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Tobacco-specific nitrosamines – Determination of *N*-nitrosoanabasine, *N*-nitrosoanatabine, *N*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in urine by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2019

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Biomonitoring methods

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and validated the presented biomonitoring method.

This analytical method permits the determination of tobacco-specific nitrosamines (TSNA) in urine using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The parameters in question are *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is a metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Due to its sensitivity, this method is suitable for the detection of the aforementioned analytes in the urine of smokers. NNAL can also be quantified in the urine of passive smokers.

The analytes NAB, NAT, NNN and NNAL are present in urine in both free and glucuronidated forms. For the determination of the total TSNA level in urine, the glucuronides are cleaved by enzymatic hydrolysis and then the analytes are isolated and concentrated using solid phase extraction (SPE). Two sorbent materials are used for sample preparation via SPE, first a material based on molecularly imprinted polymers and then a mixed-mode cation exchange polymer. Analysis is performed by LC-MS/MS. Deuterated internal standards are used for calibration. Calibration standards are prepared in pooled urine obtained from non-smokers and are processed in the same way as the samples to be analysed.

Keywords

tobacco-specific nitrosamines; TSNA; *N*-nitrosoanabasine; NAB; *N*-nitrosoanatabine; NAT; *N*-nitrosonornicotine; NNN; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNK; urine; biomonitoring; Analyses in Biological Materials; liquid chromatography-tandem mass spectrometry; LC-MS/MS

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Tobacco-specific nitrosamines – Determination of *N*-nitrosoanabasine, *N*-nitrosoanatabine, *N*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in urine by LC-MS/MS

Matrix:	Urine
Hazardous substances:	Tobacco-specific nitrosamines
Analytical principle:	High-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)
Completed in:	May 2014

Overview of the parameters that can be determined with this method and the corresponding hazardous substances:

Hazardous substance	CAS	Parameter	CAS
N-Nitrosoanabasine (NAB)	37620-20-5	*N*-Nitrosoanabasine (NAB)	37620-20-5
N-Nitrosoanatabine (NAT)	71267-22-6	*N*-Nitrosoanatabine (NAT)	71267-22-6
N-Nitrosonornicotine (NNN)	16543-55-8	*N*-Nitrosonornicotine (NNN)	16543-55-8
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	64091-91-4	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)	76014-81-8

Summary

This analytical method permits the determination of tobacco-specific nitrosamines (TSNA) in urine using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The parameters in question are *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is a metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Due to its sensitivity, this method is suitable for the detection of the aforementioned analytes in the urine of smokers. NNAL can also be quantified in the urine of passive smokers.

The analytes NAB, NAT, NNN and NNAL are present in urine in both free and glucuronidated forms. For the determination of the total TSNA level in urine, the

glucuronides are cleaved by enzymatic hydrolysis and then the analytes are isolated and concentrated using solid phase extraction (SPE). Two sorbent materials are used for sample preparation via SPE, first a material based on molecularly imprinted polymers and then a mixed-mode cation exchange polymer. Analysis is performed by LC-MS/MS. Deuterated internal standards are used for calibration. Calibration standards are prepared in pooled urine obtained from non-smokers and are processed in the same way as the samples to be analysed.

Reliability data of the method

***N*-Nitrosoanabasine (NAB)**

Within-day precision:	Standard deviation (rel.)	s_w = 11.7%, 1.1% or 1.1%
	Prognostic range	u = 32.5%, 3.1% or 3.1%
	at a spiked concentration of 8.9 ng, 123 ng or 192 ng NAB per litre urine and where n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 3.8%, 6.5% or 3.5%
	Prognostic range	u = 9.8%, 16.7% or 9.0%
	at a spiked concentration of 8.9 ng, 123 ng or 192 ng NAB per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 88%, 91% or 97%
	at a spiked concentration of 11 ng, 100 ng or 180 ng NAB per litre urine and where n = 5 determinations	
Detection limit:	1.1 ng NAB per litre urine	
Quantitation limit:	5.0 ng NAB per litre urine	

***N*-Nitrosoanatabine (NAT)**

Within-day precision:	Standard deviation (rel.)	s_w = 13.2%, 1.4% or 2.2%
	Prognostic range	u = 36.6%, 3.9% or 6.1%
	at a spiked concentration of 4.3 ng, 442 ng or 1423 ng NAT per litre urine and where n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 8.8%, 6.7% or 2.5%
	Prognostic range	u = 22.6%, 17.2% or 6.4%
	at a spiked concentration of 4.3 ng, 442 ng or 1423 ng NAT per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 104%, 94% or 102%
	at a spiked concentration of 4.9 ng, 750 ng or 1350 ng NAT per litre urine and where n = 5 determinations	
Detection limit:	0.7 ng NAT per litre urine	
Quantitation limit:	2.0 ng NAT per litre urine	

***N*-Nitrosonornicotine (NNN)**

Within-day precision:	Standard deviation (rel.)	s_w = 4.4%, 1.9% or 3.1%
	Prognostic range	u = 12.2%, 5.3% or 8.6%
	at a spiked concentration of 4.3 ng, 90 ng or 225 ng NNN per litre urine and where n = 5 determinations	

Day-to-day precision:	Standard deviation (rel.)	s_w = 11.4%, 7.1% or 5.1%
	Prognostic range	u = 29.3%, 18.3% or 13.1%
	at a spiked concentration of 4.3 ng, 90 ng or 225 ng NNN per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 91%, 91% or 90%
	at a spiked concentration of 4.1 ng, 100 ng or 180 ng NNN per litre urine and where n = 5 determinations	
Detection limit:	0.8 ng NNN per litre urine	
Quantitation limit:	2.0 ng NNN per litre urine	

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

Within-day precision:	Standard deviation (rel.)	s_w = 8.3%, 11.9% or 6.0%
	Prognostic range	u = 23.0%, 33.0% or 16.7%
	at a spiked concentration of 7.8 ng, 591 ng or 1252 ng NNAL per litre urine and where n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 3.9%, 11.5% or 9.9%
	Prognostic range	u = 10.0%, 29.6% or 25.5%
	at a spiked concentration of 7.8 ng, 591 ng or 1252 ng NNAL per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 111%, 100% or 110%
	at a spiked concentration of 8.5 ng, 750 ng or 1350 ng NNAL per litre urine and where n = 5 determinations	
Detection limit:	2.0 ng NNAL per litre urine	
Quantitation limit:	5.0 ng NNAL per litre urine	

General information on tobacco-specific nitrosamines (TSNA)

TSNA include NAB, NAT, NNN and NNK. They are formed almost exclusively from tobacco alkaloids during the curing process of tobacco leaves [Hecht et al. 1981]. The main-stream smoke of cigarettes inhaled by smokers typically contains concentrations of 25 ng NAB, 230 ng NAT, 200 ng NNN and 150 ng NNK per cigarette [IARC 2004]. NNN and NNK concentrations of 4–20 and 0.8–1.5 ng/m^3 respectively, were measured in rooms where people were smoking [Tricker et al. 2009]. NNN and NNK have been classified by the IARC (International Agency for Research on Cancer) as Group 1 carcinogens ('carcinogenic to humans'). Both NAB and NAT, however, have been classified by the IARC as Group 3 carcinogens ('not classifiable as to its carcinogenicity to humans') [IARC 2004]. In animal studies, NNN leads to tumor formation in the oesophagus while NNK has been shown to be a carcinogen in lung and pancreas [Hecht and Hoffmann 1989].

The metabolism of NAB, NNN and NNK is well known, while that of NAT is less well investigated [Hecht 1998]. The main metabolite of NNK is NNAL, which is also a carcinogen. NNAL can be *O*- or *N*-glucuronidated. NNAL is excreted in urine in both free and conjugated form (approximate ratio 1 : 1). About 20–30% of the absorbed dose of NKK is excreted with the urine as NNAL [Hecht et al. 1993; Meger et al. 1999]. NAB, NAT and NNN are also excreted in free form and as glucuronides

[Stepanov and Hecht 2005]. Only about 1% of the absorbed dose of NNN is excreted in urine [Urban et al. 2009]. Since the mid-1990s, NNAL in urine (usually determined as total NNAL, free plus conjugated NNAL) has been an established biomarker for smokers, users of smokeless tobacco and passive smokers [Hecht 2002]. The simultaneous quantification of NAB, NAT, NNN and NNAL (see Figure 1) as biomarkers of exposure to TSNA is still relatively new [Kavvadias et al. 2009 a, 2009 b; Stepanov and Hecht 2005].

This method is suitable for the determination of NAB, NAT, NNN and NNAL in human urine. Table 1 summarises the mean concentrations of TSNA in urine samples of smokers (n = 83) and non-smokers (n = 25) determined in a volunteer study [Kavvadias et al. 2009 a]. The results are largely in line with literature data [Stepanov and Hecht 2005]. The biomarkers given are primarily used to assess the exposure of tobacco users to TSNA. NNAL can also be used to quantify the exposure of passive smokers to NNK.

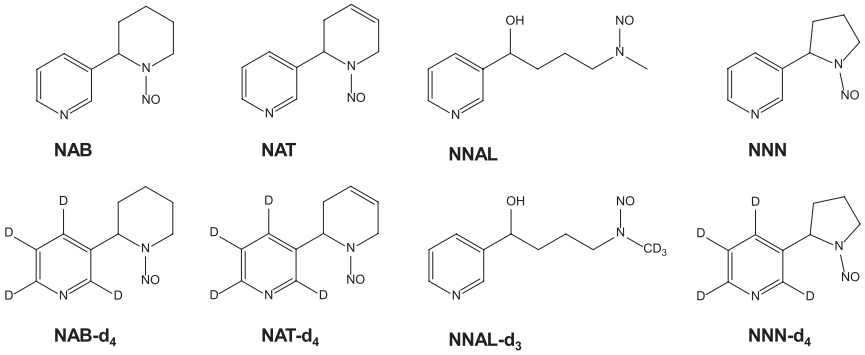

Figure 1 Chemical structures of the analytes NAB, NAT, NNN and NNAL as well as of the internal standards used.

Table 1 NAB, NAT, NNN and NNAL levels determined in the urine of smokers and non-smokers (according to Kavvadias et al. 2009 a).

	NAB	NAT	NNN	NNAL
	Median (range) [ng/L]			
Non-smokers (n = 25)	0.72 (< LOD–1.46)	0.03 (< LOD–0.21)	0.32 (< LOD–1.28)	9.9 (< LOD–39.0)
Smokers (n = 83)	47.2 (5.6–295)	210 (20.2–1234)	30.9 (0.60–1331)	126 (17.6–781)

Contents

1 General principles

This analytical method permits the determination of tobacco-specific nitrosamines (TSNA) in urine using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The parameters in question are *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is a metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Due to its sensitivity, this method is suitable for the detection of the aforementioned analytes in the urine of smokers. NNAL can also be quantified in the urine of passive smokers.

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polymers and then a mixed-mode cation exchange polymer. Analysis is performed by LC-MS/MS. Deuterated internal standards are used for calibration. Calibration standards are prepared in pooled urine obtained from non-smokers and are processed in the same way as the samples to be analysed.

2 Equipment, chemicals and solutions

2.1 Equipment

- LC-MS/MS system consisting of an HPLC system with a binary high-pressure pump, column oven with switching valve and degasser (e.g. HP 1100 by Agilent Technologies Deutschland GmbH, Waldbronn, Germany) as well as a temperature-controlled autosampler (e.g. HTC PAL by Axel Semrau GmbH & Co. KG, Sprockhövel, Germany) and a tandem mass spectrometer (e.g. API 4000 by AB Sciex Germany GmbH, Darmstadt, Germany)
- LC column: Luna 3 µm, C18(2) 100 Å, 250 × 3 mm with corresponding pre-column C18, 4 × 3 mm and pre-column holder (e.g. Phenomenex LTD, Aschaffenburg, Germany, No. 00G-4251-Y0)
- Laboratory shaker (e.g. IKA®-Werke GmbH & CO. KG, Staufen, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Incubator with integrated shaker (e.g. MMM Medcenter GmbH, Planegg, Germany)
- Vortex mixer (e.g. VWR International GmbH, Darmstadt, Germany)
- pH-Meter (e.g. Schott AG, Mainz, Germany)
- Centrifuge (e.g. Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Pasteur pipettes made of glass (e.g. Brand GmbH & Co. KG, Wertheim, Germany)
- Various pipettes and multipettes with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Various volumetric flasks, beakers and measuring cylinders (e.g. Schott AG, Mainz, Germany)
- Screw cap bottles made of glass (e.g. Schott AG, Mainz, Germany)
- 4 mL amber glass vials with screw caps (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- 14 mL screw top glass vials with plastic caps (e.g. Schuett Biotec GmbH, Göttingen, Germany) and septa (e.g. Sigma-Aldrich Chemie GmbH, München, Germany)
- Amber glass autosampler vials (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- SupelMIP® SPE cartridges 50 mg, 10 mL (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, Supelco™ Analytical, No. 53221-U)
- Oasis® MCX SPE cartridges, 60 mg, 3 mL (e.g. Waters GmbH, Eschborn, Germany, No. 186000254)
- SPE cartridge vacuum manifold (e.g. Separtis GmbH, Grenzach-Wyhlen, Germany)
- Vacuum concentrator Jouan (e.g. Thermo Electron Corp., Waltham, USA)
- UV lamp (e.g. Konrad Benda Laborgeräte, Wiesloch, Germany)
- Urine containers (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

2.2 Chemicals

Unless otherwise specified, all chemicals must be at least p.a. grade.

- *N*-Nitrosoanabasine (NAB) (e.g. Toronto Research Chemicals Inc, Canada, No. N524250)
- *N*-Nitrosoanabasine-d_4 (NAB-d_4) (e.g. Toronto Research Chemicals Inc, Canada, No. N524252)
- *N*-Nitrosoanatabine (NAT) (e.g. Toronto Research Chemicals Inc, Canada, No. N524750)
- *N*-Nitrosoanatabine-d_4 (NAT-d_4) (e.g. Toronto Research Chemicals Inc, Canada, No. N524752)
- *N*-Nitrosonornicotine (NNN) (e.g. Toronto Research Chemicals Inc, Canada, No. N535000)
- *N*-Nitrosonornicotine-d_4 (NNN-d_4), 1.0 mg/mL in acetonitrile (e.g. Toronto Research Chemicals Inc, Canada, No. KIT0730)
- 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (e.g. Toronto Research Chemicals Inc, Canada, No. M325740)
- 4-(Methyl-d_3-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL-d_3) (e.g. Toronto Research Chemicals Inc, Canada, No. M325742)
- Acetonitrile with 0.1% formic acid (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. 34668)
- Ammonium hydroxide solution, 25% in water (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. 09857)
- Dichloromethane, picograde (e.g. LGC Standards GmbH, Wesel, Germany, No. SO-1185-B025)
- Disodium hydrogen phosphate, ≥ 99.5% (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. 71636)
- *β*-Glucuronidase from E. coli, type IX-A (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. G7396)
- *n*-Heptane, 99% (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. 650536)
- *n*-Hexane, picograde (e.g. LGC Standards GmbH, Wesel, Germany No. SO-1244-B010)
- Hydrochloric acid, ≥ 37% (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. 30721)
- Methanol, picograde (e.g. LGC Standards GmbH, Wesel, Germany, No. SO-1263-B025)
- Potassium dihydrogen phosphate (e.g. Merck, No. 1.04873)
- Toluene, ≥ 99.9% (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. 650579)
- Ultrapure water (e.g. Sigma-Aldrich Chemie GmbH, München, Germany, No. 39253)
- Ultrapure water with 0.1% ammonium acetate (e.g. VWR International GmbH, Darmstadt, Germany, No. PRLS89781.320)

2.3 Solutions

- Ultrapure water, UV-irradiated
  Prior to use, the ultrapure water is irradiated with ultraviolet light at 254 nm for at least 12 h. For all further steps, only this UV-irradiated water is used.

- Ultrapure water with 0.1% ammonium acetate, UV-irradiated
  Prior to use, the ultrapure water with 0.1% ammonium acetate is irradiated with ultraviolet light at 254 nm for at least 12 h. For all further steps, only this UV-irradiated water with 0.1% ammonium acetate is used.

- Ammonium acetate solution, 10 mM
  60 mL ultrapure water are added to 200 mL ultrapure water with 0.1% ammonium acetate in a 500 mL screw cap bottle. The solution is thoroughly mixed.

- Dichloromethane toluene mixture, 1 : 1 (v/v)
  500 mL dichloromethane are placed into a 1000 mL screw cap bottle and 500 mL toluene are added. The solution is thoroughly mixed.

- Phosphate buffer, pH 7.2
  Solution A: Exactly 90.73 g potassium dihydrogen phosphate are weighed into a 1000 mL volumetric flask and dissolved in ultrapure water. The flask is then filled up with ultrapure water.
  Solution B: Exactly 94.64 g disodium hydrogen phosphate are weighed into a 1000 mL volumetric flask and dissolved in ultrapure water. The flask is then filled up with ultrapure water.
  To prepare the phosphate buffer 1 L of solution B is poured in a 2 L beaker. A pH meter is used for pH measurement and solution A is added under stirring to adjust the pH to 7.2 (ratio B : A approx. 4 : 1, v/v).

- Enzyme suspension (β-glucuronidase), 250 U/µL
  The total amount of β-glucuronidase (440.7 mg solid, approx. 50% protein, 3660000 U/g protein) is suspended in 3224 µL of the phosphate buffer in the original vial.

- Hydrochloric acid solution, 0.1 M
  Approximately 700 mL ultrapure water are placed into a 1000 mL volumetric flask and 8 mL of concentrated hydrochloric acid are added. The flask is then filled up with ultrapure water.

- Methanol in dichloromethane, 10% (v/v)
  Approximately 700 mL dichloromethane are placed into a 1000 mL volumetric flask and 100 mL methanol are added. The flask is then filled up with dichloromethane.

- Methanolic ammonium hydroxide solution
  Approximately 700 mL methanol are placed into a 1000 mL volumetric flask and 100 mL of the 25% ammonium hydroxide solution are added. The flask is then filled up with methanol.

The aqueous solutions and the methanolic ammonium hydroxide solution are stable for at least three months when stored in the refrigerator at +4 °C. All other solvent mixtures are stable for at least twelve months when stored in the refrigerator at +4 °C. The enzyme suspension is stable for at least two months when stored at −20 °C.

2.4 Internal standards (ISTD)

- ISTD stock solution I (1000 mg/L)
  Exactly 10 mg each of the standard substances NAB-d_4, NAT-d_4 and NNAL-d_3 are weighed into a 10 mL volumetric flask each and dissolved in methanol. The volumetric flasks are filled up with methanol.
- ISTD stock solution II (100 mg/L)
  1 mL each of the ISTD stock solutions I of NAB-d_4, NAT-d_4 and NNAL-d_3 as well as 1 mL of the purchased NNN-d_4 stock solution are pipetted into a 10 mL volumetric flask each. The volumetric flasks are filled up with methanol.
- ISTD spiking solution I
  0.1 mL each of the ISTD stock solutions II of NAB-d_4, NNAL-d_3 and NNN-d_4 as well as 1 mL of the ISTD stock solution II of NAT-d_4 are pipetted into a 10 mL volumetric flask. The flask is filled up with methanol (concentration 1 mg/L each of NAB-d_4, NNAL-d_3 and NNN-d_4 as well as 10 mg/L of NAT-d_4).
- ISTD spiking solution II
  0.5 mL of the ISTD spiking solution I are pipetted into a 20 mL volumetric flask. The flask is filled up with water (concentration 25 µg/L each of NAB-d_4, NNAL-d_3 and NNN-d_4 as well as 250 µg/L of NAT-d_4).

The ISTD stock and spiking solutions are stable for at least two years when stored at −20 °C.

2.5 Calibration standards

- Stock solution I (2000 mg/L)
  Exactly 20 mg each of the standard substances NAB, NAT, NNAL and NNN are weighed into a 10 mL volumetric flask each and dissolved in methanol. The volumetric flasks are filled up with methanol.
- Stock solution II (100 mg/L)
  0.5 mL each of the stock solution I of NAB, NAT, NNAL and NNN are pipetted into a 10 mL volumetric flask. The volumetric flask is filled up to the mark with methanol.
- Spiking solution I (10 mg/L)
  1 mL of stock solution II is pipetted into a 10 mL volumetric flask and the flask is filled up with water.

- Spiking solution II (100 µg/L)
  0.1 mL of spiking solution I is pipetted into a 10 mL volumetric flask and the flask is filled up with water.
- Spiking solution III (10 µg/L)
  1 mL of spiking solution II is pipetted into a 10 mL volumetric flask and the flask is filled up with water.
- Spiking solution IV (0.2 µg/L)
  0.2 mL of spiking solution III are pipetted into a 10 mL volumetric flask and the flask is filled up with water.

The stock solutions are stable for at least twelve months when stored at –20 °C. The spiking solutions are stable for at least three months when stored at –20 °C.

The calibration standards are prepared in pooled urine obtained from non-smokers. To prepare the pooled urine, spot urine samples are collected in a suitable container, thoroughly mixed and stored at –20 °C until preparation of the standards and the control material.

Calibration standards in a concentration range between 2 and 1500 ng/L are prepared by diluting the spiking solutions with pooled urine according to the pipetting scheme shown in Table 2. Additionally, the pooled urine used is included as a blank value.

Table 2 Pipetting scheme for the preparation of calibration standards used to determine NAB, NAT, NNN and NNAL in urine.

Calibration standard	Spiking solution used	Volume of the spiking solution	Volume of the pooled urine	Concentration of the analytes
		[µL]	[µL]	[ng/L]
Blank value	–	–	6000	–
1	IV	60	5940	2
2	IV	150	5850	5
3	IV	300	5700	10
4	III	15	5985	25
5	III	30	5970	50
6	III	60	5940	100
7	III	180	5820	300
8	II	45	5955	750
9	II	90	5910	1500

3 Specimen collection and sample preparation

3.1 Specimen collection

The urine samples are collected in suitable urine collection containers and stored at –20 °C until analysis.

3.2 Sample preparation

Prior to analysis, the samples are thawed at room temperature in the dark and thoroughly mixed. For sample preparation, 6 mL of the urine samples are transferred into 14 mL screw top vials and 2 mL phosphate buffer (pH 7.2), 20 µL of the ISTD spiking solution II and 40 µL of the enzyme suspension are added. The samples are thoroughly mixed using a vortex and incubated overnight at 37 °C for about 16 h in the dark while shaking (approx. 100 rpm). Afterwards, the samples are centrifuged at 3300 × *g* for 20 min.

For the first solid phase extraction procedure, the SupelMIP-SPE cartridges are washed with 10 mL each of 10% (v/v) methanol in dichloromethane. To this end, the solvent is passed through the cartridges at a flow rate of about 0.5 mL/min. The cartridges are then vacuum-dried (20–30 min at 500–600 mbar). The SPE columns are then sequentially conditioned with 1 mL methanol and 1 mL ultrapure water each. The cartridge material must not be allowed to run dry. Afterwards, the supernatant of the hydrolysed urine samples are transferred to the cartridges using a pasteur pipettes. The flow rate should not exceed 0.5 mL/min. This flow rate should also be maintained with all further rinsing steps. It may be necessary to apply vacuum and adjust it if the sample flow is very slow. After loading the samples, the cartridges are washed with 3 mL of the 10 mM ammonium acetate solution and then vacuum-dried (20–30 min at 500–600 mbar). Afterwards, the cartridges are first rinsed with 2 mL *n*-heptane and then with 1 mL *n*-hexane and then again vacuum-dried (20–30 min at 500–600 mbar). The analytes are then eluted with 3 mL of the dichloromethane toluene mixture into 4 mL amber glass vials at an elution rate of max. 0.2 mL/min. The eluates are evaporated to dryness in a vacuum concentrator and then dissolved in 1 mL of the phosphate buffer (pH 7.2).

Subsequently, the second solid phase extraction procedure using MCX cartridges takes place. The cartridges are successively conditioned with 2 mL methanol, 1 mL of the methanol ammonium hydroxide solution and 2 mL ultrapure water each. The sample extracts dissolved in the phosphate buffer are then completely transferred onto the cartridges, which are then rinsed successively with 2 mL ultrapure water, 2 mL of 0.1 M hydrochloric acid solution and 2 mL methanol, each. A flow rate of up to 5 mL/min is acceptable for all conditioning and rinsing steps. However, a flow rate of 1.0 mL/min should not be exceeded during sample transfer and analyte elution. The analytes are eluted with 2 mL of the methanol-ammonium hydroxide solution into 4 mL amber glass vials. The eluates are evaporated to dryness in a vacuum concentrator. The residues are then dissolved in 100 µL methanol, transferred to amber glass autosampler vials and can be directly injected into the LC-MS/MS system for analysis.

4 Operational parameters

Analysis was performed using an HPLC system coupled with a tandem mass spectrometer.

4.1 High performance liquid chromatography

Analytical column:	Luna 3 µm, C18(2) 100 Å, 250 × 3 mm
Mobile phase:	Mobile phase A: water with 0.1% ammonium acetate Mobile phase B: acetonitrile with 0.1% formic acid
Flow rate:	0.45 mL/min
Column temperature:	50 °C, isothermal
Injection volume:	5 µL
Autosampler temperature:	10 °C
Gradient program:	see Table 3

Table 3 Gradient program for the determination of NAB, NAT, NNN and NNAL in urine.

Time [min]	Mobile phase A [%]	Mobile phase B [%]	Flow rate [mL/min]
0	90	10	0.45
1.0	90	10	0.45
5.0	40	60	0.45
6.0	30	70	0.45
10.0	30	70	0.45
11.0	90	10	0.45
17.0	90	10	0.45

4.2 Tandem mass spectrometry

Ionisation mode:	positive electrospray ionisation (ESI+)
Source temperature:	650 °C
Detection mode:	MRM (Multi Reaction Monitoring)
Curtain gas:	25 psi
Nebulizer gas:	45 psi
Heater gas:	45 psi
Entrance potential:	10 V
Ion spray voltage:	1500 V
Parameter-specific MRM settings:	see Table 4

Table 4 Parameter-specific MRM settings for NAB, NAT, NNAL and NNN and the corresponding internal standards (DP: declustering potential, CE: collision energy, CXP: collision exit potential).

Analyte	Retention time [min]	Q1 [m/z]	Q3 [m/z]	Measurement duration [ms]	DP [V]	CE [V]	CXP [V]
NAB	9.30	192.0	162.0[a]	100	51	17	28
			105.9	25	51	47	18
NAB-d_4	9.28	196.0	166.0[a]	100	46	19	10
NAT	9.15	189.9	160.0[a]	100	41	15	28
			79.0	25	41	39	14
NAT-d_4	9.13	194.0	164.0[a]	100	41	15	10
NNAL	7.92	210.0	180.0[a]	50	56	17	16
			93.0	50	56	27	16
NNAL-d_3	7.92	213.0	183.0[a]	50	46	17	16
NNN	8.50	178.0	148.0[a]	100	46	17	8
			120.0	25	46	27	20
NNN-d_4	8.48	182.0	152.0[a]	100	41	15	12

[a] Quantifier

All ion source settings and MRM parameters are instrument-specific and must be adjusted individually by the user. The specified parameters are therefore intended as a rough guide only.

5 Analytical determination

5 µL each of the samples prepared as described in Section 3 are injected into the LC-MS/MS system. Identification of the analytes is based on the retention times and their specific mass transitions. The retention times of the analytes and internal standards as well as the ion transitions used are presented in Table 4. To confirm NAT and NNAL peak identity, the quantifier/qualifier ratio is calculated. A deviation of ± 35% from the reference value is acceptable. Here, the mean quantifier/qualifier ratio of the calibration solutions in the relevant concentration range is used as reference value. The qualifier signals of NAB and NNN are routinely only evaluated from 100 ng/L upwards, as matrix components interfere to varying degrees in the usual, low concentration range. One reagent blank (ultrapure water instead of the urine sample) and at least two quality control samples (see Section 8) are included in each analytical run.

The retention times given in Table 4 are intended as a rough guide only. Users must ensure proper separation performance of the HPLC column used influencing the resulting retention behaviour of the analytes. Figures 2 to 5 (in the Appendix) show chromatograms of a processed smokers' urine sample.

6 Calibration

The calibration standards described in Section 2.5 are prepared and processed in the same way as the samples (cf. Section 3) and analysed using LC-MS/MS (cf. Section 4). Calibration graphs are obtained by plotting the quotients of the peak areas of the analytes and of the respective isotope-labelled internal standards against the concentration of the calibration standards. Any blank values in the unspiked pooled urine are taken into account by subtraction and the interceptions of the calibration graphs are set to zero. Under the analytical conditions described, the calibration graphs are linear in the concentration range from 2 to 1500 ng/L. Figures 6 to 9 (in the Appendix) show calibration graphs of the analytes as examples.

7 Calculation of the analytical results

The analyte concentrations in the urine samples are calculated using the calibration functions of the respective analytical run (Section 6). To calculate the analyte concentrations in a urine sample, the peak area of the respective analyte is divided by the peak area of the corresponding internal standard. The quotients thus obtained are entered in the calibration functions according to Section 6 to give the respective analyte concentration in ng/L.

8 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundesärztekammer (German Medical Association) and in a general chapter of the MAK Collection for Occupational Health and Safety [Bader et al. 2010; Bundesärztekammer 2014]. To check precision, at least two quality control samples with known analyte concentrations are analysed within each analytical run. As material for quality control is not commercially available, it must be prepared in the laboratory. To this end, pooled urine obtained from non-smokers is spiked with the standard solutions of the analytes. The resulting analyte levels of the control materials should lie within the relevant concentration range. 6 mL-Aliquots of these samples are stored at –20 °C and included in each analytical run as quality control samples. The nominal value and the tolerance ranges of the quality control materials are determined in a pre-analytical period (one analysis of the control materials each on ten different days) [Bader et al. 2010].

9 Evaluation of the method

The reliability of the method was verified by comprehensive validation as well as by implementation and validation of the method in a second, independent laboratory.

9.1 Precision

Within-day precision was determined using pooled urine obtained from non-smokers, which was spiked with the analytes at three different concentration levels. These samples were then processed five times in parallel and analysed. The precision data obtained are presented in Table 5.

Day-to-day precision was determined using the same spiked pooled urine samples, which were processed on six different days and analysed. The precision data obtained are presented in Table 6.

Table 5 Within-day precision for the determination of NAB, NAT, NNAL and NNN in urine (n = 5).

Analyte	Spiked concentration	Standard deviation (rel.) s_w	Prognostic range u
	[ng/L]	[%]	[%]
NAB	8.9	11.7	32.5
	123	1.1	3.1
	192	1.1	3.1
NAT	4.3	13.2	36.6
	442	1.4	3.9
	1423	2.2	6.1
NNAL	7.8	8.3	23.0
	591	11.9	33.0
	1252	6.0	16.7
NNN	4.3	4.4	12.2
	90.0	1.9	5.3
	225	3.1	8.6

9.2 Accuracy

To determine the accuracy of the method, recovery experiments were performed based on pooled urine samples obtained from non-smokers spiked with the analytes at three different concentration levels. The relative recovery rates obtained are presented in Table 7.

Table 6 Day-to-day precision for the determination of NAB, NAT, NNAL and NNN in urine (n = 6).

Analyte	Spiked concentration	Standard deviation (rel.) s_w	Prognostic range u
	[ng/L]	[%]	[%]
NAB	8.9	3.8	9.8
	123	6.5	16.7
	192	3.5	9.0
NAT	4.3	8.8	22.6
	442	6.7	17.2
	1423	2.5	6.4
NNAL	7.8	3.9	10.0
	591	11.5	29.6
	1252	9.9	25.5
NNN	4.3	11.4	29.3
	90.0	7.1	18.3
	225	5.1	13.1

Table 7 Relative recovery rates for the determination of NAB, NAT, NNAL and NNN in urine (n = 5).

Analyte	Spiked concentration	Mean relative recovery r	Range
	[ng/L]	[%]	[%]
NAB	11.0	88.2	85.5–89.1
	100	90.8	86.4–93.9
	180	97.2	93.8–102
NAT	4.9	104	89.8–116
	750	93.5	80.1–98.4
	1350	102	99.0–105
NNAL	8.5	111	97.6–122
	750	100	97.2–106
	1350	110	97.6–121
NNN	4.1	91.1	85.4–95.1
	100	90.8	86.5–95.9
	180	90.3	86.6–97.0

9.3 Absolute recovery

To determine the absolute recovery, pooled urine samples obtained from non-smokers were spiked with the analytes at three different concentration levels and compared with a reference sample. The same urine matrix was used as reference. However, the reference sample was spiked with the analytes after processing, directly before sample injection into the LC-MS/MS system. The absolute recovery rates obtained are given in Table 8.

Table 8 Absolute recovery rates for the determination of NAB, NAT, NNAL and NNN in urine (n = 6).

Analyte	Spiked concentration	Mean absolute recovery *r*	Range
	[ng/L]	[%]	[%]
NAB	11.0	42.0	40.9–42.6
	100	61.6	58.5–63.6
	180	76.3	73.2–79.3
NAT	4.9	46.3	41.1–53.3
	750	60.4	51.3–63.0
	1350	77.4	75.5–80.1
NNAL	8.5	39.1	35.0–43.9
	750	32.1	30.9–33.7
	1350	45.5	40.7–50.5
NNN	4.1	37.9	35.4–39.4
	100	40.2	38.4–41.8
	180	56.8	54.3–60.8

9.4 Matrix effects

The matrix effects of the method were investigated using three individual urine samples. The urine samples were processed unspiked and were spiked with the internal standards and the analytes at a low and a high concentration just before the actual LC-MS/MS analysis. The matrix effects were calculated by comparing the peak areas with the signals in pure solvent. Data on the matrix effect are given in Table 9.

Table 9 Matrix effects for the determination of NAB, NAT, NNAL and NNN in urine (n = 3).

Analyte	Spiked concentration	Mean matrix effect	Range
	[ng/L]	[%]	[%]
NAB	5.0	78.6	70.4–82.8
	80.0	78.8	73.0–83.5
NAB-d_4	83.0	73.5	61.4–80.3
NAT	52.0	89.8	81.7–94.3
	600	95.4	91.6–101
NAT-d_4	833	86.4	79.9–93.2
NNAL	5.0	75.9	69.3–80.3
	600	72.2	65.6–75.7
NNAL-d_3	83.0	70.1	61.6–75.3
NNN	2.0	67.1	52.4–75.5
	80.0	66.3	55.7–77.7
NNN-d_4	83.0	63.4	47.9–77.0

9.5 Limits of detection and limits of quantitation

The limits of detection (LOD) were calculated by determining the signal-to-noise ratio in different urine samples. To determine the LOD, three different analyte-free matrix samples were spiked at a low concentration level and the signal-to-noise ratio was calculated. The LOD was set at a signal-to-noise ratio of three. The limit of quantitation (LOQ) was set by determining the precision and accuracy in six different urine samples spiked with standard solutions at a concentration level near the expected LOQ. The following criteria must be met: precision of max. 20% and accuracy of 80–120%. The values determined for the analytes in urine are given in Table 10.

Table 10 LOD and LOQ levels for the determination of NAB, NAT, NNAL and NNN in urine.

Analyte	LOD	LOQ
	[ng/L]	[ng/L]
NAB	1.1	5.0
NAT	0.7	2.0
NNAL	2.0	5.0
NNN	0.8	2.0

9.6 Stability

All stability tests were performed on urine samples freshly spiked with the analytes at a low and a high concentration level. The stability of the analytes in the matrix was tested by storing the samples for 24 h at room temperature (short-term stability) and for a longer period (8.5 months) at –20 °C (long-term stability) as well as after three freeze-thaw cycles (freeze-thaw stability). Furthermore, stability of processed samples was tested under autosampler conditions (10 °C) for six days. The analytes NAB, NAT, NNAL and NNN were adequately stable under all conditions tested, with the exception of NNAL, which showed a clear increase in concentration after 8.5 months (long-term stability). Thus, the long-term stability of NNAL is guaranteed for about six months only.

9.7 Sources of error

Carryover effects in the chromatographic system, such as injection carryover effects, were investigated by multiple injections of high and low concentration samples. A low concentration matrix sample was analysed three times, each after five injections of a high concentration sample. No interference was observed at the retention times of the analytes or the internal standards.

A potential artificial formation of the analytes NAB, NAT and NNN during sample preparation was investigated by analysing a matrix sample spiked with the precursors. Thus, a non-smoker urine sample was spiked with 215 ng/mL anabasine, 217 ng/mL anatabine and 253 ng/mL nornicotine and then processed and analysed in duplicate as described. No artificial formation of NAB, NAT and NNN was observed. In preliminary tests with the same spiked non-smoker urine sample, however, an artificial formation of NAT and NNN was observed when the solid phase extraction was performed in changed order: first MCX-SPE, then MIP-SPE.

Chromatographic interferences between the analytes and the matrix were observed in highly concentrated urine samples. This may considerably affect quantification, particularly in samples with low analyte levels. In some urine samples analysed so far, the signals of the analytes and ISTD were almost completely suppressed. Given a sufficient analyte concentration, analyte quantification was possible even in these samples, when the urines were processed with a significantly reduced sample volume (1 mL). In rare cases (< 1% of the samples), significantly elevated NNN levels (> 100 ng/L) were determined in urine samples of smokers. It can be assumed that artificial NNN formation from the precursors nornicotine and nitrite/nitrate took place in these samples during sample storage. Such artefact formation can be prevented by adding a strong base (e.g. sodium hydroxide) or ammonium sulfamate to the collected urine [Shah and Karnes 2010].

10 Discussion of the method

This method is the first multi analyte method for the simultaneous determination of tobacco-specific nitrosamines (TSNA) in human urine. It is suitable for the detection of exposure to TSNA among smokers and smokeless tobacco users. The application

to the assessment of TSNA exposure of non-smokers exposed to second-hand smoke is currently restricted to the analyte NNAL. In principle, however, TSNA exposure measurement from second-hand smoke can also be extended to the determination of NAB, NAT and NNN in urine, as these biomarkers could also be measured in urines of some non-smokers, using this method (cf. Table 1, Kavvadias et al. 2009 a). However, further systematic studies are needed.

Unlike NNAL, there are hardly any data available regarding the urinary excretion of NAB, NAT and NNN in humans. In addition to the authors' studies using the method described herein [Kavvadias et al. 2009 a; 2009 b; Urban et al. 2009], results are also available from the U.S. working group led by Mr. Hecht [Stepanov and Hecht 2005; Stepanov et al. 2009 a; 2009 b]. The concentrations measured by this working group in the urine of cigarette smokers tend to be higher for NNAL and NNN as well as lower for NAB and NAT compared to the authors' studies. The reason for these differences is still unclear. In another study from the U.S. [Sarkar et al. 2010], NNN excretions among cigarette smokers were found to be in line with the data obtained by this method.

Instruments used: LC-MS/MS system consisting of an Agilent 1100 series HPLC system (Agilent Technologies Deutschlang GmbH, Waldbronn, Germany) with the modules G1312 A (binary high-pressure pump), G1316 A (column oven with switching valve), G1379 A (degasser) and the temperature-controlled HTC PAL autosampler (Axel Semrau GmbH & Co. KG, Sprockhövel, Germany) coupled with an API 4000 tandem quadrupole mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany).

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Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft: MAK Commission

12 Appendix

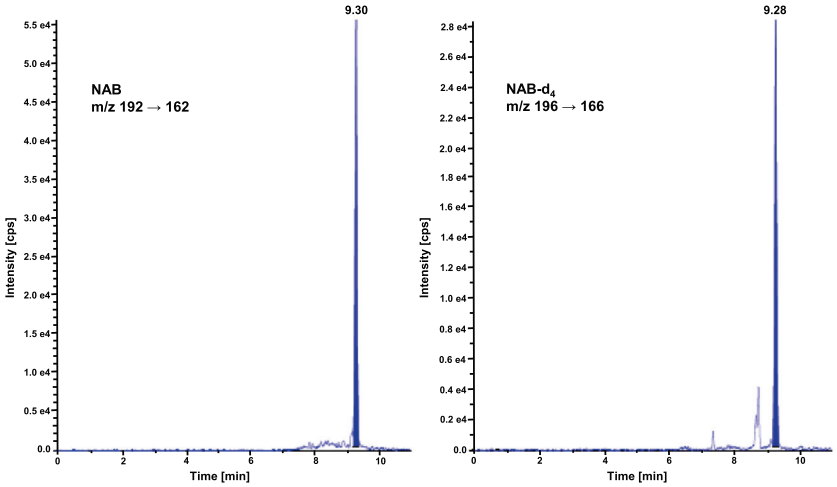

Figure 2 LC-MS/MS chromatogram of a processed smoker urine sample for the analyte NAB and the internal standard NAB-d_4 (NAB concentration determined: 70 ng/L).

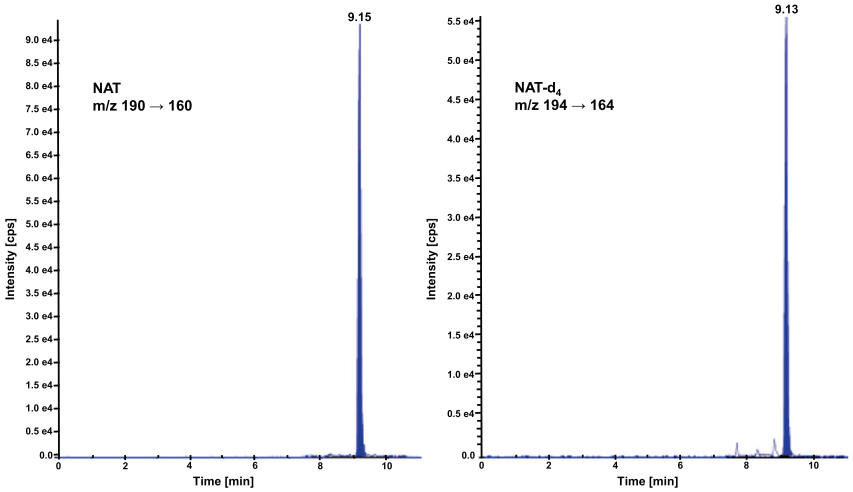

Figure 3 LC-MS/MS chromatogram of a processed smoker urine sample for the analyte NAT and the internal standard NAT-d_4 (NAT concentration determined: 327 ng/L).

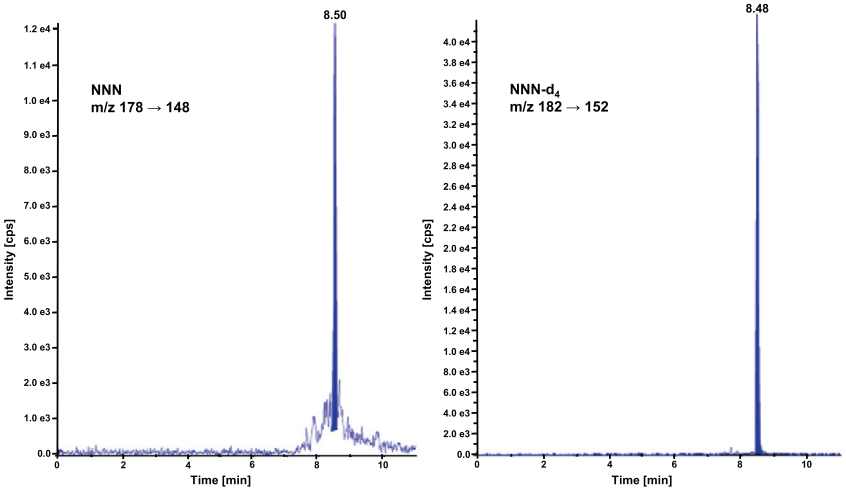

Figure 4 LC-MS/MS chromatogram of a processed smoker urine sample for the analyte NNN and the internal standard NNN-d_4 (NNN concentration determined: 17 ng/L).

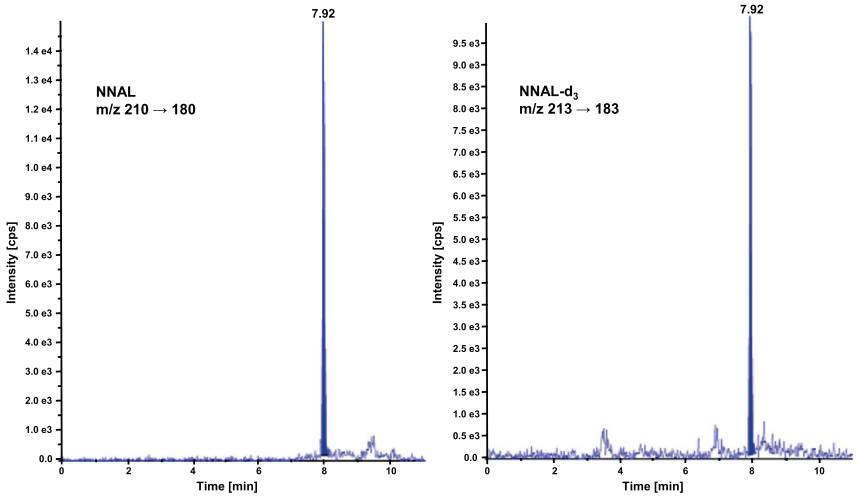

Figure 5 LC-MS/MS chromatogram of a processed smoker urine sample for the analyte NNAL and the internal standard NNAL-d_3 (NNAL concentration determined: 119 ng/L).

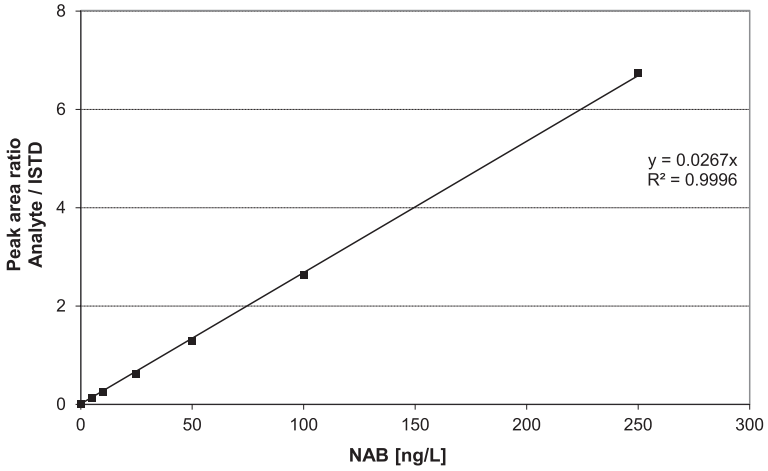

Figure 6 Calibration curve for the determination of NAB in urine (range 0–250 ng/L).

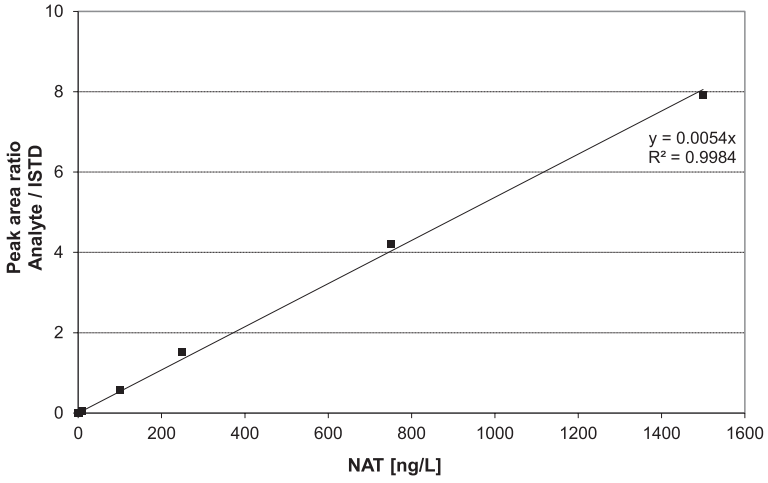

Figure 7 Calibration curve for the determination of NAT in urine (range 0–1500 ng/L).

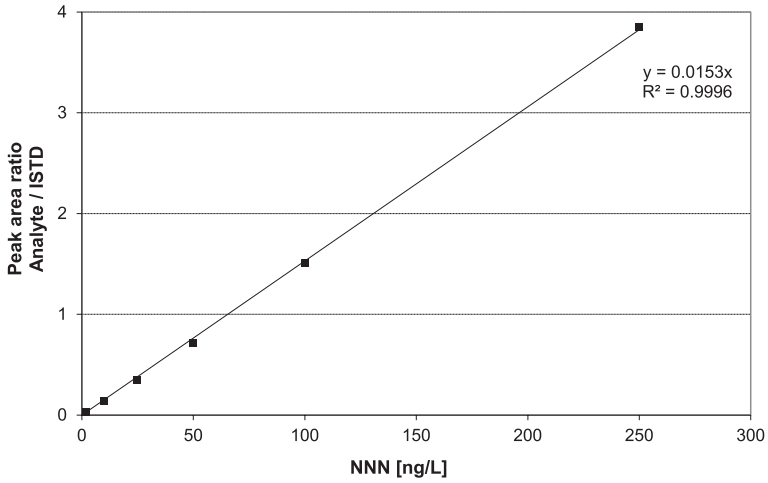

Figure 8 Calibration curve for the determination of NNN in urine (range 0–250 ng/L).

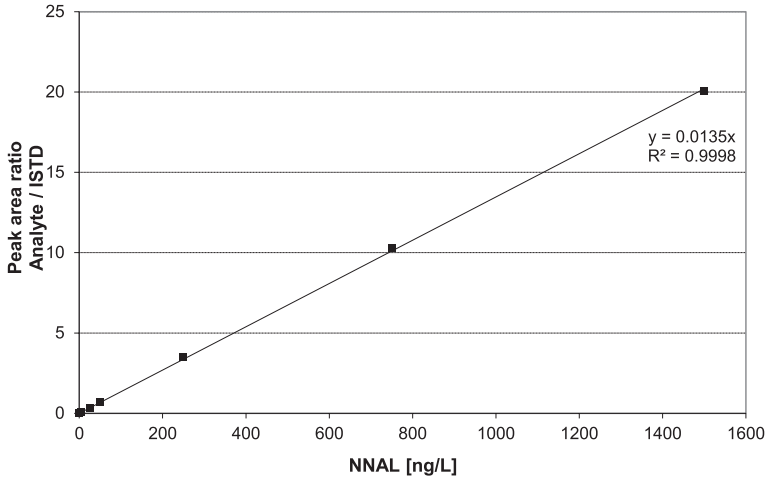

Figure 9 Calibration curve for the determination of NNAL in urine (range 0–1500 ng/L).