived limit.

4. Accuracy (Recovery)

Accuracy is assessed by spiking known amounts of nitrosamine into blank or placebo matrix at multiple levels (typically LOQ, and low/mid/high specification-relative levels) and calculating percent recovery. Published acceptance ranges are wider at the LOQ than at higher concentrations, reflecting the greater relative measurement uncertainty inherent to trace-level work: commonly cited criteria are approximately 70-130% recovery at the LOQ and 80-120% (sometimes tightened to 85-115%) at other validated levels [3].

5. Precision

Repeatability (intra-day) and intermediate precision (inter-day, inter-analyst, inter-instrument) are evaluated as relative standard deviation (RSD) of replicate determinations. Reported acceptance criteria commonly allow up to about 20-25% RSD at the LOQ, tightening to roughly 15% RSD or better at higher concentrations, consistent with the greater

variability inherent to measurements near the detection limit [3].

6. Robustness

Robustness studies deliberately vary parameters such as mobile phase composition, column temperature, flow rate, and extraction hold time to confirm the method tolerates normal operational variability without meaningful loss of accuracy or precision. Given the sensitivity of MS ionization to small chromatographic changes, robustness testing is considered particularly important for nitrosamine LC-MS/MS methods.

7. Matrix Effect and Recovery Studies

Beyond standard ICH Q2 parameters, nitrosamine-specific validation packages increasingly include explicit matrix effect (ion suppression/enhancement) assessment, typically by comparing analyte response in neat solvent versus post-extraction spiked matrix, as demonstrated in the rifampin/rifapentine case study where undetected matrix effects were identified as the root cause of an inaccurate earlier method [16].

8. Solution and Sample Stability

Given the trace concentrations involved and the potential for light-induced or thermal degradation of both nitrosamines and derivatized extracts, stability of standard solutions and prepared sample extracts (e.g., stored at 4°C in amber vials) is routinely verified over the intended hold time of the analytical run [13].

Table no.3: Summary of Typical Acceptance Criteria

Parameter	Typical Acceptance Criterion	Basis
Linearity (r^2)	≥ 0.99	ICH Q2(R2)
Range	~50–150% of AI-based specification	USP <1469> / ICH Q2(R2)
Accuracy at LOQ	~70–130% recovery	USP <1469>
Accuracy (other levels)	~80–120% recovery	USP <1469> / ICH Q2(R2)
Precision at LOQ	≤ 20–25% RSD	USP <1469>
Precision (other levels)	≤ 15% RSD	USP <1469>
LOQ	At or below a defined fraction of the AI limit	FDA Nitrosamine Guidance / ICH Q2(R1)

Note: Acceptance criteria vary by nitrosamine, matrix, and applicable AI limit, and should be scientifically justified for each method rather than applied as fixed defaults [3].

Applied Case Studies

1. Losartan–Hydrochlorothiazide Fixed-Dose Combination

An UPLC-TQ-MS/MS method was developed and validated for eight genotoxic nitrosamine impurities in a losartan-hydrochlorothiazide fixed-dose combination tablet, motivated by the observation that losartan-containing products accounted for a disproportionate share of nitrosamine-related recalls in the FDA database [12]. The method used a diphenyl-phase column with formic-acid-modified aqueous/methanol gradient elution and was reported to meet specificity, accuracy, and precision requirements across all eight target analytes.

2. Rifampin and Rifapentine: A Matrix-Effect Case Study

A published investigation into LC-MS/MS quantitation of a nitrosamine impurity in rifampin

and rifapentine drug substances and products found that a sample preparation protocol adapted from FDA's generic LC-HRMS method, though adequate in its original application, introduced significant matrix effects when applied to these particular matrices [16]. The authors documented their approach to identifying and mitigating the matrix effects and reported final validated method details for both products, illustrating that method transfer between matrices cannot be assumed to preserve accuracy without re-verification [16].

3. Mexiletine Formulations

A recently published LC-MS/MS method for mexiletine formulations targeted seven minor nitrosamines using a C18 column and gradient elution, validated according to USP <1225> and ICH criteria, reporting linearity above r^2=0.999 and accuracy in the range of approximately 95-105% [10]. The authors described a systematic, statistically informed method-development approach intended to ensure robustness ahead of formal validation [10].

4. Metformin: Distinguishing NDMA from a Co-Occurring Solvent Impurity

A comparative study of five commercial metformin tablet formulations used both LC-MS and LC-MS/MS to quantify NDMA, finding trace levels below the regulatory limit in all samples, while also identifying appreciable levels of dimethylformamide (DMF), a synthesis-related solvent impurity that had previously been implicated in inconsistent NDMA results reported to the FDA [5]. The study is notable for demonstrating that an interfering co-impurity (DMF) can materially affect the accuracy of nitrosamine quantitation if not adequately resolved, a discrepancy previously cited as the reason FDA's own investigation revised its estimate of non-compliant metformin products from 42% to 21% of tested batches [5].

5. Multi-Analyte HILIC-SPE/LC-HRMS Screening

A broader-scope method combining HILIC-based SPE cleanup with LC-HRMS detection was validated for simultaneous quantification of 15 regulated nitrosamines and applied across 26 marketed drug products spanning multiple APIs and combination products [15]. NDMA was the most frequently detected nitrosamine in this survey, and notably was found only in expired drug products, suggesting a possible relationship between nitrosamine formation and product aging or degradation over shelf life. The method's design, intended to be adaptable to newly identified NDSRIs through adjustment of the SPE elution protocol, was highlighted as a practical response to the continually expanding list of regulated nitrosamines.

Persistent Challenges

1. NDSRIs and Reference Standard Availability

Nitrosamine drug substance-related impurities, formed from the API's own structure rather than a generic small molecule, continue to be identified across new drug classes, often outpacing the availability of certified reference standards and isotopically labeled internal standards needed for accurate LC-MS/MS quantitation [2,17]. In their absence, laboratories must rely on structurally related surrogate standards, matrix-matched calibration, or standard addition, each of which introduces additional validation burden and uncertainty [17].

2. Matrix Complexity and Ionization Effects

As illustrated by the rifampin/rifapentine and metformin case studies above, matrix-derived ion suppression/enhancement and co-occurring interferents remain the most frequently cited source of inaccurate nitrosamine quantitation, underscoring that method validation cannot rely solely on generic or literature-derived sample preparation protocols without matrix-specific verification [5,16].

3. Regulatory Harmonization

While FDA, EMA, and Health Canada guidance are broadly aligned on general principles, differences remain in specific AI limits, in the treatment of less-than-lifetime dosing scenarios, and in recommended methodologies, creating a compliance burden for products marketed across multiple regions [11,9]. FDA has, for example, historically declined to accept the less-than-lifetime dosing approach used elsewhere for setting nitrosamine specifications, a divergence that directly affects how validation acceptance limits are derived for globally marketed products [9].

4. A Moving Target: Evolving AI Limits

Because AI limits are periodically revised as new toxicological and carcinogenic potency data become available (as occurred between the FDA's 2021 and 2024 nitrosamine guidance revisions), previously validated methods may require re-justification of their LOQ and specification range whenever a relevant AI limit changes, adding a recurring lifecycle burden to nitrosamine method validation that is less pronounced for impurities governed by static specification limits [6,9].

Future Directions

- Continued adoption of automated and online SPE-LC-MS/MS or SPE-LC-HRMS platforms to reduce analyst variability and solvent consumption while maintaining sub-ppb sensitivity.
- Expansion of HILIC-based and other generic-but-adaptable cleanup platforms capable of accommodating newly identified NDSRIs without full method redevelopment.
- Greater use of LC-HRMS non-targeted acquisition to enable retrospective data mining as new nitrosamines are identified, reducing the lag between hazard identification and analytical readiness.
- Development of additional isotopically labeled reference standards for NDSRIs to reduce reliance on surrogate calibration approaches.
- Progress toward greater cross-regional regulatory harmonization of AI limits and validation acceptance criteria, reducing duplicative validation for globally marketed products.

CONCLUSION

The framework for validating analytical methods used to identify nitrosamine impurities has significantly improved since the 2018 valsartan incident. Previously, these investigations were done on a case-by-case basis, but now the approach is more systematic and based on risk. This is supported by guidelines such as ICH M7(R2) and Q2(R2), USP , and specific advice from the FDA and EMA on nitrosamines. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is still widely used for regular testing. However, gas chromatography with mass spectrometry (GC-MS/MS) is preferred for volatile substances, and high-resolution mass spectrometry (LC-HRMS) is used when multiple substances or unknown impurities need to be

checked. Sample preparation has become more important than ever, as it often limits how sensitive a method can be. This has developed into a specialized area with techniques like HILIC-based extraction, automated processes, and strategies to reduce matrix effects, which were developed in response to past failures in method transfer. Even though progress has been made, the field is still constantly changing. New NDSRIs (Nitrosamine Drug Substance Related Impurities) are being discovered regularly, guidelines are updated, there is not enough agreement between regions, and there is a shortage of labeled standards for new impurities. This means that validated nitrosamine testing methods should be treated as ongoing tools that require regular review, not as one-time solutions. Therefore, a lasting strategy for validation involves following ICH Q2(R2) and USP standards closely while staying flexible to incorporate new regulatory and toxicological information as it becomes available.

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