QUALIMETRIX
Analytical Laboratories - Quality Control

Nitrosamines in pharmaceuticals

by QUALIMETRIX

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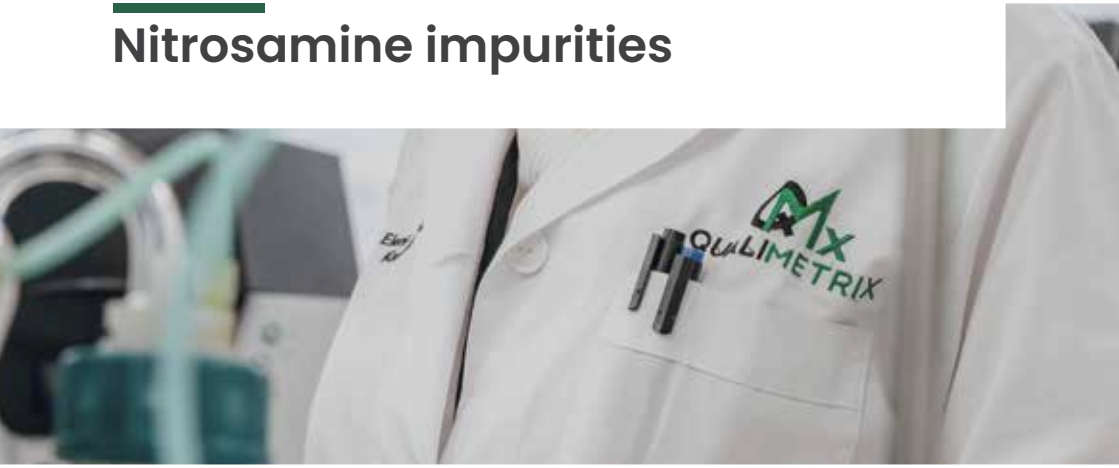
Nitrosamine impurities



In 2018, nitrosamine impurities, including N-nitrosodimethylamine (NDMA), were found in a number of blood pressure medicines known as 'sartans'. This led to some product recalls and to a regulatory review, which set strict new manufacturing requirements for these medicines. Subsequently, a nitrosamine impurity has been detected in batches of ranitidine, a medicine used to treat heartburn and stomach ulcers, and the Agency's Committee for Medicinal Products for Human Use (CHMP) has started a review.

Nitrosamines (NAs) are chemical compounds classified as probable human carcinogens on the basis of animal studies. However, there is a very low risk that nitrosamine impurities at the levels found could cause cancer in humans. In September 2019, a 'call for review' was launched for medicinal products containing chemically synthesised active pharmaceutical ingredients (APIs) to request MAHs to review their manufacturing processes in order to identify and, if necessary, mitigate the risk of presence of nitrosamine impurities and report the outcome back to authorities. This exercise was started while the review by CHMP under Article 5(3) for Nitrosamine impurities in human medicinal products was ongoing.

Following the conclusion of the review under Article 5(3), the CHMP considered that there is also a risk of presence of nitrosamines in biological medicinal products, in particular for the biological medicines with the following risk factors:

- biologicals containing chemically synthesised fragments, where risk factors similar to chemically synthesised active substances are present;
- biologicals using processes where nitrosating reagents are deliberately added;
- biologicals packaged in certain primary packaging material, such as blister packs containing nitrocellulose.

For the above reasons the call for review was extended to include also all biological medicinal products for human use. The call for review consists of the steps depicted in the following figure:

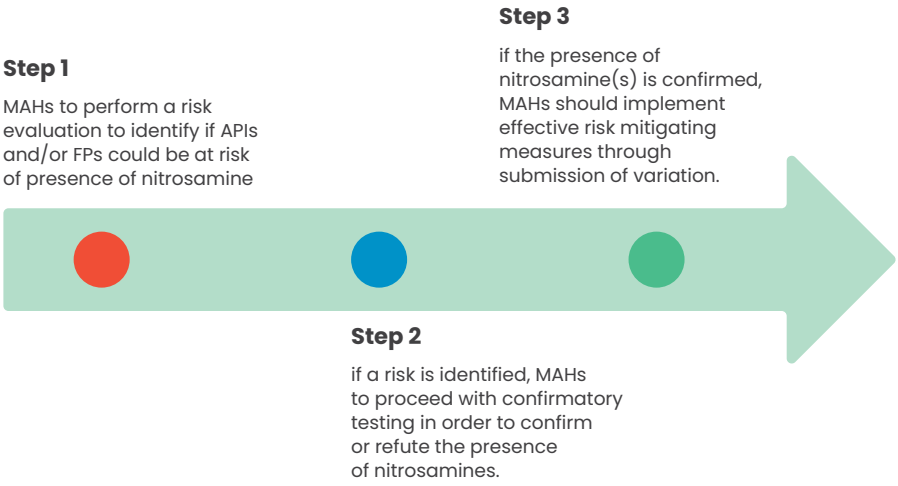


Figure 1: "Call for review" process

The following sections aim to provide a summary of the theoretical background along with an overview of the strategy applied by QMx to address the complexities inherent to the assessment of the risk for nitrosamine presence in human medicinal products

Nitrosamine formation and potential sources

In general, the formation of N-nitrosamines is only possible in the presence of a secondary or tertiary amine and nitrite or other nitrosating agent, usually under acidic reaction conditions. Additionally, the presence of impurities that can't be formed as part of the process, based on the conditions used, can be explained to an extent by cross-contamination and/or the use of recovered solvents or equipment contaminated with N-nitrosamines formed outside of the declared synthetic process. An indicative list of common nitrosating agents and classes of amines that can be nitrosated is given the in following figure:

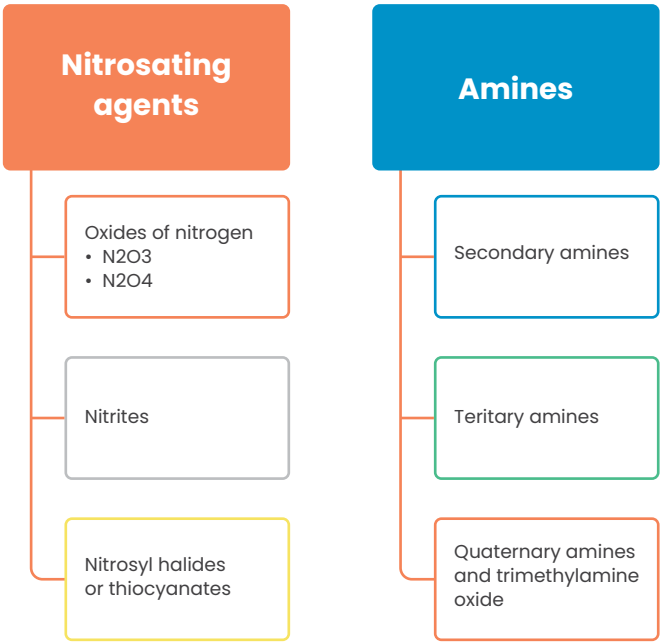


Figure 2: Amines and Nitrosating agents

Formation of the most common types of N-Nitrosamines

For the formation of N-Nitrosodimethylamine (NDMA), the presence of the secondary amine dimethylamine (DMA) is important. A possible route to the formation of DMA is the decomposition of dimethylformamide (DMF) at high temperature to DMA as depicted in Figure 3.

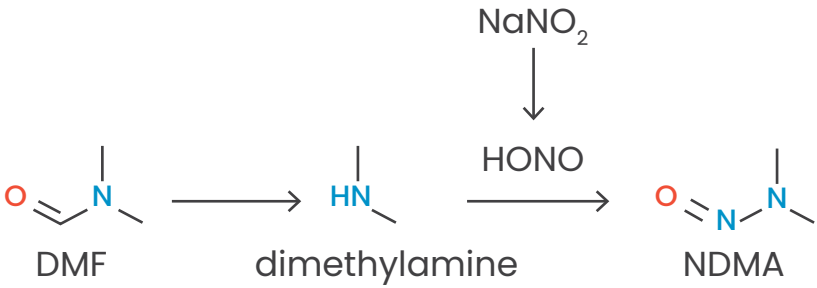


Figure 3: Formation of NDMA from DMF

An alternative possibility is that DMA is present as an impurity in DMF since it is a precursor in the industrial DMF synthetic process. It may also be a degradant formed during storage of the solvent, potentially present as the formate salt. N-Nitrosodiethylamine (NDEA) may be generated from diethylamine (DEA) by analogy to the formation of NDMA from DMA (Figure 4).

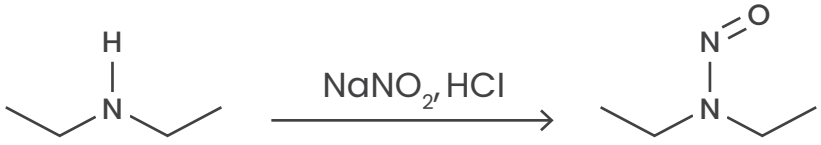


Figure 4: Formation of NDEA from DEA

Analogous to DMA formation, DEA could be formed by degradation of triethylamine (TEA) or exist as impurity in TEA raw material.
Alternatively, direct nitrosation of TEA may occur via a nitrosoiminium ion, resulting in the generation of an aldehyde and a secondary amine,1 which reacts with further nitrous acid to form a nitrosamine (Figure 5).

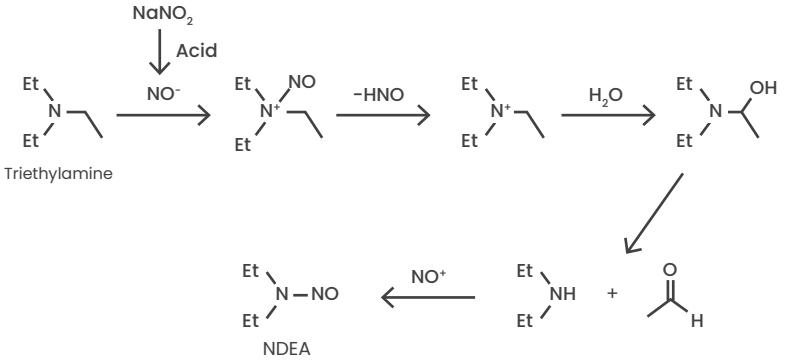
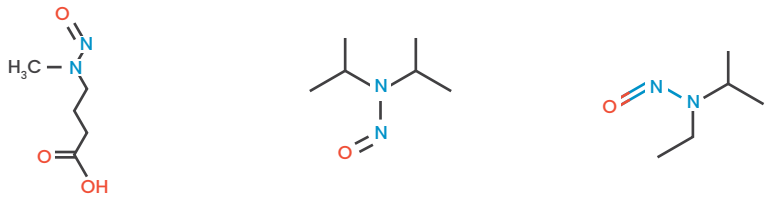


Figure 5: Nitrosative cleavage of TEA to DEA followed by nitrosation to NDEA

Potential contamination with other N-nitrosamines is also possible. Such impurities could be generated if different sources of secondary or tertiary amine are present at the same time as nitrite. Some common organic solvents (e.g. NMP which could give rise to 4-(methyl)(nitroso)amino)butanoic acid = NMBA) and amine bases (e.g. diisopropylamine = DIPEA which could give rise to N-Nitrosodiisopropylamine (DIPNA) and N-Nitrosoethylisopropylamine (EIPNA)) would present such risks. This list of amine-derived solvents and bases is not exhaustive. Therefore, all other potential sources of N-nitrosamines should be taken into account during the review of the processes.



4-(methyl)(nitroso)amino)butanoic acid (NMBA)

N-Nitrosodiisopropylamine (DIPNA)

N-nitrosoethylisopropylamine (EIPNA)

Figure 5: Nitrosative cleavage of TEA to DEA followed by nitrosation to NDEA

The EMA has recently published an updated Q&A document[1] (28 July 2023) entitled **Questions and answers on "Information on nitrosamines for marketing authorization holders"**. According to the answer in Question 4: *"What are the currently identified root causes for presence of nitrosamines?"* the root causes for N-nitrosamines in medicinal products can be grouped as risk factors linked exclusively with the manufacturing process and storage of active substance and/or as risk factors associated with manufacture and storage of the finished product. Moreover, there are risk factors specifically linked to GMP aspects.
The following figure summarizes the main factors that should be considered in the frame of the risk assessment exercise:

Risk factors related to the manufacture of the active substance

- Presence of nitrosating agents (or precursors) and "nitrosatable" amines (or precursors) under favorable conditions to form nitrosamines
- Use of contaminated materials (e.g. starting materials, intermediates, solvents, reagents, etc.)

Risk factors also related to the finished product

- Presence of "nitrosatable" amines in API or impurities and nitrosating agents in components of the Final product (e.g. excipients)
- Degradation process of API
- Packaging materials

Risk factors related to GMP aspects

- Cross-contamination
- Carry-over of impurities and use of contaminated or recycled materials

Figure 7: Potential sources of nitrosamines

[1] *EMA/409815/2020 Rev.17 Corr.**

One of the major risk factors, is the reaction of nitrosatable nitrogen functionality in APIs or their impurities/degradants with nitrosating agents that are present in components of the FP during formulation or storage. A particular risk of formation of nitrosamines exists for active substances that contain a nitrosatable amine functional group. Several examples have been reported where the amine functionality was shown to be vulnerable to nitrosation and formation of the corresponding N-nitroso impurity (i.e. NO-API). Secondary amines appear particularly vulnerable to this reaction although some cases with tertiary amines have also been observed.

Vulnerable amines could also be formed by degradation (e.g. hydrolysis) during formulation or storage. Nitrites have been identified as impurities in many common excipients. To this end, MAHs should be aware that N-nitroso API impurities can form at levels exceeding the AI even if nitrite levels in the excipients are very low. As it has been reported that N-nitroso impurities can form from APIs or their impurities/degradants (containing amine functionality or susceptible to degradation to reveal amines) during manufacture of the finished product, as well as during storage, the stability of the finished product should be considered in order to ensure that the AI of any N-nitrosamine impurity is not exceeded until the end of shelf life of the FP.

QMx stepwise approach & strategy

Qualimetrix is a customer-driven CRO that employs a set of quality management tools in order to design and implement optimized processes with the aim of transforming customer inputs and requirements into "customer value". As such, the first and probably the most critical factor for a successful project is its proper definition in terms of both customer and technical requirements. To this end, a comprehensive study request form is provided to the customer with the following objectives:

- The definition of the type and scope of the study
- The provision of critical product information
- The determination of the most suitable, expedient and cost-effective approach

The source of the "sartan" contamination appears to be an understudied and unvalidated production change, resulting in the creation of excess quantities of nitrosamine contaminants, exacerbated by three other factors:

- A complex and poorly understood supply chain
- Lax QC testing on incoming raw materials, including solvents
- Ineffective cleaning or cleaning validation testing.

In addition to the above, the fact that it is not yet agreed upon whether the nitrosamines found in ranitidine and nizatidine is a contamination problem similar to that of the "sartans", or if it is a degradation issue (either in packaging or in the body) further highlights the multivariate nature of nitrosamine contamination and the necessity for applying 'due diligence' during the risk assessment exercise.

Qualimetrix can assist MAHs as well as other parties involved in the supply chain of a pharmaceutical product in properly addressing the "nitrosamine issue" by fully undertaking the completion of the first two steps outlined above, namely the risk evaluation and confirmatory testing. However, prior to proceeding with confirmatory testing that is often intended to cover a large number of diverse and in many cases unlikely to occur, nitrosamine species, it is recommended to follow the process outlined in figure 8 by answering a number of questions aiming to refine and focus the testing process at what is really important. The suggested scheme is mainly applicable to the so-called **Nitrosamine Drug Substance Related Impurities (NDSRIs)** due to their increased probability of occurrence and the lack of compound-specific limits. According to the work of Schlingemann et al. [2] who performed an in-silico analysis of more than 12,000 small molecule drugs and their related impurities, approximately 40% of the analyzed APIs and 30% of the API impurities are potential nitrosamine precursors.

The **first three questions** of the scheme are addressed in the frame of the risk assessment process. In case that the outcome of the latter is that there is indeed a risk of nitrosamine formation the **following four questions** should also be answered prior to proceeding with confirmatory testing.

As noted above, in case that the identified species correspond only to well-known nitrosamine species with established limits and their estimated levels are not well below 10% of their acceptable intake (AI), then one should proceed directly to confirmatory testing.

[2] *Journal of Pharmaceutical Sciences, 112 (2023), 1287-1304*

Regarding the option to "waive" confirmatory testing, based on a "fate and purge" rationale (i.e. estimated levels well below 10%) it should be borne in mind that, according to ICH M7, an Option 4 control strategy should be based on thorough process understanding and confidence regarding the estimated levels of nitrosamines. This is reflected in both EMAs assessment report[3] and FDA's *"Stakeholder Questions for May 4th FDA-Industry Meeting to Discuss Nitrosamine Impurities in Pharmaceuticals[4]"* which state that theoretical purge calculations should be confirmed with analytical data for nitrosamines in order to provide confidence that there is negligible risk that the impurities will be present above AI.

The updated Q&A No. 10 regarding the limits that should be applied to new nitrosamines (i.e. nitrosamines with no established AI), has introduced two very important tools that significantly facilitate the proper assessment of the related safety risks and the selection of the most appropriate limit. The first one is the **Carcinogenic Potency Categorization Approach (CPCA)** for assigning an N-nitrosamine impurity (including NDSRIs) to a predicted carcinogenic potency category, with a corresponding acceptable intake (AI) limit, based on an assessment of activating (i.e. associated with an increase in carcinogenic potency) or deactivating (i.e. associated with a decrease in carcinogenic potency) structural features present in the molecule. The second tool is the **enhanced Ames test** that allows control of a N-nitrosamine at 1.5 µg / day in case that the result is negative. The same tools are described in FDA's guidance "Recommended Acceptable Intake Limits for Nitrosamine Drug Substance-Related Impurities (NDSRIs) Guidance for Industry – August 2023[5]" with the only difference being in the recommended AI for Carcinogenic Potency Category 1 (i.e. **26.5 ng/day**) which is based on the most potent, robustly tested nitrosamine, N-nitrosodiethylamine (NDEA) vs the class-specific TTC for nitrosamine impurities (i.e. **18 ng/day**) adopted by the EMA.

[3] EMA/369136/2020
[4] FDA Stakeholder Questions
[5] Recommended Acceptable Intake Limits for NDSRIs

To this end, once all possible nitrosamine species have been identified (excluding "well-known") and their worst-case levels estimated, the structure-activity relationship concepts of the CPCA can be applied in order to conclude on the potency category of each species and evaluate their content against the corresponding AI. In case that the estimated value of the content exceeds 10% of the latter there are **two additional questions** that should be addressed as a safeguard against unnecessary of analytically challenging confirmatory testing. The latter is especially true in the case of NDSRIs considering that, so far, the Pharmacopoeias do not offer any NDSRI compendial standards. Despite the fact that the coverage with commercial standards has increased over the past months, it is far from adequate or complete. It is estimated that only approximately 5% of all potential NDSRIs are currently available. Moreover, many of the offered compounds are not in stock but will only be synthesized upon request. The problem is augmented by the fact that in many cases all efforts to synthesize the NDSRI are unsuccessful or the provided standard is extremely labile and therefore not amenable to analytical testing.

According to the guidance provided on how confirmatory tests should be conducted (Q&A No. 8):

> *"If, despite extensive efforts, it becomes apparent that the relevant nitrosamine impurity cannot be synthesised, then this could be an indication that the nitrosamine either does not exist or that there is no risk of it being formed. In such cases, it may not be necessary to conduct confirmatory testing. This should be justified thoroughly on a case-by-case basis according to appropriate scientific principles. The justification could include relevant literature, information on structural/stereo-electronic features and reactivity of the parent amine, stability of the nitrosamine and experimental data to illustrate the efforts made to synthesise and to analyse the impurity".*

The IQ consortium has established a set of three conditions that represent a worst case for drug substance and drug product manufacturing scenarios with trace nitrite contamination. These three conditions:

i. use an excess of nitrosating agent,

ii. are orthogonal (i.e. use inorganic and organic nitrite),

iii. performed at room temperature to avoid the known nitrite decomposition at elevated temperature,

iv. are in solution phase and

v. any potential N-nitrosamine formation is assessed down to 0.5% using MS detection.

To this end, considering the comprehensiveness of the three conditions, the absence of formation of a N-nitrosamine from the corresponding amine precursor leads to the conclusion that the drug substance and / or drug product is free from any N-nitrosamine risk.
QMx has extensive experience in the application of the workflow described above and the confirmation of the expected structure, especially in cases where multiple stage MS (MSn) and / or additional techniques (e.g. NMR) need to come into play in order to elucidate structural isomers.

However, in case that the hypothesized species (most often a NDSRI) can be formed and remain stable, then the enhanced Ames test could serve as a useful tool, prior to confirmatory testing e.g. in situations where there are valid reasons to believe that the CPCA tool results in a potency category that does not reflect its actual carcinogenic potency and a negative Ames outcome is likely. In case this is confirmed and provided that the estimated worst-case daily intake is below 150 ng (i.e. 10% of 1500 ng/day applicable for species testing negative in a GLP-compliant enhanced Ames test), confirmatory testing could be waived.

QMx has extensive experience in conducting GLP Ames testing according to OECD's Test Guideline No. 471 and the Enhanced Testing Scheme (EAT), recently proposed by CMDh, which significantly improves the sensitivity of the test for N-nitrosamines.

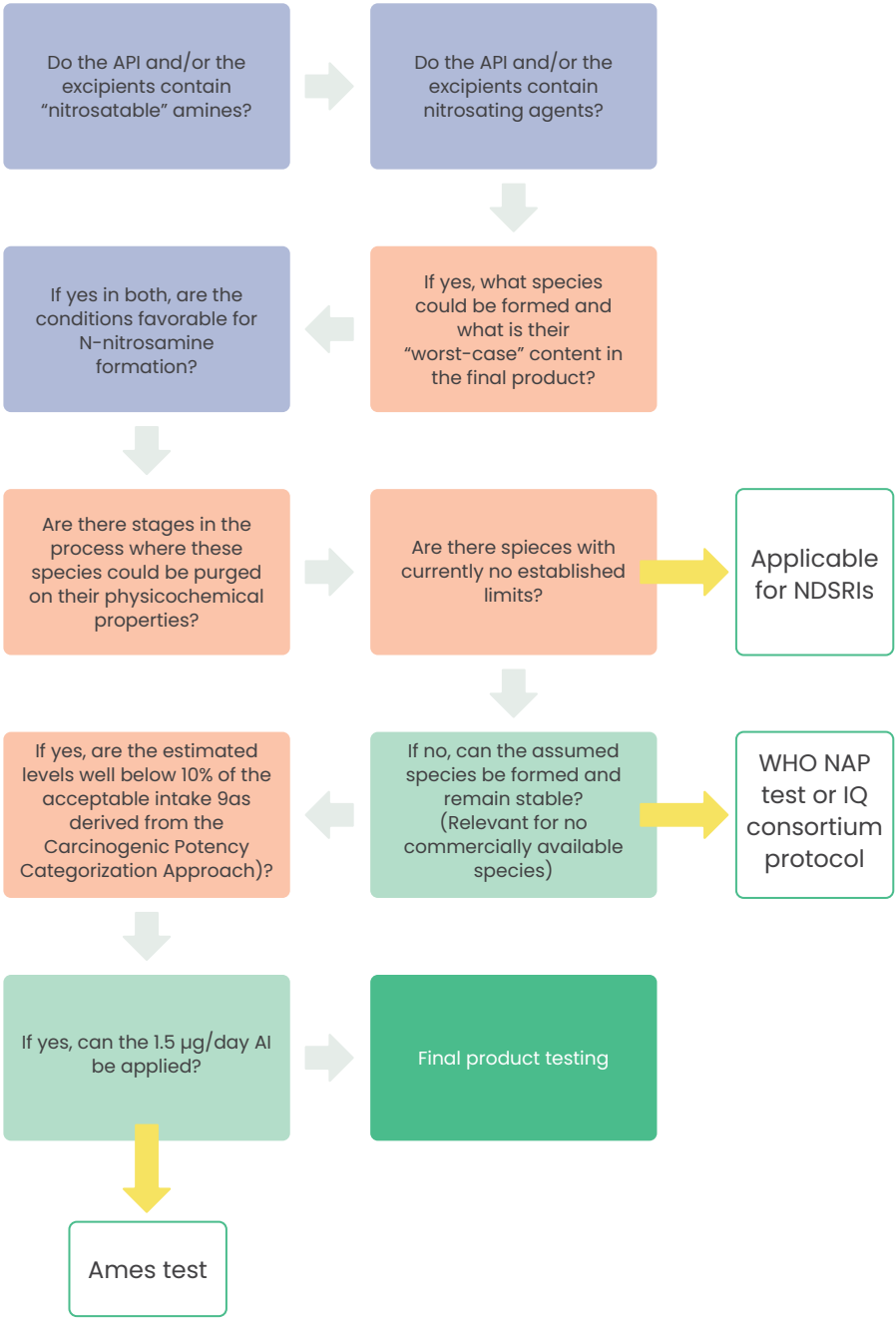


Figure 8: Questions to answer prior to confirmatory testing

Risk Assessment Strategy

The model employed for the Risk Assessment exercise is based on the Risk Ranking and Filtering method. Risk ranking and filtering is a tool for comparing and ordering risks. The Risk ranking of complex systems involves the evaluation of multiple diverse quantitative and qualitative factors for each risk. The tool employed involves breaking down the basic risk question:

"Is there a possibility of N-nitrosamines being present in the final product?"

into the number of components required to capture factors involved in the risk. These factors that are closely related with the basic risk question and determine the probability of nitrosamine presence in the final product are graphically depicted by means of an Ishikawa (Fishbone) diagram in Figure 9 below.

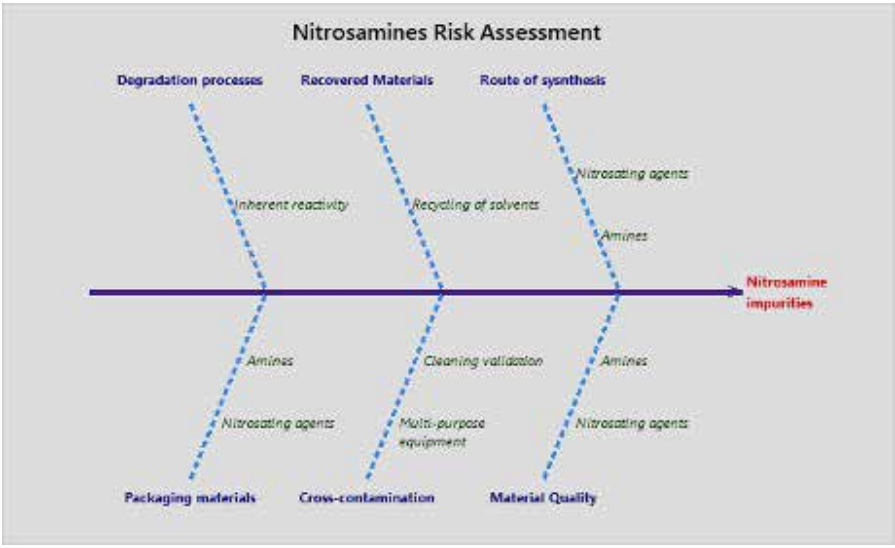


Figure 9: Sources of nitrosamines

In order to properly address each of the risk factors listed above, all relevant information pertaining to the potential formation of nitrosamine impurities and the potential for cross-contamination should be provided by all the involved manufacturers and suppliers.

MAHs are responsible for the quality, safety and efficacy of their products, including the quality of the APIs, excipients and raw materials used in the manufacture of finished products.

MAHs should therefore ensure (via quality agreements) that they and the holder of the manufacturing authorisation have access to relevant information from the API manufacturers concerning potential formation of nitrosamine impurities and the potential for cross-contamination.

To this end, a questionnaire developed in line with EMA's "Information on nitrosamines for marketing authorisation holders" is provided to the corresponding MAH in order to obtain the information and data that is essential for performing the risk assessment and evaluation. In case that information by the involved parties is not available or provided, alternative sources are sought in the publicly available scientific literature.

The filled questionnaire serves as the basis for assigning risk scores to each of the risk factors based on the combination of different response scenarios. Each of these scenarios are graded with a numerical value reflecting the level of associated risk and a weight factor to account for these factors that are associated with a higher probability of resulting in the presence of nitrosamine impurities in the final product. An indicative scenario for one of the risk factors along with the corresponding risk scores is presented in Table 1 below.

Table 1: Risk Calculation for "Cross-contamination"

Cross-contamination		Impact of potential risk: Choose presented risk and enter corresponding value into the Risk column (R)				Weight factor
Question	Risk questions / Failure modes	10	8	5	3	1.5
11 12 13 14	.....? .....? .....? .....?	If “Yes” in questions 11, 12 & 13	If "Yes" in question 11 and either question 12 or 13	a) If "Yes" in question 11 and "No" in questions 12, 13 & 14 b) If information is not available	a) If "No" in question 11 b) If "Yes" in questions 11 & 14 and "No" in questions 12 & 13	

The rationale for the selection of the values employed for “quantifying” the risk is based on the fact that the root cause for the nitrosamine contamination is associated with a high degree of uncertainty. While the root cause in the original “sartans” contamination is believed to have been identified, in the case of ranitidine and nizatidine, the source appears much more difficult to track down and characterize, meaning that it might not be easily solvable.

Based on the above, a conservative approach has been applied with respect to the risk scores. Two values are employed to characterize a "**High risk**", one value to represent a "**Medium risk**" and one value for the "**Low Risk**". A "Medium risk" score is assigned in cases where no relevant information is available for answering each of the risk questions. The values along with their associated risk level are presented in Table 2 below.

Table 2: Risk Levels and scores

Risk Level	Risk score
High	10
High	8
Medium	5
Low	3

The final risk score is calculated as the sum of the weighted risk scores for each of the 6 identified Risk Factors divided by the sum of their corresponding weights, according to the formula provided below:

$$Final\ Risk\ Score = \frac{(wf_1 \times RFS_1) + (wf_2 \times RFS_2) + (wf_3 \times RFS_3) + (wf_4 \times RFS_4) + (wf_5 \times RFS_5) + (wf_6 \times RFS_6)}{wf_1 + wf_2 + wf_3 + wf_4 + wf_5 + wf_6}$$

where,

wf_n: Weight factor associated with each Risk factor ranging from 1.0 to 2.0

RFS_n: Risk Score assigned to each of the Risk Factors ranging from 3 to 10

A Final Risk Score that is equal to or greater than **5.0** signifies the existence of a significant risk of presence of nitrosamines in the final product and that confirmatory testing should be carried out using appropriately validated and sensitive analytical methods.

A final risk score of **3.0** would result into an acceptable risk decision while a score between 3.0 and 5.0 should be assessed with the appropriate level of "due diligence" and the decision for accepting such a risk without confirmatory testing should be based on a sound scientific rationale. The Risk evaluation table is presented below.

Table 3: Risk Evaluation

Final Risk Score	Risk Level	Actions
Risk score ≥ 5.0	High (Unacceptable)	Confirmatory testing
3.0 > Risk score > 5.0	Medium ("Borderline")	Confirmatory testing or strong justification for waiving the need for testing
Risk score = 3.0	Low (Acceptable)	Confirmatory testing can be waived

The calculated risk addresses the direct contribution of the starting materials, intermediates, raw materials (solvents, reagents, catalysts etc.) and finished drug product manufacturing processes (multipurpose equipment, recycling solvents etc.) in N-nitrosamine impurities while also the indirect contribution of the aforementioned factors in nitrosamine precursors which in the course of finished drug product manufacturing could result in N-nitrosamine in-process formation.

The control strategy is evaluated in terms of the existent risk mitigating actions which include but are not limited to:

1. Quality management procedures / GMP measures such as on-site audits of the involved suppliers, excipient specifications, regulatory assessments disclosure (Master Files), technical agreements, cleaning procedures and environmental monitoring.

2. Factors related to nitrosamine formation chemistry and parameters such as inherent amine reactivity, manufacturing conditions, steric factors, stoichiometry, purification steps that can reduce the levels of nitrosamines.

The final outcome of the risk evaluation process consists of the following elements:

- Nitrosamine species that could potentially be formed for which the current control strategy is not adequate and therefore confirmatory testing should be performed.

- Additional risk mitigation measures that could further reduce or eliminate the presence of nitrosamine impurities in the final product.

Determination of Nitrosamines – Confirmatory and Routine Testing

Both the European Medicines Agency (EMA) and the U.S Food and Drug Administration (FDA) have issued guidelines and requirements regarding the determination of nitrosamines in pharmaceutical products. These can be summarized in the following figure.

EMA	FDA
Validated quantitative methods	Validated quantitative methods
Sensitivity (i.e. LOQ ≤ 10% of the acceptable limit)	Sensitivity (i.e. LOQ ≤ 0.03 ppm - If more than one nitrosamine is detected or the MDD exceeds 880 mg/day the LOQ should be lower…)
Selectivity if the same method is employed for the determination of multiple nitrosamines	Specificity
	Chromatographic separation

Figure 10: Analytical method requirements

EMA's Assessment Report highlights the following technical aspects that need to be carefully considered during the development of analytical methods, given the trace levels of nitrosamines that need to be measured:

- Interference caused by presence of trace amounts of nitrosamines in testing materials utilised (e.g. water, airborne sources, plastics products and rubber/elastomeric products);
- Contamination during sample preparation (avoiding cross contaminations from gloves, membranes, solvents etc.) which could lead to false positive results;
- In situ formation of nitrosamines during analysis;

- Use of accurate mass techniques are required (MS/MS or high-resolution accurate mass systems) in order to overcome interference in the identification of the specific peak of a certain nitrosamine (e.g. false positives have been observed from DMF co-eluting with NDMA).

The analysis of ppm level, low-molecular weight nitrosamine species in pharmaceuticals with an often burdened and complex matrix without a properly developed and validated quantitative method could lead to inaccurate results. The following are characteristic cases of interference and in situ formation reported in the literature.

Keire's[6] team at the U.S. FDA demonstrated in detail that when NDMA in metformin was analyzed, DMF co-eluted with NDMA. Without sufficient mass accuracy in data acquisition or sufficient mass tolerance in data analysis, the quantity of NDMA could be overcalculated.

Lee et al.[7] reported that artifactual NDMA levels in ranitidine, nizatidine, and metformin markedly increased with incubation temperature and time. Under the high-temperature chromatographic condition, these pharmaceuticals can immediately convert to NDMA even when directly injected. Furthermore, the US FDA claimed that GC approaches are problematic in ranitidine NDMA analyses because ranitidine is thermally unstable[8].

Hitherto, the most efficient way to detect Nitrosamines is provided by GC-MS and LC-MS, enabling LODs and LLOQs in the lower ppb range. Whilst GC-MS measurements span the analytical range of low molecular and volatile compounds, like NDMA, LC-MS approaches facilitate the detection of moderate and/or highly polar, non-volatile NAs. Nonetheless, LC-MS is applicable to both groups, making it favorable for multi-target screening procedures. Enrichment steps via Solid Phase Extraction (SPE) prior to analysis are described and allow the trace determination in the ng per liter range. The most efficient adsorption resins, in particular for challenging NDMA, are charcoal based materials. However, a major challenge of low trace multi-target approaches in pharmaceutical analysis is to reduce the burden of matrix, in particular excess of API and additives, by simultaneously mitigating any detrimental impact on the analytes by losses during work-up or ion suppression.

[6] *The AAPS Journal (2020) 22: 89 DOI: 10.1208/s12248-020-00473-w*
[7] *Anal. Methods 13 (2021) 3402–3409*
[8] *https://www.fda.gov/media/130801/download*

Considering the above, QMx has established an analytical control strategy to ensure the suitability of the method employed that can be summarized in the following figure

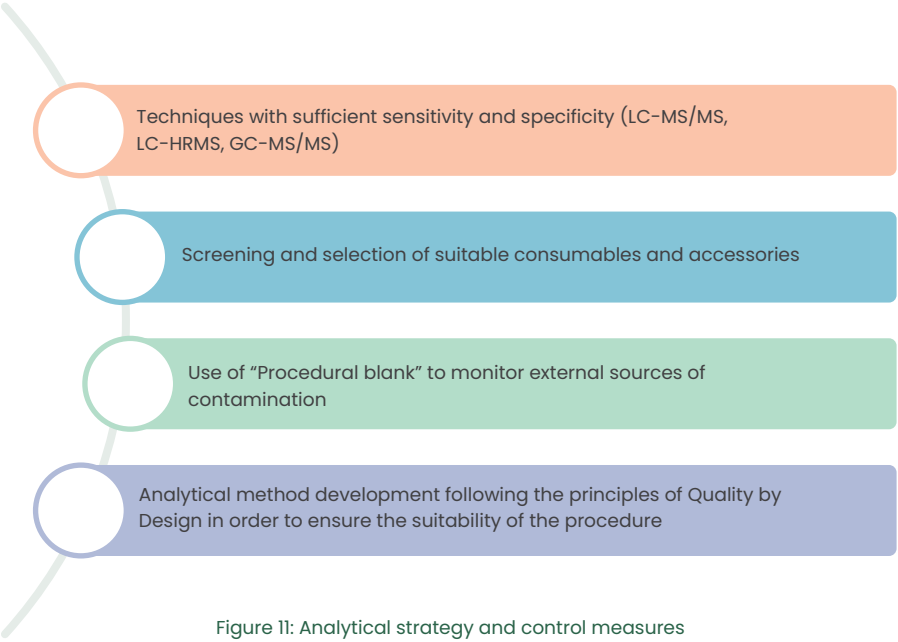


Figure 11: Analytical strategy and control measures

The design and development of an analytical method is the most critical stage during its lifecycle. The procedure design encompasses procedure development, which consists of the analytical technology and sample preparation. It includes understanding that is gained through knowledge gathering, systematic procedure development experiments, and risk assessments and associated lab experiments. The basic pillars constituting an analytical methodology for the determination of NAs are depicted in Figure 12.

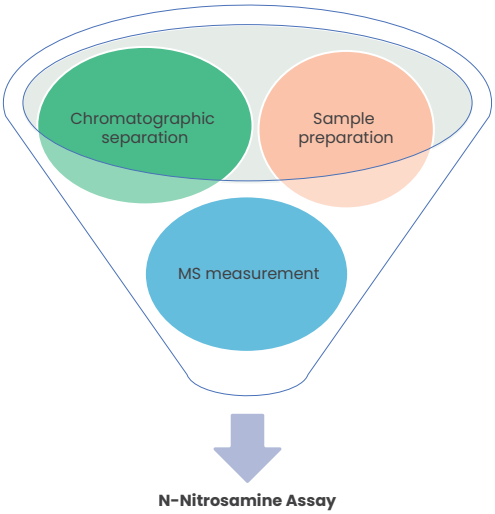


Figure 12: Method pillars

During the design stage, one should consider the physical and chemical characteristics of individual nitrosamine impurities and the analytical matrices included in APIs and drug products. The goal is to minimize the burden of pharmaceutical excipients and API that in many cases are responsible for extensive ion suppression. Moreover, the careful choice of chromatographic columns and conditions is of paramount importance in avoiding misinterpreting signals at the retention times of interest. To this end and in order to facilitate and expedite the often time-consuming and laborious process of method development, QMx has developed the following structured workflow and decision flow chart.

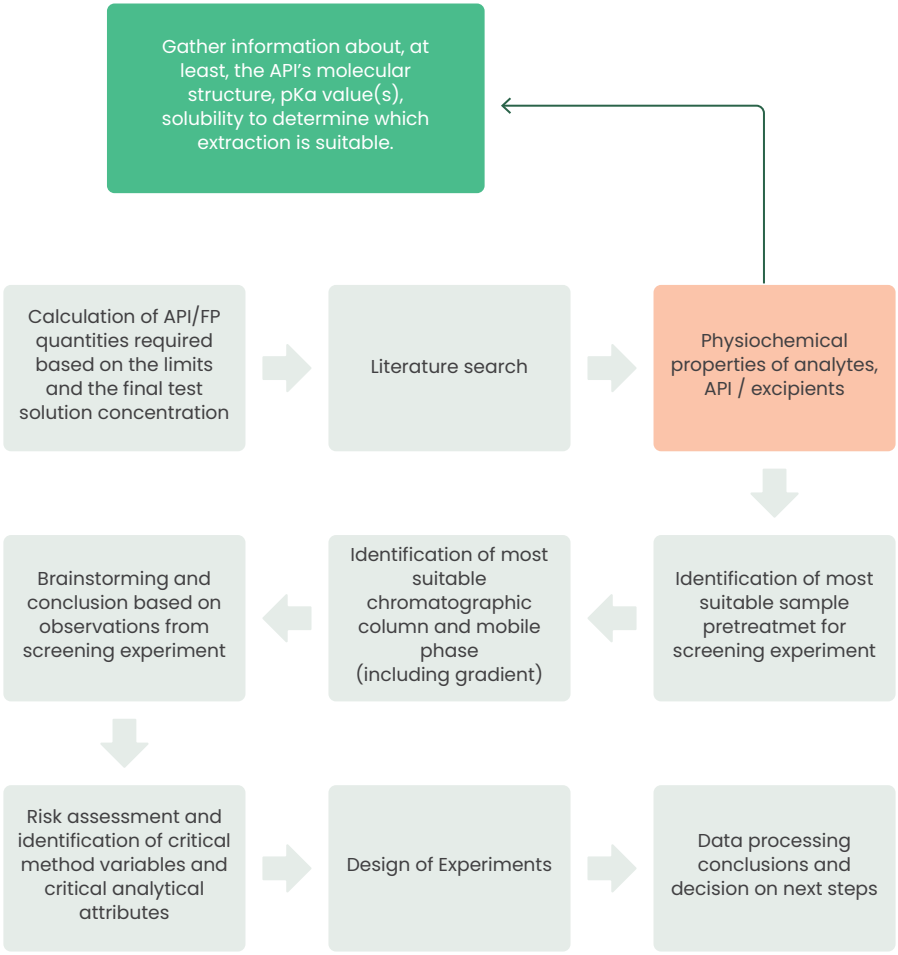


Figure 13: Analytical Development Workflow

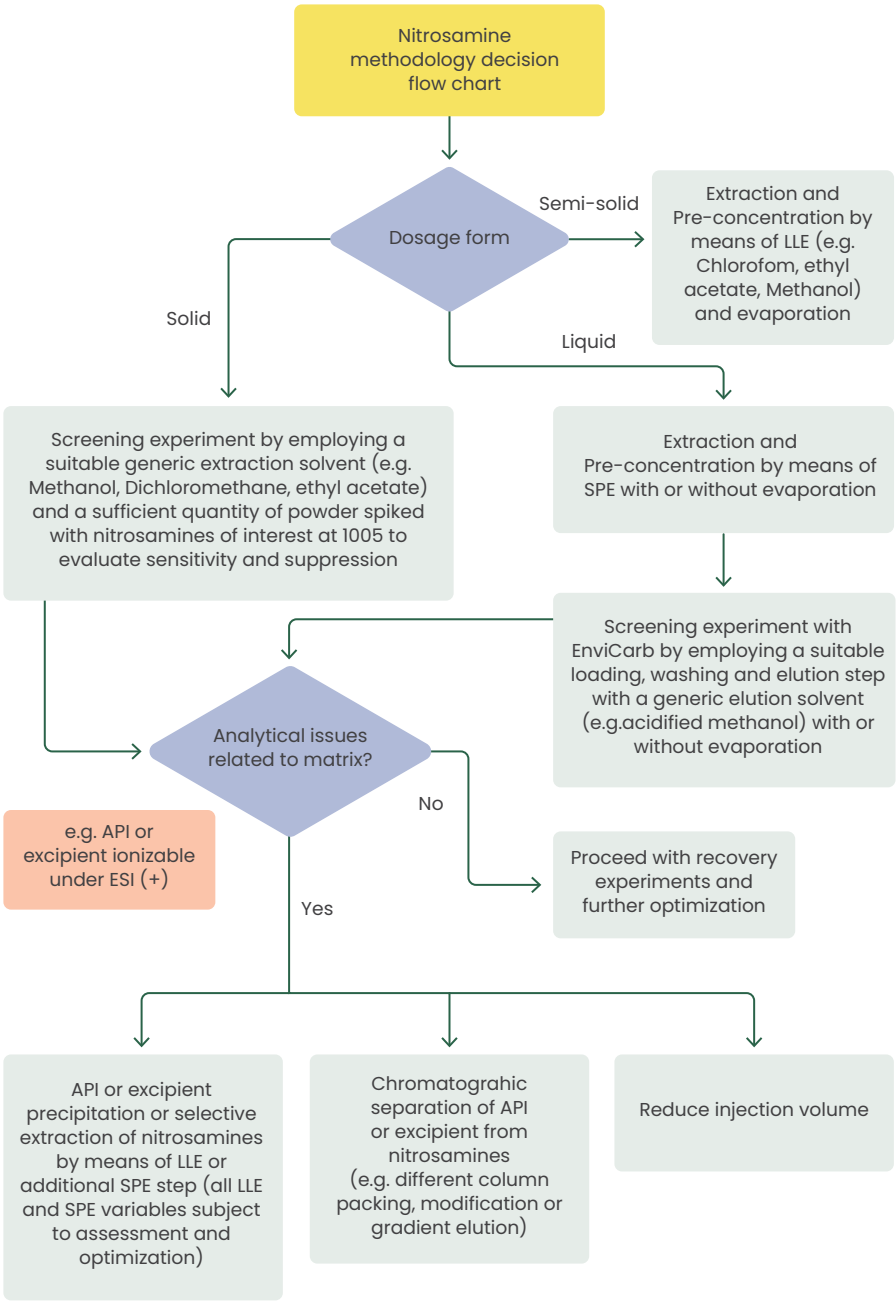


Figure 14: Decision flowchart

The structured approach illustrated above minimizes the risk of failures during the following stage of method validation (or procedure performance qualification) and the need to revisit the initial step of method development.

The following stage of method validation or procedure performance qualification, aims to demonstrate the suitability of the method for its intended purpose by confirming that all performance characteristics are within their acceptance criteria. The experimental scheme and strategy applied for the various scenarios of procedure qualification are depicted in the following table.

Table 4: Experimental scheme for procedure qualification

Case / Scenario	Means of establishing suitability
New method developed in house or another site	Method validation
New method developed in-house for a "worst-case matrix" representative that is intended for application to "simpler" matrices	Method validation for the "worst-case matrix" representative (e.g. Losartan/Hydrochlorothiazide tablets) and verification / waiver for the simpler matrices (e.g. Losartan tablets and Losartan API) along with a rationale, based on the principles of risk management, justifying the omission of additional experiments for certain characteristics of the method (e.g. robustness, linearity, etc.)
Method validated at another site	Method transfer or verification
Method validated in-house that needs to be applied without modifications for a similar final product or API	Method verification: The verification should provide a rationale based on the principles of risk management justifying the omission of additional experiments for certain characteristics of the method (e.g. robustness, linearity, etc.)
API compendial monograph	Method verification (it applies only for the initial implementation)

When it comes to confirmatory testing, ideally one should aim at developing and validate a common method for nitrosamine determination. This is indeed a challenging task, especially when the number of analytes is large (e.g. > 10), due to several factors, including their diverse physicochemical properties and the existence of the more complex NDSRIs. Nitrosamine compounds can exhibit a wide range of physicochemical properties, including differences in solubility, polarity, volatility, and reactivity. These differences can affect their behavior during analytical techniques, making it difficult to establish a single universal method that can effectively analyze all nitrosamines. To this end, different analytical techniques may be required to detect and quantify nitrosamine species with varying properties. For example, some nitrosamines may be amenable to gas chromatography-mass spectrometry (GC-MS), while others may require liquid chromatography-mass spectrometry (LC-MS). Developing a single method that accommodates all these variations is complex and may compromise the sensitivity and specificity of the analysis. Moreover, achieving high sensitivity and selectivity in detecting nitrosamine species is critical for ensuring the safety of pharmaceuticals. However, nitrosamines are often present in minute concentrations, and their detection can be hindered by interference from other compounds in complex matrices. Finally, the stringent limits and requirements for these "cohort of concern" impurities, further complicate the task, especially for products with high daily dose (e.g. > 1g). The following figure reflects a "real-world" approach in terms of goal-setting when developing a method for confirmatory testing.

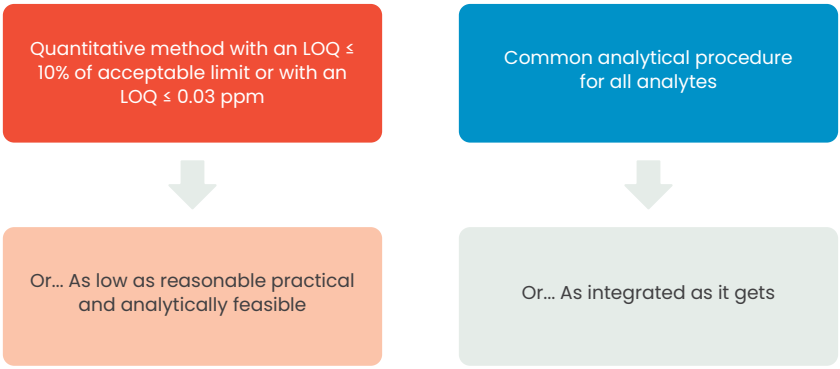


Figure 15: Goals to achieve during method development

QMx participates in Proficiency Testing Schemes (PTS) organized by the EDQM. Proficiency testing is a tool for measurement of the performance of laboratories based on inter-laboratory comparisons. Participation in Proficiency Testing Schemes (PTS) provides laboratories with an objective means of assessing and demonstrating the reliability of the data they produce. Thus, participation in a PTS provides independent verification of the competence of a laboratory and shows commitment to the maintenance and improvement of performance. Proficiency testing covers the overall performance of a laboratory. This includes the entire process from reception and storage of samples, the experimental work in the laboratory, the interpretation and the transcription of the data, the conclusions drawn from the data and the production of reports. Failure at any of these stages affects the competence of the laboratory.

The following figure exhibits the results of QMx and other participants in **PTS227: NDMA in valsartan tablets** held in October 2022. The aim of the study was to assess the performance of the laboratories with regard to determination of nitrosamines (specifically of N-nitroso-dimethylamine, NDMA) in a medicinal product.

Proficiency Testing Scheme on NDMA in valsartan tablets (PTS227)
Table 1. Data From Participants and Scoring

		Assigned Value = 32.5 ppm NDMA / tablet Target RSD = 9.5%								µg NDMA / tablet			ppm NDMA / tablet claim		
Lab	Method	Rep.1	Rep.2	Rep.3	N	Mean	SD	RSD	z-Score	N	Mean	SD	N	Mean	SD
1	GC-MS	42.8	41.4	39.7	3	41.3	1.6	3.9	3.9	3	39.4	1.1	3	33.2	0.5
2	LC-MS	30.81	30.34	29.73	3	30.3	0.5	1.8	-0.7	3	30.9	0.4	3	65.2	1.2
3	LC-MS	31.79	31.09	31.20	3	31.7	0.4	1.3	-0.3	3	31.8	0.3	3	66.1	0.9
4	LC-UV	41.01	39.43	41.08	3	40.5	0.9	2.3	2.8	3	37.9	0.9	3	87.1	2.0
5	LC-UV	32.15	32.44	32.76	3	32.5	0.3	0.9	0.02	3	32.3	0.2	3	69.6	0.7
6	LC-MS	32.36	32.21	32.12	3	32.2	0.1	0.4	-0.2	3	32.2	0.1	3	69.3	0.3
7	GC-MS	5.06	5.84	4.94	3	5.0	0.1	1.3	8.8	3	3.5	0.04	3	10.8	0.1
8	GC-MS	38.33	38.67	36.86	3	37.9	0.9	2.5	1.7	3	35.1	0.6	3	81.3	1.9
9	LC-MS	36.523	36.349	37.099	3	36.7	0.4	1.1	1.3	3	35.2	0.3	3	78.8	0.8
10	LC-MS	18.6809	18.3537	19.0371	3	18.8	0.2	1.2	-4.5	3	12.6	0.2	3	40.1	0.5
11	LC-UV	0.41	0.40	0.42	3	0.4	0.00	3.7	-10.4	3	0.4	0.01	3	0.4	0.01
12	LC-MS	31.23	30.75	30.87	3	31.0	0.2	0.8	-0.3	3	45.0	0.4	3	90.7	0.8
13	LC-MS	26.37	25.79	26.02	3	26.1	0.3	1.1	-4.2	3	17.9	0.2	3	50.1	0.6
14	GC-MS	34.26	34.22	34.46	3	34.3	0.1	0.4	0.6	3	23.6	0.1	3	73.8	0.3
15	LC-MS	32.39	32.84	32.42	3	32.6	0.05	0.2	0.04	3	22.9	0.03	3	69.6	0.1
16	LC-MS	34.01	33.96	33.75	3	33.9	0.1	0.4	1.1	3	24.7	0.1	3	77.1	0.5
17	GC-MS	3.68	2.96	3.18	3	3.3	0.4	11.3	-9.3	3	2.3	0.3	3	7.0	0.8
18	LC-UV	36.52	36.57	36.72	3	36.6	0.1	0.3	1.3	3	25.2	0.1	3	78.7	0.2
19	LC-MS	29.57	29.87	29.70	3	29.7	0.2	0.5	-0.3	3	20.5	0.1	3	63.9	0.3
20	LC-MS	30.86	30.59	30.36	3	30.6	0.3	0.8	-0.8	3	21.1	0.2	3	65.8	0.5
21	GC-MS	33.95	33.38	33.64	3	33.7	0.3	0.8	0.4	3	23.2	0.2	3	72.6	0.6
22	LC-MS	29.22	30.05	29.46	3	29.7	0.3	1.0	-0.9	3	20.6	0.2	3	64.5	0.6
23	GC-MS	34.88	34.14	33.90	3	34.1	0.2	0.6	0.5	3	23.5	0.2	3	73.4	0.5
24	LC-MS	31.482	30.454	30.777	3	30.9	0.5	1.7	-0.5	3	21.3	0.4	3	66.5	1.1
25	LC-MS	31.35	32.40	32.73	3	32.8	0.4	1.2	0.1	3	22.6	0.3	3	70.7	0.8

Figure 16: PTS Results

A z-score of – **0.7** in our case is indicative of the reliability of the provided value.

The z-score depicted in Figure 15 gives a bias estimate of the result. Absolute z-scores less than 2 are acceptable. A zone of doubtful performance exists for absolute z-scores between 2 and 3. Those do not necessarily have to be unacceptable since there is some uncertainty how close the consensus value is to the true value. An absolute z-score of 3 or more can be interpreted as an unacceptable performance, requiring corrective actions

Equipment

Qualimetrix is equipped with state-of-the-art instrumentation spanning a wide range of analytical techniques combined with analytical expertise and experience.

UPLC-MS/MS (triple quadrupole, QqQ)

Several triple quadrupole mass spectrometers hyphenated with UPLC chromatographic systems are employed within our lab. The latter is the technique of choice for the reliable identification and quantitation of trace level analytes that are contained within a complex matrix, such as the determination of N-Nitrosamines in pharmaceutical products. Through the Multiple Reaction Monitoring (MRM) mode, it provides higher Signal-to-Noise, allowing thus selective and sensitive identification and quantitation, as well as wide linear range.

The Sciex Triple Quadrupole Mass Spectrometers employed and especially the highly sensitive 7500 model, provide unparalleled performance and are valuable and well-suited tools for the determination of nitrosamines due to their exceptional sensitivity, selectivity, speed, and reliability that are necessary for ensuring the safety and compliance of pharmaceutical products.

Gas Chromatographic systems with a single quadrupole or tandem Mass Spectrometer (GC-MS, GC-MS/MS)

Gas chromatographic systems combined with singe quadrupole (GC-MS) and triple quadrupole (GC-MS/MS) mass analyzers are available at QMx. They are employed for the determination of volatile & semi-volatile nitrosamine species in cases where the interferences by common residual solvents that could potentially lead to false-positive results or overestimated levels of nitrosamines can be effectively mitigated.

Ultra-Performance Liquid Chromatographic system with High Resolution Mass Spectrometer (UPLC - HRMS)

QMx possesses cutting edge Orbitrap HRMS instrumentation by Thermo Scientific. These are hybrid Ion Trap- Orbitrap Mass Spectrometers, with very high resolving power, high speed, sensitivity and advanced fragmentation information. These instruments provide a reliable alternative for nitrosamine determination in cases where the analytes of interest are expected to have similar mass-to-charge ratios as other compounds in the sample matrix. HRMS can help resolve such isobaric interferences by distinguishing between species with similar masses based on their accurate mass measurements. It is also the instrument of choice during the investigation of whether a hypothesized species can be formed (by application of the IQ consortium protocol previously described) for the detection and confirmation of its presence (or absence).

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