Overview of Testing Methods for *N*-nitrosamines Monitoring: Regulatory Requirements and Analytical Challenges

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200 usp
The standard of trust

Agenda

- Sensitivity Requirements: Regulatory Recommendations (EMA and FDA)
- Factors which impact sensitivity and selectivity
- Techniques used:
  - Nitrosamines in different matrices,
  - Nitrite/Nitrate in excipients
- Analytical Challenges
- USP <1469> Nitrosamine Impurities: Testing Methods
- Case Studies

Analytical Procedure for *N*-Nitrosamines

What should be the required sensitivity for analytical methods?

Nitrosamine Level ≤ **Acceptable Intake (AI)**
toxicologically required limit

e.g.: NDMA in Valsartan (96ng/day / 320mg/day)
AI= 0.3ppm (0.0003mg/mL)

- Limit of Quantification (LOQ) - ICH Q2 (R1): provides the minimum level at which an analyte can be quantified with acceptable accuracy and precision
  - LOQ preferred over LoD for impurity testing and decision-making
  - LOQ should be used to define the required analytical sensitivity for impurity testing.
- Limit of Detection (LOD) - ICH Q2 (R1): LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value
  - Experts recommended not to use LOD for setting limits and not to use technical limits as nitrosamines may not be avoidable completely in many cases

As a minimum requirement the method should have: LOQ ≤ AI and meet regulatory recommendations for sensitivity...

Analytical Procedure for *N*-Nitrosamines

Required sensitivity for analytical methods

- FDA Control of Nitrosamines Impurities in Human Drugs - Guidance for Industry - FDA February 2021
  - Products with MDD < 880 mg/day: LOQ ≤ 0.03ppm
  - Products with MDD > 880 mg/day: LOQ as low as reasonably practical
  - LOQ < Test Result ≤ Acceptable Intake
- EMA Assessment Report - Nitrosamines Impurities in human medicinal products - EMA 25June20
  - LOQ ≤ Acceptable limit for the respective nitrosamine impurities, taking into account the purpose of testing
    - Routine control: LOQ ≤ Acceptable Limit
    - Justify skip testing: LOQ ≤ 30% of AL
    - Justify omission from the specification: LOQ ≤ 10% of AL
  - Exceptions may be needed depending on the maximum daily dose (MDD) or if more than one nitrosamine is expected to be present. Such cases should be discussed with the relevant competent authorities.

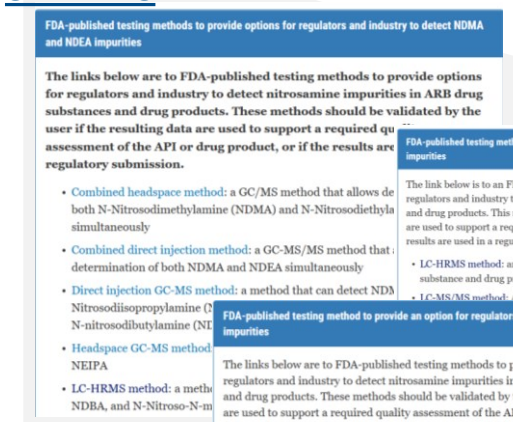
Analytical Procedure for *N*-Nitrosamines

Published Methods for Nitrosamines monitoring in APIs and DP

The low levels at which the nitrosamine impurities occur creates challenges for testing

To assist in the testing of samples the US FDA, Official Medicines Control Laboratories (OMCLs) Network of the Council of Europe, Health Canada and have also published several testing methods for nitrosamines

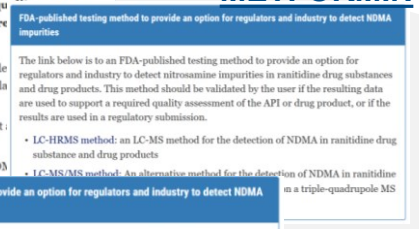
SARTANS


FDA-published testing methods to provide options for regulators and industry to detect NDMA and NDEA impurities

The links below are to FDA-published testing methods to provide options for regulators and industry to detect nitrosamine impurities in ARB drug substances and drug products. These methods should be validated by the user if the resulting data are used to support a required qu... assessment of the API or drug product, or if the results are ... regulatory submission.

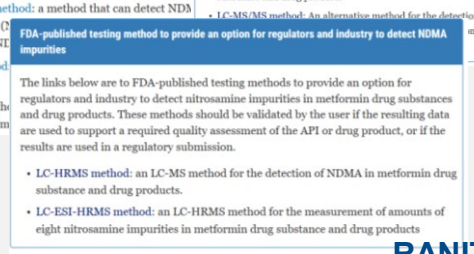
- Combined headspace method: a GC/MS method that allows de... both N-Nitrosodimethylamine (NDMA) and N-Nitrosodiethyla... simultaneously
- Combined direct injection method: a GC-MS/MS method that ... determination of both NDMA and NDEA simultaneously
- Direct injection GC-MS method: a method that can detect NDA... Nitrosodiisopropylamine (N... N-nitrosodibutylamine (ND...
- Headspace GC-MS method... NEIPA
- LC-HRMS method: a metho... NDBA, and N-Nitroso-N-m...

METFORMIN


FDA-published testing method to provide an option for regulators and industry to detect NDMA impurities

The link below is to an FDA-published testing method to provide an option for regulators and industry to detect nitrosamine impurities in ranitidine drug substances and drug products. This method should be validated by the user if the resulting data are used to support a required quality assessment of the API or drug product, or if the results are used in a regulatory submission.

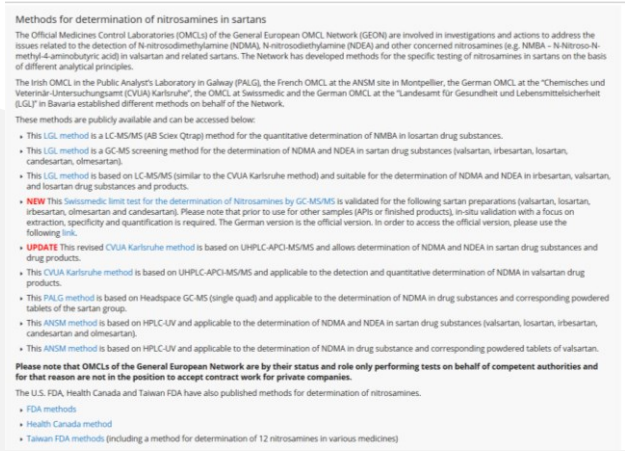
- LC-HRMS method: an LC-MS method for the detection of NDMA in ranitidine drug substance and drug products
- LC-MS/MS method: An alternative method for the detection of NDMA in ranitidine ... a triple-quadrupole MS


FDA-published testing method to provide an option for regulators and industry to detect NDMA impurities

The links below are to FDA-published testing methods to provide an option for regulators and industry to detect nitrosamine impurities in metformin drug substances and drug products. These methods should be validated by the user if the resulting data are used to support a required quality assessment of the API or drug product, or if the results are used in a regulatory submission.

- LC-HRMS method: an LC-MS method for the detection of NDMA in metformin drug substance and drug products.
- LC-ESI-HRMS method: an LC-HRMS method for the measurement of amounts of eight nitrosamine impurities in metformin drug substance and drug products

RANITIDINE


Methods for determination of nitrosamines in sartans

The Official Medicines Control Laboratories (OMCLs) of the General European OMCL Network (GEON) are involved in investigations and actions to address the issues related to the detection of N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA) and other concerned nitrosamines (e.g. NMBA - N-Nitroso-N-methyl-4-aminobutyric acid) in valsartan and related sartans. The Network has developed methods for the specific testing of nitrosamines in sartans on the basis of different analytical principles.

The Irish OMCL in the Public Analyst's Laboratory in Galway (PALG), the French OMCL at the ANSM site in Montpellier, the German OMCL at the "Chemisches und Veterinär-Untersuchungsamt (CVUA) Karlsruhe", the OMCL at Swissmedic and the German OMCL at the "Landesamt für Gesundheit und Lebensmittelsicherheit (LGL)" in Bavaria established different methods on behalf of the Network.

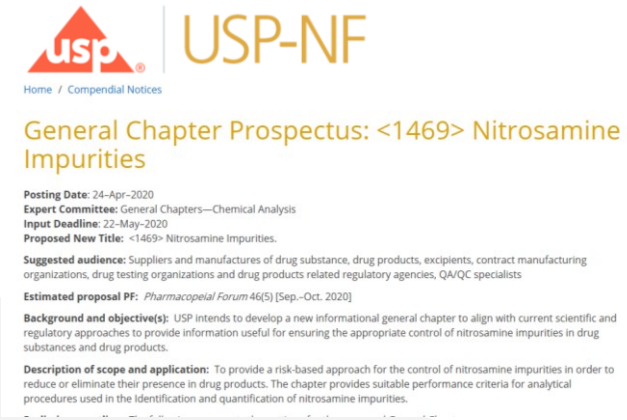
These methods are publicly available and can be accessed below:

- This LGL method is a LC-MS/MS (AB Sciex Qtrap) method for the quantitative determination of NMBA in losartan drug substances.
- This LGL method is a GC-MS screening method for the determination of NDMA and NDEA in sartan drug substances (valsartan, irbesartan, losartan, candesartan, olmesartan).
- This LGL method is based on LC-MS/MS (similar to the CVUA Karlsruhe method) and suitable for the determination of NDMA and NDEA in irbesartan, valsartan, and losartan drug substances and products.
- **NEW** This Swissmedic limit test for the determination of Nitrosamines by GC-MS/MS is validated for the following sartan preparations (valsartan, losartan, irbesartan, olmesartan and candesartan). Please note that prior to use for other samples (APIs or finished products), in-situ validation with a focus on extraction, specificity and quantification is required. The German version is the official version. In order to access the official version, please use the following link.
- **UPDATE** This revised CVUA Karlsruhe method is based on UHPLC-APCI-MS/MS and allows determination of NDMA and NDEA in sartan drug substances and drug products.
- This CVUA Karlsruhe method is based on UHPLC-APCI-MS/MS and applicable to the detection and quantitative determination of NDMA in valsartan drug products.
- This PALG method is based on Headspace GC-MS (single quad) and applicable to the determination of NDMA in drug substances and corresponding powdered tablets of the sartan group.
- This ANSM method is based on HPLC-UV and applicable to the determination of NDMA and NDEA in sartan drug substances (valsartan, losartan, irbesartan, candesartan and olmesartan).
- This ANSM method is based on HPLC-UV and applicable to the determination of NDMA in drug substance and corresponding powdered tablets of valsartan.

Please note that OMCLs of the General European Network are by their status and role only performing tests on behalf of competent authorities and for that reason are not in the position to accept contract work for private companies.

The U.S. FDA, Health Canada and Taiwan FDA have also published methods for determination of nitrosamines.

- FDA methods
- Health Canada method
- Taiwan FDA methods (including a method for determination of 12 nitrosamines in various medicines)


USP-NF

Home / Compendial Notices

General Chapter Prospectus: <1469> Nitrosamine Impurities

Posting Date: 24-Apr-2020
Expert Committee: General Chapters—Chemical Analysis
Input Deadline: 22-May-2020
Proposed New Title: <1469> Nitrosamine Impurities.

Suggested audience: Suppliers and manufactures of drug substance, drug products, excipients, contract manufacturing organizations, drug testing organizations and drug products related regulatory agencies, QA/QC specialists

Estimated proposal PF: *Pharmacopeial Forum* 46(5) [Sep.–Oct. 2020]

Background and objective(s): USP intends to develop a new informational general chapter to align with current scientific and regulatory approaches to provide information useful for ensuring the appropriate control of nitrosamine impurities in drug substances and drug products.

Description of scope and application: To provide a risk-based approach for the control of nitrosamine impurities in order to reduce or eliminate their presence in drug products. The chapter provides suitable performance criteria for analytical procedures used in the Identification and quantification of nitrosamine impurities.

US FDA
LC-ESI-HRMS, GC-MS/MS, LC-APCI-QqQ

NDMA, NDEA, NDIPA, NDBA, NEIPA, NMBA, NMPA, NDPA

Health Canada/EMA
GC-MS/MS, LC-APCI-QTrap, LC-APCI-QqQ

NDMA, NDEA

USP General Chapter <1469> Nitrosamine Impurities – USP/NF- 1st June 2021
(LC-ESI-HRMS, GC-MS/MS, LC-APCI-QqQ)

NDMA, NDEA, NDIPA, NDBA
NEIPA, NMBA, NMPA

Overview of Testing Methods: FDA and USP Methodologies

Different types of nitrosamines found in ARB drugs:
Valsartan, Losartan, Irbesartan etc.

2018

June — Nitrosamine found in Valsartan DS

11Dec — **GC-MS/MS** NDMA/NDEA in **Valsartan**

2019

28 Jan — **GC-MS (Headspace)** NDEA in **Valsartan**

19 Apr — **GC-MS/MS (Direct injec.)** NDMA, NDEA, NDIPA, NDBA, NEIPA in **Valsartan DS/DP**

29 Apr — **GC-MS/MS (Headspace)** NDMA, NDEA, NDIPA, NEIPA in **Valsartan DS/DP**

21 May — **LC-HRMS ESI-QOrbitrap Kinetex F5** NDMA, NDEA, NDIPA, NDBA, NEIPA, NMBA in **Losartan DS/DP**

Sep — Nitrosamines found in Ranitidine DP

24 Jul — **RapidFire-MS/MS** graphitic carbon SPE cartridge NDMA, NDEA, NDIPA, NDBA, NEIPA, NMBA in **ARB drugs**

17 Oct — **LC-MS/MS APCI-QqQ** NDMA in **Ranitidine DS and DP**

13 Sep — **LC-HRMS ESI-QOrbitrap ACE C18AR** NDMA in **Ranitidine DP**

Nov/Dec — NDMA found in metformin

4 Feb — **LC-HRMS ESI-QOrbitrap XSelect CSH C18** NDMA in **Metformin**

6 Mar — **LC-HRMS ESI-QOrbitrap Kinetex Biphenyl** NDMA, NDEA, NDIPA, NDBA, NEIPA, NMBA, NMPA, NDPA in **Metformin**

2020

July — Release of USP nitrosamines standards
NDMA
NDEA
NDIPA
NDBA
NEIPA
NMBA
NMPA*
NDMA-d6*
**May2021*

1 Sep — *USP PF46(5)* GC <1469> *Nitrosamin Impurities*

2021

1 June — *USP-NF* GC <1469>

1 June — **USP-NF Issue 3 - GC <1469> became online**
NDMA, NDEA, NDIPA, NEIPA, NMBA, NDBA and NMPA in **Sartans**
LC-HRMS ESI-QOrbitrap/L43 Column (PFP)
NDMA, NDEA, NDIPA, NEIPA, NMBA and NDBA in **Sartans**
LC-APCI-QqQ / L1 Column (C18)

NDMA, NDEA, NDIPA, NEIPA in **Sartans**
GC-MS/MS Headspace (GC-EI-QqQ)
NDMA, NDEA, NDIPA, NEIPA, NDBA and NMPA in **Sartans**
GC-MS/MS Direct. Inj. (GC-EI-QqQ)

Legend:
LC: Liquid Chromatography
GC: Gas Chromatography
HRMS: High-Resolution Mass Spectrometry
ESI: Electrospray Ionization
APCI: Atmospheric Pressure Chemical Ionization
QqQ: Triple quadrupole

Testing Methods Published by Regulators

Sartan-Based Drugs

U.S. FDA

https://www.fda.gov/media/131868/download
https://www.fda.gov/media/142092/download

Health Canada

https://healthycanadians.gc.ca/recall-alert-rappel-avis/hc-sc/2020/72963a-eng.php

Council of Europe

edqm
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

Funded by the European Union and the Council of Europe
EUROPEAN UNION

COUNCIL OF EUROPE
CONSEIL DE L'EUROPE

Implemented by the Council of Europe

https://www.edqm.eu/en/ad-hoc-projects-omcl-network

Taiwan FDA

https://www.fda.gov.tw/ENG/siteList.aspx?sid=10360

FDA
食品藥物管理署
Food and Drug Administration

Ranitidine-Based Drugs

Council of Europe

Methods for determination of nitrosamines in ranitidine

The German OMCL at the "Landesamt für Gesundheit und Lebensmittelsicherheit (LGL)" in Bavaria and the German OMCL at the "Chemisches und Veterinär-Untersuchungsamt (CVUA) Karlsruhe" established the following methods:

- This LGL method is a GC-MS screening method for NDMA in ranitidine drug substances.
- This CVUA Karlsruhe method is based on UHPLC-APCI-MS/MS and allows determination of NDMA in ranitidine drug substances and drug products.

https://www.edqm.eu/en/ad-hoc-projects-omcl-network

U.S. FDA

FDA-published testing method to provide an option for regulators and industry to detect NDMA impurities

The link below is to an FDA-published testing method to provide an option for regulators and industry to detect nitrosamine impurities in ranitidine drug substances and drug products. This method should be validated by the user if the resulting data are used to support a required quality assessment of the API or drug product, or if the results are used in a regulatory submission.

- LC-HRMS method: an LC-MS method for the detection of NDMA in ranitidine drug substance and drug products
- LC-MS/MS method: An alternative method for the detection of NDMA in ranitidine drug substance and drug products. This method is based on a triple-quadrupole MS platform.

https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-ndma-zantac-ranitidine

Health Science Authority Singapore

Date of release	Title
12 Sep 2019	Test method for determination of N-nitrosodimethylamine (NDMA) in ranitidine products by LC-MS/MS 154 KB
18 Sep 2019	Test method for identification of six nitrosamine impurities in western medicines by LC-HRMS 514 KB

https://www.hsa.gov.sg/announcements/safety-alert/updates-on-impurities-in-ranitidine-products

What can be tested and how?

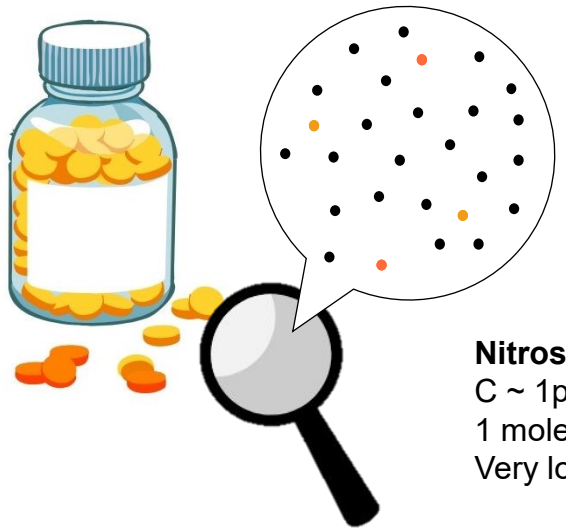
8. How should confirmatory tests be conducted by MAHs and manufacturers?

For the purpose of confirmatory testing as part of step 2 of the call for review to MAHs, testing should generally be carried out on the FP. Testing of the API, its intermediates, starting materials, solvents, reagents, excipients or any other raw materials for nitrosamines, amines, nitrites or other compounds with potential to generate nitrosamines is also recommended, if the risk assessment indicates that they are a potential source of nitrosamine impurities in the FP. In such cases, the results of testing API, intermediates or other relevant materials may be used to support root cause investigations and the development of a justified control strategy for nitrosamine impurities.

EMA Q&A - Article 5(3) of Regulation (EC) on nitrosamine impurities - July 2023

Analytical Procedure for *N*-Nitrosamines

Performance Characteristics*



- Active ingredient
- Nitrosamine impurity
- Endogenous Matrix Compound

Nitrosamine Impurity
C ~ 1ppm
1 molecule out of 1,000,000 molecules
Very low concentration!

1. Sensitivity:

- Sensitive to detect and quantify *N*-nitrosamines in DPs and APIs at ppm level
- Sensitive enough to meet the proposed regulatory recommendations
- Neither LOD nor LOQ are constant values and can change over time depending on: equipment, laboratories, personnel, sample preparation and many other factors.

2. Selectivity:

- Selective for target* nitrosamines (Same method applicable to different DP? And APIs?) **those possible to be formed during the API/DP manufacturing process, storage etc – after risk assessment*

3. Accuracy and Precision: Challenges: extraction efficiency suitable recovery of trace impurities, matrix effects

4. Linearity:

- Linear within the proposed concentration range. Ex.: LOQ, 50%, 75%, 100%, 125%, 150% (including the AI)
- Note: LOQ as low as reasonably practical or ≤ 10% of AI if possible)

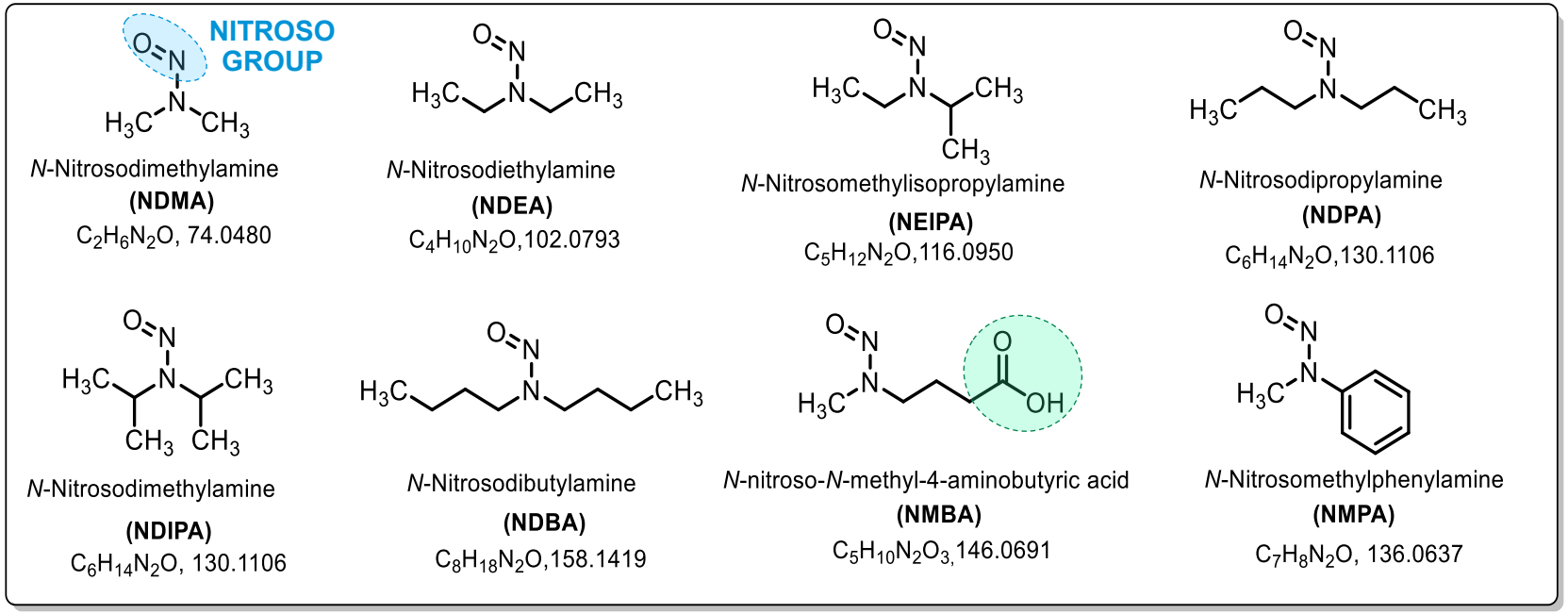
5. Robustness: ensure consistent/reproducible results

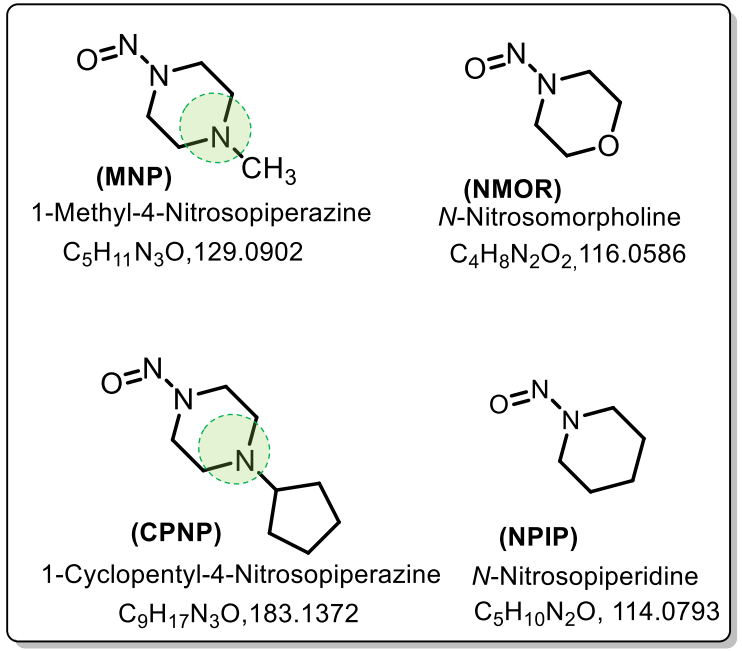
ICH guideline Q2(R1) - VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY

*"**Specificity** is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.*

** for quantitative analysis*

Physicochemical Properties





- The majority of *N*-nitrosamine impurities reported/found in pharmaceutical products are polar compounds
- Ionizable compounds?
  - Majority are neutral: NDMA, NDEA, NEIPA, NDPA, NDIPA, NDBA, NMPA, NMOR, NPIP
  - Basic compounds: MNP and CPNP
  - Acidic Compounds: NMBA
- Solubility: While NDMA and NDEA show a high-water solubility, the water solubility of NDPA and NDBA is lower. Typical solvents used are dichloromethane, methanol and acetone.
- Some nitrosamines may degrade at high temperature
- Evaluate physico-chemical properties of APIs:
  - Can degrade forming secondary amines? Etc.

- Majority of Nitrosamines are volatile and semi-volatile: analysis by GC-MS!

N-Nitrosamine	Boiling Point
NDMA	151°C
NDEA	175°C
NDIPA	195°C
NEIPA	217°C
NDBA	235°C

N-Nitrosamine	Boiling Point
NMPA	185°C
NMBA*	364°C
NDPA	206°C
MNP	236°C
CPNP*	316°C

Which analytical techniques can be used?

Nitrosamines

- **LC-UV**
  - Nitrosamines that have only the N-NO group as chromophore group N-NO have low molar absorptivity (2 maxima of absorption at 230 and 330 nm).
  - HPLC-UV will have a low detectability and poor sensitivity and may not be suitable to control nitrosamines at or below the established acceptable limits.

- **Gas Chromatography-Mass Spectrometry (GC-MS)**
  - It may increase sensitivity and selectivity
  - Many nitrosamines are volatile/semi-volatile
  - Thermally labile APIs (e.g., rantidine) may degrade at high temperature which may form nitrosamine as artifact (in the presence of nitrite and acid).
  - Limitations: Non-volatile nitrosamines, thermal degradation

- **Liquid Chromatography-Mass Spectrometry (LC-MS):**
  - It may increase sensitivity and selectivity
  - Broad range of nitrosamines (even those not so volatile)
  - Thermal degradation of nitrosamines using APCI? Use of lower temperature in APCI.

- **Gas Chromatography-Thermal Energy Analyzer (GC-TEA)**
  - Great sensitivity for compounds with *N*-nitroso group
  - Lack of selectivity for organic nitrites, *N*-nitroso, *C*-nitroso, nitrates and inorganic nitrite.

- **LC-FLD With Pre-column Fluorescence Labeling:**
  - Use derivatization protocols and analysis by HPLC-FLD

Mei Zhao et al. Chromatographia (2016) 79:431–439. DOI 10.1007/s10337-016-3040-1

To enhance sensitivity and selectivity:

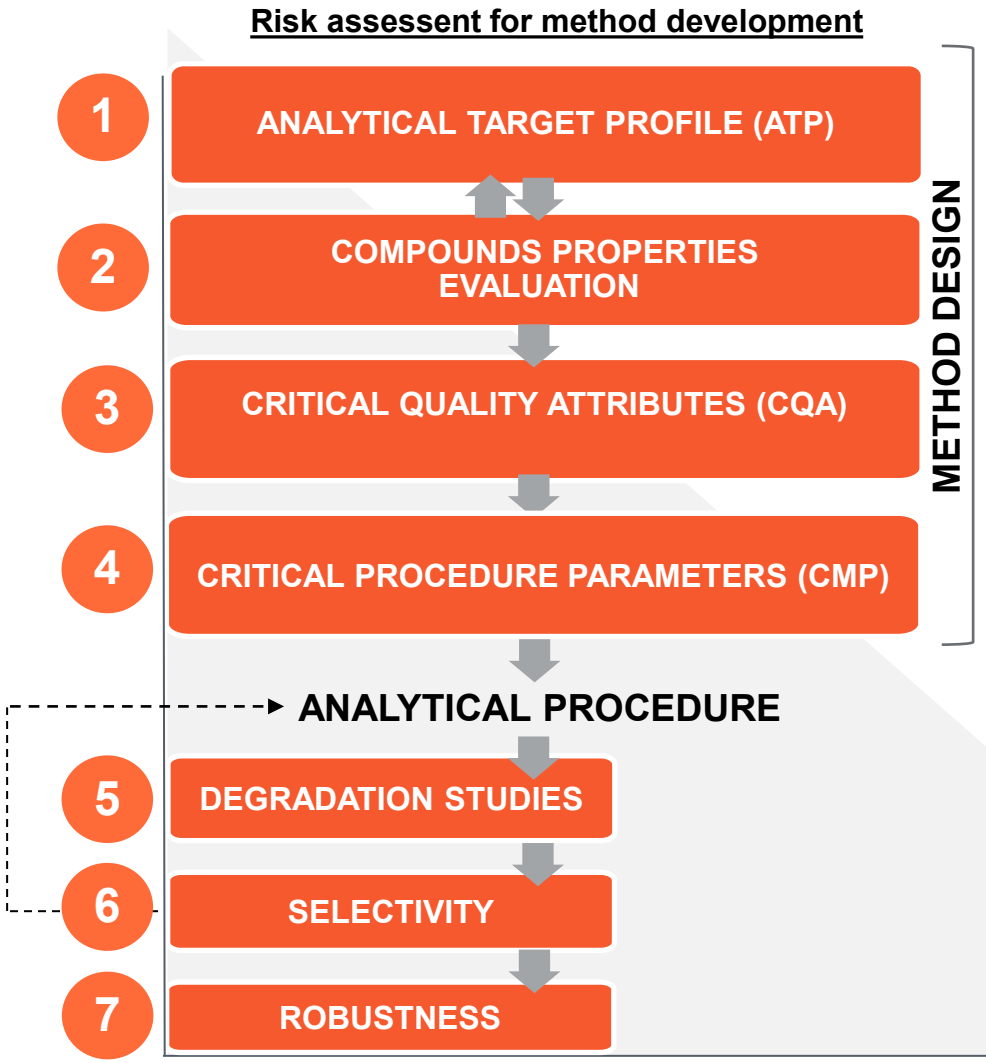
GC-MS: In case nitrosamines are volatile/semi-volatile and API does not degrade at high temp

LC-MS: Broad range of nitrosamines (even those not so volatile) Thermally labile APIs, which may form nitrosamine as artifact

Review article on published methods: Shaik, K.M. et al. Regulatory Updates and Analytical Methodologies for Nitrosamine Impurities Detection in Sartans, Ranitidine, Nizatidine, and Metformin Along with Sample Preparation Techniques, (2020): Critical Reviews in Analytical Chemistry

Analytical Procedure for *N*-Nitrosamines

Analytical Procedure Development



CQA

1. Selectivity
- Resolution between target compounds and adjacent peaks (chromatography)
- Mass Resolution between isobaric compounds (MS)

2. Sensitivity
- Sensitive to detect at ppb level

3. Accuracy & Precision

4. Retention Factor (k):
- Ideal: $1 < k < 10$,
- Acceptable: $0.5 < k < 20$

CPP

SAMPLE PREPARATION
- Selective extraction *x* Total API/target compounds dissolution?
- Sonication, extraction time, extraction repetition?
- Solid Phase Extraction (SPE) to concentrate trace impurities enhancing sensitivity?
- **Reduce/minimize matrix effect**

CHROMATOGRAPHY
- Stationary phase: chromatographic columns
- Organic solvent, pH of mobile phase
- Use of additives (ion-pair reagents, acid etc)
- Column temperature (especially for ionizable compouds)
- **Reduce/minimize matrix effect**

MASS SPECTROMETRY
- Ionization Source (sensitivity and selectivity)
- MS resolving power (selectivity)
- Acquisition data strategies (enhance sensitivity and selectivity)
- **Reduce/minimize matrix effect**

DATA PROCESSING
- Data Processing: mass tolerance settings (selectivity)

Which analytical techniques can be used?

Nitrites and Nitrate

- Nitrite is everywhere
  - Nitrosamines can be formed at any stage of drug product manufacturing
  - Reagents, solvents, APIs, excipients, packaging materials are relevant as nitrite sources
- Nitrite as analyte
  - Very polar
  - Low UV absorbance
  - Reactive
  - Ubiquitous nitrite makes trace level analysis challenging
  - Many methods rely on derivatization: Griess, DAN

Methods for the detection and determination of nitrite and nitrate: A review

Qiu-Hua Wang [a 1], Li-Ju Yu [b c 1], Yang Liu [c], Lan Lin [c], Ri-gang Lu [d], Jian-ping Zhu [d], Lan He [a c], Zhong-Lin Lu [a]

Wang, Q. H., et al (2017). Methods for the detection and determination of nitrite and nitrate: A review. In *Talanta* (Vol. 165, pp. 709–720). Elsevier B.V. https://doi.org/10.1016/j.talanta.2016.12.044

169 novel methods for nitrite detection 2001-2017

Which analytical techniques can be used?

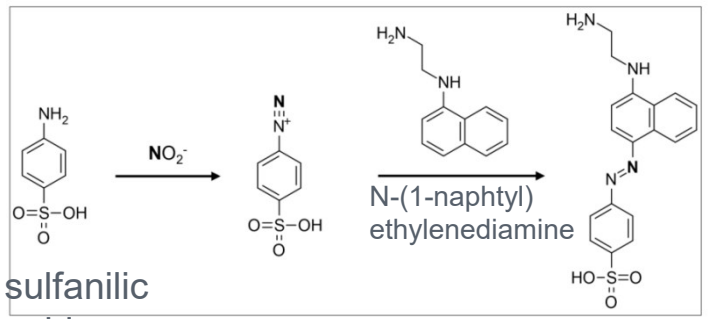
Nitrites and Nitrate

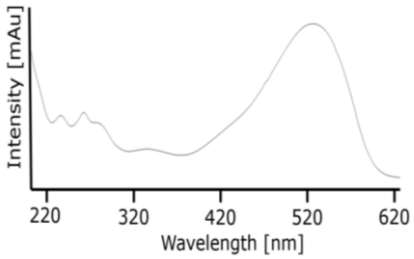
Analytical technique	~ LOQ [ng/g]	Prevalence of instrumentation	Cost (instrument + reagents)	Selectivity	Limitations	Advantages
HPLC-UV	20-30*	high	low	high	co-elution Griess inhibition	Easy, generic workflow
HPLC-MS/MS	20-30*	scarce	high	highest	Griess inhibition	Highest sensitivity & selectivity, generic workflow, SIDA
IC-CD	100-150	medium	medium	lowest	co-elution, method development (sample prep. & chromatography) required	Direct method, nitrate data available
IC-PCD	20-30*	scarce	medium	high	(co-elution)	Generic workflow, most versatile

Challenge ahead:
Test hundreds of chemicals at low ng/g level for nitrite

*For Griess derivatization-based methods, the LOQ is not limited by instrument sensitivity but by ubiquitous nitrite background contamination!

- Griess derivatization
  - Limited UV absorbance of nitrite (max. at 210 nm) → poor sensitivity and selectivity (UV detection)

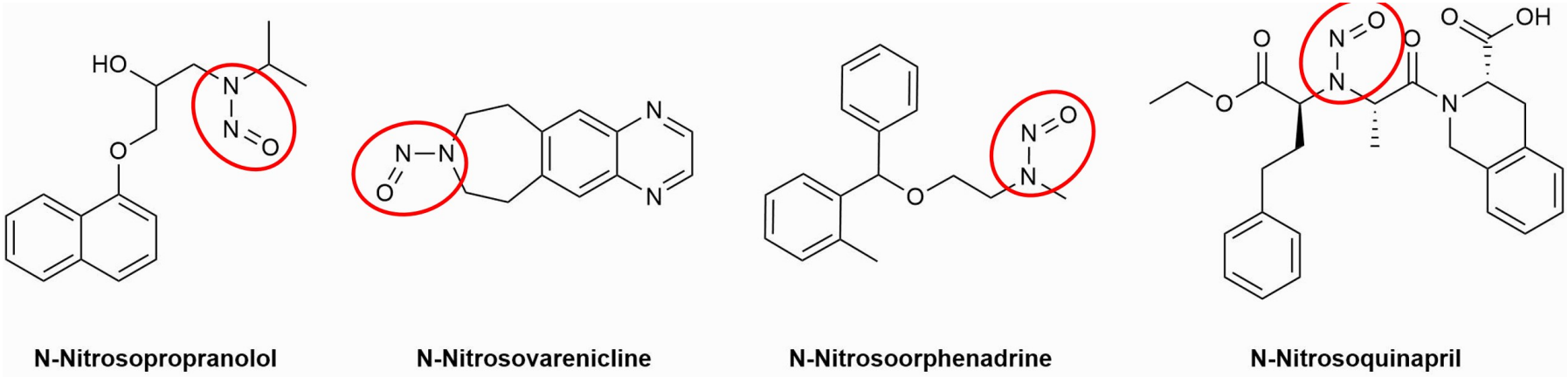




Source: Stephanie Wetzel, Merck

NDRSIs

- It was recently shown that up to 40% of common APIs and 30% of API impurities are potential NA precursors, as they contain vulnerable amine moieties.
- If only the more reactive secondary amines are considered, still 13−15% of APIs are potentially at risk.
- Not surprisingly, NDSRIs have become the focus from both an industry and regulatory perspective
- Which technique can be used to monitor NDRSIs?



N-Nitrosopropranolol | N-Nitrosovarenicline | N-Nitrosoorphenadrine | N-Nitrosoquinapril

Analytical sensitivity requirements - Examples of required LOQs to prevent routine testing

LOQ expressed as ng/g product - Assumes 10% drug loading

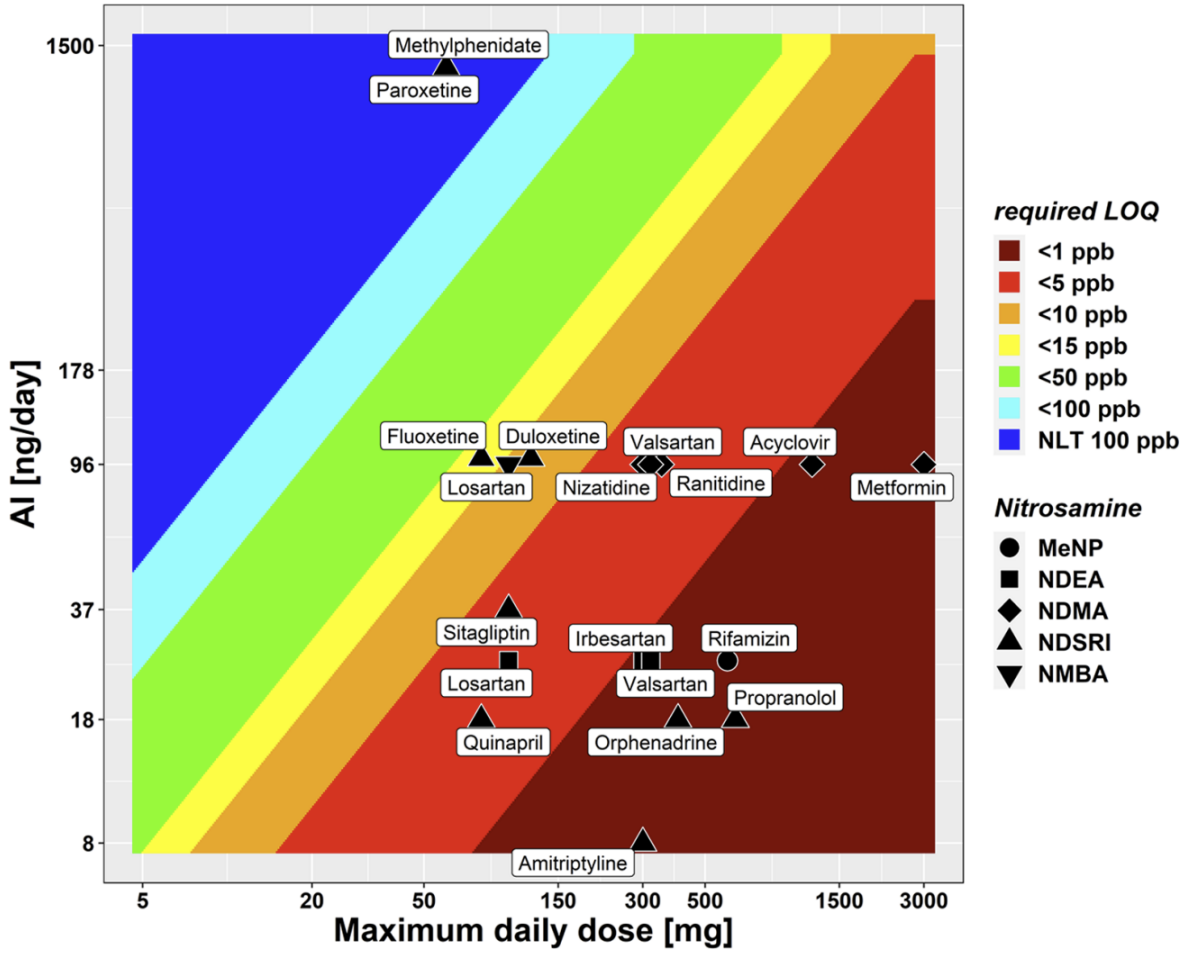


Figure 5. Interrelation of NA AI, API MDD, and required method LOQ, expressed as nanograms per gram of product and assuming a drug loading of 10%. For NDSRIs, calculations were done based on a worst-case AI of 18 ng/day, unless a compound-specific AI is listed in the EMA Q&A, as for nitrosoamitriptyline.

Nudelman, R., et al. (2023). The Nitrosamine "Saga": Lessons Learned from Five Years of Scrutiny. Organic Process Research & Development. https://doi.org/10.1021/acs.oprd.3c00100

Few Challenges

- Interference caused by presence of trace amounts of nitrosamines in testing materials used (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 and/or sample preparation
- Selectivity: Use of accurate mass techniques may be 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).
- Recovery issues (due to matrix effect) – artificially lower quantitation results
- Sensitivity – detection at ppm level

Mass Spectrometry Fundamentals

Mass Spectrometry Overview

SAMPLE INTRODUCTION

- LC
- GC
- ICP
- CE
- Direct Injection
- Flow Injection Analysis (FIA)

IONIZATION SOURCE

- EI *(Electron Ionization)*
- CI *(Chemical ionization)*
- API *(Atmospheric Pressure Ionization)*
  - ESI *(Electrospray Ionization)*
  - APCI *(Atm. Pressure Chemical Ionization)*
  - APPI *(Atm. Pressure Photoionization)*
- MALDI
- *DESI *(Desorption Electrospray Ionization)*
- *DART (*Direct Analysis in Real Time)*
- FAB *(Fast Atom Bombardment)*

DATA ACQUISITION STRATEGIES
Data-Dependent Acquisition (DDA)
Data-Independent Acquisition (DIA)
Targeted Data Acquisition (TDA)
etc.

ANALYZER

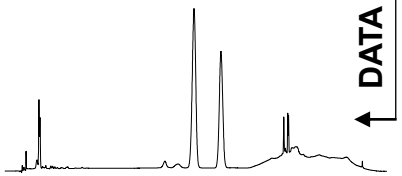
- Quadrupole
- Ion Trap
- ToF *(Time-of-Flight)*
- FT-ICR *(Fourier Transformed-Ion Cyclotron Resonance)*
- Orbitrap
- Tandem MS (MS/MS)
  - Triple Quadrupole (QqQ)
  - Ion-Trap (IT): 3D or linear
  - QToF/IT-ToF
  - Q-Orbitrap
  - LIT-Orbitrap
  - Qq-Linear Ion Trap
  - Q-Orbitrap-LIT

DETECTOR

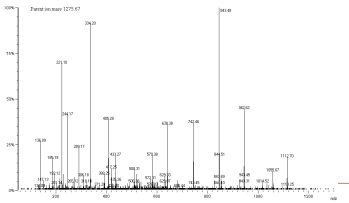
DATA SYSTEM

DATA PROCESSING

CHROMATOGRAMS



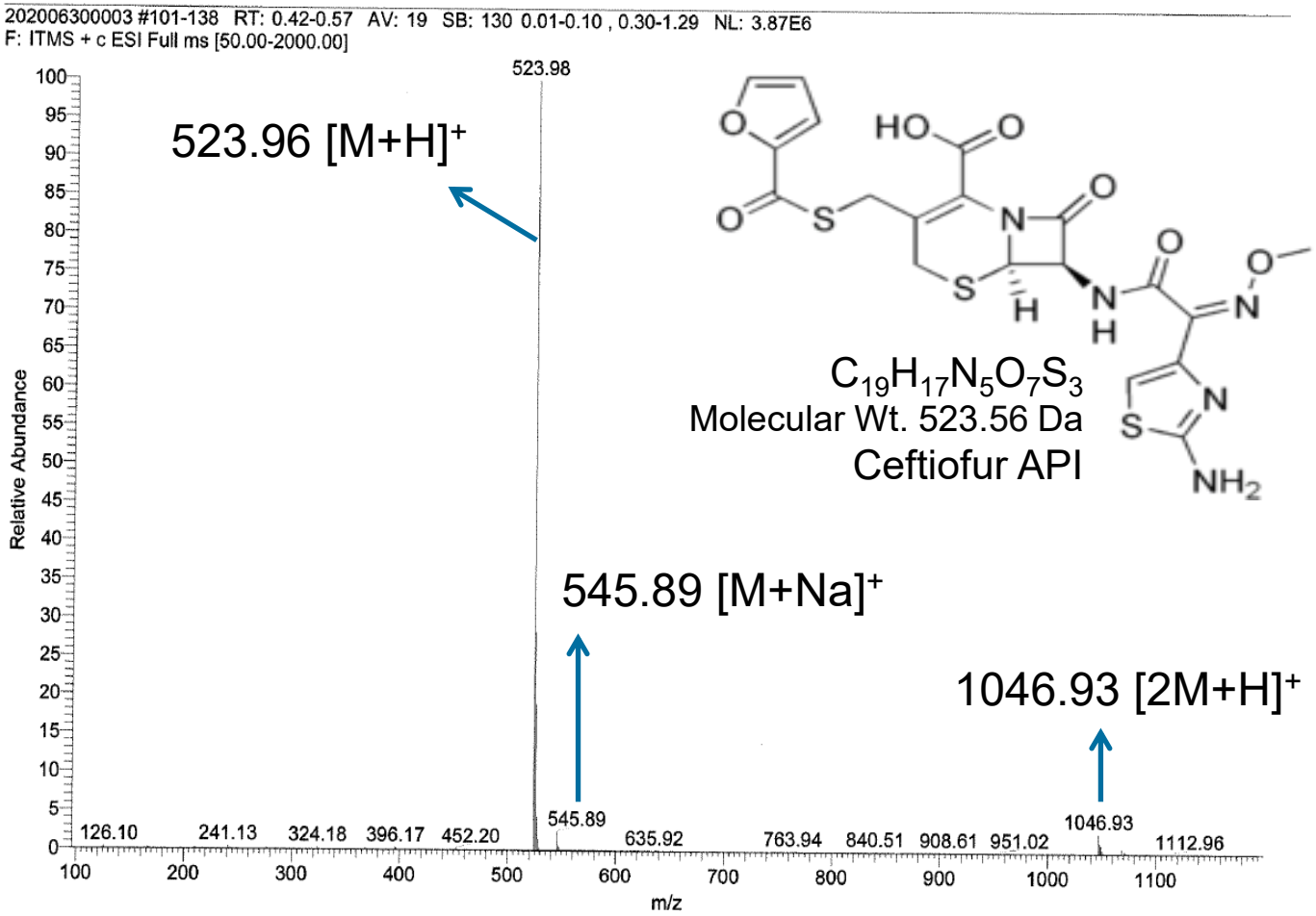
MASS SPECTRUM



Mass Spectrometry Fundamentals

Mass Spectrum

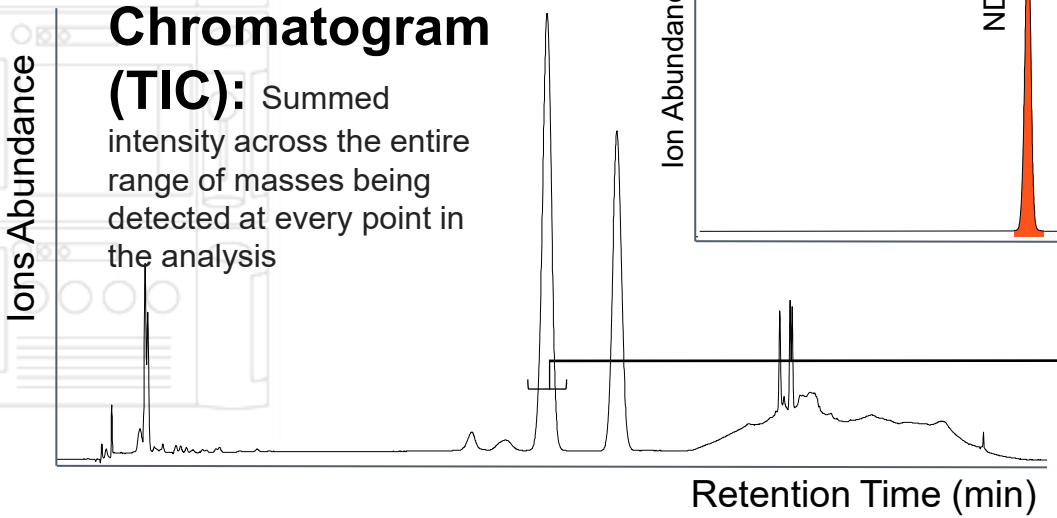
- The mass spectrum is typically displayed as a plot of *m/z* on the abscissa versus ion intensity as the ordinate.
- The mass-to-charge ratio of each component of a sample
- The relative abundance of each ion
- Depending on the ionization procedure, the different types of ionic species may be formed and are indicative of molecular mass of the analyte:
  - Molecular ion ($M^{+\bullet}$, $M^{-\bullet}$)
  - Protonated or deprotonated molecules $[M+H]^+$ or $[M-H]^-$
  - Adduct ions, etc.



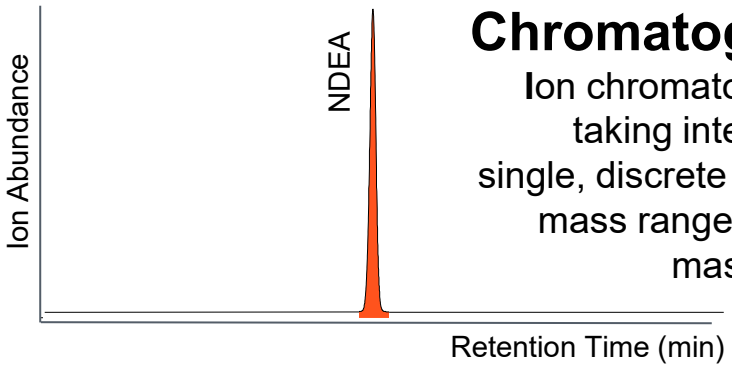
USP <1736> Applications of Mass spectrometry

Mass Spectrum and Chromatograms

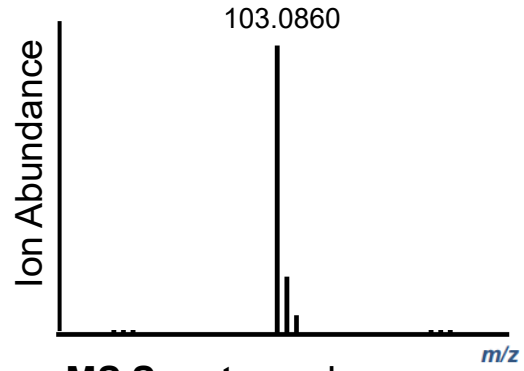
- **Total Ion Current Chromatogram (TIC):** Summed intensity across the entire range of masses being detected at every point in the analysis



Ex.: Chromatogram of ion *m/z* 103.08 ±15ppm



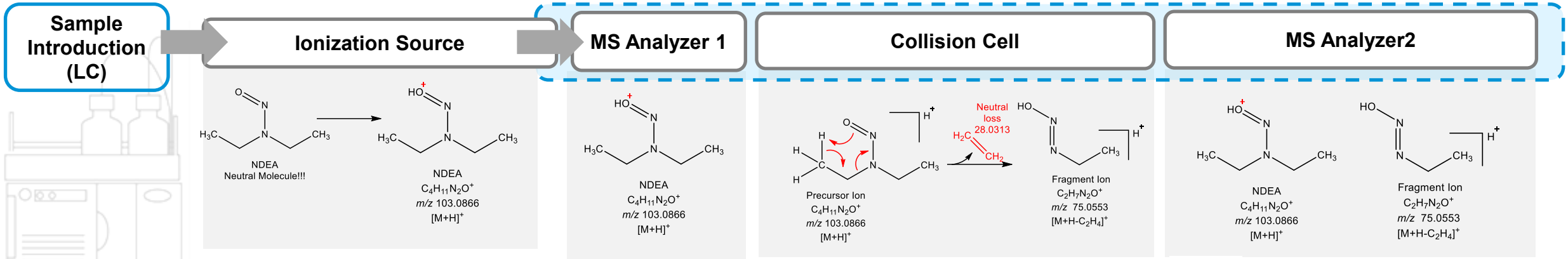
- **Extracted Ion Chromatogram (EIC):** Ion chromatogram created by taking intensity values at a single, discrete mass value, or a mass range, from a series of mass spectral scans



- **MS Spectrum:** Ion abundance x mass/charge (m/z) of detected ions

Mass Spectrometry Fundamentals

Mass Spectrum and Chromatograms



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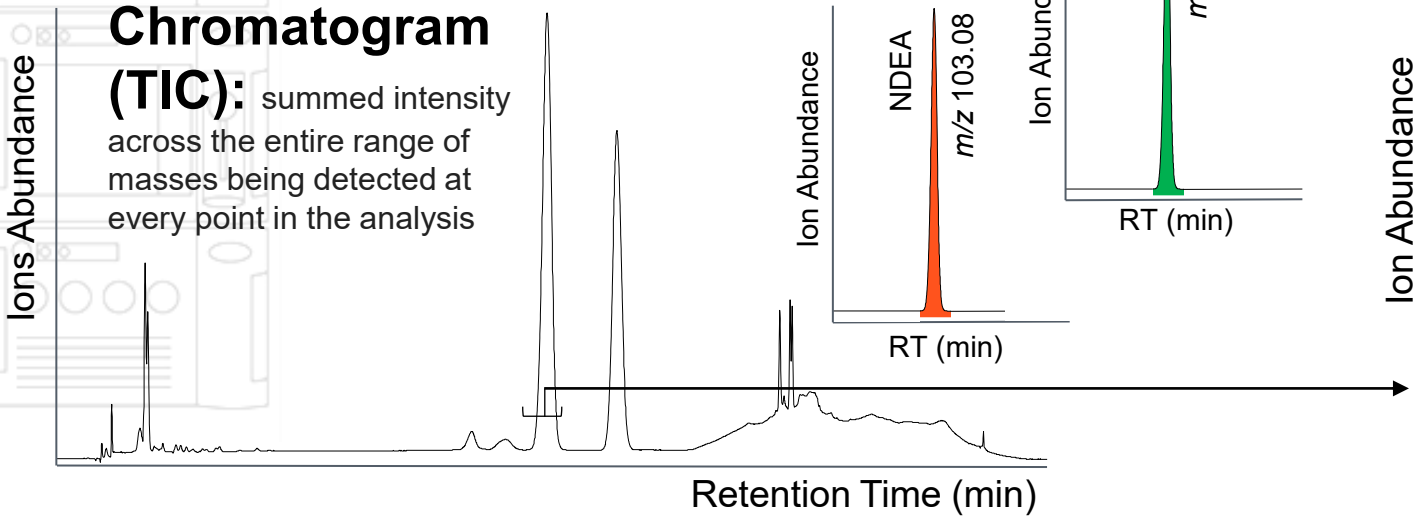
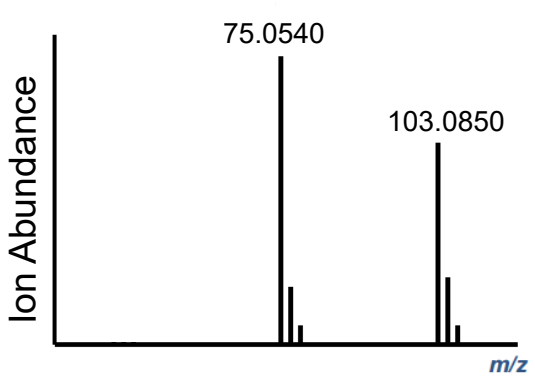
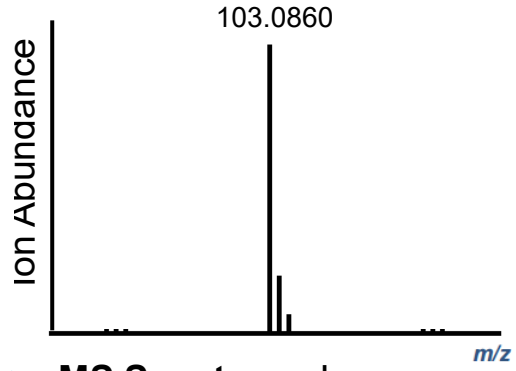


- **EIC**



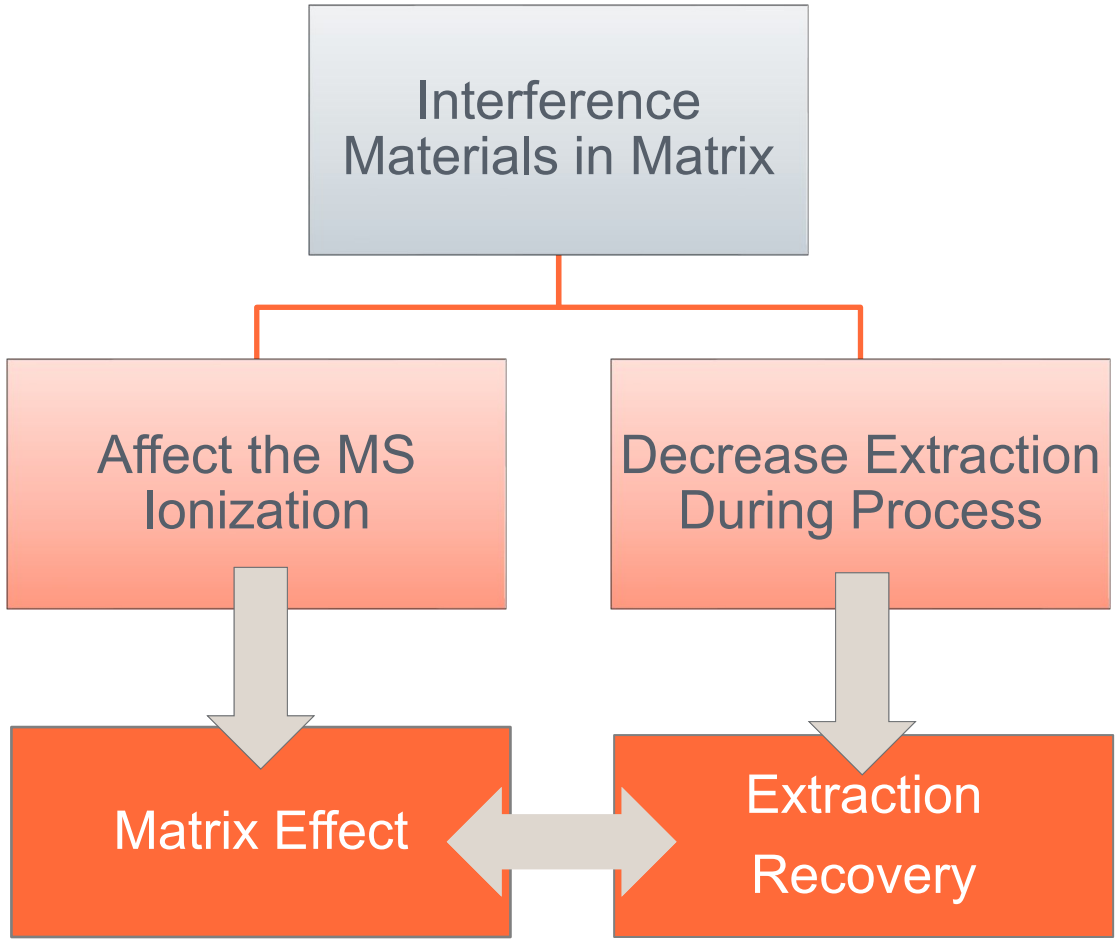
- **MS/MS Spectrum:** Ion abundance x mass/charge (*m/z*) of detected ions



- **MS Spectrum:** Ion abundance x mass/charge (*m/z*) of detected ions

Matrix Effects

- **Matrix effect** refers to a difference in MS response for an analyte in standard solution *vs* the response for the same analyte in a matrix
- **Matrix effect** is often caused by the alteration of ionization efficiency of target analytes in the presence of co-eluting compounds in the same matrix.
- The ionization process can be affected by different factors causing ion suppression or enhancement that negatively affect the measurement of quantity.



Matrix Effects

Matrix Effect in LC-ESI-MS

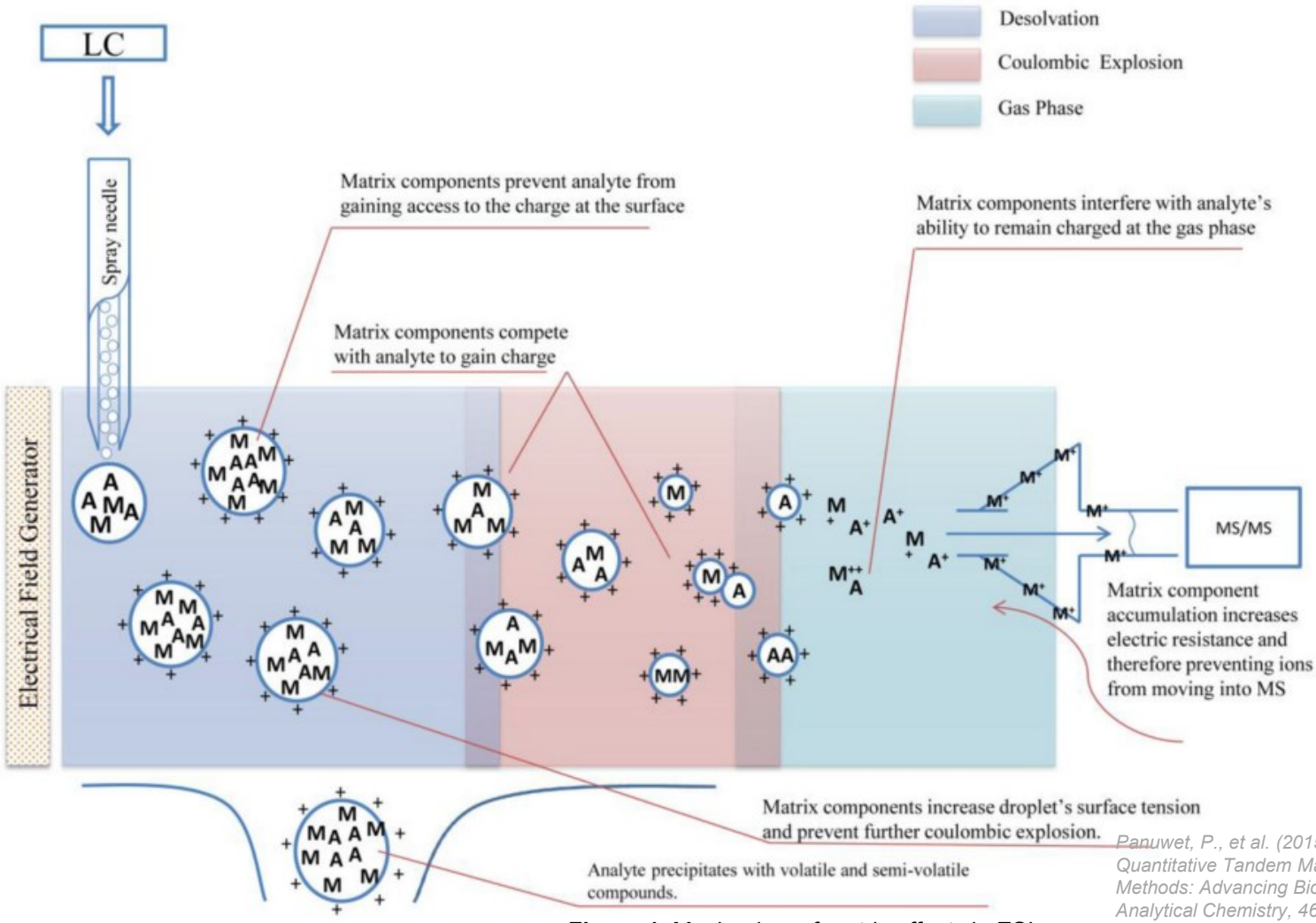


Figure 1. Mechanism of matrix effects in ESI

Panuwet, P., et al. (2015). Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring. Critical Reviews in Analytical Chemistry, 46(2), 93–105. doi:10.1080/10408347.2014.980775

Ionization Sources

Ionization Source: ESI x APCI (nitrosamines)

Lee J-H. et al. Analysis of nine nitrosamines in water by combining automated solid-phase extraction with HPLC-APCI-MS/MS. International Journal of Environmental Analytical Chemistry, 2013

Table 1. The SPE and LC-MS/MS conditions for target nitrosamines.

Parameter	Condition				
SPE (final method)	Solid-phase: HLB (top) – UCT (bottom) Conditioning: Dichloromethane (10 mL) → methanol (10 mL) → water (20 mL) Sample loading: 3 mL min^{-1}/Drying: 30 min using N_2 gas Elution: dichloromethane (20 mL)				
Column	YMC-C8 (2.0 mm I.D × 150 mm × 5.0 µm)				
Ionization mode	ESI (+)			APCI (+)	
Mobile phase	A: 2 mM Ammonium acetate in Methanol B: 2 mM Ammonium acetate in water			A:Methanol B: Water	
Flow rate	0.3 mL min^{-1}			0.5 mL min^{-1}	
Gas temp.	350°C			300°C	
Gas flow	10 L min^{-1}			5 L min^{-1}	
Vaporiser Temp.	–			350°C	
Corona Current pos	–			5 µA	
Capillary voltage	4000 V			2000 V	
Nebuliser	45 psi			30 psi	
MS/MS		Product ions (m/z)		Fragment	Collision
	Compound	Precursor ion (m/z)	Quantification	Confirm	(V)	(V)
	NDMA	75	43	58	90	12
	NMOR	117	45	86	90	16
	NPYR	101	55	29	90	16
	NMEA	89	61	43	70	10
	NPIP	115	41	69	90	20
	NDEA	103	29	75	90	12
	NDPA	131	43	89	100	10
	NDBA	159	103	57	90	9
	NDPhA	199	169	168	80	12

APCI: ionization efficiency is about **2–20 times** higher than ESI for some NITROSAMINES!

LOD ESI+: 2.80–14.2 pg,
LOD APCI+:0.40– 3.80 pg

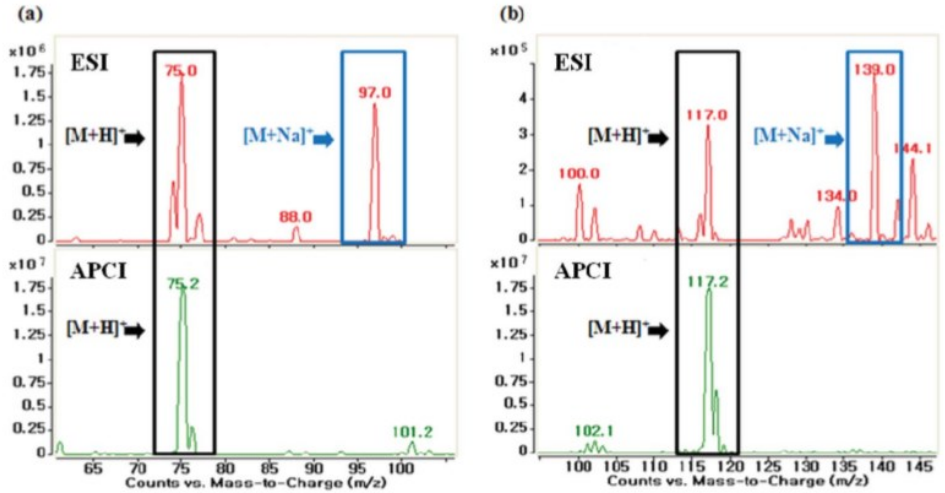


Figure 1. The full scan mass spectrum of NDMA, NMOR in ESI and APCI: (a) NDMA, (b) NMOR.

Analytical Procedure for *N*-Nitrosamines

MS Analyzers

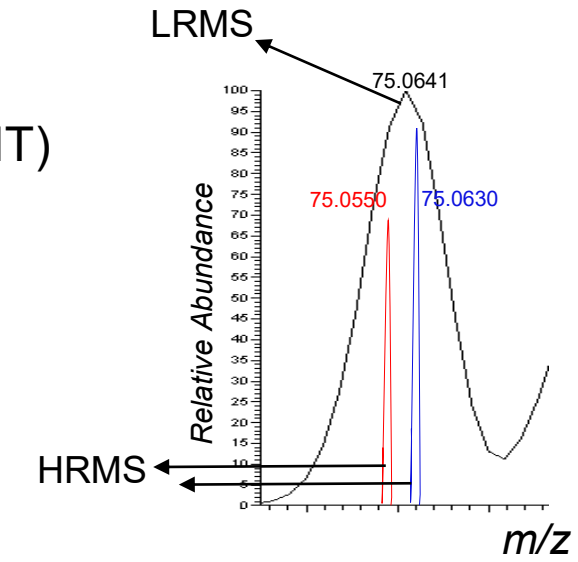
SAMPLE INTRODUCTION → IONIZATION SOURCE → ANALYZER → DETECTOR

***Low Resolution* – LRMS**

- Quadrupole
- Ion-Traps 3D and Linear Ion-Traps (LIT)

High Resolution - HRMS*

- Time-of-Flight (ToF)
- Orbitrap
- Fourier Transform Ion Cyclotron Resonance (FT-ICR)



MS/MS Analyzers (Tandem MS)

- Triple Quadrupole (QqQ)
- Ion-Trap (IT): 3D or linear
- QToF/IT-ToF*
- Q-Orbitrap*
- LIT-Orbitrap*
- Qq-Linear Ion Trap
- Q-Orbitrap-LIT*

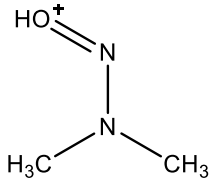
Hybrid

RESOLVING POWER IUPAC

ability of an instrument or measurement procedure to distinguish between two peaks at *m/z* values differing by a small amount

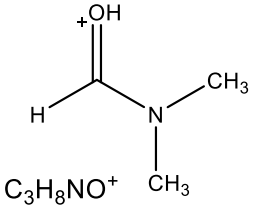
K.M et al. Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013). Pure Appl. Chem., Vol. 85, No. 7, pp. 1515–1609, 2013.

Resolving Power and Mass Accuracy: Impact on Selectivity



NDMA
$C_2H_7N_2O^+$
m/z 75.05529

DMF and NDMA co-elution



$C_3H_8NO^+$

Monoisotopic exact mass

m/z: 74.0601 (100.0%),
m/z: **75.0634 (3.2%) (A+1)**
Dimethylformamide (DMF)

Isotopic ion
m/z 75.0569 DMF ^{15}N
m/z 75.0631 DMF ^{13}C

Good Selectivity
Resolution 45,000

Lack of Selectivity: DMF ^{15}N peak partially resolved from NDMA MI
Resolution 40,000

Lack of Selectivity DMF ^{15}N peak not resolved from NDMA MI
Resolution 25,000

Lack of Selectivity DMF ^{15}N and ^{13}C peaks not resolved from NDMA MI
Resolution 10,000

**MI: monoisotopic peak*

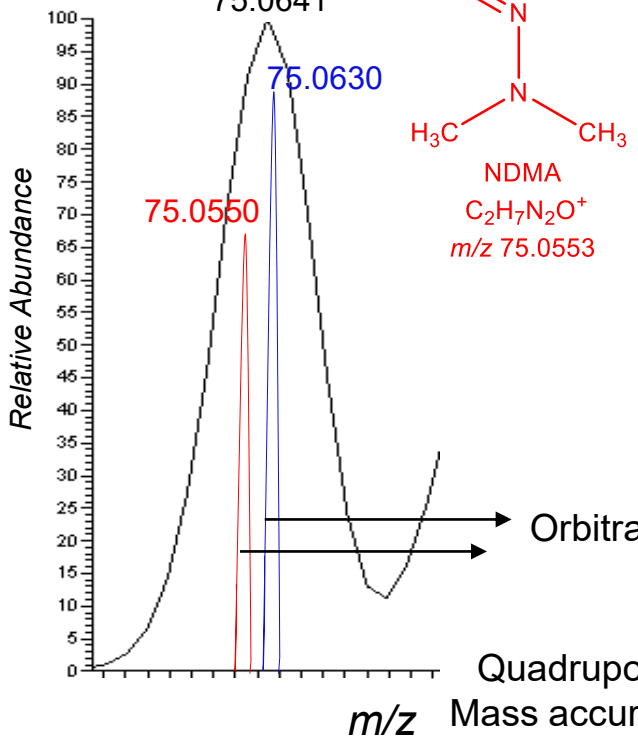
Higher resolving power leads to better accuracy in the estimation of mass!

Higher resolving power = greater selectivity

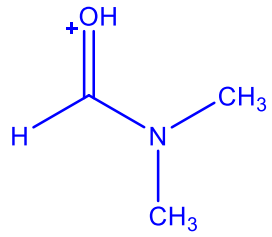
A minimum resolution of **45,000** and maximum mass tolerance of **15 ppm** are required to prevent overestimation of NDMA when quantifying NDMA using the monoisotopic ion, if NDMA and DMF are co-eluting.

Resolving Power and Mass Accuracy: Impact on Selectivity

Example: NDMA with DMF



NDMA
$C_2H_7N_2O^+$
m/z 75.0553



DMF
$C_3H_8NO^+$
m/z 74.0601 (100.0%) Monoisotopic exact mass
m/z 75.0634 (3.2%) (A+1)

Orbitrap R=40,000

Quadrupole R = 1,000
Mass accuracy = 117ppm

Higher resolving power = greater selectivity

- With low mass, resolving power only peaks differing by 1 mass unit can be separated and the recorded masses are then the nominal masses
- Mass spectrometers with insufficient mass resolving power do not allow distinguishing ions having the same nominal mass but different exact masses (i.e., isobaric ions).
- The increase in the mass resolving power narrows the peak width, allowing:
  - Peaks differing by a small *m/z* increment to be resolved
  - Better accuracy of the mass measurement
- With sufficient high mass resolving power, the contaminant could be determined from the experimentally measured accurate mass

Analytical Procedure for *N*-Nitrosamines

Mass Analyzer: Lack of selectivity

Yang J. et al. A Cautionary Tale: Quantitative LC-HRMS Analytical Procedures for the Analysis of *N*-Nitrosodimethylamine in Metformin. The AAPS Journal. 2020

Table 2. Comparison of mass spectrometry (MS) conditions used in this study (FDA) and the private laboratory method description

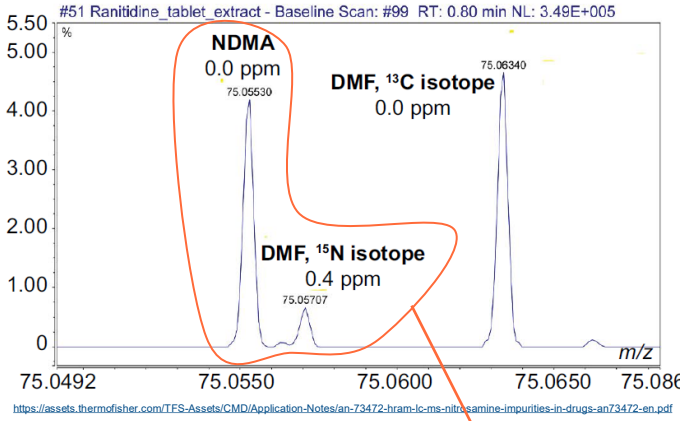
MS Conditions	Private laboratory	FDA
Instrument	QToF	Orbitrap
Ionization mode	APCI, positive	APCI, positive
Data acquisition	MRMHR	Targeted MS2
MS scan	50–450 m/z	40–90 m/z
Mass resolution	>25,000[a]	45,000[b]
Transition(s)	75.0553 → 75.0553	75.0553 → 75.0553
	83.0997 → 83.0997	83.0997 → 83.0997

- High content of NDMA in metformin drug products
- FDA analyzed the same DPs using MS system with higher resolution
- Presence of an **interfering substance (DMF)** which **coeluted** with **NDMA**:
  - insufficient mass resolution or accuracy in data acquisition
  - inappropriate mass tolerance setting in data processing: A mass tolerance window of ±15 ppm or ±30 ppm was applied to obtain the EICs

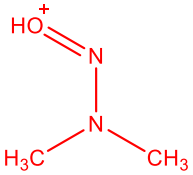
Table 1. NDMA Amounts in metformin samples reported by FDA (using FDA-1 and FDA-2 methods) and the private laboratory

Sample #	Metformin dosage and formulation	Manufacturer name as per private laboratory	Lot #	FDA-1[a,b] (ng/mg)	FDA-2 (ng/mg)	Private lab (ng/mg)
1	500 mg IR	ACI Healthcare USA, Inc.	D105061	ND[c]	ND	0.062
2	500 mg IR	ACI Healthcare USA, Inc.	C105019A	ND	ND	ND
3	500 mg IR	ACI Healthcare USA, Inc.	D105019	ND	ND	ND
4	500 mg ER	Actavis Pharma, Inc.	1376339 M	0.021[d]	0.021	0.364

INSUFICIENT MASS RESOLUTION



A minimum resolution of **45,000** and maximum mass tolerance of **15 ppm** are required to prevent overestimation of NDMA when quantifying NDMA using the monoisotopic ion.



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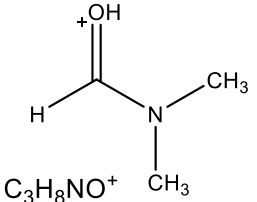

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Analytical Procedure for *N*-Nitrosamines Quantification

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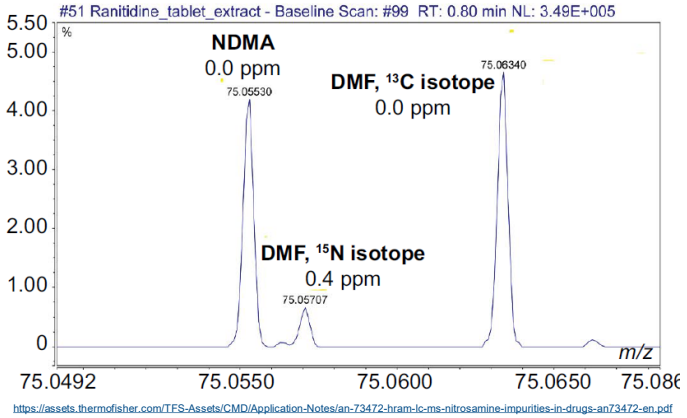
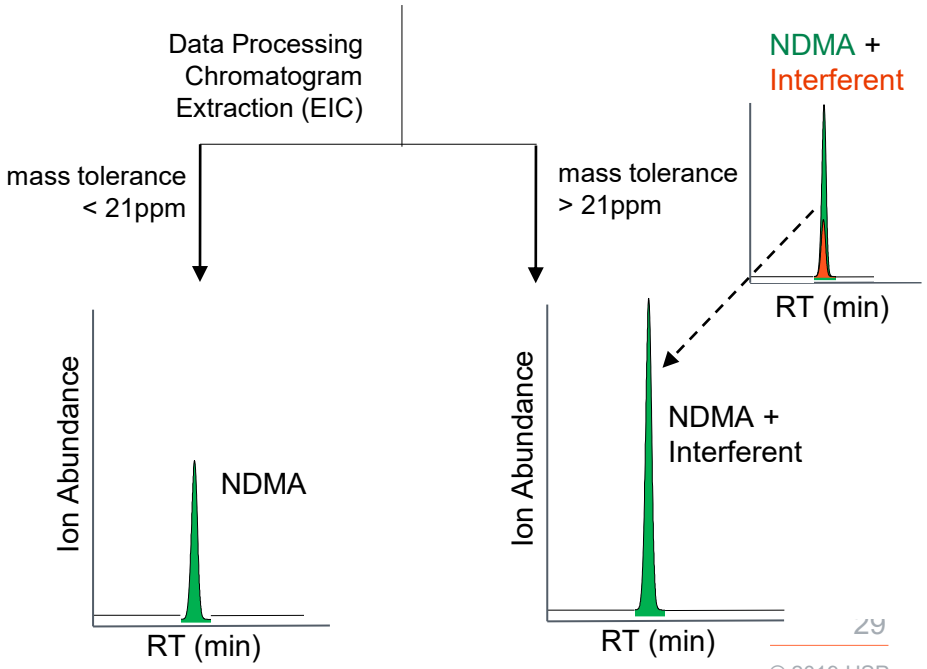
3	500 mg IR	ACI Healthcare USA, Inc.	D105019	ND	ND	ND
4	500 mg ER	Actavis Pharma, Inc.	1376339 M	0.021[d]	0.021	0.364

INNAPROPRIATE MASS TOLERANCE: DATA PROCESSING

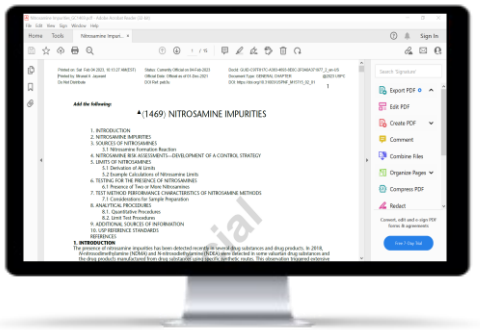


https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-73472-hram-lc-ms-nitrosamine-impurities-in-drugs-an73472-en.pdf



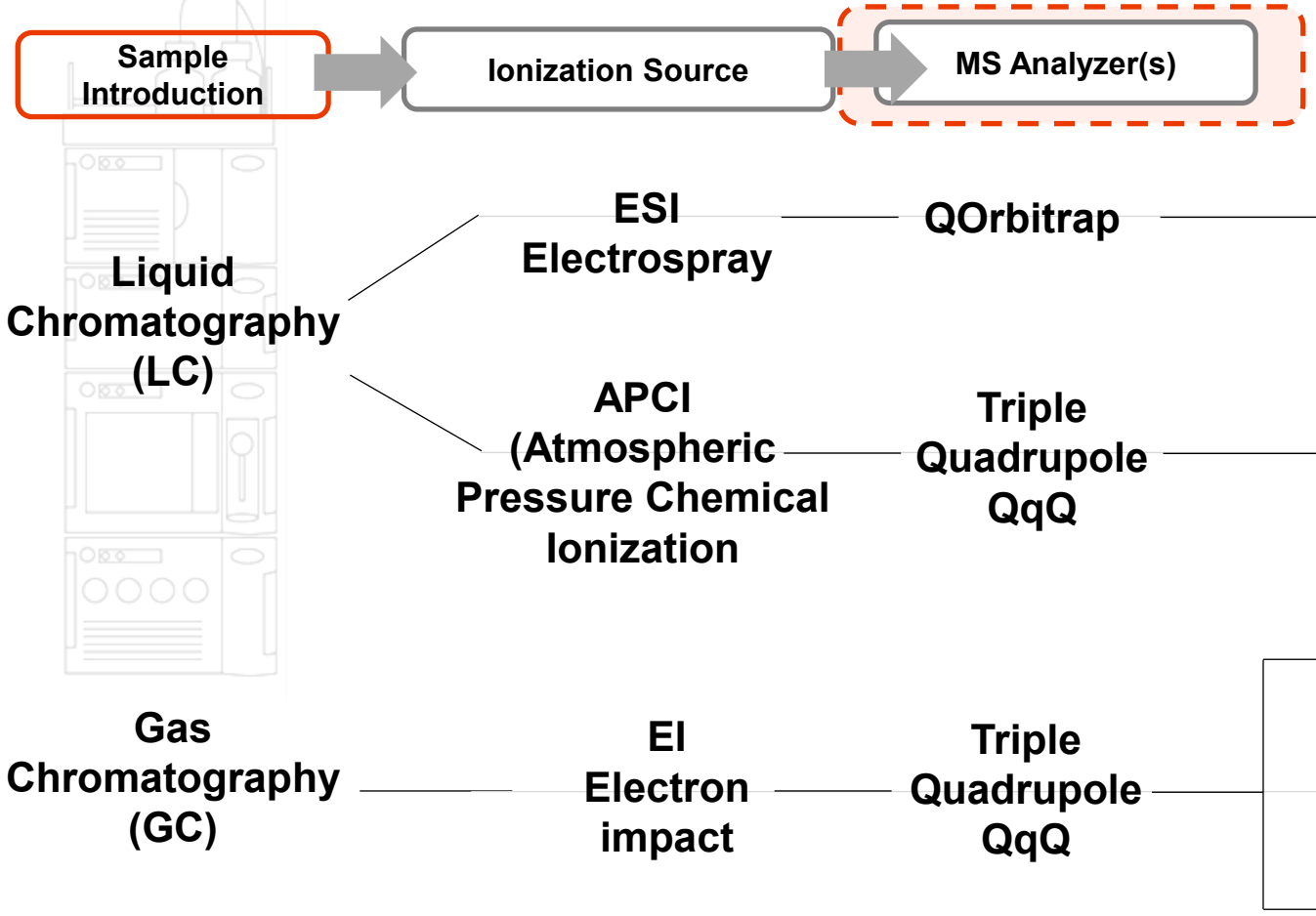
USP <1469> Nitrosamines Impurities

Content



USP <1469> Nitrosamines Impurities

8. ANALYTICAL PROCEDURES



Methods

HPLC-HRMS (QOrbitrap)
Procedure 1: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, NDBA and NMPA in selected sartans by HPLC–HRMS
LC Column: L43 - PFP

HPLC-APCI-QqQ
Procedure 3: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans by HPLC–MS/M
(Internal standards NDMA-d6, NMBA-d3, NDEA-D10 NDBA-D18)
LC Column: L1 – C18

GC-MS/MS (Headspace) GC-EI-QqQ
Procedure 2: Quantitation of NDMA, NDEA, NDIPA, and NEIPA in selected sartans by GC–MS
(Internal Standard NDMA-d6)

GC-MS/MS (Direct injection) GC-EI-QqQ
Procedure 4: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NDBA and NMPA in selected sartans by GC– MS/MS (triple-quad)
(Internal standards NDMA-C_{13}d6)

USP <1469> Procedure 1: HPLC-ESI-HRMS

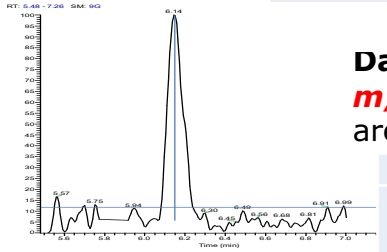
Quantitation of six nitrosamines (NDMA, NDEA, NDIPA, NEIPA, NMBA, NDBA and NMPA) in selected sartans by HPLC-HRMS*

Diluent: Methanol
Standard solution: 6.0 ng/mL(0.3ppm) each in *Diluent* of USP Nitrosamine Reference Standard (NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA)
Sensitivity solution: 1.0 ng/mL (0.05ppm) each in *Diluent* of USP Nitrosamine Reference Standard (NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA) from *Standard stock solution.*
Sample solution: 20 mg/mL of DS in *Diluent.*

Chromatographic system:
Mode: LC
Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in methanol
Column: 4.6 mm x 10-cm, 2.6 µm packing *L43 (PFP)*
Column Temperature: 40º,
Flow Rate: 0.6 mL/min
Injection Volume: 3 µL
Autosampler Temperature: 4º

System suitability requirements
Relative standard deviation: NMT 20.0% from 6 replicate injections, *Standard solution*
Signal-to-noise ratio: NLT 10, *Sensitivity solution*

EIC



Detector: High resolution mass spectrometer
MS conditions:
Ionization: Electrospray Ionization (ESI)
Data Acquisition Strategy: Selected Ion Monitoring (SIM)/Parallel Reaction Monitoring (PRM)

Scan settings **[Note -** Divert the API from the MS source during the elution**.]**

Impurity	NDMA	NMBA	NDEA	NEIPA	NDIPA	NDBA
Scan Type	SIM	SIM	PRM	SIM	SIM	PRM
Polarity	POS	NEG	POS	POS	POS	POS
Scan Start –End (min)	1.0-3.5	3.5-5.5	5.5-7.0	7.0-8.5	8.5-10.0	13.0-15.5
m/z Isolated for PRM	N/A	N/A	103.0866	N/A	N/A	159.1492
Resolution	30000	60000	30000	60000	60000	30000
Isolation Window	N/A	N/A	1.5 m/z	N/A	N/A	1.5 m/z
Scan Range	m/z 74.3-75.8	m/z 144.3 - 145.8	m/z 50.0-114.0	m/z 116.4-117.9	m/z 130.4 - 131.9	m/z 50.0-170.0

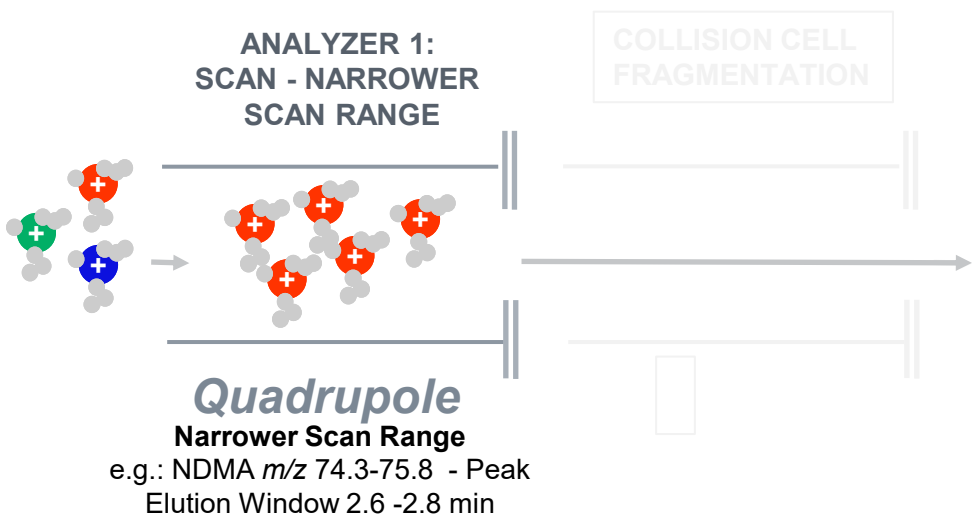
Data Processing: Peak areas in the extracted ion chromatograms (EIC) with a ***m/z* tolerance of 15 ppm** are used for quantitation. The *m/z* values extracted are listed below.

Impurity	NDMA	NMBA	NDEA	NEIPA	NDIPA	NDBA
m/z extracted	75.0553	145.0619	75.0553, 103.0866	117.1022	131.1179	57.0704, 103.0872, 159.1492

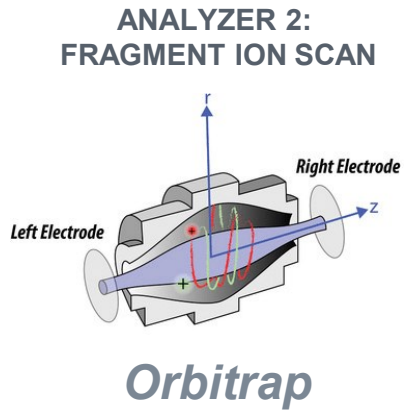
*Adapted from the LC-HRMS method published by the US FDA: https://www.fda.gov/media/125478/download

QOrbitrap (Tandem MS) – High Resolution

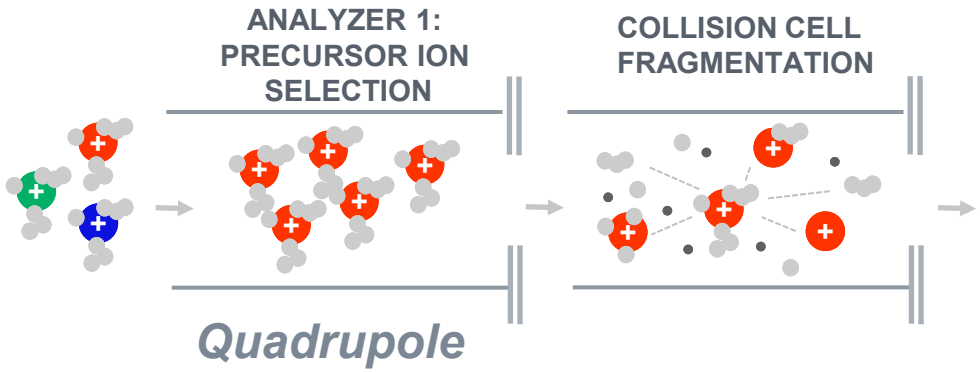
SELECTED ION MONITORING (SIM)



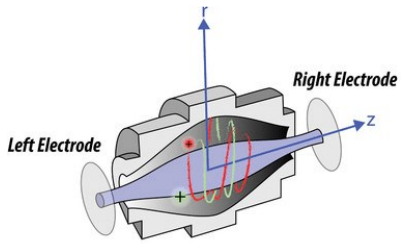
Narrower Scan Range
e.g.: NDMA *m/z* 74.3-75.8 - Peak Elution Window 2.6 -2.8 min



PARALLEL REACTION MONITORING (PRM))



NDEA
***m/z* Isolated for PRM** 103.0866
Scan Range m/z 50.0-114.0
Isolation Window 1.5 *m/z*



QOrbitrap:

- High resolution (Selectivity!)
  – higher accuracy in mass measurements
- Higher cost: not widely available
- Data aquisition strategies for quantification:
  (a) Selected Ion Monitoring (SIM):
  – records the abundance of one or more specific *m/z* values in an expected retention time window.
  – In this mode, the MS does not spend time scanning the entire mass range, but rapidly changes between *m/z* values for which characteristic ions are expected: ↑ sensitivity

(b) Parallel Reaction Monitoring (PRM)

- quadrupole selects the precursor ion (selection window is usually *m/z*≤2); the precursor ion is fragmented in the collision cell; Orbitrap scans all product ions with high resolution and high accuracy.
- ↑ sensitivity and selectivity

USP <1469> Procedure 3: HPLC-APCI-QqQ

Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans by HPLC-APCI-QqQ

Diluent: 1% formic acid in water

Internal standard solution: 10 µg/mL each of NDMA-d6 and NMBA-d3, 1 µg/mL each of NDEA-d10 and NDBA-d18 in water

Standard stock solution: 10 ng/mL each of USP Nitrosamine RS (NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA) in methanol

Standard solutions: Prepare Standard solutions at the concentration levels (L#) given in Table 13.
0.02 – 1.35ppm NDMA, NDIPA, NEIPA, NMBA, and NDBA
0.01 – 0.89ppm NDEA

Sample solution: Transfer about 80 mg of the drug substance into a 2-mL lidded centrifuge tube. Add 1188 µL of Diluent and 12 µL of the Internal standard solution. Vortex at 2500 rpm for 20 min (except for losartan potassium, which should be vortexed NMT 5 min). Centrifuge at about 10,000 rpm for 10 min, and filter into a vial using a hydrophilic polytetrafluoroethylene (PTFE) filter of 0.45-µm pore size.

Chromatographic system:
Mode: LC
Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in methanol
Column:3.0-mm × 15-cm; 2.7-µm packing L1

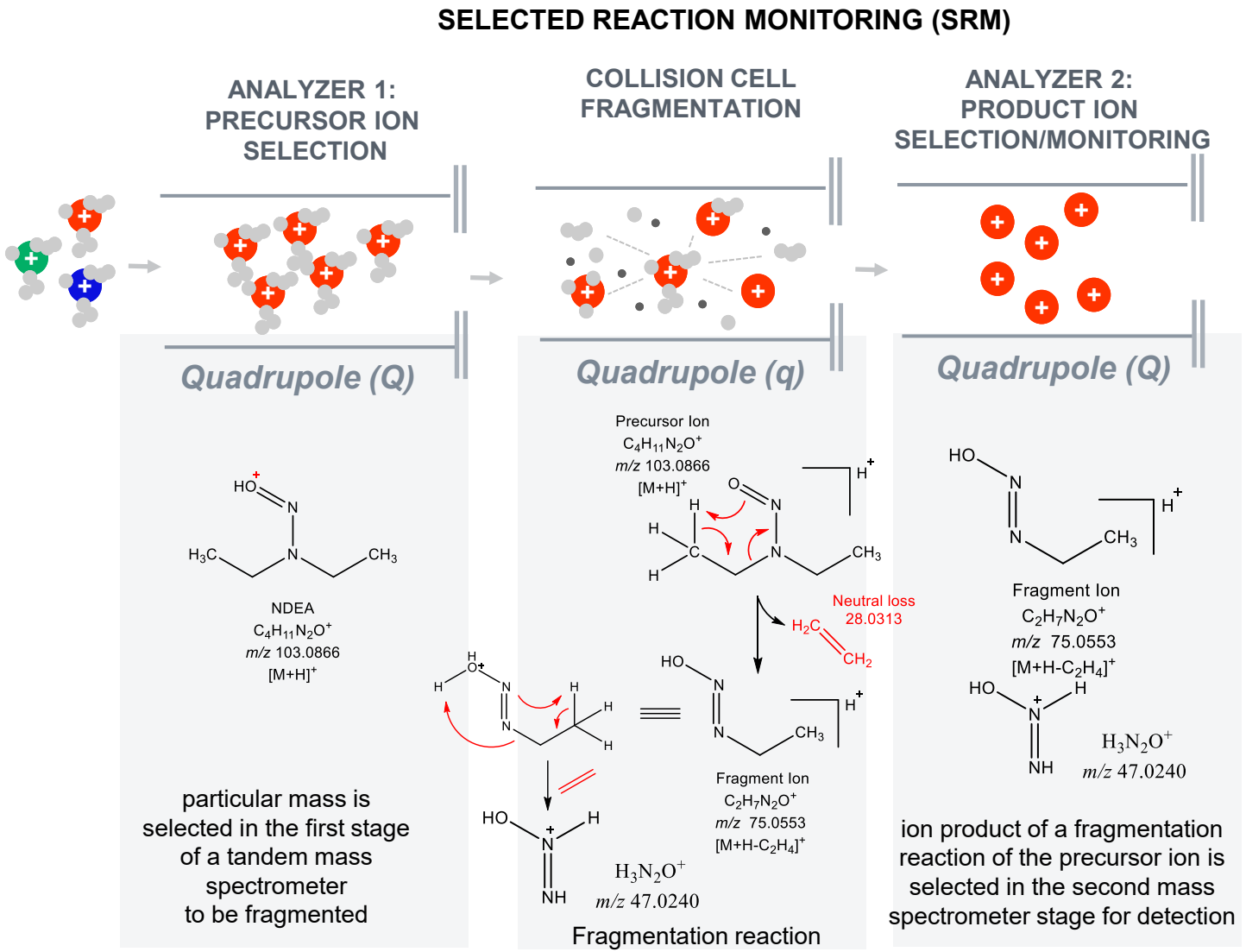
Chromatographic system:
Temperatures
Autosampler:18°
Column:60°
Flow rate: 0.5 mL/min
Flow rate to ion source: 0.5 mL/min
Injection volume: 20 µL

Detector: MS/MS (triple quadrupole mass spectrometer)
MS conditions:
Ionization: Atmospheric pressure chemical ionization (APCI)
Scan Settings:

Impurity	NDMA	NDMA-d6	NDEA	...
Acquisition mode	MRM	MRM	MRM	
Polarity	Positive	Positive	Positive	
MRM-1	*m/z* 75→43	*m/z* 81.2→46	*m/z* 103.1→75.1	
MRM-2	*m/z* 75→44.1	*m/z* 81.2→64.1	*m/z* 103.1→47.1	

System suitability requirements
Generate the response versus concentration standard curve for each nitrosamine impurity under test using the corresponding Standard solutions and perform the linear regression analysis.
Correlation coefficient:NLT 0.99
y-Intercept:NMT 25%, Standard solution L4

QqQ (Tandem MS) - Low Resolution



QqQ:

- Low resolving power
- Lower cost: triple quadrupole MS platform is more widely available than the LC-HRMS platform
- Data aquisition modes:

(a) Selected Reaction Monitoring (SRM):

- ↑ selectivity
  - less interference of co-eluting compounds and matrix
  - works like a double mass filter which drastically reduces noise and increases selectivity
- ↑ sensitivity:
  - Better Signal-to-Noise ratio (S/N) allowing quantitation with lower limits of quantitation
  - Wider linear range of quantitation

(b) Multiple Reaction Monitoring (MRM):

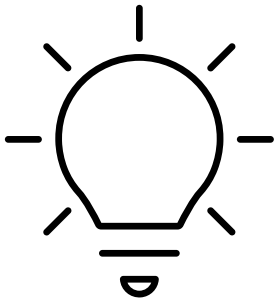
- Multiple SRM transitions are measured within the same experiment
- ↑ Selectivity: allows additional selectivity by monitoring the chromatographic coelution of multiple transitions for a given analyte.

Challenges and Recommendations

Resolution

Use of High-Resolution Mass Spectrometry is not the only option to ensure good selectivity for *N*-nitrosamines quantification!!!

Low-Resolution MS systems may also be used!!

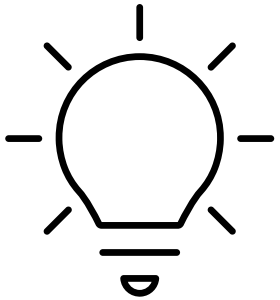


- Sample Preparation: Reducing extraction of endogenous compounds from matrix
- Chromatographic Method: Improving chromatographic resolution
- MS Analysis: Using HRMS or Tandem MS + Data Acquisition Strategies to improve selectivity (SRM, MRM, PRM…)
- Data Processing: Targeted data extraction strategy. LC-HRMS: Using a suitable mass tolerance window to obtain the EICs

Challenges and Recommendations

Sensitivity

How can the sensitivity of *N*-nitrosamines in APIs and DPs be enhanced?



- Sample Preparation: Reducing extraction of endogenous compounds from matrix—selective extraction; pre-concentration step
- Chromatographic Method: Improving chromatographic resolution—reduce impact on ionization efficiency/matrix effects
- MS Analysis:

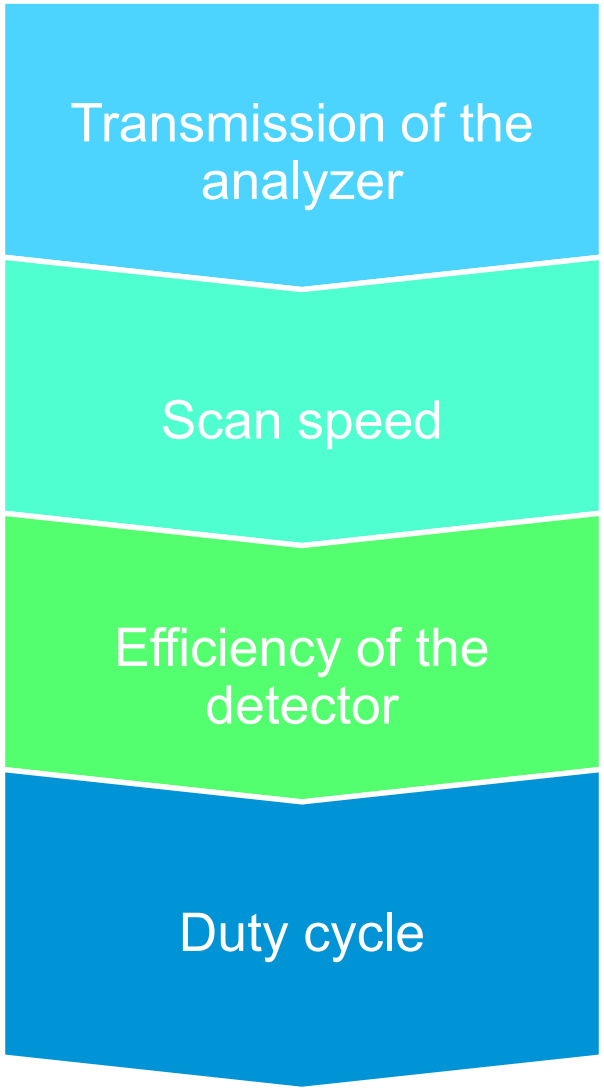
  Use high sensitivity MS systems and data acquisition strategies to improve sensitivity and scan speed (minimum of 10 points/peak)
- Data Processing:

  Averaged MS spectrum, etc. (reduce/eliminate Matrix Effect)

Challenges and Recommendations

Sensitivity

Sensitivity of an instrument is better described by a factor defined as the mass spectrometer efficiency that takes into account:



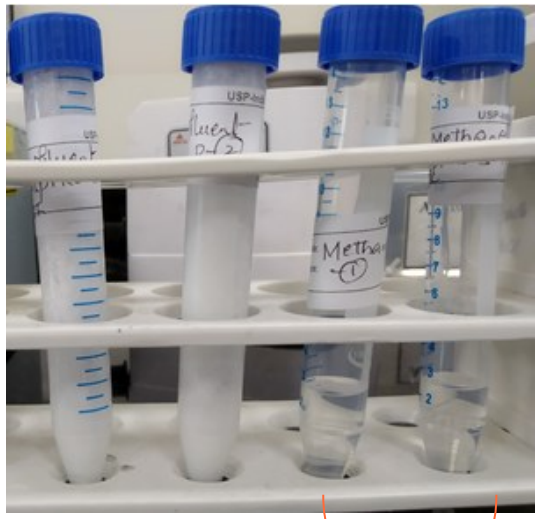
Challenges and Recommendations

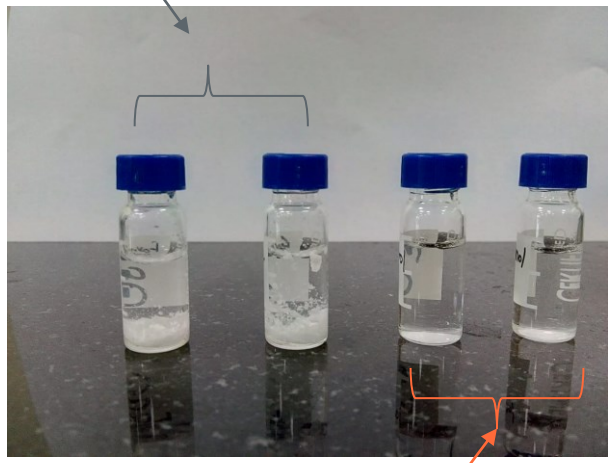
Sample Preparation
Dispersion in Extraction Solvent

- The sartans are insoluble in the diluent (1% formic acid in water).
- The sartans must be fully dispersed for efficient extraction.
- Clumps or dry spots will cause artificially lower quantification results.
- Using larger vessels allows for more of the sartans to deposit on the walls, limiting the interaction with solvent.

USP GC <1469> - Procedure 3: Diluent - 1% Formic Acid in Water
SARTANS ARE NOT SOLUBLE

Losartan Potassium in Diluent (1% Formic Acid in Water)
Larger surface area of vessel complicates extraction.





Losartan Potassium in Methanol
USP GC <1469> - Procedure 1:
Diluent MeOH
SARTANS ARE SOLUBLE

Challenges and Recommendations

Sample Preparation – Extraction Efficiency Considerations

Total Dissolution

- Drug substance is SOLUBLE in extraction solvent.
- No extraction efficiency issues.
- Must prevent the API from entering the MS by diverting LC flow to waste during elution.

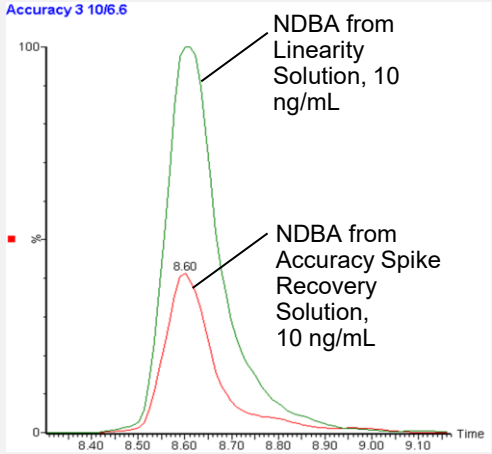
Matrix Precipitation Strategy

- Dissolve the drug substance in an appropriate solvent, then add an “anti-solvent” to precipitate the drug substances.
- Advantages :
  - guarantee nitrosamines are transferred into solution.
  - potential contamination of or damage to the mass spectrometer
  - Can be more reproducible and less time-consuming.

https://pubmed.ncbi.nlm.nih.gov/25576043/

Selective Extraction

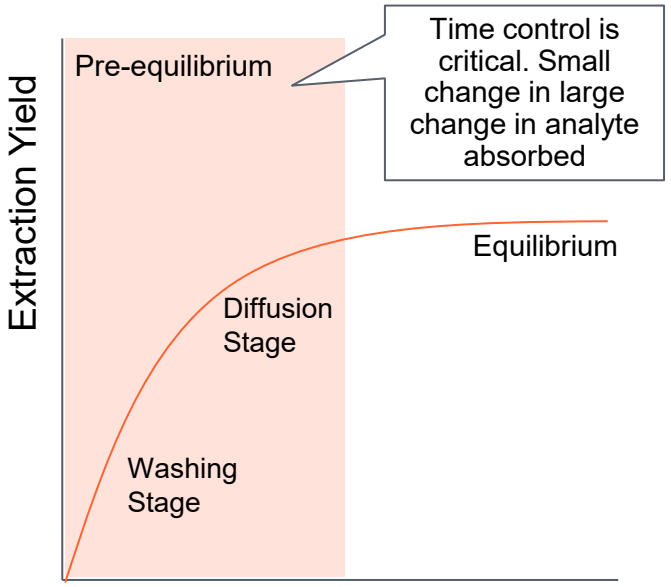
- Drug substance is **SOMEWHAT** soluble or **INSOLUBLE** in extraction solvent.
- Extraction time and mixing are critical for proper extraction.
- High potential for nitrosamines to adsorb onto the API yielding lower quantification results



- Inefficient extraction will affect LOD/LOQ and Accuracy/Precision
- Strategies for matrix effect compensation:
  - use of internal standard and appropriate equilibration
  - Matrix-matched calibration

Other Selective Extraction Protocols

- Solid Phase Extraction (SPE)
- Dispersive Liquid Liquid Extraction (DLLE)



Time effect for selective extraction

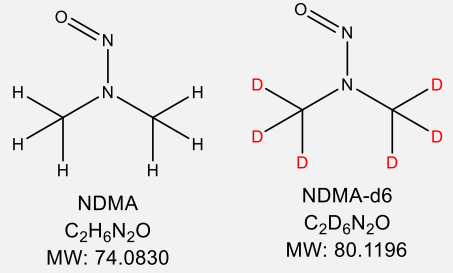
Challenges and Recommendations

Use of Internal Standard

- The analyte-to-internal standard response ratio can compensate the matrix effect and ion suppression during analysis providing for a more accurate and precise method.

- ISTD can be used:
  - during sample preparation (account for extraction AND ionization efficiency issues)
  - or prior to sample injection into the LC–MS or GC-MS (account for ionization efficiency issues due to matrix effects)

- ISTD must have ionization properties and retention time similar to the analyte:
  - isotopically labelled compounds
  - structural analogue or
  - another compound that is similar to the analyte under investigation.



USP Deutero *N*-Nitrosodimethylamine RS (NDMA-d_6)

Challenges and Recommendations

Use of Internal Standard

- Isotopically labelled compounds (IL-STD):
  - IL-STD will behave almost identically to the analyte during sample preparation, chromatographic separation and MS ionization
  - Same degree of ion suppression* or enhancement will be observed for the target analyte and its isotopically labeled analogue: the ratio of the two signals should not be affected, and correct quantification can still be achieved

- Challenges for using IL-STD:
  - high cost and difficult to obtain and/or synthesis (often unavailable)
  - lack of confidence in the isotopic purity and integrity

USP GC <1469>: Analytical Procedures

- **Procedure 2:** GC-MS/MS (Headspace) GC-EI-QqQ, ISTD: NDMA-d6
- **Procedure 3:** HPLC-APCI-QqQ
  ISTD: NDMA-d6, NMBA-d3, NDEA-D10
  NDBA-D18
- **Procedure 4**: GC-MS/MS (Direct injection)
  GC-EI-QqQ
  ISTD: NDMA-13C2-d6

Panuwet, P. et al. Critical Reviews in Analytical Chemistry, (2016) 46:2, 93-105
A. Furey et al. Talanta 115 (2013) 104-122

Heavy Isotope Effect on Chromatography*

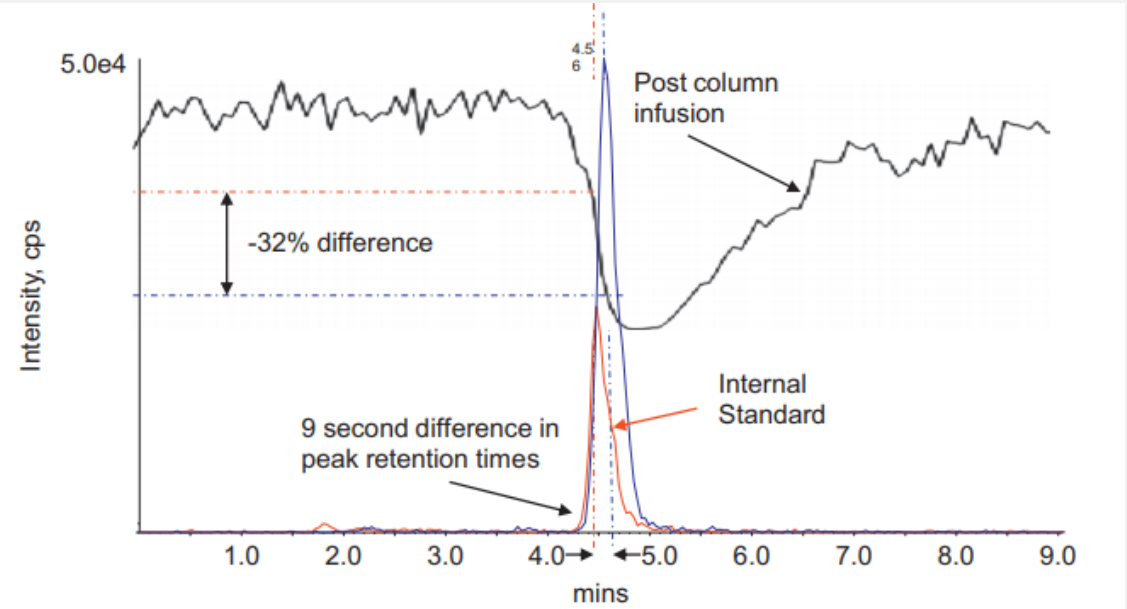
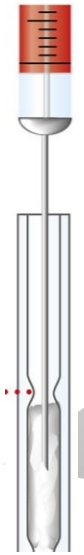


Fig. 5. Illustration of when a standard and internal standard are affected by different levels of ion suppression. This shows a 32% difference in signal response over a 9 second period of analysis.

7.1. CONSIDERATION FOR SAMPLE PREPARATION

- Appropriate sample preparation is a critical step in trace impurity analyses such as those required to evaluate the levels of nitrosamines in drug substances and drug products.
- This is particularly critical to prevent the loss or generation of nitrosamines as artifacts of the analytical procedure itself, as in the following circumstances.



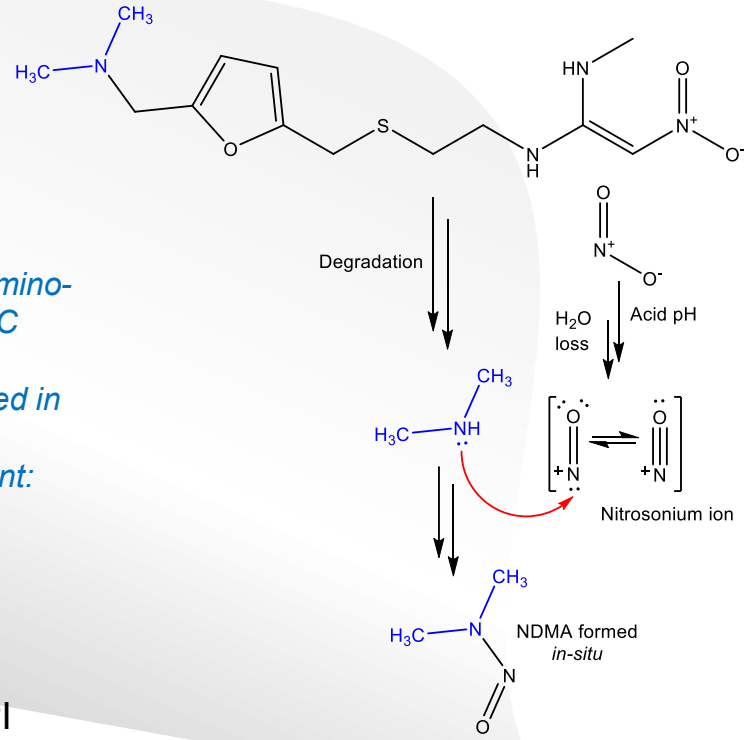
Injection port (GC System): High Temperature

In-situ formation of NDMA in GC-MS analysis

Dialkylamines (dimethylamine):

- degradation product of the API: *total dissolution of the DS containing dimethylamino-group should be avoided when applying GC techniques.*
- *High concentration of the API, when injected in the GC can generate nitrosamines in the injection port if a nitrosating agent is present: sample extractions should be modified to prevent the solubilization of the API while maintaining the extraction efficiency for nitrosamines present in the material.*
- process impurity
- counter ion of the salt form of the API

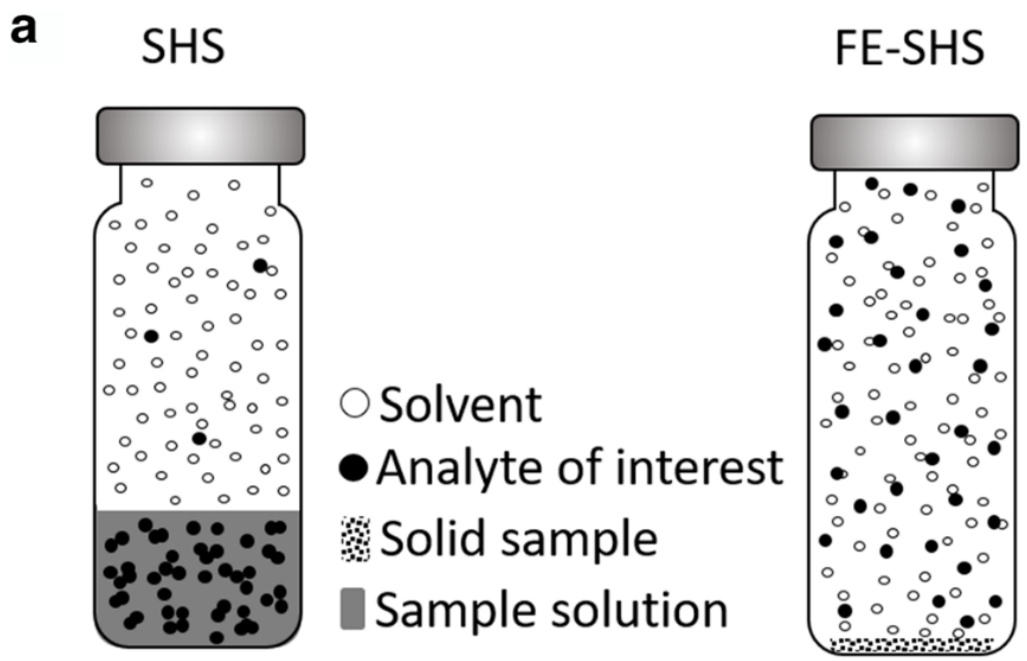
...in the presence of nitrite and acid can lead to *in situ* formation of nitrosamines as an **artifact**, especially in GC analyses.

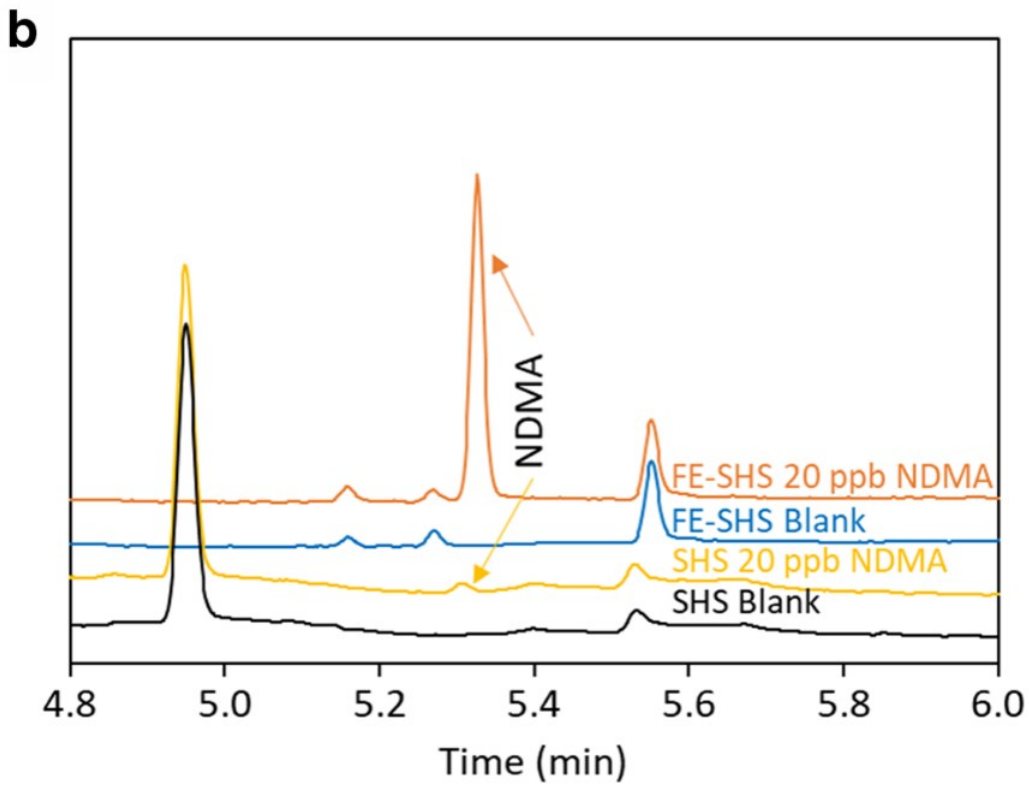


- It is highly recommended that **LC-MS** be used for determination of NDMA in Ranitidine DS and DP.

Analytical Challenges

Sample Preparation & Analysis: GC-NPD



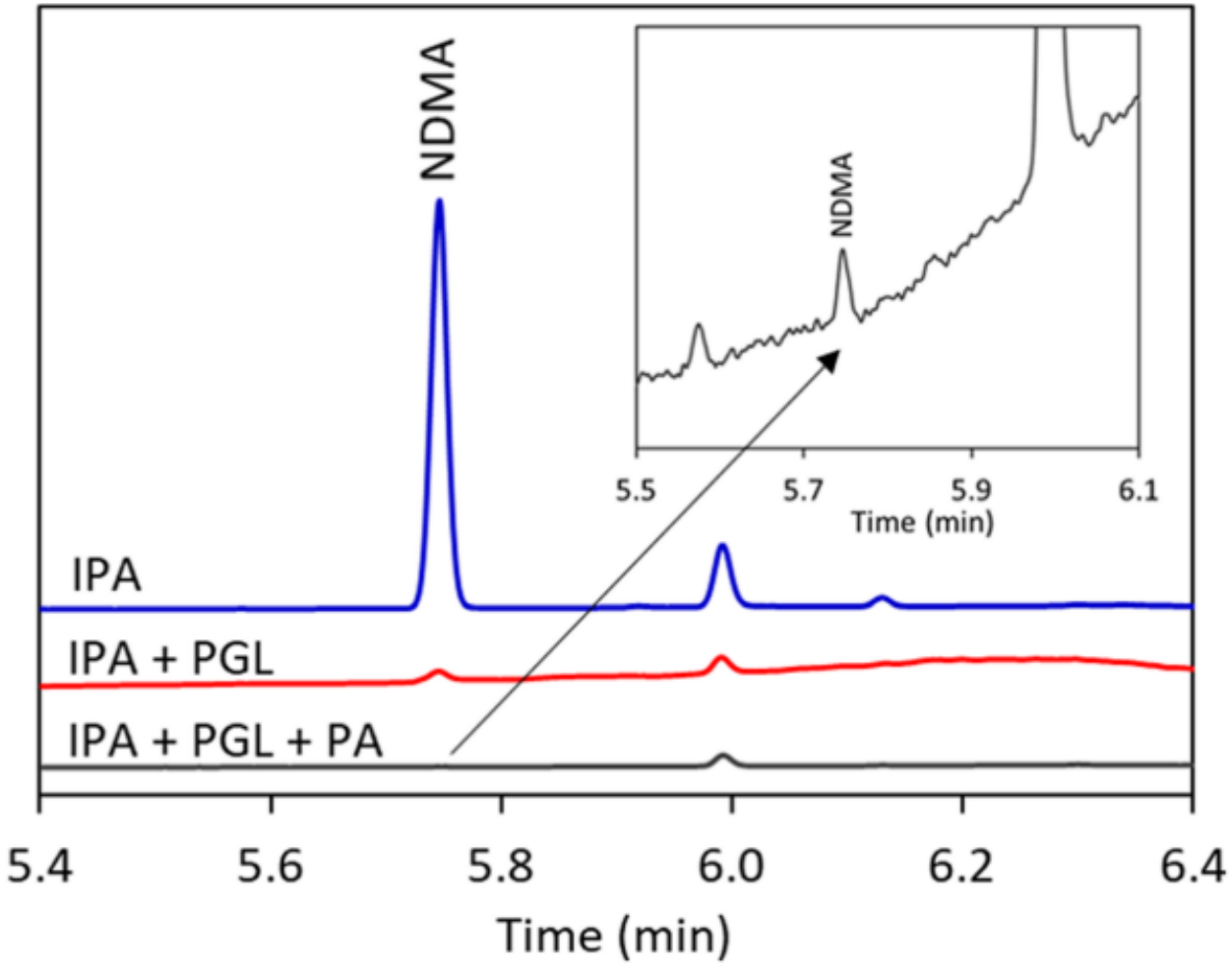


SHS: traditional static headspace sampling
FE-SHS: full evaporation static head- space (sampling

J. Zheng, C.L. et al. AAPS J. 24:23 (2022). https://doi.org/10.1208/s12248-021-00669-8

Analytical Challenges

Sample Preparation & Analysis: GC-NPD



Several scavengers for nitrosating agents evaluated:

- pyrrole,
- 2,5-dimethylpyrrol,
- pyrogallol,
- phloroglucinol,
- caffeic acid,
- catechol,
- ascorbic acid,
- hydrazine,
- propyl gallate,
- gallic acid

Figure 2. Inhibition of *in situ* formation of NDMA during analysis by FE-SHSGC-NPD. About 30 mg metformin HCl drug substance was added to the headspace vial with 50 µL diluent containing (1) isopropanol (IPA), (2) 20 mg/mL pyrogallol (PGL) in IPA (IPA + PGL), or (3) 20 mg/mL pyrogallol and 0.1% phosphoric acid (PA) in IPA (IPA + PGL + PA)

J. Zheng, C.L. et al. AAPS J. 24:23 (2022). https://doi.org/10.1208/s12248-021-00669-8

Analytical Challenges

Matrix effect and artifactual formation of NDMA during sample preparation

- Proficiency testing was performed between four different laboratories employing different sample preparation protocols and orthogonal LC-MS and GC-MS procedures used for NDMA quantification in metformin-containing drug products
  - two metformin immeadiate-release (IR) formulations and
  - one metformin extended-release (XR) formulation.

Method code	Measurement Principle	Extraction principle[1]		Executing lab
		IR	XR	
GCM	GC-MS/MS	Water, DCM	DCM	Merck (Germany)
GCM-W	GC-MS/MS		DCM + washing	Merck (Germany)
GCM-P	GC-MS/MS		DCM + Scavenger PYR	Merck (Germany)
GCM-D	GC-MS/MS		DCM + Scavenger MPD	Merck (Germany)
LCM	LC-MS/MS	water, DCM, water	Not developed	Merck (Germany)
LCE	LC-MS/MS	water	DCM, water wash, 5% MeOH	Eurofins Amatsi Analytics (France)
GCA	GC-HRMS	water, DCM	Not developed	AstraZeneca (UK)

[1]Solvents are listed in the order of their application. IR: immediate release formulations; XR: extended-release formulations; DCM: dichloromethane; PYR: pyrrolidine; MBD: 4-methylbenzene-1,2-diamine.

DOI: https://doi.org/10.1016/j.ejps.2021.106026 European Journal of Pharmaceutical Sciences

Analytical Challenges

Matrix effect and artifactual formation of NDMA during sample preparation

- Extended-Release (ER) formulations - Controlled-release behavior is in general achieved by
  - using polymer coatings on solid dosage forms or
  - by the incorporation of different types of polymer matrix systems within the formulation for instance
  - Examples: cellulose derivative HPMC in Metformin ER Tablets
- During sample preparation these high molecular weight (MW) polymers can swell and gelatinize in aqueous solutions causing extraction efficiency issues - matrix effect leading to artificial low quantification of NDMA
- If these high molecular weight polymers are extracted and sprayed into the MS source this can contaminate the MS system

DOI: https://doi.org/10.1016/j.ejps.2021.106026 European Journal of Pharmaceutical Sciences

Analytical Challenges

Matrix effect and artifactual formation of NDMA during sample preparation

- Fritzsche et al (2022) employed the following strategies to avoid extraction efficiency issues and injection of these polymers into the MS system
  - Procedure GCM: GC-MS/MS analysis using dichloromethane as extraction solvent in order to avoid extraction of HMPC and formation of a viscous gel;
  - Procedure LCE: RPLC-MS/MS and dichloromethane as first extraction solvent, followed by washing with water for the removal of residual HPMC and evaporation of DCM and resuspension in 5% MeOH.

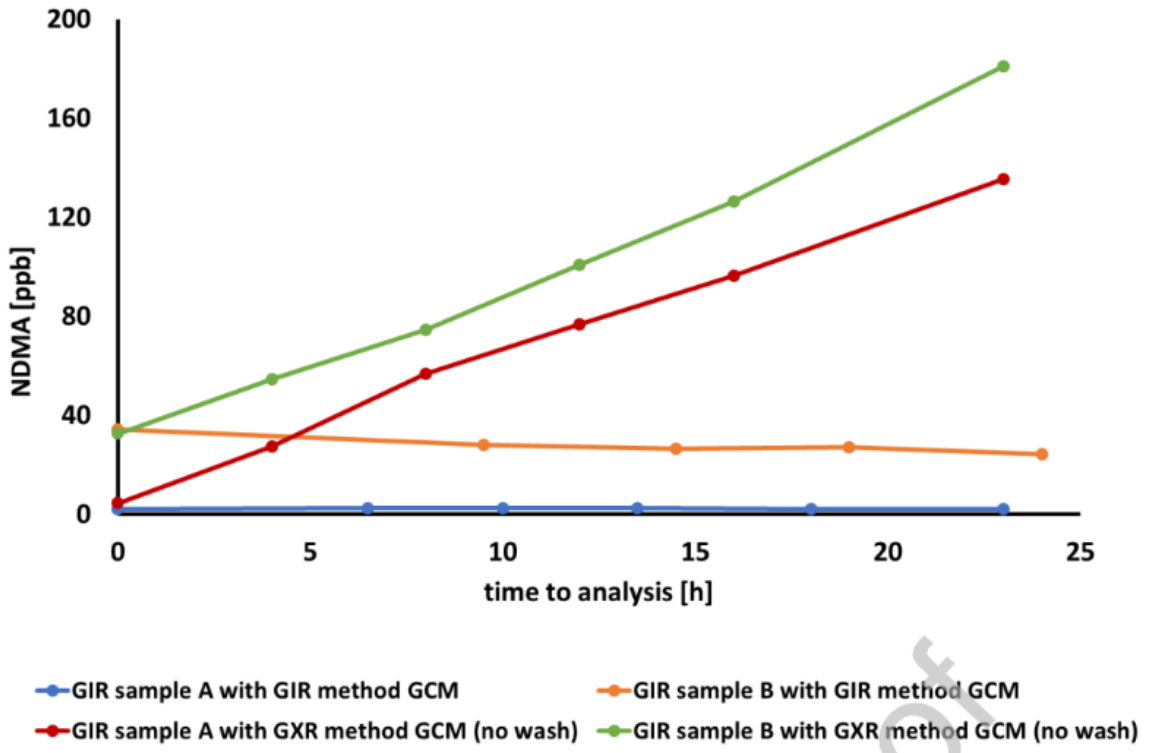


Figure 5: *Increase of NDMA values over time with two Glucophage® IR (GIR) batches (low and high initial NDMA values) when using the GXR method without washing (extraction with DCM) instead of the GIR method (extraction with water, back extraction into DCM). Similar results were obtained for the other immediate release product Glucovance® (not shown).*

DOI: https://doi.org/10.1016/j.ejps.2021.106026 European Journal of Pharmaceutical Sciences

Analytical Challenges

Matrix effect and artifactual formation of NDMA during sample preparation

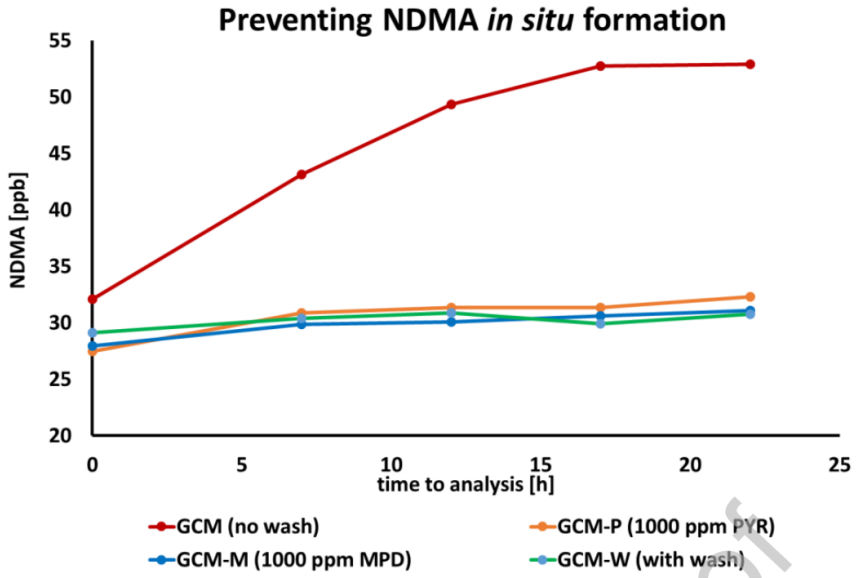
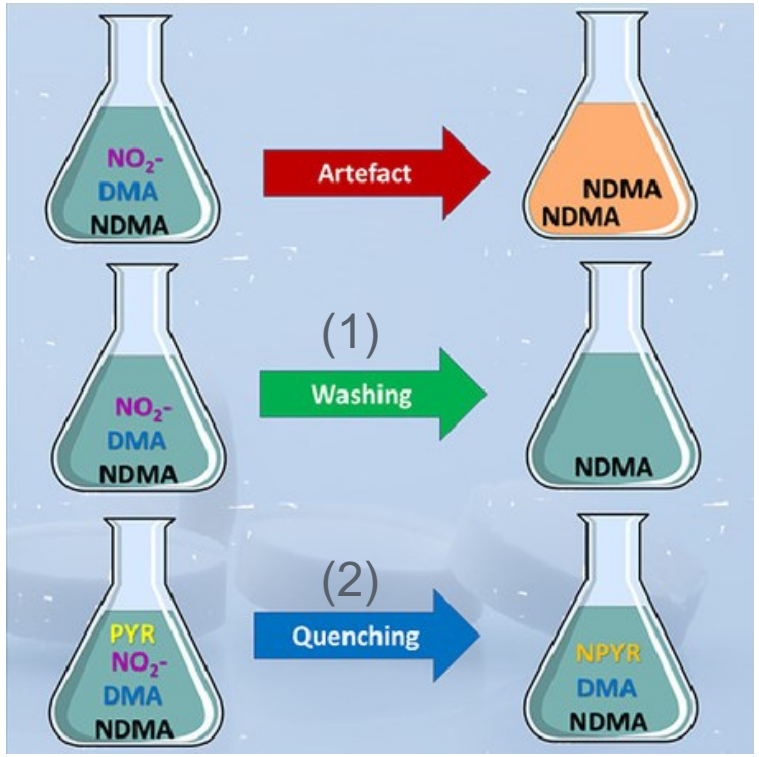


Figure 8: *Prevention of NDMA in-situ formation in the sample tube by prior removal of DMA and nitrite ("washing") or addition of a Nitrite scavenger. Behavior of NDMA values over time in samples from a batch of GXR. Both procedures prevent in-situ formation of NDMA without affecting the initial value, whereas the non-washed DCM extract shows increasing NDMA signals over time.*

DOI: https://doi.org/10.1016/j.ejps.2021.106026 European Journal of Pharmaceutical Sciences

(1) Removal of DMA and nitrite by including an additional water washing step after extraction with DCM

(2) Addition of nitrite scavengers (e.g.: 4-methylbenzene-1,2-diamine and pyrrolidine) to consume nitrite



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Thank You

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The standard of trust

USP GC <1469> 8. ANALYTICAL PROCEDURES

Procedure 2 – Headspace (HS) GC-MS/MS (QqQ) - Quantitation of NDMA, NDEA, NDIPA, and NEIPA in selected sartans.

Sample solution: 200 ± 10 mg of DS and 100 mg of imidazole in a headspace vial. Add 1.0 mL of Internal standard solution (0.016 µg/mL of NDMA-d6 in methanol) and 1.0 mL of acetonitrile. Apply the stopper, cap and crimp tightly.

Chromatographic conditions:
Mode: GC
Injector: Headspace
Injection type: Split (Split ratio, 1:1 or 1:3)
[Note—Split ratio can be modified to optimize sensitivity.]
Detector: MS/MS (QqQ mass detector)
Column: 0.32-mm × 30-m fused-silica coated with a 1.0-µm layer of phase *G16*
Column temperature:

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temp. (°)	Hold Time at Final Temp. (min)
45	0	45	3
45	10	130	3
130	15	190	-
190	40	240	10

Carrier gas: Helium
Gas Flow: Constant flow at 1.8 mL/min (adjustment and verification are necessary for other carrier gases)
Purge Flow: 3.0 mL/min or default value

MS conditions:
Ionization: Electron Impact
Scan Settings:

Impurity	NMDA	NDMA-d6	NDEA	NEIPA	NDIPA
Acquisition mode	MRM	MRM	MRM	MRM	MRM
MRM 1	*m/z* 74 → 44	*m/z* 80 → 50	*m/z* 102 → 85.1	*m/z* 116 → 99.1	*m/z* 130.0 → 42
MRM 2	*m/z* 74 → 42		*m/z* 102 → 56.1	*m/z* 99.0 → 44.1	*m/z* 130.0→ 43.1

System suitability:
Suitability requirements
Relative standard deviation: NMT 20.0% for the ratios of the impurity standard peak response to the internal standard peak response from six replicate injections, Standard solution
Signal-to-noise ratio: NLT 10 for each nitrosamine,
Sensitivity solution Blank: No interfering peaks from the blank

Analysis
Standard solution and Sample solution Calculate the concentration (ppm) of each specified nitrosamine impurity in the portion of Drug Substance taken:
Result = (1/W) x (RU/ RST) x CST

USP <1469> Procedure 2: Headspace (HS) GC-MS/MS (QqQ)

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45	10	130	3
130	15	190	-
190	40	240	10

Carrier gas: Helium
Gas Flow: Constant flow at 1.8 mL/min (adjustment and verification are necessary for other carrier gases)
Purge Flow: 3.0 mL/min or default value

MS conditions:
Ionization: Electron Impact
Scan Settings:

Impurity	NMDA	NDMA-d6	NDEA	NEIPA	NDIPA
Acquisition mode	MRM	MRM	MRM	MRM	MRM
MRM 1	*m/z* 74 → 44	*m/z* 80 → 50	*m/z* 102 → 85.1	*m/z* 116 → 99.1	*m/z* 130.0 → 42
MRM 2	*m/z* 74 → 42		*m/z* 102 → 56.1	*m/z* 99.0 → 44.1	*m/z* 130.0→ 43.1

System suitability:
Suitability requirements
Relative standard deviation: NMT 20.0% for the ratios of the impurity standard peak response to the internal standard peak response from six replicate injections, Standard solution
Signal-to-noise ratio: NLT 10 for each nitrosamine,
Sensitivity solution Blank: No interfering peaks from the blank

Analysis
Standard solution and Sample solution Calculate the concentration (ppm) of each specified nitrosamine impurity in the portion of Drug Substance taken:
Result = (1/W) x (RU/ RST) x CST

USP <1469> Procedure 4: GC-MS/MS (triple-quad)

Procedure 4 – Quantitation of NDMA, NDEA, NDIPA, NEIPA, and NDBA in selected sartans by GC-MS/MS (triple-quad)

Sample solution: Transfer 500 mg of the drug substance into a disposable 10- to 15-mL glass centrifuge tube. Add 5.0 mL of the Internal standard solution (50 ng/mL of NDMA:13C2-d6 in methylene chloride). Cap the tube. Vortex the sample for 1 min, and then place in the centrifuge. Centrifuge the sample at 4000 rpm for 2.5 min. Transfer 2 mL of the bottom methylene chloride layer to a 5-mL syringe fitted with a 0.45-µm nylon filter. Filter 1 mL of sample extract into a 2-mL GC autosampler vial and cap.

Chromatographic conditions:
Mode: GC
Injector: Split/splitless
Injection type: Splitless with purge, **Purge time:**0.5 min
Column:0.25-mm × 30-m; fused-silica coated with a 1.0-µm layer of phase G16
Carrier gas: Helium, **Flow rate:** Constant flow at 1.0 mL/min
Injection volume:2 µL
Temp. Injector: 250°
Temp. Transfer line to MS detector:220°
Temp. Ionization source:250°
Column: See Table 17.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temp. (°)	Hold Time at Final Temp. (min)
40	0	40	0.5
40	20	200	0
200	60	250	3

Detector: MS/MS (triple quadrupole mass spectrometer)
MS conditions
Ionization: Electron impact
Scan Settings:

Impurity	NMDA	NDMA-C13d6	NDEA	NEIPA	NDIPA	NDBA
Acquisition mode	MRM	MRM	MRM	MRM	MRM	
MRM 1	*m/z* 74 → 44	*m/z* 82 → 48	*m/z* 102 → 85.1	*m/z* 116 → 99	*m/z* 130 → 88	*m/z* 158 → 99
MRM 2	*m/z* 74 → 42		*m/z* 102 → 56	*m/z* 99.0 → 44.1	*m/z* 130.0→ 42	*m/z* 84→ 56

System suitability Samples: Generate the response versus concentration standard curve for each nitrosamine impurity under test using the corresponding Standard solutions and perform the linear regression analysis.

Suitability requirements
Correlation coefficient: NLT 0.98
Signal-to-noise: NLT 10 for the impurity peak, Standard solution Cal 2

Sample Introduction: Chromatography

Liquid Chromatography

- Coverage Range
  - Volatility not required: Volatile, semi- and non-volatile NAs
  - Can minimize potential in situ formation of NAs due to thermal degradation of APIs which may form nitrosamines precursor reagents (secondary amine, nitrosating agents, etc.)
  - Can minimize thermal degradation of nitrosamines
    - Note: APCI - use of lower source temperature
- Chromatographic Resolution/Reproducibility: Critical Factors
  - Mobile phase compatible with MS (reduce impact on ionization efficiency of target NAs)
  - Chromatographic columns for polar compounds, etc.

Gas Chromatography

- Coverage Range:
  - Volatility required : Volatile and semi-volatile NAs
  - The presence of dialkyl amines (process impurity, counter ion of the salt form of the API, degradation product) in the presence of nitrite and acid (or other nitrosating agent) can lead to in situ formation of nitrosamines as an artifact.
  - Thermally labile API (use selective extraction of NAs) with dialkylamines group
- Chromatographic Resolution: Critical Factors
  - Chromatographic columns
    - Caution with column/septum bleeding: ↑ s/n - ↓ sensitivity
  - Injection modes
  - Type of liners used, etc.

Summary of Factors Impacting on Sensitivity & Selectivity

General Notes

SENSITIVITY

SAMPLE PREPARATION PROTOCOL

- Matrix effect? Can we improve sample preparation to reduce matrix effects and increase sensitivity?
- Low extraction efficiency? Are the NAs being totally extracted from matrix?
- Selective extraction protocols – Strategy to improve selectivity: reduce extraction of possible interfering compounds
- Total dissolution extraction?
- Concentration step using SPE after selective extraction?
- Good repeatabilty? Good recovery?

CHROMATOGRAPHIC METHOD

- Chromatographic method with good resolution between target compounds and interfering compounds: Can reduce ion supression
- Columns: improve analysis efficiency/resolution
- GC: caution with column/septum bleeding
- LC: use mobile phase compatible with MS, change pH value to optimize ionization efficiency

IONIZATION SOURCE

Optimize Ion Source Parameters

ESI

- Majority of nitrosamines are not basic - lower ionization efficiency compared to APCI, exception: NMBA (acidic group) great ionization efficiency in ESI(-) mode, MNP/CPNP - ESI+
- Less tolerant to Matrix effects (ME). Is ME impacting on ionization efficiency?

APCI

- Higher ionization efficiency for SOME nitrosamines when compared to ESI (non basic and acidic NAs)
- More tolerant to matrix effect

MASS ANALYZER

Low Resolution MS

- Tandem MS: Improve analysis sensitivity by acquiring data in Multiple Reaction Monitoring (MRM) mode allowing quantitation with lower limits of quantitation
- Single quad: Selected Ion Monitoring (SIM) mode

High Resolution MS

- Improve analysis sensitivity by acquiring data in: PRM (QOrbitrap)/ MRM (QToF) / Selected Ion Monitoring (SIM) versus Full Scan

MASS ANALYZER & DATA ACQUISITION

- Higher Sensitivity *vs* Lower Sensitivity Instruments: Ion transmission and detector efficiency
- Higher mass resolution analysis can lead to sacrifice in sensitivity:
  - ToF: Loss of sensitivity
  - FTICR/Orbitrap - Increased scan times, slight loss in sensitivity (need of lower scan speeds)
- Scan Speed – Instrument-specific/optimized by the user (acquire more than 10 points/peak)
- Duty Cycle – can be optimized by the user

DATA PROCESSING

- Averaged MS Spectrum

SYSTEM MAINTENANCE AND CLEANING

- To prevent loss of instrumental sensitivity the instrument should be subject to:
  - Preventive maintenance frequently
  - System cleaning after analysis

Summary of Factors Impacting on Sensitivity & Selectivity

General Notes

SELECTIVITY

SAMPLE PREPARATION PROTOCOL

- Selective extraction protocols – Strategy to improve selectivity: reduce extraction of possible interfering compounds

CHROMATOGRAPHIC METHOD

- Chromatographic method with good resolution between target compounds and adjacent peaks!

MASS ANALYZER

Tune and calibrate the system prior analysis

High Resolution MS

- High accuracy in mass measurement: provides greatly increased selectivity and confidence for identification of compounds
- Data acquisition strategy:
  - QOrbitrap hybrid systems: Parallel Reaction Monitoring mode (PRM), Selected Ion Monitoring mode (SIM)
  - QToF systems: Multiple Reaction Monitoring Mode

Low Resolution MS

- Poor accuracy in mass measurement may lead to false detection of nitrosamines - lack of selectivity
- Tandem MS can improve selectivity: Selected Reaction Monitoring (SRM) mode /Multiple Reaction Monitoring (MRM) mode will enhance selectivity
- Single quadrupole: Work on sample preparation protocol and chromatographic separation along with larger injection quantities (either by volume or concentration)

DATA PROCESSING

- Targeted data extraction strategy = Extract the ion Chromatogram for the specific ion (*m/z*)
- LC-HRMS: Use a suitable mass tolerance window to obtain the EICs (not so broad window in ppm/amu)

Summary of Factors Impacting on Sensitivity and Selectivity

General Notes

ACCURACY & PRECISION

SAMPLE PREPARATION PROTOCOL

- Matrix effects? Strategie to minimize Matrix effects
  - Internal standards, matrix-matching calibration
- Non-reproducible extraction efficiency?
  - Internal standards: account for possible losses during workup or thermal instability and volatility inherent to several *N*-nitrosamines
- Selective extraction protocols: reproducible protocol and loss of NAs?
  - Internal standards

CHROMATOGRAPHIC METHOD

- Good resolution and robust conditions
- Strategy to minimize Matrix effects

MS ANALYSIS

- LC-MS/MS: broader range of applicability; preferred if the API may degrade due to high temperature (APCI: use ↓ temperature)
- GC-MS/MS: Inlet: high temperature - Degradation of API? Formation of nitrosamines in-situ (inlet/headspace)
- Robust conditions

MASS ANALYZER & DATA ACQUISITION

- Scan Speed – Instrument-specific/optimized by the user (acquire more than 10 points/peak) – improve definition of the peaks
- Higher mass resolution analysis can lead to sacrifice in sensitivity and need for lower scan speeds

DATA PROCESSING

- Peak smoothing can assist reproducibility: use suitable smoothing algorithms
- Averaged MS Spectrum