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Overcoming Analytical Challenges in Nitrosamine Detection:

An LCGC International Peer Exchange

By Aaron Acevedo, Assistant Editor, *LCGC International*

Impurity analysis comes with many complex analytical and regulatory challenges. Nitrosamines are a large group of *N*-nitroso compounds (NOCs) that bear common functional >N–N=O groups. NOCs can be divided into two classes: *N*-nitrosamines and *N*-nitrosamides and related compounds (1). These compounds can be found in various substances, including pharmaceuticals. If humans are exposed to nitrosamines above acceptable levels and over long periods of time, these impurities can increase the risk of cancer (2).

Detecting acceptable intake limits, or the acceptable amount of impurity, such as nitrosamines, in a drug, can be difficult due to the limited availability of safety data for an impurity (3). When safety data is unavailable for nitrosamine impurity, information from nitrosamine comparators, or structurally similar compounds, can be used to identify acceptable intake limits. However, oftentimes appropriate comparators are not available. Further, default acceptable intake limits present challenges to both industry and regulators, significantly impacting drug supply chains.

To discuss these complex issues, the editors of *LCGC International* organized a peer exchange of experts to discuss the intricate nature of nitrosamine analysis. The panel was moderated by Aloka Srinivasan, principal and managing partner of Raaha, and featured Mayank Bhanti, senior director of the Compendial Development Laboratory at the United States Pharmacopeia (USP), and Amber Burch, the senior manager of Technical Business Development at Purisys (4).

The Big Analytical Challenges

The main analytical challenges associate with nitrosamine analysis can be roughly divided into five categories, Bhanti said. One of the main challenges with nitrosamine detection is the increasingly low sensitivities being required by regulatory agencies. This requires the use of techniques such as liquid chromatography–tandem mass spectrometry (LC–MS/MS) technology (5). These instruments can help achieve the target sensitivities, but robustness and reproducibility rates may be a challenge, Bhanti said, especially when handling low levels of the compounds.

"These metrics post a lot of interferences, especially from a mass spectrometry point of view," Bhanti said (5). Ion sources, by comparison, could potentially lead to an underestimation of nitrosamine impurities.

Sample preparation is also an important part of nitrosamine analysis. Optimizing extraction and clean-up processes may be necessary to help achieve lower sensitivities. Achieving chromatographic resolution can be especially challenging with nitrosamine drug substance-related impurities (NDSRIs), which are a class of nitrosamine impurities that have been found in both drug products and active pharmaceutical ingredients (APIs) (6). These impurities can form during drug synthesis, storage, or degradation, and hold potential carcinogenic risks. Bhanti calls for higher-resolution technologies, such as high-resolution mass spectrometers (HRMS) to be used for these procedures. "Pharmaceutical companies have to be flexible in their approach and have to have to change their methods in order to meet the requirements of different regulatory agencies," he said (5). "Overall, I think a multifaceted approach needs to be adopted to really be able to overcome all these challenges and to get in a nitrosamine-free drug product for the patients."

Burch emphasized the importance of inter- and intra-laboratory precision of methods. "Making sure that a method is able to be executed accurately by multiple analysts or by multiple organizations is very key to assuring that the method was going to be suitable for the life of the product," she said (5). "[Ensuring] that the method can be executed accurately by multiple analysts or by multiple

organizations is key to assuring that the method is going to be suitable for the life of the product."

Supply Chain Considerations

The point at which nitrosamines are introduced during drug manufacturing has been a longstanding question among experts. During the manufacturing process, for example, nitrite-containing excipients can introduce nitrosamines into the drug (7). The FDA has indicated these compounds have been found in a range of medicines including heartburn products, diabetes medicines, and antibiotics (8). The best approach is for organizations to work closely with stakeholders throughout the process.

"I think it is really important for companies to take a multi-pronged approach to the problem and working collaboratively with both drug product and drug substance suppliers," Burch said. Proper risk assessment can help mitigate problems, and with the right plans, it can ensure that the materials are free of nitrosamines, Bhanti said.

"I think the industry has to adopt a more proactive approach," Bhanti said (7). "It's a responsibility of the product manufacturer, but they have to work collaboratively with active pharmaceutical ingredient (API) manufacturers and to make sure that the product is free from nitrosamines."

Looming Deadlines

A major concern for drug manufacturers worldwide is the rapidly approaching deadlines set by regulatory agencies—such as the U.S. Food and Drug Administration (FDA)—regarding confirmatory testing for nitrosamine drug substance-related impurities (NDSRIs) in drug products. These regulations require companies to rigorously assess and, if necessary, reformulate their products to ensure safety and compliance. The complexity and scope of the testing, coupled with the high stakes of non-compliance, have placed significant pressure on manufacturers to act swiftly while navigating scientific, logistical, and regulatory challenges.

"It's a nightmare," Srinivasan said (7). "You can't resolve this. Even for simplest of the simple products, and even if you have five products in the market which are nitrosamine prone.

While Bhanti noted that many industries are now working to catch up and complete their confirmatory and risk assessment testing for nitrosamines, he emphasized that these efforts are only part of a broader set of challenges. "August 1, 2025, is going to be challenging, but I think the industries are now trying to catch up, and they are finalizing the confirmatory testing and risk assessment

parts, trying to achieve that date. But as you mentioned, it's not that easy, and it is going to be a lot more challenging" (7).

The panel's discussion of these complexities not only shed light on the current landscape but also aimed to spark further dialogue about potential solutions and paths forward for both industry and regulators.

You can watch the full peer-exchange here.

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Q&A: N-Nitrosamine Impurities and FDA's Recommendations for Acceptable Intake Limits

Josh Hoerner, general manager of Purisys, provides his perspective on FDA's recommendations for acceptable intake limits for N-nitrosamine impurities.

By Susan Haigney

Josh Hoerner, general manager of Purisys

Nitrosamines occurring in pharmaceuticals became a developing problem in 2018, and regulatory agencies have been working with pharmaceutical companies since to mitigate the risk of these cancer-causing chemicals in drug products (1). In January 2025, the United States Food and Drug Administration (FDA) posted "Determining Recommended Acceptable Intake Limits for N-nitrosamine Impurities in Pharmaceuticals: Development and Application of the Carcinogenic Potency Categorization Approach" in the Spotlight on CDER Science on the FDA website (2).

We spoke with Josh Hoerner, general manager of Purisys, which specializes in a small volume custom synthesis and specialized controlled substance manufacturing, to gain his perspective on FDA's recommendations for acceptable intake limits for N-nitrosamine impurities.

What are nitrosamine drug substance-related impurities (NDSRIs)?

Hoerner: NDSRIs are nitrosamine impurities that share structural similarities with the API and are generally unique to each API. These impurities are considered more complex and require more sophisticated testing methods to detect and quantify. The presence of NDSRIs is considered a higher risk,

especially when the impurity forms as a result of the API's chemical structure or the processes used during production.

The advanced testing conducted to determine the presence of NDSRIs involves sophisticated and sensitive mass spectrometry (MS) technology, including triple quadrupole technology, which enables liquid chromatography-tandem mass spectrometry (LC-MS/MS). A high-sensitivity, low-level quantitation technology is particularly advantageous for detecting nitrosamines and other impurities that must remain within low permissible levels (based on safety data, a comparator, or the Carcinogenic Potency Categorization Approach [CPCA] Potency score).

What can you tell us about the recommendations FDA published in January 2025 for the development and application of carcinogenic potency categorization approach to nitrosamines?

Hoerner: Safety data (animal carcinogenicity data) for many potential NDSRIs or comparators are not readily available. FDA worked with the Nitrosamine International Technical Working Group (NITWG) to develop a methodology called the CPCA. The approach is based on a training set of 81 nitrosamines to establish five potency categories (1 high–5 low), reflecting carcinogenic risk. Essentially, the CPCA applies the knowledge that the α-hydroxylation mechanism of metabolic activation and the associated number of α-hydrogens as well as associated activating and deactivating features, drives nitrosamine potency. This allows a potency score for a potential NDSRI to be calculated along with the associated acceptable intake without direct safety data. Using this framework, a drug product manufacturer can then assess their drug products for NDSRIs below the acceptable intake and ensure patients are not exposed to levels exceeding or approaching the acceptable intake.

What makes determining acceptable intake so challenging?

Hoerner: There is a lack of direct safety data on NDSRIs and comparator compounds to establish a basis for potency. Prior to the CPCA framework, one might default to higher acceptable intake limits such as the 1500 ng/day, whereas those with higher potency scores have higher carcinogenicity risk, and therefore, should be controlled to lower acceptable intake levels.

What can pharma manufacturers do to manage the nitrosamine risk?

Hoerner: There are two key guidelines included in the FDA-published compliance recommendations that define the framework for nitrosamine assessment and control:

- **Risk assessment and control of nitrosamine formation.** Manufacturers should evaluate and control two types of nitrosamine impurities:
  - Small-molecule nitrosamines (structurally different from the API)
  - NDSRIs, structurally similar to the API.

- **Acceptable daily intake limits.** Risk assessments should determine if nitrosamines are likely present, followed by testing. If testing shows nitrosamine impurity is present at a level 10% above the acceptable intake limit for that specific nitrosamine, FDA recommends that manufacturers develop specifications to keep nitrosamine levels within limits.

A comprehensive approach includes an initial paper-based assessment of technical risks (e.g., chemistry, solvents, excipients), followed by testing according to USP [United States Pharmacopeia] General Chapter 1469.11. Risk ratings (low, medium, high) are assigned based on the likelihood of nitrosamine formation, with secondary amines posing the highest risk.

Manufacturers should partner with experienced contract development and manufacturing organizations (CDMOs) that offer a comprehensive solution for testing, synthesis, and risk assessment to ensure compliance with these complex requirements.

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Analytical Method Lifecycle of SFC Methods from Development Use to Routine QC Implementation *Supporting Small Molecule R&D and Commercialization*

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Selection of the correct analytical technique from the outset of method development is key in securing the optimal and most robust method to be used throughout a product's development journey from R&D to commercialization. In this article, we review some key considerations for chromatographic technique selection and method development across the full drug process—from early-stage API synthesis to routine commercial release activities. We describe the implementation of supercritical fluid chromatography (SFC) in the pharmaceutical industry, especially for chiral, water sensitive analytes, and low to high LogP and LogD hydrophobic compounds, while demonstrating high levels of method robustness and support of "green" analytics.

The product development lifecycle in the pharmaceutical industry can be divided into three distinct phases: discovery, development, and registration and manufacturing.

In the discovery phase, the analytical impetus is normally on speed and throughput. However, once a candidate has passed the proof-of-concept stage selected for development and used in clinical trials, the analytical methodology that accompanies the development should take into consideration the overall method lifecycle, which may extend through to routine product manufacturing support. Such analytical methods need to meet requirements of *United States Pharmacopeia* (*USP*) resolution (USP <621>) (1–2) and sensitivity (limit of quantification [LOQ] and limit of detection [LOD]). Eventually, the full development to registration and manufacturing has to guarantee robustness and ruggedness, ensuring good transferability of the method. Meeting sustainability goals is also a key driver that should be considered, as these analytical methods could be used for a considerable period of time (in the order of years).

The required performance of the analytical methodology, defined in the product analytical target profile (ATP) serves as the starting point for method technique selection and method development (3). The correct marrying of "method requirement," "analyte properties," and "technique capability" is what ultimately ensures robustness of the method. Specificity, sensitivity, accuracy, and reproducibility are typical parameters that measure the performance of the analytical method. The technique should be able to meet these performance criteria versus the analyte, instrumentation and transferability across laboratories, departments and business units.

Prior knowledge or experimental verification relating to the performance of available analytical technologies enables the analyst to select the most appropriate technology to meet the requirements of the ATP. Where more than one technology has been demonstrated to meet the ATP, a review of business requirements (such as throughput, automation, downstream availability) should be performed to aid selection. Procedure specific performance indicators should be defined. These can include critical resolution of defined impurities (also known as the key predictive sample set [KPSS]), as well as specific sensitivity requirements, and this becomes the starting point of method development.

Discussion

The choice of analytical technique plays a critical role in the success of the analytical method. The key requirements from the method are defined in the ATP, and the analytical technology needs to be able to satisfy all these requirements–not only during the development, but also throughout the project lifecycle, from development to commercialization. If a non-robust or poorly optimized method is developed in the first instance, subsequent method redevelopment will be needed downstream, generating a cumulative resource burden.

In the pharmaceutical industry, reversed-phase liquid chromatography (RPLC) is by far the most widely employed analytical technique, and carries several advantages over alternative techniques, including analyst familiarity and versatility for many different molecular classes. Although alternative separation modes are often proposed during development, technique selection is often driven by business considerations, such as instrument availability across downstream or partner laboratories. In agreement with ICH-Q14 (4), other techniques should be prioritized for method development if they provide higher method robustness, instrument ruggedness, validation success, and transferability.

While the analyst can rely on the availability of a wide toolbox of separation techniques (including, but not limited to, RPLC, supercritical fluid chromatography [SFC], gas chromatography [GC], ion exchange [IEX] chromatography, capillary electrophoresis [CE], hydrophilic-interaction chromatography [HILIC], normal phase liquid chromatography [NPLC], and size-exclusion chromatography [SEC]), each of them can perform optimally only within defined constraints of physicochemical properties, such as pKa, logD, logP, or solubility. Each chromatographic mode has an operating "sweet spot," and, within this sweet spot, the optimal performance and robustness for the analytical method is unlocked. In this article. we discuss the application of SFC identified as the optimal approach to meet ATP requirements in various measurement challenges.

The Road of Enabling SFC from Development to QC

As discussed earlier, SFC may be the first choice for analytes that have compatible physicochemical "sweet spots," for example:

Retention:

- Analysis of polar compounds, (LogD < -1): While SFC provides adequate retention, RPLC risks eluting such compounds with the solvent front unless appropriate fully aqueous-compatible stationary phases are selected or ion pairing reagents utilized. This leads to additional method complexity, and often require additional method controls.
- Hydrophobic compounds (LogD > 4): With RPLC, such analytes are typically eluted later in the gradient... or not at all.

Solubility:

- SFC is a good technique for the analysis of lipophilic analytes with high solubility in organic solvents (and poor solubility in water). Selecting RPLC for such an application elicits a risk of precipitation (column blockage), diluent or mobile phase incompatibility (potentially leading to poor peak shapes), insufficient column loading, or carry over.

Stability:

- The "water-free" mobile phases employed with SFC offer the advantage of being the ideal choice for the analysis of water labile compounds.

Selectivity:

- SFC has useful orthogonality (chromatographic selectivity) to RPLC. This alternative orthogonality has been shown to offer superior positional isomer selectivity, which can be useful in resolving difficult critical pairs (4).
- SFC is Pfizer's (and many other companies) default choice for chiral separations.

The Use of SFC–MS in an Open Access Environment

In our laboratories, an open access (OA) SFC–MS instrument has been in use since 2021 by both chemists and analysts. The availability of easy-to-use generic method screens (chiral and achiral) facilitate high throughput screening of a large number of process chemistry samples.

In 2022, over 3000 samples were analyzed on this open access SFC. The OA SFC screen has significantly decreased method development time, and, by incorporating SFC earlier on in the development workflow (at the initial process chemistry stage), has led to the organic growth of the technique in the department. The inclusion of an open access SFC has therefore lowered the energy-barrier for wider SFC application in development at Pfizer.

SFC for Drug Product Development

Due to its applicability to analytes with low solubility in aqueous environments, SFC also has application in drug product (DP) analysis. The application detailed here is for an injectable DP that required an oil-based formulation. Castor oil, soybean oil, sesame oil, and oleic acid were explored by the formulation team as potential excipients for an injectable solution of an Active Pharmaceutical Ingredient (API). A risk assessment of a potential RPLC method highlighted several risks, such as the accumulation of the oil matrix or risk of precipitation of the API on column, the risk of inaccurate analyte recovery, intensive sample preparation, tighter system control requirements, and higher maintenance burden. With SFC being a "water-free" chromatographic technique, and with the API and formulation oils being soluble in organic solvent and CO_2 mobile phases, most of these risks were mitigated. On a polar stationary phase, the API peak eluted in the middle of a 2% to 42% organic modifier gradient. As the formulation oils were unretained, good separation of the oil matrix and the API was achieved, with no impurity co-elution issues and with simpler sample preparation than the RPLC method.

Figure 1a and 1b shows the overlay of the chromatograms of API (black trace) dissolved in dichloromethane (DCM, red trace) versus the formulation matrix oils

(blue trace). Acetone was later selected as the method diluent to replace DCM as a "greener" option (5).

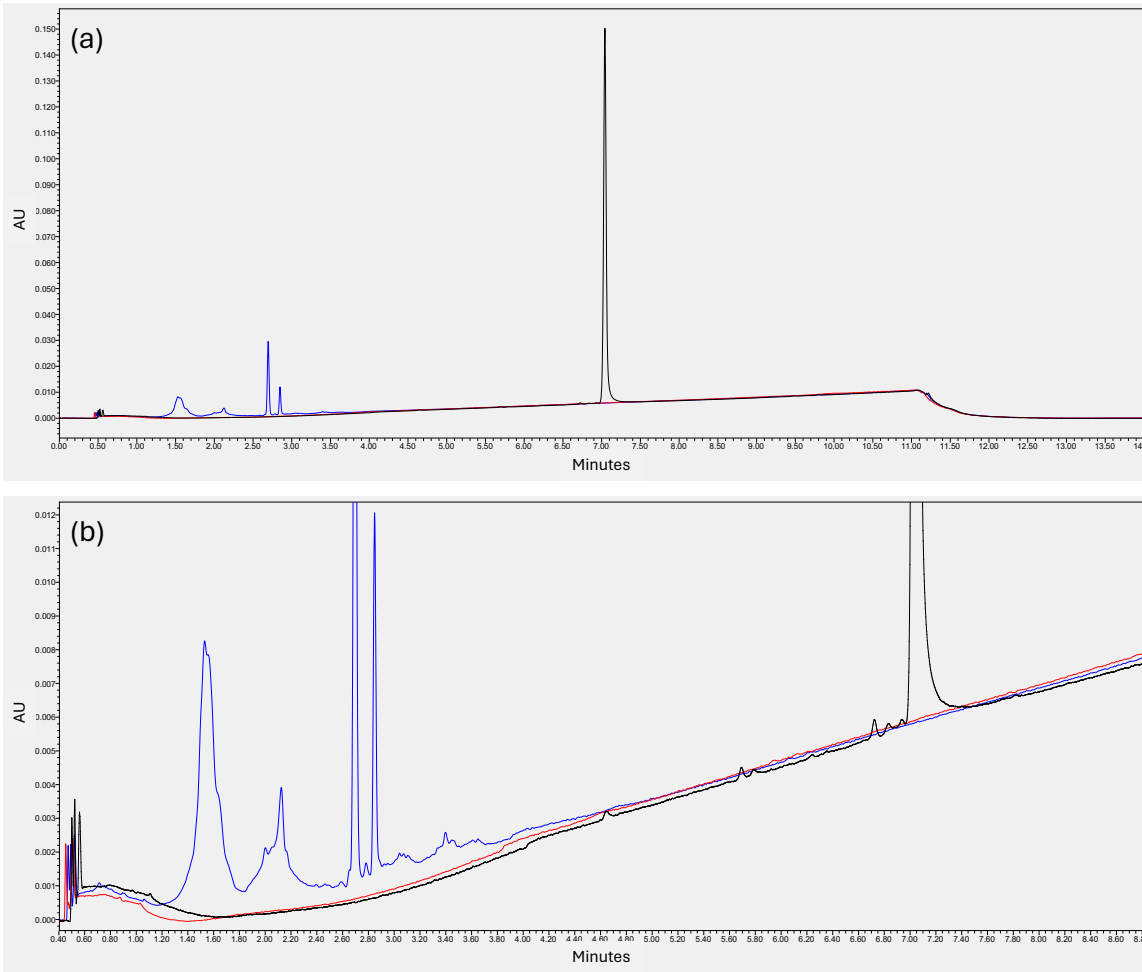

FIGURE 1: Overlay of the SFC chromatograms of an API (black trace), its formulation excipients (blue trace), the sample diluent (DCM) - blank (red trace). (a) full scale, and (b) zoom (Torus DIOL (100 x 3.0 mm, 1.7 µm), methanol 10 mM ammonium formate (2%, hold 0.5 min, to 42% in 10 min), 40 °C, 120 bar, 1.7 mL/min, 2 µL, 292 nm).

The drug product application of SFC detailed above started with a risk assessment of the sample characteristics versus the ATP. It is an example of a science-based approach to successful method development. The method development took no more than one day, compared to several weeks dedicated to RPLC method development and troubleshooting. This highlights the alternative utility of SFC in pharmaceutical analytical laboratories with challenging matrices.

SFC in the QC Laboratory: API Manufacture and Release Testing

The superiority of SFC for chiral separations is well known and widely described in the literature (6-9). SFC has been the technique of choice for decades

in compound discovery departments and for preparative and purification separations. It is only recently that SFC technology became reliable enough that its use could satisfy the strict requirements for validation, method transfer, and ultimately implementation in the QC labs. Several reports have been presented in the literature highlighting GMP applications in recent years (10–13).

Implementing modern SFC instrumentation in QC laboratories has added a new platform for the chiral control strategy of APIs which were predominantly being analyzed using normal-phase LC (NPLC). While the use of NPLC for chiral measurements can be satisfactory, due to long analysis times and toxic or non-green mobile phases incorporating organic solvents such as hexane or heptane, it is rarely the optimum separation approach.

Figure 2 displays the increase in number of batches tested with SFC as part of Pfizer's API clinical manufacturing control strategy (Sandwich, UK R&D site) from 2016 to 2023. As can be seen, SFC has replaced NPLC for clinical API manufacturing support almost entirely over this timespan.

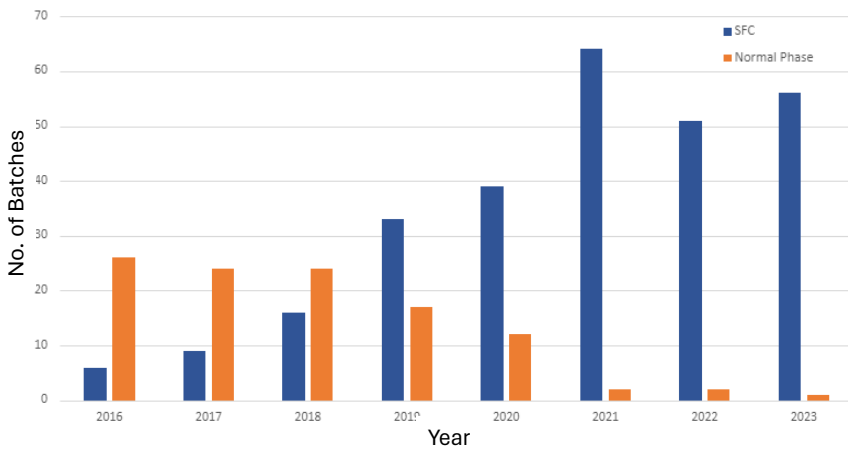

FIGURE 2: SFC vs. NPLC in Pfizer, GMP Analytics (Sandwich) for release of Clinical API, Intermediats and IPCs. Bar graph shows the number of batches tested at Pfizer's API clinical manufacturing (Sandwich, UK R&D) site from 2016 to 2023 by SFC (blue bars) and by NPLC (orange bars).

The confidence in the robustness of SFC methods created during development, allowed Pfizer to transfer SFC methods to commercial operations. Between 2016 and 2020, SFC chiral methods were redeveloped to NPLC equivalents at point of registration or commercial transfer due to lack of SFC systems in manufacturing units. Since then, three marketed products have been registered with SFC methods as part of their control strategy:

- **Abrocitinib (cibinqo):** Determinaiton of process impurity content in water-sensitive intermediate by SFC where NPLC gave poor and unreproducible chromatography.
- **Ritlecitinib (litfulo):** API identity and chiral purity evaluation by SFC.
- **Nirmatrelvir (paxlovid):** Determination of API stereoisomer content by SFC.

The transfer of abrocitinib in 2020 from clinical to commercial manufacture opened the door for SFC to Pfizer's commercial manufacturing partners.

Two subsequent commercial chiral API methods were successfully transferred to Pfizer commercial API sites, without method transfer issues. Furthermore, the SFC methods were well received by the QC teams.

Since its implementation in the Ringaskiddy (Ireland) API manufacturing site, over 110 individual release runs were completed using SFC since 2020, over half of which were for Nirmatrelvir. Modern SFC instrumentation has proven to be very reliable in this environment.

Nitrosamine Determination

In 2020, the FDA published guidance (14) discussing the risk of nitrosamine drug substance related impurities (NDSRIs) and mitigation strategies. In 2023, the FDA published recommended acceptable intake limits for these mutagenic impurities (15). While a risk assessment and mitigation approach can be taken for the control of NDSRIs, identification and quantitation by analytical methods are still required to appropriately support the risk assessment. Here, we consider SFC as a technique to aid in the control strategy of NDSRIs (16).

Sensitivity to low- or sub-ppm levels are required to reliably determine levels of NDSRIs, often requiring mass spectrometric (MS) and tandem mass spectrometric (MS/MS) detection. While nitrosamines are often analyzed by GC or RPLC, SFC can also be considered for the following reasons:

1. **Orthogonal selectivity to RPLC:** Nitrosamine derivative of the API often are eluted after the API with RPLC, whereas they often are eluted prior to the API using SFC which can, in turn, aid quantification.
2. **Compatibility of SFC with MS detection.**
3. **Often enhanced MS sensitivity due to simpler mobile phase desolvation.**
4. **High on column drug loading:** High concentration samples are possible through dissolution in organic solvents, such as acetone. These strong organic strength solutions are highly compatible with SFC.

Figure 3 shows the total ion chromatogram (TIC) plots of an (a) SFC–MS chromatogram, and (b) RPLC–MS chromatogram. The nitrosamine derivative is eluted before the main component in SFC–MS providing a reduced risk of matrix effects in the ion source and improved quantification.

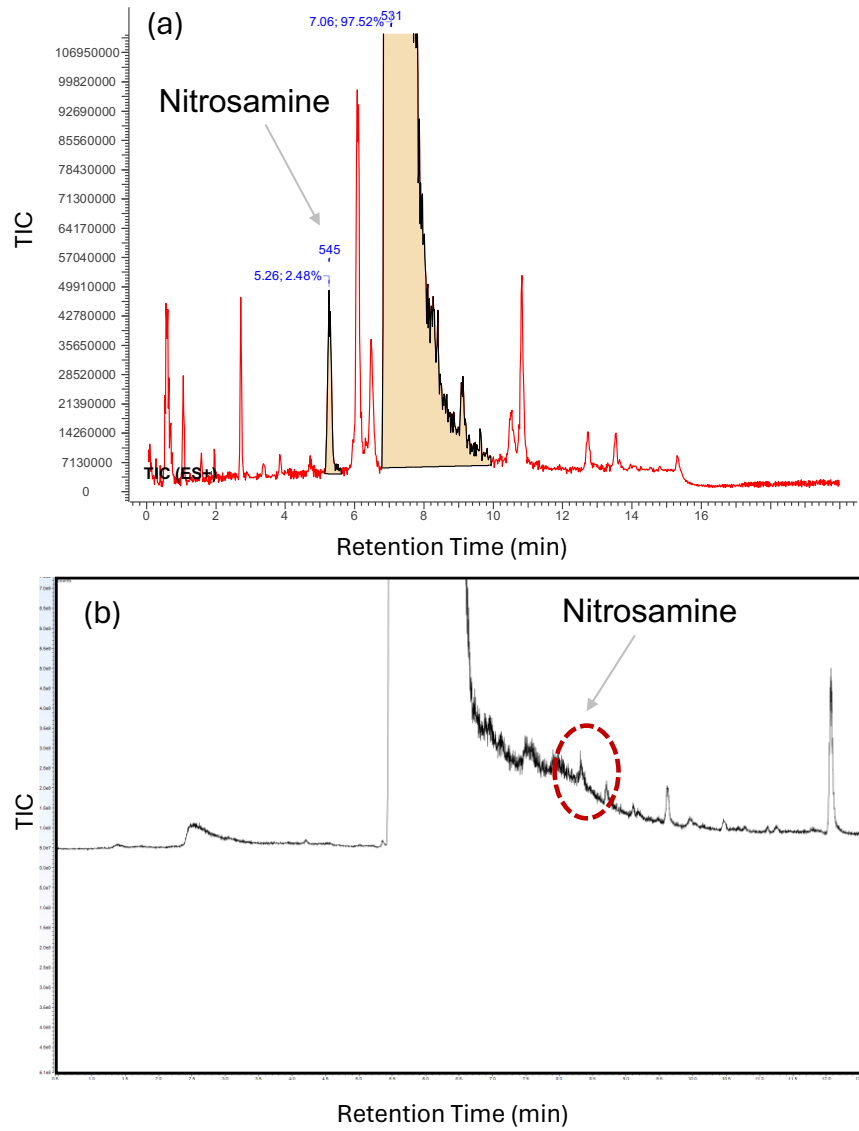

FIGURE 3: Total Ion Chromatograms (TIC) - API (30 mg/mL) spiked with NO-Am (0.05 mg/mL) for (a) an SFC-MS (Torus DIOL (100 x 3.0 mm, 1.7 µm), methanol 10 mM ammonium formate [5% to 20% in 20 min], 40 °C, 120 bar, 2.0 mL/min, 10 µL); and (b) LC-MS, RPLC (Zorbax Eclipse Plus [100 x 2.1 mm, 1.8 µm], MPA: 10 mM ammonium acetate, pH 4.5, MPB: acetonitrile, gradient 20% hold 2 min to 80% in 13 min, 0.3 mL/min, 40 °C, 10 µL injection vol.) of an API spiked with its related nitrosamine.

Conclusion

Systematic science-based method development is essential to ensuring analytical methods can successfully support drug development through the entire lifecycle. We have shared a series of applications demonstrating successful method development and validation with SFC. We have demonstrated the application of SFC in QC and commercial manufacturing support and highlight how the technique can be used beyond discovery applications. Additionally, through these examples, we have shown SFC can provide a higher degree of speed, robustness, and simplicity over alternative or more established techniques.

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What's New in Nitrosamine Research

This roundup from the editors of *LCGC International* highlights emerging trends in nitrosamine detection and separation science applied to diverse pharmaceutical formulations.

SPE and GC-MS for Analyzing N-Nitrosamine Impurities in Cough Syrups

By Aaron Acevedo

Scientists from the Mankind Research Centre in Haryana, India, recently tested how effective solid-phase extraction (SPE) and gas chromatography–mass spectrometry (GC–MS) are for detecting small molecule N-nitrosamine impurities in antitussive syrups. Their findings were published in the *Journal of Chromatography A* (1).

Nitrosamines are organic compounds that stem from chemical reactions. They are organic compounds that exist at low levels in water and foods, but some can also form in drugs during manufacturing. These chemicals, called N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA), and N-Nitroso-N-methyl-4-aminobutyric acid (NMBA), may increase the risk of cancer if people are exposed to them above acceptable levels and over long time periods (2). Because of the highly toxic nature of these compounds, various regulatory agencies have recommended steps to establish control and limits regarding acceptable intake (AI) to monitor their presence in various drug substances and products.

Since nitrosamine impurities often have low AI, analysis is typically carried out with the help of instruments like liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS). In this article, the scientists present the application of SPE technique with GC–MS to enrich and

analyze the nitrosamine impurities present in cough syrups. A quantitative testing method was developed for analyzing low-molecular-weight (small molecules) nitrosamine impurities in cough syrups using SPE on strong cation-exchange functionalized polymeric sorbent cartridges followed by GC–MS.

Solid-phase extraction is used to extract nitrosamine impurities from large sample volumes of cough syrup to achieve result recovery and reproducibility at lower standard concentrations. SPE is a non-equilibrium procedure that combines non-linear modes of chromatographic separation; these modes include sample loading/retention followed by stepwise sample desorption/elution or gradient dependent desorption for targeted analysis. Compared to liquid–liquid extraction (LLE), SPE is a targeted form of sample preparation that separates the analyte of interest from interfering compounds that may be present in samples. Altogether, SPE can help purify, fractionate, and concentrate targeted analytes with superior column loading and reduced mobile phase/solvent consumption.

The matrix spike recoveries of the nitrosamine impurities from the cough syrup samples were observed to be within the range of 90–120%. Limit of detection (LOD) achieved for N-Nitrosodimethylamine (NDMA) and N-Nitroso morpholine (NMOR) was about 0.1 ng/mL while the LOD for N-Nitrosodiethylamine (NDEA), N-Nitrosodiisopropylamine (NDIPA), and N-Nitrosoisopropylethylamine (NIPEA) impurities was about 0.02 ng/mL. The method was evaluated and found to meet the acceptable criteria as per the ICH Q2 guidelines for a working concentration range of 0.02 ng/mL to 1.2 ng/mL for the analyzed impurities. The selectivity of the nitrosamine impurities against the presence of drug product was established using multiple reaction monitoring (MRM) transitions during analysis.

Using SPE for sample clean-up and quantitative analysis of nitrosamines was deemed suitable for its intended purpose, owing to limitations such as lower drug content, parts per million level limit determinations, and sample interference. This method met acceptable limits for the evaluated criteria, with the method's sensitivity ensuring low-level determination of nitrosamine impurities in the sample. This approach could potentially be extended to various ranges of sample matrices, all while considering SPE developmental guidelines as shown in this study.

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HILIC-SPE and LC-HRMS Method Used for N-Nitrosamine Detection in Pharmaceuticals

By Kate Jones

The 2018 discovery of N-nitrosodimethylamine (NDMA) in valsartan (1,2) changed the pharmaceutical landscape forever. The discovery of NDMA during routine quality control at an average level of 66.5 parts per million forced the pharmaceutical industry to re-evaluate their manufacturing processes. Several global recalls have meant that this interest has continued, and nitrosamine drug substance-related impurities (NDSRIs) have been reported recently (3). A team of researchers from the University of Ljubljana in Slovenia, has introduced a method that joins hydrophilic interaction chromatography (HILIC)-based solid-phase extraction (SPE) with liquid chromatography–high-resolution mass spectrometry (LC–HRMS) (4) to determine N-nitrosamine (NA) in pharmaceuticals.

Potentially highly carcinogenic, NAs are formed during synthesis or degradation and pose significant health risks even at trace levels. Highly rigorous processes are therefore required to determine them. While existing methods address single active pharmaceutical ingredients (APIs), there is a lack of a versatile method to detect multiple NAs in a range of drug products. This new approach using a HILIC-based SPE step allows multiple APIs and polar excipients to be retained while NAs pass through. This clean-up step significantly mitigates matrix interference and removes excessive amounts of API.

The SPE process employs sequential elution steps optimized for different NAs. This ensures good recovery while maintaining high precision, even in complex matrices. After sample clean-up, LC–HRMS was used in the analysis and demonstrated linearity (R^2 > 0.999), accuracy (85–115%), and repeatability (RSD < 10%). Recoveries exceeded 80% and detection limits were as low as 42.5% of regulatory thresholds, underscoring the method's efficacy. These parameters were tested across 59 APIs and validated across 15 tested NAs, including multi-API formulations.

The researchers tested the method on a variety of commercially available and expired drug products (DPs). While no NAs exceeded limits in active DPs, expired ranitidine products contained NDMA levels up to 32 times higher than acceptable. These findings underscore the importance of proper storage and immediate sample preparation to prevent degradation-related NA formation.

The team concluded that the adaptability of the method is one of its best features. It is the first method to overcome the difficulties involved with analyzing such a class of compounds, such as the differing physicochemical properties of the NAs, the low concentrations relative to the API, and the complexity of the matrix (4). By modifying the SPE elution protocol, the method can accommodate newly

identified NDSRIs and tailor detection for specific APIs. As the pharmaceutical landscape continues to evolve in response, this method provides flexibility in routine quality control and regulatory compliance.

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LC-MS/MS for Quantifying Nitrosamines in Olmesartan Tablets

By Aaron Acevedo

A recent study led by scientists from the Mithibai College of Arts in Mumbai, India, shows the effects of using a modified version of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for detecting nitrosamines in Olmesartan tablets. Their findings were published in the *Journal of Chromatography A* (1).

Mutagenic and carcinogenic substances, which can cause health risks to humans, including increasing cancer risks, typically stem from the manufacturing processes of drugs, food processing, and water treatment. N-Nitroso compounds, which are known for mutagenic and carcinogenic properties, have been found in various products, including dairy, vegetables, alcoholic beverages, and meats. These compounds can also appear in pharmaceutical products. N-nitrosamines are viewed as genotoxic impurities that can directly or indirectly damage cellular DNA. In particular, the U.S. Food and Drug Administration (FDA) highlighted N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA), N-Nitroso-N-methyl-4-aminobutyric acid (NMBA), N-Nitrosoisopropylethylamine (NEIPA), N-Nitrosodiisopropylamine (NDIPA), and N-Nitrosomethylphenylamine (NMPA). NDMA, NDEA, NMBA, NEIPA, and NMPA have been detected in drug substances or products, with NDMA and NDEA specifically being primary nitrosamine contaminants whose presence have led to immediate recalls of pharmaceutical products from the market.

In this study, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was tested for concurrently measuring nitrosamines in Olmesartan tablets, which are used to treat high blood pressure. Olmesartan works by blocking substances in the body that cause blood vessels to tighten (2). It relaxes the blood vessels, lowering blood pressure and increasing the supply of blood and oxygen to the heart.

LC-MS/MS offers minimal sample consumption, suitability for thermally labile analytes, and heightened sensitivity for detecting nitrosamines in pharmaceutical matrices. Conventional LC-MS/MS often needs extensive and time-consuming sample preparation, though this was addressed in this study by developing more efficient and streamlined sample preparation that reduced variability and improved recovery rates (1). With this method, internal standards were not needed to overcome problems while analyzing nitrosamines.

The method applied effective chromatographic separation and optimized parameters for mass spectrometric detection. Detection was carried out using APCI positive ion mode. Chromatographic separation was achieved using a 150 mm x 4.6 mm, 2.6-µm column, with a simple gradient elution of mobile phase consisting of 0.1% formic acid in water (mobile phase A) and methanol (mobile phase B). The total run time was 20 min, with a flow rate of 0.800 mL/min.

Overall, the established method showed excellent linearity (R^2 > 0.99) and sensitivity for all the nitrosamines. The detection and quantification limits were sufficiently low for trace nitrosamine levels, holding good signal-to-noise (S/N) ratios. When tested on Olmesartan tablet samples, the method showed good accuracy, with recovery ranges between 80–120%. This analytical approach showed notable repeatability and reliability, making it possible to precisely quantify nitrosamine levels in Olmesartan tablets in a single analytical run. This work can help pave the way for more significant advancements, allowing for the facilitation of more precise and accurate analysis in routine quality control analysis.

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Detection and Identification of Total Nitrosamines Using Automated Total Nitrosamine Analysis (ATNA) and GC-TEA

Nitrosamines have long been established as potent carcinogenic compounds, predominantly formed when secondary amines react with nitrosating agents such as nitrites, nitrates, or nitrosyl compounds. These reactions are catalysed under acidic or reducing conditions or elevated temperatures, although nitrosamines can also form under ambient storage conditions. Given their widespread occurrence in pharmaceuticals, personal care products, foods, beverages, and packaging materials, robust and reliable analytical methods are imperative to detect and quantify nitrosamines accurately, ensuring consumer safety and regulatory compliance.

IMAGE 1: Ellutia ATNA System

Unexpected nitrosamine contamination incidents, notably with widely prescribed pharmaceuticals such as ranitidine and sartans, have led to significant regulatory interventions and market withdrawals. The formation and presence of nitrosamines in medications such as metformin and nizatidine have been of particular concern due to their high consumption rates, thus posing substantial public health risks. Regulatory bodies globally, including the US FDA and European Medicines Agency, have emphasised stringent testing and monitoring protocols, heightening the demand for efficient, sensitive, and specific analytical solutions.

Historically, nitrosamine analyses have employed chromatography coupled with mass spectrometry, specifically LC-MS/MS and GC-MS/MS methods. While these methods offer high specificity and sensitivity for targeted analytes, they require extensive method development and validation due to the potential for false negatives arising from closely eluting peaks and overlapping daughter ion fragments. They can work well if the only known contamination comes from a nitrosamine that has already been identified and an analytical standard readily available to calibrate against it. However, what if contamination from another nitrosamine was not expected or the standards required are not readily available?

ATNC (Apparent Total Nitrosamine Content) Analysis offers a unique approach to tackling this issue. This uses a chemical reaction and chemiluminescent detection of the nitric oxide (NO) from the nitrosamine to give a single value for all apparent nitrosamine content. So, there is no

separation and identification of individual nitrosamines, but all nitrosamines will be detected regardless of their makeup.

Historically, this has been performed manually via a glassware system connected to a TEA detector. However, recent advances have meant the Ellutia ATNA system can automate this analysis. The ATNA system utilises a dynamic headspace approach wherein nitrosamines, nitrites, and nitrates present in the sample are chemically reacted to release nitric oxide (NO), which is subsequently quantified. The molar detection of NO allows universal detection of nitrosamines without prior knowledge or availability of specific standards.

- **Analytical Throughput and Automation:** The ATNA facilitates automated sampling, capable of analyzing up to six samples per hour, with a standard vial capacity supporting 120 vials, enabling extensive unattended sample analysis.
- **Sensitivity and Detection Limits:** The system demonstrates a robust analytical sensitivity, capable of detecting nitrosamines such as NDMA at concentrations as low as 1 ppb. This sensitivity is critical for pharmaceutical products, where even trace levels of nitrosamines can represent significant health risks.
- **Flexible Application:** ATNA's design enables analysis across diverse matrices, including raw materials, finished pharmaceutical formulations, solvents, packaging, and personal care products.

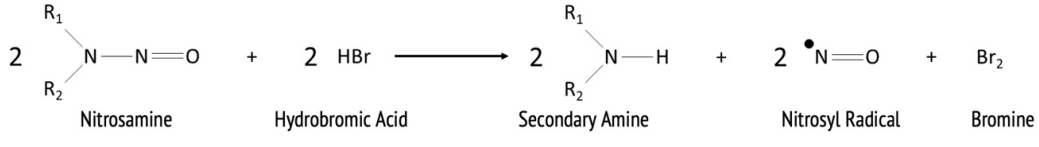
Technical Mechanism of Nitrosamine Detection by the ATNA

The ATNA system employs a chemical reaction between nitrosamines and hydrobromic acid, liberating the nitrosyl radical (NO). Subsequent reaction with ozone in the Thermal Energy Analyzer (TEA) generates excited-state nitrogen dioxide (NO_2^*). This excited molecule relaxes, emitting a photon measured by a photomultiplier tube, correlating directly to the nitrosamine concentration. This reaction mechanism ensures specificity and robustness, significantly reducing the likelihood of false negatives.

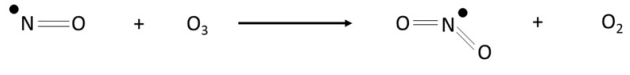
Differentiation of Nitrites and Nitrosamines

A critical analytical challenge in this type of nitrosamine testing is distinguishing between nitrites, nitrates, and nitrosamines, as nitrites can serve as precursors for

- The chemical strip reaction.



- The 800 TEA reaction.



- The 800 TEA detection.

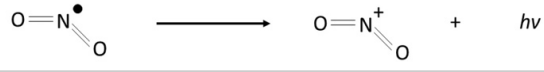
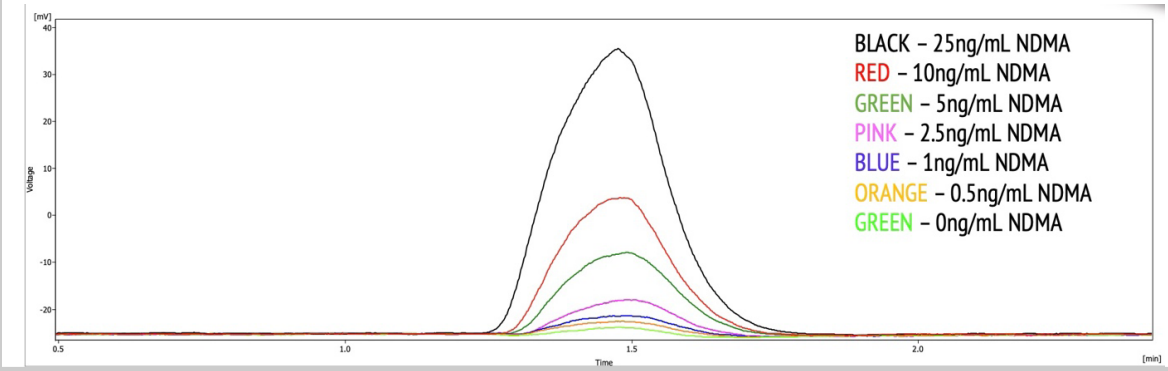


IMAGE 2: Chemical Reaction of ATNA Analysis

IMAGE 3: Calibration results from NDMA standards from 0-250ppb



nitrosamine formation. Traditionally, just using the vial chemistry shown in image 2 can cause potential false positives at any nitrites present will also release NO which would be detected.

Ellutia has optimised the vial chemistry, enabling the ATNA to effectively remove interference from nitrites at concentrations as high as 10 ppm while maintaining accurate detection of nitrosamines down to 10 ppb of NDMA. This is done by successfully reducing the nitrite content before the ATNA analysis. As seen in image 4, the response from samples with nitrite in up to 10 ppm treated with this vial chemistry gives a similar response to a blank. However, if nitrosamine is present, the response is still linear, as shown in image 5.

This optimisation significantly enhances the analytical clarity of ATNC measurements, providing a much clearer picture into the presence and concentration of actual nitrosamines. Whilst the original analysis showing Total Nitrosamine and nitrite content can also be a helpful measurement, highlighting the nitrite content in the sample which may also be of concern as a potential nitrosating agent.

GC-TEA for Speciated Nitrosamine Analysis

The same detector used in the ATNA system (800 Series TEA) can also be interfaced with a GC to provide targeted chromatographic separation for confirmatory testing. In this configuration The GC-TEA system utilizes a pyrolyser interface, where analytes eluted from the GC column are thermally decomposed, releasing NO radicals which

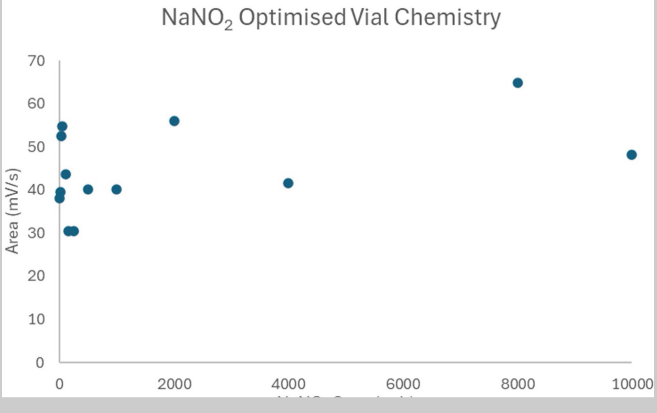


IMAGE 4: Response from samples of nitrite treated with vial chemistry

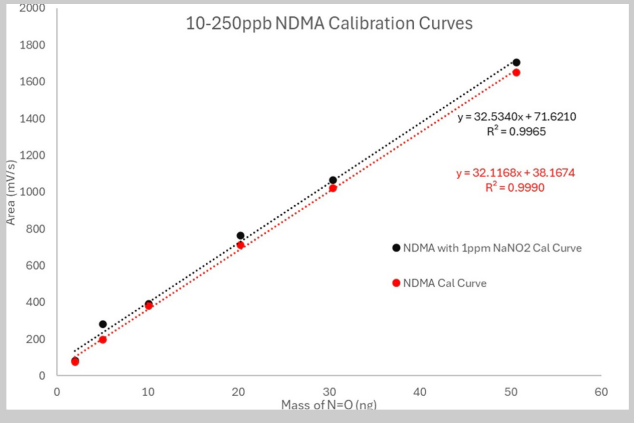
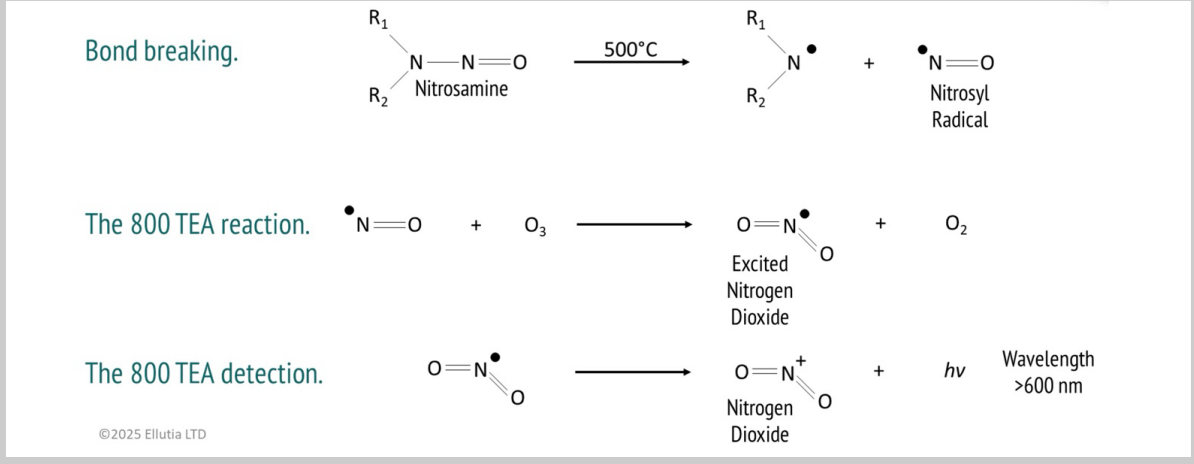


IMAGE 5: Calibration results from NDMA and NDMA with nitrite with vial chemisty

IMAGE 6: GC-TEA reactions



are subsequently detected using the TEA mechanism identical to that in ATNA. The pyrolytic process ensures that the detector response is specific only to nitrosamines, thus eliminating common analytical interferences and enabling detection of both known and unknown nitrosamine compounds.

Practical Application

A practical example demonstrated the combined use of ATNA and GC-TEA systems in analysing pharmaceutical samples for unknown nitrosamine contamination. This sample had been initially analysed by LC-MS/MS for expected nitrosamines which had identified them at

IMAGE 7: Chromatogram of Sample and of Calibration Standard

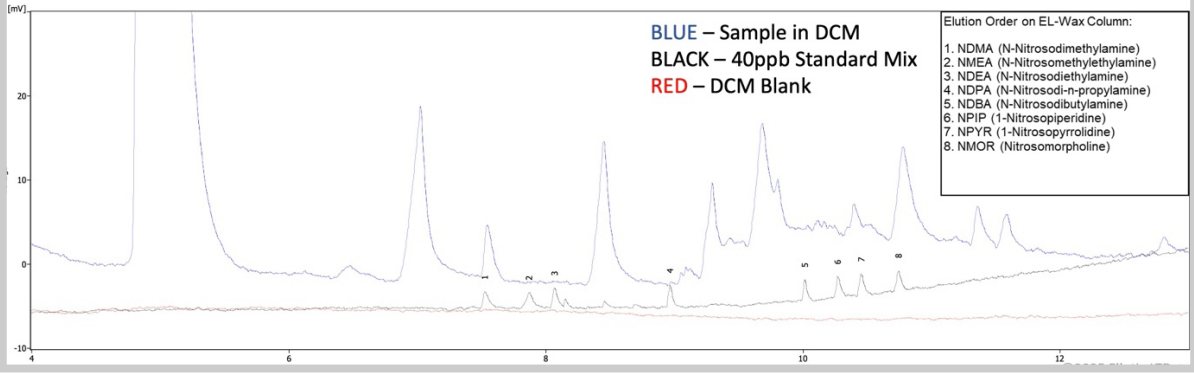
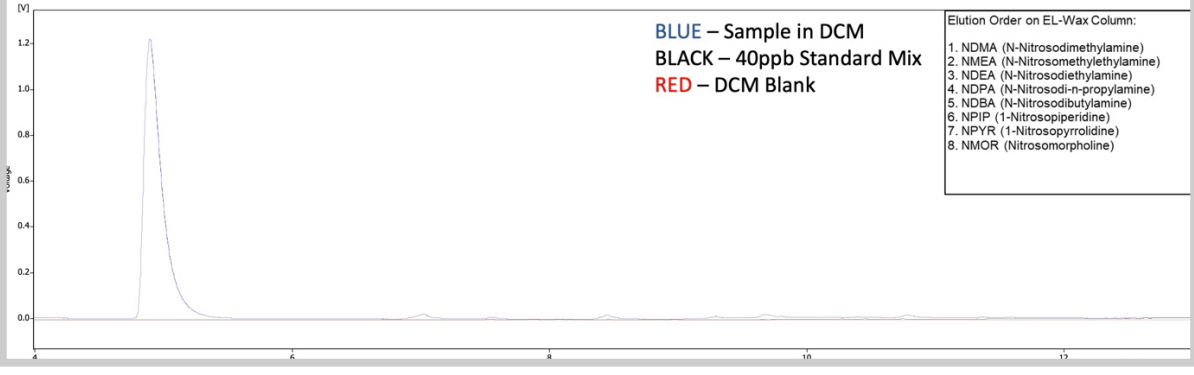


IMAGE 8: Chromatogram of sample zoomed out showing size of unidentified nitrosamine.



low levels. However the sample was then flagged for high ATNC values when analysed on the ANTA System. This same sample was then run in a GC-TEA system, which revealed multiple nitrosamine peaks, including previously unidentified nitrosamines not detected by the LC-MS/MS. As can been seen in Images 7 and 8 the size of the unidentified contamination was significant compared to the expected nitrosamines present.

This case study shows the GC-TEA's and ATNA's superior capability to highlight unknown nitrosamines, emphasising the importance of complementary use of screening and speciated analytical techniques.

Confidently detect and quantify trace nitrosamines and NDSRIs

Proven analytical workflows to address evolving regulatory requirements

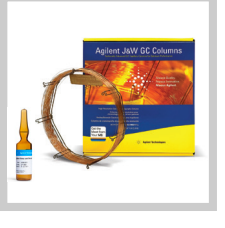
- N-nitroso impurities have been a focal point for regulatory bodies like the FDA and EMA due to their genotoxic and potentially carcinogenic nature, even in trace amounts.
- Highly sensitive analytical strategies are essential to meet stringent regulatory requirements.
- Agilent's comprehensive workflow support and offerings, including GC/MS, LC/MS, and high-throughput LC/MS systems, provide the precision and reliability needed for accurate detection and quantification of these nitrosamines and NDSRIs in APIs and drug products.

1290 Infinity III LC

6495D Triple Quadrupole LC/MS

7010D Triple Quadrupole GC/MS

LC/MS Consumables



GC/MS Consumables

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KEY APPLICATION NOTES

LC/MS-based strategies:

- Quantitation of N-Nitroso Dabigatran Etexilate Impurity Using the Agilent 6495 LC/TQ
- Quantitation of N-Nitroso Dabigatran Etexilate Impurity
- Improved LC/MS Performance for the Determination of Polar Nitrosamines by Means of the Agilent 1260 Infinity II Hybrid Multisampler

GC/MS-based strategies

- Quantification of Nitrosamine Impurities in Sartan Drugs Using an Agilent GC/TQ with Hydrogen Carrier Gas
- Screening of Nitrosamine Impurities in Drug Products and Drug Substances Using Agilent GC/MS/MS Instrumentation
- Quantification of Nine Nitrosamine Impurities in Sartan Drugs Using an Agilent GC-TQ