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Levels of nicotine and tobacco-specific nitrosamines in oral nicotine pouches

Nadja Mallock, Thomas Schulz, Sebastian Malke, Nadine Dreiack, Peter Laux, Andreas Luch

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Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Correspondence to
Dr Nadja Mallock, Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), 10589 Berlin, Germany; nadja.mallock@bfr.bund.de

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ABSTRACT

Background Nicotine pouches without tobacco are new products that deliver nicotine into the body via the oral mucosa. There is a lack of independent research on the chemical composition and product characteristics of these products, contributing to uncertainties regarding product regulation. This study sought to address knowledge gaps by assessing levels of nicotine and screening for tobacco-specific nitrosamines (TSNAs) in a sample of these products.

Methods Nicotine pouches (n=44) and nicotine-free pouches (n=2) from 20 different manufacturers were analysed regarding their contents of nicotine and TSNAs by gas chromatography with flame ionisation and liquid chromatography–tandem mass spectrometry, respectively. Product labelling and pH values of aqueous extracts were determined.

Results Nicotine contents of products ranged from 1.79 to 47.5 mg/pouch; median product weight, pH, and proportion of free-base nicotine were 0.643 g, 8.8, and 86%, respectively. A clear labelling of the nicotine content was missing on 29 products and nicotine strength descriptions were ambiguous. TSNAs were detected in 26 products, with a maximum of 13 ng N-nitrosonornicotine/pouch.

Conclusion Although nicotine pouches may potentially be a reduced risk alternative for cigarette smokers or users of some other oral tobacco products, nicotine contents of some pouches were alarmingly high. Presence of carcinogenic TSNAs in the nicotine pouches is of serious concern. Better manufacturing processes and quality control standards should be implemented. Labels of nicotine strength on most products are misleading. A strict regulation regarding nicotine contents and its labelling would be advisable.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Nicotine pouches without tobacco leaf material are described in the recent scientific literature with up to 12 mg nicotine per pouch.
⇒ Most published studies were conducted by nicotine pouch manufacturers.
⇒ Carcinogenic tobacco-specific nitrosamine (TSNA) content has been investigated in one study with negative results.

WHAT THIS STUDY ADDS

⇒ In this study, some nicotine pouches were found to have nicotine contents approaching 50 mg per pouch.
⇒ TSNAs were detected in more than half of the samples.
⇒ Most products lacked a label with the nicotine content in milligram per pouch or per gram.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Specific regulation for nicotine pouches is advisable, including limits and label requirements for nicotine contents.
⇒ Further research on nicotine delivery of products with high nicotine content and on product toxicology is necessary.

INTRODUCTION

Nicotine pouches without tobacco leaf material in the final product have been present on the US market since 2016[1] and in Europe since 2018.[2 3] These products resemble pouched snus in their appearance and use. Similar to snus, the pouches are placed between upper lip and gum. Released nicotine can be absorbed via the oral mucosa. In contrast to pouched snus, these new nicotine pouches do not contain tobacco.[4] Instead, they are based on plant fibres supplemented by nicotine, flavourings and other ingredients.[4] An example is shown in figure 1.

A representative survey among Dutch adults and adolescents has identified 0.06% current and 0.56% ever users of nicotine pouches; 6.88% of participants were aware of this product group.[5] Among current or former smokers and/or e-cigarette users in the UK, 2.7% were current and 4.4% were ever users with an awareness of 15.9%.[6] The European Tobacco Products Directive (TPD) bans tobacco products for oral use such as snus from the European Union (EU) market with an exception for Sweden (Article 17).[7] However, nicotine pouches without tobacco do not fall into the scope of the TPD and are thus unaffected by this provision.[2] Consequently, there are uncertainties regarding their regulation.

Additionally, many jurisdictions such as most of the EU have no special regulation for packaging and contents of nicotine pouches.[8] In the absence of regulation, nicotine contents may not be clearly labelled in milligram per pouch or per gram. As displayed in figure 1C, an arbitrary score may be used to indicate nicotine strength, with no relatability to nicotine contents. Nicotine strength may also be indicated using only a strength descriptor such as 'medium' or 'strong'. For labelling of chemical hazards such as acute toxicity, the European Directive for Classification, Labelling and Packaging (CLP) applies in the EU.[9] Consequently, GHS (Globally Harmonized System for Classification and Labelling of Chemicals) pictograms for acute toxicity are required for certain nicotine

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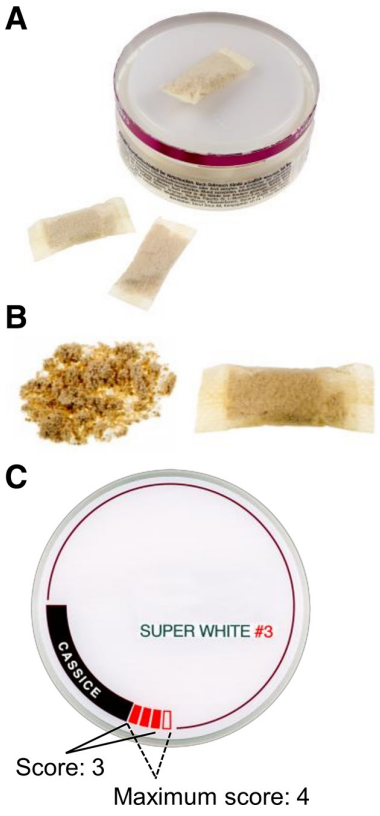

Figure 1 (A) Nicotine pouch in its package with removed lid, (B) content of an opened pouch and (C) package lid (brand name removed) with the nicotine strength labelled using a scoring system.

concentrations in the EU. Nicotine contents above 2.5 mg/g require the GHS07 label (harmful) that is depicted by an exclamation mark. Above 16.7 mg/g nicotine, the GHS06 label (toxic) depicted by skull and crossbones is required. Calculations for the acute toxicity categories of nicotine pouches are provided in the online supplemental material of this manuscript.

Little is known about the new products' toxicity and addictive potential, and parameters affecting these properties, such as nicotine content and chemical composition. A limited number of studies have investigated the chemical composition of these new nicotine pouches.[10–12] These studies included very limited numbers of brand varieties and were conducted mainly by the products' manufacturers.[10 11] Consequently, tobacco control regulators require reliable information in order to take science-based action.

The German Federal Institute for Risk Assessment was requested by the German Federal Ministry of Food and Agriculture to assess potential risks of new nicotine pouches. For this purpose, an intramural research project was initiated starting with the chemical characterisation of these products. The goal of the study was to analyse levels of constituents such as nicotine, unprotonated nicotine and to screen for tobacco-specific nitrosamines (TSNAs) in 46 brand varieties of pouches with and without nicotine. Measured nicotine content was compared with the labelled nicotine on the product packaging and warning labels were assessed for each product.

MATERIAL AND METHODS

Nicotine pouches, chemicals and standard substances

A convenience sample of nicotine pouches was bought from January to May 2021 at German online shops or at online shops located in other countries shipping to Germany. In total, 46 different pouch samples from 20 different producers were obtained. All chemicals or standard substances used for this assessment were of analytical or higher purity grade. Further details on chemicals and standard substances are provided in the online supplemental material.

Assessment of product labelling

Product labels were visually examined regarding labelled nicotine content of the products and/or labelled nicotine strength. Further, the presence or absence of hazard symbols according to CLP regulation[9] and/or warnings directed to vulnerable groups (eg, minors, pregnant women) was assessed.

Analysis of pouch weight and total nicotine content

Nicotine pouches were placed into Erlenmeyer flasks with stoppers and weighed with an analytical scale (LE225D-0CE, Sartorius, Göttingen, Germany). Nicotine was extracted from the pouches using liquid-liquid extraction (LLE) prior to quantification using gas chromatography with flame ionisation (GC/FID). The LLE protocol was adapted from WHO TobLabNet SOP 4 for nicotine determination from tobacco filler.[13] To the Erlenmeyer flask containing one complete pouch, 10 mL ultra-pure water, 5 mL sodium hydroxide solution (2 M) and 20 mL extraction solution containing the internal standard (2 g/L n-hexadecane in n-hexane) were added. After 75 min extraction on an orbital shaker (GFL 3005, Lauda-GFL, Lauda-Königshofen, Germany) at 350 rpm, the phases were allowed to separate, assisted with sonication (Sonorex Digitec DT 255H, BANDELIN electronic GmbH & Co KG, Berlin, Germany) if necessary. Analysis was performed using GC/FID as described in the online supplemental material. Method validation included the parameters precision, accuracy, linearity and storage stability. Limit of detection (LOD) and limit of quantification (LOQ) were determined using the calibration method according to DIN 32645:2008.[14] Procedures for validation and preparation of nicotine stock solution are described in the online supplemental material. Nicotine content per gram was calculated by dividing nicotine content per pouch by the weight. Nicotine content and pouch weight are reported as mean and SD of triplicate analysis.

Analysis of pH of the aqueous extract and calculation of the proportion of free-base nicotine

Analysis of pH of smokeless tobacco products is mainly performed after extraction with deionised water.[15 16] A similar approach was chosen for analysis of pH of nicotine pouches. Pouches were placed into vials and 10 mL of ultra-pure water was added. After extraction for 15 min on an orbital shaker at 350 rpm, pH of the aqueous extract was measured with a calibrated pH metre (765 Calimatic; Knick, Berlin, Germany). Proportion of free-base nicotine was calculated with the Henderson-Hasselbalch equation using the pKa of 8.01 of the pyrrolidine moiety of nicotine as published by Barlow and Hamilton.[17] As the used ultra-pure water was not degassed, an influence of remaining acidic CO_2 is possible. Pouch pH is reported as mean of duplicate analysis.

Screening for TSNAs

The TSNAs NNN (N-Nitrosonornicotine), NNK (4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone), NAT (N-Nitrosoanatabine) and NAB (N-Nitrosoanabasine) were determined according to ISO 21766:2021 using liquid chromatography tandem mass spectrometry (LC-MS/MS).[18] Nicotine pouches were placed into Erlenmeyer flasks with stoppers. A 200 µL of internal standard solution (100 ng/mL NNN-d_4, NNK-d_4, NAT-d_4, NAB-d_4, in

Table 1 Summary of product characteristics assessed in this study

	Labelled nicotine*	Nicotine strength*	Pouch wet weight (g)†	Nicotine (mg/pouch)†	Nicotine (mg/g wet weight)‡	pH§	Free-base (%)‡	Warnings minors (M), pregnancy (P)	GHS label
1	Nicotine free	–	0.617 (0.032)	Not detected	–	9.6	–	–	–
2	Nicotine free	–	0.575 (0.012)	Not detected	–	8.8	–	–	–
3	–	1/4	0.398 (0.003)	1.79 (0.07)	4.48	8.0	49	M	–
4	–	1/4, 'easy'	0.425 (0.016)	3.47 (0.16)	8.18	8.8	86	M	07
5	–	1/4, 'easy'	0.696 (0.064)	3.55 (0.28)	5.11	8.8	86	M	07
6	–	3/4	0.373 (0.004)	3.85 (0.06)	10.3	8.4	71	M	–
7	–	2/4	0.579 (0.027)	3.99 (0.11)	6.90	8.7	83	M	–
8	6 mg/pouch	2/4	0.487 (0.017)	4.53 (0.25)	9.30	8.9	89	M	–
9	–	2/4	0.508 (0.019)	4.80 (0.62)	9.46	7.6	28	M	–
10	6 mg/pouch	2/4	0.520 (0.012)	4.83 (0.25)	9.28	8.8	86	M	07
11	–	3/5, 'medium'	0.872 (0.020)	5.25 (0.22)	6.02	5.5	0.3	M	–
12	–	3/4, 'strong'	0.577 (0.005)	5.32 (0.44)	9.22	8.7	83	M	–
13	6 mg/pouch	2/4, 'medium'	0.664 (0.024)	5.42 (0.22)	8.15	9.0	91	M	07
14	20 mg	5/5, 'extra strong'	0.305 (0.027)	5.72 (0.58)	18.7	8.3	66	M, P	07
15	8 mg/pouch	4/5	0.501 (0.011)	5.88 (0.25)	11.7	8.7	83	M	07
16	10 mg/pouch	3/4, 'strong'	0.487 (0.029)	6.12 (0.36)	12.6	9.8	98	M	07
17	20 mg	5/5, 'extra strong'	0.351 (0.007)	6.24 (0.07)	17.8	9.1	92	M, P	07
18	–	5/5, 'extra strong'	0.403 (0.029)	6.66 (0.50)	16.5	7.7	33	M, P	07
19	6 mg/pouch	2/4	0.667 (0.005)	7.09 (0.13)	10.6	8.0	49	M	07
20	–	1/5	0.449 (0.013)	7.14 (0.14)	15.9	9.0	91	M, P	06
21	–	3/4	0.674 (0.012)	7.20 (0.13)	10.7	8.7	83	M	–
22	–	4/5, 'strong'	0.407 (0.010)	7.61 (0.17)	18.7	8.5	76	M	07
23	–	3/4, 'strong'	0.712 (0.030)	9.17 (0.45)	12.9	8.8	86	–	07
24	–	–	0.672 (0.060)	9.48 (0.36)	14.1	10.3	99	M	07
25	–	–	0.720 (0.018)	9.48 (0.53)	13.2	9.8	98	M	07
26	11 mg/pouch	4/4, 'x-strong'	0.454 (0.012)	9.85 (0.09)	21.7	8.2	61	M	07
27	10 mg/pouch	3/4, 'strong'	0.621 (0.027)	10.0 (0.6)	16.1	9.2	94	M	07
28	–	4/4	0.681 (0.021)	10.4 (0.5)	15.3	8.0	49	M, P	–
29	–	4/4, 'x-strong'	0.676 (0.006)	11.2 (0.2)	16.6	8.2	61	–	07
30	16 mg/g	–	0.807 (0.019)	11.5 (0.5)	14.3	9.5	97	M	07
31	–	'Extra strong'	0.685 (0.010)	11.7 (0.1)	17.1	9.9	99	M	–
32	–	5/6, 'ultra'	0.864 (0.036)	12.1 (0.5)	14.0	8.2	61	M	07
33	20 mg/pouch	'Power'	0.902 (0.009)	12.7 (0.3)	14.1	10.2	99	M	07
34	–	5/6, 'ultra'	0.919 (0.047)	13.0 (0.3)	14.1	9.0	91	M	07
35	–	–	0.910 (0.062)	16.0 (1.7)	17.6	10.2	99	–	–
36	–	4/4, 'extreme strong'	0.691 (0.004)	16.8 (1.3)	24.3	8.1	55	M	–
37	–	–	0.746 (0.025)	17.2 (1.9)	23.1	8.0	49	M	07
38	–	'Danger strong'	0.735 (0.013)	19.0 (1.1)	25.8	9.9	99	M, P	–
39	–	6/6, 'max'	1.246 (0.010)	20.2 (0.1)	16.3	8.4	71	M	07
40	–	'Extreme'	0.459 (0.004)	20.5 (0.9)	44.7	10.0	99	M, P	06
41	–	'Hard'	0.341 (0.012)	25.7 (1.3)	75.5	8.7	83	M, P	06
42	–	'Hard'	0.408 (0.021)	27.0 (1.7)	66.1	9.9	99	M, P	06
43	25 mg	–	0.597 (0.065)	27.9 (3.6)	46.9	9.7	98	M	–
44	–	'Hard'	0.491 (0.043)	31.2 (1.6)	63.8	9.7	98	M, P	06
45	50 mg/pouch	'Brutal'	0.668 (0.028)	43.4 (4.6)	64.8	10.1	99	M	06
46	50 mg/pouch	'Brutal'	1.064 (0.081)	47.5 (3.7)	44.7	10.5	100	M	06

Warning labels: M: no use by minors; P: no use during pregnancy; GHS06: toxic; GHS07: harmful.
*As labelled by the manufacturer.
†Mean and SD of triplicate analysis.
‡Calculated using mean values.
§Mean of duplicate analysis.
GHS, Globally Harmonized System for Classification and Labelling of Chemicals.

methanol) was added, followed by 20 mL of extraction solution (0.1 M ammonium acetate in ultra-pure water). After flasks were shaken at 200 rpm for 1 hour, the extracts were filtered through a PTFE syringe filter (0.45 µm, Merck KGaA, Darmstadt, Germany). Filtered extracts were analysed using LC-MS/MS as described in the online supplemental material. For quantification, isotope-labelled standards of all four analytes were used. Method validation included the parameters precision, accuracy,

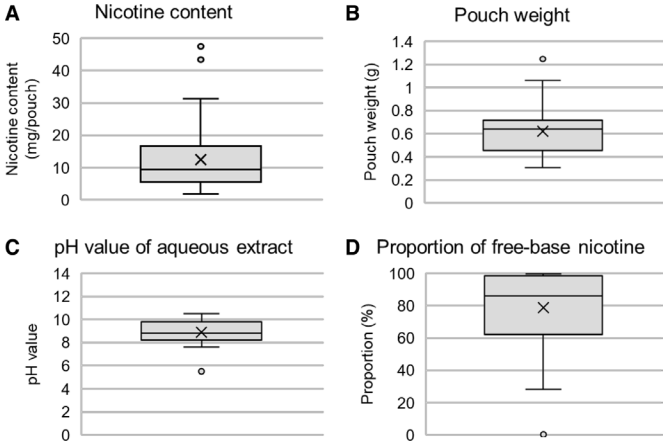

Figure 2 Product characteristics for the 44 nicotine-containing pouches analysed in the study: (A) nicotine contents, (B) pouch weights, (C) pH values of aqueous extracts and (D) the proportion of free-base nicotine.

linearity, stability on the autosampler, storage stability, LOD and LOQ. Procedures are described in the online supplemental material. Reported results stem from a single screening analysis.

RESULTS

Product labelling

Of the 46 analysed products, 2 were labelled as nicotine free. Of the remaining 44 products, only 15 had the nicotine content labelled in either milligram nicotine per pouch or per gram. Thirty-eight products indicated the nicotine strength using an arbitrary scoring system (see figure 1C) or a descriptor. The descriptors 'easy', 'medium', 'strong', 'extra strong', 'x-strong', 'extreme strong', 'ultra', 'power', 'max', 'extreme', 'hard' and 'brutal' used by the manufacturers to indicate nicotine strength were identified on 28 products. Labelled nicotine contents and/or strengths are summarised in table 1.

All but three products had a warning label for people below the age of 18 years. Ten packages had a label advising against use during pregnancy. As seen in table 1, some products have a measured nicotine content higher than 16.7 mg/g wet weight, requiring a GHS06 label (skull and crossbones), but do not have either label 06 or 07. One of the products (number 35) was provided by an online shop as a free sample containing neither any labelling on ingredients and the nicotine content nor any instructions or warnings.

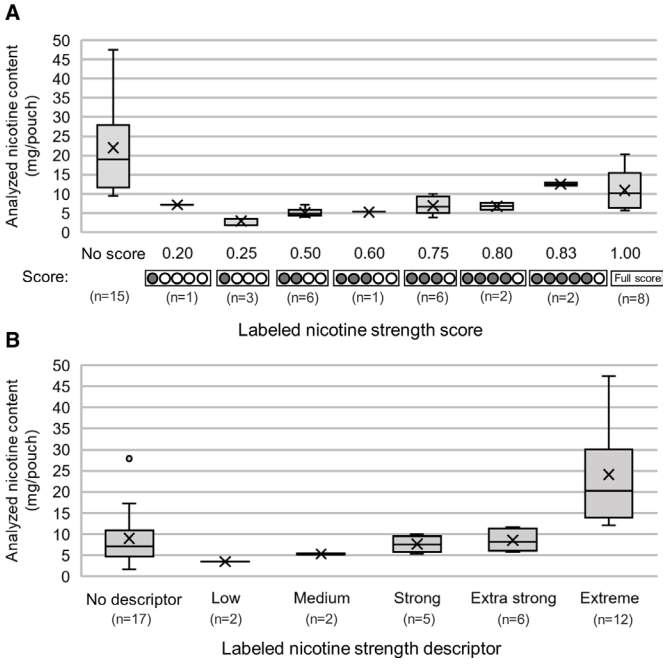

Figure 3 Comparison between analysed nicotine contents (y-axis) and nicotine strengths indicated on the packages (x-axis) using (A) an arbitrary score (score divided by maximum and as labelled) or (B) strength descriptors (grouped into categories). The number of products in the respective category is given in brackets.

Nicotine contents, pouch weights and pH of extracts

Nicotine contents in pouches, weights of pouches and pH values of aqueous pouch extracts are shown in table 1. Summary statistics are illustrated in figure 2. Median nicotine content was 9.48 mg/pouch with an IQR between 5.50 and 16.6 mg/pouch. Two pouches (sample numbers 45 and 46) had much higher nicotine contents of above 40 mg/pouch. The lowest detected nicotine content was 1.79 mg/pouch (sample number 3). Median pouch weight was 0.643 g (IQR 0.455–0.718 g). All but one pouch had an alkaline pH of the aqueous extract with a median pH value of 8.8 (IQR 8.2–9.8). Proportion of free-base nicotine was calculated from the pH value using the Henderson-Hasselbalch equation. Median was 86% (IQR 62%–98%).

Relationship between labelled and measured nicotine contents

Most of the nicotine pouches did not reveal a clear labelling of nicotine contents. On 29 products, the nicotine strength was indicated by the manufacturer using an arbitrary score (see figure 1C). Corresponding nicotine contents behind these scores are neither clarified nor harmonised. Figure 3A presents a comparison of measured nicotine contents and the respective scoring on the label (reached score divided by maximum score and depicted as labelled). As mentioned above, on 28 products the nicotine content was indicated using a descriptor. A comparison between analysed nicotine contents and the used descriptor is shown in figure 3B. For this, descriptors were grouped as followed: low comprising 'easy' (n=2), medium comprising 'medium' (n=2), strong comprising 'strong' (n=5), extra strong comprising 'extra strong' and 'x-strong', and extreme comprising 'max', 'ultra', 'power', 'extreme', 'extreme strong', 'danger strong', 'hard', and 'brutal'. Four products had no indication of nicotine content at all, neither in milligram nor with a score or descriptor.

TSNAs in nicotine pouches

TSNAs were detected in 26 nicotine pouches as summarised in table 2. NNN was detected in 24 pouches, 9 of them were above the detection limit but below the quantification limit (BQL). NNK was detected in three pouches. NAT and NAB were detected in six and five pouches, respectively, with three and two pouches as BQL. The highest amounts of TSNAs determined per pouch were 12.9 ng for NNN, 5.4 ng for NNK, 2.7 ng for NAT and 5.6 ng for NAB.

Results of method validation

Methods for analysis of nicotine and TSNAs were adapted based on standard methods and validated for analysis of nicotine pouches. Validation procedures and results are summarised in more detail in the online supplemental material. Mean recoveries

Table 2 TSNAs in nicotine pouches detected during TSNA screening using one pouch per sample

Sample no	NNN (ng/pouch)	NNK (ng/pouch)	NAT (ng/pouch)	NAB (ng/pouch)
4	BQL (<0.4)	n.d.	n.d.	n.d.
5	0.9	n.d.	n.d.	n.d.
6	n.d.	5.4	n.d.	n.d.
7	BQL (<0.4)	0.9	n.d.	n.d.
8	1.0	n.d.	n.d.	n.d.
9	BQL (<0.4)	n.d.	n.d.	n.d.
13	2.9	n.d.	n.d.	BQL (<1.1)
14	BQL (<0.4)	n.d.	n.d.	n.d.
19	0.5	n.d.	n.d.	n.d.
20	0.9	n.d.	n.d.	n.d.
21	1.1	n.d.	n.d.	n.d.
26	3.5	n.d.	1.3	n.d.
27	7.4	n.d.	BQL (<0.7)	1.4
28	0.9	n.d.	n.d.	n.d.
29	BQL (<0.4)	n.d.	n.d.	n.d.
30	BQL (<0.4)	n.d.	n.d.	n.d.
31	BQL (<0.4)	n.d.	n.d.	n.d.
32	4.0	n.d.	0.8	BQL (<1.1)
34	0.9	n.d.	n.d.	n.d.
36	BQL (<0.4)	n.d.	n.d.	n.d.
37	n.d.	0.5	n.d.	n.d.
39	7.0	n.d.	BQL (<0.7)	1.2
40	1.9	n.d.	BQL (<0.7)	n.d.
42	1.0	n.d.	n.d.	n.d.
43	BQL (<0.4)	n.d.	n.d.	n.d.
44	13	n.d.	2.7	5.6

BQL, below quantification limit (limit provided); NAB, N-Nitrosoanabasine; NAT, N-Nitrosoanatabine; n.d., not detected; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N-Nitrosonornicotine; TSNAs, tobacco-specific nitrosamines.

ranged from 102.9% to 126.1% for nicotine, from 110.2% to 123.9% for NNN, from 110.4% to 117.6% for NNK, from 113.7% to 121.0% for NAT, and from 115.0% to 127.2% for NAB. Precision was below 10% for all analytes. One important aim during method development was to achieve a high sensitivity for TSNAs. LODs were 0.12, 0.12, 0.22, and 0.34 ng/pouch for NNN, NNK, NAT, and NAB, respectively.

DISCUSSION

We analysed 44 nicotine pouches and 2 pouches without nicotine. Nicotine contents ranged from 1.79 to 47.5 mg/pouch, the median was 9.48 mg/pouch. This is much higher than the maximum nicotine content of 6.73 mg/pouch determined by Stanfill *et al* focusing on nicotine pouches sold in the USA by bigger brands.[12] The highest nicotine content determined in a study by a manufacturer was 11.9 mg/pouch.[11] Thus, we are the first to report products with nicotine contents of up to almost 50 mg/pouch. This is partly because we did not solely focus on products known to be from big brands but also purchased samples from smaller brands in online shops. Such nicotine contents are concerning as they are expected to induce and maintain addictive behaviour in users. Further, nicotine is toxic upon ingestion and has negative effects on the cardiovascular system.[19]

In the absence of an appropriate regulation, product labelling is not harmonised and was mostly not informative for the consumer. Only a few products had clear labelling of the actual nicotine content in milligram per pouch or per gram. Even some high-nicotine pouches with more than 20 mg nicotine did not have such a label. Nicotine strengths were indicated on most packages using arbitrary scores or adjectives that were not clearly relatable to certain nicotine contents. This could lead to an unwanted high exposure to nicotine or an underestimation of the risks associated with the product. While most products had a warning label for minors, a warning against use during pregnancy was present in only 10 products. Non-medicinal nicotine products should not be used during pregnancy due to negative effects on the developing brain.[20] GHS warning labels 07 (harmful) and 06 (toxic) are required in the EU by CLP regulation at nicotine contents above 2.5 and 16.7 mg/g, respectively, accounting for the acute toxicity of nicotine upon ingestion (see online supplemental material).[9] Although most packages contained a GHS label, the required label was still missing on some packages.

For the biokinetics of nicotine products, pH values play an important role. Nicotine is an alkaline alkaloid with a pKa value of 8.01 at which half of nicotine molecules are protonated and half are unprotonated.[17] At pH values above 8, the proportion of unprotonated and thus uncharged nicotine molecules increases. This free-base nicotine can pass biomembranes more rapidly resulting in a faster absorption of nicotine via the oral mucosa, faster nicotine delivery into the blood with higher blood levels.[21 22] Analysed pH values of pouch extracts ranged from 5.5 to 10.5 with a median of 8.8. Median proportion of free-base nicotine was 86% thus facilitating fast absorption. This is similar to findings by Stanfill *et al*.[12] Consequently, the high nicotine contents present in the products are likely to be quickly taken up into the bloodstream, potentially increasing addictiveness of the products.

Smokeless tobacco products usually do not contain typical products of cigarette combustion. However, some substances of toxicological concern are already present in unsmoked tobacco. One such group are TSNAs with the two carcinogens, NNN and NNK.[23] Exposure to NNN is associated with the promotion of oesophageal tumours.[23] This is of special relevance for nicotine pouches that are used in the oral cavity. TSNAs are formed from tobacco alkaloids during curing and processing of tobacco. Nicotine that is added to pouches may be derived from tobacco plant extracts,[4] although synthetic nicotine is also already available.[24] Thus, it is possible that the products contain traces of TSNAs. In spit-free snus, levels of up to 1190 ng NNN and 120 ng NNK per pouch have been found.[25] In contrast to this, in oral nicotine replacement therapy (NRT) products, TSNAs are only present in trace amounts. A study from 2005 has reported 2 ng NNN per g wet weight in a 4 mg nicotine gum, while no TSNAs were detected in a 2 mg nicotine lozenge.[26] In smoke of various US commercial cigarettes generated using the Health Canada Intense puffing regime, NNN and NNK ranged from 33 to 323 ng/cigarette and 40 to 246 ng/cigarette, respectively.[27] In our screening for TSNAs in nicotine pouches, we have detected traces of TSNAs in 26 products. Results were mostly below or close to quantification limits. In 17 products, detected NNN or NNK was quantifiable. The highest measured concentrations of NNN and NNK were 13 ng and 5.4 ng/pouch, respectively, much lower compared with most cigarettes[27] and snus.[25] It should be noted that these results stem from a single measurement and that precision at the instrument's lower limit is unsteady. However, comparison with the lower traces detected in NRT shows that traces of TSNAs in some nicotine pouches are still problematic and should be eliminated. In a study published by a manufacturer, no TSNAs were quantified in the four nicotine pouches investigated.[11] However, their method was with an LOQ of 10 ng/g, much less sensitive than our method. In addition, endogenous

formation of TSNAs is expected to play a role for oral nicotine pouches. NNN was detected in urine of exclusive users of NRT[28] and in saliva of exclusive e-cigarette users.[29] Oral nicotine products that remain in the mouth for approximately 20–60 min may be particularly prone to TSNA formation in saliva.

Currently, nicotine pouches without tobacco are not regulated as tobacco products in the EU. State authorities in Germany have classified these products as foodstuffs thereby applying the corresponding requirements.[30 31] The European Food Safety Agency has established an acute reference dose of 0.0008 mg/kg bodyweight for nicotine in food due to its pharmacological effects on the cardiovascular system.[32] The acceptable daily intake (ADI) has been set to 0.0008 mg/kg bodyweight as well, resulting in an acceptable intake of 0.048 mg nicotine per day for an individual with 60 kg bodyweight.[32] In their clinical study, Lunell *et al* have analysed the remaining nicotine content in nicotine pouches after 60 min of use.[33] The extracted fraction of nicotine was between 50% and 59%.[33] With an approximated nicotine uptake of 60%, the ADI for a 60 kg person is already exceeded 22-fold when using the nicotine pouch with the lowest nicotine concentration (1.79 mg). Consequently, the nicotine pouches are not compliant with current European regulation for foodstuffs and cannot be marketed as such.

Due to their reduced exposure to hazardous substances, nicotine pouches are under discussion as potentially being a risk-reducing alternative to smokeless tobacco products[34] or cigarette smoking. This is of importance as tobacco smoking still claimed the lives of 7.69 million people worldwide in 2019.[35] Manufacturers already advertise the products as alternatives to cigarettes.[36] However, the overall public health effect of these new products is unclear. For example, the appeal of nicotine pouches to youth and their potential to induce addiction are not known yet. In light of these uncertainties and our findings, we believe that an appropriate regulation of nicotine pouches is advisable, including the following measures:

- The nicotine content should be limited. This limit should be based on clinical studies and should be as low as needed to reduce craving in cigarette smokers. Since users consume the product per pouch and not per gram, the limitation of nicotine per pouch could not be bypassed by just selling higher weight pouches. Since products with 16.7 mg/g are considered acute toxic category 3 via the oral route (see online supplemental material), the nicotine limit should be lower than 16.7 mg/pouch.
- Only ingredients of high purity should be used in the manufacture of nicotine pouches to reduce impurities such as carcinogenic TSNAs in the final product to technically unavoidable amounts.
- The product packaging should carry a label clearly indicating the nicotine content in mg/pouch.
- The product packaging should carry warning labels for pregnant or breastfeeding women and should contain a warning statement for people suffering from cardiovascular diseases.
- Strict marketing and sales restrictions to prohibit sales to individuals younger than legal age.

Manufacturers should implement a quality control system using sensitive analytical methods to avoid any traces of TSNA or their precursors (eg, nornicotine). Carcinogenic substances such as NNN and NNK should not be present. Nicotine contents as well as the occurrence of other toxic substances should be routinely monitored in nicotine pouches. The potential product addictiveness should be investigated. Research is needed to learn more about the product's appeal to and harm perceptions, particularly in young people. Flavours contribute to attractiveness and promote use initiation of smokeless tobacco products.[37 38] Thus, studies on flavours used in nicotine pouches and their effects on abuse liability are needed. It might be advisable to regulate advertisement, package design, or to ban certain flavours to lower the attractiveness and thus to improve protection of the young. Other constituents of toxicological concern, for example, heavy metals, need to be investigated. Further, nicotine delivery into the bloodstream after consumption of high nicotine level pouches should be addressed in a clinical study.

CONCLUSION

This study demonstrates that in the absence of an appropriate regulation, new nicotine pouches are available with concerning nicotine contents approaching 50 mg/pouch and that labelling of nicotine contents is mostly insufficient. Further, traces of carcinogenic TSNAs were detected which should be eliminated by application of better quality control standards. Overall, although toxicant levels are lower compared with tobacco products, nicotine pouches are still associated with concerns regarding product toxicity and addictiveness.

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Contributors NM, TS, PL and AL contributed to the implementation and design of this study. Project administration was carried out by NM. NM, TS, PL and AL supervised the study. NM, SM and ND established and validated the analytical methods and performed the experiments and data analysis. NM drafted the manuscript. All authors have reviewed and refined the manuscript. NM is the guarantor of this work.

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ORCID iD

Nadja Mallock http://orcid.org/0000-0002-7109-0766

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Supplementary Material

Levels of nicotine and tobacco-specific nitrosamines in oral nicotine pouches

Nadja Mallock*, Thomas Schulz, Sebastian Malke, Nadine Dreiack, Peter Laux, Andreas Luch

German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, Berlin, Germany

* Corresponding author: Nadja.Mallock@bfr.bund.de

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1. Acute toxicity classification of nicotine products

According to the European Union Regulation on classification, labelling and packaging of substances and mixtures (CLP Regulation)[1], ANNEX I, Part 3: Health Hazards, acute toxicity estimates (ATE) may be used for classification of substances regarding their acute toxicity. In case of mixtures, if there are no data available on the acute toxicity, ATE of the mixture can be calculated using equation 1.

Equation 1. Additivity formula for calculation of a mixture's acute toxicity estimate (ATE_{mix}) in mg/kg bodyweight as described in the CLP Regulation.

$$\frac{100}{ATE_{mix}} = \sum_{n} \frac{C_i}{ATE_i}$$

C_i = concentration if ingredient i (% w/w or % v/v)
i = individual ingredient from 1 to n
n = number of ingredients
ATE_i = Acute toxicity estimate of ingredient i in mg/kg bodyweight

Based on its ATE, the substance or the mixture can be allocated to a toxicity category. Definitions for acute oral toxicity categories are presented in Supplementary Table 1.

Supplementary Table 1. Categories for acute toxicity hazards via the oral route and the defining acute toxicity estimates (ATE) in mg/kg bodyweight as described in the CLP regulation.

	Category 1	Category 2	Category 3	Category 4
Acute toxicity via the oral route	ATE ≤ 5	5 < ATE ≤ 50	50 < ATE ≤ 300	300 < ATE ≤ 2000

Nicotine has an ATE for acute oral toxicity of 5 mg/kg bodyweight and a classification of pure nicotine as acute toxic cat. 2.[2] A mixture of nicotine with other substances requires an ATE_{mix} of at least 300 mg/kg bodyweight to be allocated to category 3. ATE_{mix} for an allocation to category 4 would be at least 2000 mg/kg bodyweight.

Under the assumption that nicotine is the only substance with acute oral toxicity in the mixture, equation 1 can be solved for C_i by applying nicotine's ATE (5 mg/kg bodyweight) as ATE_i. When setting ATE_{mix} as 300 mg/kg bodyweight, the resulting C_i will be the concentration of nicotine (in % w/w or v/v) from which the mixture has to be allocated to acute toxicity category 3. For the nicotine concentration (in % w/w or v/v) that leads to an allocation to category 4, 2000 mg/kg bodyweight should be set as ATE_{mix}.

Consequently, a mixture containing 2.5 mg/g nicotine or more leads to a classification as acute toxic cat. 4 (oral). These mixtures have to be labeled with the GHS07 symbol (warning). A mixture containing 16.7 mg/g nicotine or more is classified as acute toxic cat. 3 (oral) and requires labeling with the GHS06 symbol (toxic).

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2. Methods and method validation

2.1. Chemicals and standard substances

All used chemicals or standard substances were of analytical or higher purity grade. Nicotine (purity ≥99 %), n-hexadecane (99%), N-nitrosonornicotine (NNN, 1.0 mg/mL in methanol, 99,995 %), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 1.0 mg/mL in methanol, 99,995 %), N'-nitrosoanatabine (NAT, 1.0 mg/mL in acetonitrile, 99,995 %), N-nitrosoanabasine (NAB, 1.0 mg/mL in methanol, 99,995 %), n-hexane, sodium hydroxide, glacial acetic acid, ammonium acetate, acetonitrile, and methanol were obtained from Merck KGaA (Darmstadt, Germany). NNN-d4, NNK-d4, NAT-d4, and NAB-d4 were bought from Toronto Research Chemicals (Toronto, Canada). Ultra-pure water was prepared with Milli-Q Integral Water Purification System (Merck KGaA, Darmstadt, Germany).

2.2. Determination of total nicotine content

2.2.1. Gas chromatography – flame Ionization (GC/FID) settings

Following extraction of the samples, 1 µL of the organic phase was injected into the split/splitless injector of the GC/FID system (G1530A series from Agilent Technologies/Hewlett Packard, Agilent Technologies, Waldbronn, Germany). Injector temperature was 225°C and a split ratio of 1:50 was used. Separation was carried out with a constant flow of 1.5 mL/min hydrogen (purity 99.999%, Linde, Pullach, Germany) on a DB-ALC1 capillary column (30 m length, 320 µm inner diameter, 1.80 µm film thickness, 10 m pre-column, Agilent Technologies, Waldbronn, Germany). The temperature program started with 140 °C for 5 min, followed by a 40 °C/min ramp to 250 °C with 4 min hold. FID was operated at 260 °C with a hydrogen flow of 30 mL/min, air flow of 300 mL/min, and a nitrogen (purity 99.999%, Linde, Pullach, Germany) make up flow of 20 mL/min.

2.2.2. Preparation of nicotine stock solutions

The nicotine stock solution that was used to prepare the calibration standards was prepared following a protocol adapted from WHO TobLabNet SOP 4.[3] 200 mg of nicotine standard was weighed into an erlenmeyer flask with stopper. 50 mL ultra-pure water, 25 mL sodium hydroxide solution (2 M), and 100 mL extraction solution containing the internal standard (2 g/L n-hexadecane in n-hexane) were added. After 75 min extraction on an orbital shaker at 350 rpm, the phases were allowed to separate. The organic phase was used to prepare the calibration standards.

2.2.3. Method validation

Validation of the method included the parameters precision, accuracy, linearity, storage stability, limits of detection (LOD), and limits of quantification (LOQ). The results are summarized in Supplementary Table 2. Quality control samples were prepared in matrix using nicotine-free pouches. Nicotine-free pouches were placed in erlenmeyer flasks with stoppers. The liquid-liquid extraction procedure for samples was followed except for the addition of ultra-pure water. Instead, 10 mL of different nicotine solutions in water were added with the target concentrations of 1, 5, 20, and 32 mg nicotine per pouch. Quality control samples were produced in sextuplets on two different days. LOD and LOQ were determined using the calibration method according to DIN 32645:2008.[4]

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Supplementary Table 2. Summary of method validation for determination of nicotine using GC/FID.

Parameter	Determined via	Acceptance criteria	Summary of results
Working range and linearity	Regression coefficient	$R^2 > 0.995$	1 – 40 mg/pouch $R^2 > 0.9999$
Trueness	Recovery of 4 different quality control samples in sextuplets at 2 days	Recovery: 85 – 115 % Lower limit of quantification (LLOQ) (1 mg/pouch): 70 – 130 %	Mean recoveries, day 1: 1 mg/pouch: 119.5 % 5 mg/pouch: 107.0 % 20 mg/pouch: 102.9 % 32 mg/pouch: 105.4 % Mean recoveries, day 2: 1 mg/pouch: 126.1 % 5 mg/pouch: 105.8 % 20 mg/pouch: 103.0 % 32 mg/pouch: 103.7 %
Precision	Standard deviation of six quality control measurements at 2 days	Standard deviation < 15 % LLOQ (1 mg/pouch): < 20 %	Standard deviations, day 1: 1 mg/pouch: 2.8 % 5 mg/pouch: 2.0 % 20 mg/pouch: 1.1 % 32 mg/pouch: 1.3 % Standard deviations, day 2: 1 mg/pouch: 1.4 % 5 mg/pouch: 1.5 % 20 mg/pouch: 0.7 % 32 mg/pouch: 0.6 %
Storage stability	Recovery of quality control samples after storage at +4 °C for 1 and 3 weeks.	Recovery: 85 – 115 % LLOQ (1 mg/pouch): 70 – 130 %	Mean recoveries, after 1 week: 1 mg/pouch: 108.4 % 5 mg/pouch: 102.2 % 20 mg/pouch: 100.0 % 32 mg/pouch: 100.7 % Mean recoveries, after 3 weeks: 1 mg/pouch: 108.2 % 5 mg/pouch: 98.0 % 20 mg/pouch: 92.6 % 32 mg/pouch: 94.3 %
Limits of detection and quantification	Calibration method according to DIN 32645:2008	As low as possible	Limit of detection: 0.08 mg/pouch (0.004 mg/mL sample solution)

			Limit of quantification: 0.3 mg/pouch (0.015 mg/mL sample solution)

2.3. Determination of tobacco-specific nitrosamines (TSNAs)

2.3.1. Method description

TSNAs were analyzed using liquid chromatography coupled with electrospray ionization and tandem mass spectrometry. 10 µL of filtered extract were injected into the LC system (LC-20AD pumps, DGU-20As degasser, SIL-20AC HT auto sampler, CTO-20AC column oven, CBM-20A communication bus module; Prominence series, Shimadzu, Kyoto, Japan) coupled with a triple quadrupole MS (API4000QTrap, ABSciex, Framingham, MA, USA) equipped with an electrospray ionization source. Separation was performed at 40 °C on a Luna Phenyl-Hexyl column (100 mm length, 2.0 mm inner diameter, 3 µm particle size, 100 Å pore size) with an according guard column (both Phenomenex, Torrance, CA, USA). Solvent A was 10 mM ammonium acetate and 0.1% acetic acid in ultra-pure water, solvent B was 10 mM ammonium acetate and 0.1% acetic acid in methanol. Solvent gradient started from 10 % B for 1 min, followed by an increase over 2.5 min to 95 % B, after a hold for 2.5 min, the gradient decreased to 10 % B over 1 min with a final hold for 4 min. Flow rate was 0.2 mL/min. Mass spectrometric detection was performed in positive mode using multiple reaction monitoring (MRM). At the ion source, the following conditions were used: ion spray voltage, 5500 V; temperature, 650 °C; curtain gas, N_2 with 12 psi; ion source gas 1, N_2 with 90 psi; ion source gas 2, N_2 with 8 psi; entrance potential, 10 V. Mass spectrometric detection was performed using multiple reaction monitoring (MRM) with a dwell time of 70 ms. MRM parameters are summarized in Supplementary Table 3. The software ScieX OS (Version 1.4.0.18067, AB Sciex, Framingham, MA, USA) was used for data analysis.

Supplementary Table 3. Multiple reaction monitoring parameters for detection of tobacco-specific nitrosamines

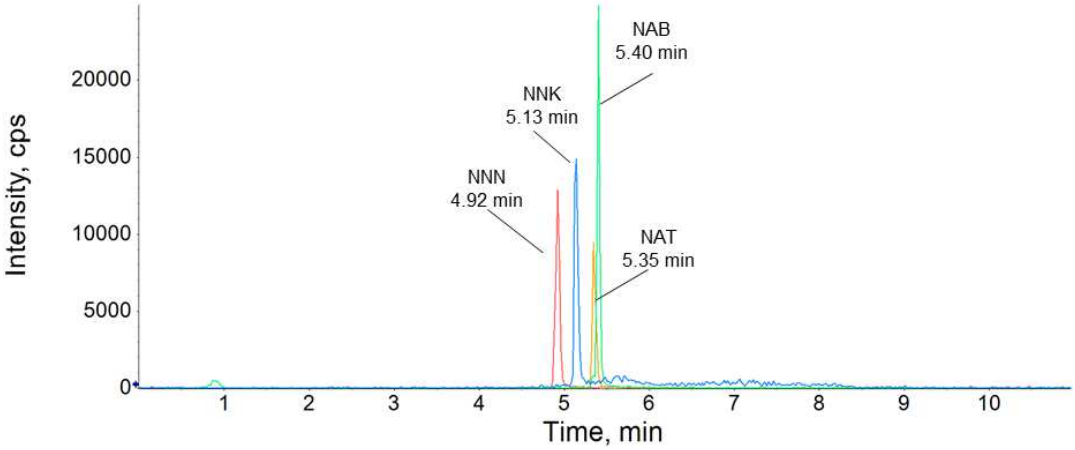
Substance			Quantifier			Qualifier		
Name	Q1 mass (Da)	DP (V)	Q3 mass (Da)	CE (V)	CXP (V)	Q3 mass (Da)	CE (V)	CXP (V)
NNN	178.2	46	148.2	15	8	120.1	29	22
NNN-d_4	182.2	46	152.3	15	8	109.1	25	20
NNK	208.1	41	122.0	17	6	106.2	31	18
NNK-d_4	212.2	51	125.9	17	6	83.3	57	14
NAT	190.1	41	160.2	15	10	106.2	25	18
NAT-d_4	194.1	31	164.0	15	10	83.1	39	14
NAB	192.1	41	162.1	17	10	133.2	31	24
NAB-d_4	196.1	36	166.2	17	10	137.1	33	8

NNN: N-Nitrosonornicotine; NNK: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT: N-Nitrosoanatabine; NAB: N-Nitrosoanabasine; Q1: precursor ion; Q3: product ion; DP: declustering potential; CE: collision energy; CXP: collision exit potential

2.3.2. Example chromatogram

An example chromatogram derived from a matrix sample is shown in Supplementary Figure 1.

Supplementary Figure 1. Quantifier traces for NNN, NNK, NAT, and NAB of quality control sample 3 (2.5 ng/mL) with retention times.



2.3.3. Method validation

Validation of the method included the parameters precision, accuracy, linearity, stability on the autosampler, storage stability, LOD, and LOQ. The results are summarized in Supplementary 4. Quality control samples were prepared in matrix. As an exemplary matrix, nicotine pouches have been selected that were tested for the absence of analytes. TSNA-free nicotine pouches were placed in erlenmeyer flasks with stoppers. Analyte solutions were added to obtain four different levels (0.1 ng, 0.3 ng, 2.5 ng, and 5.0 ng per pouch for all analytes). After addition of 200 µL internal standard solution (100 ng/mL of all internal standards), the spiked pouches were extracted applying the same procedure as for samples. Quality control samples were produced in sextuplets on two different days. LOD and LOQ were determined using the calibration method according to DIN 32645:2008.[4]

Supplementary Table 4. Summary of method validation for determination of tobacco-specific nitrosamines using LC-MS/MS.

<table>
<tr><th>Parameter</th><th>Determined via</th><th>Acceptance criteria</th><th>Summary of results</th></tr>
<tr><td>Working range and linearity</td><td>Regression coefficient</td><td>$R^2 > 0.995$</td><td>2 – 100 ng/pouch
$R^2 > 0.999$</td></tr>
<tr><td>Trueness</td><td>Recovery of 4 different quality control samples in sextuplets at 2 days</td><td>Recovery:
75 – 125 %</td><td>Mean recoveries (%), day 1:
<table>
<tr><td>ng/mL</td><td>NNN</td><td>NNK</td><td>NAT</td><td>NAB</td></tr>
<tr><td>0.1</td><td>117.0</td><td>111.9</td><td>115.8</td><td>115.0</td></tr>
<tr><td>0.3</td><td>115.4</td><td>113.0</td><td>118.1</td><td>122.3</td></tr>
<tr><td>2.5</td><td>123.9</td><td>116.2</td><td>121.0</td><td>127.2</td></tr>
<tr><td>4.0</td><td>117.5</td><td>110.5</td><td>114.6</td><td>118.8</td></tr>
</table>
Mean recoveries (%), day 2:</td></tr>
</table>

<table>
<tr><td></td><td></td><td></td><td>
<table>
<tr><td>ng/mL</td><td>NNN</td><td>NNK</td><td>NAT</td><td>NAB</td></tr>
<tr><td>0.1</td><td>113.9</td><td>117.6</td><td>120.9</td><td>122.1</td></tr>
<tr><td>0.3</td><td>110.2</td><td>112.0</td><td>119.5</td><td>116.7</td></tr>
<tr><td>2.5</td><td>111.0</td><td>111.6</td><td>113.7</td><td>116.7</td></tr>
<tr><td>4.0</td><td>111.3</td><td>110.4</td><td>116.1</td><td>115.1</td></tr>
</table>
</td></tr>
<tr><td>Precision</td><td>Standard deviation of six quality control measurements at 2 days</td><td>Standard deviation < 15 %</td><td>
Standard deviations (%), day 1:
<table>
<tr><td>ng/mL</td><td>NNN</td><td>NNK</td><td>NAT</td><td>NAB</td></tr>
<tr><td>0.1</td><td>6.0</td><td>7.2</td><td>7.1</td><td>9.5</td></tr>
<tr><td>0.3</td><td>2.3</td><td>2.6</td><td>4.9</td><td>3.2</td></tr>
<tr><td>2.5</td><td>3.0</td><td>3.2</td><td>4.4</td><td>4.8</td></tr>
<tr><td>4.0</td><td>4.8</td><td>1.5</td><td>2.7</td><td>3.3</td></tr>
</table>
Standard deviations (%), day 2:
<table>
<tr><td>ng/mL</td><td>NNN</td><td>NNK</td><td>NAT</td><td>NAB</td></tr>
<tr><td>0.1</td><td>7.7</td><td>9.6</td><td>4.4</td><td>5.4</td></tr>
<tr><td>0.3</td><td>4.0</td><td>2.6</td><td>3.2</td><td>3.6</td></tr>
<tr><td>2.5</td><td>1.7</td><td>2.3</td><td>2.0</td><td>1.9</td></tr>
<tr><td>4.0</td><td>1.6</td><td>3.3</td><td>2.1</td><td>2.9</td></tr>
</table>
</td></tr>
<tr><td>Storage stability</td><td>Recovery of quality control samples after storage at -18 °C for 1 and 2 weeks.</td><td>Recovery:
75 – 125 %</td><td>
Mean recoveries (%), after 1 week:
<table>
<tr><td>ng/mL</td><td>NNN</td><td>NNK</td><td>NAT</td><td>NAB</td></tr>
<tr><td>0.3</td><td>116.0</td><td>113.6</td><td>117.3</td><td>110.0</td></tr>
<tr><td>4.0</td><td>113.1</td><td>108.5</td><td>115.9</td><td>113.3</td></tr>
</table>
Mean recoveries (%), after 2 weeks:
<table>
<tr><td>ng/mL</td><td>NNN</td><td>NNK</td><td>NAT</td><td>NAB</td></tr>
<tr><td>0.3</td><td>114.5</td><td>116.9</td><td>120.2</td><td>106.5</td></tr>
<tr><td>4.0</td><td>110.5</td><td>107.8</td><td>117.6</td><td>108.8</td></tr>
</table>
</td></tr>
<tr><td>Stability under autosampler conditions</td><td>Recovery of quality control samples on the autosampler at +4 °C.</td><td>Recovery:
75 – 125 %</td><td>
Mean recoveries (%), after 19 h:
<table>
<tr><td>ng/mL</td><td>NNN</td><td>NNK</td><td>NAT</td><td>NAB</td></tr>
<tr><td>0.3</td><td>120.4</td><td>110.7</td><td>110.6</td><td>116.2</td></tr>
<tr><td>4.0</td><td>119.7</td><td>109.5</td><td>115.7</td><td>111.8</td></tr>
</table>
</td></tr>
<tr><td>Limits of detection and quantification</td><td>Calibration method according to DIN 32645:2008</td><td>As low as possible</td><td>
Limits of detection:
NNN: 0.006 ng/mL sample solution
NNK: 0.006 ng/mL sample solution
NAT: 0.011 ng/mL sample solution
NAB: 0.017 ng/mL sample solution

Limits of quantification:
NNN: 0.022 ng/mL sample solution
NNK: 0.021 ng/mL sample solution
NAT: 0.035 ng/mL sample solution
NAB: 0.056 ng/mL sample solution
</td></tr>
</table>

NNN: *N*-Nitrosonornicotine; NNK: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT: *N*-Nitrosoanatabine; NAB: *N*-Nitrosoanabasine

3. References

1. European Parliament. Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation), 2008.
2. RAC. Opinion proposing harmonised classification and labelling at EU level of Nicotine: ECHA, 2015:15.
3. WHO TobLabNet SOP 4. Standard operating procedure for determination of nicotine in cigarette tobacco filler. Geneva: World Health Organization, 2014.
4. DIN 32645. Chemische Analytik - Nachweis-, Erfassungs- und Bestimmungsgrenze unter Wiederholbedingungen - Begriffe, Verfahren, Auswertung Berlin: Deutsches Institut für Normung, 2008.