Bachelor's project

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Literature review on Nitrosamines – Formation, Health and Environmental Impact and the Ways of Monitoring and Analysis

Bachelor's project in Chemical Engineering
Supervisor: Eirik Sundby (NTNU)
Co-supervisor: Huiling Liu (Chiron AS/Client)
May 2021

NTNU
Kunnskap for en bedre verden

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Litteraturstudie av nitrosaminer - Syntese, helse- og miljøpåvirkninger og hvordan de kan kontrolleres og analyseres

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PREFACE

The presence of nitrosamines in the environment and their carcinogenicity in humans are a well-known issue studied since the 1950's. In recent years increased attention has also been drawn to their occurrence in pharmaceutical products causing numerous drug product recalls. Hopefully, this review will raise awareness on the hazard of nitrosamines and the benefit of quality assured reference materials for analysis and monitoring.

The opportunity to investigate this issue was provided by Chiron AS, a Norwegian based and leading producer and supplier of chemical products for research and analysis. I want to thank Huiling Liu at Chiron AS for providing me insight and being adaptable when changes due to Covid-19 had to be done, giving me the opportunity to contribute. A thanks also go to Eirik Sundby at NTNU for support and guidance during my work on this thesis.

Trondheim, May 20th, 2021

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ABSTRACT

More than 10 congeners of nitrosamines are now classified as carcinogenic, probably, or possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC). Secondary nitrosamines are stable compounds shown to be persistent in our environment. They occur in sources of human intake like tobacco, food, personal care products, waters, and pharmaceutical products. Because of this, there is a need for pure analytical reference materials for analysing and monitoring of their occurrences.

The literature has been reviewed, and shown that nitrosamines occur in numerous sources, and that their general chemistry is well described. The focus is mainly on exogenous formation, but evidence for endogenous formation is also well studied. In the human body the biotransformation of nitrosamines damages the DNA by generation metabolites who form DNA-adducts. This can disturb the DNA replication, leading to tumours. Especially two nitrosamines, N-Nitroso nornicotine (NNN) and nicotine-derived nitrosamino ketone (NNK), are carcinogenic to humans.

The trends in analytical methods for analysing and monitoring are the use of solid phase extraction before analysing on GC, and HPLC for the non-volatile compounds. GC-TEA (Gas Chromatography – Thermal Energy Analyzer) is highly sensitive and selective for nitrosamines, and GS-MS/MS has also shown low limits of detection of 0.5 µg/L in water analyses. Evidence based methods for syntheses of many nitrosamines are available through SciFinder^n. An easy and classical method showing excellent yields of 100% for synthesis of N-Nitrosodimethylamine (NDMA) was found using SciFinder^n. A suggested procedure for synthesis of NNK, with an estimated yield of 30%, was found with SciFinder^n's retrosynthesis tool.

Institutions like e.g. the U. S. Food and Drug Administration (FDA) have available guidance's for industry, and values for nitrosamine limitations in some sources are set. Even though their presence is monitored and regulated within some industries, further research is needed to set acceptable intake limits for more compounds. This is especially true for tobacco products containing NNN and NNK, where the response from the industry to reduce the nitrosamine levels has been slow. This review suggest that the development of intake limits also need to take the total nitrosamine burden into account, considering that they are relatively widespread. Further research should also investigate endogenous vs. exogenous formation of nitrosamines in calculating the total nitrosamine burden. Validated methods for analyses and reference materials are available on the marked. They are presented in this review to increase attention, and inspire to further research and development of nitrosamine monitoring. A maintenance of the analytical reference standard supply is crucial for monitoring, and maintenance of safe products on the marked especially within the pharmaceutical industry.

SAMMENDRAG

International Agency for Research on Cancer (IARC) har klassifisert mer enn 10 nitrosaminer som kreftfremkallende, sannsynligvis eller muligens kreftfremkallende for mennesker. Sekundære nitrosaminer er stabile forbindelser som har vist seg å være vedvarende i vårt miljø. Mennesker inntar nitrosaminer gjennom tobakksprodukter, mat, vann, hygieneprodukter eller farmasøytiske produkter. For å kunne analysere og kontrollere deres forekomst i vårt miljø er det et behov for rene, analytiske referansematerialer av nitrosaminer.

En gjennomgang av litteraturen har vist at nitrosaminer forekommer i utallige kilder til menneskelig inntak, og deres generelle kjemi er godt studert og beskrevet. Primært er fokuset på dannelsen av nitrosaminer før inntak via forskjellige produkter, men endogen dannelse av nitrosaminer er også godt beskrevet. Når nitrosaminer brytes ned i kroppen dannes det mellomprodukter som kan skade DNA ved å forstyrre replikasjonen, og føre til ukontrollert celledeling og tumorer. Spesielt to forbindelser som forekommer i tobakk, N-Nitroso nornikotin (NNN) og nikotin-avledet nitrosaminketon (NNK), har vist seg å være kreftfremkallende i mennesker.

For analyse og kontroll av nitrosaminer brukes hyppigst solid-fase ekstraksjon før analysering på GC, og HPLC for lite flyktige komponenter. GC-TEA er en metode som er svært selektiv for nitrosaminer, og GC-MS/MS har også vist lav deteksjons-grense på 0.5 μg/L i vannprøver. Syntesemetoder for referansematerialer av nitrosaminer er tilgjengelig via SciFinder^n sine databaser. Dette ble brukt til å finne en metode for syntese av N-nitrosodimetylamin (NDMA) med utbytte på 100%. SciFinder^n sin retrosyntese-planlegger ble også brukt for å finne et forslag til syntese av NNK med et utbytte på 30%.

Det foreligger retningslinjer for industrien, og begrensende verdier for nitrosaminer for noen produkter via institusjoner som f.eks. U. S. Food and Drug Administration (FDA). Selv om forekomsten av nitrosaminer kontrolleres i noen industrier er det et behov for videre forskning for å få satt akseptable inntaksgrenser. Dette gjelder særlig for NNN og NNK hvor industrien har reagert sakte med tanke på å senke innholdet av nitrosaminer. Dette litteraturstudiet peker også på behovet for å ta hensyn til den totale eksponeringen for nitrosaminer med i disse beregningene, og undersøke eksogen vs. endogen dannelse av nitrosaminer videre. Validerte metoder for analyse og syntese av referansematerialer er tilgjengelig, og er presentert her for å øke bevisstheten rundt og inspirere til en videre utvikling på feltet. En opprettholdelse av tilgangen på referansematerialer er nødvendig for kontroll av forekomst, og særlig kritisk innen farmasøytisk industri for å beholde trygge produkter på markedet.

CONTENT

List of appendices:

ABBREVIATIONS

Abbreviation	Meaning
API	Active Pharmaceutical ingredient
APCI-MS	Atmospheric Pressure Chemical Ionization – Mass spectrometry
DBDMH	1,3-dibromo-5,5-dimethylhydantoin
DCDMH	1,3-dichloro-5,5-dimethylhydantoin
DCM	dichloromethane
EMA	European Medicines Agency
EPA	Environmental Protection Agency
FDA	U. S. Food and Drug Administration
GC-TEA	Gas chromatography – thermal energy analyser
HS-injection	Head Space injection
IARC	International Agency for Research on Cancer
IEC	International Electrotechnical Commission
ISO	the International Organization for Standardization
Iso-NNAC	4-(methylnitrosamino)-4-(3-pyridyl) butyric acid
Iso-NNAL	Iso-4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
LPME	Liquid-phase microextraction
NAB	N-Nitrosoanabasine
NAT	N-Nitrosoanatabine
NDBA	N-Nitrosodibutylamine
NDEA	N-Nitrosodiethylamine
NDELA	N-Nitrosodiethanolamine
NDIPA	N-Nitrosodiisopropylamine
NDMA	N-Nitrosodimethylamine
NDPHA	N-nitrosodiphenylamine
NIPEA	N-Nitrosoisopropylethylamine
NMBA	*N*-Nitroso-N-methyl-4-aminobutyric Acid
NMEA	N-Nitrosomethylethylamine
NMOR	N-Nitrosomorpholine
NMPA	N-Nitrosomethylphenylamine
NNA	Nicotine-derived nitrosaminoaldehyde
NNAL	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	Nicotine-derived nitrosamino ketone
NNN	N-Nitrosonornicotine
NPIP	N-Nitrosopiperidine
NPYR	N-nitrosopyrrolidine
RS	Reference standard
SPME	Solid-phase microextraction
TNA burden	Total nitrosamine burden
TSNA	Tobacco specific nitrosamine
USDA	U.S. Department of Agriculture
WHO	World Health Organization

1 INTRODUCTION

Nitrosamines (also referred to as N-nitrosamines) are organic compounds with the chemical structure $R_2N\text{-}N{=}O$, where R_1 and R_2 are alkyl or aryl groups [1]. Due to their possible carcinogenic hazard to humans their occurrence in tobacco, foods, personal care products and waters have been widely studied since the mid 1950's. In recent years they have also been detected in pharmaceutical products leading to multiple medications being recalled from the marked [2, 3]. More than 10 congeners of nitrosamines are now classified as carcinogenic, probably or possibly carcinogenic to humans by IARC [4]. Nitrosamine's health and environmental impact provides the need for quality assured monitoring of their occurrence in sources of human intake. This includes validated analytical methods and reference standards for detection and monitoring of nitrosamine levels in a wide range of products ingested by humans orally or through the skin. In addition to exogenous formation of nitrosamine the total nitrosamine (TNA) burden on humans may be underestimated due to endogenous formation of their precursors.

The background for this bachelor thesis was a need for pure analytical reference standards of nitroso (NO^+) compounds, meant to be synthesised by nitrating secondary amines. This would be performed in cooperation with Chiron AS, and the nitrosamine reference standards would be used for environmental and food analysis. Chiron AS is a Norwegian based company with more than 30 years' experience, and leading producer and supplier of chemical products for research and analysis. Due to the Covid-19 pandemic the practical syntheses could not be performed within the scope of this project, and this thesis will instead present a review of nitrosamines covering their formation, health and environmental impact, and the ways of monitoring and analysis. As part of the bachelor a brochure text (a biomarker focus) about nitrosamines supplied by Chiron AS was prepared, added in Appendix A. Structures for their available nitrosamine compounds were also drawn.

To provide a better understanding of nitrosamine's properties, their general chemistry and formation will be presented at the very beginning. This will function as a basis for an understanding of their formation and occurrences in sources of human intake presented later. As a response to the increased need for monitoring of nitrosamines in pharmaceutical products, this review has also dedicated a section to this issue. Finally, the review will present analytical approaches for characterization, analysis, and monitoring of nitrosamines in general, before suggesting methods for the syntheses of nitrosamine reference standards. Primary nitrosamines are not stable, and the original plan was to nitrite secondary amines. Therefore, the secondary N-nitrosamines will be the focus throughout this review. A lot of abbreviations will appear throughout the review, and a list of abbreviations is added prior to the introduction (page vi) for an easy access to their meanings. The aim of this thesis is to provide a summarized literature review on the topic of nitrosamines in our environment. Together with the addition of analytical and synthetical methods, hopefully this review will increase awareness on the hazard of nitrosamines, and inspire further research and development of reference materials for safe and quality assured monitoring. A "popular science" article about nitrosamines is also added in Appendix B.

2 METHODS

2.1 PROCEDURE FOR LITERATURE SEARCHES

Literature searches were conducted within NTNU's library database Oria containing the library's' printings and online database. Through NTNU, Scopus was also accessed, an online database containing full text peer reviewed articles in science subjects. Different search strings were developed for each sections in the review to find targeted literature. As an example, to get an overview over nitrosamines history and occurrence, and information about their presence in pharmaceuticals the following strings were used, respectively:

- (nitrosamin* OR N-nitro*) AND (occurrence OR found OR identified OR history).
- (nitrosamin* OR N-nitro*) AND (pharmaceutical* OR drug* OR medic*)

Applied filters in all searches were: peer reviewed, English, Norwegian, and a preference for matches in the title of documents. Previous works on the issue of nitrosamines also functioned as an inspiration to investigate their primary references.

2.2 HOW DRAWINGS AND SYNTHETICAL METHODS WERE DEVELOPED

For application of personal style preferences, all figures and sketches in this review have been modified in Chem Draw Professional 16.0. All structural sketches have also been cross-checked in SciFinder^n according to their CAS-numbers.

Synthetical methods for NDMA (N-Nitrosodimethylamine) were found using SciFinder^n's search tool on reactions. Searches for desired product were done using CAS-number with added filters: 90-100% yield, dichloromethane (DCM) as solvent (according to Chiron AS for respective compound), and English as language. In choosing between proposed procedures for syntheses simplicity, costs and practical convenience were emphasized. For the suggested NNK (Nicotine-derived nitrosamino ketone) synthesis, SciFinder^n's retrosynthesis tool was used to find the predicted steps. The retrosynthesis plan suggested in SciFinder^n is attached in appendix C (page vi). In selecting between scientific evidence for the predicted steps high yields were emphasized.

3 GENERAL CHEMISTRY OF NITROSAMINES – STRUCTURE, PROPERTIES AND FORMATION

3.1 NITROSAMINE STRUCTURE AND FORMATION

N-nitrosamines have the general chemical structure shown in Figure 3-1, and is obtained from N-N bond formation between an amino compound and NO^+ (nitrosonium ion) as a free ion or as part of compounds carrying NO^+. Formation of these compounds occur when amines are in the presence of nitrite salts under acidic conditions, in which nitrite salts can form nitrous acid (HNO_2) which then act as a nitrosating agent on amines to form N-nitrosamines [1, 5].

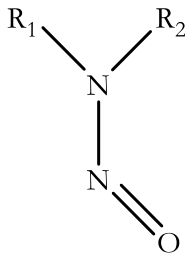
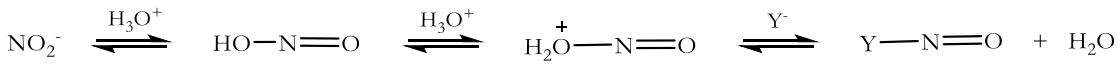


Figure 3-1 General structure of N-nitrosamines. R_1 and R_2 are alkyl or aryl groups in secondary N-nitrosamines. Inspired by Challis and Challis (1982) [1].

Free NO^+ can be directly obtained in strong acids or solid salts but N-nitrosamines more often form in the presence of carriers of NO^+ [1]. Carriers of NO^+ can form what is called nitrosating agents through activating transformations giving them the NO^+ functionality. As an example neither nitrous acid or the nitrite ion (NO_2^-) interacts directly with amines but form the nitrosating agent Y-NO in aqueous acidic solutions as shown in Scheme 3-1 [1]. Possible sources of nitrosating agents are nitrites ($R\text{-}NO_2^-$), nitrates ($R\text{-}NO_3^-$) and nitro compounds ($C\text{–}NO_2$). They can form N-nitrosamines in the presence of amines, hydroxylamine or amine peroxides, and other nitrogen containing substances [1, 6].



Scheme 3-1 Rapid equilibrium steps in formation of the nitrosating agent Y-NO, where Y is a nucleophilic catalyst (anion, e.g., Cl^- or NO_2^-) or neutral molecule. Collected from Challis and Challis (1982) [1].

When primary amines are in the presence of nitrosating agents, the N-nitrosamine yield is very low. Primary N-nitrosamines are also generally unstable compounds. However, heteroaromatic primary N-nitrosamines are known to be more stable when electron withdrawing substituents are present [1, 7]. A list of primary N-nitrosamines and their

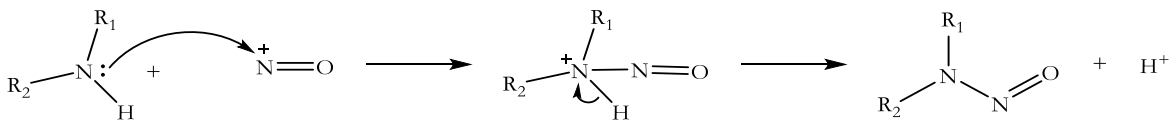
general structure is presented in Table 3-1, but these compounds are not well studied and will not be further discussed in this review. Since the focus of this review is nitrosation of secondary amines, nitrosation of tertiary and quaternary amines will not be discussed in detail either.

Table 3-1 Primary N-nitrosamines, their R-groups, and general properties [1].

Primary N-nitrosamines	R-groups	Some general properties
Aromatic	H and aryl	Unstable and rapidly decompose
Heteroaromatic	H and heteroaryl	More stable with electron-withdrawing substituents
Aliphatic	H and alkyl	Decompose below room temperature

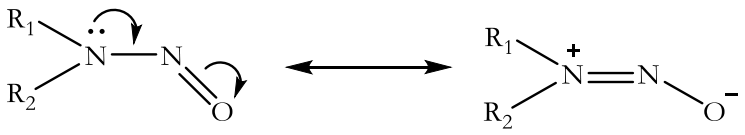
3.2 NITROSATION OF SECONDARY AMINES AND THE PROPERTIES OF N-NITROSAMINES

Numerous precursors of secondary amines and nitrosating agents makes det formation of N-nitrosamines in situ possible [3, 8]. Secondary N-nitrosamines form quickly and easily, especially under weakly acidic conditions with nitrates and nitrites, or in the presence of sodium nitrite [7]. For simplicity nitrosation of secondary amines is shown in Scheme 3-2 with a free nitrosonium ion. When explaining how chemical reactions happen, organic chemistry refers to nucleophiles and electrophiles. Nucleophiles are electron rich, and electrophiles are electron-deficient atoms and molecules. Nucleophiles and electrophiles are attracted to each other, and this attraction is the force behind chemical reactions. Which reaction mechanism different compounds undergo is determined by their functional groups. As described by Challis and Challis (1982, p. 1154), many nitrosation reaction can be depicted as S_N2 reactions, which are biomolecular substitution reactions where a nucleophile substitutes for an atom or a group [1, 9].



Scheme 3-2 Nitrosation of secondary amines by a free nitrosonium ion acting as an electrophile. Arrows show the movement of electrons, with two barbs signifying the movement of two electrons. Inspired by Challis and Challis (1982) [1].

Secondary aliphatic, heterocyclic, and aromatic N-nitrosamines are generally highly stable compounds. This is because the lone pair of amino-N electrons are delocalised through the π-electron system of the nitroso function, as shown in Scheme 3-3. This is supported by electron diffraction studies on gaseous NDMA (N-Nitrosodimethylamine), showing bond angles close to 120° (making NDMA planar), and that both the N-N and N=O bond lengths are intermediates between single and double bonds between respective atoms. This gives them bond orders about 1.5, implying an intermediate between the resonance structures shown in Scheme 3-3 [1].



Scheme 3-3 The two main resonance structures of secondary N-nitrosamines. Inspired by Challis and Challis (1982) [1].

In hydrogen bonding, N-nitrosamines are too weak to act as acids (proton donors). They are also much weaker bases than their parent amines and are very stable in water and alkaline conditions. In strongly acidic and aqueous conditions (pH < 3) they can undergo protonation, with the oxygen as the most basic atom. Some general properties of secondary N-nitrosamines are listed in Table 3-2, but their physical properties depend on their substituent groups and varies greatly. As an example, NDMA is an oily liquid dissolving in organic solutions, while N-nitrosodiphenylamine is a solid with very low solubility in water. In general N-nitrosamines are resistant to temperature and light but can be subjects to homolysis of the N-N bond, especially thermolysis in high temperature conditions, and photolysis has been reported [1, 7].

Table 3-2 Secondary N-nitrosamines and some general properties [1].

Secondary N-nitrosamine	Properties
Aliphatic	Yellow liquids or low-melting solids Soluble in water and organic solvents
Heterocyclic	Yellow liquids or low-melting solids Soluble in water and organic solvents
Aromatic	Usually low-melting solids Insoluble in water Thermally unstable

Different reactions with N-nitrosamines are well described by Challis and Challis (1982) [1]. This includes nucleophilic reactions with halogens and phosphorous oxychloride, reactions with inorganic acids and organometallic reagents, reduction, and oxidations. A further explanation of their biological properties and carcinogenicity in humans will be given in section 5.

4 HISTORY AND OCCURRENCE OF NITROSAMINES IN SOURCES OF HUMAN INTAKE

The interaction between nitrous acid and primary aromatic amines was discovered and discussed already in 1864 and 1870, and the term "nitrosamines" were introduced in the literature in 1878 by Otto Witt [8]. It was not until about 100 years later Magee and Barnes first discovered N-nitroso compounds as carcinogenic. In 1956 they observed liver tumours in rats due to NDMA, and later it was shown that NDMA transformed to an active form which methylated proteins and nucleic acids in the liver of rats [2, 10]. At this point NDMA was used as a solvent in dry cleaning, and later reports from Norway also showed that sheep fed with "herring meal" preserved in sodium nitrite developed liver damages [2]. Early on the link between sodium nitrite and NDMA was debated, because it was thought that nitrite could react with dimethylamine and form NDMA only under acidic conditions, and the herring was preserved under neutral or alkaline conditions. In 1973 though it was shown that this reaction also could take place at pH 7 in the presence of formaldehyde [11].

Since then, numerous N-nitroso compounds have shown carcinogenic characteristics after an extensive amount of research on the field. Over 300 congeners of N-nitrosamines are now identified, and they occur in tobacco, foods, personal care products, pharmaceuticals and water [8, 12]. In this review nitrosamines carcinogenic hazard to humans will often refer to IARCs classification groups listed in Table 4-1. A complete list of all mentioned N-nitrosamines, their structure and IARC classification is also available in Table A-1, appendix D (page xi).

Table 4-1 IARC classification groups for identification of carcinogenic hazards to humans [4].

Group	Description
Group 1	Carcinogenic to humans Supported by sufficient evidence
Group 2A	Probably carcinogenic to humans Sufficient evidence for their effect on experimental animals, but limited evidence for humans
Group 2B	Possibly carcinogenic to humans Limited evidence in both experimental animals and humans
Group 3	Not classifiable as to its carcinogenicity to humans Inadequate data on carcinogenicity

4.1 NITROSAMINES IN TOBACCO, THEIR PRECURSORS AND HOW THEY FORM

Tobacco smoke contains more than 4000 chemicals, and about 100 of them is identified as carcinogenic to humans and associated with tumours of the lung, oral cavity, pancreas, and oesophagus. Tobacco smoke is a highly complex mixture of chemicals, but the occurrence of nitrosamine is well studied and understood with research going back to 1974 [13]. Stephen Hecht have conducted comprehensive research on TSNAs and their presence in tobacco, their biochemistry, biology and carcinogenicity [14].

Among the chemicals in tobacco, we find the tobacco-specific nitrosamines (TSNAs) derived from multiple alkaloids in the tobacco plants, depicted in Figure 4-1 [15]. Most of the nitrosamines form during the curing process, but they also form during combustion (smoking). It has been show that a manipulation of the curing parameters and a reduction of fertilization levels can lower the TSNA levels in tobacco [16]. More nitrosamines have been detected in tobacco products, but the research has mainly focused on TSNAs due to their abundance, and because some of them have known carcinogenic effect in laboratory animals [15]. Two of the TSNAs are also classified in IARC's group 1 as carcinogenic to humans, namely NNN (N-Nitrosonornicotine) and NNK (nicotine-derived nitrosamino ketone) [4, 15].

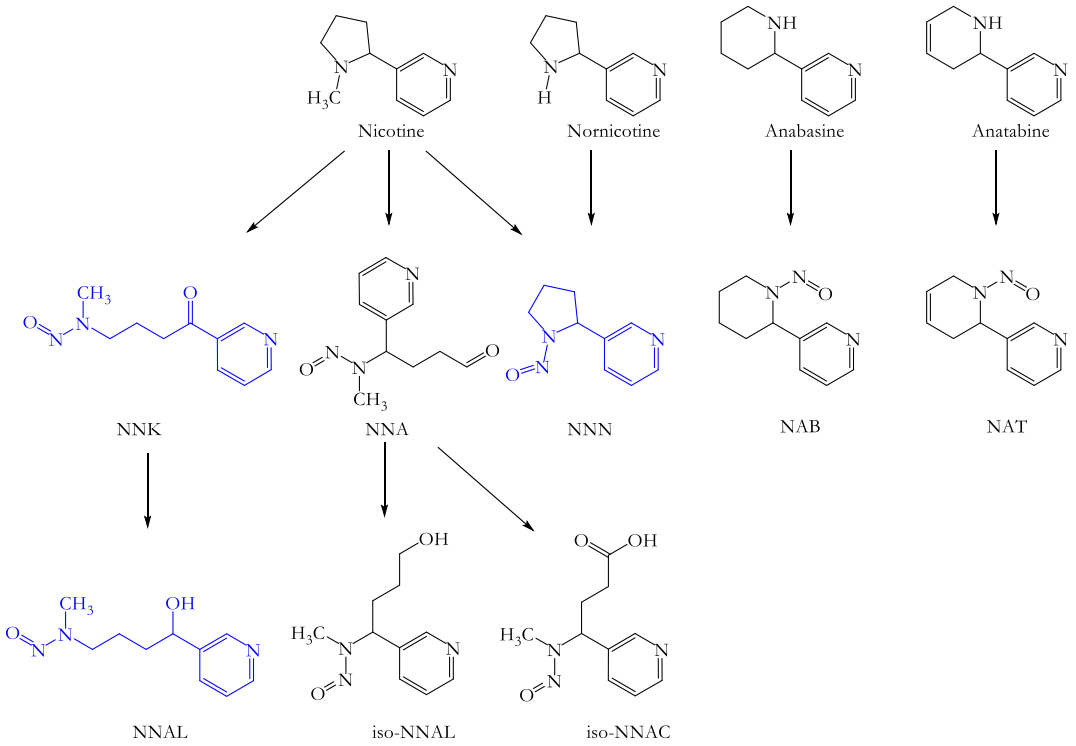


Figure 4-1. Structures of tobacco-specific nitrosamines and tobacco alkaloid precursors, inspired by Hecht 1998. The more carcinogenic are marked as blue. Apart from NNA, all have been detected in tobacco products. Full chemical names are listed in abbreviations [14].

Alkaloids is a group of nitrogen containing organic molecules made by plants [17]. In tobacco the most abundant alkaloid is nicotine (a tertiary amine), and minor alkaloids such as nornicotine, anatabine and anabasine (secondary amines) [15]. These alkaloids form TSNAs through different nitrosation reactions. In secondary amines there is a substitution of NH by N-N=O as previously shown in Scheme 3-2, and for tertiary amines there is an oxidation where a C-N bond is cleaved.

The formation of TSNAs in tobacco can follow different pathways, including reactions with nitrous acid. During smoking (combustion) nitrous acid can degrade to NO and NO_2, which then can lead to the formation of N_2O_3. Another pathway for TSNA-formation then become the reaction between N_2O_3 and especially nornicotine as shown in Figure 4-2 to form NNN [18].

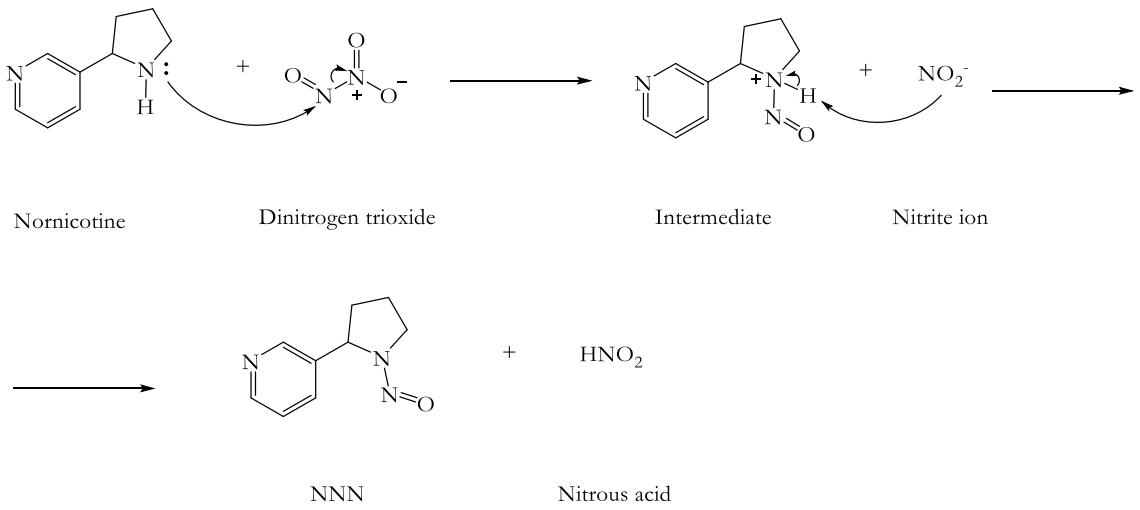


Figure 4-2 Reaction mechanism between nornicotine and dinitrogen trioxide to form NNN. Inspired from Moldoveanu and Borgerding (2008) [18].

Organ specificity is typical for nitrosamines and when it comes to tobacco, NNKs organ specificity for lung cancer in rats er especially notable [13]. NNN and NNK are classified as group 1(carcinogenic to humans) by IACR, while NAB (N-nitrosoanabasine) and NAT(N-nitrosoanatabine) are classified as group 3 (not classifiable as to its carcinogenicity to humans) [19]. A study conducted in 2011/2012 on about 50 different cigarette brands in the USA showed vide variation in TSNAs levels, but NNN and NAT were the most abundant across all brands [20]. This also applies to electronic cigarettes. In development and validation of a LC-MS/MS method, Kim and Shin (2013) analysed 105 brands of replacement liquids for electronic cigarettes. NNN, NNK, NAT and NAB were detected with a frequency of 93,3 % [21].

4.2 COMMON NITROSAMINES IN FOODS AND THEIR PRECURSORS

Since the acknowledgements of nitrosamines as possible carcinogens in the 1960's by Magee and Berners, extensive research has been done on the field of nitrosamines in foods. Their formation and metabolism have been well described by Rostkowska et. al. (1998). Nitrosamines occur in foods due to a reaction of nitrogen oxide with amines, and the sources of both are many because their precursors are found in among other pesticides, herbicides, and nitrogen fertilizers [6, 22]. Nitrite has been used as an additive in different foods, especially processed meats, because of its preservative properties and effects on taste and colour. Nitrite can also inhibit the growth of the bacteria Clostridium botulinum, which can produce botulinum toxin, a highly toxic compound to humans causing paralysis. The use of nitrite as additive in foods are now limited but is still allowed in restricted amounts as preservatives in some meats such as bacon [23]. In acidic conditions, nitrite can be hydrogenated to $H_2NO_2^+$ (nitrosooxonium) which then can react with another nitrite ion to form N_2O_3 known to react with secondary amines to form nitrosamines as shown above in Figure 4-2.

Typical food sources of nitrosamines are bacon, sausages, and hams, while non-processed meats have shown low or no amounts at all. They have also been found in fish, alcoholic beverages, and water (see section 4.4), but it is worth mentioning that the amounts in beers have decreased when changes have been done in the malting process. Nitrosamines are also present in cheeses as they are rich in secondary amines and some are also preserved with sodium nitrite [9, 22]. The seven most reported nitrosamines in foods, shown in Figure 4-3, are NDMA, N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR) and N-nitrosomorpholine (NMOR) [22].

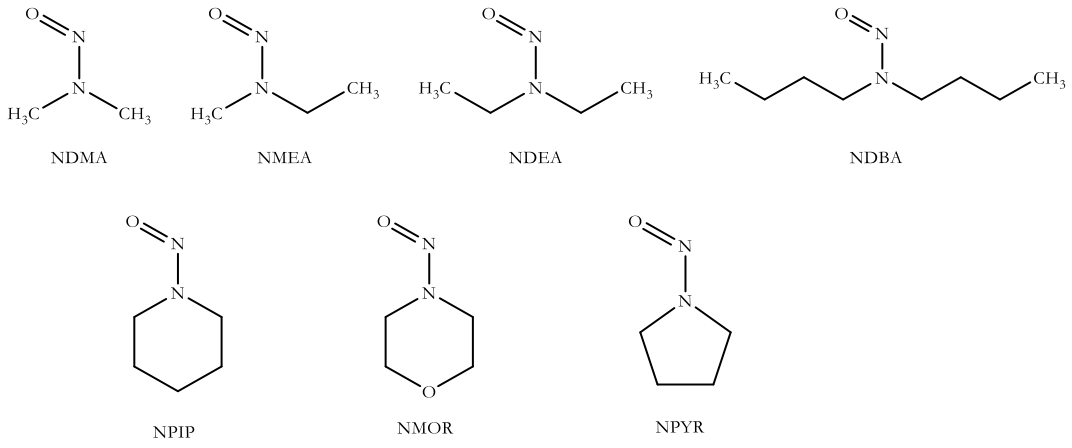


Figure 4-3. Structures of common nitrosamines found in food. Full chemical names are shown in the list of abbreviations, page vi. Drawn in Chem Draw Professional 16.0 according to CAS numbers.

Despite the well documented presence of different nitrosamines in food, it is not yet sure whether these levels are hazardous to humans. The common compounds in Figure 4-3 are listed by IARC as group 2A (NDMA and NDEA) and 2B congeners of nitrosamines [4, 9]. Different institutions have also set limitations on the content of some nitrosamines in food, presented in Table 4-2 [24].

Table 4-2 Limitation of nitrosamines by various institutions, collected from Bian, Y., et al (2020) [24].

Institution or country	Matrix	Standards limits
EPA[a]	Drinking water	NDMA: 7 ng/L, NMEA: 20 ng/L, NDEA: 2 ng/L
USDA	Cured meat products	Total volatile nitrosamines: 10 mg/kg
USDA	Fish and related products	NDMA: 4 and 7 mg/kg
USA	Retail products	NPYR: 10 μg/kg
Canada	Retail products	NDMA: 10 μg/kg
WHO	Fresh foodstuff	Nitrosamines: 0.002 mg/kg
WHO	Smoked foodstuff	Nitrosamines: 0.004 mg/kg
FDA and EMA[b]	Maximum acceptable amount per day	NDMA: 96 ng/day, NDEA 26.5 ng/day

[a] Environmental Protection Agency, [b] European Medicines Agency

Besides nitrite as a directly source of nitrosamine precursor, nitrate (NO_3^-, found in high amounts in many vegetables) can also be reduced to nitrite by microorganisms, making it impossible to reduce the amount of nitrite in foods entirely [23]. Microorganisms also play an important role in the formation of nitrosamine precursors. In fermentation processes, microorganisms can degrade proteins to secondary amines, and creates acidic conditions which is beneficial for the nitrosation reactions. The optimum pH for nitrosation is 3-4, and endogenous formation of nitrosamines in the human stomach has been reported (see section 0).

The levels of nitrosamines ingested by humans can also vary due to differences in cooking methods and diets. Higher temperatures have shown to accelerate the amount of NPYR in bacon. Higher amounts of NPYR and NDMA have been detected in fat tissue of bacon compared to the lean tissue when cooked. This have been attributed to

the non-polar lipid phase in the fat tissue that provides a suitable environment for nitrosamine formation [25, 26]. Nitrosamines generally form at elevated temperatures and are detected after processing, e.g. smoking, baking and frying of different foods [24, 27].

4.3 Nitrosamines in Personal Care Products and Their Precursors

In 1977 Fan *et. al.* investigated the presence of N-Nitrosodiethanolamine (NDELA), now classified in group 2B by IARC, in a variety of common personal care products in the USA. Products such as shampoos, lotions, and different cosmetics was analysed and the presence of NDELA varied from 1 to 48,000 ng/g. These findings were alarming because although intake of nitrosamines through human skin was not well investigated, at this point NDELA was known to induce liver tumours in rats and to be carcinogenic to all species that had been tested so far. The estimated daily use of a shampoo, a lotion and a cosmetic product was suggested to give a possible daily exposure to NDELA of about 3 μg, 2 μg, and 50-100 μg, respectively. The contaminating source to the formation of NDELA was assumed to be a reaction of diethanolamine (secondary amine), or triethanolamine (tertiary amine) commonly used as an emulsifiers in industry, with a nitrosation agent [28].

The permeability and absorption of NDELA through human skin was soon investigated. Three frequent ingredients assumed to be important carriers of NDELA through the skin (water, propylene glycol and isopropyl myristate) showed a greater absorption when the more lipoidal isopropyl myristate was used as a carrier [29]. Another study by Edwards et. al., measuring the amount of NDELA in human urine after application of a cosmetic product, found a total of 17.3 μg after 21.5 hours. This finding clearly demonstrated the absorption of NDELA through the skin when using cosmetics. It was also stated that their findings were biased towards low, suggesting that the actual levels of NDELA in the urine were higher than 17.3 μg [29, 30].

4.4 Nitrosamines in Waters and Their Precursors

NDMA was the first discovered nitrosamine in waters, in 1994, Canada [31]. Other nitrosamines are now also detected, such as NMEA, NMOR, NPIP, NDEA and more. The main sources of nitrosamine formation are nitrogen in wastewaters from several industries, and has been a growing problem du to agricultural, industrial, and municipal wastewater [7, 31]. In aquatic ecosystems, nitrogen has a significant effect and is one of the most important elements not only for the water quality, but also for the organisms living in these ecosystems. A change in the amount of nitrogen in waters poses a threat to the entire ecosystem and to human health. The formation of nitrosamines occurs both in the aquatic ecosystems and within the living organisms there, which makes nitrosamines precent in both drinking water and in foods obtained from these ecosystems. The stable chemical property of secondary nitrosamines also makes them persistent in the environment [7].

A wide variety of industries contribute to the build-up of nitrosamine precursors in waters, including pharmaceuticals receiving increased attention as a newly discovered source. Pedemonte et al. have presented the amount of nitrogen compunds from different industries, presented here in Table 4-3. The increased amount of nutritients in waters can lead to eutrophication, a frequent form of water pollution, and a significant source of nitrosamine formation [7, 32].

Table 4-3 Amount of nitrogen compounds [mg/L] in wastewaters from different industries, collected from Pedemonte et al. (2020) [33].

Indusrty	Nitrogen compounds [mg/L]
Poultry slaughterhouses	10-190
Distillery	1200
Brewery	25-35
Seafood Processing	25-405
Dairy product	60-310
Personal care and pharmeceutical products	15-130
Meat processing	90-700

In disinfection of drinking waters with high levels of nitrogen compounds chlorination can also lead to nitrosamine formation. The mechanisms behind this have been demonstrated by Park et al. (2009) to be a degradation of polymers to dimethylamine, which is easily nitrosated to NDMA as demonstrated in Scheme 3-2 [34]. Ozonation is another water treatment which has shown to increase the NDMA levels in drinking water. A common by-product in this method is formaldehyde, thought to be a catalyst in in the nitrosamine formation. Yang et al. have also proposed that the nitrogenous intermediates hydroxylamine and N_2O_4 play important roles in nitrosation reactions during ozonation [35]. The removal of nitrosamines is complex, and it has been shown to be more effective and cheaper to prevent NDMA formation by degrading or destroying the precursors [7, 31].

4.5 Nitrosamines in Pharmaceutical Products and Their Precursors

The possibility of nitrosation in pharmaceuticals was discussed already in the early 1970's. This concern led to research on possible endogenous nitrosation, and in 1978 the presence of NDMA in aminopyrine-containing drugs was detected, and later confirmed in 1980 by Castegnaro, Pignatelli and Walker in their analysis of volatile N-

nitrosamines in commercial drugs [36]. Using GC-TEA (Gas Chromatography-Thermal Energy Analysis) they analysed drugs containing aminopyrine, oxytetracycline and disulfiram. NDMA was detected in levels ranging from 1-900 μg/kg in thirty-seven aminopyrine containing products and not detectable (ND) to 7.0 μg/kg in nine oxytetracycline containing products. NDEA was detected in disulfiram containing products ranging from 94-980 μg/kg, and it was suggested that further epidemiological studies of N-nitrosamine in humans should include possible nitrosation in drugs.

In the past few years there has been several product recalls due to the presence of nitrosamines in drugs, and the issue has gained increased attention. Several batches of Valsartan were recalled in 2018 due to NDMA contamination, apparently first discovered by incident when performing other tests [5, 27]. In September 2020 FDA (U. S. Food and Drug Administration) published a guidance document for control of nitrosamine impurities in human drugs. They have presented acceptable intake limits of nitrosamine impurities in drugs presented in Table 4-4 [5]. In Bharate's *Critical Analysis of Drug Product Recalls due to Nitrosamine Impurities* (2021) it was found that FDA's database showed more than 1400 product recalls the last two years due to the presence of nitrosamine impurities [3].

Table 4-4 FDA's acceptable intake limits for NDMA, NDEA, NMBA, NMPA, NIPEA, and NDIPA from active pharmaceutical ingredients [5].

Nitrosamine	Acceptable intake limit [ng/day]
NDMA	96
NDEA	26.5
NMBA	96
NMPA	26.5
NIPEA	26.5
NDIPA	26.6

Common active pharmaceutical ingredients (APIs) associated with the nitrosamine impurities are valsartan, irbesartan, losartan, metformin, ranitidine, and nizatidine, shown in Figure 4-4 [3]. Parr and Joseph lists a total of 14 API structures reported to contain NDMA, and 19 additional NDMA precursors [27]. Most of the product recalls have been due to the presence of NDMA, NDEA and NMBA (*N*-Nitroso-N-methyl-4-aminobutyric Acid) and is related to the API production or storage and packing conditions. FDA also suggest four more impurities that theoretically can be present in pharmaceutical products, namely NMPA (N-Nitrosomethylphenylamine), NIPEA (N-Nitrosoisopropylethylamine), NDIPA (N-Nitrosodiisopropylamine) and NDBA, where NDIPA and NDBA are the only ones not yet detected in drug substances. About 81%

of the product recalls in Bharate's analysis contained "sartans", which are a common API in drugs used to treat high blood pressure and is especially useful for patients also diagnosed with diabetes [3, 37].

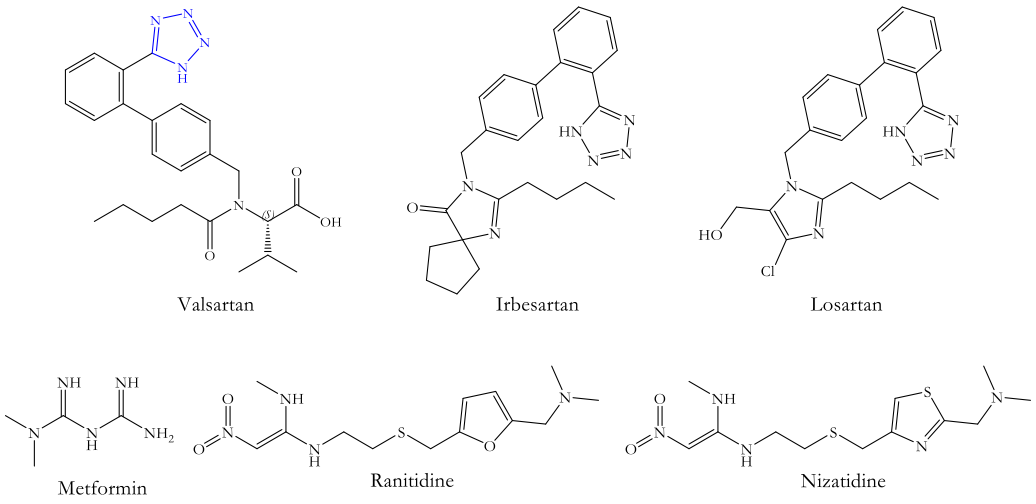


Figure 4-4 Common active pharmaceutical ingredients products associated with nitrosamine impurities in pharmaceutical products, inspired by Bharate (2020). The tetrazole ring in sartans, connected to the use of nitrous acid, is marked as blue on Valsartan [3].

FDAs guidance for industries suggests several causes for nitrosamine impurities in APIs. Process conditions where both nitrites and secondary, tertiary, or quaternary amines are present under acidic conditions make nitrosation possible, as shown in Scheme 3-2 of nitrosation of a secondary amine. Due to possible carryover into subsequent steps, there will be a risk for nitrosamine impurities under these conditions despite purification steps in the manufacturing process. In many API manufacturing processes, nitrous acid is also used for removal of reagents in the presence of nitrosamine precursors such as amines. This will increase the risk for nitrosation, and an example of this is the removal of azide (N_3^-). Azide is commonly used in the formation of the tetrazole ring present in many sartans, and marked as blue on Valsartan in Figure 4-4 [5]. In Valsartan production dimethylformamide (DMF) have been used as a solvent and appears to be the main source of NDMA formation, as illustrated in Figure 4-5. DMF can degrade to dimethylamine, especially under high reaction temperatures, which is easily nitrosated to form NDMA [27].

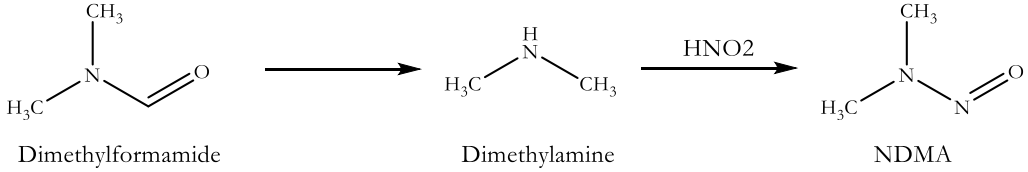


Figure 4-5 Formation of NDMA from DMF (used as a solvent in Valsartan production), inspired by FDA's Guidance for Industry [5].

Since amines have a wide range of applications as reagents, their sources are many. Secondary and tertiary amines can be present as functional groups on the API, intermediates or regents, and tertiary and quaternary amines is sometimes used as reagents or catalysts. Secondary amines can also appear as impurities in amide solvents or be formed when amide solvents degrade under certain conditions (such as DMF). Among multiple impurity sources FDA also highlights the possibility of contaminants coming from raw materials from vendors, recovered materials, inadequate cleaning procedures and degradation during storage [5].

5 ENDOGENOUS NITROSAMINE FORMATION AND THEIR BIOLOGICAL EFFECT IN HUMANS

Besides the intake of nitrosamines formed exogenously, the total intake of nitrosamines is also affected by the endogenous formation. Because of this, it has been discussed that nitrosamines carcinogenic effect might be underestimated, and Tricker (1997) reported that the endogenous formation could contribute to 45-75% of the TNA burden [38].

The issue of endogenous formation of nitrosamines and the possible carcinogenic hazard was discussed already in 1985 by Challis [39]. Since the mildly acidic condition of the stomach is suitable for nitrosation, the precursors of nitrosamines have also gained attention [39]. Rostkowska et al. described the metabolism of nitrosamines in detail in 1998 [6]. They pointed out that the active intermediates from degradation of nitrosamines are important from a toxicological view due to their carcinogenic activity. In short nitrosamines go through biotransformation in several parts of the body, including the liver, intestine, lungs, kidney, brain, skin, and placenta. Microorganisms and enzymes are responsible for the initial phases of the biotransformation at first mainly involving hydroxylation and dealkylation reactions. In the next phase polar metabolites are formed in reactions with specific enzymes. During biotransformation of nitrosamines a very reactive carbocation is formed, which causes the possibility of a complex mixture of products [6]. Besides endogenous formation in the stomach, in vivo formation may also happen due to the production of nitric oxide (NO) by intestinal bacteria and inflammatory cells, leading to nitrosation of secondary amines [3].

The carcinogenicity of TSNAs are now well documented, and besides inhalation the precursors can also be subjects to nitrosation by nitrate found in saliva [15]. TSNAs are activated by several P450 enzymes when the body try to detoxify and solubilise them. This generates DNA-reactive diazoalkane metabolites. These metabolites can eventually cause the formation of DNA-adducts, compounds bound to the DNA-molecule and preventing precise DNA-replication, and therefore often causing carcinogenic mutations [3, 40, 41]. It has also been shown that nitrosamines can damage and cause mutations in the p53 gene. p53 is a gene-regulating protein important in the regulation of the cell cycle, and when damaged the cell growth can become uncontrolled, causing tumours [3, 42]. Specifically, Cheng et al. (2015) have suggested that NNK suppress the gene for lysyl oxidase (LOX) which inhibit tumour growth [43].

6 ANALYTICAL METHODS FOR NITROSAMINE DETECTION AND MONITORING

6.1 ANALYTICAL PRINCIPLES IN CHROMATOGRAPHY

The term chromatography was first introduced by Mikhail Tswett in1906, and today multiple techniques are developed on the concept of separating sample components in a column. The basic principles in all chromatography are transport of a sample through a column carried by a mobile phase. The column consists of a stationary phase, and different components in a sample travel through the column at different speed due to different interactions with the stationary phase. The components will elute from the column at different times, where components retained most by the stationary phase will elute at last. The time from injection to elution and detection is referred to as the retention time (t_R). The t_R for a compound can vary within the same method (e.g., GC, HPLC) because it is affected by factors such as flow rate, column length, and temperature. Before different compound can be accurately analysed it is therefore necessary to develop and validate a method for its' intended use [44].

In method validation repeatable t_R is required, and many parameters must be investigated such as concentration limit of detection (cLOD), concentration limit of quantitation (cLOQ), linearity, repeatability, stability, robustness and more. Sample preparation (preconcentration and purification) is necessary, and the properties of solvents, mobile phase and stationary phase have to be considered carefully to fit the sample of interest, and intended goal of the analyse [44].

6.2 COMMON PRE-TREATMENTS OF NITROSAMINES BEFORE ANALYSIS

Sample preparation can be a crucial and time-consuming part of an analysis. It is necessary to remove interfering components in the sample, gain a concentration high enough for detection in sample sizes compatible with the separation system and remove components that can damage the system [44]. The more complex sample, the more steps are typically necessary for extraction and purification to reach the LOD (limit of detection) for a separation system and increase the accuracy and precision of the analysis [24]. Bian, Y., et al.(2020) have investigated the trends and procedures in pre-treatment of nitrosamine samples since 2010 and found that common pre-treatmenst include solid-phase extraction (SPE), liquid-liquid extraction (LLE) and various microestraction methods such as solid-phase microextraction (SPME) and liquid-phase microextraction (LPME).The most popular technique is SPE, and its common steps are shown in Figure 6-1. SPE have simple operation steps, is effective in preconsentration, have low costs and time consumption and automatical technologies are developed. The most popular sorbent in SPE of nitrosamines, also proposed in EPA method 521 (Environmental Protection Agency), is activated carbon [24].

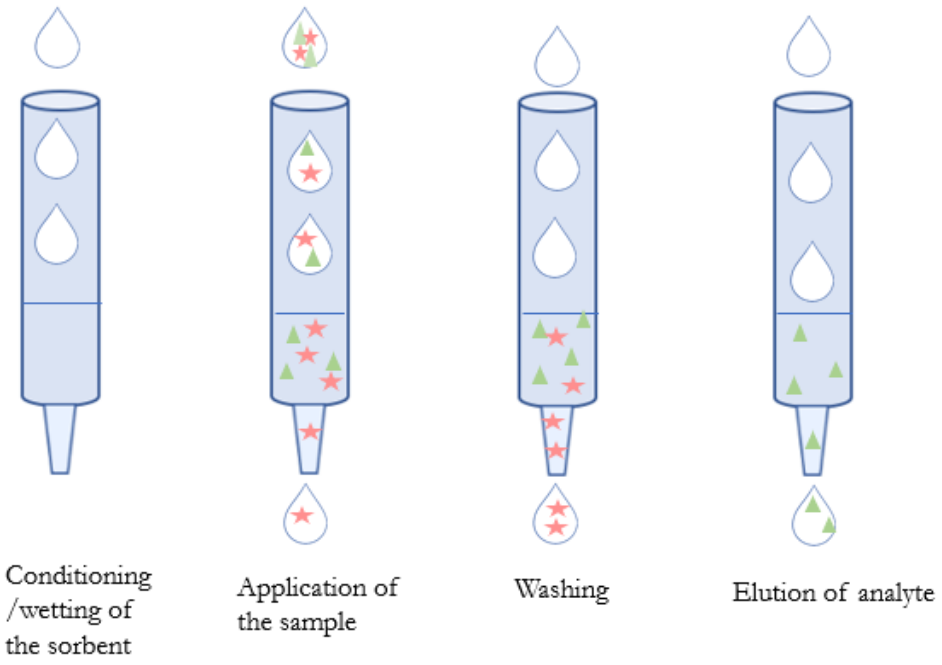


Figure 6-1 Common steps in solid phase extraction (SPE), inspired by Bian et al. (2020) [24].

In sample preparation on nitrosamines, one must be aware of the possibility for both under- and overreporting of the nitrosamine's concentration due to the preparation method. DCM is the most prominent solvents in extraction of nitrosamines and can contain NDMA. Together with nitrosation of amines already present in the sample, this can lead to an increase in the reported nitrosamine concentration [27].

On the other hand, nitrosamines are pH unstable and prone to photolysis, which can cause an underreporting of the nitrosamine concentration in samples [1, 27]. In choosing extraction method it is also necessary to be aware of the fact that nitrosamines can be both volatile and non-volatile. The volatile nitrosamines can be extracted by distillation and analysed on gas chromatography, while the non-volatile cannot [45].

6.3 Gas Chromatography (GC) on Nitrosamines

In detection and monitoring of nitrosamine GC is the most reported method in literature. Moderate to high polarity stationary phases are used. FDA end EPA recommend using a DB-wax column or HP-5 column, respectively, with DB-wax columns being ideal for analytes with low boiling points [27, 46]. GC-TEA (gas chromatography – thermal energy analyser) is frequently used in detection of nitrosamines [27]. A simplified overview of a TEA-detector is depicted in Figure 6-2. In the TEA-detector nitrosamines are pyrolyzed, which releases nitric oxide (NO) as the N-NO bond breaks. All nitrosamines can decompose on heating, but a relatively high temperature is required for nonaromatic compounds (ca. 300°C) [1, 47]. Ozone (O_3) is

used to oxidize NO in a vacuum chamber, generating electronically excited NO_2. When NO_2 falls back to its ground state, the emitted radiation is detected by a light detector. The detected emission is proportional to the concentration of the nitrosamine concentration, and the TEA-detector is highly sensitive and selective for nitrosamines, showing low LOQs [27, 47].

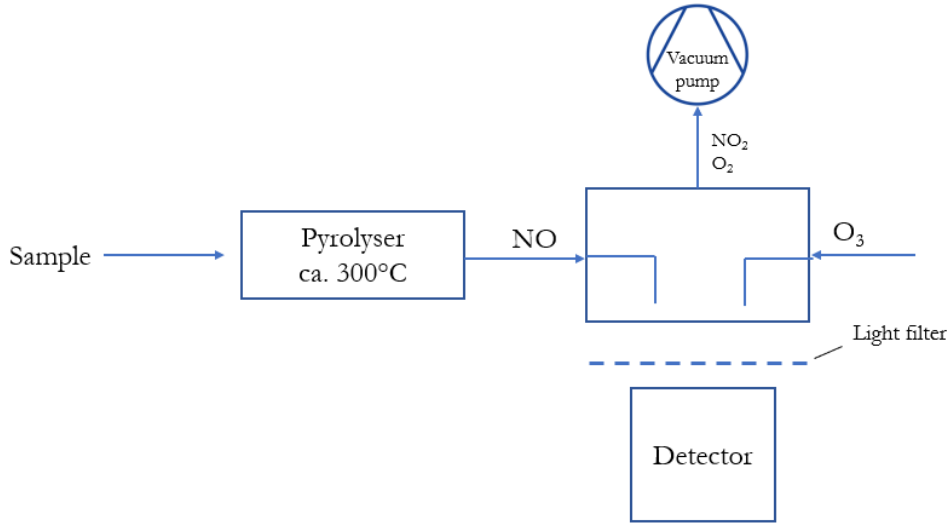


Figure 6-2 Simplified scheme of a Thermal Energy Analyzer (TEA), inspired by Fine, Lieb and Rufeh (1975) [48].

The use of headspace injection (HS-injection) has shown to give better results than the use of split/splitless injectors and given very low LOD and LOQ for NDMA, but this method needs to be further developed [27, 49]. HS-injection is a common technique in analysing volatile analytes in aqueous samples. In short, a gas is used to transport the volatile compounds to a trap prior to injection on the GC-column [44].

Other frequently GC-methods used are GC-MS and GC-MS/MS, especially on monitoring of drinking water. GC-MS/MS has shown very low LOD when connected to positive chemical ionization (PCI). When compared to GC-MS, GC-MS/MS has shown 100-times lower LOD on nitrosamine detection in environmental water, with 0.5 µg/L compared to 50.0 µg/L in GC-MS [27]. So far there are few publications on the issue of NDMA-detection in drugs. FDA have demonstrated good results in Valsartan analyses using HS-injection coupled with GC-MS, and electron ionization detection, showing low LOQ of 0.3 mg/kg. Besides this, GC-MS with the standard addition method and HS-GC-MS, have also been used in analysing pharmaceutical products for nitrosamines [27].

6.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ON NITROSAMINES

As an alternative to GC, HPLC methods on reverse phase columns (hydrophobic) are also used in nitrosamine analysis, with RP, C-8 and C-18 columns being popular [27]. Coupled with a diode array detector (DAD, a UV-detection method) at a wavelength of 233 nm, Li et al. showed LODs for nitrosamines in food in the range of 0.08-0.55 µg/kg [50]. An effective reverse phase HPLC-UV method have also been validated and separated volatile N-nitrosamines, with LODs and LOQs presented in Table 6-1 [51].

Table 6-1 LODs and LOQs of nitrosamines analysed with a RP-HPLC-UV method by Al-kaseem, Al-Assaf and Karabeet (2014). Collected from Al-Kaseem et al. (2014) [51].

Compound	LOQ [µg/mL]	LOD [[µg/mL]]
NDMA	1.616	0.48
NDEA	2.029	0.61
NDPA	1.097	0.33
NDBA	1.550	0.46
NPIP	1.011	0.30
NPYR	1.775	0.53

Considering pharmaceutical, HPLC-APCI-MS/MS (atmospheric-pressure chemical ionization) and a HPLC-UV method have been used in analysing nitrosamines. The latter are so far the most successful method with the lowest LOD of 0.1 mg/kg, quantified using standard addition [27].

7 SYNTHETICAL METHODS FOR NITROSAMINE REFERENCE MATERIALS

7.1 HOW TO ENSURE HIGH QUALITY REFERENCE MATERIALS

The accuracy of an analysis is depended on among other the purity of involved reagents. To meet this need different suppliers develop and sell reference standards (RS), which are substances that have been carefully analysed according to accreditation guidance's [52]. Reference standards are developed to fit intended uses and often to meet the need of customers. They need to be of high purity, be sufficiently homogenous and stable considering specific properties [53]. Culbert and Johnson (2004) have based on the literature presented a broad definition for RS:

> A reference standard is broadly defined as certified material or substance, supplied by a certifying body, which exhibits one or more properties that are sufficiently well established (and assigned) that it may be used for calibration of an apparatus, assessment of a measurement method, and assigning values to materials (p. 120) [54].

RS can be categorized into analytical reference standards (ARS), working reference standards (WRS), and authentic materials (AM) [54]. They are only described in short here, and the main difference between them are their level of purity. ARS have the highest purity (> 99.5%), and can also be referred to as primary standards because their purity is established without reference to other compounds [54]. They are highly purified which the accuracy of many methods relies on. Besides high purity some other requirements are atmospheric stability, absence of hydrate water (to avoid composition changes), relatively large molar mass and good solubility. ARS are thoroughly analysed so that there is a complete understanding of their properties and identity (characterization, stability, purity etc.) [52, 54]. The purity can be mathematically calculated with Equation 7.1, given by Culbert and Johnson in their description of how to assign purity to an API. MW_{API} is the molecular weight of the API, and MW_{DS} is the molecular weight of the drug substance.

$$Purty_{theory}\% = \frac{MW_{API}}{MW_{DS}} \cdot 100\% \tag{7.1}$$

WRS have lower purities, though typically also high (> 95%), and can be referred to as secondary standards because their purity is usually established with reference to primary standards. They can be used for standardization and analytical comparisons, and are often the only available RS, especially for relatively newly discovered compounds. AM are also qualified for many analytical purposes, but their purity only needs to be >80% and are not used in quantitative analyses.

The ARS have the highest requirements in qualification processes, but the guidance documents and processes provided by different institutions apply to all RS. When suppliers develop RS the International Organization for Standardization (ISO) also differ between RS and certified RS. The certified RS have undergone validating

procedures for one or more properties, and holds a certificate with values of the specific properties and associated uncertainty, including a metrological traceability [53, 54]. When methods and materials are certified by e.g. ISO, the individual companies are responsible of developing their own procedures to assure inspectors they are according to regulations and guidance's [54].

Reference standards of nitrosamines are developed for analysis and monitoring, and to ensure high quality they can be developed according to procedures set by the ISO, in addition to internal quality assurance procedures within companies. Being accredited according to ISO-standards is one way a company can ensure their costumers they are competent suppliers of RS [55]. ISO/IEC 17025:2017 provides an overview over *General requirements for the competence of testing and calibration laboratories*, while NS-EN ISO 17034:2016 provides *General requirements for the competence of reference material producers*. The latter includes general requirements on among others production control, material handling and storage, measuring equipment, characterization and control of quality and technical records [53, 56]. In Table 7-1 common techniques used in the qualification process of RS are listed, where GC and HPLC are techniques also discussed in section 6 about detection and monitoring of nitrosamines. In the development of reference materials nuclear magnetic resonance (NMR) is a technique worth mentioning. NMR is based on physical concepts and the magnetic fields associated with charged particles, and is a useful method in determining purity, structure, and physical properties of compounds [57].

Table 7-1 Common techniques in the qualification of reference standards, collected from Culbert and Johnson (2004) [54].

Appearance	Purity	Identification	Assay
Visual inspection	Loss on drying	Elemental analysis	Titration
Optical microscopy	Karl Fischer titration	UVA/Visible spectroscopy	Phase solubility analysis
	Residue on ignition	Infrared spectroscopy	
	Thermogravimetric analysis	Raman spectroscopy	
	Differential scanning calorimetry	X-ray diffraction	
	Ion chromatography	High-resolution mass spectrometry	
	High-performance liquid chromatography	Nuclear magnetic resonance spectroscopy	
	Electrophoretic separations	Optical rotatory dispersion	
	Supercritical fluid chromatography		
	Thin-layer chromatography		
	Chiral chromatography		
	Gas chromatography		

7.2 SYNTHETICAL METHOD FOR NDMA REFERENCE STANDARD

Zolfigol et. al (2000) have presented efficient methods for nitrosation of secondary amines in the presence of $NaNO_2$ (sodium nitrite), wet SiO_2 (silika), silica supported inorganic acids or inorganic salts [58]. Isolated yields of 100% of NDMA was obtained when the inorganic salts $Mg(HSO_4)_2$ or $NaHSO_4 \cdot H_2O$ were used as solid acid reagents, and the suggested method for NDMA synthesis presented here is collected from and inspired by their work. Reaction conditions are listed in Table 7-2 and the use of both inorganic salts as reagents followed the same general procedure. The procedure requires only normal glassware in organic chemistry labs, a magnetic stirrer, and is conducted at room temperature.

Table 7-2 Reaction conditions for nitrosation of dimethylamine to NDMA in the presence of $NaNO_2$, an inorganic salt in wet SiO_2 (50% w/w), and dichloromethane [a] [58].

Inorganic salt/reagent	$\boldsymbol{Reagents\ [mmol]}$		Time [h]	Yields [%]
	$\boldsymbol{Salt}$	$\boldsymbol{NaNO_2}$		
$Mg(HSO_4)_2$	40	20	1	100
$NaHSO_4 \cdot H_2O$	20	20	1	100

[a] All physical data have previously been published by and are collected from Zolfigol et. al. (2000).

Dimethylamine (10 mmol), sodium nitrite and solid acid reacted in DCM (20 mL) and wet silica (50% w/w, 2.0 g) and were put on vigorously magnetically stirring at room temperature. The reaction was followed by TLC (thin layer chromatography), and after completion silica gel (5 g) and petroleum ether (20 mL) were added. Unwanted precipitates could then be removed through filtration and washed with petroleum ether and DCM (1:1, 20 mL). To isolate NDMA from the solution, it was evaporated, and for further purification (if necessary) flash chromatography is suggested [58].

A similar procedure has been demonstrated by Niknam and Zolfigol but with 1,3-dibromo-5,5-dimethylhydantoin (DBDMH) and 1,3-dichloro-5,5-dimethylhydantoin (DCDMH) rather than $Mg(HSO_4)_2$ and $NaHSO_4 \cdot H_2O$ [59]. The yield was maintained at 100%, smaller amounts of reagents were necessary, and the time was markedly reduced to 5 minutes.

7.3 SUGGESTION FOR SYNTHESIS OF NNK REFERENCE STANDARD

The suggested procedure for NNK synthesis here is based on the scientific work of Tian et al. (2014), and Pathak et al. (1990), and consist of 3 overall steps presented in Figure 7-1 [41, 60]. These steps are collected from the retrosynthetical plan generated in SciFinder^n, attached in appendix C.

Step 1

The first step in this suggested NNK production is the synthesis of l-methyl-3-nicotinoyl-2-pyrrolidone **(3),** described by Tian et al. and giving a yield of 81% [60]. Prior to the first step shown in Figure 7-1 they synthesised ethyl nicotinate **(2)** by the following procedure: They first dissolved nicotinic acid (10 g) in ethyl alcohol (80 mL). Concentrated sulfuric acid (10 mL) was added gradually before heating the mixture in an oil bath at 85°C for 4 hours. Distillation was used to remove parts of the ethyl alcohol. After cooling on an ice water bath and adjustment of pH to 7-8 with concentrated NaOH, the ethyl nicotinate product was extracted using ethyl acetate (400 mL). The product was further washed with sodium bicarbonate and dried over anhydrous sodium sulphate, giving 10.3 g of oily ethyl nicotinate product.

Without further purification all the ethyl nicotinate was dissolved together with 1-methylpyrrolidin-2-one (**1**, 6.5 mL) in dry toluene (200 mL) [60]. Sodium hydride (4 g) was added, and the solution was stirred for 30 hours under reflux, a method commonly used to increase the rate of the reaction without loss of vaporising reagents [60, 61]. The solution was cooled to room temperature (20-25°C) before adding methanol (5 mL), pouring it into diluted HCl (1 mol/L, 70 mL). The product (compound **3**) was extracting with CH_2Cl_2 and washed with saline. After concentrating 11.3 g of oily product was obtained.

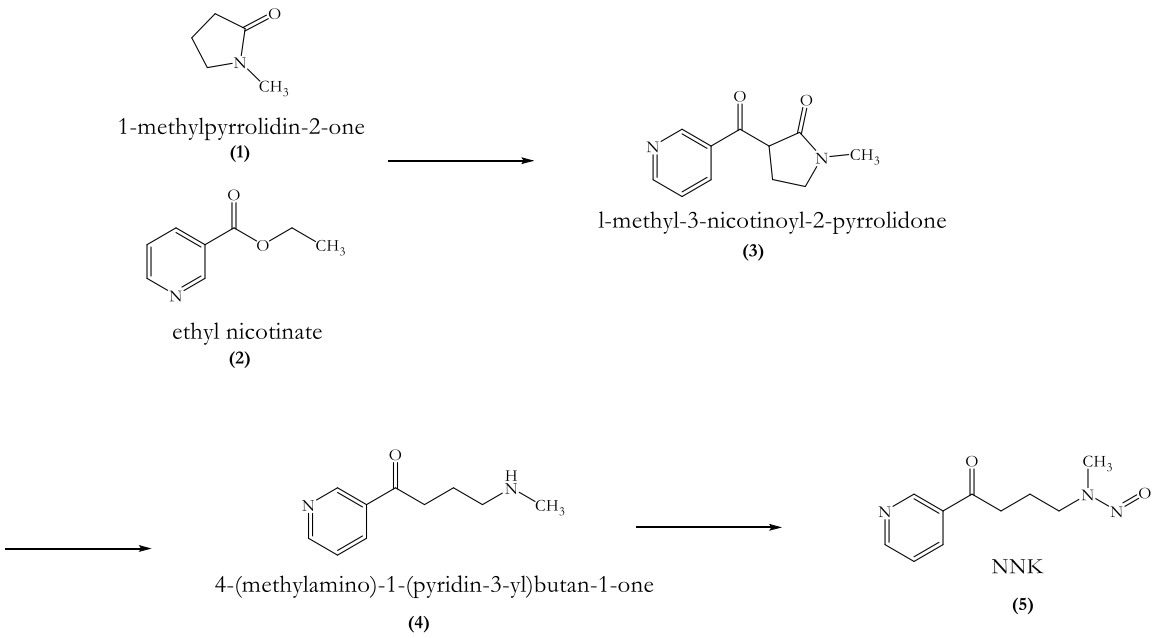


Figure 7-1 Overall procedure steps for NNK synthesis, inspired by the retrosynthesis plan from SciFinder[n], which is depicted in Appendix C.

Step 2

l-methyl-3-nicotinoyl-2-pyrrolidone **(3)** obtained from the procedure of Tian et. al. can be further applied in step 2-3 based on the work of Pathak et al. (1990) [41]. Their procedure is based on the research of Hecht (1983) with moderation towards milder conditions by using lower molarity of HCl and a decrease in refluxing time.

Compound **3** (2 g) in HCl (1M, 100 mL) was heated on reflux for 72 hours, cooled down and evaporated for drying. The remaining solid was dissolved in water before filtration, concentration and recrystallisation from a methanol-ether mixture. This gave a yield of 87% of 4-(methylamino)-1-(pyridin-3-yl) butan-1-one **(4)**.

Step 3

In the final step compound **4** (1.8 g) was dissolved in water (25 mL) and cooled to 0°C, before adjusting the pH to 4 with NaOH (1M). $NaNO_2$ (12 g) in water (17 mL) was added dropwise and the solution was put on stirring at room temperature (20-25°C) for 16 hours. CH_2Cl_2 (5x15 mL) was used for extraction before washing with NaOH (4M, 2x15 mL) and then water (3x15 mL). $MgSO_4$ was used for drying before concentrating the solution in vacuum. This gave an oil they recrystallized from CH_2Cl_2 and gave a yield of 43%.

8 DISCUSSION AND FURTHER PERSPECTIVES

8.1 HUMAN EXPOSURE TO NITROSAMINES

As presented in this review nitrosamines occur in numerous places in our environment, and the sources of human intake are so widespread that a complete avoidance seems highly unlikely. It is clear though, that lifestyle choices can have a huge impact on the daily intake of nitrosamines [8]. As presented in Table 8-1, tobacco products are by far the biggest contributor to the daily nitrosamine intake. Tobacco is also the source of NNN and NNK, the only two nitrosamines classified in group 1 by IARC [4].

Table 8-1 Estimated daily intake of nitrosamines based on habits in the USA, collected from Gushgari and Halden (2018) [8].

Source	Amount nitrosamines [ng/day]
Tobacco products	21 800 ± 4350
Food	1800 ± 350[a] to 1900 ± 380[b]
Beer and other malt containing beverages	1000 ± 200
Portable water	120 ± 24

[a]Vegetarian diet. [b]Western diet

Lifestyle choices put aside, the World Health Organization (WHO) have estimated 8 million deaths each year because of tobacco consumption, and it is a worldwide social concern [62]. As stated in section 4.1, changes in the curing process can lower the TSNA levels in tobacco. Despite this, a study from 2012 investigated new popular tobacco products (cigarettes) over the last decade. They could not find any substantial measures to reduce or control the TSNA levels in these brands [16, 63]. Stephen Hecht, which has been a great contributor to the research on nitrosamines in tobacco, put this issue in perspective by comparison with how the food industry have handled nitrosamines in cured meat. After concerns about nitrosamines in cured meat and beers, the levels were controlled and lowered to generally less than 10 ppb. Hecht promotes that control and modification of processing methods for tobacco are also possible, and that cigarettes should be produced with 100 ppb or less of NNN and NNK. A requirement to reduce levels of carcinogens would probably also be the case if other products of human consumption contained such high levels of carcinogens, as tobacco does of NNN and NNK [64].

The possibility of reducing TSNA levels in tobacco and the health benefits from this can also be demonstrated by a recent study on smokeless tobacco, which showed a great difference in the related global disease burden. South and Southeast Asia accounts for >85% of the disease burden associated with the use of smokeless tobacco products. The products in South Asia are also considered to pose a higher health risks compared to similar products in Nordic countries, where many toxins are removed from the final

product. As an example, Swedish snus (tobacco product) contains TSNAs ranging from 601-723 (ng/g), while the common Southeast Asian product Khaini contains 21 600 – 23 900 (ng/g) [65].

Validated methods for analysis of TSNAs are already available, e.g. LC-MS/MS [21]. With possibilities to reduce the TSNA levels in the production of tobacco products, and reference standards being available from multiple suppliers (53 according to NNK retrosynthesis, appendix C), a responsibility lies with both tobacco producers and controlling institutions for guidance and monitoring. In 2008 WHO did in fact recommend mandated lowering of NNN and NNK to 0.114, and 0.072 μg/mg nicotine, respectively, in international brands [66]. This was though, prior to the 2012 study mentioned here, and Hecht's assertion on the need for regulation of TSNAs in tobacco in 2014. This indicates a slow response from the industry and regulatory agencies responsible for guidance and setting requirements. This is also in contrast to the pharmaceutical industry which have drawn back a large amount of nitrosamine contaminated drugs. [3] The intake of vital drugs is not a lifestyle choice, and the safety of patients are important. The different approaches to nitrosamine contamination different industries have though, are still noteworthy.

The values collected in this review presented in Table 4-2 do not contain values for NNN and NNK, or limitations for tobacco products. FDA's acceptable intake limits of nitrosamines from APIs, presented in Table 4-4, are calculated based on the carcinogenicity in rodents and explained in their guidance for industry [5]. Both limitation values in products and acceptable intake limits are important for all nitrosamines occurring in sources of human exposure. They should be easily available for both the industry and consumers, e.g. in WHO's information note on nitrosamines. In addition, the acceptable intake limits from FDA does not take into account the possibility of more than one nitrosamine being present in a drug [5]. In further development of acceptable intake limits of nitrosamines, it is important to not neglect the possible total nitrosamine burden being higher.

Choosing a lifestyle without tobacco products and malt containing beverages will severely decrease the daily nitrosamine intake. Looking at Table 8-1 they are still very present also in foods, even in vegetarian diets though processed meats have been assumed to be the main source of nitrosamine in this matrix [8, 22]. Cooking methods have also shown to affect the amounts of nitrosamines in foods [25, 26], but if this is the case for vegetarian diets needs to be further investigated. The values in Table 8-1 are collected from Gushgari and Halden's comprehensive review on human exposure to nitrosamines (2018), and as they point out the amounts of nitrosamines could be both over- and underestimated [8]. Most studies on nitrosamines in foods have treated non-detectable concentrations as zeros, causing a possible underestimation of the actual amounts of nitrosamines in foods. On the other hand, there has been reports of extremely high values of nitrosamines in foods. If the extreme cases are outliers and not

representative, the actual amounts of nitrosamines in foods may have been over-estimated [8]. Dietary habits are also very varied around the world, and the analysis of total nitrosamine exposure to humans are complex. The study by Fan et al. also showed a great variability in the amount of NDELA in different personal care products [28]. Guidelines considering limitation of nitrosamines in different sources of human intake, as presented in Table 4-2, therefore seems like a reasonably solution. This gives both the industry and monitoring institutions some requirements to comply, and may prevent extreme levels of nitrosamines to occur in some products in the future.

With that said about the occurrence of nitrosamines in our environment, it has been notified that endogenous formation can account for a significant part of the exposure to nitrosamines [8]. The biochemistry behind this has been well described, but the literature mainly focuses on the exogenous formation, and not on their precursors to endogenous formation [3, 6]. To better understand the total nitrosamine burden further research is necessary within endogenous vs. exogenous formation, and human exposure.

8.2 Limitations and Contributions of This Review to the Literature

The occurrence of nitrosamines has in this review shown to be a relatively widespread and comprehensive problem. Within the scope of this bachelor project some limitations have been done regarding the content. Nitrosamine's occurrence in the environment due to post-combustion CO_2 capture have been omitted from this review. In connection to CO_2 capture formation and occurrence of nitramines is also a related environmental problem. Both nitrosamines and nitramines form due to amine degradation in CO_2 capture technologies, and are by-products which could have been explained in more detail to get a better overview of the nitrosamine, and nitramine occurrences [67]. A high concentration of nitrosamines are also measured in the rubber industry, which is not mentioned in this review. Studies on rubber workers have shown an increased mortality due to cancer, and there is also a need for monitoring of nitrosamines within this industry [68].

This review has also mainly focused on the formation of nitrosamines exogenously, and because of nitrates and amines present in the environment or production processes. Other contaminating sources of precursors have been discussed in the literature. As an example, rubber gloves used to handle foods and pharmaceuticals could be sources of nitrosamine contamination, and increase the human intake [69]. When seafood was analysed before and after storage in refrigerators for 72 hours, an increase in NDMA levels was detected. Inappropriate storage conditions of pharmaceuticals have also been discussed as a possible source of nitrosamines [3, 70]. A further investigation of other possible contaminating sources and the effect of storage conditions would be an interesting approach. This could maybe contribute to a broader understanding of nitrosamines presence in products of human intake, and where their precursors occur and form into nitrosamines.

Another limitation in this review is that the proposed synthetical procedures have not been carried out by the author to ensure the actual results. This would be especially interesting in the proposed plan for NNK synthesis. With that said, the synthetical plans from SciFinder^{n} is based on well-established evidence from experienced scientists, and are considered as reasonable suggestions [41, 58, 60].

This review has gathered information on some major sources of nitrosamines leading to human intake. The unnecessary high amount of NNN and NNK in tobacco products have been put into context with a reminder about the possibility for TSNA reduction, and the comparison of smokeless tobacco products in South Asia and Nordic countries [64, 65]. This will hopefully promote a debate on the requirements for nitrosamine reduction, where the total nitrosamine burden also is taken into account.

This review has also added sections on analytical methods which have shown good results, and presented procedures for syntheses of two common nitrosamines ingested by humans. The sections on analysis and synthesis are thought to give insight on how monitoring of nitrosamines can be done. As presented in section 7.2, Niknam and Zolfigol also improved a classical NDMA synthesis [59]. Hopefully, this will inspire to a further development of pure reference materials and method validations. This is essential in the development of guidelines and requirements for the industry, and a safe monitoring of the environment. Monitoring of nitrosamines presence in API synthesis has shown to be especially important to avoid post-drawbacks, and a shortage in the drug supply of vital medications [3]. When new drugs are launched the request for reference standards also typically increases dramatically [54]. Since nitrosamines in pharmaceutical products are now on the agenda, a maintenance of the reference standard supply is crucial for monitoring, and maintenance of safe products on the marked.

9 CONCLUSION

Nitrosamines are widespread in the environment and occur in numerous sources of human intake. Secondary nitrosamines are stable compounds persistent in the environment, and especially NNN and NNK from tobacco can cause tumour formation in humans. The response from the tobacco industry to decrease the TSNA levels, which is possible, have also shown to be slow. Most validated methods for analyses and monitoring are performed on GC, and HPLC for non-volatile compounds. GC-TEA and GC-MS/MS have shown good results in nitrosamine analyses. Methods for synthesis of reference materials are available through SciFinder^{n}. They can also be purchased from accredited suppliers, following e.g. ISO/ES standardisation procedures.

Some limitations for nitrosamine content in products and acceptable intake limits are set by different institutions. There is still a need for further development of limitation requirements for the industry. These should take the total nitrosamine burden into account, and compare endogenous vs. exogenous formation of nitrosamines. Validated methods for analysing and reference materials have shown to be especially crucial within the pharmaceutical industry to avoid drawback of widely used and vital drugs.

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APPENDIX A

Biomarker focus (BMF) text for Chiron AS

The suggested BMF text made for client (Chiron AS). Font is according to Chiron's other BMFs, but Chiron will add its own graphics and full list of compounds. Structures for a full nitrosamine list have been drawn in ChemDraw Professional 16.0 on behalf on the client. Only parts are added here as an example.

BMF [NR.] Nitrosamines and Nitramines | v? | mm/yy

Nitrosamines and nitramines

Nitrosamines are organic compounds shown to be persistent in our environment, with both known and suspected carcinogenic effect on humans.[1] Nitrosamines easily form when secondary amines are in the presence on nitrites under weakly acidic conditions. They are released into the environment from agricultural, industrial, and municipal wastewater.[2] They occur in products of human exposure through tobacco, food, personal care products, and waters. In recent years there have also been numerous drug product recalls due to nitrosamine impurities.[3]

Nitrosamines and nitramines are also released to air as by-products from CO_2 capture, where amines are commonly used as solvents. [5]

International Agency for Research on Cancer (IARC) have classified multiple nitrosamines as carcinogenic, probably, or possibly carcinogenic to humans.[1] Besides intake of nitrosamines formed exogenously, the endogenous formation of nitrosamines from their precursors are now also well described.

Two nitrosamines are classified as carcinogenic to humans, namely N-Nitrosonornicotine (**NNN)**, and nicotine derived nitrosamino ketone (**NNK)**. They are both tobacco specific nitrosamines (TSNAs) occurring in nicotine-containing products. Prominent in tobacco are also N-nitrosoanatabine (**NAT**) and N-nitrosoanabasine (**NAB**), so far with inadequate data on their carcinogenicity.[1] In the USA the nitrosamine intake from tobacco products is estimated to be 21.800 ± 4350 ng/day. This is followed by food as the second largest source, also in vegetarian diets .[4]

In the body TSNAs are activated by P450 enzymes when the body try to detoxify and solubilise them. This can eventually cause the formation of DNA-adducts who disturb the DNA-replication, causing tumours. It has also been shown that nitrosamines can damage the p53 gene, causing uncontrolled cell-growth and tumours.[3]

The presence of nitrosamines in the environment and sources of human intake must be monitored. For this there is a need for pure analytical reference materials. This is especially true within the pharmaceutical industry to avoid drawbacks, and maintain the supply of widely used and vital drugs. Several batches of Valsartan were recalled in 2018 due to N-nitrosodimethylamine (**NDMA**) contamination.[3] Until 2021 sartans (angiotensin II receptor blockers used to threat high blood pressure) have accounted for >85% of the product recalls. Besides NMDA mainly two other impurities are reported: N-nitrosodiethylamine (**NDEA**) and N-nitroso-N-methyl-4-aminobutyric acid (**NMBA**).[3]

Chiron AS has a strong record of reference materials and continuously develop new products. For nitrosamines and nitramines crystalline and standardized solutions, including internal standards, are available.

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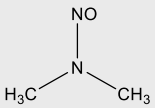
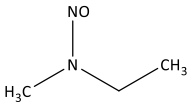
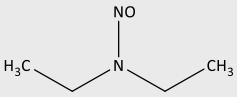
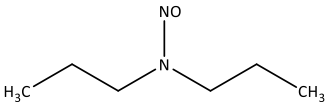
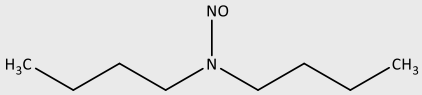
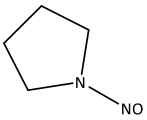
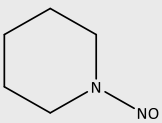
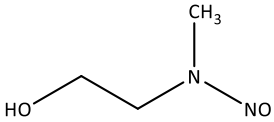
Nitramine reference materials

Chiron AS nitramine list of reference materials.
Including Chiron no., structure, name, CAS, and concentratio

Nitrosamine reference materials

Chiron AS nitrosamines list of reference materials including Chiron no., structure, name, and CAS. Grouped as: Internal standards for EPA 521, other, native, nicotine, and maybe group those common in pharmaceutical, and food).

Example/part of list:

Chiron No.	Structure	Name	CAS
	Native Nitrosamines for EPA 621:		
9004.2		N-Nitrosodimethylamine	62-75-9
9005.3		N-Nitrosomethylethylamine	10595-95-6
9006.4		N-Nitrosodiethylamine	55-18-5
9007.6		N-Nitrosodi-n-propylamine	930-55-2
9008.8		N-Nitrosodi-n-butylamine	924-16-3
9009.4		N-Nitrosopyrrolidine	930-55-2
9010.5		N-Nitrosopiperidine	100-75-4
11210.3		N-Methyl(nitrosoamino)-ethanol	26921-68-6

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NITROSAMINES – A Carcinogen Hard to Avoid

Every day all of us eat, drink, and take care of us self with numerous products. But did you know that your bacon breakfast, shampoo, body lotion or even your medications can contain compounds that the International Agency for Research on Cancer (IARC) has classified as carcinogenic to humans? Namely nitrosamines.[1]

Another article about what to avoid. It can become too much to deal with, and for many people all the scaremongering is starting to get boring and hard to take in. With that said, moderation in all things also applies to nitrosamines, and they cannot be avoided entirely. Consumers should also be entitled to information about the content in everyday products, especially when they ingest vital products prescribed by their caretakers. So, let us recognize the issue, do our best to minimize their presence in the environment, and agree upon how to monitor them.

What are nitrosamines and where do they come from?

Nitrosamines are the general term for numerous compounds that can form in acidic conditions when amines (e.g. proteins) are in the presence of nitrites.[2] Nitrites have been widely usen in preserving foods and are now mostly present in processed meats. In the environment they can also build up due to agricultural, industrial, and municipal wastewater containing nitrogen compounds.[3]

In agriculture we have fertilisers as a known source to nitrites. Also, some vegetables are rich in nitrate which can be reduced to nitrite by microorganism naturally present in the environment.[4] Together with all these possibilities for exogenous formation (in the environment) our stomach has an acidic condition suitable for endogenous formation (within the organism). The stage is set for nitrosamine formation and they cannot, especially in foods, be avoided completely. [4, 5]

Vegetables are an essential part of our diet even though they contain amines as possible precursors to nitrosamines, and these amines are not a hazard to humans. Vegetables are obviously still a healthy diet choice. What can become problematic though, is an uncontrolled use of nitrite sources which can be regulated or replaced, and a careless use of nitrites together with compounds known to be subjects of nitrosation (the reaction leading to nitrosamines).

Why are nitrosamines carcinogenic to humans?

Most nitrosamines are classified by IARC as probably or possibly carcinogenic to humans and have shown du induce tumours in animal studies. Two nitrosamines referred to as

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NNN[a] and NNK[b], derived from nicotine, on the other hand have a known carcinogenic effect on humans.[1] The issue of endogenous formation of nitrosamines and the possible carcinogenic hazard was discussed already in 1985 by Challis, and nitrosamines biological behaviour have been well studied by among others Hecht (1998).[5, 6]

In short nitrosamines go through biotransformation in several parts of the body, including the liver, intestine, lungs, kidney, brain, skin, and placenta.[7] The mixture of products are complex, but the general hazard to humans are the ways some of the products can damage the human DNA. NNN and NNK are activated by enzymes in the body, eventually causing the formation of DNA-adducts – compounds bound to the DNA-molecule and preventing precise DNA-replication.[8, 9] This can lead to mutations and an uncontrolled cell growth giving tumours. Another known source to tumour formation is mutation in the p53 gene which is important in the regulation of the cell cycle. [8, 10]

How can scientist monitor the presence of nitrosamines in our environment?

Different institutions like EMA[c] have set limitations on the content of various nitrosamines in e.g. foods, and FDA[d] have developed a guidance for industry on how to control nitrosamine impurities in drugs. [11, 12] The monitoring of nitrosamines in our environment relies on quality assured methods for analysing, tackled by decades of research within the field of chromatography. The aim of these methods is to separate and detect the amounts of nitrosamines in different products.

These methods also depend on the development of pure samples of nitrosamines, referred to as reference materials.[13] For the future it is important to gain knowledge about different nitrosamines occurrences and their chemistry, so that reference materials can be effectively developed. Reference materials function as a basis of comparison when scientists are monitoring and analysing the presence of nitrosamines in our environment.

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1. IARC. *Agents Classified by the IARC Monographs, Volumes 1–129*. 2021. [cited 02.04. 2021]; Available from: https://monographs.iarc.who.int/agents-classified-by-the-iarc/.
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10. Fossum, S. *p53 at Store medisinske leksikon*. 2019 [cited 05.05. 2021]; Available from: https://sml.snl.no/p53.
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12. Bian, Y., et al., *Progress in the pretreatment and analysis of N-nitrosamines: an update since 2010*. Critical reviews in food science and nutrition, 2020: p. 1-35.
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[a] N-Nitrosonornicotine
[b] Nicotine-derived nitrosamino ketone
[c] European Medicines Agency
[d] U. S. Food and Drug Administration

APPENDIX C

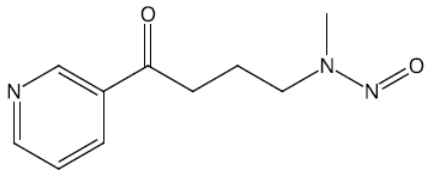
NNK Retrosynthesis sheet from SciFinder^n

CAS SciFinder^n

Retrosynthesis

View Plan in SciFinder^n

Target Structure

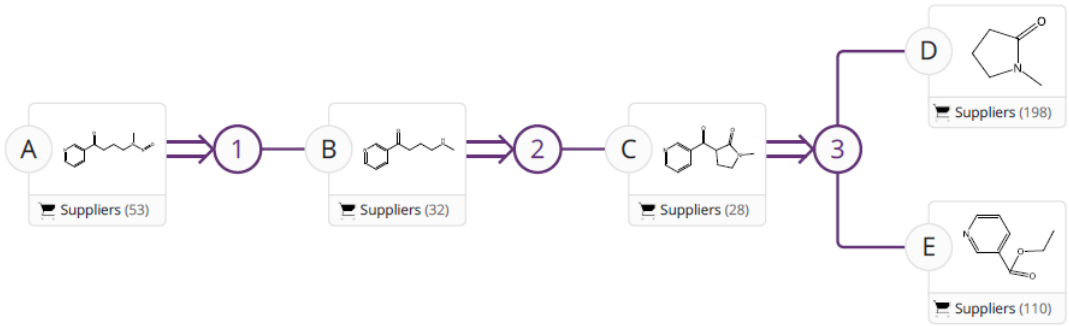


Plan Options

Synthetic Depth: **3**
Supporting Predicted Reactions: **Common**
Break & Protect Bonds: **No**

Plan Information

At the time of export, your plan included:
Experimental and Predicted Steps
Estimated Yield: **30%**
Overall Price: **$34.36 (USD per 100 grams)**
Commercially Available Substances: **A, B, C, D, E**
Total Steps: **3**



Step Key: **Experimental** ⇒ **Predicted** ⇢

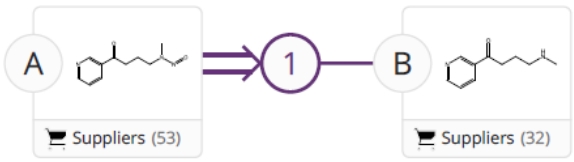
Steps

Step		Type	Yield	Evidence	Alternative Steps	Commercially Available
1	A ⇒ B	Experimental	Max.: 43%	2,718	49	A, B
2	B ⇒ C	Experimental	Max.: 87%	2	69	B, C
3	C ⇒ D + E	Experimental	Max.: 81%	3	21	C, D, E

Step 1 View Plan in SciFinder^{n}

A ⇒ B (Experimental)

Steps: **1** | Maximum Yield: **43%** | Evidence: **2,718** | Alternative: **1 of 49**

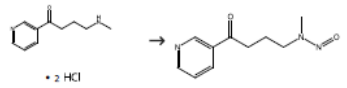


Step 1 (Evidence: 2,718) View All Evidence in SciFinder^n

A ⇒ B (Experimental)

Scheme 1 (1 Reaction)

Steps: **1** Yield: **43%**



• 2 HCl

Suppliers (32)

Suppliers (53)

Reaction Summary

Steps: **1** Yield: **43%**

1.1 **Reagents:** Sodium hydroxide, Sodium nitrite
Solvents: Water

Synthesis of [4-$^2H_2$]-, (4R)[4-$^2H_1$]- and (4S)[4-$^2H_1$]-4-(methylnitrosamino)-1-(3'-pyridyl)-1-butanone, C-4 deuterated isotopomers of the procarcinogen NNK

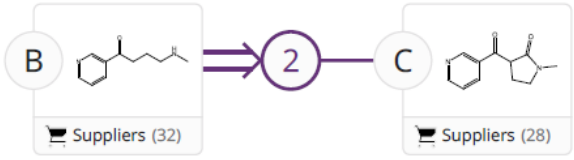
By: Pathak, Tanmaya; et al

Tetrahedron (1990), 46(5), 1733-44.

Step 2 View Plan in SciFinder^n

B ⇒ C (Experimental)

Steps: **1** | Maximum Yield: **87%** | Evidence: **2** | Alternative: **1 of 69**



Suppliers (32)

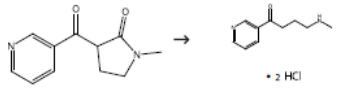
Suppliers (28)

Step 2 (Evidence: 2) View All Evidence in SciFinder^n

B ⇒ C (Experimental)

Scheme 1 (2 Reactions)

Steps: **1** Yield: **48-87%**



• 2 HCl

Suppliers (28) Suppliers (32)

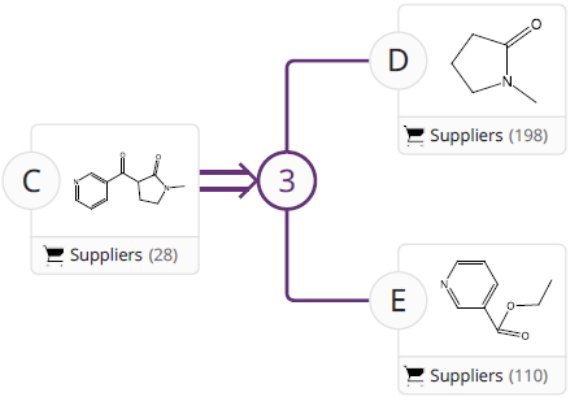
Reaction Summary	Steps: **1** Yield: **87%**	**Synthesis of [4-$^2H_2$]-, (4R)[4-$^2H_1$]- and (4S)[4-$^2H_1$]-4-(methylnitrosamino)-1-(3'-pyridyl)-1-butanone, C-4 deuterated isotopomers of the procarcinogen NNK**
1.1	**Reagents:** Hydrochloric acid **Solvents:** Water	By: Pathak, Tanmaya; et al Tetrahedron (1990), 46(5), 1733-44.

Reaction Summary	Steps: **1** Yield: **48%**	**A Chemo-Enzymatic Route to Enantiomerically Pure Cyclic Tertiary Amines**
1.1	**Reagents:** Hydrochloric acid **Solvents:** Water; 72 h, reflux	By: Dunsmore, Colin J.; et al Journal of the American Chemical Society (2006), 128(7), 2224-2225.
Experimental Protocols		

Step 3 View Plan in SciFinder^n

C ⇒ D + E (Experimental)

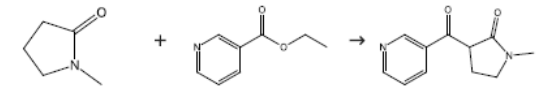
Steps: **1** Maximum Yield: **81%** Evidence: **3** Alternative: **1 of 21**



Step 3 (Evidence: 3) View All Evidence in SciFinder^n

C ⇒ D + E (Experimental)

Scheme 1 (3 Reactions) Steps: **1** Yield: **51-81%**



Suppliers (198) | Suppliers (110) | Suppliers (28)

Reaction Summary	Steps: **1** Yield: **81%**	**Process for preparing racemic nicotine**
1.1	**Reagents:** Sodium hydride **Solvents:** Toluene; 30 h, reflux; reflux → rt	By: Tian, Guanghui; et al
1.2	**Reagents:** Methanol	World Intellectual Property Organization, WO2012100722 A1 2012-08-02
		PATENTPAK available

Reaction Summary	Steps: **1** Yield: **70%**	**Synthesis of [4-$^2H_2$]-, (4R)[4-$^2H_1$]- and (4S)[4-$^2H_1$]-4-(methylnitrosamino)-1-(3'-pyridyl)-1-butanone, C-4 deuterated isotopomers of the procarcinogen NNK**
1.1	**Reagents:** Sodium hydride **Solvents:** Tetrahydrofuran	By: Pathak, Tanmaya; et al
		Tetrahedron (1990), 46(5), 1733-44.

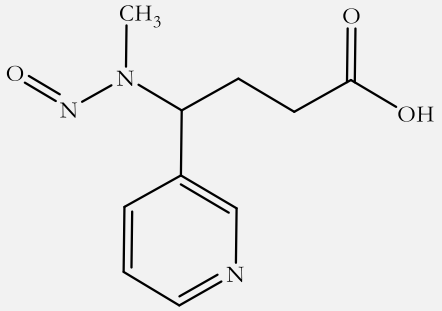
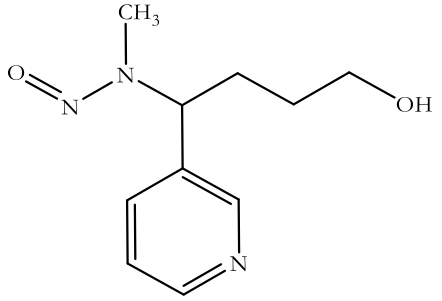
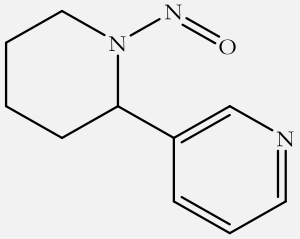
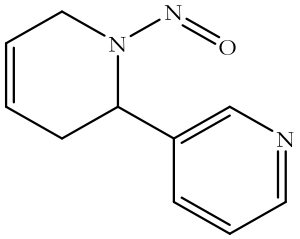
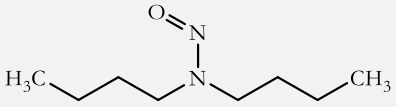
Reaction Summary	Steps: **1** Yield: **51%**	**A Chemo-Enzymatic Route to Enantiomerically Pure Cyclic Tertiary Amines**
1.1	**Reagents:** Sodium hydride **Solvents:** Tetrahydrofuran; 15 min, rt	By: Dunsmore, Colin J.; et al
1.2	**Solvents:** Tetrahydrofuran; 24 h, reflux; cooled	Journal of the American Chemical Society (2006), 128(7), 2224-2225.
1.3	**Reagents:** Hydrochloric acid **Solvents:** Water; 0 °C	
1.4	**Reagents:** Sodium hydroxide; pH 4	
Experimental Protocols		

APPENDIX D

Complete list of N-nitrosamines in the review

In Table A-1 a complete list of nitrosamines mentioned in this review are presented with CAS-numbers, names, structures and IARC classification on carcinogenicity. The IARC classifications are collected from IARC's *Agents Classified by the IARC Monographs, Volumes 1–129*, which is also described in Table 4-1, section 4. [4]

Table A- 1 Complete alphabetic list of N-nitrosamines in the review with structure and IARC classification. [4]

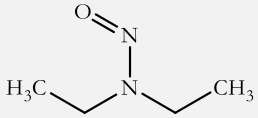
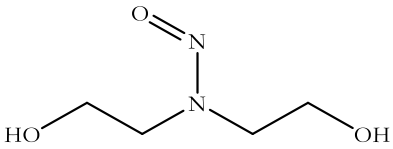
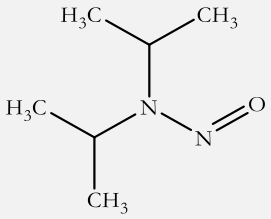
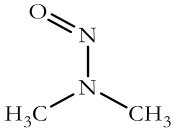
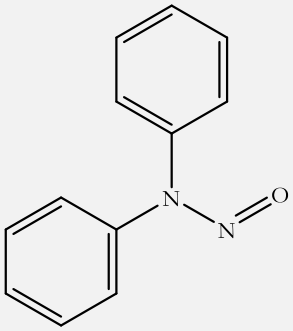
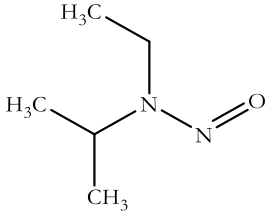
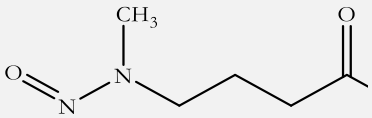
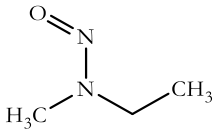
CAS	Compound name	Structure	IARC classification
123743-84-0	**Iso-NNAC** 4-(methylnitrosamino)-4-(3-pyridyl) butyric acid		NC
59578-66-4	**Iso-NNAL** Iso-4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol		NC
1133-64-8	**NAB** N-Nitrosoanabasine		3
71267-22-6	**NAT** N-Nitrosoanatabine		3
924-16-3	**NDBA** N-Nitrosodibutylamine		2B

NC = Not Classified, or not yet available through *Agents Classified by the IARC Monographs* [4]

1: Carcinogenic to humans, 2A: Probably carcinogenic to humans, 2B: Possibly carcinogenic to humans, 3: Not classifiable as to its carcinogenicity to humans.

All structures are drawn in Chem Draw Professional 16.0 according to SciFinder^n and CAS numbers.

Table A-1 Continued

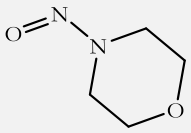
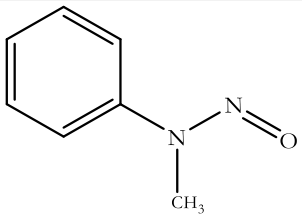
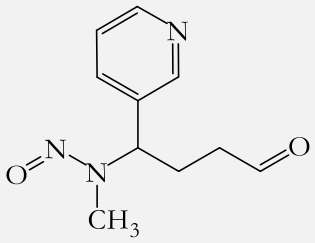
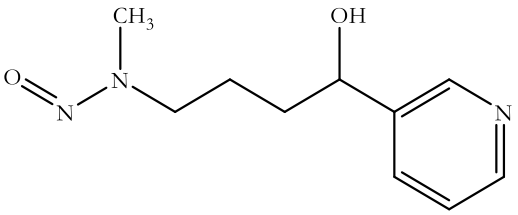
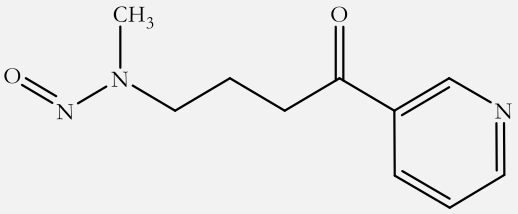
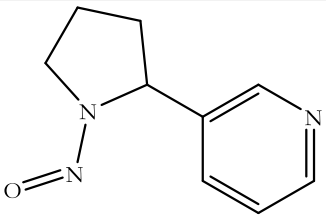
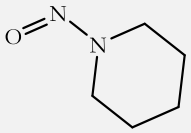
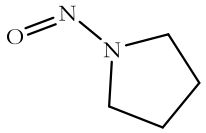
CAS	Compound name	Structure	IARC classification
55-18-5	**NDEA** N-Nitrosodiethylamine		2A
1116-54-7	**NDELA** N-Nitrosodiethanolamine		2B
601-77-4	**NDIPA** N-Nitrosodiisopropylamine		NC
62-75-9	**NDMA** N-Nitrosodimethylamine		2A
86-30-6	**NDPHA** N-nitrosodiphenylamine		3
16339-04-1	**NIPEA** N-Nitrosoisopropylethylamine		NC
61445-55-4	**NMBA** 4-(Methylnitrosoamino)butanoic acid		NC
10595-95-6	**NMEA** N-Nitrosomethylethylamine		2B

NC = Not Classified, or not yet available through *Agents Classified by the IARC Monographs* [4]

1: Carcinogenic to humans, 2A: Probably carcinogenic to humans, 2B: Possibly carcinogenic to humans, 3: Not classifiable as to its carcinogenicity to humans.

All structures are drawn in Chem Draw Professional 16.0 according to SciFinder^{n} and CAS numbers.

Table A-1 Continued

CAS	Compound name	Structure	IARC classification
59-89-2	**NMOR** N-Nitrosomorpholine		2B
614-00-6	**NMPA** N-Nitrosomethylphenylamine		NC
64091-90-3	**NNA** Nicotine-derived nitrosaminoaldehyde		NC
76014-81-8	**NNAL** 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol		NC
64091-91-4	**NNK** Nicotine-derived nitrosamino ketone		1
16543-55-8	**NNN** N-Nitrosonornicotine		1
100-75-4	**NPIP** N-Nitrosopiperidine		2B
930-55-2	**NPYR** N-nitrosopyrrolidine		2B

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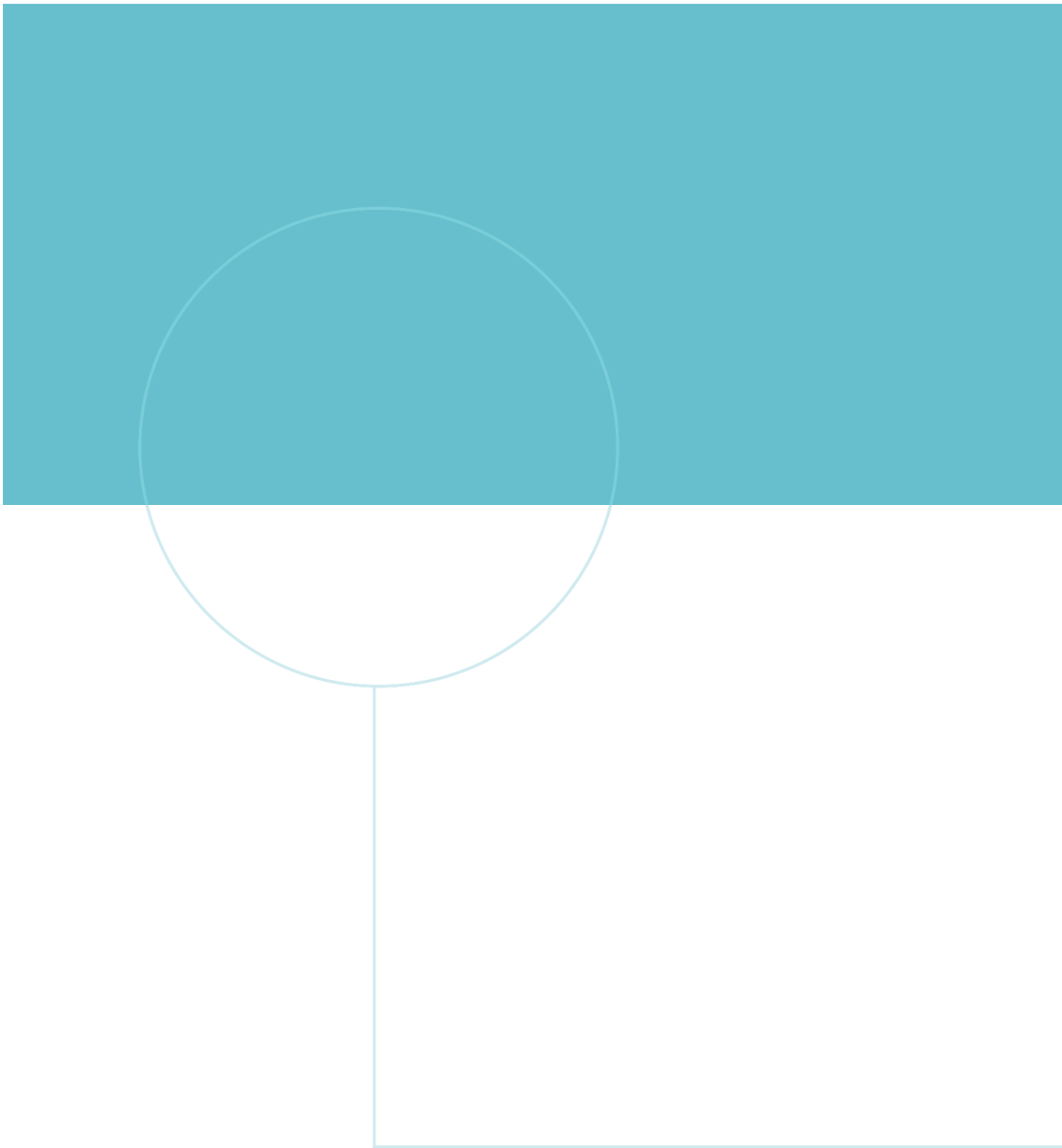

NTNU

Kunnskap for en bedre verden