Development and validation of LC-MS/MS method for N-nitrosamines analysis in pharmaceutical products

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Graduate thesis

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SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
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Razvoj i validacija LC-MS/MS metode za određivanje *N*-nitrozamina u farmaceutskim proizvodima

Diplomski rad

Rijeka, 2023.

Mentor rada: prof.dr.sc. Milan Mesić, redoviti profesor

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1. Jelena Ban, PhD, Associate professor, Committee Head
2. Stribor Marković, PhD, Assistant professor, Committee member
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The thesis has 84 pages, 65 pictures, 22 graphs, 19 tables and 60 references.

Saţetak

N-nitrozamini su klasa organskih spojeva s kemijskom strukturom koja sadrži nitrozo (-NO) funkcionalnu skupinu vezanu na dušik amina. Ovakav amin može imati jednu ili dvije alkilne skupine vezane na dušikov atom. Prema različitim provedenim studijama na životinjama, potvrđeno je da je većina nitrozamina kancerogena, a potencijalno je to slučaj i kod ljudi. Opisana je veća učestalost karcinoma želudca i jednjaka kod ljudi koji su više bili izloženi nitrozaminima u hrani. Osim toga, jedno drugo istraživanje navodi da radnici u gumarskoj industriji, koji su bili izloženi povećanim razinama nitrozamina, su također imali povećanu incidenciju karcinoma usne šupljine, jednjaka i grla. Ovaj mehanizam je povezan s citokromom P450 koji je posrednik oksidacije spoja nitrozamina koji potom proizvodi alkilirajuće sredstvo koje može reagirati s genetskim materijalom.

Od lipnja 2018. Američka agencija za hranu i lijekove (Food and Drug Administration, kao i Europska agencija za lijekove (European Medicines Agency) prate prisutnost nitrozaminskih onečišćenja u raznim lijekovima i uvode pravila o maksimalnim dopuštenim razinama. Cilj ovog rada bio je razviti kromatografsku, LC-MS/MS metodu(e) za kvalitativno i kvantitativno određivanje šest nitrozaminskih onečišćenja u šest različitih lijekova na tržištu. Kao materijal za analizu koristiti će se konačni oblici lijekova (gotove formulacije), a standardi njihovog aktivnog farmaceutskog sastojka (Active Pharmaceutical Ingredient - API) će se koristiti prilikom razvoja metode. Pregledom literature nađeno je više različitih metoda s obzirom na tehniku razdvajanja i analize, kao i neke metode koje se koriste za više različitih onečišćenja. U ovom radu cilj je razviti metodu(e) prilagođenu što većem broju potencijalnih nitrozaminskih onečišćenja u aktivnim supstancama.

N-nitrozamini, koji su predmet izučavanja u ovom radu, moraju se dobro kromatografski odvojiti međusobno, kao i od glavnog vrha aktivne supstance koja se analizira. *N*-nitrozamini od interesa koji će biti izučavani su *N*-nitrozodimetilamin, *N*-nitrozodietilamin, *N*-nitrozo-*N*-metil-4-

aminomaslačna kiselina, *N*-nitrozoetilizopropilamin, *N*-nitrozo-diizopropilamin i *N*-nitrozodibutilamin.

Lijekovi i standardi aktivnih supstanci korištene u ovom radu su azitromicin, betahistin, metformin, metronidazol, simvastatin, sitagliptin i vildagliptin u obliku različitih soli ili slobodnih baza.

Razvijene su dvije kromatografske metode korištenjem tri različite kromatografske kolone, te su izrađene kalibracijske krivulje za sve kombinacije metoda i kolona. Također, za svako nitrozaminsko onečišćenje te za svaku metodu i vrstu kolone određene su granice detekcije i granice kvantifikacije. Za *N*-nitrozo-*N*-metil-4-aminomaslačnu kiselinu zadovoljavajuća osjetljivost detektora je postignuta korištenjem samo jedne metode i jedne kolone.

Ključne riječi: nitrozamini, lijekovi, tekućinska kromatografija, masena spektrometrija, genotoksična onečišćenja

Summary

N-nitrosamines are a class of organic compounds with a chemical structure that contains a nitroso (-NO) functional group attached to the amine nitrogen. Such an amine can have one or two alkyl groups attached to the nitrogen atom. According to various studies, most nitrosamines have been confirmed to be carcinogenic in various animal studies but potentially also in humans. For example, a higher incidence of gastric and oesophageal cancer has been described in people who were more exposed to nitrosamines in food. Additionally, another study reported that workers in the rubber industry who were exposed to increased levels of nitrosamines also had an increased incidence of mouth, oesophagus, and throat cancers. This mechanism is associated with cytochrome P450, which mediates the oxidation of nitrosamine compounds that then produce an alkylating agent that can react with genetic material.

Since June 2018, the US Food and Drug Administration (FDA), as well as the European Medicines Agency (EMA), have monitored the presence of nitrosamine contaminants in various drugs and introduced rules on maximum permissible levels. The aim of this work was to develop LC-MS/MS method(s) for the qualitative and quantitative determination of six nitrosamine contaminants in six different drugs on the market. The final forms of the drug (Finished Dosage From – FDF) and the standards of their active pharmaceutical ingredient (API) will be used during the development of the method. A review of the literature found several different methods regarding the technique of separation and analysis, as well as some methods that are used for several different pollutants. In this paper, the goal is to develop a method(s) adapted to as many potential nitrosamines as possible impurities in active substances.

N-nitrosamines, which are the subject of study in this work, must be well chromatographically separated from each other as well as from the main peak of the active substance being analysed. The *N*-nitrosamines of interest

that will be studied are *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitroso-*N*-methyl-4-aminobutyric acid, N-nitrosoethylisopropylamine, *N*-nitroso-diisopropylamine and *N*-nitrosodibutylamine.

Medicines and standards of active substances used in this work are Azithromycin, Betahistine, Metformin, Metronidazole, Simvastatin, Sitagliptin and Vildagliptin in the form of various salts or free bases.

Two chromatographic methods were developed using three different chromatographic columns, and calibration curves were created for all combinations of methods and columns. Also, detection limits and quantification limits are determined for each nitrosamine contamination and for each method and type of column. For *N*-nitroso-*N*-methyl-4-aminobutyric acid, satisfactory detector sensitivity was achieved using only one method and one column.

Keywords: nitrosamines, drug products, liquid chromatography, mass spectrometry, genotoxic impurities

List of abbreviations

AI	Acceptable Intake
APCI	Atmospheric Pressure Chemical Ionisation
API	Active Pharmaceutical Ingredient
ARB	Angiotensin Receptor Blockers
CID	Collision-Induced Dissociation
CIP	Cahn-Ingold-Prelog
DEA	*N,N*-diethylacetamide
DMA	*N,N*-dimethylacetamide
DMF	*N,N*-dimethylformamide
DNA	Deoxyribonucleic Acid
EMA	European Medicines Agency
EP	European Pharmacopeia
ESI	Electrospray Ionisation
FDA	Food and Drug Administration
FDF	Finished Dosage Form
GC	Gas Chromatography
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HFTB	1,1,1,3,3,3-hexahluoro-2-methyl-2-propanol
HPLC	High Performance Liquid Chromatography
HPW	High Purity Water
HRMS	High Resolution Mass Spectrometry
HSS	High Strength Silica
IARC	International Agency for Research on Cancer
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
i.d.	Internal Diameter
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MDD	Maximum Daily Dose
mg	Milligram
mL	Millilitre
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry

MS/MS	Tandem Mass Spectrometry
NDBA	*N*-nitrosodibutylamine
NDEA	*N*-nitrosodiethylamine
NDMA	*N*-nitrosodimethylamine
NDIPA	*N*-nitrosodiisopropylamine
NEIPA	*N*-nitrosoethylisopropylamine
ng	Nanogram
NMBA	*N*-nitroso-*N*-methyl-4-aminobutyric acid
NMP	*N*-methylpyrrolidone
NTFB	Nonafluoro-*tert*-butyl alcohol
PFP	Pentafluoro phenyl
PP	Perfluoropinacol
ppb	Part-per-billion
ppm	Part-per-million
QbD	Quality by Design
QC	Quality Control
QqQ	Triple Quadrupole MS Detector
Q-ToF	Quadrupole Time-of-Flight
%RSD	Percentage of Relative Standard Deviation
RT	Retention Time
SD	Standard Deviation
SFC	Supercritical Fluid Chromatography
SIR	Single Ion Recording
S/N	Signal to Noise Ratio
SRM	Selected Reaction Monitoring
SST	System Suitability Test
TFA	Trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TQMS	Tandem Quadrupole Mass Spectrometer
TTC	Threshold of Toxicological Concern
UPLC	Ultra Performance Liquid Chromatography
UHPLC	Ultra High Performance Liquid Chromatography
USP	United States Pharmacopeia
WHO	World Health Organisation

Table of contents

1. Introduction

N-nitrosamines (often called nitrosamines) are organic compounds with a chemical structure containing a nitroso functional group (–NO) bonded to an amine. This amine nitrogen atom might have one or two alkyl groups attached to itself. According to different studies, most nitrosamines are carcinogenic in non-human species and probable mutagenic and carcinogenic in humans.[1,2] In a systematic review by Jakszyn and González of published cohort and case-control studies, there has been a suggested connection between nitrite and nitrosamine intake with gastric and oesophageal cancer. The origin of nitrites has been found in meat and processed meat, which are rich with nitrites and nitrates since these compounds are used in the processing of meat. Also, the intake of preserved fish, vegetables, and smoked food can be connected with gastric cancer, which could be rationalised by the presence in such processed food.[3] Also, Straif *et al.* explore the connection between cancer mortality in rubber industry workers exposed to *N*-nitrosodimethylamine (NDMA) for prolonged periods. They found that excess cancer mortality, which can be associated with *N*-nitrosamine exposure, is similar to the data of the various animal studies of *N*-nitrosamines carcinogenicity.[4]

The organic chemistry of nitrosamines is well understood regarding their synthesis, structures, and reactivity.[5,6] Nitrosamines formation is the result of nitrous acid, HNO_2, and various secondary amine reactions. The nitrous acid is usually synthesised by the protonation of a nitrite group. This synthesis method is similar to the formation of nitrosamines under some biological conditions. Structurally, the functional group of nitrosamines is planar, as determined by X-ray crystallography. In NDMA, one of the most straightforward members of a large class of *N*-nitrosamines, distances between two nitrogen atoms and nitrogen and oxygen atoms are 132 ppm and 126 pm, respectively.[7]

Nitrosamines are not directly carcinogenic. Cytochrome P450 enzymes shown in Figure 1 are responsible for the activation of nitrosamine compounds and converting nitrosamines to alkyl-diazonium ions, alkylating agents that are highly carcinogenic. Cytochrome P450 enzymes are a superfamily of heme-containing mono-oxygenases found in all kingdoms of life and show extraordinary diversity in their reaction chemistry. Estabrook *et al.* described the role of cytochrome as a catalyst in the synthesis of steroid hormones and various drug metabolism, including different kinds of toxins.[8]

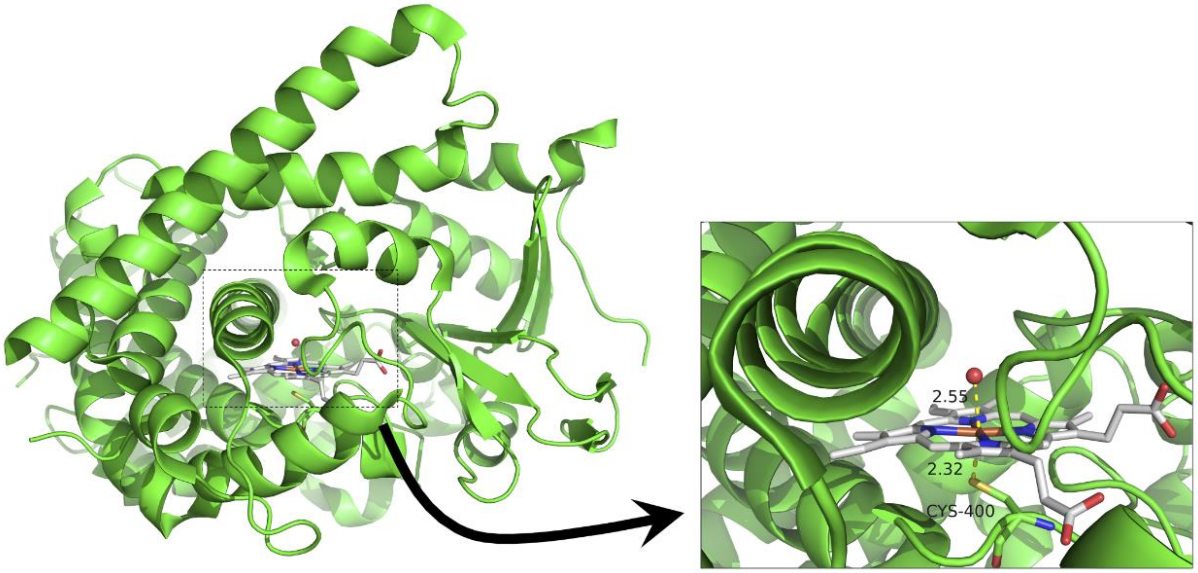


Figure 1. Cytochrome P450 enzyme with heme prosthetic group magnified on the right

Depending on the source, several different cytochromes are involved in *N*-nitrosamines activation, like P450 2E1, according to Yang *et al.*,[2] or P450 2A6 by Wong *et al.*[9] and Chowdhury, Calcut and Guengerich.[10] Nevertheless, these cytochrome enzymes contain the prosthetic heme group, the core of enzyme catalytic activity, whose oxidation cycle is shown in Figure 2. This group has an iron atom in the middle of the heme, which in resting form, is in the Fe(III) oxidation state. After substrates (*N*-nitrosamines) bind to it, , molecular oxygen is bonded and immediately reduced to a coordinated hydroperoxide, as shown in Figure 2. A substrate

is depicted as S-H. A proton which is relayed from glutamic acid through a chain of hydrogen-bonded water molecules, induces the cleavage of the peroxide bond. One of the assumed roles of cysteine sulphur is electron donation, which weakens the peroxide bond. After breaking the peroxide bond, a stable water molecule and a highly reactive oxidising intermediate are induced. Various evidence supports an oxo-iron(IV) porphyrin radical cation formation, which is the species responsible for cleaving even strong bonds in substrates, like C-H bonds. This cleavage is a one-electron process where the proton of the substrate is transferred to the ferryl oxygen to produce specie 2 and a radical –S, derived from the substrate. Finally, the collapse of this complex affords the hydroxylated product (S-OH) and the enzyme enters into the resting state to complete the cycle.[11]

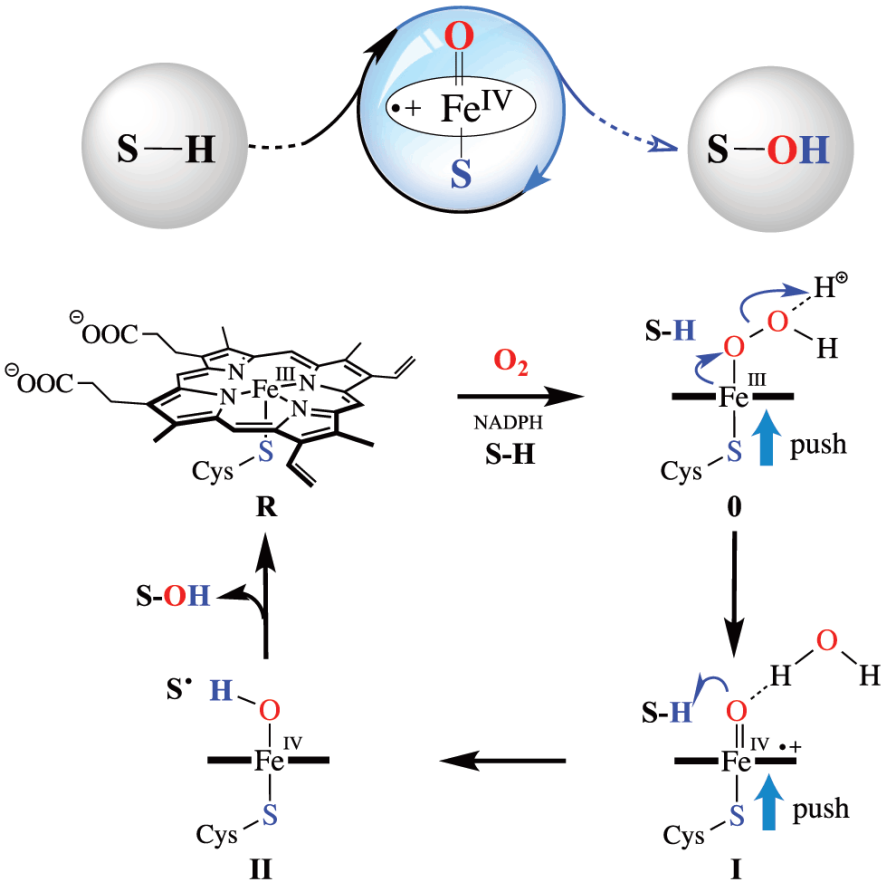


Figure 2. Cytochrome P450 heme catalytic cycle[11]

Enzymatic α-hydroxylation by cytochrome P450, as shown in Figure 3, is the first step of activation of *N*-nitrosamines. *N*-nitrosamines are subsequently modified to form reactive alkyl-diazonium ion, capable of alkylation of DNA and induction of mutations and promote genetic toxicity. The type of alkylating agent varies with the R-group of specific nitrosamine, but they all contain alkyl diazonium centres.[12,13]

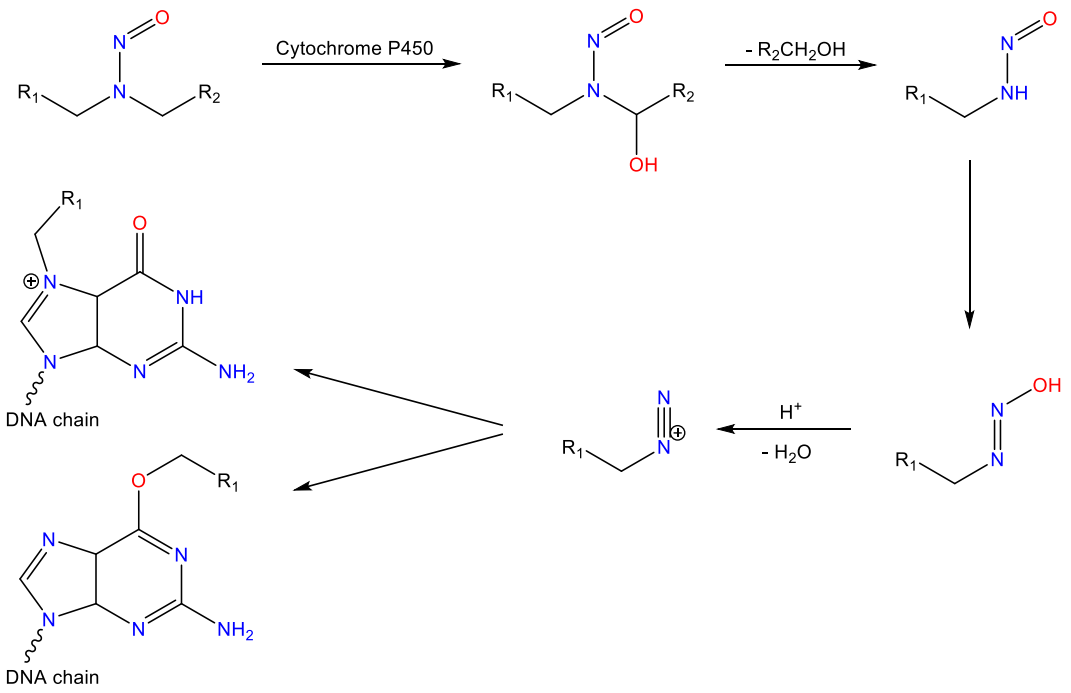


Figure 3. Metabolic activation of the nitrosamine by cytochrome P450, enabling α-hydroxylation in the first step, and subsequent formation of an alkylating agent, which can potentially react with guanidine from DNA chain[12,13]

1.1 Nitrosamines in medications

In June 2018 first discovery of nitrosamine, namely NDMA, a probable human carcinogen in Valsartan led to high efforts of the regulatory agencies, as well as the pharmaceutical industry, to find and better understand the root causes of the presence of this impurity in pharmaceutical products.[14,15]

In September 2019 US Food and Drug Administration (FDA) became aware that standard heartburn medications, Ranitidine and Nizatidine, contained high levels of NDMA.[16] Agency immediately recommended to manufacturers a recall of Ranitidine and Nizatidine products containing NDMA in levels above acceptable limits, as discussed in paragraph *1.5 Acceptable intake limits*.[14,17,18] Also, after stability testing, there have been findings which suggested that NDMA levels in some Ranitidine products at ambient temperature can increase with time above acceptable levels. Preliminary results after forced degradation studies showed higher levels of NDMA in all products after two weeks of study. In addition, the test results suggest that NDMA levels further increase with time in storage. Therefore, in April 2020, FDA issued a directive that all Ranitidine drug products are to be pulled from the market in the United States. However, as *N*-nitrosamine impurities issue extends well beyond the US drug supply, regulatory authorities worldwide have been sharing information, coordinating inspections, developing and testing analytical methods to detect known and identify unknown nitrosamines and create immediate solutions to ensure the quality and safety of the drug supply for the patients worldwide.

In December 2019, regulatory agencies worldwide also became aware of elevated amounts of NDMA in Metformin diabetes medicines from various countries.[19] Considering this information, agencies tested various Metformin samples for NDMA. Samples tested by February 2020 showed NDMA in some samples but within acceptable limits. However, further testing in May 2020 revealed several lots of extended-release formulations of Metformin containing NDMA above the acceptable intake limit. Based on the last test results, agencies requested that manufacturers voluntarily recall affected lots. Regulatory agencies worldwide continue to investigate NDMA and other nitrosamine impurities in Metformin as well as in other drug products and advise manufacturers on actions to be taken.

Regulatory agencies have identified nitrosamine impurities that, in theory, could be found in various drug products: *N*-nitrosodimethylamine (NDMA),

N-nitrosodiethylamine (NDEA), *N*-nitroso-*N*-methyl-4-aminobutanoic acid (NMBA), *N*-nitrosoisopropylethyl amine (NEIPA), *N*-nitrosodiisopropylamine (NDIPA), *N*-nitrosodibutylamine (NDBA), and *N*-nitrosomethylphenylamine (NMPA) shown in Figure 4. Five of these impurities, such as NDMA, NDEA, NMBA, NEIPA, and NMPA, have already been found in various drug substances and drug products.

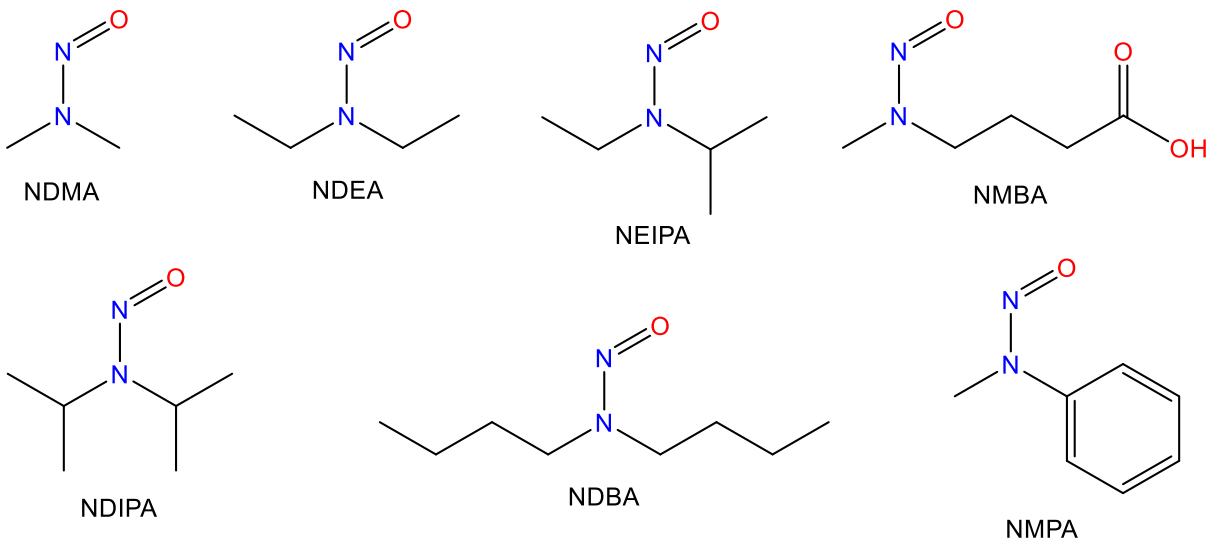


Figure 4. Structures of potential nitrosamine impurities which could be found in medicines

Apart from medicines regulatory agencies, other institutions worldwide have also been exploring the nitrosamine impurities problem at hand, International Agency for Research on Cancer (IARC), part of the World Health Organisation (WHO), classified various nitrosamine compounds as very potent genotoxic impurities in several animal species. Some of them are also classified as potential human carcinogens.[20] Also, these are referred to as "cohort of concern" compounds according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (shortened abbreviation: ICH) guidance for industry M7(R1),[21] Assessment and control of all potential DNA reactive (mutagenic) impurities in pharmaceutical products to limit carcinogenic risk in March of 2018. This guidance recommends control of any known or suspected

mutagenic carcinogen, in this case, *N*-nitrosamines, at or below level such that there should be a negligible risk of resulting in human cancer associated with the exposure. All relevant regulatory agencies update their recommendations periodically, and manufacturers must check for any updates. Following the discovery of nitrosamine contaminants in angiotensin receptor blockers (ARB), the FDA, followed by other regulatory agencies, published acceptable interim limits for nitrosamine impurities.[22] Agencies also recommended manufacturers to take all necessary actions to quantify levels of nitrosamine impurities in their drugs and reduce or remove, if possible, these impurities when above the limit. Agencies use these interim limits to guide immediate decision-making for further evaluation and possible product recalls[14,16] while balancing the risk of long-term carcinogen exposure and undesired disruption to patient care.

1.2 General conditions for nitrosamine formation

The formation of *N*-nitrosamines in some kinds of drugs is likely to happen in the presence of amines and nitrites while under acidic conditions. These acidic conditions facilitate the formation of nitrous acid from nitrite salts, which can further react with an amine (secondary, tertiary or quaternary) to form a nitrosamine compound, as seen in Figure 5. There is even higher risk of nitrosamine formation if, in the reaction workup procedure, quenching of residual azides is done using nitrous acid in the presence of precursor amines. Azides are highly explosive compounds and reagents commonly used to form tetrazole rings or introduce azide functionality into a molecule.[23]

Nitrites used as reagents in synthesis can be carried over to subsequent steps, despite purification efforts, and then react with amines that are used in the next step(s) to generate nitrosamine impurities. Therefore, carryover into subsequent steps must be considered when nitrite salts are present. Generally, processes using nitrite salts in the presence of various amines significantly increase the risk of generating *N*-nitrosamine impurities.[24]

1.3 Sources of amines

Amines may be used throughout a production process for many different purposes. The API's, reaction intermediates and starting materials can contain various amine functionalities. Additionally, amines are also used as reagents or catalysts in reactions. [25] These amines could react with nitrous acid as well as other nitrosating agents to form *N*-nitrosamine impurities.

Apart from amines, various amide solvents that are susceptible to degradation are another potential source of nitrosamine impurity forming secondary amines. For example, under high temperatures and for an extended period of time, *N*,*N*-dimethylformamide (DMF) decomposes into dimethylamine, which can react with nitrous acid to form NDMA, as shown in Figure 5. In addition, *N*-methylpyrrolidone (NMP), *N*,*N*-dimethylacetamide (DMA), and *N*,*N*-diethylacetamide (DEA) have very similar decomposition pathways obtaining secondary amines which could, in the same manner, react with nitrous acid to form their respectful nitrosamine impurities. Additionally, secondary amines could be present as impurities of amide solvents which can also react with nitrous acid to form *N*-nitrosamine impurities.

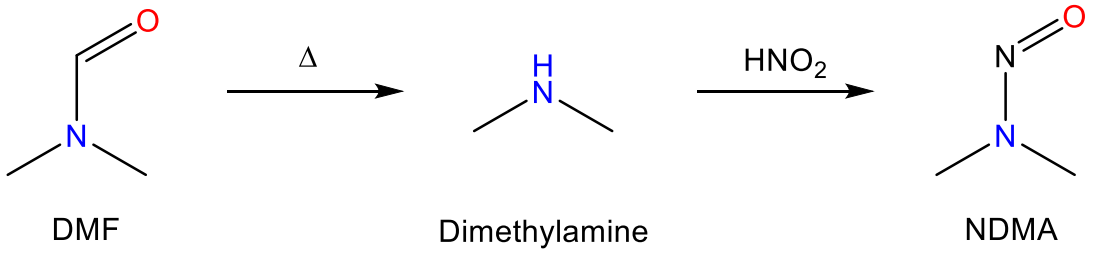


Figure 5. Formation of NDMA from *N*,*N*-dimethylformamide

Tertiary and quaternary amines used as reactants or reagents in the API manufacturing process may contain other amine impurities. For example, tertiary amines, such as triethylamine, have been known to contain small amounts of other secondary amines (*N*,*N*-diisopropylamine and *N*-isopropyl-*N*-ethylamine). Also, secondary and tertiary amines could be

present as impurities or degradants formed by the dealkylation of quaternary amines, like, for example, a commonly used phase-transfer catalyst, tetrabutylammonium bromide, could contain tributyl- and dibutyl-amine impurities. The level of amine impurities that could lead to the formation of nitrosamine impurity and, subsequently, contamination of the API is process dependent and has to be evaluated by each manufacturer.[26]

As mentioned earlier, this list of sources needs to be completed, as amine reagents are used in a wide range of synthetic processes. Therefore, manufacturers must evaluate other reagents which contain amine functionality for the risk of the potential *N*-nitrosamine impurity formation, as well as final API's which could also be transformed into nitroso impurity.

1.3.1 Raw reaction materials

Nitrosamine impurities can also be introduced with different vendor-sourced materials, such as starting and raw materials, which can be contaminated. Understanding the supply chain relevant to manufacturing is essential in preventing and controlling contamination.[26] For example, sometimes API producers are not aware of nitrosamine impurities present in raw or starting materials that are acquired from third-party vendors. In such case, an API producer whose production process is usually not susceptible to the formation of nitrosamines may need to realise that outsourced material could have impurities introduced during the production of this material or transport.

1.3.2 Recycled solvents, reagents or catalysts

Materials recovered from performed reactions, such as solvents, reagents, and catalysts, may contain nitrosamine impurities precursors due to residual amines. If the workup or recovery process involves a quenching procedure, i.e. quenching of azides using nitrous acid, nitrosamines formed could contaminate the recovered solvent. These nitrosamines can easily be introduced to the solvents if they have similar properties to the recovered

materials, depending on recovery and eventual purification procedure (e.g., aqueous washes or evaporation).[26] Since the smallest nitrosamines, NDMA and NDEA are both highly miscible with water and soluble in organic solvents and, therefore, can easily be introduced into the final product. Because of this fact, various drug products using API's manufactured by low-risk processes were also contaminated by *N*-nitrosamines.

1.3.3 Quenching process

As mentioned before, a significant risk of the formation of nitrosamine impurities when quenching azides excess using nitrous acid is performed in the reaction mixture. This procedure allows nitrous acid to react with potential amine residues in raw materials used in the process. *N*-nitrosamine impurities formed in this manner can be carried over to the subsequent step or steps if there is inadequate removal of impurities during purification and if the purification procedure is not optimised for removing specific impurities.[26] These impurities can even contaminate the entire downstream processes once formed. Therefore, even if the quenching is performed outside the main reaction mixture, a significant risk of contamination of recovered materials being introduced into the primary process remains.

1.3.4 Lack of process optimisation and control

Another potential source of the formation of *N*-nitrosamine impurities is the need for more optimisation of the whole manufacturing process for API's if some of the reaction conditions like temperature, pH, or the order of adding reagents or intermediates are inappropriate. There have been reported cases describing reaction conditions that varied significantly between batches and even between different manufacturing equipment in the same production facility for the same API, which might lead to a significant quantity of different nitrosamines produced.[26]

1.4 Nitrosating impurities in drug products

Various nitrites are common nitrosating impurities found in many excipients used in formulations at ppm (part-per-million) levels. Nitrite impurities found in commonly used excipients could lead to the formation of *N*-nitrosamine impurities in Finished Dosage Forms (FDF) during the drug product manufacturing process or the shelf-life storage period. Supplier qualification programs should consider that nitrite impurities, in most cases, vary across excipient lots but may also vary by manufacturer.[27]

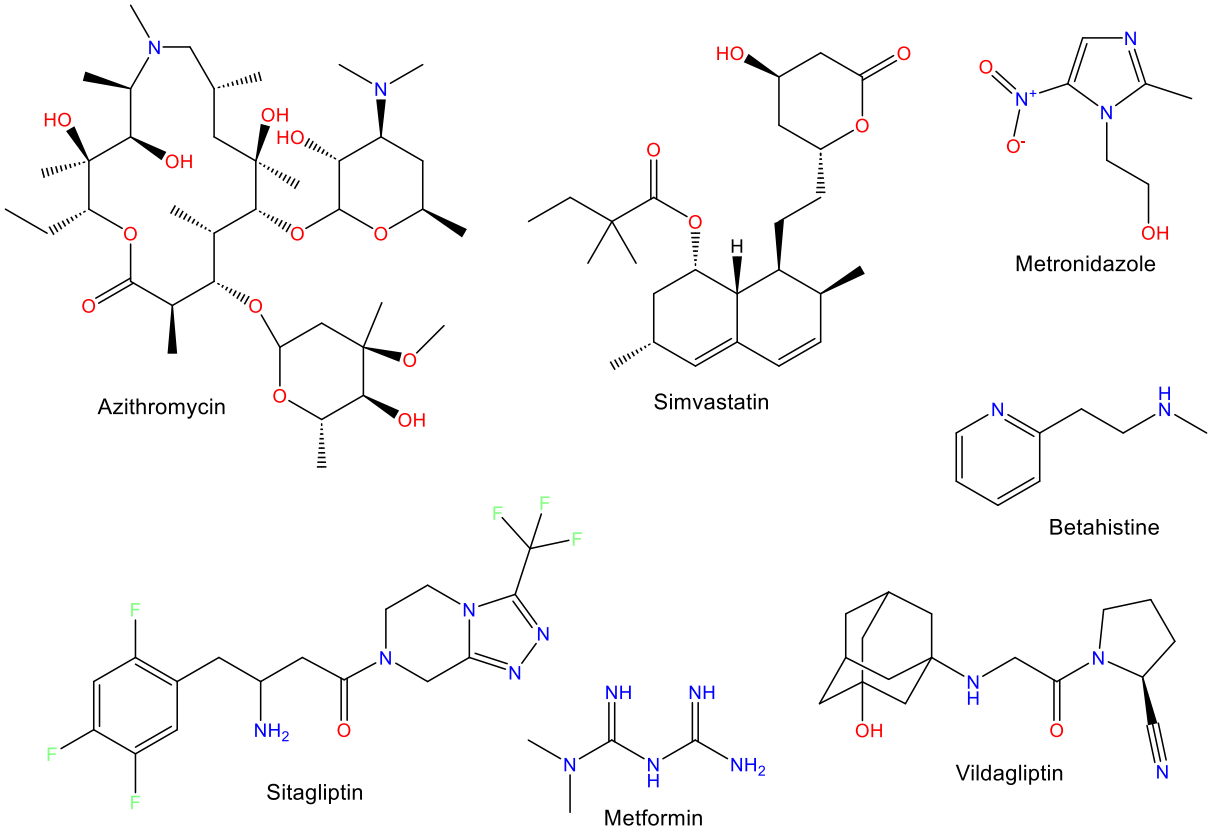


Figure 6. Structures of the API's used in method development

Drug product manufacturers, even formulators developing formulations, should bear in mind that nitrites, as well as nitrosamine impurities, could be present to some extent in the water used in the process. Also, there have been reports that some drug products may become subject to degradation pathways that form nitrosamine impurities; this could occur during drug product storage. These various multiple root causes of

nitrosamine contamination listed above can occur within the same API manufacturing process. Therefore, various strategies may be needed to identify and mitigate all potential sources of contamination. Typical routine tests for API purity, identity, and known impurities are highly unlikely to detect the presence of nitrosamine impurities. Furthermore, each failure or error during all of the synthetic steps could generate different nitrosamine impurities in different amounts across batches of the same process and API manufacturer, with contamination detected in some batches.

Compounds used in this study (Figure 6) were chosen according to literature examples in which these compounds were analysed for the traces of nitrosamine impurities.[28,29]

1.5 Acceptable intake limits

Regulatory agencies recommend acceptable intake (AI) limits of the following *N*-nitrosamine impurities, NDMA, NDEA, NMBA, NEIPA, NDIPA, NDBA and NMPA, as stated in Table 1. Term acceptable intake (AI) is used and described in ICH Guidelines M7(R1)[21] to indicate the threshold value of toxicological concern (TTC), which is considered for any impurity that is associated with negligible risk of carcinogenicity or any other toxic effects. Also, agencies recommend manufacturers use these AI values when determining limits for *N*-nitrosamine impurities in both API's and drug products. Apart from drug manufacturers, API manufacturers have to control nitrosamine impurities in their products to ensure their API's used in drug products meet the recommended AI limits. The AI limit is defined as a daily exposure limit to potentially carcinogenic compounds such as aforementioned nitrosamine impurities, which approximates a 1 in 100,000 risk of cancer after 70 years of continuous exposure. The conversion of the AI limit into ppm is calculated based on the maximum daily dose (MDD) of the drug as reflected in the drug label claim (ppm = AI (ng)/MDD (mg)) and varies by product. These limits are applicable only if a single nitrosamine is detected in a drug product. In other cases, if more than one

of the nitrosamine impurities is identified and the total quantity of found nitrosamine impurities exceeds 26.5 ng/day, which represents the AI for the most potent nitrosamines (NDEA, NEIPA, NDIPA and NDBA), based on the MDD, the manufacturer should contact the regulatory body for further evaluation. For any drug product with an MDD under 880 mg/day, a recommended LOQ for total nitrosamines of 0.03 ppm is under 26.5 ng/day and is acceptable.

Table 1. AI limits for NDMA, NDEA, NMBA, NEIPA, NDIPA, NDBA and NMPA in drug products

Nitrosamine	AI limit (ng/day)
N-nitrosodimethylamine, NDMA	96.0
N-nitrosodiethylamine, NDEA	26.5
N-nitroso-*N*-methyl-4-aminobutanoic acid, NMBA	96.0
N-nitrosoisopropylethyl amine, NEIPA	26.5
N-nitrosodiisopropylamine, NDIPA	26.5
N-nitrosodibutylamine NDBA,	26.5
N-nitrosomethylphenylamine, NMPA	26.5

For any drug product with an MDD above 880 mg/day, the limit for total detected nitrosamine impurities should be adjusted not to exceed the recommended limit of 26.5 ng/day. In case new nitrosamine impurities without published AI limits are detected in drug products, manufacturers should use the approach described in ICH M7(R1)[21] to evaluate the risk associated with nitrosamine impurity and contact the relevant regulatory body and check the acceptability of any proposed limit.

Generally, highly sensitive analytical methods with a limit of quantitation (LOQ) in the low parts-per-billion (ppb) range are necessary to meet these low AIs recommended by the regulatory bodies for nitrosamine impurities. Manufacturers of API's and drug products should develop, validate and use methods with LOQ's at or below 0.03 ppm. In this case, LOQ can be set as

a limit for reporting the quantity of the impurities found. Manufacturers should develop methods for which the limit of detection (LOD) and LOQ are as low as possible but reasonably practical for products for which the maximum daily dose is higher than 1 g. In case more than one *N*-nitrosamine impurity listed in Table 1 is detected, the analytical method has to be validated for LOQ's below 0.03 ppm for each of the nitrosamines detected and to accurately quantify a total nitrosamine level which should not exceed 26.5 ng/day. Regulatory agencies worldwide have a public webpage that includes information about news, risks, and validated analytical methods developed for detecting and quantifying *N*-nitrosamine impurities in different API's and products of interest.[16,23]

1.6 Considerations for method development and validation

Recently all major pharmaceutical manufacturers are rushing to develop analytical methods and production processes to ensure compliance using suitable instrumentation. The ability to detect low-level nitrosamine impurities in their products will lead to safer medicines, but the margin of error is minimal. Considering the significant consequences of inaccurate nitrosamine analysis, any results produced must be accurate and consistent. On the other hand, false positive results could generate substantial financial losses due to unnecessary product recalls and time consumed during investigations and even more for reformulation. In comparison, false negative results presume that these compounds remain undetected or detected with falsely acceptable levels and therefore delivered to the patients that can cause significant health issues and subsequently high legal costs.

In method development and validation for nitrosamine impurity analysis, consideration of the complex product matrix, compound physical-chemical properties, and possible sources of cross-contamination and interference can help avoid erroneous results. For example, nitrosamines could be formed during sample preparation if precursors are present in the sample

matrix.[27] Also, many rubber and plastic materials used in sample preparation and sample handling can contain traces of *N*-nitrosamine impurities which could corrupt the results of the analysis. By knowing and understanding chemistry, as well as potential sources of *N*-nitrosamine impurity generation, analysts can better fit their methods to avoid bad results. Except to test for already generated *N*-nitrosamine impurities, analytical laboratories can screen the finished drug product and formulation excipients for known precursor chemicals, such as nitrites and secondary amines, that could generate *N*-nitrosamine impurities. By regularly testing for nitrosamine precursors, laboratories can identify and subsequently control potential sources of *N*-nitrosamine impurity formation. For example, if an *N*-nitrosamine impurity is detected in the drug product downstream, laboratories have the option to inspect nitrite levels in various excipients to identify any correlations.

1.6.1 Comparing chromatography and mass spectrometry technologies

When evaluating instruments and techniques for nitrosamine impurity analysis, analytical laboratories will check various sources, such as published methods in peer-reviewed articles and regulatory bodies guidelines regarding detecting nitrosamine impurities in drug products.[30] These methods can be a good initial point when developing and validating new methods for *N*-nitrosamine impurity analysis, but the methods must be validated by the in-house laboratory for the tested drug product. Methods published use a variety of platforms, such as liquid chromatography coupled with tandem quadrupole mass spectrometry (LC-MS/MS), gas chromatography coupled with mass spectrometry (GC-MS) and liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS). Each technique has its advantages counting on the type of *N*-nitrosamine and drug product being tested and must be considered when evaluating and selecting analytical technologies and instrumentation.

Widely used systems for *N*-nitrosamine analysis, in terms of a detector type, are HRMS and MS/MS. An HRMS instrument can be particularly beneficial for initial screening analysis in order to characterise any unknown *N*-nitrosamine impurity where a standard, especially a deuterated standard, is not yet available. Also, it is preferred for samples with highly complex matrices since the higher resolving power of such instrumentation, compared to MS/MS instruments, can selectively detect the *N*-nitrosamine impurity of interest by its isotopic specific pattern, even in the presence of various co-eluting interferences. On the other hand, MS/MS is the first choice for high-throughput work in a quality control (QC) environment because of its higher sensitivity compared to HRMS, the robustness of the technique and ease of use with regard to the specific MS expertise needed to interpret HRMS data.

However, higher time and resource allocation could be needed to develop chromatography methods for MS/MS workflows compared to high-resolution MS, which is far less dependable on chromatographic separation. Nevertheless, HRMS and MS/MS will both need inlets like GC or LC to separate the sample components before MS analysis. Still, for HRMS it is not crucial to achieving a level of separation as for MS/MS. Therefore, these orthogonal techniques can be used in the early phases of drug development and screening to detect and quantify *N*-nitrosamines in the drug product matrix more accurately.

Compared to LC systems, a higher level of sensitivity and better chromatographic separation is achieved with GC. However, it is necessary to consider several potential issues. For instance, the heat generated during GC headspace sampling can potentially generate nitrosamine impurities *in situ* and must be carefully used. In addition, GC analysis requires analytes to be volatile but stable, which is often contradictory. Achieving stability, for example, for *N*-nitrosodiphenylamine is possible only with various time-consuming derivatisation steps.[31] Ideally, and especially in a high-throughput QC environment where simplicity is crucial, derivatisation is

preferred to be avoided. Also, derivatisation prolongs the time for analysis and is more complex for low-abundance impurities.

Having a high mass resolution instrument can be important when considering two closely eluting compounds, as well as two structurally similar compounds, such as NDMA and the residual solvent DMF, as shown in Figure 7. Generally, if high or even ultra-performance LC cannot chromatographically resolve these compounds, a high mass resolution is needed to distinguish between the two.

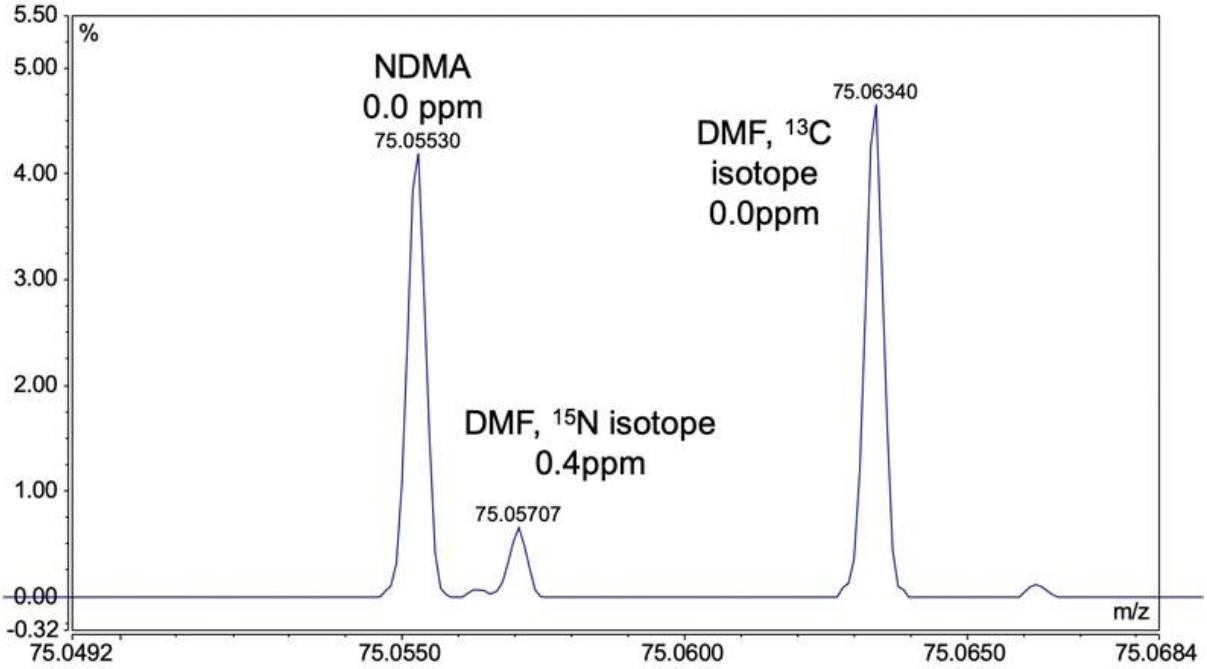


Figure 7. Mass spectrum of a sample of Ranitidine showing separation of NDMA and DMF in HRMS

The potential problem was detected and described in a 2020 paper published by scientists from the FDA when they reviewed the results of a private analytical laboratory which overestimated the levels of NDMA in Metformin.[31] Notation of Figure 7 stating *0.0 ppm* is the mass error of the measurement. This common notation used in HRMS data processing shows how precise the instrument is compared with the theoretical mass calculated from the compound structure. For structure elucidation purposes

industry standard in the pharmaceutical industry is 2 ppm, but higher resolving instruments can achieve under 100 ppb of mass error. The analytical technique used in this case was high-resolution Quadrupole Time-of-Flight (Q-ToF) mass spectrometry; however, DMF interfered with the NDMA measurement with an insufficient resolution, which could have been avoided if a higher-resolution mode of operation for the instrument had been used. Also, instead of selecting only one instrument for *N*-nitrosamine impurity analysis, many analytical laboratories are prone to choose both MS/MS and HRMS instruments to support the complex requirements for *N*-nitrosamine analytics. A high-resolution MS instrumentation will appeal for its high-resolving power and selectivity, typically providing more structural data than MS/MS instrumentation. This fact is particularly beneficial during early method development procedures, initial screenings, or while analysing samples with very complex matrices. In comparison, MS/MS can provide a higher sensitivity and accuracy that is well-suited to targeted analysis in a QC environment. Also, MS/MS instruments are more robust, which is an advantage in routine testing environments.

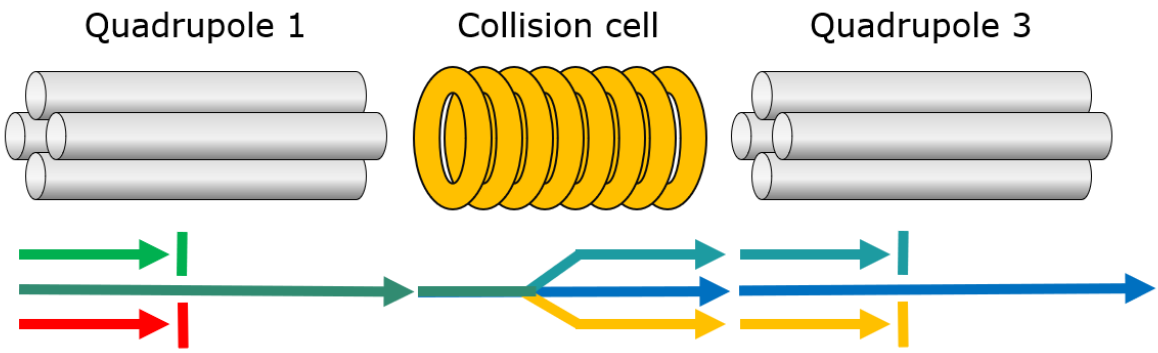


Figure 8. Schematic of Tandem quadrupole (TQ) MS when recording MRM experiment; precursor ion (dark green arrow) is transmitted through Q1 towards collision cell where it is fragmented, and fragments are transmitted into Q2 where only selected ions (blue arrow) are only ones detected

Multiple reaction monitoring (MRM) MS experiment, also known as selected reaction monitoring (SRM), is a method used in tandem mass spectrometry

in which an ion of a particular mass is selected in the first quadrupole of a tandem quadrupole mass spectrometer and an ion product of a fragmentation reaction of the precursor ions is selected in the second quadrupole of the mass spectrometer for detection as shown in Figure 8. A selected ion, precursor ion, is transmitted through the first quadrupole, fragmented in the collision cell, and a specified fragment ion, product ion, is then transmitted through the second quadrupole while all other ions drift off the ion path and into the one of the Q2 rods.

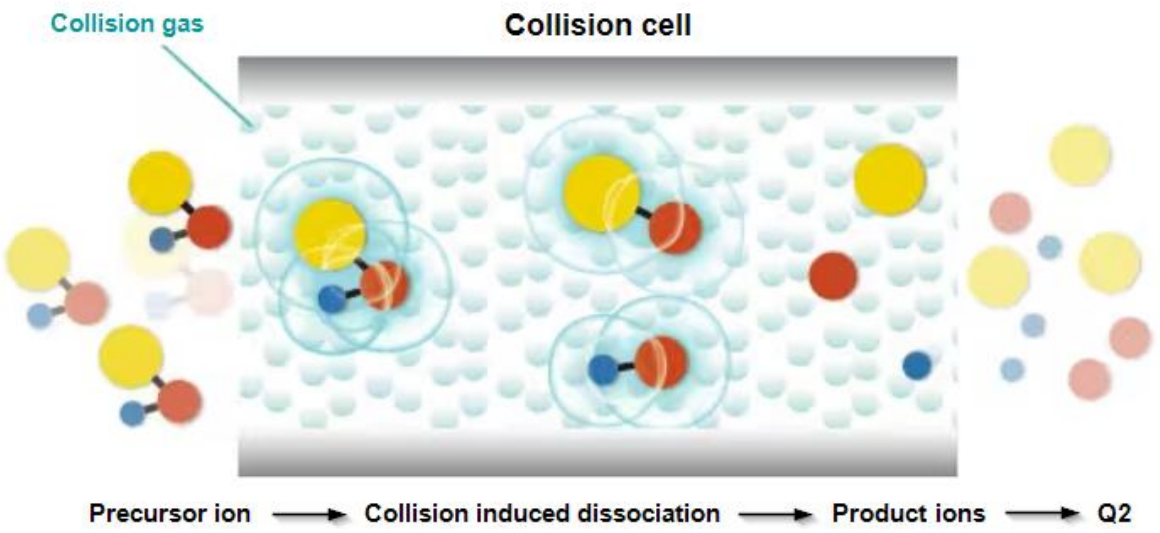


Figure 9. Schematic of collision-induced dissociation in collision cell of MS

Fragmentation in collision cells, or collision-induced dissociation (CID), is achieved by a collision of inert molecules of gas with gas phase ions of the analyte. Gases like nitrogen and argon are usually used for this purpose. When gaseous ions travel from the ion source through the first quadrupole and collision cell filled with gas like argon, these molecules collide, resulting in the breaking of their bonds and producing ion fragments that are seen in the detector, as shown in Figure 9.

There are two types of fragmentation mechanisms, homogenous fragmentation and heterogenous fragmentation mechanism. If the homogenous fragmentation is occurring, radicals are produced, but if the

bond breaks heterogeneously, cation and anion are produced, depicted in Figure 10.

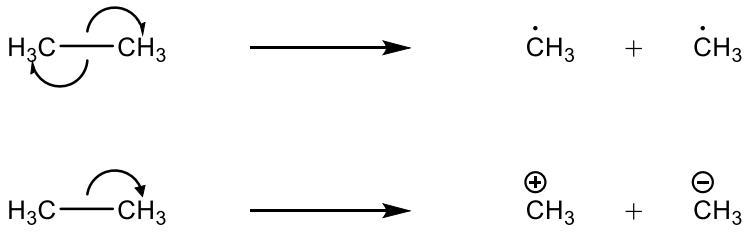


Figure 10. Homogenous and heterogenous fragmentation mechanisms.

This method, MRM, is more selective and sensitive than a single ion recording experiment (SIR) due to the analyte-specific transition needed for response to be seen and less interference by co-eluting background ions of the same mass. No mass spectra are generated by MRM experiments.

2. Purpose of the thesis

The objective of this work was to develop and validate method(s) suitable for the determination of the quantity of nitrosamine impurities in drug products, FDF's. Methods efficiently separated nitrosamine impurities from each other as well as from active pharmaceutical ingredient(s). Method had to able to resolve six nitrosamine impurities; NDMA, NDEA, NMBA, NEIPA, NDIPA, NDBA and six API's; Azithromycin, Betahistine, Metformin, Metronidazole, Simvastatin, Sitagliptin and Vildagliptin.

After method development, method validation was performed to ensure that the method performed well according to established good practices in analytical laboratories. Validation parameters tested in this study were precision, selectivity, the limit of detection (LOD), the limit of quantitation (LOQ), linearity, accuracy and method linear range. After validation was performed, system suitability test (SST) parameters were determined for the method. Method development and validation were performed using LC-MS/MS system to achieve faster analysis which can be easily transferred to the quality control laboratory.

Analytical runs were run to test methods in actual conditions as well as to determine if impurities were present in several commercially available drug products. Products chosen for this test were various compounds that could contain some amount of nitrosamine impurities. Drug products used in this test contained the following API's: Azithromycin, Betahistine, Metformin, Metronidazole, Simvastatin, Sitagliptin and Vildagliptin. These compounds were chosen because of the possibility of generating nitrosamine impurities during the manufacturing process.[28,29]

3. Materials and methods

3.1 General information

3.1.1 Solvents, reagents and standards

Solvents used were commercially available; acetonitrile LC-MS grade (Honeywell – Riedel-de Haën, Acetonitrile CHROMASOLV, cat. no. 34967), methanol LC-MS grade (Honeywell – Riedel-de Haën, Methanol CHROMASOLV, cat. no. 34966). The modifier used is formic acid LC-MS grade (Honeywell - Fluka eluent additive for LC-MS, cat. no. 60-048-227). High-purity water (HPW) was produced using Merck Millipore MilliQ IX 7003 water purification system.

Active pharmaceutical ingredients standards used in this study were also commercially available from Merck; Azithromycin dihydrate (European Pharmacopeia (EP) Reference Standard, cat. no. Y0000306), Betahistine dichloride (United States Pharmacopeia (USP) Reference Standard, cat. no. 1065618), Metformin hydrochloride (EP Reference Standard, cat. no. M0605000), Metronidazole (EP Reference Standard, cat. no. M1850000), Simvastatin (EP Reference Standard, cat. no. S0650000), Sitagliptin phosphate monohydrate (EP Reference Standard, cat. no. Y0001812), Vildagliptin (ACS Reagent, cat. no. SML2302).

Nitrosamine impurities standards were purchased from Sigma-Aldrich, part of Merck; NDMA (USP Reference Standard, cat. no. 1466674), NDEA (USP Reference Standard, cat. no. 1466652), NMBA (USP Reference Standard, cat. no. 1466696), NEIPA (USP Reference Standard, cat. no. 1466685), NDIPA (USP Reference Standard, cat. no. 1466663), NDBA (USP Reference Standard, cat. no. 1466641).

3.1.2 Mobile phase preparation

Mobile phase A was prepared by adding 1 mL of formic acid into the 1 L volumetric flask and filling it with high-purity water (HPW) to the mark. In

the same manner, mobile phase B was prepared using acetonitrile or methanol. Those two mobile phase B were prepared for the purpose of method development.

3.1.3 Standards preparation

Nitrosamine impurity standards were bought neat but were also available in solution for more practical use. A stock solution of each nitrosamine impurity standard was prepared in a concentration of 500 ng/mL with HPW in a 25 mL volumetric flask. From the stock solution, to make the calibration curve, dilutions up to the following concentrations were made; 0.5 ng/mL, 1 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL and 100 ng/mL. The standards used for SST during the analysis of the drug products were in concentrations of 5 ng/mL. The stock solution of these standards was stored in the refrigerator for a maximum of one week.

Stock solutions of API standards, in a concentration of 1 mg/mL, were prepared by dissolving them in methanol. Then, stock solutions were diluted to achieve the final 0.01 mg/mL solution for injection. The stock solution of API standards was stored in a refrigerator for a maximum of one week.

3.1.4 Drug product sample preparation

Depending on the label claim, the corresponding number of tablets was crushed in a mortar or tablet crusher to obtain a fine homogenous powder. The powder is then weighed in sufficient quantity to prepare 5-10 mL of a solution, a concentration of 30 mg/mL of active substance in HPW. The suspension is stirred briefly on a vortex mixer and then for 15 minutes in an ultrasonic bath at room temperature. It is then centrifuged at 4000 rpm for 15 min. The resulting supernatant was filtered through a syringe filter (PTFE membrane, 0.22 µm) by discarding the first two millilitres. Samples were prepared fresh daily since the stability in the solution was unknown.

3.2 Method development

Methods were developed using Shimadzu Nexera X3 UHPLC system coupled with Shimadzu LCMS-8045 Tandem quadrupole Mass Spectrometer. Nexera X3 UHPLC comprised of two solvent delivery pumps LC-40B X3, degassing unit DGU-405, autosampler SIL-40 C, column oven CTO-40S, photodiode array detector SPD-M40 and system controller SCL-40. In addition, Shimadzu LCMS-8045 MS was equipped with Electrospray Ionisation (ESI) source and Atmospheric Pressure Chemical Ionisation (APCI) source.

3.2.1 LC method

To achieve the best possible selectivity for all the nitrosamine and API standards, different LC method parameters, including different mobile phases, were used before the best methods were developed. Two final methods were chosen, *LC-method 1*, described in Table 2. and *LC-method 2*, described in Table 3. After testing and a literature search for compatibility of column chemistry and mobile phase solvents, methanol was chosen as an organic solvent with 0.1% formic acid and used in both LC methods.

Table 2. General parameters of the *LC-method 1*

Gradient table and LC parameters						
min	2.0	6.0	12.0	15.0	15.1	18.0
%B	5	10	90	90	5	5
Flow rate	0.7 mL/min					
Injection volume	30.0 µL					
Column temperature	40°C					
Autosampler temperature	5°C					

Also, methanol is the solvent of choice in order to facilitate π-π interactions between phenyl column chemistry and analyte, compared to acetonitrile which is showing suppression of these interactions.[32]

Table 3. General parameters of the *LC-method 2*

Gradient table and LC parameters									
min	3.0	5.0	6.0	10.0	13.0	13.1	15.0	15.1	18.0
%B	5	10	60	60	80	95	95	5	5
Flow rate	0.4 mL/min								
Injection volume	30.0 µL								
Column temperature	30°C								
Autosampler temperature	5°C								

3.2.2 Columns

In preparation for LC method development and for the purpose of choosing adequate columns, physical and chemical characteristics of *N*-nitrosamine compounds of interest were evaluated. For this purpose, *Chemicalize* web-based software (https://chemicalize.com/app/calculation) was used to calculate theoretical physical and chemical properties based on the compound structure. Using these properties and knowing the structure of the analytes, the best columns could be chosen, and the best performance of the column and method could be achieved.

From a pool of columns available in-house, several columns were tested. As a result, three different columns were used in the final LC methods; Shimadzu Shim-pack GIST C18 5 µm, 4.6 x 150 mm (part no. 227-30017-07) (*Column 1*), Phenomenex Kinetex BiPhenyl 2.6 µm, 3.0 x 150 mm (part no. 00F-4622-Y0) (*Column 2*) and Waters Cortecs Phenyl 2.7 µm, 3.0 x 150 mm (part no. 186008332) (*Column 3*).

3.2.3 MS method

During MS method development and optimisation, both ESI and APCI sources were tested, and the final method was developed using the APCI source. Also, various source parameters were tested to achieve the best

possible sensitivity of the instrument for each MRM experiment. During the method development process, an MS scan experiment was also performed to scan the range of molecular masses and determine the API's retention time (RT) so the correct time for the waste valve switch can be used, which is diverting API peak to waste during drug product analysis. In this mode of operation, a high concentration of API is not introduced into the ion source or mass analyser to prevent contamination and subsequent instrument downtime. The parameters of the *MS-method* are shown in Table 4.

Table 4. General parameters of the *MS-method*

Source and gas parameters	
Ion mode and polarity	APCI, positive
Nebulizing Gas	3 L/min
Interface temperature	350 °C
DL Temperature	200 °C
Heating Block Temperature	200 °C
Drying Gas	5 L/min

All of the transitions, precursor ion to quantifier and precursor ion to qualifier, were optimised using software and instrument automatic optimisation to determent instrument parameters specific for each nitrosamine impurity to achieve the best possible signal intensity.

Table 5. MRM parameters used in *MS-method 1*

Impurity	**Precursor ion**	**Quantifier transition**	**Qualifier transition**
NDMA	75.100	75.100 → 43.000	75.100 → 58.100
NDEA	103.000	103.000 → 29.200	103.000 → 75.000
NMBA	147.100	147.100 → 117.050	147.300 → 44.000
NEIPA	117.000	117.000 → 75.100	117.200 → 27.200
NDIPA	131.200	131.200 → 89.050	131.200 → 43.100
NDBA	159.200	159.200 → 29.200	159.200 → 103.100

Table 5 shows transitions tuned during instrument tuning for the analysis of these *N*-nitrosamine impurities, while Figure 11 shows the structures of the fragments that are corresponding masses shown during instrument tune and monitored during analysis. Predominant fragmentation pattern for these compounds is α-cleavage, a type of fragmentation in which bond between α-carbon, carbon adjacent to atom carrying functional group, in this case nitrogen.

Time switches for each nitrosamine impurity and combination of API are determined individually and based on the retention timetable, Table 8. The smallest possible windows were used during sample analysis due to high concentrations; peaks of API's are expected to be very broad. Some API's (i.e. Azithromycin) showed peaks eluting as early as at the start of the chromatogram, probably due to inadequate stationary phase. However, it was acceptable to determine API's retention and solvent switching times since the main focus of method development is *N*-nitrosamine impurities.

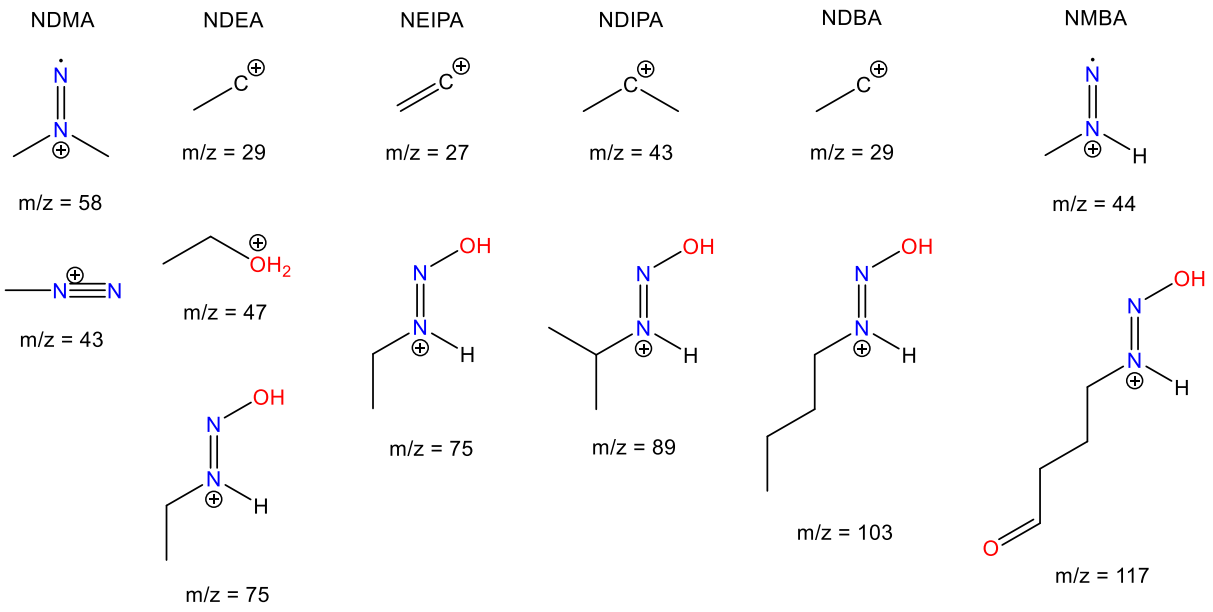


Figure 11. Structures of fragments of *N*-nitrosamine impurities found during MRM optimisation experiment and suggested in literature[33,34,35]

3.3 Method validation

Validation of the methods included the following parameters: precision, selectivity, the limit of detection, the limit of quantitation, range, linearity and accuracy.

3.3.1 Precision

In order to determine the precision of both method and column, repeatability test results were assessed. For the test, nitrosamine concentrations in test mixtures were 5 and 10 ng/mL. The primary acceptance criterion for repeatability of six consecutive injections of standards is %RSD on area ≤10%.

3.3.2 Selectivity/Specificity

Method specificity was confirmed by analysing a mixture of API standards and *N*-nitrosamines standards. The acceptance criterion for specificity was the lack of unknown peaks and API peaks at the retention time of nitrosamine detection.

3.3.3 Limit of detection

To evaluate the LOD for the methods and instrumentation used, standard mixtures were analysed with concentrations of *N*-nitrosamines of 0.1 ng/mL, 0.2 ng/mL, 0.4 ng/mL, 0.6 ng/mL, 0.8 ng/mL and 1.0 ng/mL. The acceptance criterion for the LOD was a signal-to-noise (S/N) ratio of 3. Signal to noise ratio is defined as the height of the peak detector response divided by the response of baseline noise.

3.3.4 Limit of quantitation

The LOQ of the methods and instrumentation used was determined with the analysis of standard mixtures with concentrations of *N*-nitrosamines of 0.2

ng/mL, 0.4 ng/mL, 0.6 ng/mL, 0.8 ng/mL and 1.0 ng/mL. The acceptance criterion for this parameter was a S/N ratio of 10.

3.3.5 Range

The range will be determined as the range between the LOQ and the highest concentration shown to be precise, linear and accurate.

3.3.6 Linearity

Solutions prepared in concentrations of 0.5 ng/mL, 1 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL and 100 ng/mL were injected once. The calibration curve should exhibit $R^2 \geq 0.99$, and Y-intercept should be insignificant according to the t-Student statistical test with 95% confidence.

3.3.7 Accuracy – recovery

Method accuracy was assessed using a recovery of analyte used from three different spiked samples. Recovery should be in the range of 70 – 130 %. %RSD between parallels should be no more than 10%. Value was calculated using external calibration standards of nitrosamine impurity spike solutions with concentrations of 0.5 ng/mL, 1 ng/mL, 5 ng/mL and 10 ng/mL.

3.4 System suitability test parameters

Several parameters were established as essential in order to verify instrument and method performance. These parameters ensure that the method and instrument perform as expected to get as accurate results as possible. System suitability parameters determined for these methods are:

- %RSD of the peak area of each nitrosamine impurity in the first six injections of standard solution should be less than 10%.
- The cumulative %RSD of the peak area of each nitrosamine impurity should be less than 15%. The cumulative %RSD of the peak area is calculated by combining the initial six injections of

the standard solution and each subsequent bracketing standard. A bracketing standard should be injected every six injections or 80 minutes, whichever happens first, depending on the method duration. In this case, the runtime for the methods developed is 18 minutes, meaning that 4 runs can be performed in between bracketing standards.

- The retention time relative standard deviation of any *N*-nitrosamine impurity in the analysed samples should not exceed 1% of the retention time of the corresponding standard.
- The cumulative %RSD of retention time of any *N*-nitrosamine impurity monitored, tested in the same manner as %RSD of area, should be less than 2%.
- Column theoretical plates should be no less than 5000. The theoretical plate number is an index that indicates column efficiency. It describes the number of plates as defined according to plate theory and can be used to determine column efficiency based on calculation in which the larger the theoretical plate number, the sharper the peaks and higher the resolution between peaks.
- The tailing factor should be between 0.8 and 1.2. The tailing factor is a coefficient that shows the degree of peak symmetry. If the number is smaller than 1, then the peak is "fronting", and if it is larger than 1, the peak is tailing. Too much fronting or tailing can influence resolution and is a sign of column degradation.

3.5 Data interpretation and calculation

3.5.1 Quantifier and qualifier parameters according to EU regulative

For all nitrosamine impurities, a second precursor ion pair, or transition, is used to verify the results, a qualifier. The relative intensity of the quantifier over the qualifier from the calibration measurements is compared to the

qualifier ratio of the samples, where the software automatically determines the intensity ratio. This ratio between quantifier and qualifier is essential so the analyte can be positively identified and not be mistaken by matrix or co-eluting impurity. The maximum accepted relative ion intensity tolerance, which states acceptable deviation, is +/- 20%, as per EUR-Lex Directive regarding analytical method development and validation procedures.[36]

3.5.2 Testing chromatographic separation of DMF and NDMA

Reports of the investigation into the cause of the discrepancy revealed that DMF could interfere with NDMA measurements if not separated chromatographically or if the resolution of the mass spectrometer is inadequate.[31] Since, in this study, TQ MS was used, adequate chromatographic separation of DMF and NDMA had to be achieved. To this purpose, a small amount of DMF was injected to ensure chromatographic separation from NDMA.

3.5.3 Drug sample analysis

Drug samples were analysed using developed methods in order to test the functionality of methods and to check if there were any nitrosamine impurities present. The injection sequence used for determining the quantity of each nitrosamine impurity is described in Table 6. From this sequence, all relevant system suitability parameters were calculated to verify the performance of the instrument and method. For the system suitability test (SST) purposes, the standard at the nominal level is injected six consecutive times and %RSD on retention time and peak area is determined and tested, shown in line 2 in Table 6.

If the %RSD of retention time is below 1%, the system and method are performing within the parameters. Also, in lines 5, 7 and 10, another injection of the standard is performed (same vial) to confirm that the instrument is still performing within limits. If the %RSD on first six injections and the latter, so-called bracketing injections, is under 2%, the

system and method performed well. Apart from retention time, peak area is monitored in a similar manner, with a somewhat wider window, 10% and 15%, respectively, in more detail explained in paragraph *3.4 System suitability test parameters*.

Sample preparation is described in the method description, and in sequence, there are usually triplicates. A sample is prepared three times to check if the results are consistent and that the preparation procedure is done accordingly. Also, all of the samples prepared are injected three times so the performance of the instrument and method can be re-checked, taking into account the matrix effect, which is not present with standards.

Table 6. Injection sequence used for determining the quantity of nitrosamine impurities in drug products

Number	Solution	Number of injections
1	Blank	1
2	SST STD (5 ppb)	6
3	Blank	1
4	Sample preparation 1	3
5	SST STD (5 ppb)	1
6	Sample preparation 2	3
7	SST STD (5 ppb)	1
8	Sample preparation 3	3
9	Blank	1
10	SST STD (5 ppb)	1
…	Blank	1

3.5.4 Calculation and reporting the amount of impurity in drug product

Detected nitrosamine impurity content is to be reported in ppm with three significant figures relative to API in case the value is ≥ LOD. If there is no

nitrosamine impurity detected or the value is < LOD, it is reported as *not detected* or *ND*.

The formula used for the calculation of the nitrosamine impurity amount in the drug product sample:

$$Nitrosamine\ impurity\ (ppm) = \frac{A_{sample}}{A_{STD}} \cdot \gamma_{STD} \cdot \frac{1\ mg}{1 \cdot 10^6\ ng} \cdot \frac{1}{30\ mg/mL} \cdot 10^6$$

where,

- **A_{sample}** peak area of nitrosamine impurity in drug product sample.
- **γ_{STD}** concentration of nitrosamine impurity standard injected six consecutive times (ng/mL).
- **A_{STD}** average peak area of the nitrosamine impurity standard injected six consecutive times.

4. Results

4.1 Determination of analyte properties

In order to choose columns for the purpose of method development, the physical and chemical properties of analytes were evaluated. Some of the properties calculated were solubility, logP and pKa. Results and graphs are shown for each relevant analyte in Table 7. These properties are relevant to predicting the analyte's interaction with the stationary phase and, therefore, to selecting the right columns for the method development.

Table 7. Some of the calculated physical and chemical properties of analysed *N*-nitrosamines as well as API's

Impurity	Strongest pKa		logP
	Acidic	Basic	
NDMA	n/a	3.52	0.039
NDEA	n/a	3.32	0.752
NMBA	4.78	3.40	-0.038
NEIPA	n/a	3.22	1.169
NDIPA	n/a	3.12	1.585
NDBA	n/a	3.30	2.686
Azithromycin	12.46	11.16	2.183
Betahistine	n/a	9.77	0.632
Metformin	n/a	12.33	-0.918
Metronidazole	15.41	3.03	-0.459
Simvastatin	14.91	n/a	4.458
Sitagliptin	n/a	8.66	1.257
Vildagliptin	14.71	8.78	-0.217

In Figures 12 through 17 pKa graphs for *N*-nitrosamine impurities analysed in the scope of this work are shown. It is noticeable that most compounds exhibit similar basic pKa values, which suggest similar compound behaviour on the same column chemistry.

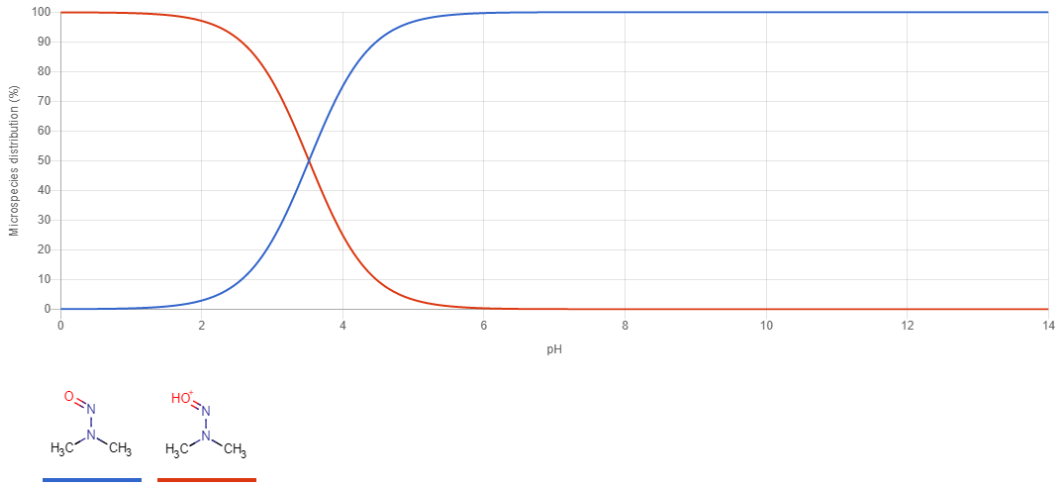


Figure 12. pKa graph of NDMA

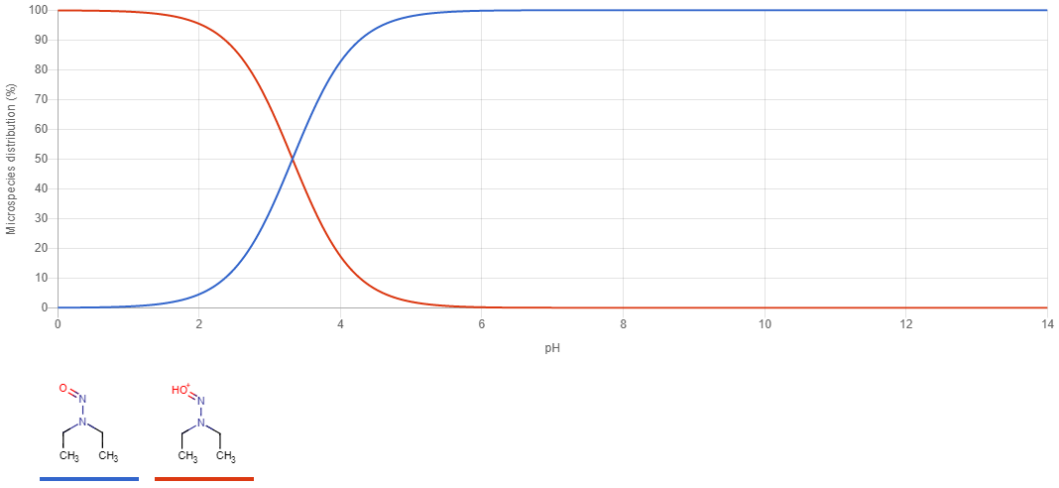


Figure 13. pKa graph of NDEA

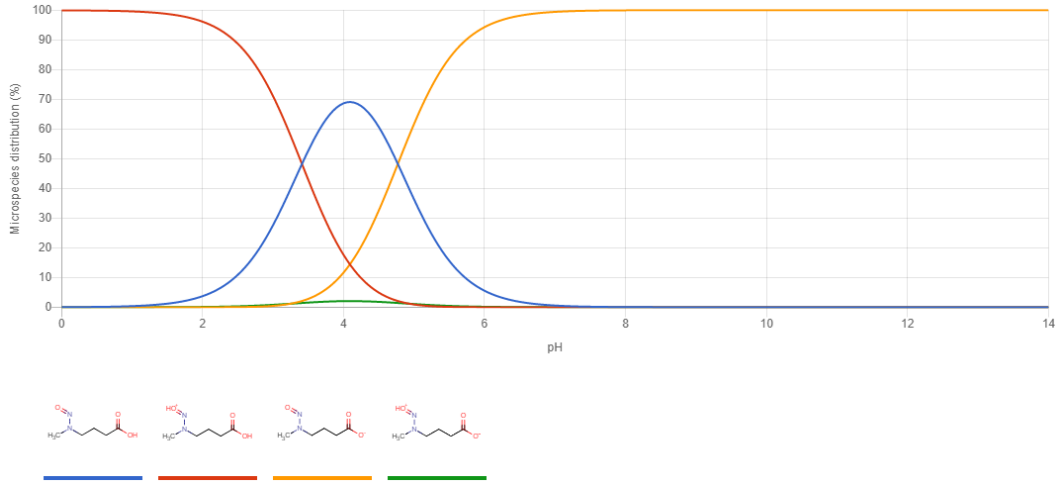


Figure 14. pKa graph of NMBA

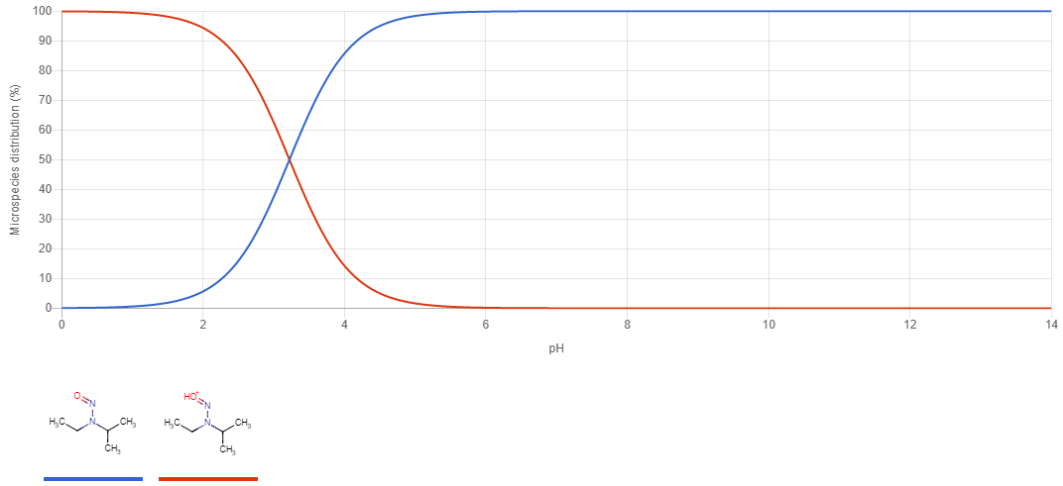


Figure 15. pKa graph of NEIPA

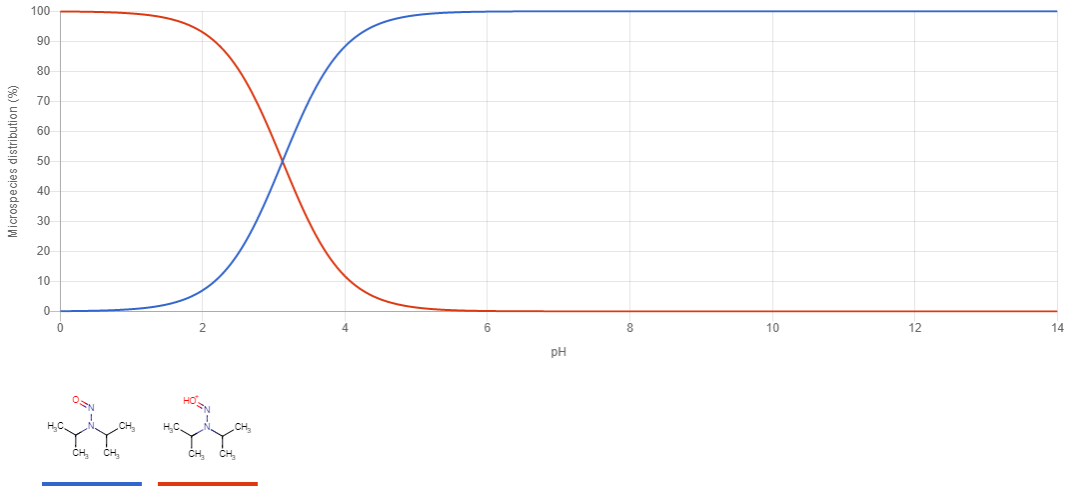


Figure 16. pKa graph of NDIPA

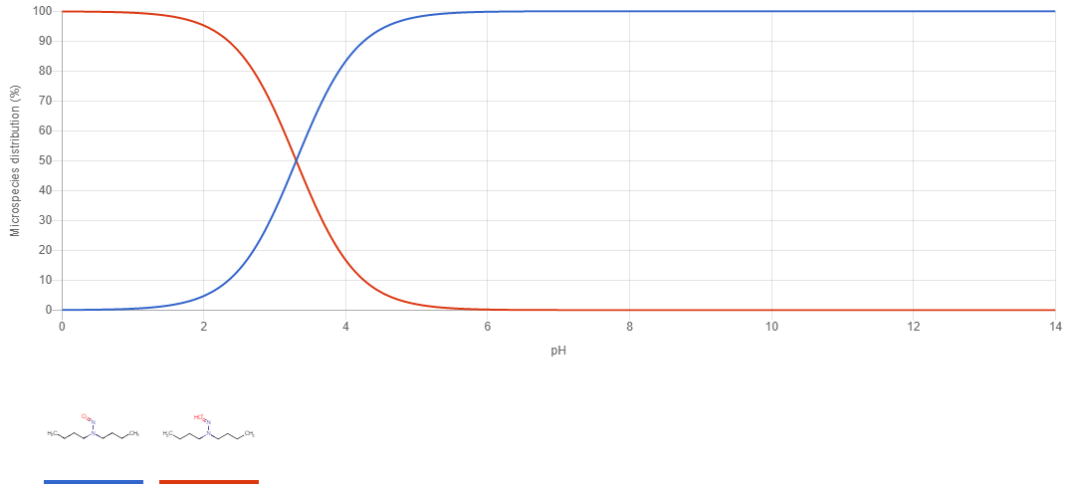


Figure 17. pKa graph of NDBA

4.2 Method development

Two methods using three different columns were developed for the analysis of six *N*-nitrosamine impurities in six different FDF's, a total of seven API's since two products are a combination of two API's. Columns used were chosen with different selectivity to broaden the field of their use. Using only one method and one column combination is not sufficient for all possible combinations of *N*-nitrosamine impurities and API's; however, combining two LC-methods and three columns gave good results.

4.3 Method validation

Methods were successfully validated across all previously determined parameters. Methods showed good linearity and range for analyses of nitrosamine impurities in final dosage forms in order to achieve limits set by regulatory agencies worldwide.

4.3.1 Precision

The precision of the method was determined as %RSD on the area of impurity on two levels, 5 ng/mL and 10 ng/mL.

Table 8. Precision as average area SD and %RSD for all tested impurities using *LC-method 1* and *Column 1*

Impurity	Average area		SD		%RSD	
	5 ppb	10 ppb	5 ppb	10 ppb	5 ppb	10 ppb
NDMA	269610	519608	1192.98	6207.18	0.442	1.195
NDEA	237279	475433	7141.08	340.95	3.010	0.716
NMBA	n/a	n/a	n/a	n/a	n/a	n/a
NEIPA	251942	503667	1358.04	5306.03	0.539	1.053
NDIPA	204049	409420	1244.63	1095.58	0.610	0.268
NDBA	412926	825623	942.44	929.81	0.228	0.113

Results in Table 8. show the calculated %RSD under the limit set in acceptance criteria for all the nitrosamine impurities when tested using *LC-method 1* and *Column 1*. Table 9. and Table 10. show precision data for *LC method 2* using *Column 2* and *Column 3*, respectively. Data for NMBA are shown only in Table 9.

Table 9. Precision as average area SD and %RSD for all tested impurities using *LC-method 2* and *Column 2*

Impurity	Average area		SD		%RSD	
	5 ppb	10 ppb	5 ppb	10 ppb	5 ppb	10 ppb
NDMA	72993	145476	2223.39	494.33	0.307	0.340
NDEA	213636	452324	3098.15	2114.19	1.450	0.467
NMBA	51591	90766	530.68	565.25	1.029	0.623
NEIPA	255093	508273	7634.91	1655.30	2.993	0.326
NDIPA	177894	344261	1374.66	2190.88	0.773	0.636
NDBA	210971	421996	1156.18	802.48	0.548	0.190

Table 10. Precision as average area SD and %RSD for all tested impurities using *LC-method 2* and *Column 3*

Impurity	Average area		SD		%RSD	
	5 ppb	10 ppb	5 ppb	10 ppb	5 ppb	10 ppb
NDMA	38554	73211	461.44	316.47	1.197	0.432
NDEA	220542	447818	1637.77	1873.77	0.741	0.418
NMBA	n/a	n/a	n/a	n/a	n/a	n/a
NEIPA	218538	435118	1753.98	2277.41	0.803	0.523
NDIPA	144383	272885	1600.50	1855.31	1.109	0.680
NDBA	303111	611566	1220.07	2088.10	0.403	0.341

4.3.2 Selectivity/Specificity

The specificity of the method was confirmed by overlaying chromatograms of the blank solution and sample of APIs and nitrosamine impurity

standards. MS scan of the API standards and nitrosamine impurities standards were used to determine the retention times of all compounds included in this study. This data showed no interferences between any of the API's and nitrosamine impurities, except for similar retention time of Azithromycin and NDEA using *LC-method 2* and *Column 3*, but the other two methods and column combination retention time window is wide enough to facilitate analysis.

Table 11. Retention times of API's standards and nitrosamine standards

Compound	m/z	Retention time (min)		
		LC-method 1 Column 1	*LC-method 2 Column 2*	*LC-method 2 Column 3*
Azithromycin	749	12.19	8.02	7.91
Betahistine	137	2.36	1.64	1.66
Metformin	130	2.42	1.64	1.63
Metronidazole	172	11.23	7.92	7.61
Simvastatin	419	16.91	15.72	15.38
Sitagliptin	408	4.24	3.41	3.38
Vildagliptin	304	10.81	7.78	7.51
NDMA	75	6.59	3.99	2.63
NDEA	103	12.61	8.45	7.99
NMBA	147	n/a	7.75	n/a
NEIPA	117	8.41	9.08	8.41
NDIPA	131	13.80	9.99	8.92
NDBA	159	14.87	13.77	12.14

Possible interferences with the content of formulation were taken into account using drug products available, but tests showed no problematic interference with analytes. Chromatograms showing peaks and transitions of the tested nitrosamine impurities are shown below. Also, mass spectra of transitions for each impurity are shown next to chromatograms.

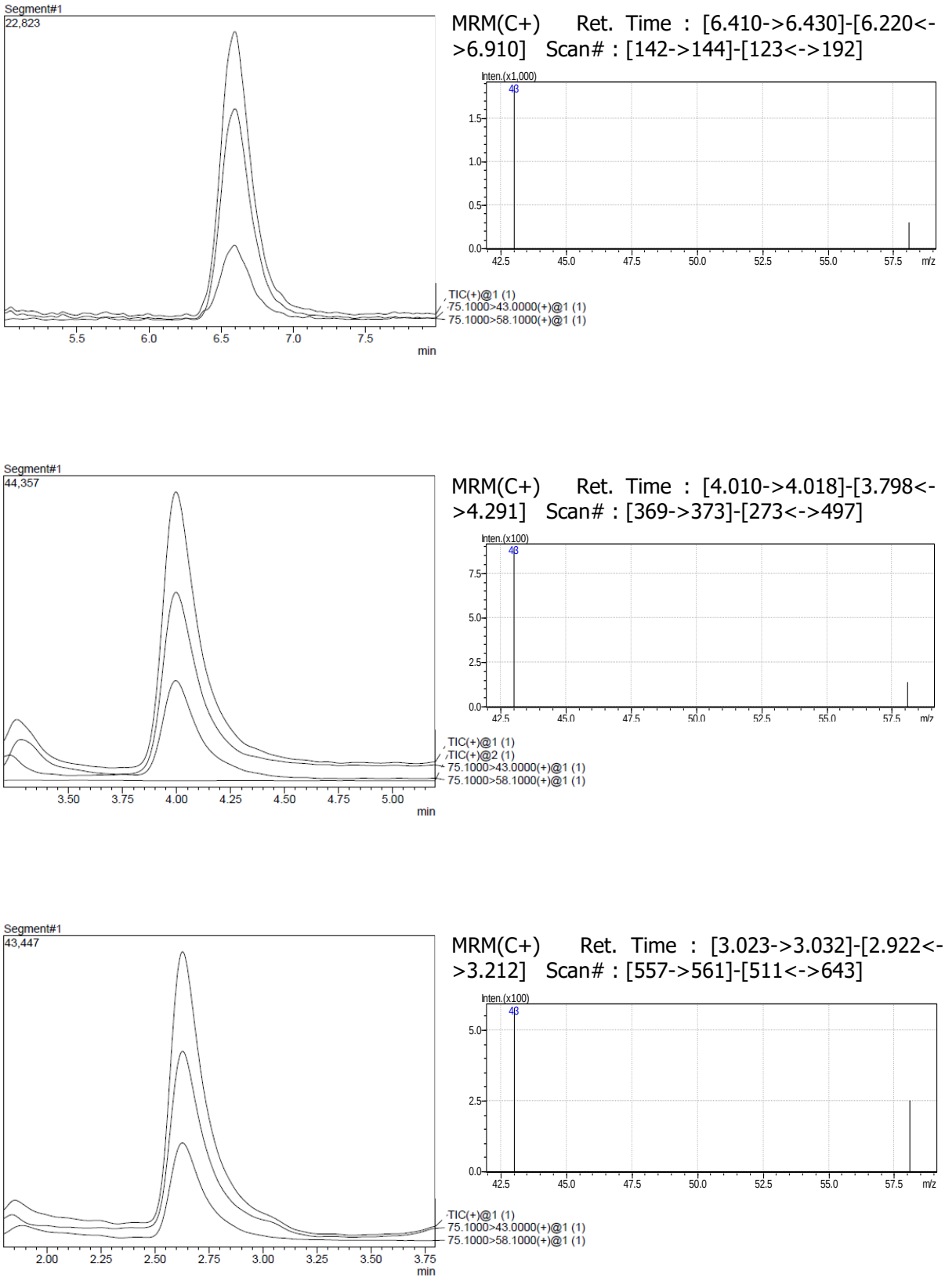


Figure 18. Chromatograms and mass spectra of NDMA using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); c) using *LC-method 2* and *Column 3* (bottom)

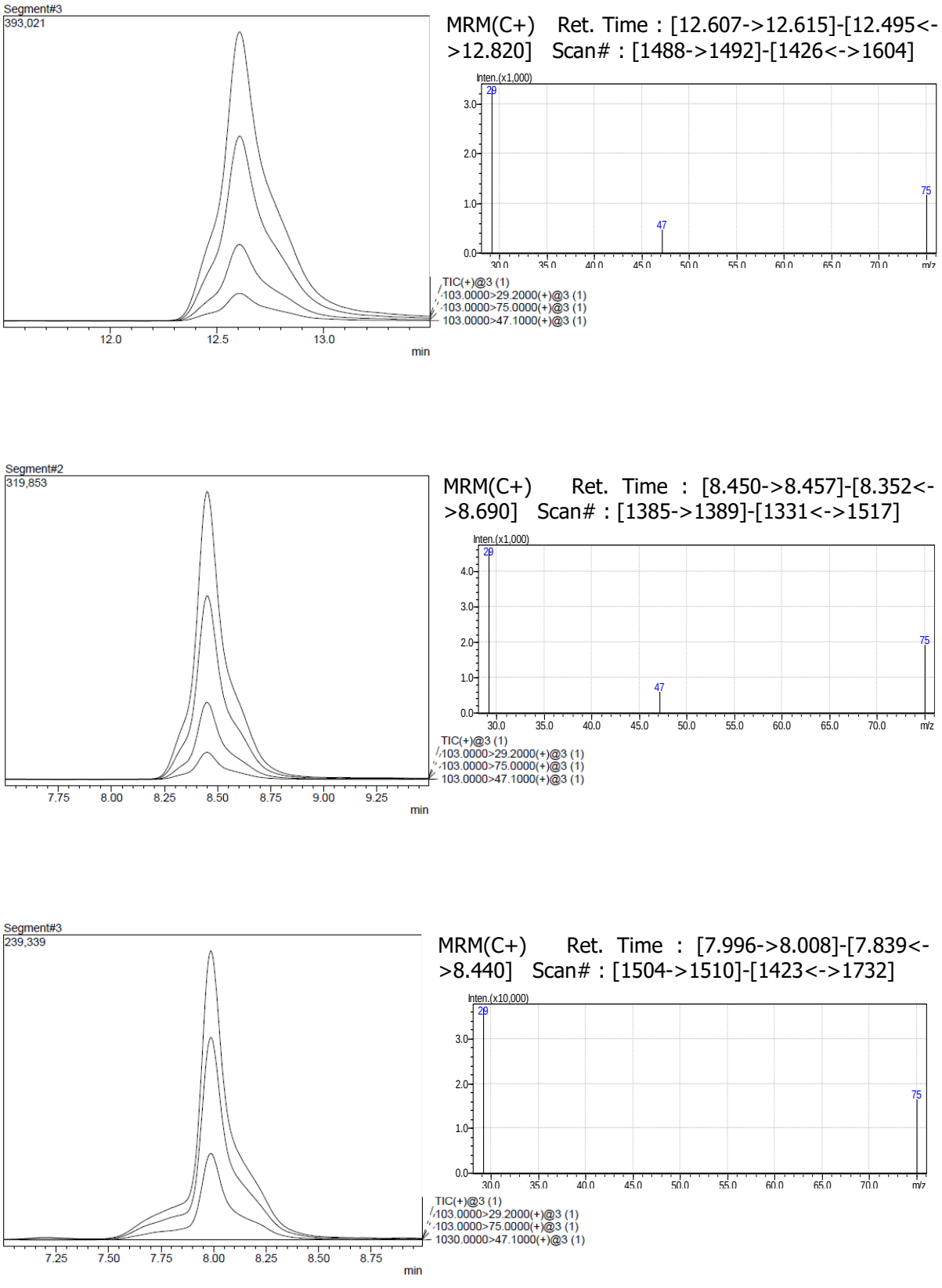


Figure 19. Chromatograms and mass spectra of NDEA using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); c) using *LC-method 2* and *Column 3* (bottom)

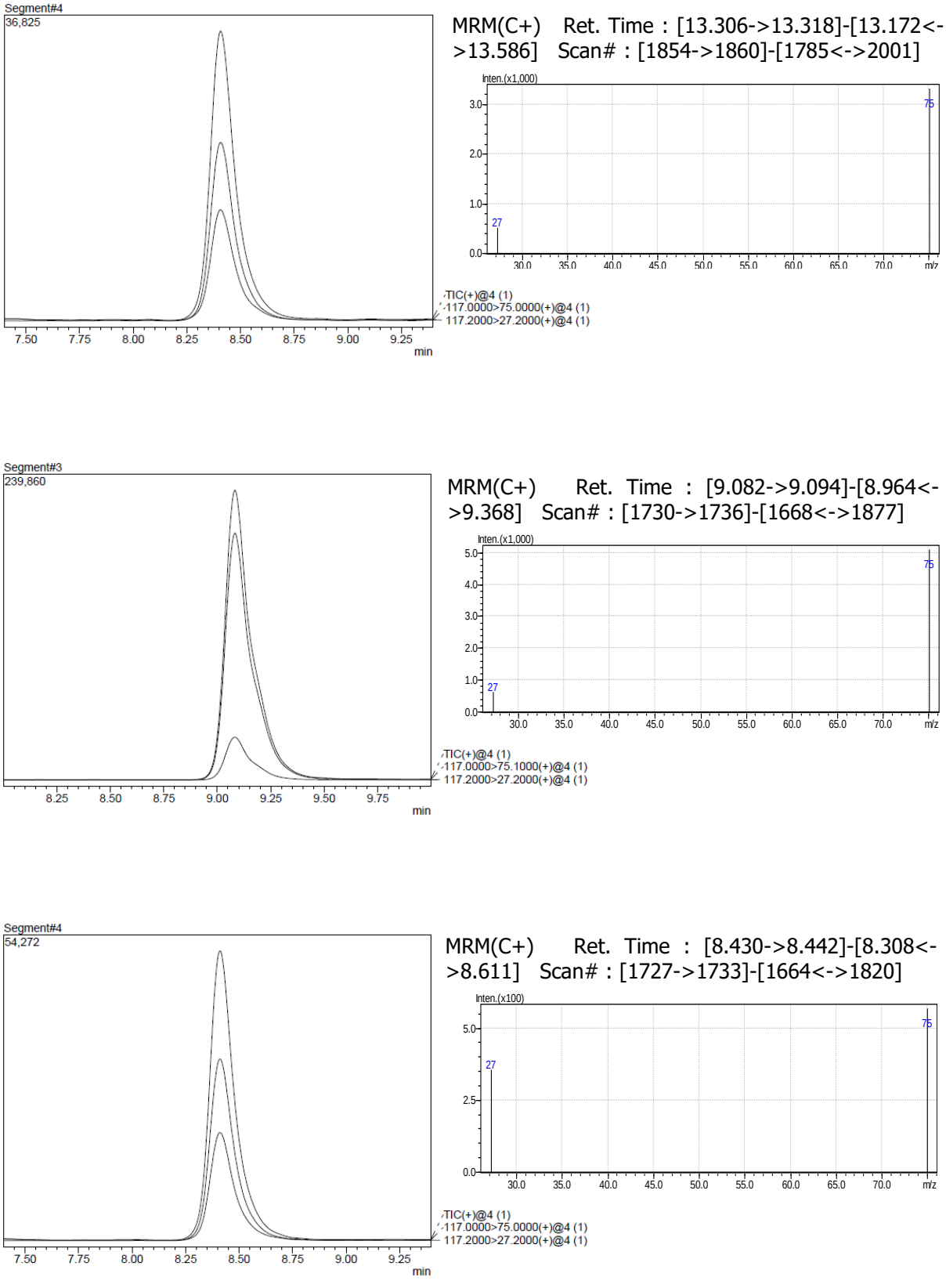


Figure 20. Chromatograms and mass spectra of NEIPA using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); c) using *LC-method 2* and *Column 3* (bottom)

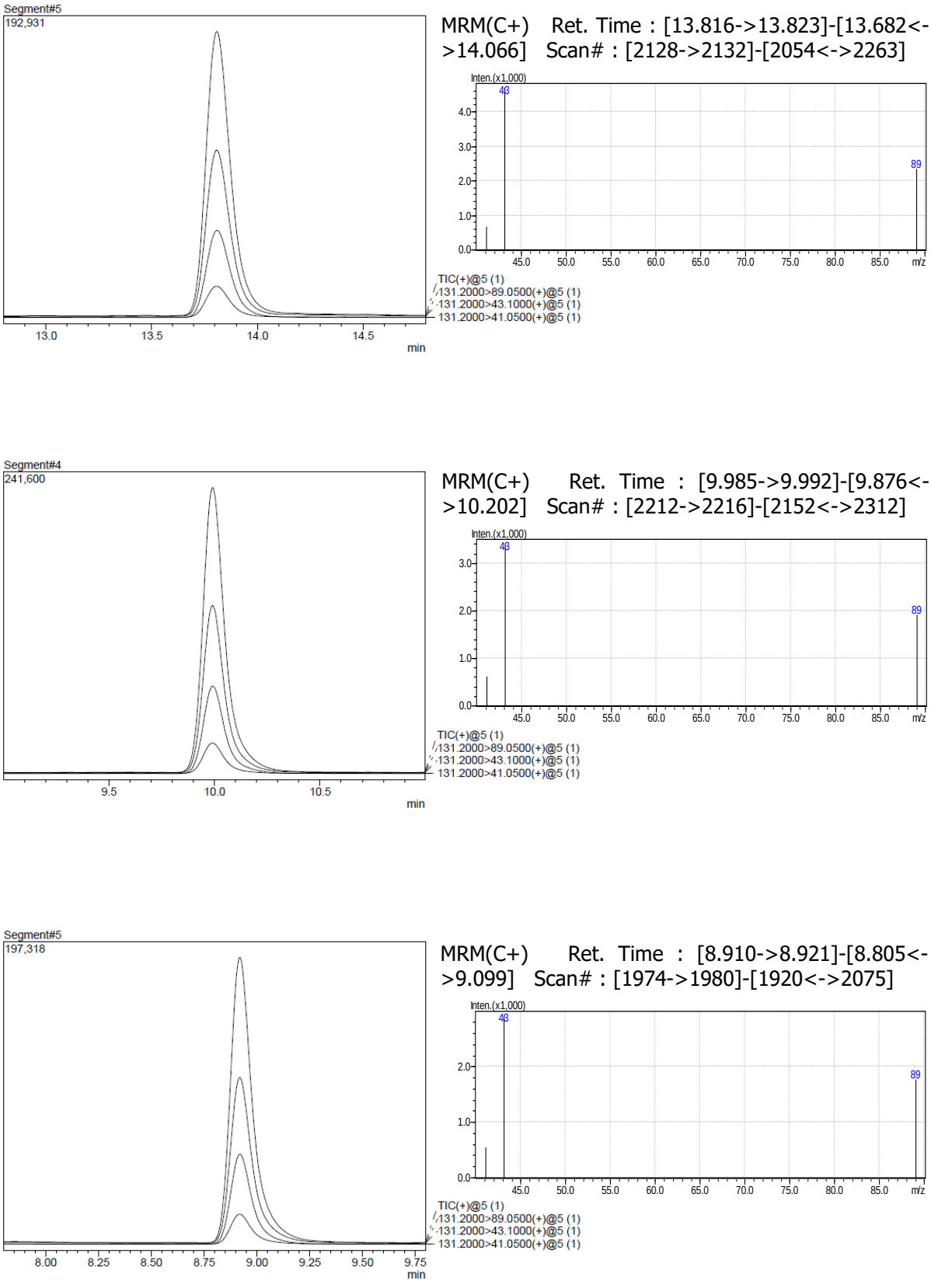
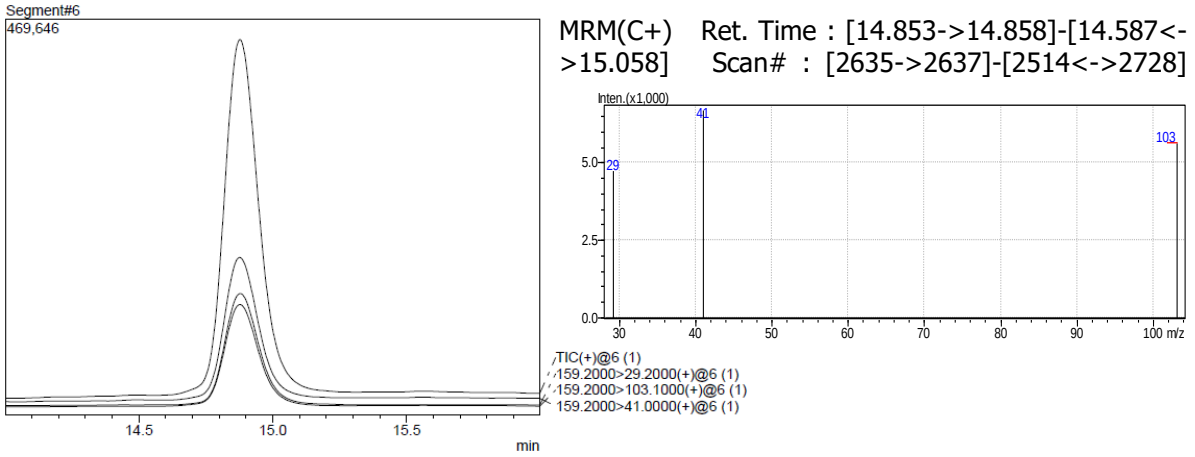
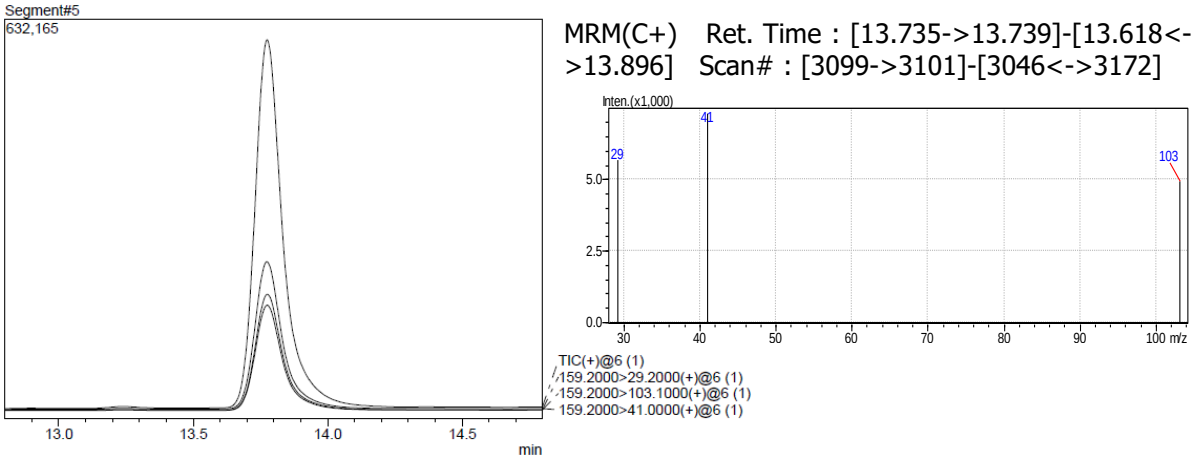


Figure 21. Chromatograms and mass spectra of NDIPA using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); c) using *LC-method 2* and *Column 3* (bottom)





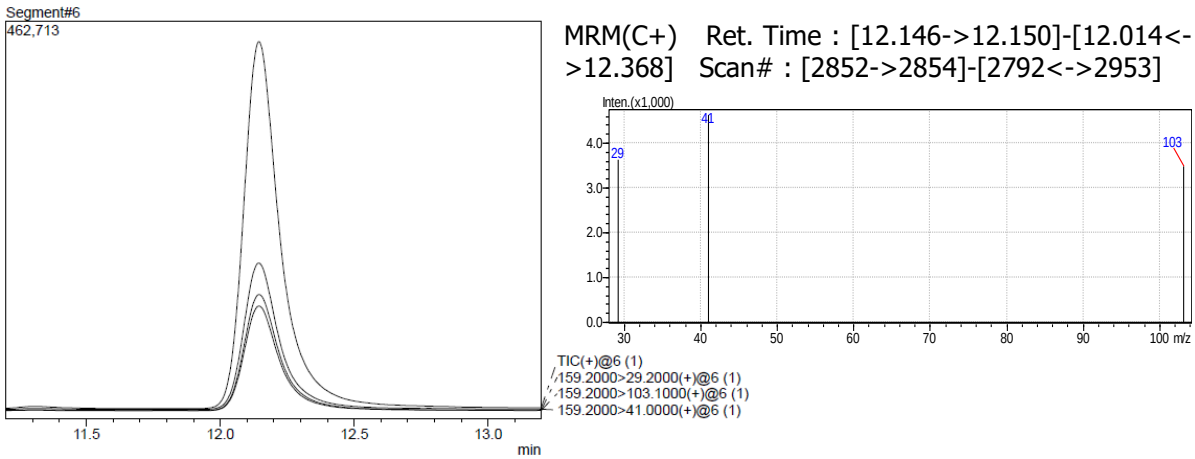


Figure 22. Chromatograms and mass spectra of NDBA using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); c) using *LC-method 2* and *Column 3* (bottom)

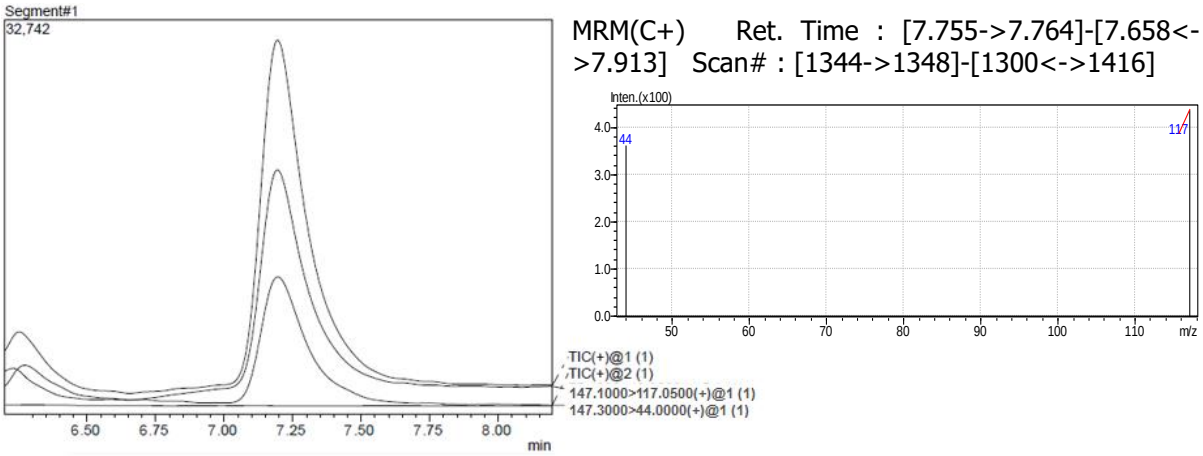


Figure 23. Chromatogram and mass spectra of NMBA; using *LC-method 2* and *Column 2*

Since there were several indications of false positive results generated by the presence of DMF in samples, special attention has been given to this fact. DMF samples were prepared at similar concentrations as nitrosamine standards in order not to overload the instrument.

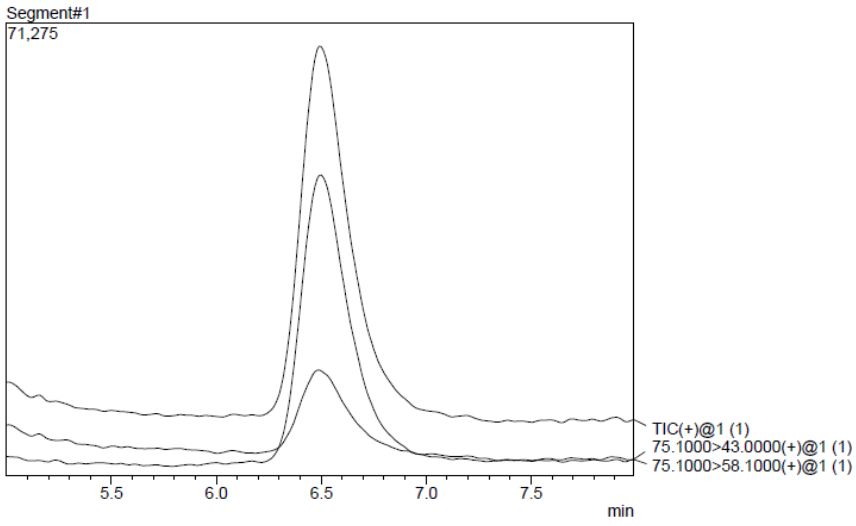


Figure 24. Chromatogram of NDMA using *LC-method 1* and Column *1*

The generated data showed that the use of sufficient mass accuracy in the data acquisition and appropriate mass tolerance setting in the data processing to assure the selectivity of mass spectrometry measurements of NDMA in the presence of co-eluting DMF was necessary to prevent

overestimation of the level of NDMA in metformin drug products. Therefore, the DMF sample was recorded using *LC-method 1* and *Column 1* to test chromatographic separation, which can help avoid false positive results.

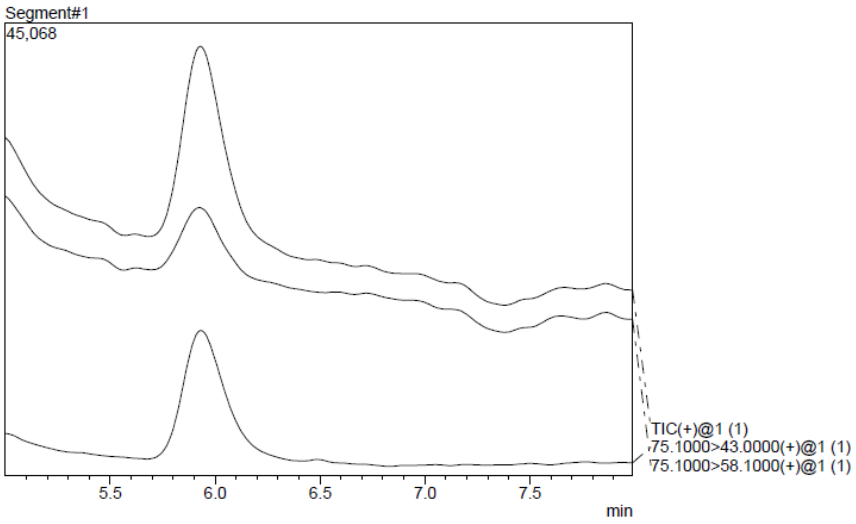


Figure 25. Chromatogram of DMF using *LC-method 1* and *Column 1*

It is visible from Figure 24 and Figure 25 above that NDMA and DMF have different retention times using the same method and column. NDMA has a retention time of around 6.5 minutes, while DMF has a retention time of around 5.9 min, which is a good margin to prevent false positive results.

LC-method 1 with *Column 1* was determined to have the lowest LOD and LOQ (Table 12.) for testing of NDMA. Also, the selectivity of NDMA and all of API's is good according to the selectivity test of this method and column combination; the separation of NDMA and DMF on other methods and columns was not tested.

4.3.3 LOD, LOQ and range

The limit of detection, the limit of quantitation and analysis ranges were determined for each nitrosamine impurity using each of the methods developed and columns used. In the case of *N*-Nitroso-*N*-methyl-4-aminobutyric acid (NMBA), as mentioned before, only one calibration curve was created, and one LOD and LOQ were determined. For all other

nitrosamine impurities, three sets of data were generated. LOD, LOQ and range determined for each nitrosamine impurity are shown in Table 12. for *LC-method 1, Column 1*.

Table 12. LOD and LOQ for nitrosamine impurities using *LC-method 1* and *Column 1*

Impurity	LOD	LOQ	Range	s/n @ LOQ
NDMA	0.40	0.80	0.80-100	10.4
NDEA	0.20	0.40	0.40-100	10.9
NMBA	n/a	n/a	n/a	n/a
NEIPA	0.20	0.40	0.40-100	13.3
NDIPA	0.20	0.40	0.40-100	13.1
NDBA	0.20	0.60	0.60-100	10.7

Table 13. shows LOD, LOQ and range determined for nitrosamine impurities using *LC-method 2* and *Column 2*. Somewhat higher LOD and LOQ values are observed for NDMA and NDIPA, while for others, the values are lower.

Table 13. LOD and LOQ for nitrosamine impurities using *LC-method 2* and *Column 2*

Impurity	LOD	LOQ	Range	s/n @ LOQ
NDMA	0.60	2.00	2.00-100	10.3
NDEA	0.10	0.20	0.20-100	12.6
NMBA	0.10	0.40	0.40-100	14.8
NEIPA	0.10	0.20	0.20-100	12.3
NDIPA	0.10	0.20	0.20-100	12.6
NDBA	0.10	0.60	0.60-100	12.2

LOD, LOQ and range determined for nitrosamine impurities using *LC-method 2* and *Column 3* are shown in Table 14. This method showed lower values than *LC-method 1* only for NDBA.

Table 14. LOD and LOQ for nitrosamine impurities using *LC-method 2* and *Column 3*

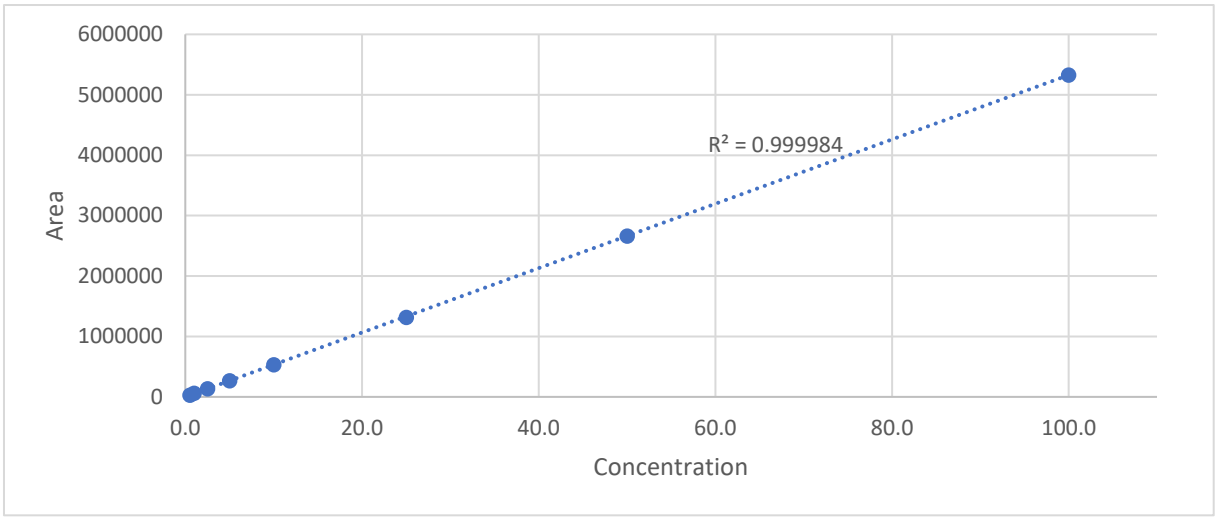
Impurity	LOD	LOQ	Range	s/n @ LOQ
NDMA	0.40	1.20	1.20-100	10.2
NDEA	0.60	2.00	2.00-100	10.9
NMBA	n/a	n/a	n/a	n/a
NEIPA	0.10	0.40	0.20-100	10.3
NDIPA	0.10	0.40	0.40-100	11.1
NDBA	0.10	0.40	0.40-100	12.7

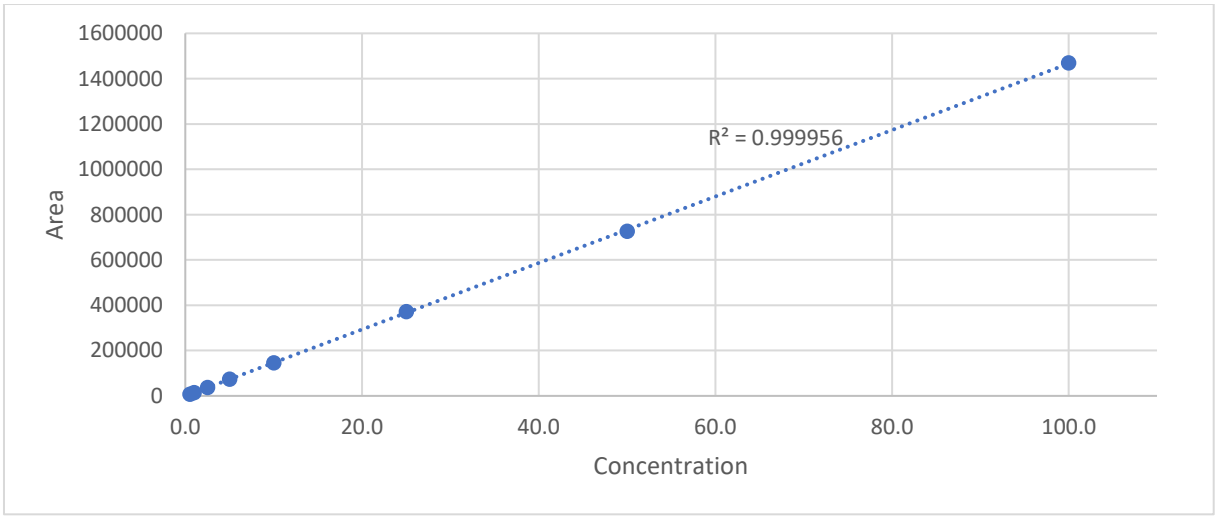
Since the standards for the determination of LOD, LOQ and ranges were prepared in concentrations of 0.2 ng/mL, 0.4 ng/mL, 0.6 ng/mL, 0.8 ng/mL and 1.0 ng/mL, which were not sufficient for some impurities and some conditions, as seen in Table 12., Table 13. and Table 14., larger concentrations had to be prepared in these cases. Also, there were cases in which the lowest concentration for determining LOD was too high, so new samples were prepared. In these cases, as seen in Table 12. through Table 14., S/N for LOD was well above the limit of 3, so the concentration of samples was reduced to more accurately determine LOD.

Methods robustness tests were not performed at this point, as %RSD on retention time and area are relatively low, which generally indicates that methods and analytical procedures are robust.

4.3.4 Linearity

All methods developed gave good linearity for all nitrosamine impurities tested, as shown in calibration diagrams; R^2 values for all nitrosamine impurities are shown in graphs. In the case of NMBA, only one method showed sufficient chromatography and sensitivity, so only one calibration curve was generated for this impurity (Figure 31.). Other nitrosamine impurities have three calibration curves (Figure 26. - Figure 30.).





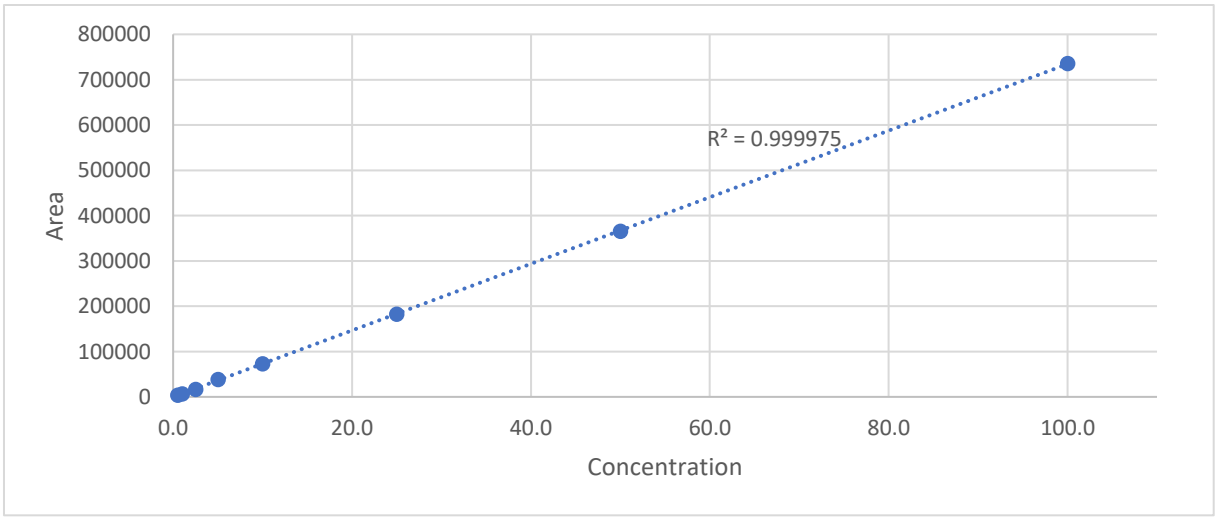
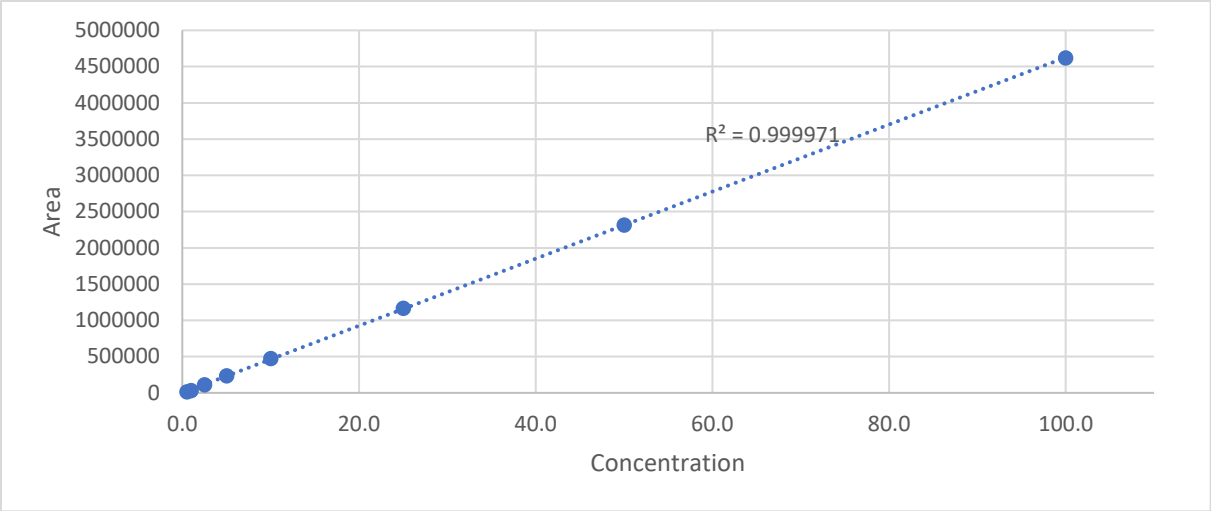
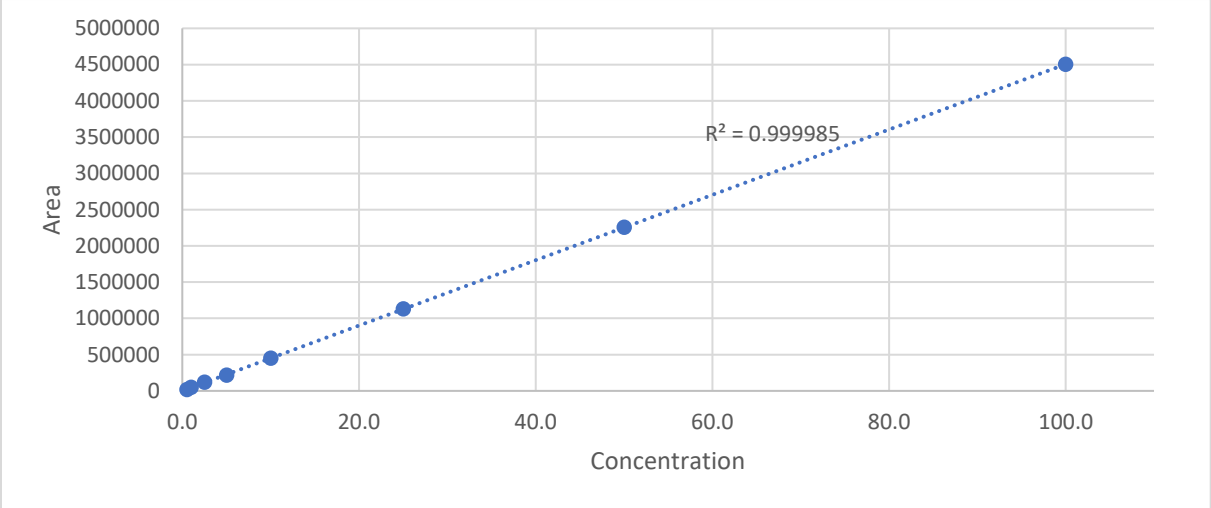


Figure 26. Calibration curves with R^2 values for NDMA standard using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); using *LC-method 2* and *Column 3* (bottom)





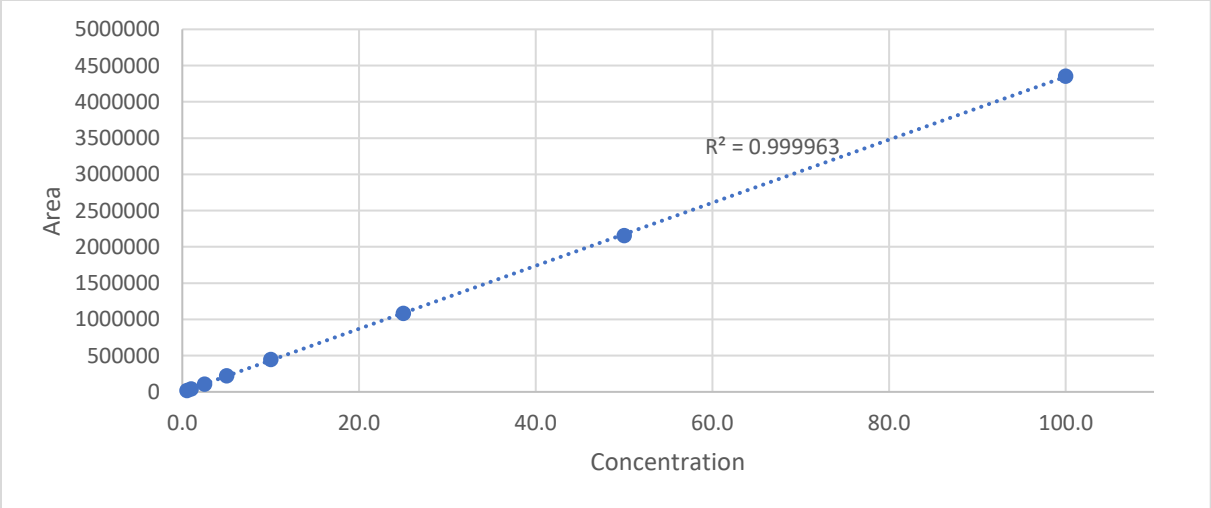
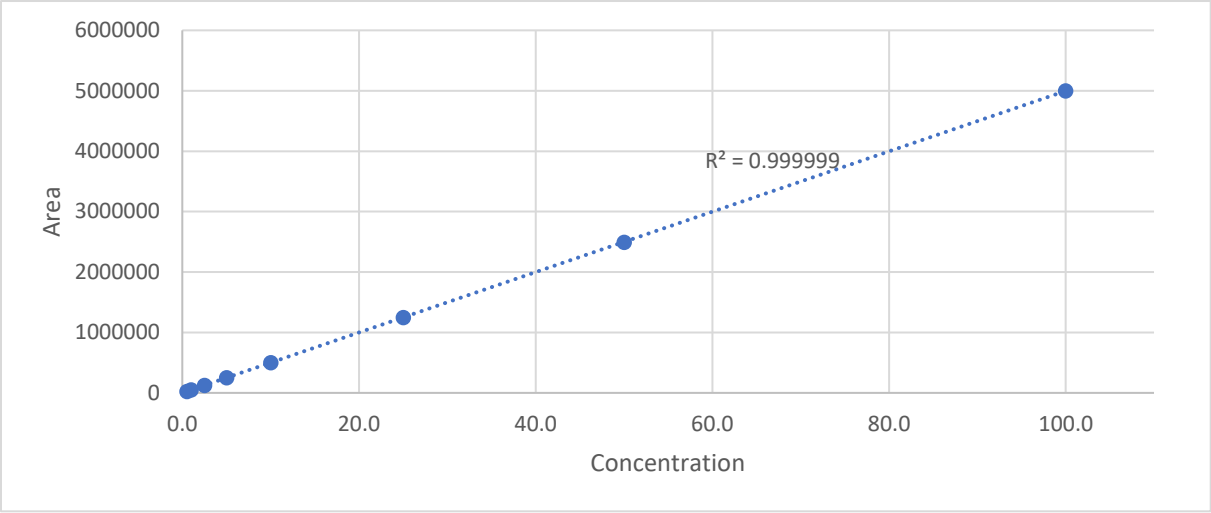
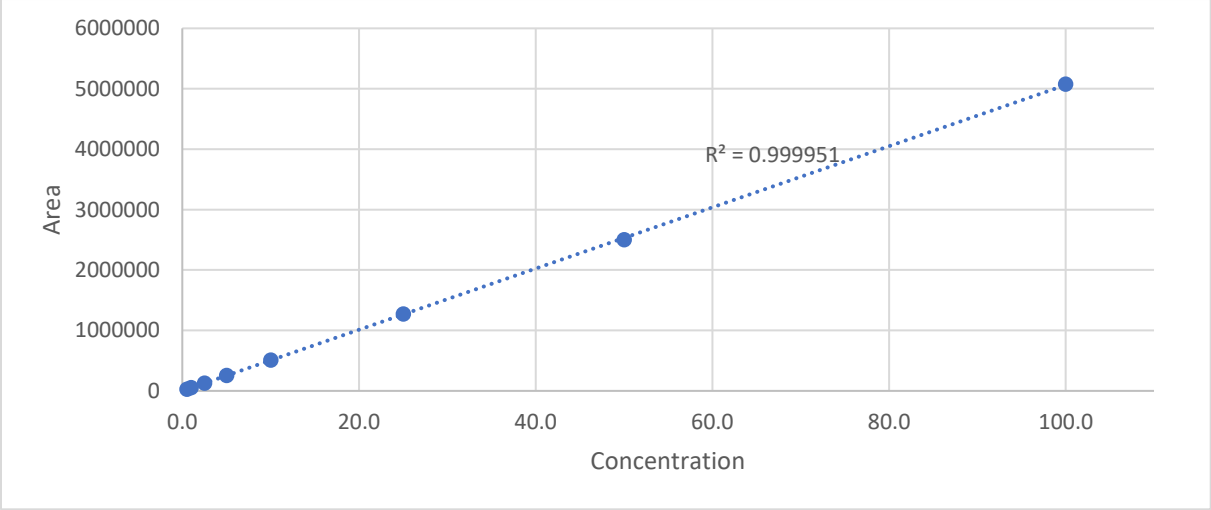


Figure 27. Calibration curves with R^2 values for NDEA standard using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); using *LC-method 2* and *Column 3* (bottom)





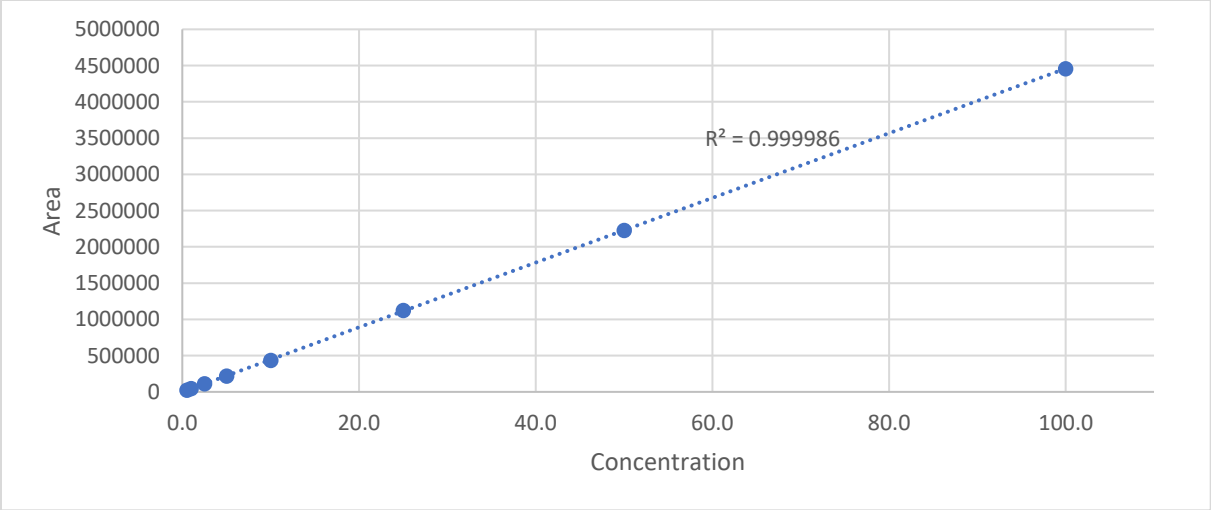
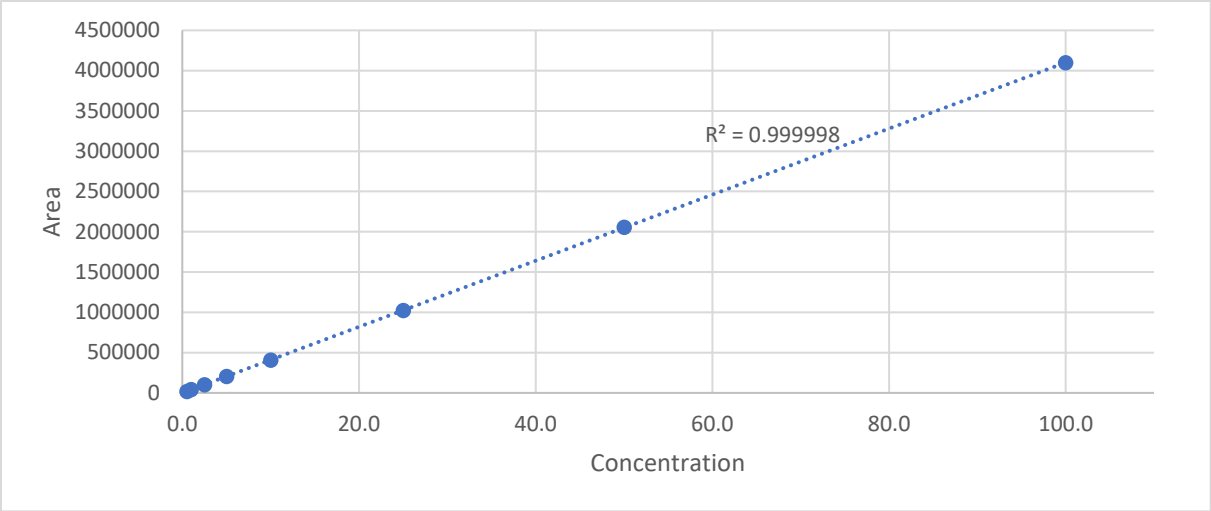
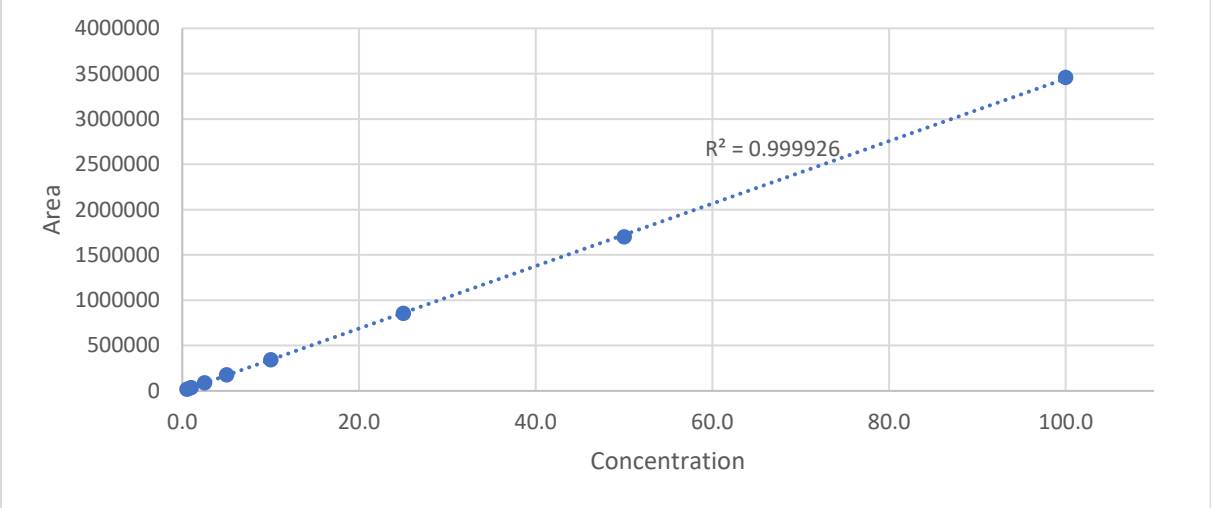


Figure 28. Calibration curves with R^2 values for NEIPA standard using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); using *LC-method 2* and *Column 3* (bottom)





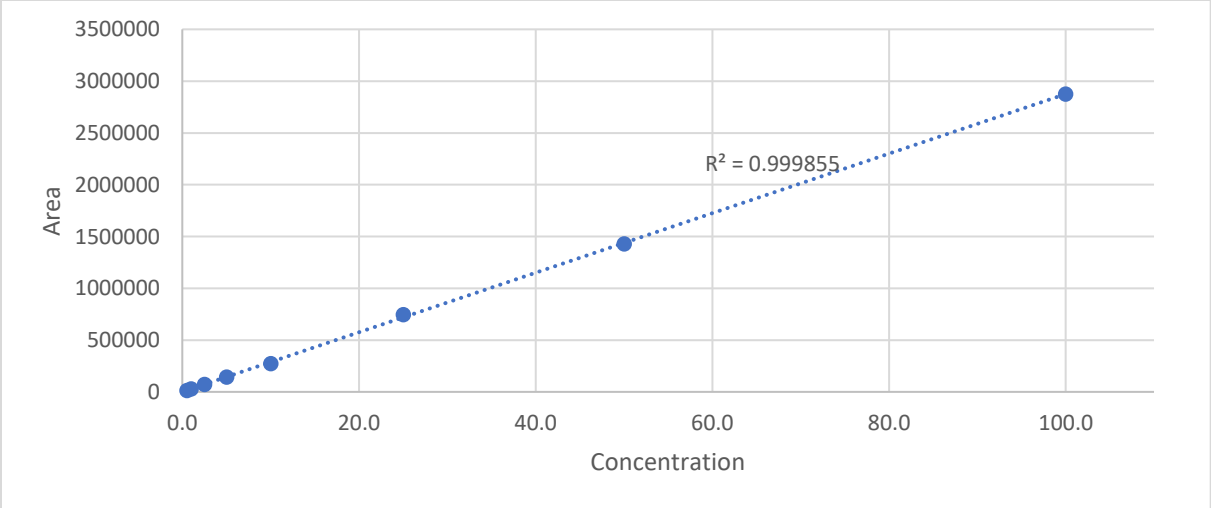
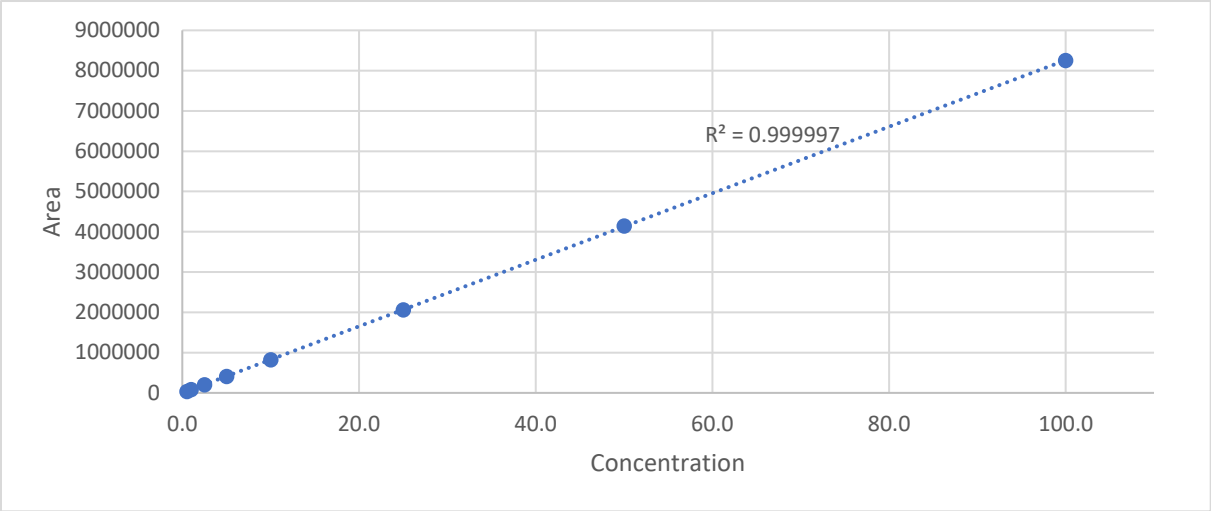
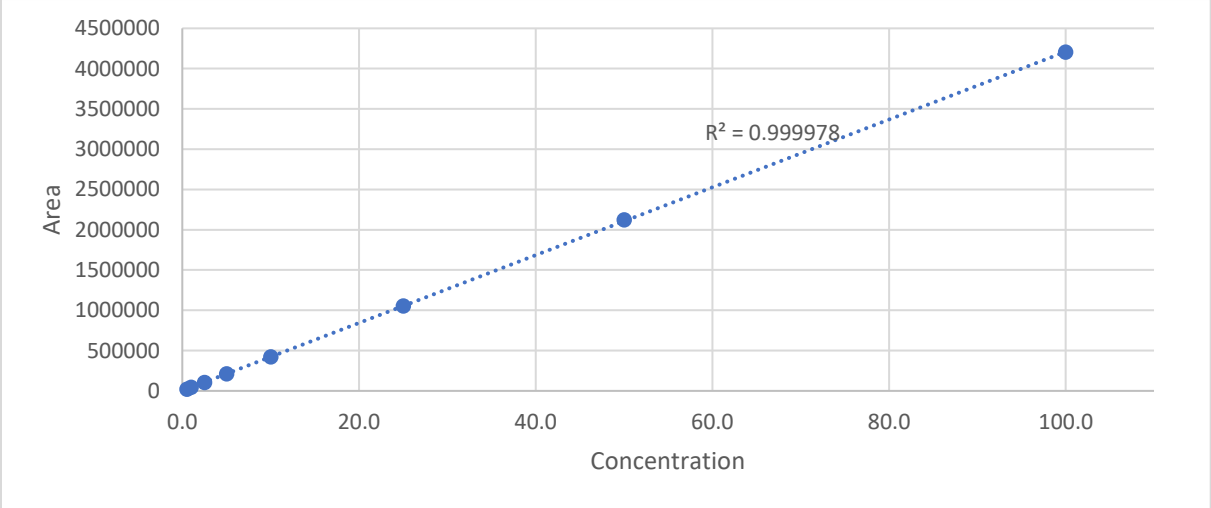


Figure 29. Calibration curves with R^2 values for NDIPA standard using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); using *LC-method 2* and *Column 3* (bottom)





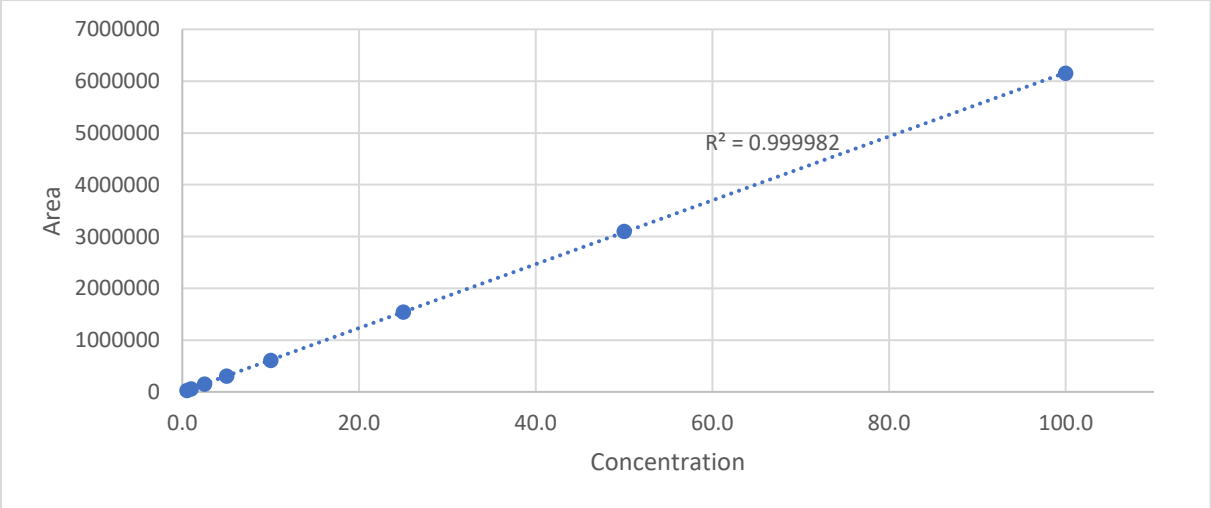


Figure 30. Calibration curves with R^2 values for NDBA standard using *LC-method 1* and *Column 1* (top); using *LC-method 2* and *Column 2* (middle); using *LC-method 2* and *Column 3* (bottom)

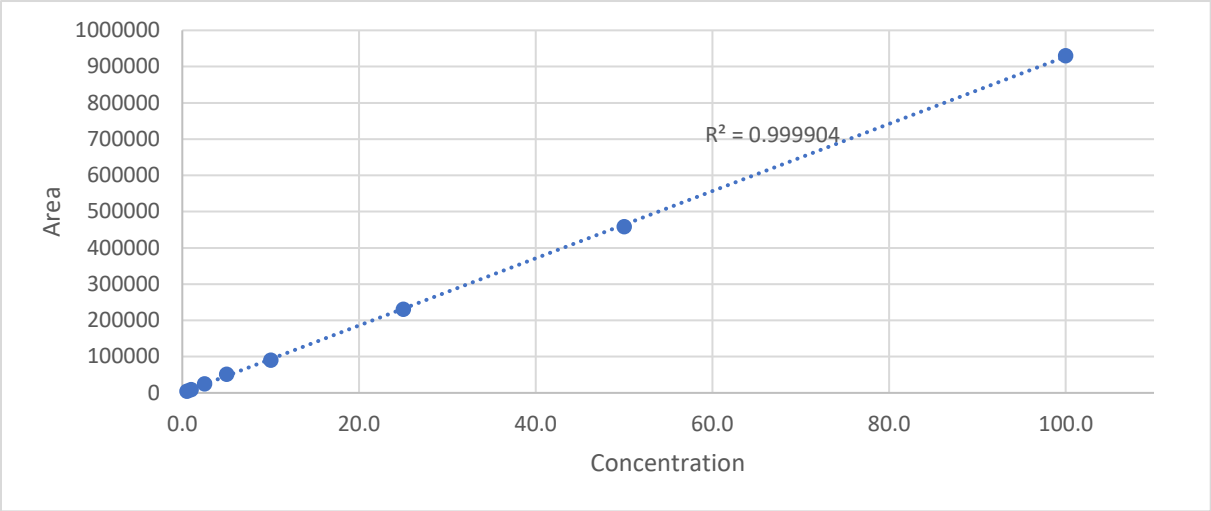


Figure 31. Calibration curve with R^2 value for NMBA standard using *LC-method 2* and *Column 2*

4.3.5 Accuracy – recovery

Recovery was tested for all nitrosamine impurities and all the methods.

Table 15. Results of the recovery test on *N*-nitrosamine standards

Compound	Level	Recovery (%)		
		LC-method 1 Column 1	*LC-method 2 Column 2*	*LC-method 2 Column 3*
NDMA	LOQ	95.90	96.51	98.12
	5 ng/mL	101.24	103.01	98.50
NDEA	LOQ	105.95	101.74	98.62
	5 ng/mL	99.19	97.85	101.15
NMBA	LOQ	n/a	103.27	n/a
	5 ng/mL	n/a	99.50	n/a
NEIPA	LOQ	101.08	105.86	94.84
	5 ng/mL	99.78	98.21	99.49
NDIPA	LOQ	98.04	101.25	99.53
	5 ng/mL	104.01	99.69	98.05
NDBA	LOQ	101.80	97.33	100.61
	5 ng/mL	99.68	102.69	101.43

Two different levels of spike were used, LOQ for each impurity and 5 ng/mL. Recovery results were within the parameters desired and are stated in Table 15. Results showed good method accuracy and showed that method could accurately be used for nitrosamine impurity analysis.

4.4 System suitability results

Previously established system suitability parameters were tested during the validation of the method. These parameters were incorporated into the method to be used when analysing samples using these methods. For all the methods, these parameters were the same. System suitability parameters requirements and results after testing for these methods are shown in Tables 16 through 18.

Table 16. System suitability parameters for *LC-method 1* and *Column 1*

	%RSD area 6 inj.		**%RSD area 6+1 inj.**		**%RSD RT 6 inj.**	
Level	5 ng/mL	10 ng/mL	5 ng/mL	10 ng/mL	5 ng/mL	10 ng/mL
NDMA	0.442	1.195	0.468	1.274	0.029	0.014
NDEA	3.010	0.716	3.105	0.733	0.012	0.012
NMBA	n/a	n/a	n/a	n/a	n/a	n/a
NEIPA	0.539	1.053	0.547	0.992	0.013	0.011
NDIPA	0.610	0.268	0.793	0.325	0.029	0.009
NDBA	0.228	0.113	0.416	0.190	0.034	0.007

Tables are show data for %RSD of peak area on 6 consecutive injections of the standard at two levels, 5 ng/mL and 10 ng/mL. Except for this data, there is %RSD of 6 consecutive injections of standards and one bracketing injection performed after samples were injected. Apart from %RSD on the peak area, %RSD on retention time was determined for standards injected during sample analysis. For all these tests, criteria have been set according to general analytical procedures and guidelines. The primary purpose of these testing is to verify analytical procedures and instrumentation.

Table 17. System suitability parameters for *LC-method 2* and *Column 2*

	%RSD area 6 inj.		%RSD area 6+1 inj.		%RSD RT 6 inj.	
Level	5 ng/mL	10 ng/mL	5 ng/mL	10 ng/mL	5 ng/mL	10 ng/mL
NDMA	0.307	0.340	0.581	0.382	0.024	0.029
NDEA	1.450	0.467	1.513	0.452	0.054	0.017
NMBA	1.029	0.623	1.497	0.850	0.017	0.021
NEIPA	2.993	0.326	2.742	0.331	0.009	0.019
NDIPA	0.773	0.636	0.848	0.895	0.013	0.014
NDBA	0.548	0.190	0.630	0.724	0.010	0.009

This data shows good performance of instrument, method and columns for this application. Column performance parameters were well above requirements for column theoretical plates and tailing factor and were not in focus at this point.

Table 18. System suitability parameters for *LC-method 2* and *Column 3*

	%RSD area 6 inj.		%RSD area 6+1 inj.		%RSD RT 6 inj.	
Level	5 ng/mL	10 ng/mL	5 ng/mL	10 ng/mL	5 ng/mL	10 ng/mL
NDMA	1.197	0.432	1.222	0.460	0.036	0.028
NDEA	0.741	0.418	0.891	0.470	0.014	0.012
NMBA	n/a	n/a	n/a	n/a	n/a	n/a
NEIPA	0.803	0.523	0.783	0.538	0.015	0.018
NDIPA	1.109	0.680	1.513	0.712	0.009	0.011
NDBA	0.403	0.341	0.459	0.381	0.010	0.009

4.5 Quantity of nitrosamine impurities in drug products

Several commercially available FDF's were tested to explore how these methods are performing in realistic conditions. Among all analysed nitrosamines, only NDMA was found in tested drug products. The NDMA was found in combined drug formulations of antidiabetics Metformin/Vildagliptin

and Metformin/Sitagliptin. However, the amount calculated from the analysis data was within FDA and other regulatory agencies guidelines for this impurity. MDD for Metformin drug product is 2000 mg/day for extended-release formulation. The amount of impurity is calculated accordingly.

Table 19. Amount of NDMA in drug products using *LC-method 1* and *Column 1*

API	NDMA (ppm)	NDMA in MDD (ng)
Azithromycin	Not detected	Not detected
Betahistine dichloride	Not detected	Not detected
Metformin/sitagliptin	0.0164	32.87
Metformin/vildagliptin	0.0046	9.22
Metronidazole	Not detected	Not detected
Simvastatin	Not detected	Not detected

Other nitrosamine impurities were not detected in any of the drug products tested, and if present, they are below LOD.

4.5.1 Drug sample analysis

Integrated chromatograms of detected NDMA in two tested metformin drug products are shown in Figure 32 and Figure 33. Two different formulations of Metformin were tested, one with Sitagliptin and one with Vildagliptin.

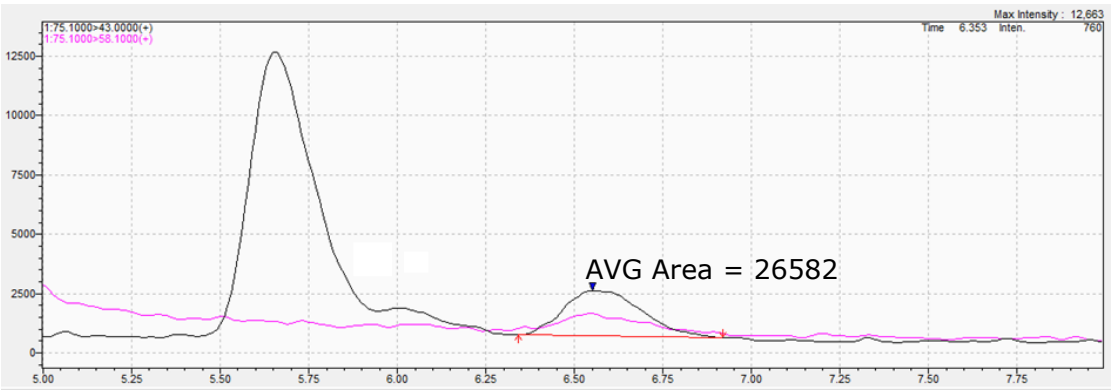


Figure 32. Detected NDMA in Metformin/Sitagliptin drug product

Samples were prepared in triplicates as per procedure in the concentration of 30 mg/mL. Average areas of three consecutive injections of each sample preparation are noted on each chromatogram and used in calculations.

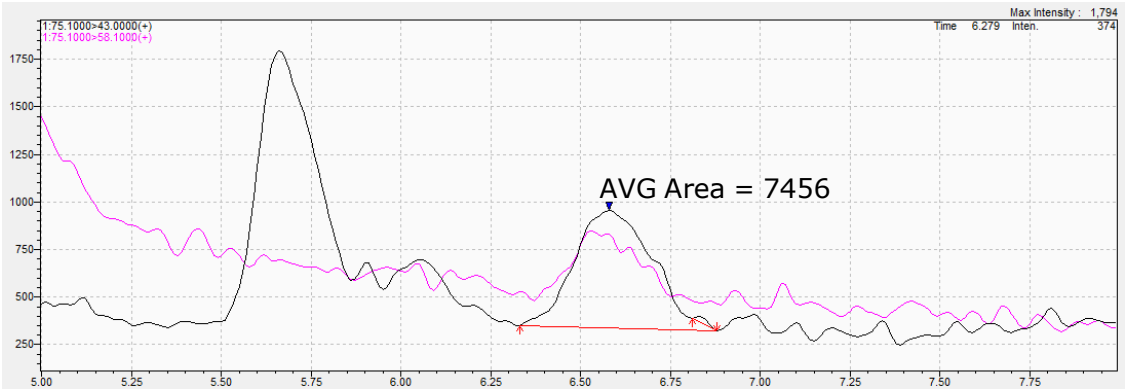


Figure 33. Detected NDMA in Metformin/Vildagliptin drug product

On both chromatograms peak at approximately 6.00 min is present. This peak could be a small amount of DMF present in drug products, visible using the same transitions as for NDMA. This fact can explain false positive results for NDMA in the past. Since this method is developed to achieve chromatographic separation between NDMA and DMF, this peak is not an issue. These results are significant in the analysis of not just drugs selected in this work but samples from different sources (medications, food and environmental matrices) where DMF might be present. Clearly, in this method, only NDMA concentration is determined. To confirm this peak as DMF, samples of DMF should be injected and recorded.

5. Discussion

Since the discovery of nitrosamine impurities in various drug products on the market in 2018, these compounds have been the focus of the pharmaceutical industry, scientific institutions, testing laboratories and regulatory agencies. Technology innovators and manufacturers monitor the situation and cooperate to find new and better solutions for analysing these impurities. All of them are working together in order to control these potentially dangerous substances.

The process of method development for identification and quantification of nitrosamine impurities (NDMA, NDEA, NMBA, NEIPA, NDIPA and NDBA) was divided into essential steps, with the major parts being the development of the method for chromatographic separation and settings of optimal ionisation to achieve the lowest possible level of detection in the mass spectrometer.

5.1 LC method development

Researchers from various institutions and industries have published numerous methods for nitrosamine impurity monitoring. These methods, like those published by Malihi and Wang[37] are good starting points for further development and optimisations.

An essential benchmark of developing a chromatographic separation method was the possibility of separating the API from nitrosamine impurities. Achieving this goal significantly increases the method's selectivity. It also reduces the possibility of reduced MS detector sensitivity due to contamination with high concentrations of non-target components, such as active pharmaceutical ingredients if the chromatographic separation is insufficient to prevent API's entrance into the mass spectrometer. Since the nitrosamine impurities are relatively polar compounds and their retention on the column poses a significant challenge, especially for NDMA,

careful selection of mobile phase and the column had been performed to achieve this. Another valuable but often overlooked parameter of the chromatographic separation method is the duration of the analysis. The shortest analysis time possible is preferential to transfer method to the routine quality control to analyse as many samples as possible within a limited time.

5.1.1 Column selection

Chromatographic columns were selected using mainly literature research through publications describing the method development for nitrosamine impurity analysis in various pharmaceutical substances.[38] Also, in the review article by Shaik *et al.*, other polar compound retention columns like Phenomenex Synergi Polar and Phenomenex Kinetex F5 are mentioned as columns of choice.[39] Several literature examples used the Waters HSS T3 C18 column as the column of choice.[37,39,40] This column is specially designed to retain very polar compounds and could be used as the next step in optimising these methods.

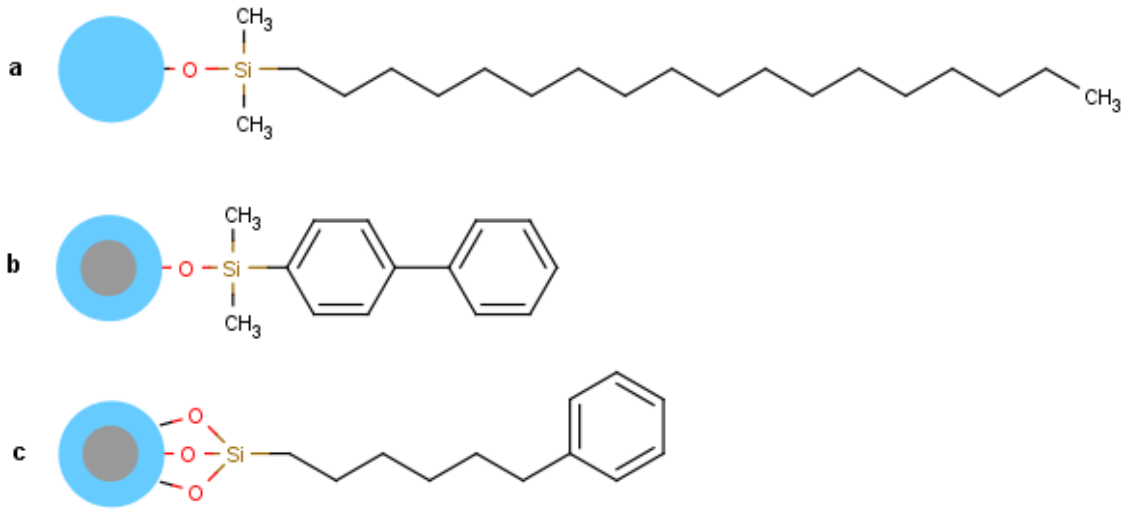


Figure 34. Column chemistries used in this study; (**a**) Shimadzu Shim-pack GIST C18, (**b**) Phenomenex Kinetex BiPhenyl and (**c**) Waters Cortecs Phenyl

Also, the higher activity of silanol groups of the non-end-capped 100% silica HSS particles could provide advantages and much higher selectivity regarding NDMA retention compared to the end-capped columns.[37]

When choosing columns, there are several parameters that need to be taken into account, and one of these parameters is particle size. Since the modern LC techniques use columns of very small particle diameter, 1.7 µm, compared to the 5 µm being used 20 years ago, this can result in better separation explained by the Van Deemter plot in Figure 35.[41]

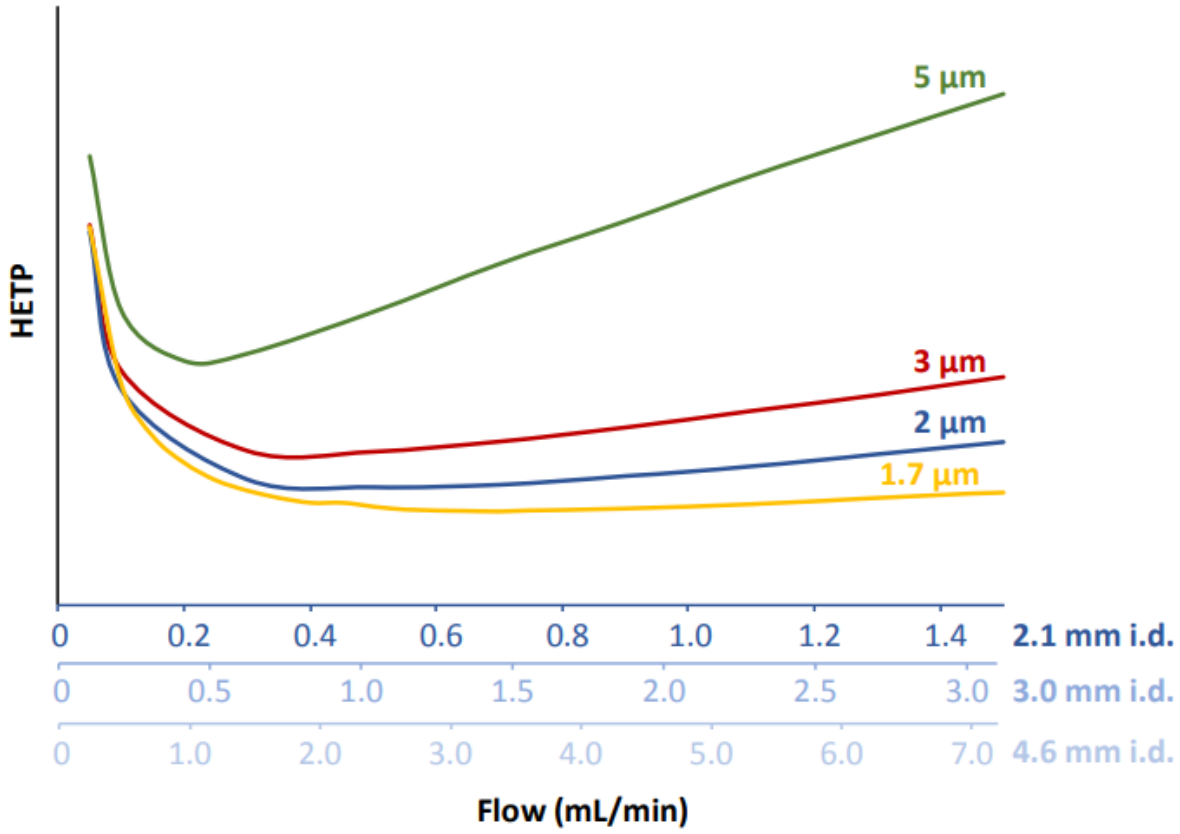


Figure 35. Van Deemter plot; minimum of each cure shows the highest efficiency and optimal flowrate for that particle size

On the ordinate of the Van Deemter plot, there is the height of one theoretical plate, and lower the single plate is, on the length of the column, we can get a higher plate count, hence higher efficiency of the column. Abscissa shows the optimal flow rate for columns given their internal

diameter (i.d.) and particle size. It is noticeable that columns with smaller particles have a more linear curve in the curve minimum, achieving better performance in a broader range. The result of reducing particle size is higher backpressure generated by the column. It has been noticed that backpressure can be reduced when using solid core particles, which enables higher flow rates, lower analysis time, smaller particles but equal backpressure to that of larger particles and, as a most important benefit, better column efficiency. Two of the three columns used were solid core columns, as shown in Figure 34. Solid core particles, shown in Figure 36, are known from the 1970's, but the advances in technology which enabled to create much smaller particles are still used nowadays in modern LC systems.[41,42]

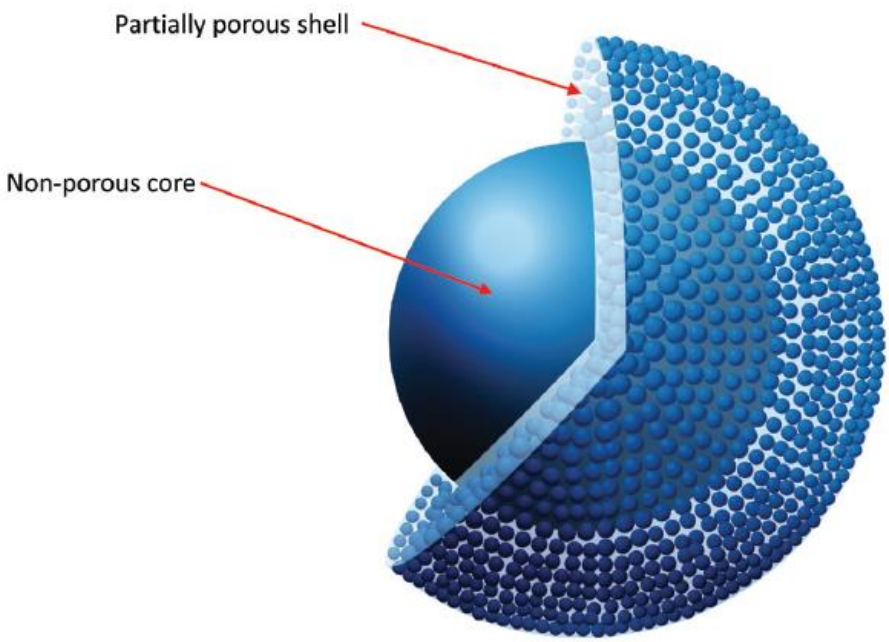


Figure 36. Solid core particle

Three columns with somewhat different selectivity were chosen for this study to evaluate their performance and to cover as much of the selectivity field as possible, given the characteristics of the compound analysed. These columns are Shimadzu Shim-pack GIST C18, Phenomenex Kinetex BiPhenyl and Waters Cortecs Phenyl. During method development, an unexpected

situation occurred with NMBA, *N*-Nitroso-*N*-methyl-4-aminobutyric acid, which, when analysed using *Column 1* and *Column 3*, did not achieve suitable chromatography. Since there was insufficient chromatography using these columns, only *Column 2* was used to analyse this compound. Since NMBA has a carboxylic functional group and nitroso one, one electron pair of each oxygen atom is sp^2 hybridised and can create conjugate with the carbonyl group's π orbital system, needs a different mechanism of retention than the aliphatic sp^3 hybridised compounds.[43] Phenomenex Kinetex BiPhenyl column is designed to retain polar compounds using π-π interactions of these oxygens with bi phenyl column chemistry, enabling good retention of compounds containing these moieties, especially NMBA. On the other hand, the Waters Cortecs Phenyl column has only one phenyl ring per active spot and probably cannot achieve enough interactions to retain these polar compounds efficiently.

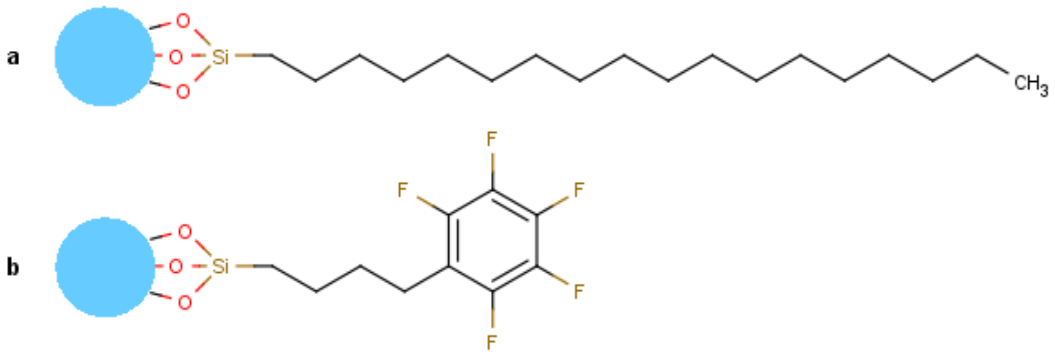


Figure 37. Column chemistries proposed for future method optimisation, (**a**) Waters HSS T3 C18 and (**b**) Waters HSS PFP

The π-π interactions of nitroso functional groups with the column stationary phase are great instruments to retain the smaller, more polar *N*-nitrosamine impurities.[31] Suggestions for the future method optimisation could be using Waters HSS T3 C18 column and Waters HSS PFP, pentafluoro phenyl column. Waters HSS T3 C18 is an end-capped, tri-bonded (T3) column designed especially for polar analytes retention. It has lower ligand density,

which makes it a better choice for retaining polar compounds.[44,45] This column does not benefit π-π interactions with the nitroso functional group. However, it enables a high percentage of aqueous phase that will retain polar compounds. On the other hand, Waters HSS PFP is a non-end-capped silica-based column for low pH separations. As already mentioned, the non-end-capped silica enables higher silanol activity. In contrast, fluorophenyl groups could enable π-π interactions with nitroso functionality and excellent selectivity and retention of these compounds. The structure of these two columns is in Figure 37.

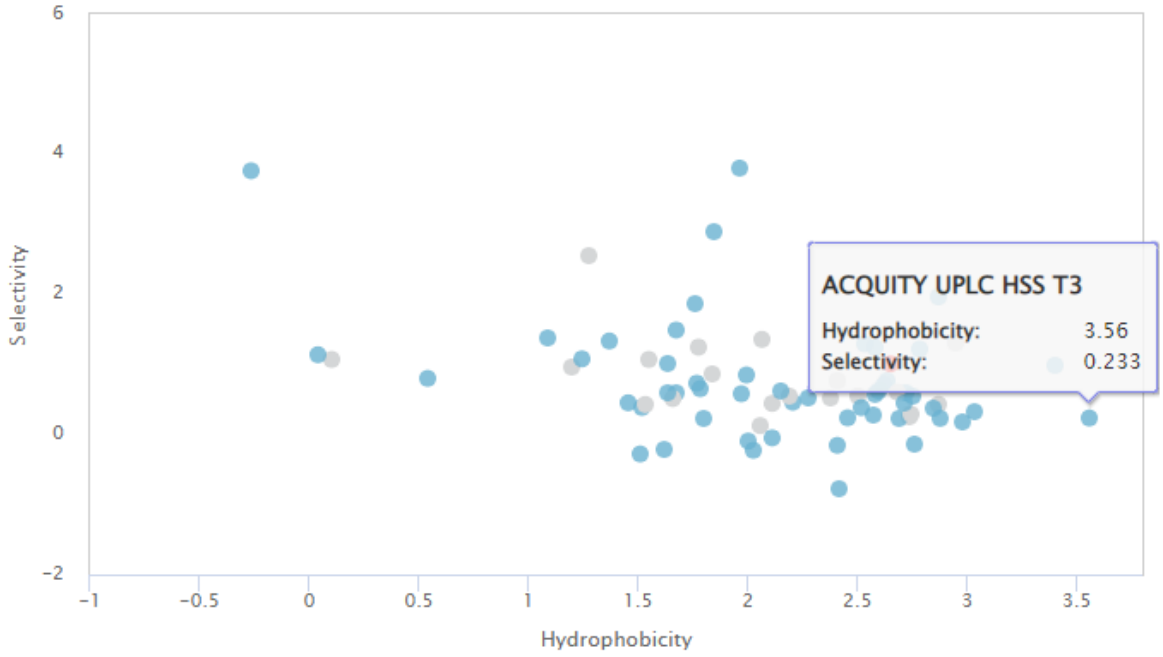


Figure 38. Waters column selectivity chart[46]

An excellent tool for comparing column selectivity is the Waters Column Selectivity chart.[46] This chart, as shown in Figure 38, shows different columns and the correlation between the column hydrophobicity and selectivity. These parameters are calculated for each column based on the experimental results obtained by measuring the retention of the standard compounds set. This helps to decide which column to choose for the analyte that must be analysed.

5.1.2 Mobile phase considerations

In order to select the initial mobile phase for the development of this method, a few combinations of organic solvents were tested, and methanol was chosen over acetonitrile due to a somewhat higher polarity and better retention of NDMA, which is the smallest and most challenging to retain of all analytes. Since methanol has a higher viscosity than acetonitrile, a slightly higher column temperature of 40° C was considered. *Column 2* and *Column 3* are solid core particle columns,[47] which generally generate lower backpressure, and for these columns, a temperature of 30° C was used. For a fully porous column, *Column 1*, 40° C setting was used. Yang *et al.*, in their study, showed the impact of different organic solvents, specifically methanol and acetonitrile, on the chromatographic separation of compounds using a phenyl chemistry chromatographic column. The authors noticed that acetonitrile, in high concentration, has a significant impact on the retention of compounds which could achieve $\pi-\pi$ interactions with the stationary phase. When methanol is used, $\pi-\pi$ interactions and, subsequently, good retention was once again achieved. An explanation of acetonitrile blocking $\pi-\pi$ interactions can be found in the interaction of acetonitrile nitrile functionality with the phenyl stationary phase, which competes with the analyte. When the ratio of acetonitrile increases enough to occupy all active sites of the stationary phase, loss of retention has been noticed. At this point, when $\pi-\pi$ interactions between the analyte and stationary phase are blocked, the only retention mechanism remaining are hydrophobic interactions, and the column is acting as a C6 column; not even the C18 performance can be achieved. To enable additional retention mechanisms when using phenyl or bi-phenyl columns, like in our case, methanol is recommended as an organic solvent of choice since it is likely to give the maximum selectivity difference when compared to that of the C18 column.

Formic acid was the only modifier tested in this study, given the limited time frame and the fact that it was the most widely used modifier.

Furthermore, since most nitrosamine impurities described in this work ionise in positive mode, protonating, an acidic additive is a logical choice to promote and enhance this kind of ionisation. Nevertheless, it was noticed that various authors tested different modifiers to enhance either column retentivity or signal strength in MS.

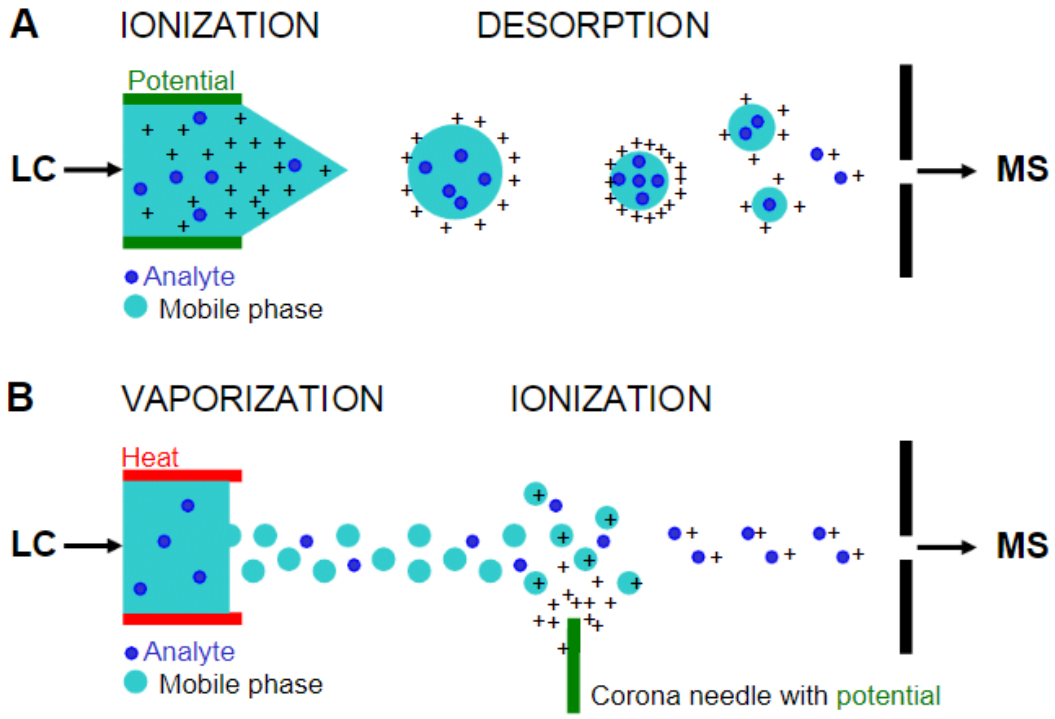


Figure 39. Comparison of ESI (A) and APCI (B) ions formation

In the event of future method optimisation, different additives could be tested, which could increase retention time. Veigure *et al.* used several fluorinated additives for LC-MS analysis. These additives include 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFTB), 2,2,2-trifluoroethanol (TFE), perfluoropinacol (PP) and nonafluoro-*tert*-butyl alcohol (NTFB).[48] Authors state increased retention factor using these additives for some of the tested compounds with pKa values over 9.5 and using C18 columns. Since nitrosamine compounds of interest have significantly lower pKa values of around 3.5, as shown in the *Results* section, this is not useful for these compounds. However, this could be further explored using other column chemistries,

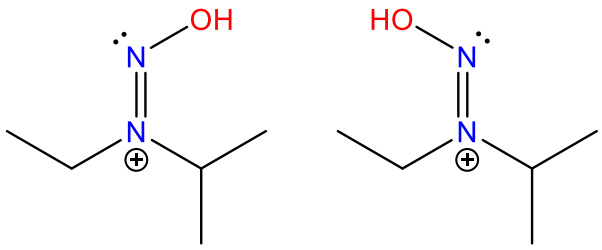
especially superficially charged ones. The authors further noticed that sensitivity in MS, on the other hand, changed. By adding additives and after saturation of the stationary phase, there has been a noticeable drop in sensitivity. This drop in sensitivity is contributed to the constant elution of additives which then reduces ionisation efficiency in the ESI source by competing for charge with the analyte in droplets. Since the APCI source vaporises solvent first and then charges gaseous molecules, this effect could be less noticeable. Figure 39 shows different ion formation in ESI and APCI. Also, in the case of future method optimisations, Malihi and Wang reported that the TFA-associated ion pairing effect and significant signal suppression of the analyte were not observed.[37] Instead, higher temperatures in the APCI source appeared to overcome the TFA ion pairing effect and improved the signal-to-noise ratio.

5.1.3 Cis-trans isomerisation (E, Z)

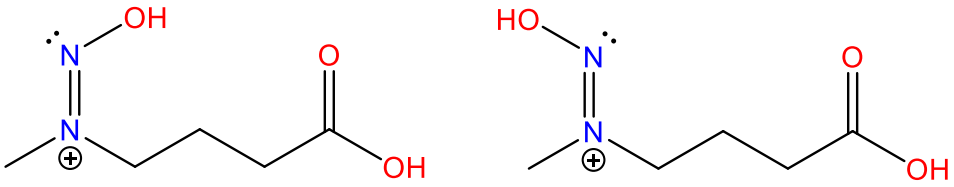
It has been reported in various publications that *N*-nitrosamine impurities with different substituents on amines (such as NMBA, NEIPA and NMPA) are subjected to *cis-trans* isomerisation.[37] The *cis-trans* isomerisation of nitrosamines is known, and rotation barrier energies were previously studied for different cyclic and aliphatic substituents by NMR.[49,50,51]

However, although throughout the literature these isomers are referred to as *cis* and *trans* isomers, it is not accurate nomenclature according to Cahn-Ingold-Prelog (CIP) system. In order to isomers to be *cis* and *trans* relative to the double bond, at least two of the identical substituents should be bonded on carbon atoms that are connected with the double bond. When the identical substituents are on the same side of the double bond axis, this isomer is *cis*, and if the substituents are on the opposite side of a bond axis, the isomer is *trans*. Since in the case of nitrosamines after protonation, the nitrogen of the nitroso group has only one substituent and free electron pair, this pair has the lowest priority possible, as shown in Figure 40. Therefore, it is more accurate to use Cahn-Ingold-Prelog system to

determine the names of these rotamers relative to the double bond. Using the CIP system, when naming compounds relative to a double bond, substituents on each side of double bonds are prioritised using a set of rules when the priority substituents on each side of a double bond are determined, depending on the position of the substituent of the same priority, *E* or *Z* prefix is added, *Z* from German *zusammen*, meaning together or *E* from German *entgegen*, meaning opposite.



E and *Z* isomers of NEIPA



E and *Z* isomers of NMBA

Figure 40. *E* and *Z* isomers of NEIPA and NMBA

Based on the data collected from previous studies, even though the barrier energy to rotation around the N-N bond in *N*-nitrosamines are not high, elevated temperatures are required to induce the N-N bond rotation and subsequently to increase speed effectively to eliminate the *E* to *Z* equilibrium. Reaching these temperatures is outside the column thermal range of LC instrument capability. Therefore, it is important to integrate both isomers if this phenomenon is noticed and accurately report the sum of both values to calculate the exact amount of *N*-nitrosamine impurities. The secondary amine is more prone to protonation compared to primary amine during ionisation, but this protonated species can be tautomerised, which locks rotamers formed while N-N bond rotation was possible, as

shown in the proposed mechanism of formation of these rotamers in Figure 41.

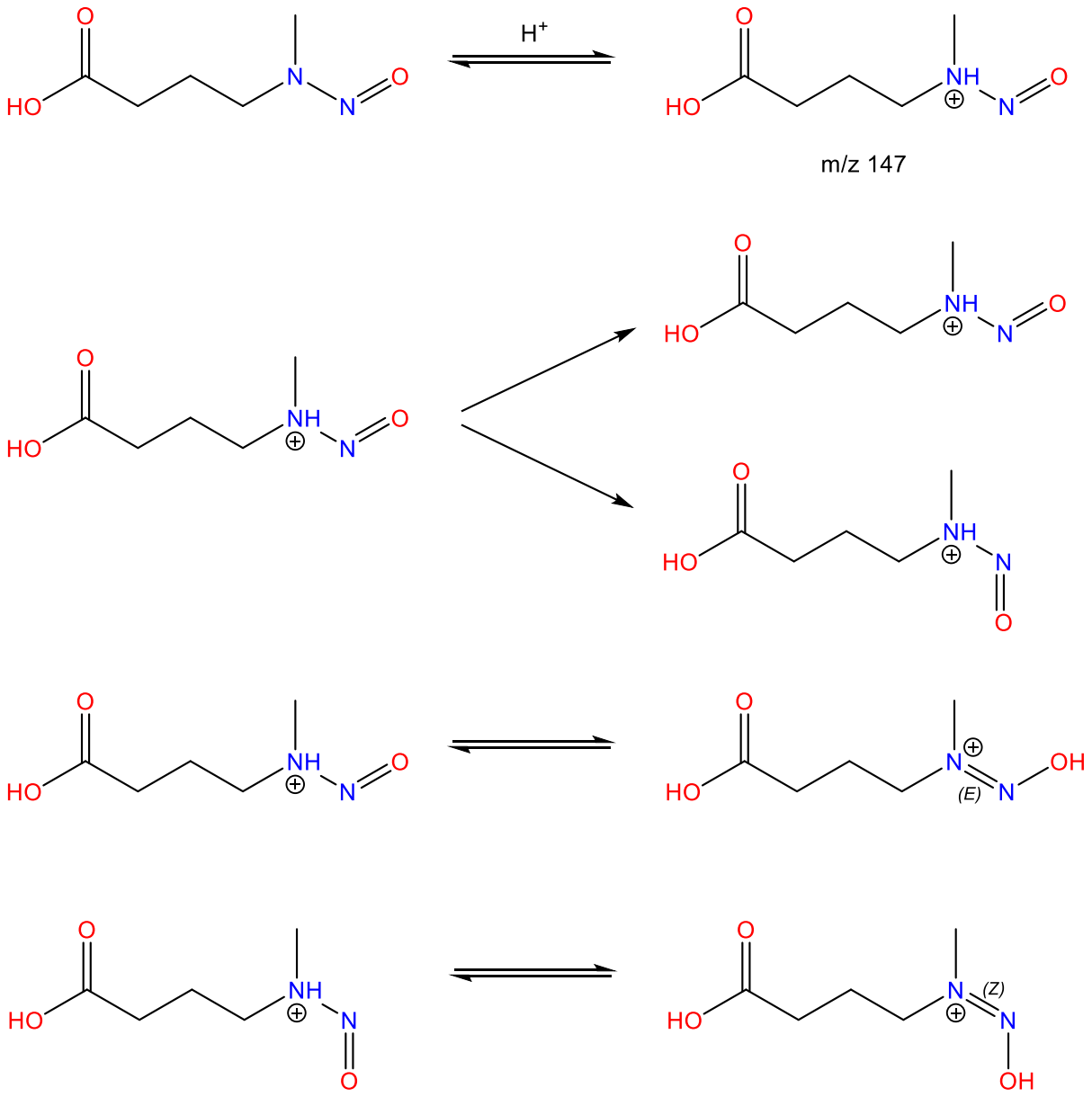


Figure 41. Proposed mechanism of rotamer formation of NMBA

The abundance of these rotamers in the chromatogram or spectrum is based on intramolecular hydrogen bonding capability and each rotamer's hindrance to creating a more thermodynamically stable form. Larger substituents on nitrogen generate more steric hindrance; therefore, more energy is needed to achieve a stable form. In the case of NMBA from the image above, there is the smallest methyl substituent and larger, but not

branched, substituent of butyric acid. Considering the sheer size of each substituent, it is safe to assume that *E* rotamer is more stable.

5.2 MS method development

Since the *N*-nitrosamine molecules are smaller in size compared to most compounds analysed in drug discovery and development, and the fact that they lack a soft ionisation centre, atmospheric pressure chemical ionisation (APCI) mode was found to generate better signals than electrospray (ESI) mode. However, there are examples, like a paper published by Kadmi *et al.* describing the usage of ESI sources in nitrosamine analysis in water, which is a significantly different type of matrix with low or any interference that might complicate analysis.[52] Various authors report the APCI signal of nitrosamine compounds being significantly better than the signal in ESI. For example, Lee *et al.* reported that the results of each target ion indicate that all of the nitrosamine substances tested showed higher sensitivity and abundance in APCI than in ESI.[53] Depending on the compound analysed, signs were two to ten times higher using APCI than the ESI ion source. Moreover, in the ESI full scan mode, $[M+Na]^+$ peak was observed in all nitrosamines, but no sodium adducts were observed in APCI. Also, APCI showed a higher abundance of protonated species than ESI in the same concentration. All these results suggest that APCI is a better choice than ESI for nitrosamine mass analysis in terms of sensitivity.

There are applications for nitrosamine analysis, as mentioned before that are done using ESI. However, like stated before, this is done when analysing samples with less complex matrices like environmental water samples. These findings are consistent with Ripollés *et al.*, which got similar results earlier regarding each higher sodium adduct abundance when using ESI and higher sensitivity when using APCI. Only when a small amount of formic acid was added to the vial used for instrument tuning in ESI abundance of protonated species sensitivity was higher than with the ions with sodium adducts. This could be explained with more protons that were available at

this point.[54] This is also the reason why we used the flow of mobile phase with additives and combined flow of tune solution which helped ionisation of the standard being tuned.

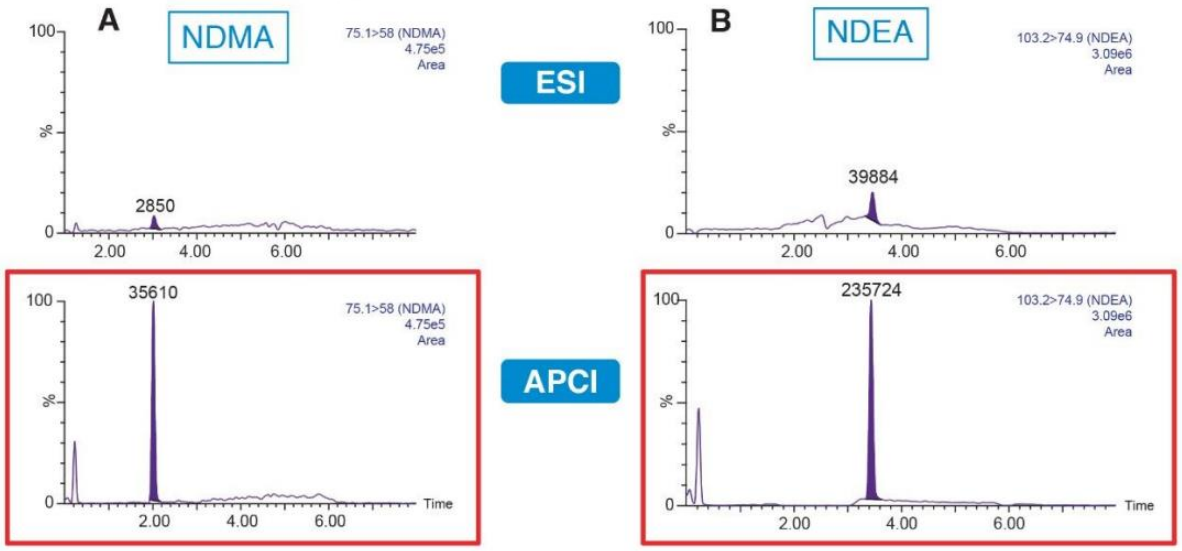


Figure 42. Comparison of NDMA and NDEA ionised using ESI probe (upper half) and APCI probe (lower half)[40]

Lame and Hatch noticed that apart from a significant signal strength increase when using APCI, the signal intensity is also subject to temperature change.[40] When using probe and source temperature on somewhat lower settings, the signal strength increases, as shown in Figure 42 and Figure 43. Once molecular masses were optimised, daughter ions for *N*-nitrosamine impurities were defined and used in experiments using the automatic tune option. The complete list of daughter ions for MRM experiments is presented in Table 5. For further MS method optimisation, using different ion sources, like Waters UniSpray™, which combines ESI and APCI sources, could be an option.[55]

Regarding low NMBA sensitivity in MS, it could be increased in negative ion mode. NMBA is carboxylic acid, and acids are more prone to negative ion mode ionisation by deprotonating the carboxylic group. Usually, negative ion mode are more sensitive in MS but also experience high noise levels.

This impacts the overall signal-to-noise ratio and is not the first choice. In this case, further optimisation could be somewhere in that direction, especially with new instruments developing, like Waters TQ Absolute which has very low levels of negative ion mode noise. This test, however, was not performed due to the limited timeframe available for this study, but it most definitely should be tested in the future during further method optimisation.

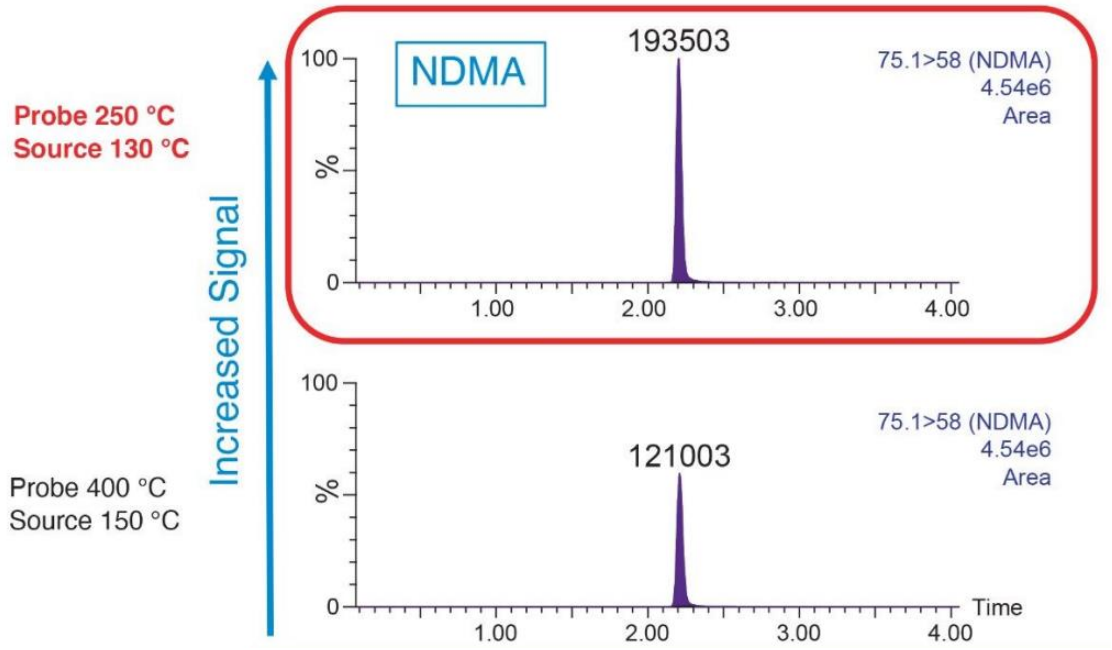


Figure 43. Comparison of different APCI probes and MS source temperature relative to signal strength[40]

Fragments of analytes noticed during MRM development are shown in Figure 9 for each of the compounds used in this study. Most of these fragments are self-explanatory, but it is worth discussing some of the fragments since they are somewhat specific. Most fragments formed during MRM experiments are formed in collision cell using argon gas. Argon molecules collide with analyte ions in the gas phase and fragment them. For example, Kulikova *et al.* mention the methyl-diazonium ion, *m/z* 43, that is formed during CID during the fragmentation of NDMA in the collision cell of MS. This proposed fragmentation pattern is shown in Figure 44.[35] In this case first step is dihydroxylation of NDMA, followed by α-cleavage.

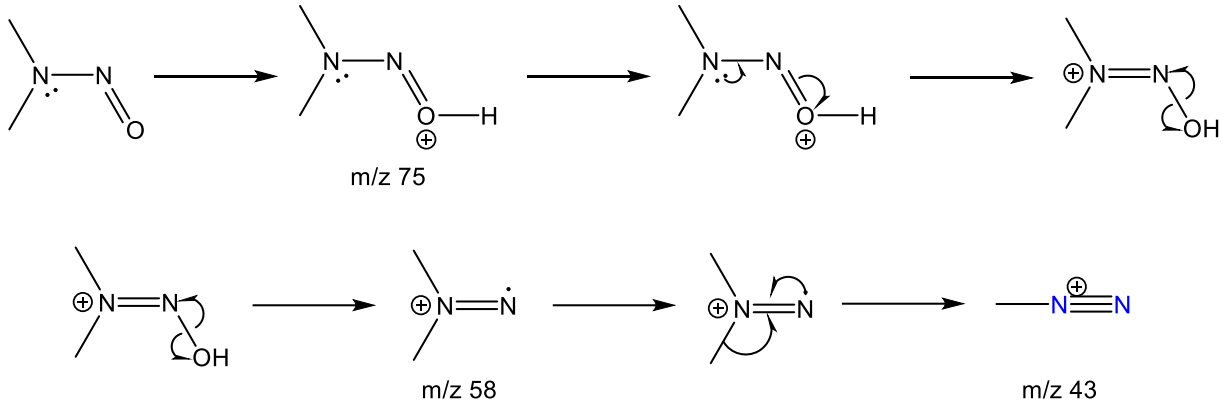


Figure 44. Proposed pathway of collision-induced dissociation of NDMA[35]

Another somewhat interesting ion is the ethenyl ion, *m/z* 27, which is not very abundant type of products of fragmentation using ESI and APCI modes of ionisation, usually ethyl ion, *m/z* 29, is more abundant one. However, during the fragmentation of NEIPA, the ethenyl ion is found to be more abundant than the ethyl ion.

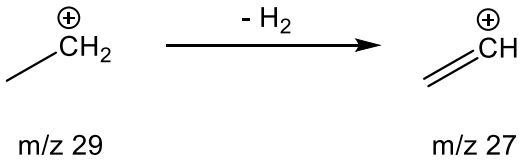


Figure 45. Proposed pathway of collision-induced dissociation of NEIPA[33]

According to a paper by Field, there are two possible mechanisms of this ion formation during fragmentation. The first one is direct, involving the abstraction of a hydride ion from the ionised species if chemically feasible. The other one is a two-step mechanism which involves the protonation of a compound followed by the loss of hydrogen. In the case of NEIPA, since the ion is already formed in the source prior to fragmentation in the collision cell, a direct mechanism is the most probable, shown in Figure 45.[33] There is structurally similar product of McLafferty rearrangement[56], but in this case, product is neutral molecule, ethene. Also, for this rearrangement, usual intermediate is six membered ring, for which, in case of NEIPA there is not enough atoms.

5.3 Method validation

Method validation was completed with optimised conditions per recommended ICH guidelines,[57] and all critical parameters were established to show the efficiency of the developed methods. The specificity of the method showed good selectivity for all of the nitrosamine impurities and API. The results showed no interference of any API's with all six nitrosamine impurities. Linearity was determined for all six nitrosamine impurities, and the range was established for each impurity individually and up to 100 ng/mL. The regression coefficients, slopes and intercepts are determined using data processing software, and for all of the nitrosamines analysed, calibration curves were very accurate, all with $R^2 > 0.999$. In the same manner, LOD and LOQ were determined for each impurity and based on S/N ratios of 3 and 10, respectively. Accuracy and recovery were evaluated and were within defined limits. The robustness of the methods was not tested at this time since this test was not essential for the scope of this work. However, in the future, robustness tests in the form of changing method conditions, flow and column temperature should be performed. Repeatability was tested and confirmed using %RSD on the area at LOQ. Results showed the method to be specific, linear in the desired range, accurate, robust and reliable.

5.4 Technology improvements and new techniques

Another possibility for further development of the method is a paper by Schmidtsdorff and Schmidt which shows that these nitrosamine analytes can be analysed using Supercritical Fluid Chromatography (SFC).[58] The authors used Fusion Quality by Design (QbD) software to developed a method for this analysis. This tremendous statistical tool helps in method development by suggesting various variables in method parameters and generating so-called design space. This gives a whole new space for progress in analysing nitrosamine impurities.

Aforementioned the UniSpray™ ion source is a great option to increase sensitivity. The spray the probe generates in the source is targeted to a charged metal rod in front of the ion inlet, as seen in Figure 46. Under these conditions, the gas flow becomes attached to a portion of the curved surface and results in asymmetric gas streamlines in the wake that are directed towards the ion inlet orifice. This flow phenomenon is known as the Coandă effect.[55,59] Under the influence of the Coandă flow field, ions and charged droplets are directed towards the ion inlet, which enables more ions to enter the ion inlet and increase sensitivity.

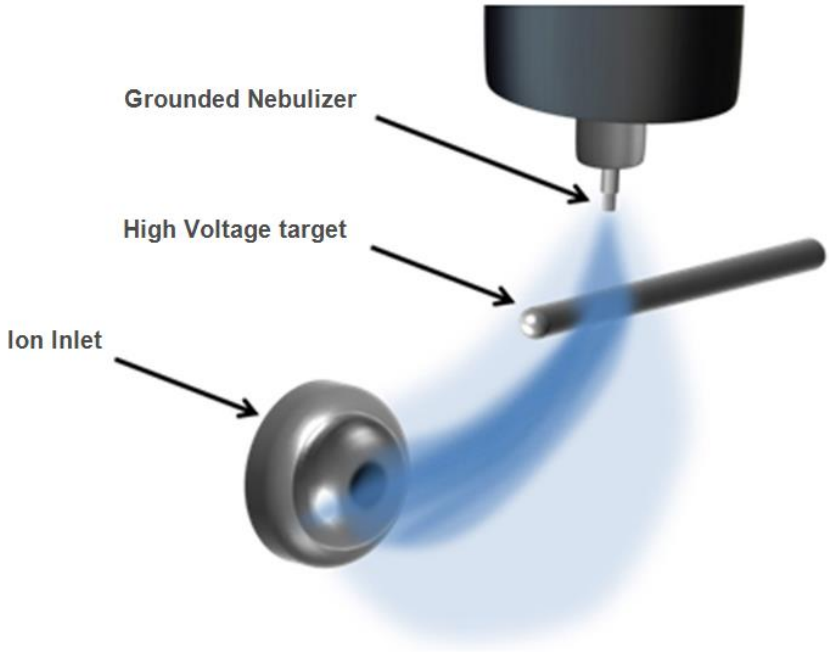


Figure 46. Schematic of Waters UniSpray™ ion source

Already mentioned Waters TQ Absolute MS shows even lower limits of detection and qualifications of these compounds. However, a study by Maziarz *et al.* showed that this instrument has 10 to 100 times better sensitivity depending on the analyte.[60] This fact allows us to further increase the safety of the medicines produced and to ensure higher accuracy for these genotoxic and carcinogenic impurities analyses in food, drugs and environmental applications.

6. Conclusion

In summary, two LC methods using three different columns and an MS method have been developed for the simultaneous determination of six nitrosamine impurities: *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitroso-*N*-methyl-4-aminobutanoic acid (NMBA), *N*-nitrosoisopropylethyl amine (NEIPA), *N*-nitrosodiisopropylamine (NDIPA) and *N*-nitrosodibutylamine (NDBA). The method can determine these impurities in six API's: Azithromycin, Betahistine, Metformin, Metronidazole, Simvastatin, Sitagliptin, Vildagliptin and their drug substances.

LC methods were developed to resolve the nitrosamine impurities from each other and API in corresponding drug substances. Three different columns were used in order to improve selectivity when needed. Analysis of all nitrosamines in all of the API's is possible using *LC-method 2* and *Column 2*, but in that case, limits for some impurities are higher. To achieve better sensitivity in case this is required by companies or regulatory bodies, other columns and method combinations are more suitable in cases described in the *Results* section.

APCI ion source was used rather than ESI since nitrosamine impurities analysed are small volatile molecules with better ionisation response when using APCI. Furthermore, tuning the instrument to correct MRM transitions helped eliminate any coeluting peaks if present, buts still, the signal suppression by matrix effect remained. Finally, the LC-MS/MS technique allowed us to quantify a maximum number of impurities compared to other detection techniques like GC-MS/MS, where there is a limitation to ionise impurities like NMBA.

In this work, we have established all the critical validation parameters to test the efficiency of these methods. The determined LOQ and LOD values are low, which shows the sensitivity performance of the method and

instrument used. Also, the methods are all linear and exhibit great sensitivity, which can quickly fulfil the requirements of regulatory agencies worldwide for these very common nitrosamines and tested drug products or APIs. Apart from that, the method proved to be robust and accurate within the parameters suggested in ICH Guidelines. In summary, the validated methods can routinely quantify all six nitrosamine impurities in six drug products.

The final result of analysis of the six commercially available final dosage forms showed the presence of NDMA in two of them, Metformin/Sitagliptin and Metformin/Vildagliptin drug products. However, in both cases, the amount of NDMA found was under limits determined by regulatory agencies.

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