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NITROSAMINES
– A REAL HEALTH CONCERN

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In recent years, the primary concern for human safety has arisen from the presence of nitrosamines found in a variety of products spanning pharmaceuticals, cosmetics, foods and food supplements. Nitrosamines are organic chemical substances that form under specific conditions when a nitrosating agent reacts with amino-based substances. The term "nitrosamine" describes a class of compounds with a chemical structure of a nitroso group bonded to an amine (R1N(-R2)-N=O). The International Agency for Research on Cancer (IARC) classifies these reaction products as probable human carcinogens due to their highly mutagenic and genotoxic potential, as evidence in several animal studies.

The beginning of a series of regulatory and safety assessments on nitrosamines in pharmaceuticals was commenced in 2018, when analytical reports revealed the presence of N-nitrosodimethylamine (NDMA) in valsartan drug substance from one manufacturer in June 2018. The FDA and EMA, as two of the world's largest drug regulatory bodies, extensively reviewed and assessed the toxicological profiles of specific nitrosamines found in pharmaceutical products. Understanding the conditions and environments conducive to the formation of these carcinogenic impurities was crucial, leading to the publication of a specific guidelines to instruct manufacturers on how to minimize the nitrosamine content in the final product.

The Scientific Committee on Consumer Safety (SCCS) published its opinion on nitrosamines in cosmetics in 2012. This opinion led to the prohibition of personal care products (cosmetics, hair products, lotions, shampoos, soaps) containing nitrosamines, including N-nitrosodiethanolamine (NDELA) from the EU/EEA market under the Cosmetics Directive 76/768/EEC.

A pressing need today is to develop sensitive analytical methods capable of delivering repeatable results with very low quantification levels on nitrosamines. Given their high toxic potential, the EU/EEA cosmetic market has regulated nitrosamines with a maximum limit of 50 parts per billion (ppb). However, no current EU legislation exists to impose an upper limit on nitrosamine content in food or drinking water.

Nitrosamines are regulated in the European Union for their presence in elastomer or rubber teats and soothers, cosmetic products and in toys. As indicated in the Directive 2009/48/EC on the toys' safety, levels of N-nitrosamines are limited to ⩽ 10 µg/kg in articles made with elastomers. The German Bundesinstitut für Risikobewertung (BfR) estimated a daily exposure of < 0.19 ng, assuming a maximum release limit of 0.05 mg/kg of rubber is met (EMA, 2020).

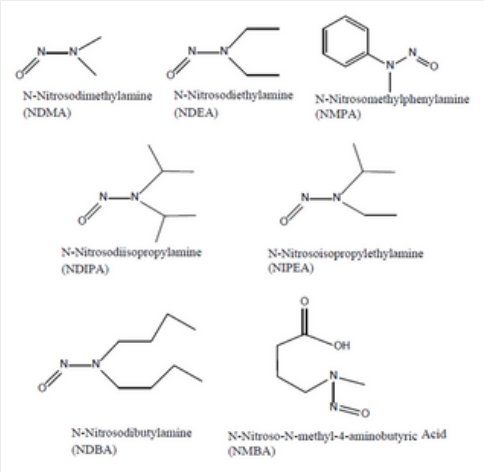


Figure 1
Seven potential nitrosamine impurities, FDA, 2021

CHEMISTRY BEHIND NITROSAMINE FORMATION

N-Nitrosamines, characterised by the R1R2N-NO functional group, are created through a process called N-nitrosation. During this chemical reaction, alkyl amines, like primary, secondary or tertiary amines, are converted to N-nitroso derivates in the presence of nitrosating agents (nitrites or nitrogen oxides), such as nitrous acid (HNO2), oxides of nitrogen (nitrous anhydrite or nitrite), or any other compound that can release a ion NO2+ (Fiume et al., 2013).

Organic nitroso compounds are divided to C-Nitroso, when the nitroso radical is attached to the carbon, and N-Nitroso, when it is attached to the nitrogen. C-Nitroso compounds are generally less toxic with low potency of carcinogenicity due to its isomerisation or dimerization. Primary amines can be nitrosated, but tend to be short-lived, highly reactive diazonium ions. Secondary amines are the most reactive nitrosating agent to form stable N-Nitrosamines (ASCC, 2003). These decompose to give molecular nitrogen by substitution, elimination, and molecular rearrangement pathways. However, nitrosamines occasionally arise from secondary processes (SCCS, 2012).

A particular concern in cosmetics is the formation of N-nitrosamines through the conversion of secondary amines, such as diethanolamine which has two ethanol moieties attached to the -NH. Triethanolamine, known as trolamine or TEA, is a common ingredient in cosmetic and pharmaceutical formulations, and it is a tertiary alkyl amine, where three ethanol groups are attached to N. This group of amines don't have the tendency to form nitrosamines directly in the presences of N-nitrosating agents. However, there is the risk from tertiary amines, such as TEA to generate diethanolamine when undergoing nitrosative cleavage. The diethanolamine formed can be N-nitrosated and generate nitrosamines, such as NDELA. Accordingly, TEA can react, in a formulation or in vivo, with nitrites or oxides of nitrogen to form a nitrosamine. Nitrous anhydride is the oxide of nitrogen that most commonly initiates nitrosation in vivo (Fiume et al., 2013).

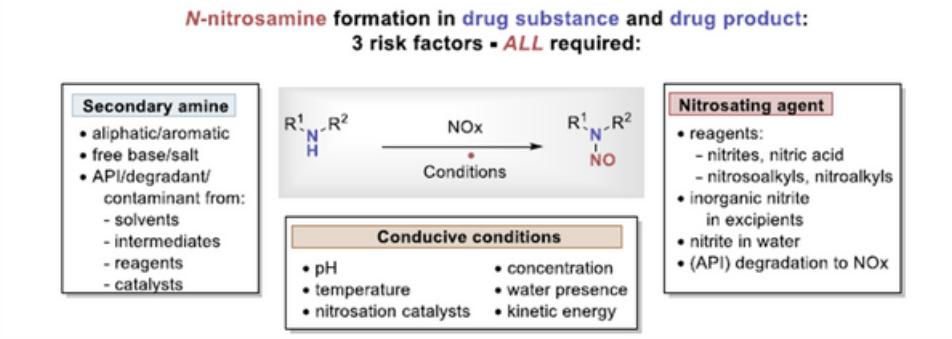


Figure 2

Condition for N-Nitrosamine Formation in Drug Substance and Drug product, extracted from Cioc et al., 2023

The nitrosating reaction depends on the acidity of the medium and, in emulsions, the dielectric constant of the oily layer and nature of the anionic surfactant. In all mechanisms of nitrosamine formation, the actual nitrosating agent in the oily phase is the nitrosonium ion, NO+, or its hydrated form, the nitrous acidium ion, H2O+NO. Thus, nitrosation by nitrate in aqueous solutions is enhanced in acidic pH with a maximum value around pH 3.4. On the other hand, in the presence of catalysts, such as chloral or an aldehyde (e.g., formaldehyde), nitrosation proceeds readily up to a pH of 11. The Figure 2 above shows some factors that influence the formation of nitrosamine in a Active Pharmaceutical Ingredient (API) or a product.

Notably, secondary amines can undergo nitrosation more rapidly by dinitrogen trioxide (N2O3), and dinitrogen tetroxide (N2O4) as nitrosating agents in basic solutions compared to acidified nitrites. The chemical pathway of the nitrosamines creation and the influencing factors can act in favor of this reaction is of great interest so manufactures can design their production and manufacturing process accordingly.

Consequently, three factors are required for N-nitrosamine formation:

1. the presence of a nitrosatable aminet
2. the presence of a nitrosating agent,
3. the conditions that favor the N-nitrosamine formation.

If one of those parameters can be under control the nitrosamines content can be minimized (Cioc et al., 2023).

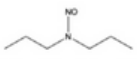
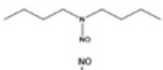
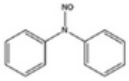
Analyte[a]	CAS number	Chemical structure	Molecular weight (g mol^{-1})	Log $K_{o/w}$
NDMA	62-75-9		74.08	−0.50
NMEA	10 595-95-6		88.11	0.01
NDEA	55-18-5		102.14	0.52
NDPA	621-64-7		130.19	1.54
NDBA	924-16-3		158.24	2.56
NPIP	100-75-4		114.15	0.44
NPYR	930-55-2		100.12	−0.09
NMOR	59-89-2		116.12	−0.59
NDPhA	86-30-6		198.22	3.13

Table 1

Chemical structure and relevant data of the target N-nitrosamines, Shettino et al.,2023

CARCINOGENIC POTENTIAL AND MECHANISM OF ACTION

The carcinogenic potential of nistrosamines is closely related to the genotoxicity and mutagenicity of the formed compounds. Though, not all nistrosamines formed have the potential to be genotoxic or mutagenic. A critical step to exhibit genotoxic properties is the metabolic activation of nitrosamines, often through CYP-450 dependent hydroxylation at the alfa-position. The resulting unstable alpha-hydroxynitrosamine, after removing the aldehyde group, quickly converts into the genotoxic form, binding to DNA, causing mutations and cancer. Acyclic N-nistrosaminses with dimethyl- and diethyl-groups were reported to be more genotoxic and mutagenic than N-Nitrosamines with longer chains and cyclic N-Nitrosaminess. In general, there should be other pathways of the carcinogenic potential of nitrosamines, but this need further observations and evaluation (SCCS, 2012).

Studies in rodents have demonstrated that the target tissues for carcinogenicity activity are the liver, followed by the gastrointestinal tract and respiratory tract. However, epidemiological studies in humans have not confirmed the same target tissues, likely due to differences in absorption, distribution, metabolism, and excretion between species and in bioactivation and DNA repair mechanisms.

Analysis of 900 human colorectal cancer (CRC) cases identified the mutational signature of DNA O6-alkylguanine, the most mutagenic adduct induced by N-Nitrosamines. This signature was associated with the development of CRC and with high intakes of processed or unprocessed red meat (EFSA, 2023). The exact fate of N-nitrosamines within the human body remains to be fully elucidated. Detection of those substances have been reported in human blood, urine, gastric juice and breast milk. There is also the possibility for endogenous creation during physiological chemical reactions in the human organism.

In human studies, only trace amounts of N-nitrosamines were detected in biological fluids after a known amount of ingested nitrosamine.

It was found that ethanol may decrease the hepatic clearance of NDMA, as demonstrated in rodents. Data collection from N-nitrosamines found in food, show that are mostly biotransformed during metabolic process by by CYP2E1 and 2A6, while CYP2B1 and CYP1A1 are involved to a lesser extent (EFSA, 2023).

NITROSAMINES' EXPOSURE

- ### FOOD

The human diet contains nitrates, nitrites and possibly nistrosamines, primarily from fruits and vegetables, but also in processed food as preservatives and flavorings such as meat, fish, ham, bacon. In recent years, due to the development of food processing technology, especially in processed meats, have led to increased consumption of nitrates and nitrites, correlating with a rise gastric cancer. When nitrates and nitrites react with proteins produce nitrsosamines, such N-nitrosodimethylamine (NDMA), a common nitrosamine in dietary products. Animal studies have revealed that NDMA is genotoxic, with the liver, lung, and stomach being its primary target tissues. Dietary nitrates are associated with a statistically reduced risk of gastric cancer, as they are mainly coming from vegetables and fruits, which also contain fibers, vitamins, ascorbic acid and other antioxidants and block any malignant possible effect (Song et al., 2015). More well-designed large prospective studies are needed to help us understand these substances in the etiology of gastric cancer (Song et al., 2015).

The European Food Safety Authority (EFSA) collected and reviewed data from humans' dietary exposure in nitrosamines. Their 2023 assessment report identified and characterized the hazard associated with thirty-two nitrosamines found in food samples, with risk assessments conducted for ten nitrosamines known to have carcinogenic potential, including NDMA, N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosodi-n-butylamine (NDBA), N-nitrosomethylamine (NMA), N-nitrososarcosine (NSAR), N-nitrosomorpholine (NMOR), N-nitroso-1-(3-pyridyl)-1-piperidine (NPIP), and N-nitrosopyrrolidine (NPYR).

As mentioned earlier, cured meat products, processed fish, as well as quantifiable amounts in beverages, cheese, soy saucesauce, oils, processed vegetables, and even human milk contained notable levels of nitrosamines. NDMA can also unintentionally form in beer due to the reaction of specific amines found in germinated barley (hordenine and dimethylamine) and atmospheric nitric oxides during the kilning process. Water used in food production facilities can also serve as a potential source of nitrosamines.

An intriguing discovery is the correlation between temperature and nitrosamine production. Unprocessed and uncooked meat has trace amounts of nitrosamines compared to those found after cooking processes like baking, frying, grilling, and microwaving. Other factors found to influence the nitrosamine production are pH, processing conditions (i.e. raw material and storage) and the presence of free amines, particularly biogenic amines (EFSA ANS Panel, 2017).

Detectable amounts of nitrosamines can also be found in cheese, due to the action of the enzyme xanthine oxidase and the large availability of secondary and tertiary amines in this kind of product, particularly if sodium or potassium nitrate is added to cheese milk (EFSA, 2023). After different types of cooking, such as baking, frying, boiling, N-Nitrosamines (NDMA and NDEA) were detected in potatoes, due to

Picture 1
Beer might contains trace amounts of nitrosamines

the high level of nitrate in the raw materials. In addition, the possible source of N-Nitrosamine presence in non-alcoholic beverages is the water used for their production (EFSA, 2023).

In 2017, the EFSA ANS Panel performed a risk assessment of nitrates and nitrites as food additives (EFSA ANS Panel, 2017a,b) assumed that nitrite maylead to the formation of N-nitrosamines, endogenously after food consumption and in food matrices. For the endogenous formation of N-nitrosamines, the ANS Panel measured the theoretical amount of N-nitrosodimethylamine (NDMA) after consumption of nitrite at the nitrite ion per day with an ADI (0.07 mg/kg bw).

- ## COSMETICS

The investigation of nitrosamines in cosmetic products began in March 1977 when a scientific paper was submitted to the American Chemical Society.. Although the results of this study were initially questioned due to the analytical technique used, they triggered extensive research, leading to the development of more advanced analytical techniques for nitrosamine quantification in the cosmetic industry. Subsequent research provided clear evidence that secondary alkanolamines and alkylamines are precursors of nitrosamine formation.

From a long study in the nitrosamines' formation, it was identified that those are formed when secondary or tertiary amines react with nitrosating agents, such as nitrites or nitrogen oxides. NDELA has been reported in cosmetics as a derivative from the nitrosation of diethanoliemine under certain conditions. In case, that nitrosamines found in many cosmetic finished products, manufacturing process and cosmetic formulations should not use ingredients containing or releasing nitrite ions, but if they are employed, nitrosation reaction inhibition systems should be used (such as the use of a-tocopherol, ascorbic acid and other substances (Schettino et al., 2023).

The European Cosmetic and Perfumery Association (Colipa), published in 2008, a guideline to inform cosmetic manufacturers with minimizing measures in formulation and production of cosmetic products, taking into consideration all the factors that can contribute into nitrosamine nitrosamine formation to the finished product (Colipa, 2008). Later, in 2012, the Scientific Committee on Consumer Safety published an opinion on Nitrosamines and Secondary Amines in Cosmetic Products (SCCS, 2012). The SCCS based the ranking of the NOCs assessed with respect to their carcinogenic potential using three dose descriptors common in carcinogenic risk assessment, T25, BMDL10 and TD50. A good correlation was found between the carcinogenic potency and the in vivo genotoxic potency. After this safety assessment, the European Cosmetic Directive (76/768/EEC) was amended to cover nitrosamines in Annex II (prohibited substances) and III (substances with restrictions).

In general, if Good Manufacturing Practice (GMP) is followed, nitrosamines that are technically unavoidable in the finished product are accepted in trace amounts provided the finished product conforms with Article 2 of the Cosmetic Regulation (i.e., it does not cause damage to human health when applied under normal or reasonably foreseeable conditions of use). The level of specific nitrosamines, mono and tri- alkylamines, alkalolamines and their salts, fatty acid dialkylamines and dialkanolamides is set at maximum concentration of 50 ppb in Annex III. Manufacturers should be aware that these substances may be present as impurities in other ingredients.

In compliance with the SCCS opinion and EU Cosmetic Regulation, the cosmetic industry has implemented quality analyses to control the presence of these carcinogenic substances in finished products that contain any precursor substances that could inadvertently produce nitrosamines. Various analytical methods for identifying and determining N-nitrosamines in cosmetic products have been developed, with many focused on the determination of NDELA (e.g., ISO 10130:2009 and ISO 15819:2014).

The most common nitrosamines found in cosmetics are N-Nitroso- diethanolamine (NDELA) and the N-Nitroso-bis (2-hydroxypropylamine) (NBHPA), other nitrosamines found only very rarely in cosmetic products and/or their raw materials are presented below (SCCS, 2012):

NDMA N-Nitroso-dimethylamine,
CAS 62-75-9
NDEA N-Nitroso--diethylamine,
CAS 55-18-5
N-Nitroso-N-methyl-N-dodecylamine,
CAS 55090-44-3
NMOR N-Nitroso- morpholine,
CAS 59-89-2
NPYR N-Nitroso- pyrrolidine,
CAS 930-55-2
NPABA N-nitroso-Para amino benzoic acid esters

N-nitrosated sunscreen agents, such as the NOC of 2-ethylhexyl-p-N,N-dimethylamino benzoate have been reported to occur in sunscreen formulations from the US market. It was found, that under specific conditions, some preservatives, such as Bronopol and Bronidox, might enhance the nitrosating reactions, acting as catalysts in the presence of secondary amines. It is generally advised to cosmetic formulators to include both hydrophobic and hydrophilic nitrosation inhibitors, so they can act in both phases. Substances acting as inhibitors of nitrosation are ascorbic acid/ascorbates and other water soluble antioxidants, oil soluble inhibitors may include ascorbyl palmitate, tocopherols, butylated hydroxytoluene / hydroxyanisole (BHT/BHA), gallate esters, amongst others.

In line with Colipa guidelines on the reduction of nitrites sources the manufacturers are advised to use purified water, using nitrite-free packing for storage, minimize the time with air contact during the manufacturing process, separation of hydrocarbon fuel equipment and open flames, and controlling the unnecessary nitrates/nitrites from raw materials (Colipa, 2008). In addition to the above, Cosmetic Regulation, had set a specific restriction on the use sodium nitrite, when secondary and/or tertiary amines are present.

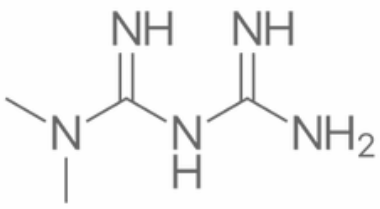
Picture 2
Nitrosamines can be found in cosmetic products.

• DRUGS

Back in 2018, pharmaceutical manufacturers reported to the FDA and EMA, the presence of NDMA, as an impurity in some batches of the pharmaceutical product Valsartan. The FDA and EMA has since conducted in depth investigations on the identification of nitrosamines in pharmaceutical products, and the results showed other drugs such as angiotensin II receptor blockers (ARBs), Ranitidine Nizatidine, and Metformin containing nitrosamines in unacceptable levels. his situation necessitated the implementation of a risk assessment strategy to address the potential presence of nitrosamines in pharmaceutical products (FDA, 2021).

It was also found that NDMA levels can be increased during the storage of drug products at room temperature. FDA's preliminary results using accelerated stability testing demonstrated elevated levels. Both the EMA and FDA advise manufacturers that, in cases where a nitrosamine is found in their product without a reported Accepted Intake (AI) limit, they should follow the approach described in ICH M7 (R1) to determine the risk.

For the accurate quantitation of nitrosamines, analytical methods with high sensitivity are required to meet the low acceptable intake levels recommended for nitrosamines. The limits of quantitation (LOQ) of the most toxic nitrosamines are as low as parts per billion. Manufacturers of active pharmaceutical ingredients and pharmaceutical products should establish analytical methods with LOQs and limit of detection (LOD) at or below 0.03 ppm to accurately quantify a total nitrosamine level of not more than 26.5 ng/day. For example, if the maximum daily dose (MDD) for an active substance is 1200 mg, the LOQ should be below 0.02 ppm (EMA, 2020).



Picture 3
Chemical structure of metformine

As discussed previously for cosmetics and processed food, the same instructions regarding the use of potable water usually should apply in pharmaceuticals, as may contain low levels of nitrite and even nitrosamines from environmental contamination. Therefore, to avoid the presence of nitrosamines in APIs, manufacturers should analyze nitrite and nitrosamine levels in processing water used. If any nitrosamine impurity is detected in water samples above the LOQ, manufacturers should establish a protocol to control this nitrosamine within the AI limit.

In 2020, the European Medicines Agency's (EMA's) Committee for Medicinal Products for Human Use (CHMP) published an opinion on the presence of nitrosamines in pharmaceuticals due to contaminated raw materials or APIs used or any related reaction during the manufacturing process. Within this report, EMA states to the manufacturer to be aware of the nitrosamine minimization measures and techniques, so as they can ensure that the levels of these compounds stay below the acceptable control limits that have been defined for specific nitrosamines (EMA, 2020).

The EMA/CHMP, based their approach on the guidelines for the assessment and control of DNA-reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk (ICH M7 (R1), 2015). They set acceptable intake (AI) for each specific nitrosamine belonging to the 'cohort of concern' in human medicinal products. The toxicological value of TD50, which indicates the lifetime dose at which half of the tested animals have developed tumors, and the maximum daily dose of the medicinal product was used for the derivation of those AI limits. Based on TD50 values, N-nitrosamines with a TD50 below 1.5 mg/kg/day (cohort of concern chemicals) would be listed according to their TD50 as follows: NMPEA > NDEA > NDMA > NMEA > NNK > NNN > NMOR > NMA > NDPA > NDBA > NPYR > MNNG > NMBA > NPIP.

The FDA and EMA follow the same strategy and have impose the below daily intake limits for the below reported nitrosamines:

NDMA – 96 ng per day
NMBA – 96 ng per day
NDEA - 26.5 ng per day
EIPNA - 26.5 ng per day
DIPNA - 26.5 ng per day
MeNP - 26.5 ng per day
NDBA - 26.5 ng per day

Manufacturers should ensure that the total risk level of the sum of all identified N-nitrosamines in a given finished product does not exceed 1 in 100.000 life-time risk. When N-nitrosamines are detected with insufficient toxicological data to extract a substance specific limit for lifetime exposure as recommended in ICH M7(R1), the toxicological values of 18 ng/day should be used as the Threshold of Toxicological Concern (TTC) for nitrosamines as the default value (EMA, 2020).

• WATER

An interesting finding of the recent years is the presence of nitrosamine in processed and treated water. Usually, nitrosamines are the derivative chemicals from water disinfection process with chloramine and sodium hypochlorite, ozonation and/or anion-exchange process. In addition to that, drinking water can be polluted as well, because of environmental pollution when it is pumped and received from the ground. California has already imposed a drinking water notification level of 10 ng/L for NDMA and two other N-nitrosamines. The nitrogen-containing waste comes from animal-feeding operations, wastewater treatment plants, dimethylhydrazine degradation, emissions from diesel, sewage sludge application, pesticides etc (WHO, 2008).

Domestic sewage has a very complex matrix, as it can contain chemicals from pharmaceuticals (ranitidine, methadone), to consumer products, such as shampoos, hand soaps and dish soaps. Tertiary-amine chemical compounds are characterized as an important source of nitrosamines and specially NDMA, as during chloramination (a water treatment process) can be yielded up to 90% (Zeng and Mitch, 2023). Ozonation of certain hydrazine, sulfamide, hydrazone, and carbamate-containing industrial chemicals can form NDMA at high yields.

Results from the study of Zeng and Mitch showed that chemical household products affect the N-nitrosamine formation.

Especially, they found that hand soap and dish soaps are assumed to be chloramine-reactive and ozone-reactive precursors for NDMA and other N-nitrosamines. Chemical analysis from a variety of greywater streams has proven that there is a uniformity of the results on nitrosamine content, that explains the fact that N-nitrosamine precursors were related to the functioning groups of chemicals contained in the product (e.g., quaternary ammonium compounds in shampoos) (Zeng and Mitch, 2023).

These findings underscore the importance of monitoring and controlling nitrosamine levels in water treatment processes to safeguard water quality and public health. Addressing the sources and precursors of nitrosamines in water is crucial to mitigate potential risks associated with their presence in drinking water and wastewater.

Picture 4
Domestic sewage can be a potential source for environmental pollution with nitrosamine impurities.

CONCLUSION

The collected information and data from chemical analyses of various consumer products have revealed the presence of N-nitrosamines, raising concerns about daily human exposure to these toxicants. Not all N-nitrosamines are potential toxic compounds, and those that are classified as carcinogens by IARC is due to their proven mutagenicity from several animal studies. Regulatory bodies like the FDA and EU have taken measures to regulate the presence of N-nitrosamines in raw materials and finished products, aiming for levels as low as detectable if their presence cannot be technically avoided. However, the specific dermal toxicity of N-nitrosamines is yet to be fully determined.

Manufacturers must be aware that nitrosamines can be present in pharmaceuticals, medical devices, cosmetics, and foods due to various factors such as contaminated raw materials, chemical reactions during manufacturing processes, storage conditions, or by absorption of nitrosamine precursors from the environment, including packaging. Scientific opinions have been published in order to help manufacturers to understand the root causes for nitrosamine formation so as they can control their presence and mitigating the risk. Important step on the detection and quantification of these substances is to establish sensitive analytical techniques that can be as low as the acceptable intake levels calculated for specific nitrosamines.

Moreover, there is limited information on the correlation of carcinogenic potential of nitrosamines in between animal species and humans, as the mechanism of action and the activation of nitrosamines to act as mutagens is not clearly specified. Therefore, further non-clinical and clinical studies are crucial to shed light on these aspects. Additionally, exploring different routes of exposure, such as dermal exposure, is essential for a comprehensive understanding of human exposure to nitrosamines through various pathways. Conducting epidemiological studies is also necessary to assess potential health risks associated with additive exposure to nitrosamines in daily life.

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