Supplementary Material

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

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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

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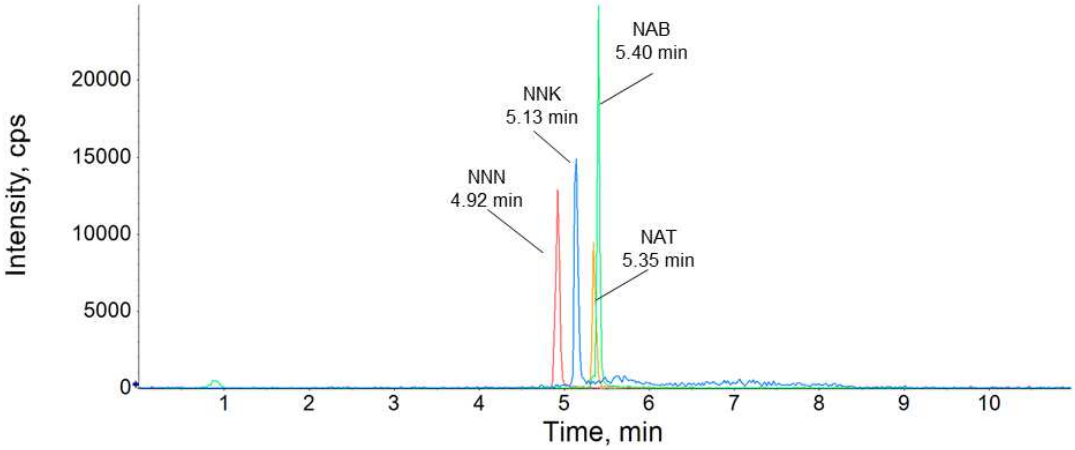
2.3.2. Example chromatogram

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

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3. References

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