



Nitrosoamine Impurities: Current Status, Challenges and Future Perspectives

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Abstract: N-nitrosamine (NA) impurities have emerged as a critical safety concern in pharmaceutical manufacturing since the 2018 discovery of N-nitrosodimethylamine (NDMA) contamination in valsartan, which triggered global recalls and revealed similar issues in metformin, ranitidine, and rifampicin. Classified as Class 1 mutagenic carcinogens under ICH M7(R1) and Group 2A carcinogens by IARC, these compounds form through nitrosation reactions between secondary/tertiary amines and nitrosating agents. Contamination can arise from a number of sources including API synthesis (solvent degradation, azide quenching in sartan manufacture), excipient-drug interactions leading to complex nitrosamine drug substance-related impurities (NDSRIs), packaging materials (rubber components, blister packs), environmental water treatment processes, dietary sources (cured meats, tobacco) and endogenous gastric formation. Nitrosamines are zwitterionic resonance structures which are bioactivated by cytochrome P450 into reactive diazonium ions, which form carcinogenic DNA adducts, especially O6-methylguanine, leading to mutations and epigenetic silencing of tumour suppressor genes. Regulatory agencies (FDA, EMA, WHO) have adopted the Carcinogenic Potency Categorisation Approach (CPCA) in which compounds are placed in five potency categories with allowable intakes of 18 to 1500 ng/day with required risk assessment, confirmatory testing and remediation. Detection is carried out using very sensitive GC-MS/MS and LC-MS/MS platforms capable of quantifying contaminants at parts per billion levels and with rigorous sample preparation using nitrite scavengers to prevent artifactual nitrosation. Despite regulatory progress, major gaps remain in the toxicological characterisation, human-specific ADME data, and low-dose combined exposure effects of NDSRIs. Continued research, in silico prediction, green chemistry strategies, and international regulatory harmonisation are needed to protect the integrity of the drug supply and patient safety.

Keywords: N-Nitrosamines; NDSRIs; Genotoxicity; CPCA; LC-MS/MS; Regulatory control.

1. Introduction

Background N-nitrosamine (NA) impurities, containing a nitroso functional group (N=N=O) attached to an organic amine moiety, have been a major safety concern in the global pharmaceutical regulatory landscape. These compounds are classified as high potency mutagenic carcinogens in ICH M7(R1) and as Group 2A (probably carcinogenic to humans) by the IARC.^[1,12] The worldwide crisis began in July 2018 when the angiotensin-II receptor blocker (ARB) valsartan was found to be contaminated with N-nitrosodimethylamine (NDMA). This finding sparked a series of product recalls and enquiries that uncovered contamination in other popular medicines such as metformin, rifampicin and ranitidine. Nitrosamines are typically formed during the manufacture of active pharmaceutical ingredients (APIs) or during the storage of formulations by nitrosating reactions between secondary or tertiary amines and nitrous acid under acidic conditions^[1]. The problem is now dominated by

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complex nitrosamine drug substance-related impurities (NDSRIs) which arise when APIs containing secondary or tertiary amines react directly with trace nitrites in excipients, and which have taken the place of simple, low-molecular-weight nitrosamines^[13]. According to *in silico* analyses, up to 40.4% of thousands of drugs under investigation may theoretically form these complex NDSRIs.

Nitrosamine contamination is a difficult problem. Given the ultra-trace activity of NAs, analytical methods such as gas chromatography-mass spectrometry (GC-MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are required for parts-per-billion sensitivity^[16]. The analytical techniques required for the quantitative determination of impurities present in complex drug matrices have to be very sensitive, accurate and selective. In addition, APIs with susceptible amines are subject to nitrosation *in situ* during sample preparation, which may result in false positives, or in the very acidic gastrointestinal environment following oral ingestion^[13]. Regulatory agencies (e.g. USFDA, EMA, ICH, WHO) use the Carcinogenic Potency Categorisation Approach (CPCA) to set structure-based Acceptable Intake levels and to validate mutagenic potential using advanced Ames test (EAT) methods.^[2] Pharmaceutical manufacturers should avoid co-existence of nitrosating agents and amines, use nitrite scavengers during formulation, select low nitrite excipients and use strong screening techniques in an effort to protect the drug supply and patient health.

2. Source And Mechanism of Nitrosoamine Formation

N-nitrosamines (NAs) and nitrosamine drug substance-related impurity (NDSRI) pollution can be caused by many chemical, environmental, nutritional and physiological factors^[1]. Based on the articles reviewed, the main sources of these mutagenic carcinogens are described below:

1) Chemical Manufacturing and Synthesis of Active Pharmaceutical Ingredients (APIs):

Direct Amine-Nitrite Reactions: Amine precursors may serve as starting materials, intermediates, reagents or intentionally added catalysts. The most common pathway to nitrosamine formation during drug manufacturing is a nitrosation reaction of amine groups (secondary, tertiary or quaternary amines) with nitrous acid or nitrite salts in an acidic media^[1].

Solvent Degradation: Polar aprotic solvents that are widely used in the API manufacturing such as N, N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), N, N-dimethylacetamide (DMA) and tributylamine (TBA) can degrade or dealkylate under severe reaction conditions, especially at high temperatures, to form secondary amines such as dimethylamine or diethylamine. **Azide Quenching:** Sodium azide and polar solvents are used in the synthesis of drugs that contain a tetrazole ring, such as 1xwangiotensin-II receptor blockers or sartans like valsartan and losartan.^[14] To destroy the dangerous leftover azides, sodium nitrite (NaNO_2) is added under acidic conditions, generating nitrous acid.^[3] Any nitrous acid carried through to subsequent steps will nitrosate any remaining secondary amine impurities, e.g. dimethylamine in DMF, to form NDMA or NDEA.

Methodology and Vendor Cross-Contamination:

Raw materials, starting materials, recycled or recovered solvents, catalysts, and reagents can all be a source of impurities^[4]. Furthermore, in multipurpose (rather than dedicated) equipment without appropriate cleaning protocols, there is a high risk of cross-contamination during the manufacture of pharmacological compounds. Inadequate optimisation of reaction parameters (e.g., pH, temperature or addition sequences) also promotes *in-process* nitrosamine formation.

2) Storage of Preservatives, Excipients and Formulation

- **Trace Nitrites in Excipients:** Typical pharmaceutical excipients, like pregelatinized starch, polyvinyl pyrrolidone, croscarmellose sodium, sodium starch glycolate, cross-polyvinyl pyrrolidone, and lactose, commonly contain traces of nitrites and nitrates as impurities^[1,4].
- **Excipient-API Interactions (NDSRIs)** The use of nitrite-bearing excipients in combination with APIs containing secondary or tertiary amine groups can lead to direct reactions of the amine with trace nitrites during formulation or storage resulting in complex nitrosamine drug substance-related impurities (NDSRIs)^[2].
- **Aldehyde-Catalyzed Nitrosation Degradation** of some excipients (e.g., furfuraldehyde from plant-derived cellulose excipients or formaldehyde from polyethylene glycol [PEG 300] and polysorbate 80) can lead to the formation of aldehydes.^[4] These aldehydes catalyse nitrosation of secondary amines by formation of reactive iminium ion intermediates. Formulation parameters and storage conditions. The presence of high temperature and moisture during wet granulation and subsequent drying has been shown to accelerate the reactivity of excipient nitrites with susceptible APIs (e.g., metformin forming NDMA). Storage can also lead to excipient-API interactions or *in situ* degradation of unstable drug compounds due to exposure to temperature, humidity and light, which in time can result in the formation of nitrosamine impurities^[4].

3) Packaging Materials and Containers Interaction:

- **Rubber Components:** Some of the main packing materials that provide a significant risk of contamination are elastic rubber packing, seals, and containers^[4]. Chemical accelerators and stabilisers (e.g. dithiocarbamates, thiurams, sulfonamides, thioureas) used in rubber vulcanisation.^[26] These vulcanisation chemicals can migrate directly as preformed nitrosamines (e.g. NDMA, NDEA, NDBzA) or as amine degradants, which then react with excipient nitrites in the drug product.
- **Blister Packs and Inks** Nitrocellulose blister packaging cards have been associated with nitrosamine contamination of solid formulations during storage.^[4] Also, nitrosamines or precursors may migrate from printing inks, printed aluminium foil, nasal spray pumps and eye drop containers via direct contact with the formulation^[4].

4) Sources of Water and Environmental Disinfection^[42, 47]

- Chloramination: Wastewater and municipal drinking water systems are a major environmental source of NDMA.^[1] When water is disinfected using chloramination, the chloramine species (monochloramine or dichloramine) react with secondary or tertiary organic amine precursors (e.g., ranitidine or other dimethylamine moieties) to form hydrazine intermediates that oxidise to nitrosamines.^[18] During chloramination, water treatment polymers such as polyDADMAC degrade to form secondary and tertiary amines, which are precursors of nitrosamines. Nitrosating species are also produced when nitrite-containing waters are chlorinated^[18].
- Ozonation: Ozonation of water leads to significant yields of nitrosamines if the wastewater contains specific industrial or agricultural precursors, such as N,N-dimethylsulfamide or the plant growth regulator daminozide^[15].
- Effluents, Sewage and Air: Some of the ways nitrosamines enter ecosystems are by hospital wastewater (containing medical chemicals and disinfectants), municipal wastewater effluents, landfill leachates and direct application of solids/biosolids (treated sewage sludge) to agricultural soils.^[15] Secondary atmospheric formation of particulate nitrosamines is caused by reactions of vehicular ammonia and urban nitrogen oxides (NO_x) with ambient alkylamines, especially in the winter.

5) Occupational, Tobacco and Dietary Exposures

- Cured and Preserved Foods: Sausage, salami, bacon and hams are examples of cured meats which have sodium or potassium nitrites (\$E249\$, \$E250\$) as food additives.^[4] Nitrites can react with proline and piperidine precursors or biogenic amines (cadaverine, spermine, and spermidine, which are increased in low-quality meats due to bacterial growth) to form volatile nitrosamines such as NPIP and NPYR^[29]. Other food sources include cheese, fermented soy sauce, and processed or smoked salmon.^[39, 41]
- Beverages and Dairy Products: Beer and malt are contaminated with NDMA from nitrosation of tertiary amine alkaloids (hordenine and gramine) formed during the germination of malt. In addition, trace NDMA is formed in milk proteins (casein and lactalbumin) by direct-fire heating and drying methods used in industrial dairy plants.^[28]
- Tobacco: Highly carcinogenic tobacco-specific nitrosamines (TSNAs) including N-nitrosornicotine (NNN) and 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) are found in both cigarette smoke and smokeless tobacco.^[46]
- Industrial/Occupational Exposure: High nitrosamine levels produced under high-temperature curing or metal-shaping conditions are a risk for workers in tanneries, rubber manufacture, metalworking (using cutting fluids), and foundries. Six. In Vivo (Formation Endogenous)
- Gastric Acid Synthesis: Endogenous formation in the highly acidic gastric environment of the stomach ($\text{pH} < 3$) accounts for 45–75% of human exposure to N-nitroso compounds^[24]. Protonated nitrous acid is formed from ingested nitrite, with a pK_a of 3.15–3.3. This acid then equilibrates to dinitrogen trioxide (N_2O_3), which is the principal nitrosating agent^[29]. This substance rapidly reacts in vivo with drugs containing secondary or tertiary amine groups (e.g. metformin, ranitidine or sartans) to form complex NDSRIs or carcinogenic nitrosamines.^[41, 48]
- Salivary and Bacterial Pathways: The main source of gastric nitrite is from the salivary reduction of dietary nitrate to nitrite (5% to 36% of dietary nitrate) by oral nitrate-reducing bacteria present in human saliva^[13]. In addition, in patients with urinary tract infections, active microbial nitrogen cycles increase levels of nitrosamines, causing endogenous nitrosation.^[37, 41]

3. Nitrosoamines Chemistry

N- Nitrosamines share a common molecular arrangement ($\text{R}_1\text{R}_2\text{N}-\text{N}=\text{O}$) with an organic amine moiety attached to a nitroso group^[35, 4]. Its chemical reactivity and physical properties are largely contributed by a resonance hybrid with a major zwitterionic structure ($\text{R}_1\text{R}_2\text{N}^+=\text{N}-\text{O}^-$). This resonance induces a high degree of double bond character to the nitrogen-nitrogen ($\text{N}-\text{N}$) bond, which restricts its rotation and preserves the rigidity of the $\text{C}_\alpha\text{C}_\alpha\text{NNO}$ plane^[18].

This polar resonance is in agreement with the observation that the nitrosamines are most basic at the O atom. NMR investigations^[18] show that O-protonated hydroxy diazenium salts are formed mainly by protonation at the oxygen and are ~16 kcal/mol more stable than the possible N^+ -protonated isomers. Metabolic bioactivation is the major mechanism of nitrosamine-induced carcinogenesis. The liver enzymes, cytochrome P450, catalyse α -hydroxylation of the alkyl groups adjacent to the nitroso group to form an unstable primary nitrosamine. This intermediate spontaneously decomposes to give the highly reactive diazonium ion ($\text{R}-\text{CH}_2^+$), a potent DNA-alkylating mutagenic agent that causes mutagenic transitions^[29].

4. Classification of Nitroso-Amines

Sources use three primary chemical and regulatory systems for classifying nitrosamines.

Small Molecule vs. NDSRI Origin of Structure:

1) Small-Molecule Nitrosamines: These are simple low molecular weight aliphatic molecules (e.g., NDMA, NDEA) that are often introduced from contaminated reagents or solvents and do not resemble the structure of active pharmaceutical ingredients (APIs). Nitrosamine drug substance-related impurities (NDSRIs) are larger, structurally complex impurities that have a direct relationship to the structural features of the parent API. They are formed when trace nitrites in pharmaceutical excipients react directly with APIs containing susceptible secondary or tertiary amines,^[13, 33]

2) Volatility of Volatile Nitrosamines (VNA): Low molecular weight, slightly nonpolar compounds with low boiling temperatures such as NDMA, NPYR, NPIP and NMOR^[28]

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3)Non-Volatile Nitrosamines (NVNAs) Polar, hydrophilic compounds, often substituted with a carboxyl acid group (e.g. β -nitrosoproline and β -nitrososarcosine). These are generally less hazardous because hydrophilic substituents reduce absorption and facilitate physiological excretion^[28].

Structural features have a significant effect on potency:

The Carcinogenic Potency Categorisation Approach (CPCA) is a structure-activity relationship (SAR) based framework that categorises nitrosamines into five categories (PC1 to PC5) to derive Acceptable Intake (AI) limits ranging from 18 to 1500 mg/day.

o High-risk characteristics that increase potency include methyl or ethyl groups, benzylic, allylic, or β -carbonyl groups.

o Potency-increasing features (low risk): the presence of carboxylic acid groups and steric hindrance at the α -carbon (such as isopropyl or tert-butyl groups), which greatly reduce the chance of cancer^[38].

Pharmaceutical Product Associated With Nitrosamines

Overview of the Contamination Landscape When health authorities found N-nitrosodimethylamine (NDMA) in the angiotensin-II receptor blocker (ARB) valsartan in July 2018, the pharmaceutical industry underwent a major regulatory change. This led to comprehensive risk-assessment procedures and voluntary global product recalls. Nitrosamine contamination is a process-driven concern rather than an isolated molecular problem, as investigations soon showed. It can affect a wide range of therapeutic classes^[13], from low molecular weight nitrosamines (such as NDMA and NDEA) to complex Nitrosamine Drug Substance-Related Impurities (NDSRIs) formed by direct nitrosation of active pharmaceutical ingredients (APIs) containing secondary or tertiary amines.

Significant Therapeutic Classes Impacted and Associated Impurities

- Angiotensin II Receptor Blockers (ARBs/Sartans): valsartan, losartan, irbesartan, candesartan, olmesartan, telmisartan, azilsartan^[2] Small-molecule nitrosamines such as NDMA, NDEA, NMBA, NIPEA (or NEiPA), NDIPA, and NMPA have been found to contaminate sartans.^[8, 9]
- Antidiabetic Drugs: NDMA has frequently contaminated metformin hydrochloride. In addition, sitagliptin, a DPP-4 inhibitor, is susceptible to forming NTTP (7-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-triazolo[4,3-a]pyrazine, also known as nitroso-STG-19). Metformin's structural vulnerability, along with heat, moisture, and nitrite traces in Hypromellose and other excipients used in sustained-release formulations.^[6, 8]
- Gastrointestinal Agents (H2 Antagonists): Because of elevated NDMA concentrations, ranitidine hydrochloride and nizatidine were severely regulated. Ranitidine is extremely unstable and contains a dimethylamine precursor. Its molecular breakdown during thermal processing, regular storage, or inside stomach fluid results in NDMA levels that are higher than allowable safety limits.^[15]
- Antimycobacterials (Rifamycins): It was discovered that structurally related nitrosating derivatives were present in rifampicin and rifapentine, two essential first-line medications for the treatment of tuberculosis. In particular, rifapentine includes CPNP (1-cyclopentyl-4-nitrosopiperazine), whereas rifampicin has MNP (1-methyl-4-nitrosopiperazine).^[6]

Neuropsychiatric & additional Cardiovascular Agents: To produce complicated NDSRIs, a variety of additional medications with susceptible secondary or tertiary amine moieties are nitrosated^[13]. Examples include: N-nitroso-varenicline and varenicline tartrate (smoking cessation). N-nitroso-duloxetine is linked to the antidepressant duloxetine^[2]

N-nitroso-propranolol and N-nitrosometoprolol are linked to beta-blockers propranolol and metoprolol^[33]. N-nitroso-enalapril and N-nitrosolisinopril are linked to ACE inhibitors enalapril and lisinopril^[4].

o Bumetanide, a diuretic linked to N-nitroso-bumetanide^[13]

N-nitrosorasagiline and rasagiline (Parkinson's disease)^[2]

Folic acid, a vitamin linked to N-nitroso folic acid^[13] Regulatory Context and Safe Intake limitations Strict acceptable intake (AI) limitations are required by regulatory agencies such as the USFDA and EMA since nitrosamines are categorized as "cohort of concern" mutagenic carcinogens under the ICH M7(R1) guideline^[12]. Regulators use proactive risk assessments, structure-activity relationship (SAR) modelling and strengthened analytical controls to protect patients and ensure the supply of life saving medicines. The standard thresholds are 96 ng/day for NDMA and NMBA and 26.5 ng/day for NDEA, NEIPA, NDIPA and NDBA^[1, 28]

Toxicological Significance And Carcinogenicity

N-nitrosamines are classified as Group 2A (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC) and very potent mutagenic carcinogens (Class 1 impurity) by ICH M7(R1) guideline^[12, 4]. These contaminants cause serious systemic chemical poisoning in humans. Repeated exposure has been associated with adverse effects in multiple organ systems including acute liver toxicity, neurotoxicity, immunotoxicity, and reproductive or developmental effects. In addition to metabolic disturbances that may cause Alzheimer's disease, Parkinson's disease and type-2 diabetes, these chemicals can cause cell death, chromosomal changes, loss of pregnancy and birth defects.^[8, 17, 19]

Molecular Mechanism of Genotoxic Carcinogenesis [44]:

The carcinogenicity induced by nitrosamines is mainly due to metabolic bioactivation mediated by cytochrome P450^[12].

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Nitrosamines are pro-carcinogens that are enzymatically α -hydroxylated, mainly in the liver by CYP2E1 and CYP2A6, rather than reacting directly with DNA^[29]. The reaction yields the unstable α -hydroxy-N-nitrosamine intermediate that spontaneously decomposes to formaldehyde and highly electrophilic carbonium and diazonium ions^[15]. These highly reactive alkylating agents covalently bind to macromolecules and react with the nitrogen and oxygen atoms of the nucleophilic bases in DNA, generating carcinogenic DNA adducts^[28]. The most abundant and toxicologically relevant adducts are N7-methylguanine (N7-Me-Gua) and O6-methylguanine (O6-Me-Gua).^[17, 19]

Organotropy, Mutational Signatures, and DNA Repair:

The highly specialized repair protein O⁶-alkylguanine-DNA alkyltransferase (AGT/MGMT) can repair damaged DNA by directly transferring the alkyl group from the O⁶ position of guanine to itself^[28]. However, if AGT is overexposed or depleted, the unrepaired O⁶-Me-Gua adducts persist through cell replication, resulting in miscoding that produces G > A (GC > AT) transition mutations.

Although saturation can divert nitrosamines to extrahepatic tissues, the liver is the primary target organ of tumor induction in animal models.^[28] Tumours are commonly induced in the lung, nasal cavity, oesophagus, pancreas, urinary bladder, colon, kidneys, and central nervous system. Epidemiological cohort studies have also confirmed these findings, showing a strong correlation between dietary nitrosamine exposure, the O⁶-alkylguanine mutational signature, and increased incidence of oesophageal squamous cell carcinoma (ESCC) and colorectal cancer in humans^[34,28].

Tumour Development and Epigenetic Inactivation:

Besides direct genotoxicity, nitrosamines also induce cancer by means of epigenetic alterations. As dysplasia progresses to an infiltrating carcinoma, exposure causes abnormal cytosine hypermethylation of CpG island promoter regions of important tumour suppressor genes (CDKN2A, DAPK1, FHIT, RASSF1A and SOCS3), progressively silencing their transcription. This epigenetic silencing often inactivates essential DNA repair enzymes such as MGMT itself, permanently disabling the defence mechanisms of the cell and accelerating the rapid growth of the tumour^[28].

5. Current Regulatory Status

Regulatory Framework (ICH M7) and Cohort of Concern:

The ICH M7(R1) guideline categorises N-nitrosamine (NA) impurities as Class 1 impurities (known mutagenic carcinogens) and assigns them to the high-potency “Cohort of Concern”^[11]. The standard Threshold of Toxicological Concern (TTC) limits (allowing exposures of up to 1.5 µg/day for other genotoxic impurities) do not apply to nitrosamines because of their extreme mutagenic potential.

The Standardised Risk Management Process:

To control and reduce the presence of these dangerous contaminants, international health agencies including USFDA, EMA, EDQM and Health Canada have mandated a three-step risk management procedure that all the drug producers and marketing authorisation holders (MAHs) must follow:

Step 1: Risk Assessment: The risk assessment will actively be conducted by manufacturers on their finished drug products (FPs) and active pharmaceutical ingredients (APIs).^[4,1] This step considers possible sources of contamination such as raw materials, solvents, reagents, catalysts, cross-contamination on multipurpose manufacturing lines, and degradation during storage.

Step 2: Confirmation testing: When a risk of nitrosamine formation or carryover is identified, manufacturers must conduct confirmation testing using highly sensitive validated analytical methods^[4].

Step 3: Reporting and Remediation: Any nitrosamine impurities found above the regulatory limits should be reported immediately to the relevant health authorities. Therefore, manufacturers need to apply robust control techniques and submit changes to upgrade their production processes to ensure the long term safety of patients.

CPCA Framework and Acceptable Intake (AI) Limits:

In the past, regulatory bodies used a very conservative class-specific limit of 18 ng/day as a worst-case limit for novel nitrosamines in the absence of specific toxicity data.^[33] However, regulators developed the Carcinogenic Potency Categorisation Approach (CPCA) when the carcinogenic potency of nitrosamines was found to vary by several orders of magnitude as a function of molecular shape.^[6] CPCA is a structural linkage framework^[33] which is used to classify nitrosamines and complex Nitrosamine Drug Substance-Related Impurities (NDSRIs) into five potency classes (PC1 to PC5). Each category has some Acceptable Intake (AI) limit:

- PC1 (high potency): AI limit 18 ng/day (e.g. N-nitroso-nortriptyline).

- PC2: Limit of 100 ng/d AI.

- PC3: 300 ng/day AI limit.

- PC4: AI limit of 1500 ng/day (such as N-nitroso-bumetanide).
- PC5 (Lowest Potency) 1500 ng/day AI limit^[36]. For well-known, small-molecule nitrosamines, regulatory agencies require certain compound-specific limitations when present as a single contaminant in a pharmaceutical product^[1,29]. The USFDA and EMA have set these limits at 96 ng/day for NDMA and NMBA and 26.5 ng/day for NDEA, NMPA, NIPEA and NDIPA2829. The total quantity of several nitrosamines detected together in a single product shall not exceed 26.5 ng/day (or the lowest compound-specific limit among those detected) based on the maximum daily dose (MDD), unless the agency permits a specialist multi-impurity evaluation.^[6]

The Enhanced Ames Test (EAT) Protocol:

Manufacturers are allowed by regulators to perform the Enhanced Ames Test (EAT)^[4] to settle doubts about whether a complex NDSRI is indeed mutagenic. Standard Ames test conditions are often not sensitive enough to detect nitrosamine

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mutagenicity and may lead to false negatives^[5]. Hence some changes are needed to the EAT protocol:^[17, 19]• Hamster liver S9 is used at high concentrations (generally 30%) instead of the normal rat liver S9, as hamster enzymes metabolise complex nitrosamines much more efficiently^[36].

- Including a required pre-incubation period of 30 minutes.
- Specific bacterial strains, *Escherichia coli* WP2 uvrA (pKM101) and *Salmonella Typhimurium* TA1535, were used as base-substitution.
- e.g. CPNP (1-cyclopentyl-4-nitrosopiperazine) as a strong nitrosamine positive control to verify the metabolic competence of the assay. If an NDSRI is conclusively shown to be negative in a properly done EAT^[5], it can be downgraded from a Class 1 mutagen to a ICH M7 Class 5 non-mutagenic impurity, and thus controlled by normal generic criteria.
- Agency updates and harmonisation of international pharmacopoeias:
- The standards for strict control are still being harmonised by the monographs and rules of global regulatory bodies:
- USFDA: In September 2024, the FDA released Revision 2 to its guidance, “Control of Nitrosamine Impurities in Human Drugs.” It brought in revised structural risk assessment procedures, specified timeframes for compliance and drew a clear line between small-molecule NAs and complex NDSRIs.
- European Pharmacopoeia (Ph. Eur.) & EDQM: A major structural approach was set up in January 2024 (approved at the 177th session). The unique production parts of the individual active ingredient monographs have been removed and the amended monograph 2034 general standards are now applied to all compounds to assure regulatory uniformity. The EDQM also published the first formal analytical methods for the quantification of complex NDSRIs.
- World Health Organisation (WHO): The WHO updated its Good Manufacturing Practices (GMP) appendix standards in April 2024 to improve worldwide nitrosamine contamination prevention, requiring that manufacturers of excipients, APIs, and final products carry out thorough root cause analyses^[2].
- Other Organisations CPCA limitations and Step 1-3 frameworks are fully embedded in the regional licensing systems of Health Canada (updated March 2024), the Australian TGA (updated October 2024), ANVISA (Brazil) and China National Medical Products Administration (NMPA).

Method Controls and Analytical Performance:

Traditional high-performance liquid chromatography (HPLC) or gas chromatography (GC) techniques combined with ultraviolet (UV) detectors are no longer sufficient due to low sensitivity and severe matrix interference because tolerable intakes are defined in nanogram-range levels.^[6, 7] Regulators need analytical systems to use very sophisticated hyphenated methods, namely gas chromatography-mass spectrometry (GC-MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), that can function in high-resolution or multiple reaction monitoring (MRM) modes. To successfully meet the requirements for compliance these techniques must be able to accurately quantify the contaminants at their safety limits. The limit of quantitation (LOQ) must be at least 0.03 ppm (parts-per-billion equivalent).^[1]

6. Analytical Method for Detection and Quantification

Regulatory sensitivity: overview and prerequisites:

N-nitrosamine impurities are high-risk “Cohort of Concern” due to their extraordinary carcinogenic potency, which implies that they can cause DNA damage and genetic alterations even at ultra-trace, nanogram level concentrations.^[1] Conventional analytical detection systems, such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) with ultraviolet (UV) or flame ionisation detectors, are not sufficient because of high detection limits and serious matrix interference. As a result, producers are required by international regulatory organizations (including the USFDA, EMA, and WHO) to use very sophisticated, hyphenated analytical techniques. International standards recommend that the Limit of Quantitation (LOQ) for routine release testing and confirmatory analysis should be equal to or less than 0.03 ppm (30 ppb). The LOQ sensitivity for guaranteeing that the total daily exposure does not exceed the safe limit of 26.5 ng/day is significantly lower when many nitrosamine impurities are co-detected^[1].

Gas Chromatography-Mass Spectrometry (GC-MS) Systems:

Gas chromatography with mass spectrometry is a well-established, reliable and economic method for the separation and determination of volatile nitrosamines (VNA) as N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA).^[6, 7]

- Direct injection vs. Headspace: Direct injection GC-MS using a classical capillary column (such as a highly polar DM-WAX or Stabilwax-MS capillary column) in the splitless mode is generally preferred for its simplicity, speed, repeatability and independence from complex instrumental variables. HS-GC-MS (headspace GC-MS) is often used to reduce direct matrix interference from non-volatile active pharmaceutical ingredients (APIs).
- Ionisation and acquisition modes Quantitative screening is generally performed by instrument acquisition in the Selected Ion Monitoring (SIM) or Selected Ion Recording (SIR) modes for important fragments (e.g. m/z 74 for NDMA, m/z 102 for NDEA and m/z 80 for the deuterated internal standard NDMA-d6). Samples are generally ionised by Electron Ionisation (EI) 70 Ev.
- Tandem Mass Spectrometry (GC-MS/MS): GC-MS/MS in Multiple Reaction Monitoring (MRM) mode removes doubt in complex pharmaceutical formulations. Collision induced dissociation transitions (e.g. m/z 74 $\rightarrow$ for NDMA, m/z $\rightarrow$ for NDEA) are monitored to differentiate target impurities from matrix peaks that co-elute with them.

Liquid Chromatography and Mass Spectrometry (LC-MS) Platforms^[6, 7] :

Liquid chromatography coupled with mass spectrometry is required for non-volatile, polar or thermally sensitive contaminants, such as complex Nitrosoamine Drug Substance-Related Impurities (NDSRIs) and thermolabile active substances (e.g., ranitidine), so that they do not undergo thermal degradation during analysis.

- LC-MS/MS (Tandem Mass Spectrometry) This platform is usually coupled to a triple quadrupole or QTRAP mass spectrometer and is mostly used in positive Electrospray Ionisation (ESI+) mode.^[15] To separate the API from the impurity and avoid source saturation, high-throughput gradient elution programmes utilise reversed-phase columns (e.g. C18 or specialised phenyl/C8 phases) with mobile phases consisting of mass-compatible volatile buffers (e.g. ammonium acetate or formic acid in water and acetonitrile).
- A common problem of LC-MS/MS analysis of nitrosamines is the appearance of cis-trans (geometric) isomers, which often lead to split peaks or non-resolved double peaks (e.g., N-nitrosometoprolol, N-nitrosopropranolol and N-nitrosoenalapril).^[13] For optimisation of fragmentation and to prevent in-source degradation of mesomerically stabilised systems. For example, N-nitroso melatonin (NMLT) is determined by ESI+ MRM by scanning the m/z transition^[33]
- LC-HRMS (high-resolution mass spectrometry) Ultra-high performance liquid chromatography (UHPLC) coupled with high-resolution instruments such as Q-Orbitrap or Q-TOF mass spectrometers can increase selectivity^[15,35]. High mass resolution (e.g. 70,000 FWHM) enables post hoc untargeted screening, confirmation of isotopic patterns and identification based on exact mass within a strict ± 5 ppm mass error. UHPLC-HRMS typically uses a combination of Heated Electrospray Ionisation (HESI) and Atmospheric Pressure Chemical Ionisation (APCI) to provide complementary coverage for polar and nonpolar nitrosamines.^[6, 7]

Sophisticated techniques of extraction and sample preparation:

The complexity of pharmaceutical drug matrices and the very low concentrations of nitrosamines make sample preparation critical.

- Solid-Phase Extraction (SPE): SPE is often used to concentrate nitrosamines and clean up the matrix prior to chromatographic analysis. In the offline extraction, reversed phase (C18) or hydrophilic lipophilic balanced (HLB) cartridges are commonly applied for the isolation of analytes from interfering excipients. In modern automated processes the online SPE systems are used with a divinylbenzene (DVB) sorbent column directly connected to the HPLC analytical column through a switching valve^[35].
- The In Situ Nitrosation Challenge and Scavenger Mitigation: During extraction, the API's susceptible secondary or tertiary amine groups undergo a direct reaction with trace nitrite impurities present in the sample diluents, reagents, or excipients. This is a serious analytical complication. Nitrite scavengers should be used during sample preparation to prevent this reaction, which results in artifactual nitrosamines and false positive signals and irregular peak regions^[33]. Tablets are powdered and pre-treated with 3,4-diaminotoluene, a non-explosive scavenger, to preserve the integrity of the recovery data and eliminate analytical artefacts. This ensures that trace nitrites react rapidly with the scavenger to form diazonium salts instead of reacting with the API.^[6]

Analytical Methods Validation Criteria:

All developed procedures must be rigorously validated in accordance with ICH Q2(R1) and ICH M10 requirements before they can be considered suitable for regulatory use.

- 1) System Suitability: Assessed by injecting duplicate calibration standards. Signal-to-Noise (S/N) ratio ≥ 10 for the standard at the LOQ, coefficient of determination ($R^2 \geq 0.998$) and relative standard deviation (RSD) of peak regions of six injections $\leq 5\%$
- 2) Specificity and Selectivity: demonstrated by the testing of nitrosamine-free blank matrices (e.g., gastric media, placebos, solvents) to confirm no false-positive signals or interfering chromatographic peaks at the target retention times.
- 3) Linearity: The linearity should be assessed by linear regression of peak area ratios (analyte to internal standard) with correlation coefficients ($R^2 > 0.99$). The linear response over the working range should be demonstrated^[13].
- 4) Precision and Accuracy: Assessed at low, medium, and high concentrations; relative recoveries must continuously fall between 80% and 120%. The accuracy, expressed as repeatability (intraday) and intermediate precision (inter-day) relative standard deviation (%RSD), must not exceed 15% for all testing replicates (and less than 12% for optimised GC-MS systems).

7. Future Perspectives

Standardisation of Genotoxicity and Toxicological Studies:

Future studies need to focus on the production of reliable experimental data to fill key knowledge gaps concerning ADME (absorption, distribution, metabolism and excretion) properties, especially concerning long-term, low-dose exposures. Regulators and researchers highlight the absolute necessity of thorough mutagenicity studies, such as enhanced Ames tests (EAT) and COMET assays, to provide insight into the variable toxicity profiles of complex nitrosamine drug substance-related impurities (NDSRIs).^[17, 19, 22]

Future work on accurate risk classification should explicitly employ human-derived S9 metabolic activation systems, use whole-genome sequencing to characterise mutational spectra, and investigate the repair kinetics of DNA adducts in human tissues. Moreover, hard evidence on non-volatile nitrosamines (NVNAs) is also needed to decrease the regulatory uncertainty that still exists related to their potential risks in consumer goods^[29].

In Silico Screening and Advanced Predictive Modelling:

Future workflows will need to employ integrated predictive models to deal with the thousands of APIs with vulnerable secondary and tertiary amines.^[13] The application of quantitative structure-activity relationship (QSAR) models, machine learning and molecular docking will allow high-throughput screening of potential impurity formation, ecotoxicology and human health risks in complex matrices.^[15] Proactive structural classification, such as the Carcinogenic Potency Categorisation Approach (CPCA) and expert-guided Bayesian structural-feature models, will continue to improve acceptable intake (AI) limits and avoid incorrect read-across from highly potent small-molecule surrogates like NDEA.

Lifecycle Management, Avoidance and Green Chemistry- We need to shift the paradigm from “mitigate after process” to “avoid ahead of process”.^[4] Drug makers should conduct thorough risk assessments throughout the entire product lifecycle—from raw starting materials to final formulations.^[2] Industries will systematically eliminate conditions where nitrosating agents and amine precursors exist together by applying principles of green engineering and green chemistry.^[15] The key areas for industrial optimisation will include practical techniques such as the use of nitrite scavengers during sample preparation to eliminate in situ analytical artefacts, selection of low nitrite excipients, and performance of in-depth real-time stability studies for the purpose of maximising and extending drug shelf life.

Environmental Sensors and Innovative Analytical Platforms:

Instrument makers will have to continue to develop more sophisticated, sensitive and selective tools to lower detection and quantitation limits.^[33] Future studies should use nitrosamine chemistry in low-cost, reliable, and portable real-time sensors instead of expensive laboratory LC-MS/MS and GC-MS/MS systems. To enable on-site aquatic monitoring and citizen research in affected communities, simplified surface-enhanced Raman spectroscopy (SERS) or electro-analytical equipment must be developed.^{[25, 27][31, 42]}

International Cooperation and Harmonization:

Deep, precompetitive partnerships between academic research institutions, the pharmaceutical industry, and regulatory bodies (FDA, EMA, and WHO) are necessary to address the global nitrosamine problem.^[4,12] Active joint networks are needed to coordinate inspections, standardize global regulatory boundaries, and promote open data exchange.

8. Prevention and Mitigation Strategies

Chemical Avoidance and API Process Design:

Global regulatory organizations generally agree that proactive avoidance strategies are significantly more successful than reactive downstream mitigation schemes. Manufacturers must avoid reaction conditions during the synthesis of active pharmaceutical ingredients (APIs) where secondary, tertiary or quaternary amines react with nitrosating agents. This is particularly important in the tetrazole ring synthesis of sartan drugs where residual sodium azide is quenched by sodium nitrite.^[1] In an effort to avoid dealkylation of the solvent and subsequent formation of nitrosamines when using vulnerable amide solvents such as dimethylformamide (DMF), N-methylpyrrolidone (NMP), N,N-dimethylacetamide (DMA) or tributylamine (TBA), process parameters such as pH, temperature and the order of addition of reagents need to be optimised. Also, multipurpose manufacturing equipment must be thoroughly cleaned to avoid cross-contamination between vendors and processes.^[6, 8, 9]

Formulations Design and Excipients Management:

The presence of trace amounts of nitrites and nitrates in excipients such as pregelatinized starch, polyvinyl pyrrolidone (PVP), croscarmellose sodium, sodium starch glycolate, and lactose is a major concern in the formulation of medicinal products. Also, excipients that can decompose to generate aldehydes, such as PEG 300 and polysorbate 80 (producing formaldehyde) or plant-derived cellulose (containing furfuraldehyde) need to be considered as aldehydes promote nitrosation of secondary amines through iminium ion intermediates.^[6, 10, 23]

The direct addition of some antioxidants to the formulation can greatly inhibit the formation of nitrosamine. Antioxidants such as trolox, alpha-tocopherol (vitamin E) and ascorbic acid (vitamin C), have been demonstrated to effectively prevent formation of nitrosodimethylamine (NDMA) in pharmaceutical products (such as metformin) under adverse storage conditions. However, dose optimisation is important since at high nitrite concentrations ascorbate can play two roles: it can act as a pro-oxidant producing nitrosating radicals (NO• and NO₂•) and catalytically promote the formation of nitrosamines, especially in the presence of transition metals such as iron or copper.^[6, 23]

Analysis, Storage and Packaging, Mitigation:

Another hazard is primary packaging; manufacturers should avoid nitrocellulose blister packaging materials as these can leak nitrosating chemicals when solid formulations are stored. Active ingredient deterioration and subsequent in-storage nitrosamine formation can be prevented through proper control of environmental storage factors including temperature, moisture and light exposure. Finally, trace nitrites in diluents and reagents can cause artifactual in situ nitrosation when performing quantitative analysis of susceptible APIs, which can lead to false positive results. In sample preparation procedures, this is reduced by inclusion of a non-explosive amine scavenger which rapidly reacts with trace nitrites to form stable diazonium salts, e.g. 3,4-diaminotoluene. This will prevent in situ formation of nitrosamines and guaranty the integrity of analytical data.

9. Research Gaps and Opportunities

Significant regulatory advances have been made, but major research gaps still exist in the toxicological, analytical and environmental domains:

- **Toxicological Profiling of NDSRIs and Complex Nitrosamines:** A large amount of research has been conducted on small molecule nitrosamines; however, there is a critical lack of experimental data on the toxicology, mutagenicity (Ames, COMET) and carcinogenicity of complex Nitrosamine Drug Substance-Related Impurities (NDSRIs). For non-volatile nitrosamines (NVNAs) the exact hazard profile for humans is also poorly understood, due to lack of systematic occurrence and toxicological data.
- **ADME and Epigenetic Gaps**^[17, 22, 19] **Human Specificity:** Gaps still exist in the knowledge of ADME pathways that are relevant to humans. Metabolic activation and DNA adducts should be studied in human tissues, rather than in animal models, which have significant species-specific differences in CYP enzyme metabolism. The use of human-derived metabolic activation systems and correlating the mutational spectra of whole-genome sequencing to human cancer signatures are great prospects.^[44, 48]
- **Knowledge-based research on low-dose combined exposure and epidemiology:** Knowledge-based research on the adverse effects of low-concentration combined exposures in real life is lacking, particularly concerning possible synergistic interaction below individual threshold limits. Omics studies are needed in molecular epidemiology studies to disentangle N-NA cancer correlations from confounding variables such as medication use or tobacco use.
- **Analysis and Remediation Innovation NDMA** continues to receive a disproportionate amount of attention in quantitative research. There is room to improve the extraction of polar nitrosamines and to move from expensive laboratory equipment (LC-MS/MS) to cheap, portable, real-time environmental sensors (like SERS-based arrays) for aquatic testing in the field. Lastly, industrial reliance on nitrites might be greatly decreased by research into alternate food preservation techniques, such as green tea, rosemary extracts, or bacteriocins.^{[23, 25, 27][31, 42]}

10. Conclusion

Nitrosamine contamination has evolved from an isolated manufacturing defect into a complex, systemic challenge spanning the entire pharmaceutical lifecycle. What began with the 2018 valsartan crisis has revealed that these ultra-potent carcinogens can originate from API synthesis, excipient interactions, packaging materials, environmental sources, and even endogenous physiological processes. The evolution from simple, low molecular weight nitrosamines to structurally complex NDSRIs has completely changed the regulatory and analytical landscape, requiring more sophisticated risk-assessment frameworks such as the CPCA and more sensitive detection platforms like GC-MS/MS and LC-MS/MS that are capable of ppb-level quantification.

Global regulatory harmonisation by USFDA, EMA, WHO and pharmacopeial bodies has made significant progress in protecting patients, establishing robust three-step risk management protocols and structure-based acceptable intake limits. But substantial gaps in toxicological data, particularly for NDSRIs and non-volatile nitrosamines, and limited understanding of human-specific ADME pathways and low-dose combined exposure effects mean the science is far from complete.

In the future, the field should move from reactive mitigation to proactive prevention, by leveraging green chemistry principles, predictive in silico modelling, and better excipient selection at the design stage. Continued collaboration between regulators, manufacturers and researchers will be vital to fill remaining knowledge gaps, improve analytical capacity and ultimately ensure life-saving medicines remain effective and free of unacceptable carcinogenic risk.

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References

- 1) Dobariya U, Chauhan N, Patel H, Pardeshi N. Nitrosoamine Impurities: Origin, Control and Regulatory Recommendations. *International Journal of Drug Regulatory Affairs*. 2021;9(2):77-80.
- 2) Manchuri KM, Kuril AK, Shaik MA, Gopireddy VS, Sultana N. An update on latest regulatory guidelines and analytical methodologies for N-nitrosamine impurities in pharmaceutical products–2024. *Medical Gas Research*. 2025 Apr 29;15(4):535.
- 3) Tummala SR, Amgoth KP. Development of GC-MS/MS method for simultaneous estimation of four nitrosoamine genotoxic impurities in Valsartan. *Turkish Journal of Pharmaceutical Sciences*. 2022 Aug 31;19(4):455.
- 4) Manchuri KM, Shaik MA, Gopireddy VS, Gogineni S. Analytical Methodologies to Detect N-Nitrosamine Impurities in Active Pharmaceutical Ingredients, Drug Products and Other Matrices. *Chemical Research in Toxicology*. 2024 Sep 16;37(9):1456-83.
- 5) Thomas DN, Wills JW, Burman M, Williams AN, Harte DS, Buckley RA, Urquhart MW, Bretonnet AS, Jeffries B, White AT, Harvey JS. Resolution of historically discordant Ames test negative/rodent carcinogenicity positive N-nitrosamines using a sensitive, OECD-aligned design. *Mutagenesis*. 2025 Apr;40(2):116-25.
- 6) Ping Y, Liu Y, Wen H, Wen N, Li B, Chen Y, Tian Y. Determination of nitrite in rifapentine and analysis of the formation causes of nitrosamine impurity. *Front Chem*. 2026;14:1791665. doi:10.3389/fchem.2026.1791665.
- 7) Shaik RB, Padala SKR, Gupta M, Devi DR, Basavaiah K, Rao BM. Structural and in silico safety evaluation of asciminib degradation products with a validated stability indicating HPLC method for genotoxic impurity determination. *Sci Rep*. 2026;16:14965. doi:10.1038/s41598-026-44693-0.
- 8) Adamson RH, Chabner BA. The finding of N-nitrosodimethylamine in common medicines. *Toxicol Pathol*. 2020.
- 9) Salim H, Jones AM. Angiotensin II receptor blockers (ARBs) and manufacturing contamination: A retrospective National Register Study into suspected associated adverse drug reactions. *Br J Clin Pharmacol*. 2022;88:4812-4827. doi:10.1111/bcp.15411.
- 10) Regulska K, Musiał J, Stanisław BJ. Solid-State Stability Profiling of Ramipril to Optimize Its Quality Efficiency and Safety. *Pharmaceutics*.

- 2021;13:1600. doi:10.3390/pharmaceutics13101600.
- 11) Järnholm B, Lavenius B, Sällsten G. Cancer morbidity in workers exposed to cutting fluids containing nitrites and amines. *Br J Ind Med*. 1986;43:563-565.
- 12) Bolleddula J, Gopalakrishnan S, Hu P, Dong J, Venkatakrishnan K. Alternatives to rifampicin: a review and perspectives on the choice of strong CYP3A inducers for clinical drug–drug interaction studies. *Clinical and Translational Science*. 2022 Sep;15(9):2075-95.
- 13) Vogel M, Escher SE, Weiler E, Londenberger A, Deppenmeier U, Whomsley R. Quantitative Investigation of Nitrosamine Drug Substance-Related Impurities (NDSRIs) Under Artificial Gastric Conditions by Liquid Chromatography–Tandem Mass Spectrometry and Structure–Activity Relationship Analysis. *Drug Testing and Analysis*. 2025 Sep;17(9):1772-84.
- 14) Shomali F, Talebpour Z, Abedi G, Mousavi N, Mokhtarifar S, Kohandani M, Mahdavi V, Adib N. Analysis and risk assessment of nitrosamines in sartans using GC-MS and Monte Carlo simulation. *Scientific Reports*. 2025 Jun 6;15(1):20032.
- 15) Sana B, Nedjoud G, Sihem K, Hadia H, Aslam AA, Mughran MH, Alzahrani KJ, Irfan M, Ahmed M. N-Nitrosodimethylamine as an Emerging Environmental Contaminant: Sources, Analytical Advances, and Ecotoxicological and Human Health Risks. *International Journal of Analytical Chemistry*. 2026;2026(1):1536227.
- 16) Hassanin HA, Hannibal L, Jacobsen DW, El-Shahat MF, Hamza MS, Brasch NE. Mechanistic studies on the reaction between R2N-NONOates and aquacobalamin: evidence for direct transfer of a nitroxyl group from R2N-NONOates to cobalt (III) centers. *Angewandte Chemie (International ed. in English)*. 2009;48(47):8909.
- 17) Dittberner U, Eisenbrand G, Zankl H. Cytogenetic effects of N-nitrosodiethanolamine (NDELA) and NDELA-monoacetate in human lymphocytes. *J Cancer Res Clin Oncol*. 1988;114:575-578.
- 18) Beard JC, Swager TM. An organic chemist's guide to N-nitrosamines: their structure, reactivity, and role as contaminants. *The Journal of organic chemistry*. 2021 Jan 21;86(3):2037.
- 19) Knasmüller S, Stehlik G, Mohn G. Studies on the metabolic activation of diethanolnitrosamine in animal-mediated and in vitro assays using *Escherichia coli* K-12 343/113 as an indicator. *J Cancer Res Clin Oncol*. 1986;112:266-271.
- 20) Monarca S, Scassellati Sforzolini G, Spiegelhalder B, Pasquini R, Fatigoni C. Monitoring nitrite, N-nitrosodiethanolamine, and mutagenicity in cutting fluids used in the metal industry. *Environ Health Perspect*. 1993;101:126-128.
- 21) Knasmüller S, Stehlik G, Mohn G. Studies on the metabolic activation of diethanolnitrosamine in animal-mediated and in vitro assays using *Escherichia coli* K-12 343/113 as an indicator. *Journal of cancer research and clinical oncology*. 1986 Nov;112(3):266-71.
- 22) Monarca S, Scassellati-Sforzolini G, Donato F, Angeli G, Spiegelhalder B, Pasquini R. Biological monitoring of workers exposed to N-nitrosodiethanolamine in the metal industry.
- 23) Lee SH, Kim HY. Effect of sodium nitrite, sodium chloride and concentrated seawater on physicochemical properties of meat emulsion system. *Food Sci Anim Resour*. 2020;40(6):980-989. doi:10.5851/kosfa.2020.e68.
- 24) Xu N, Goodrich LE, Lehnert N, Powell DR, Richter-Addo GB. Five- and six-coordinate adducts of nitrosamines with ferric porphyrins: structural models for the type II interactions of nitrosamines with ferric cytochrome P450. *Inorganic chemistry*. 2010 May 17;49(10):4405.
- 25) Cha KH, Wang X, Meyerhoff ME. Nitric Oxide Release for Improving Performance of Implantable Chemical Sensors – A Review. *Appl Mater Today*. 2017;9:589-597. doi:10.1016/j.apmt.2017.10.002.
- 26) Giménez-Campillo C, Pastor-Belda M, Campillo N, Hernández JD, Guillén I, Vizcaíno P, López-García I, Hernández-Córdoba M, Arroyo-Manzanares N, Viñas P. Ultrasound assisted extraction approach to test the effect of elastic rubber nettings on the N-nitrosamines content of Ham meat samples. *Foods*. 2021 Oct 24;10(11):2564.
- 27) Piknova B, Schechter AN. Measurement of Nitrite in Blood Samples Using the Ferricyanide-Based Hemoglobin Oxidation Assay. *Methods Mol Biol*. 2011;704:39-56. doi:10.1007/978-1-61737-964-2_4.
- 28) EFSA Panel on Contaminants in the Food Chain (EFSA CONTAM Panel), Schrenk D, Bignami M, Bodin L, Chipman JK, del Mazo J, Hogstrand C, Hoogenboom L, Leblanc JC, Nebbia CS, Nielsen E. Risk assessment of N-nitrosamines in food. *Efsa Journal*. 2023 Mar;21(3):e07884.
- 29) EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), Mortensen A, Aguilar F, Crebelli R, Di Domenico A, Dusemund B, Frutos MJ, Galtier P, Gott D, Gundert-Remy U, Lambré C. Re-evaluation of potassium nitrite (E 249) and sodium nitrite (E 250) as food additives. *Efsa journal*. 2017 Jun;15(6):e04786.
- 30) Tabish TA, Hussain MZ, Zervou S, Myers WK, Tu W, Xu J, et al. S-nitrosocysteamine-functionalised porous graphene oxide nanosheets as nitric oxide delivery vehicles for cardiovascular applications. *Redox Biol*. 2024;72:103144. doi:10.1016/j.redox.2024.103144.
- 31) Cha KH, Wang X, Meyerhoff ME. Nitric Oxide Release for Improving Performance of Implantable Chemical Sensors – A Review. *Appl Mater Today*. 2017;9:589-597. doi:10.1016/j.apmt.2017.10.002.
- 32) Vogel M, Escher SE, Weiler E, Londenberger A, Deppenmeier U, Whomsley R. Quantitative Investigation of Nitrosamine Drug Substance-Related Impurities (NDSRIs) Under Artificial Gastric Conditions by Liquid Chromatography–Tandem Mass Spectrometry and Structure–Activity Relationship Analysis. *Drug Testing and Analysis*. 2025 Sep;17(9):1772-84.
- 33) Yalamanchili J, Himavathi G, Suresh S. A Rapid and Sensitive LC–MS/MS Method for Quantification of NDSRI in Melatonin Drug Product via Controlled In Situ Formation. *Biomedical Chromatography*. 2026 Oct;40(10):e70591.
- 34) Zhao C, Xia Y, Shi Q, Gu Y, Zhang L, Dong R, Zhang Y, Han S, Jiang Y, Xiong W, Guo H. Mediation of sphingolipid metabolism on the relationship between human nitrosamines exposure and esophageal cancer risk. *Scientific Reports*. 2026 Apr 13.
- 35) Nardin T, Franceschini J, Martinelli F, Franciosi E, Larcher R. Comprehensive Assessment of nitrosamine formation in meat products using UHPLC-HRMS: analytical challenges and potential dietary implications. *Molecules*. 2025 Oct 16;30(20):4107.
- 36) Heflich RH, Bishop ME, Mittelstaedt RA, Yan J, Guerrero SK, Sims AM, Mitchell K, Moore N, Li X, Mei N, Elespuru RK. Optimizing the detection of N-nitrosamine mutagenicity in the Ames test. *Regulatory Toxicology and Pharmacology*. 2024 Nov 1;153:105709.
- 37) Bondonno CP, Sundqvist ML, Shinde S, Croft K, Hodgson JM, Carlström M, et al. A clinical study examining the effects of dietary nitrate on urinary N-nitrosamines. *Am J Clin Nutr*. 2026;123:101239. doi:10.1016/j.ajcnut.2026.101239.
- 38) Thomas R, Tennant RE, Oliveira AA, Ponting DJ. What makes a potent nitrosamine? Statistical validation of expert-derived structure–activity relationships. *Chemical research in toxicology*. 2022 Oct 27;35(11):1997.
- 39) Sallan S, Yilmaz Oral ZF, Kaya M. A Review on the Role of Lactic Acid Bacteria in the Formation and Reduction of Volatile Nitrosamines in Fermented Sausages. *Foods*. 2023;12:702. doi:10.3390/foods12040702.
- 40) Brender JD, Werler MM, Shinde MU, Vuong AM, Kelley KE, Huber JC Jr, et al. Nitrosatable drug exposure during the first trimester of pregnancy and selected congenital malformations. *Birth Defects Res A Clin Mol Teratol*. 2012;94(9):701-713. doi:10.1002/bdra.23060.
- 41) EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS). Re-evaluation of potassium nitrite (E 249) and sodium nitrite

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- (E 250) as food additives. *EFSA Journal*. 2017;15(6):4786. doi:10.2903/j.efsa.2017.4786.
- 42) Sana B, Grara N, Khadri S, Hemmami H, Aslam AA, AL Mughram MH, et al. N-Nitrosodimethylamine as an Emerging Environmental Contaminant: Sources, Analytical Advances, and Ecotoxicological and Human Health Risks. *Int J Anal Chem*. 2026;2026:1536227. doi:10.1155/ianc/1536227.
- 43) Tabish TA, Hussain MZ, Zervou S, Myers WK, Tu W, Xu J, et al. S-nitrosocysteamine-functionalised porous graphene oxide nanosheets as nitric oxide delivery vehicles for cardiovascular applications. *Redox Biol*. 2024;72:103144. [Duplicate of uploaded article 30].
- 44) Knasmüller S, Stehlik G, Mohn G. Studies on the metabolic activation of diethanolnitrosamine in animal-mediated and in vitro assays using *Escherichia coli* K-12 343/113 as an indicator. *J Cancer Res Clin Oncol*. 1986;112:266-271.
- 45) de Jong S, Nosal DG, Wardrop DJ. Methods for direct alkene diamination, new & old. *Tetrahedron*. 2012;68(22):4067-4105. doi:10.1016/j.tet.2012.03.036.
- 46) Gankhuyag N, Lee KH, Cho JY. The Role of Nitrosamine (NNK) in Breast Cancer Carcinogenesis. *J Mammary Gland Biol Neoplasia*. 2017;22:159-170. doi:10.1007/s10911-017-9381-z.
- 47) Darvishmotevalli M, Moradnia M, Noorisepehr M, Fatehizadeh A, Fadaei S, Mohammadi H, et al. Evaluation of carcinogenic risks related to nitrate exposure in drinking water in Iran. *MethodsX*. 2019;6:1716-1727. doi:10.1016/j.mex.2019.07.008.
- 48) Huber JC Jr, Brender JD, Zheng Q, Sharkey JR, Vuong AM, Shinde MU, et al. Maternal dietary intake of nitrates, nitrites and nitrosamines and selected birth defects in offspring: a case-control study. *Environ Health*. [Publication details as provided in uploaded article].
- 49) Perlman DH, Bauer SM, Ashrafian H, Bryan NS, Garcia-Saura MF, Lim CC, et al. Mechanistic Insights into Nitrite-Induced Cardioprotection Using an Integrated Metabonomic-Proteomic Approach. *Circ Res*. 2009;104(6):796-804. doi:10.1161/CIRCRESAHA.108.187005.