The MAK-Collection for Occupational Health and Safety

GMS PUBLISSO ZB MED-Publikationsportal Lebenswissenschaften

Hydrogen cyanide, cyanides and cyanide-releasing compounds – Determination of thiocyanate in plasma/serum, urine and saliva by GC-MS

Biomonitoring Method – Translation of the German version from 2020

Keywords

Hydrogen cyanide, cyanides, thiocyanate, plasma, serum, urine, saliva, gas chromatography, mass spectrometry, biomonitoring

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Citation Note:
Scherer G, Piller M, Riedel K, Müller M, Lange M, Göen T, Hartwig A, MAK Commission. Hydrogen cyanide, cyanides and cyanide-releasing compounds – Determination of thiocyanate in plasma/serum, urine and saliva by GC-MS. Biomonitoring Method – Translation of the German version from 2020. MAK Collect Occup Health Saf. 2020 Jul;5(2):Doc046. DOI: 10.34865/bi30204e5_2or

Manuscript completed:
14 May 2014

Publication date:
31 Jul 2020

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 verified the presented biomonitoring method.

Thiocyanate (SCN^-) is the main metabolite of cyanide and can thus be used as a biomarker of exposure to cyanide or cyanide-releasing compounds. Thiocyanate in plasma/serum, urine and saliva is a suitable biomarker especially for chronic exposure to low cyanide concentrations, such as those associated with smoking or found at some workplaces.

In the presented procedure thiocyanate is isolated from the matrix using liquid-liquid extraction in the presence of a phase transfer catalyst whilst it is simultaneously derivatised in the organic phase with PFBBr (2,3,4,5,6-pentafluorobenzylbromide). Analysis is then performed using GC-MS in NCI mode (negative chemical ionisation). This method allows the quantification of thiocyanate in occupationally exposed and non-exposed individuals.

Calibration standards are prepared in water and processed in the same way as the samples to be analysed. ^{13}C,^{15}N-labelled potassium thiocyanate is used as internal standard.

1 Characteristics of the method

Matrix Plasma/serum, urine and saliva

Analytical principle Gas chromatography with mass spectrometry (GC-MS)

Parameter and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
Hydrogen cyanide	74-90-8	Thiocyanate	302-04-5
Cyanide anions	57-12-5		
Sodium cyanide	143-33-9		
Potassium cyanide	151-50-8		
Cyanogen chloride	506-77-4		
Oxalonitrile	460-19-5		
Acetonitrile	75-05-8		
Acrylonitrile	107-13-1		

Reliability data

Thiocyanate in plasma/serum

Within-day precision:	Standard deviation (rel.)	s_w = 4.9%, 2.9% or 7.2%
	Prognostic range	u = 13.6%, 8.1% or 20.0%
	at a concentration of 0.75 mg, 2.78 mg or 10.7 mg thiocyanate per litre plasma/serum and where n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 3.4%, 2.6% or 3.3%
	Prognostic range	u = 9.4%, 7.2% or 9.2%
	at a concentration of 0.73 mg, 2.93 mg or 10.6 mg thiocyanate per litre plasma/serum and where n = 5 determinations	
Accuracy:	Recovery rate (rel.)	r = 93.5%, 92.9% or 93.6%
	at a concentration of 0.73 mg, 2.93 mg or 10.6 mg thiocyanate per litre plasma/serum and where n = 5 determinations	
Detection limit:	0.0005 mg thiocyanate per litre plasma/serum	
Quantitation limit:	0.0015 mg thiocyanate per litre plasma/serum	

Thiocyanate in urine

Within-day precision:	Standard deviation (rel.)	s_w = 5.4%, 4.4% or 5.3%
	Prognostic range	u = 15.0%, 12.2% or 14.7%
	at a concentration of 0.27 mg, 5.83 mg or 38.0 mg thiocyanate per litre urine and where n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 6.4%, 6.0% or 3.6%
	Prognostic range	u = 17.8%, 16.7% or 10.0%
	at a concentration of 0.26 mg, 5.81 mg or 35.5 mg thiocyanate per litre urine and where n = 5 determinations	
Accuracy:	Recovery rate (rel.)	r = 94.6%, 94.1% or 90.5%
	at a spiked concentration of 0.56 mg, 12.2 mg or 38.3 mg thiocyanate per litre urine and where n = 5 determinations	
Detection limit:	0.0006 mg thiocyanate per litre urine	
Quantitation limit:	0.0017 mg thiocyanate per litre urine	

Thiocyanate in saliva

Within-day precision:	Standard deviation (rel.)	s_w = 4.1%, 5.6% or 6.0%
	Prognostic range	u = 11.4%, 15.6% or 16.7%
	at a concentration of 13.6 mg, 92.1 mg or 162 mg thiocyanate per litre saliva and where n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 8.2%, 1.7% or 4.8%
	Prognostic range	u = 22.8%, 4.7% or 13.3%
	at a concentration of 12.9 mg, 91.6 mg or 157 mg thiocyanate per litre saliva and where n = 5 determinations	
Accuracy:	Recovery rate (rel.)	r = 97.9%, 89.9% or 88.4%
	at a spiked concentration of 28.9 mg, 113 mg or 209 mg thiocyanate per litre saliva and where n = 5 determinations	
Detection limit:	0.0007 mg thiocyanate per litre saliva	
Quantitation limit:	0.0020 mg thiocyanate per litre saliva	

2 General information on thiocyanate

Thiocyanate (rhodanide; SCN^-) is the detoxification product of cyanide, a potent inhibitor of cellular respiration, and can thus be used as a biomarker of exposure to cyanide or cyanide-releasing compounds. Thiocyanate in plasma/serum, urine and saliva is a suitable biomarker especially for chronic exposure to low cyanide concentrations, such as those associated with smoking or found at some workplaces.

Thiocyanate is formed by enzymatic transfer of sulphur from 3-mercaptopyruvate (via 3-mercaptopyruvate-cyanide-sulfurtransferase) or from thiosulphate (via rhodanase) to cyanide. Thiocyanate is mainly formed via rhodanase (see Figure 1) (Eben and Lewalter 1988). This conversion by rhodanase is also used for detoxification in case of cyanide poisoning. To this end, sodium thiosulphate is injected or infused as an antidote (Eyer 2004).

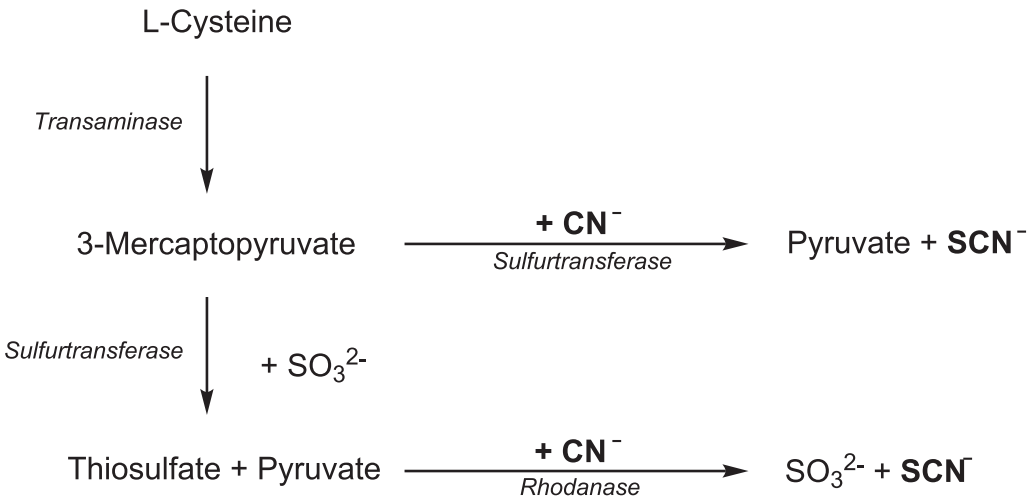

Fig. 1 Thiocyanate formation from cyanide according to Eyer (2004)

On account of the relatively long half-life of 6–14 days in plasma/serum, urine and saliva (Junge 1985; Pettigrew and Fell 1972), thiocyanate is particularly suitable for the detection of chronic cyanide exposure. In the case of acute cyanide exposure, the determination of cyanide in blood is also feasible (Eben and Lewalter 1988). The thiocyanate levels in saliva are about 20 times higher than those in plasma/serum or urine, which is probably due to active secretion of the thiocyanate ion in the salivary glands. However, the thiocyanate concentrations in saliva also depend on the salivary flow rate (Steinmaus et al. 2007) and are thus subject to major intra-individual variations.

Inhalable hydrogen cyanide, which is formed from cyanide salts even under carbonic acidic conditions, can be found wherever cyanides are used. This is the case, for example, when using electroplating baths. In addition, hydrogen cyanide is formed from nitriles such as acetonitrile, acrylonitrile and cyanohydrins and can also be formed during the combustion and pyrolysis of nitrogen-containing plastics. Moreover, hydrogen cyanide is used as pesticidal agent on ships (Eben and Lewalter 1988).

The mainstream smoke of a cigarette contains approximately 50–200 µg hydrogen cyanide (Rickert et al. 1983). Accordingly, thiocyanate levels in body fluids of smokers are about two to three times higher than those of non-smokers (see Table 1). Up into the 1980s, thiocyanate was frequently used to differentiate between smokers and non-smokers and as an objective measure of exposure to tobacco smoke. Today, cotinine is used as a much more specific biomarker of tobacco smoke exposure (Scherer et al. 2001). The specificity of thiocyanate as a marker of exposure to low cyanide concentrations at the workplace or from active smoking is limited by the fact that cyanides or thiocyanate itself can also be found in various foods. Cyanides in the form of cyanogenic glycosides can, for example, be found in almonds, nuts, pulses, bamboo shoots, beans, linseed and beer. Furthermore, the seeds of stone fruits generally contain cyanides leading to the occurrence of cyanides in fruit brandies. Preformed thiocyanate is found in cabbage, turnips, mustard and milk (Baumeister et al. 1975; Bliss and O'Connell 1984; Ockene et al. 1987; Steinmaus et al. 2007). These dietary sources make it generally difficult to evaluate thiocyanate as a biomarker of exposure.

Tab. 1 Thiocyanate levels in plasma, urine and saliva of non-smokers and smokers (mean ± standard deviation)

Non-smokers	Smokers	Reference
	Thiocyanate in plasma [mg/l]	
3.47 ± 2.39 (n = 6815)	9.09 ± 3.41 (n = 10 377)	Bliss and O'Connell 1984
3.16 ± 1.75 (n = 1356)	10.10 ± 3.22 (n = 5090)	Ockene et al. 1987
3.08 ± 1.59 (n = 3274)	10.04 ± 3.03 (n = 4553)	Ruth et al. 1991
	Thiocyanate in urine [mg/l]	
0.81 ± 0.06 (n = 260)	4.34 ± 0.65 (n = 66)	Steinmaus et al. 2007
0.77 (n = 1197)	4.71 (n = 351)	Jain 2013 a
	Thiocyanate in saliva [mg/l]	
70.9 ± 44.2 (n = 242)	158 ± 64.5 (n = 287)	Bliss and O'Connell 1984
75.5 (n = 100)	142 (n = 94)	Jarvis et al. 1984
97.0 (median) (n = 207)	170 (median) (n = 117)	Degiampietro et al. 1987

Apart from it being a biomarker of exposure to cyanide, thiocyanate is also of toxicological significance. It has been known for some time that thiocyanate can catalyse the endogenous formation of carcinogenic nitrosamines (Ladd et al. 1984; Prue et al. 1980; Tsuda and Kurashima 1991). In recent years, the role of thiocyanate in the development of thyroid dysfunction, especially in combination with an increased perchlorate intake and low iodine intake, has also been discussed (Steinmaus et al. 2013). In the U.S. NHANES studies (National Health and Nutrition Examination Survey), the levels of thiocyanate, perchlorate and thyroid hormones have been measured since 2001 to test this hypothesis (Bruce et al. 2013; Jain 2013 a, b; Steinmaus et al. 2007, 2013). Although the effect of thiocyanate on thyroid function is generally found to be small, it may be toxicologically relevant in combination with increased exposure to perchlorate, leading to a reduced iodine uptake of the thyroid.

For details on the toxicological evaluation of hydrogen cyanide, cyanides and cyanide-releasing compounds, please refer to the relevant MAK Value Documentations of the Commission (see Table 2). Table 2 lists substances that lead, among other things, to an elevated cyanide exposure of the organism and thus to a urinary thiocyanate excretion. However, it should be noted that the Commission has not derived a biological assessment value based on thiocyanate for any of the listed substances. This is mainly due to the fact that thiocyanate is a rather unspecific parameter due to dietary background levels.

The abbreviations used in Table 2 for substance classification or designation are explained in the List of MAK and BAT Values by the Commission (DFG 2019).

Tab. 2 Toxicological evaluation of hazardous substances that lead to cyanide exposure and that may be metabolised to thiocyanate

Hazardous substance	MAK value [mg/m³][a)]	Pregnancy risk group	H; Sh[b)]	Carcinogen category	MAK Value Documentation
Hydrogen cyanide	2.1	C	H	–	Greim 2003 a
Cyanides (as CN^-)	2 E	C	H	–	Greim 2003 a
Sodium cyanide	3.8 E	C	H	–	Greim 2003 a
Potassium cyanide	5.0 E	C	H	–	Greim 2003 a
Acetonitrile	17	C	H	–	Hartwig and MAK Commission 2018
Acrylonitrile	–	–	H/Sh	2	Greim 2007
3-Dimethylaminopropanenitrile	–	–	–	–	Greim 2004
Oxalonitrile	11	D	H	–	Greim 2003 b; Hartwig and MAK Commission 2019
Methyl cyanoacrylate	9.2	D	–	–	Henschler 1979
Ethyl cyanoacrylate	–	–	–	–	Henschler 1979
Cyanogen chloride	–	–	–	–	Henschler 1973
Second-hand smoke in the workplace	–	–	–	1	Greim 1999

a) *maximale Arbeitsplatz-Konzentration*; maximum concentration at the workplace
b) Danger from percutaneous absorption/of sensitisation of the skin

3 General principles

Thiocyanate is isolated from the matrix using liquid-liquid extraction in the presence of a phase transfer catalyst whilst it is simultaneously derivatised in the organic phase with PFBBr (2,3,4,5,6-pentafluorobenzylbromide) (see Figure 2). Analysis is then performed using GC-MS in NCI mode (negative chemical ionisation). This method allows the quantification of thiocyanate in occupationally exposed and non-exposed individuals.

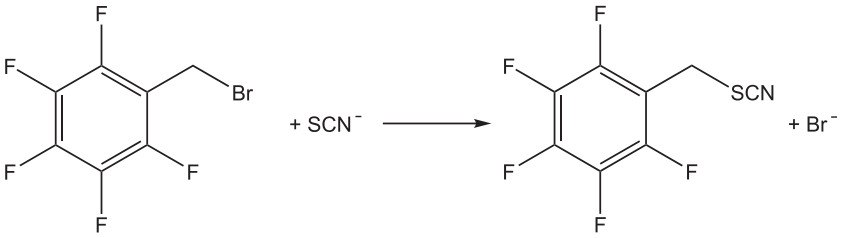

Fig. 2 Reaction of thiocyanate with PFBBr to form pentafluorobenzyl thiocyanate

Calibration standards are prepared in water and processed in the same way as the samples to be analysed. ^{13}C,^{15}N-labelled potassium thiocyanate is used as an internal standard.

4 Equipment, chemicals and solutions

4.1 Equipment

- GC-MS system (e.g. Trace GC Ultra with DSQ mass spectrometer and TriPlus, Thermo Fisher Scientific GmbH, Dreieich, Germany)
- Autosampler with cooled sample tray (e.g. Thermo Fisher Scientific GmbH, Dreieich, Germany)
- GC column (e.g. Rxi-5ms, 25 m × 0.25 mm × 0.25 µm, Restek GmbH, Bad Homburg, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Refrigerated centrifuge (e.g. Rotanta 460 R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Test tube shaker (e.g. VWR International GmbH, Darmstadt, Germany)
- Centrifugal vacuum concentrator (e.g. RC 10.22 Jouan, Thermo Fisher Scientific GmbH, Dreieich, Germany)
- Piston stroke pipettes with adjustable volumes between 10–100 µl, 100–1000 µl and 500–5000 µl, with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Various Multipettes with the matching Combitips 1.25 ml, 2.5 ml and 5 ml (e.g. Eppendorf AG, Hamburg, Germany)
- pH meter (e.g. SCHOTT AG, Mainz, Germany)
- Tube roller (e.g. VWR International GmbH, Darmstadt, Germany)
- Various beakers (e.g. SCHOTT AG, Mainz, Germany)
- Crimp cap GC vials with Teflon-coated caps (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- 4 ml screw cap vials with caps (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- 20 ml screw cap vials with caps (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- 100 ml laboratory bottles with threads (e.g. SCHOTT AG, Mainz, Germany)
- 250 ml wide-neck bottles (e.g. SCHOTT AG, Mainz, Germany)
- 10 ml and 100 ml volumetric flasks (e.g. SCHOTT AG, Mainz, Germany)
- Bottle top dispenser (e.g. Dispensette® organic, 10 ml, BRAND GMBH + CO KG, Wertheim, Germany)
- Potassium EDTA Monovettes® for blood collection (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- S-Monovettes® for blood collection (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Salivettes® for saliva collection (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Urine collection containers (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

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

- Dichloromethane, Picograde® (e.g. VWR International GmbH, Darmstadt, Germany, No. SO-1185-C011)
- Glacial acetic acid ≥ 99.0%, purum (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 45740)
- Hexadecyltrimethylammonium bromide (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. H9151)
- Methanol, HPLC grade (e.g. LGC Standards GmbH, Wesel, Germany, No. 3041)
- Sodium chloride ≥ 99.5% (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. S-9625)
- Sodium hydroxide pellets ≥ 98.0% (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 71690)
- 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr) ≥ 99.0% (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 17910)
- Toluene (e.g. LGC Standards GmbH, Wesel, Germany, No. 1350)
- Ultrapure water (e.g. Merck KGaA, Darmstadt, Germany, No. 1.16754.5000)
- Helium 5.0 (e.g. Linde AG, Pullach, Germany)
- Methane (e.g. Linde AG, Pullach, Germany)
- Potassium thiocyanate solution, 0.1 M (volumetric standard solution) (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 35194)
- Potassium thiocyanate (^{13}C, 99% ^{15}N, 98%+) (e.g. Eurisotop GmbH, Saarbrücken, Germany, No. CNLM-3952-0.5)

4.3 Solutions

- Derivatisation solution

  300 mg hexadecyltrimethylammonium bromide are weighed into a 20 ml screw cap vial. Firstly, 10 ml dichloromethane and 3 ml methanol are added followed by the addition of 130 µl PFBBr.

  The solution is stable for at least four weeks when stored at 2 °C to 8 °C.

- Acetate buffer (1 M)

  90 ml ultrapure water are placed into a beaker and exactly 5.758 ml glacial acetic acid are added. The pH of the solution is adjusted to 5.5 by adding sodium hydroxide pellets. The solution is transferred to a 100 ml volumetric flask and filled up to the mark with ultrapure water. The acetate buffer is transferred into a 250 ml wide-neck bottle for storage.

  The buffer is stored in the refrigerator and is stable for at least twelve weeks at 2 °C to 8 °C.

- Sodium chloride solution (saturated)

  50 g sodium chloride are weighed into a 250 ml wide-neck bottle and dissolved in 100 ml ultrapure water.

  The saturated sodium chloride solution is stored at room temperature and is stable for at least twelve months.

4.4 Internal standard (ISTD)

- ISTD stock solution (1.5 mM ≙ 149 mg SCN^- (^{13}C,^{15}N)/l)

  Exactly 14.87 mg KSCN (^{13}C,^{15}N) are weighed into a 100 ml volumetric flask and dissolved in ultrapure water. The flask is then made up to the mark with ultrapure water.

- ISTD spiking solution (60 µM ≙ 5.95 mg SCN^- (^{13}C,^{15}N)/l)

  4 ml of the ISTD stock solution are pipetted into a 100 ml volumetric flask. The flask is then made up to the mark with ultrapure water.

The ISTD solutions are stored at 2 °C to 8 °C. The stability of the ISTD working solution should be tested at the beginning of each analytical run (see Section 8).

4.5 Calibration standards

- Stock solution (0.1 M KSCN ≙ 5.81 g SCN^-/l)

  The 0.1 M potassium thiocyanate solution (5.81 g SCN^-/l) is purchased as a volumetric standard solution.

  The solution is stored at 2 °C to 8 °C and is stable for at least two years according to the manufacturer's specifications.

- Spiking solution 1 (SpS 1; 750 µM KSCN ≙ 43.6 mg SCN^-/l)

  75 µl of the potassium thiocyanate stock solution are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with ultrapure water.

- Spiking solution 2 (SpS 2; 93.8 µM KSCN ≙ 5.45 mg SCN^-/l)

  1250 µl of spiking solution 1 are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with ultrapure water.

- Spiking solution 3 (SpS 3; 5.86 µM KSCN ≙ 0.34 mg SCN^-/l)

  78.1 µl of spiking solution 1 are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with ultrapure water.

The standard spiking solutions are stored at 2 °C to 8 °C and are stable for at least two years.

Calibration standards in the concentration range between 0.043 mg and 43.6 mg thiocyanate per litre are prepared in ultrapure water according to the pipetting scheme shown in Table 3. Ultrapure water is included as a blank value. The calibration standards are freshly prepared for each analytical run and stored at 2 °C to 8 °C.

To establish the calibration curves in saliva, the concentrations of the calibration standards are converted (multiplication by a factor of 5), since the sample volume used for saliva is only 20 µl, but 100 µl each of the calibration standards are pipetted as for plasma/serum. The calibration standards are processed in the same way as the samples to be analysed (see Section 5.2).

Tab. 3 Pipetting scheme for the preparation of the calibration standards in water

Calibration standard	SpS 1 [µl]	SpS 2 [µl]	SpS 3 [µl]	Water [µl]	Conc. calibration standard [mg SCN^-/l]	
					plasma/serum, urine	saliva
0[a)]	–	–	–	1000	0	0
1	–	–	–	1000	0	0
2	–	–	125	875	0.043	0.213
3	–	–	250	750	0.085	0.425
4	–	–	1000	–	0.340	1.70
5	–	250	–	750	1.36	6.81
6	–	500	–	500	2.72	13.6
7	125	–	–	875	5.45	27.2
8	250	–	–	750	10.9	54.5
9	500	–	–	500	21.8	109
10	1000	–	–	–	43.6	218

[a)] Processed without the addition of ISTD

5 Specimen collection and sample preparation

5.1 Specimen collection

Plasma

After collecting blood using an EDTA Monovette, the sample is thoroughly mixed for 20 to 30 minutes on a tube roller. The sample can be stored at 4–6 °C for a maximum of 16 hours until further processing. For the preparation of plasma samples, the blood sample is centrifuged at 1200 × *g* for five minutes, the plasma is pipetted off and frozen at −20 °C until analysis. Plasma samples can be stored at −20 °C for up to 24 months without analyte losses.

Serum

For serum collection, the blood that has been collected using S-Monovettes is allowed to clot for 20 to 30 minutes. The sample is then centrifuged at 1500 × *g* for 10 minutes at room temperature, the serum is pipetted off and frozen at −20 °C until analysis. Serum samples can be stored at −20 °C for up to 24 months without analyte losses.

Urine

The polyethylene containers used for urine collection are washed with a detergent before sampling, rinsed with ultrapure water and left to dry. The urine samples collected are divided into 1 ml aliquots and frozen at −20 °C until analysis. Urine samples can be stored at this temperature for up to seven years without analyte losses.

Saliva

Saliva tubes with cotton swabs are used for saliva collection (e.g. Salivette®, Sarstedt AG & Co. KG, Nümbrecht, Germany). The cotton swab is placed in the mouth and rolled around for one to two minutes, then transferred in the tube and centrifuged for 10 minutes at 3000 × *g*. The saliva sample obtained should be processed and analysed as soon as possible, as any kind of storage could lead to false low results.

5.2 Sample preparation

Deep-frozen samples are thawed at room temperature and then mixed on a shaker.

100 µl plasma/serum or urine or 20 µl saliva are pipetted into a 4 ml screw cap vial. 300 µl ultrapure water, 25 µl acetate buffer, 50 µl of the ISTD spiking solution, 50 µl of the saturated sodium chloride solution, 25 µl toluene and 75 µl of the derivatisation solution are added. For saliva samples, 400 µl ultrapure water are used instead of 300 µl ultrapure water. The 4 ml vial is sealed and the solution is thoroughly mixed for 30 minutes on a laboratory shaker. Then, 2 ml toluene are added to the reaction mixture and the sample is again shaken on the laboratory shaker for 5 minutes. The sample is then centrifuged at 2250 × *g* for 10 minutes at room temperature. 300 µl of the toluene phase are transferred into a microvial.

The sample is reduced to a volume of 150–200 µl in the centrifugal vacuum concentrator at room temperature for about 5 minutes to remove any residual dichloromethane (originating from the derivatisation solution) which might interfere with the analysis. The analysis is then performed using GC–MS (NCl).

6 Operational parameters

Analysis is performed using a gas chromatograph coupled with a mass spectrometer operating in NCI mode.

6.1 Gas chromatography

Capillary column:	Stationary phase:	Rxi-5 ms
	Length:	25 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
Detector:	Mass selective detector	
Temperatures:	Column:	Initial temperature 50 °C, increase at a rate of 70 °C/min to 270 °C, 3 min at final temperature
	Injector:	220 °C
	Sample tray:	15 °C
Carrier gas:	Helium 5.0	
	Flow rate:	1 ml/min, constant
	Injection volume:	1 µl, split 1 : 20

6.2 Mass spectrometry

Ionisation mode:	NCI using methane (2 ml/min)
Source temperature:	150 °C
Interface temperature:	270 °C
Detector gain:	300 000
Dwell time:	20 ms
Solvent delay:	2.5 min
Detection mode:	Selected Ion Monitoring (SIM) mode

The specified operational parameters are intended as a rough guide only. Since the parameters specified above are instrument-specific, they must be adjusted individually by the user. All other parameters have to be optimised in accordance with the manufacturer's specifications.

7 Analytical determination

Identification of the analytes is based on their retention times and the specific ion traces (Table 4). Pentafluorobenzyl thiocyanate (PFBSCN) and pentafluorobenzyl thiocyanate (^{13}C,^{15}N) each have a mass fragment in which the labelled atoms of the ISTD are still present (atoms C and N in the analyte and the atoms ^{13}C and ^{15}N in the ISTD), occurring with a light and a heavy sulphur isotope. For PFBSCN these are the fragments with a mass-to-charge ratio of $m/z = 58$ and 60, for PFBSCN (^{13}C,^{15}N) the fragments with a mass-to-charge ratio of $m/z = 60$ and 62. The intensity of the fragment with the heavy sulphur isotope is approximately 5.5% of the intensity of the fragment with the light sulphur isotope and is no longer negligible at high thiocyanate levels. However, it has been shown that it is possible to quantify the fragment containing the light sulphur isotope ($m/z = 58$) for the unlabelled thiocyanate and the fragment with the heavy sulphur isotope ($m/z = 62$) for the internal standard. The measured fragment with a mass-to-charge ratio of $m/z = 60$ is only needed for validation purposes.

Tab. 4 Retention times and ion traces for the determination of thiocyanate in plasma/serum, urine and saliva

Analyte	Retention time [min]	Ion trace [m/z]
Thiocyanate	2.95	58, 60[a)]
Thiocyanate (^{13}C,^{15}N)	2.95	60[a)], 62

[a)] only needed for validation purposes

The retention times given in Table 4 are intended as a rough guide only. Users must ensure a sufficient separation performance of the analytical column used influencing the resulting retention behaviour of the analytes. Figures 3 to 5 show examples of chromatograms of a smoker's plasma sample, a non-smoker's urine sample and a non-smoker's saliva sample. The ion trace $m/z = 60$ is only needed for validation.

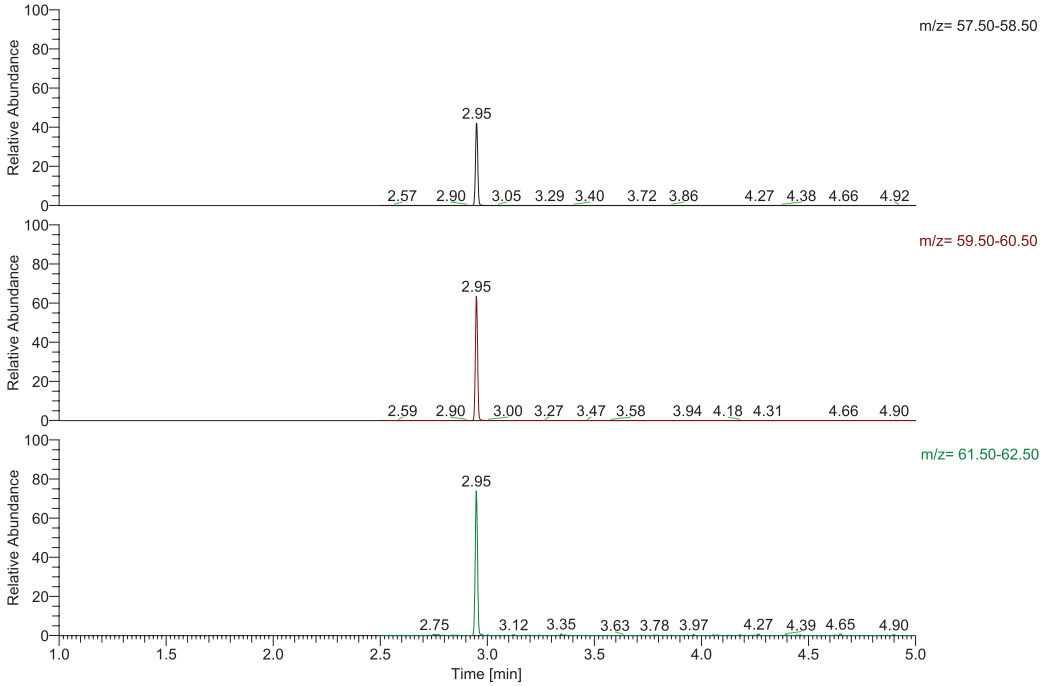

Fig. 3 Chromatogram of a smoker plasma sample with an SCN^- concentration of 205 µmol/l ≙ 120 mg/l (SCN^-: *m/z* = 58, 60; SCN^- (^{13}C,^{15}N): *m/z* = 60, 62)

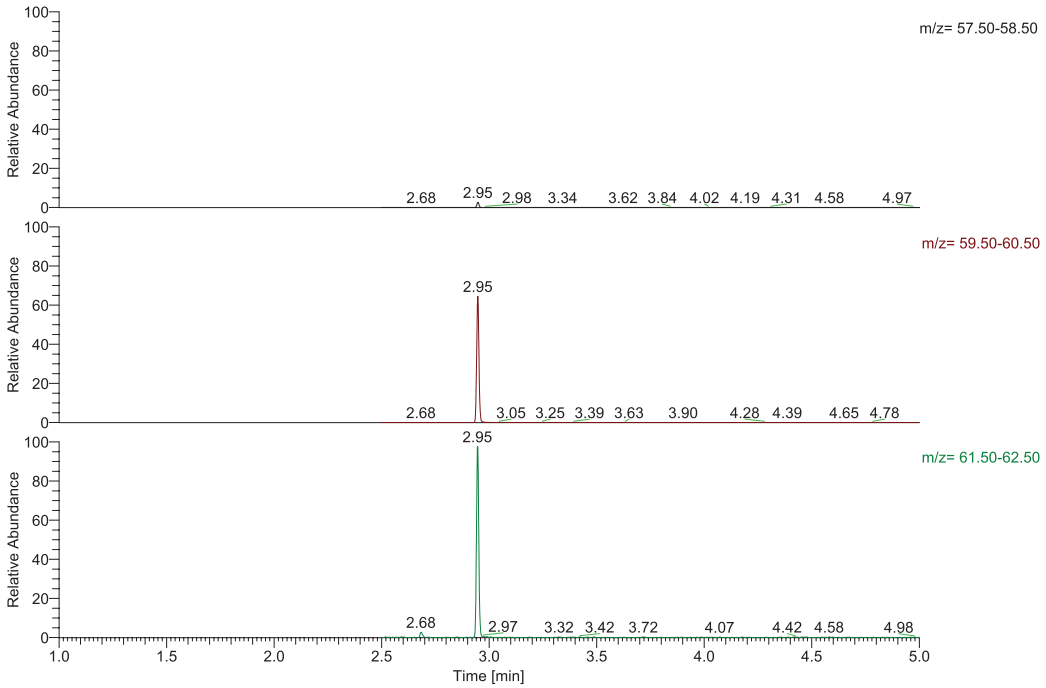

Fig. 4 Chromatogram of a non-smoker urine sample with an SCN^- concentration of 9.5 µmol/l ≙ 0.552 mg/l (SCN^-: *m/z* = 58, 60; SCN^- (^{13}C,^{15}N): *m/z* = 60, 62)

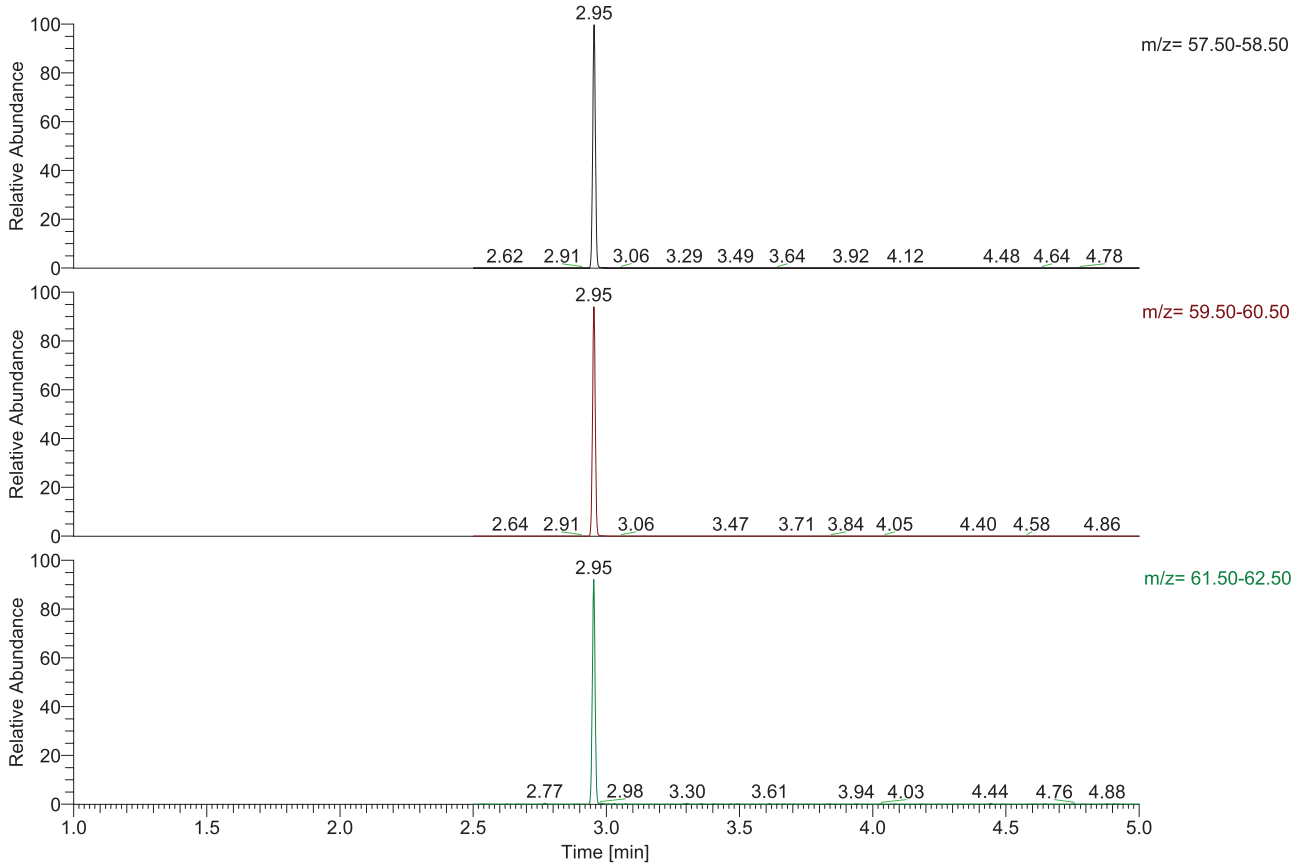

Fig. 5 Chromatogram of a non-smoker saliva sample with an SCN^- concentration of 1962 µmol/l ≙ 114 mg/l (SCN^-: *m/z* = 58, 60; SCN^- (^{13}C,^{15}N): *m/z* = 60, 62)

8 Calibration

The calibration standards described in Section 4.5 are prepared and processed in the same way as the samples (cf. Section 5.2) and analysed using GC-MS (cf. Section 6). The calibration standards are usually analysed in duplicate at each concentration level. The calibration standard 0, which is processed without the addition of ISTD, is used to check the ion trace of the internal standard for interfering peaks. In addition, in comparison with calibration standard 1, it can be seen whether there is any contamination with the unlabelled analyte.

Calibration graphs are obtained by plotting the quotients of the peak areas of the analyte and of the internal standard against the spiked concentration of the respective calibration standards. The peak area ratio of calibration standard 1 is not subtracted from the peak area ratios of the other standards, as the thiocyanate originates from the internal standard and is therefore present in each sample. In the range from the detection limit to 43.6 mg/SCN^-/l (plasma/serum and urine) and to 218 mg SCN^-/l (saliva), respectively, a linear calibration graph (weighted regression) is obtained which does not run through zero.

Figures 6 and 7 show an example of a calibration graph in plasma and saliva, respectively.

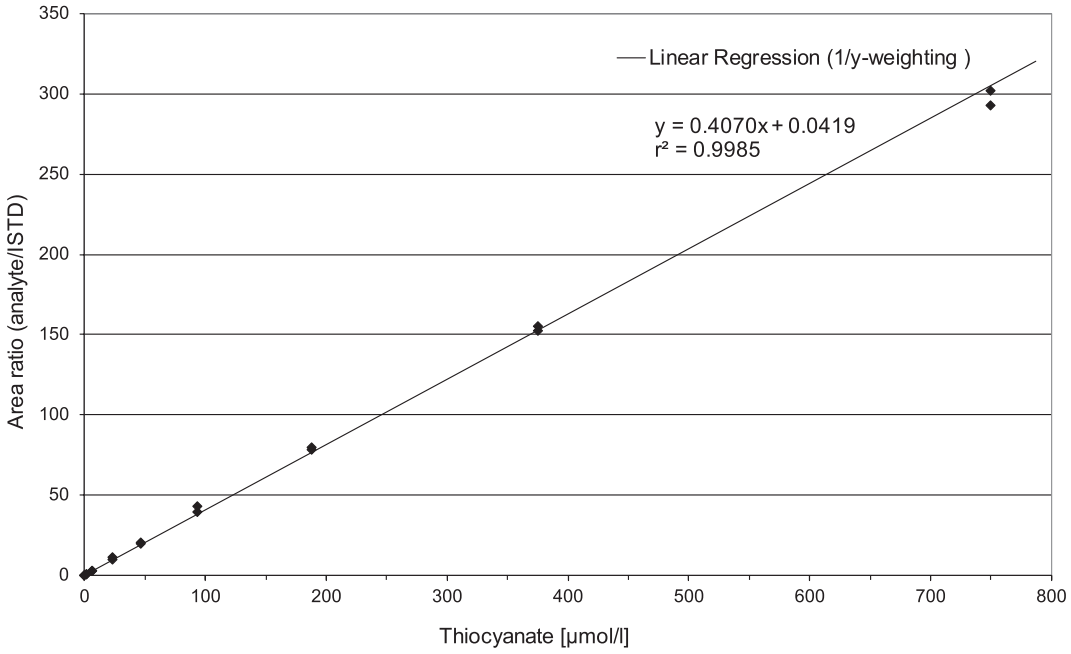

Fig. 6 Calibration curve for the determination of thiocyanate in plasma/serum (LOQ–750 µmol/l ≙ LOQ–43.6 mg/l)

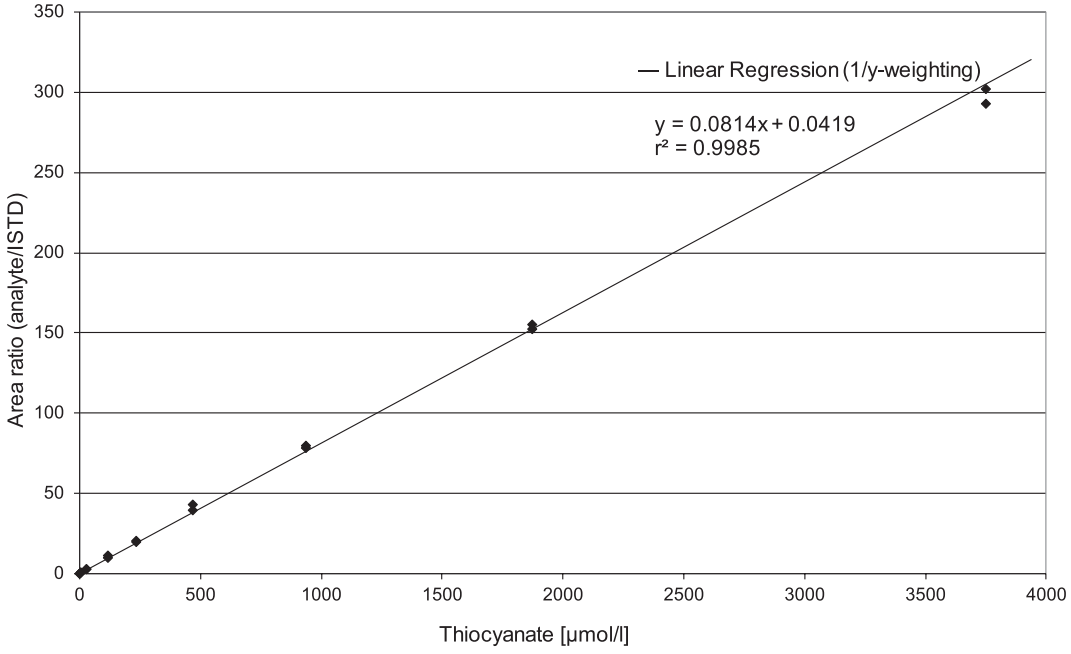

Fig. 7 Calibration curve for the determination of thiocyanate in saliva (LOQ–3750 µmol/l ≙ LOQ–218 mg/l)

9 Calculation of the analytical results

The analyte concentration of an unknown sample is determined by dividing the peak area of the analyte by the peak area of the internal standard. The quotient thus obtained is entered in the equation established according to Section 8 to give the respective analyte concentration in mg SCN^-/l.

10 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, quality control samples with known analyte levels are analysed within each analytical run. Plasma/serum, urine or saliva samples with low, medium and high concentrations (Q_{low}, Q_{medium} and Q_{high}) are used as control material.

Aliquots of these samples are stored at −20 °C and included in each analytical run as quality control samples. The nominal values and the tolerance ranges of the quality control materials are determined in a pre-analytical phase (Bader et al. 2010).

11 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.

11.1 Precision

Within-day precision was determined at three concentration levels per matrix, at a low, a medium and a high concentration. Individual samples and pooled samples, native or spiked, were used as sample material. The obtained within-day precision data are presented in Table 5.

Tab. 5 Within-day precision for the determination of thiocyanate in plasma/serum, urine and saliva (n = 5)

Analyte concentration [mg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Plasma/Serum		
0.75	4.9	13.6
2.78	2.9	8.1
10.7	7.2	20.0
Urine		
0.27	5.4	15.0
5.83	4.4	12.2
38.0	5.3	14.7
Saliva		
13.6	4.1	11.4
92.1	5.6	15.6
162	6.0	16.7

To determine day-to-day precision, sample material with a low, medium and high analyte concentration was processed on five different days and analysed. The obtained day-to-day precision data for the three matrices are presented in Table 6.

Tab. 6 Day-to-day precision for the determination of thiocyanate in plasma/serum, urine and saliva (n = 5)

Analyte concentration [mg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Plasma/Serum		
0.73	3.4	9.4
2.93	2.6	7.2
10.6	3.3	9.2
Urine		
0.26	6.4	17.8
5.81	6.0	16.7
35.5	3.6	10.0
Saliva		
12.9	8.2	22.8
91.6	1.7	4.7
157	4.8	13.3

11.2 Accuracy

The accuracy of the method was determined by spiking the relevant matrices with defined amounts of thiocyanate. Three different concentrations were each analysed. Five determinations were performed per concentration level. The results are presented in Table 7.

Tab. 7 Relative recovery rates for the determination of thiocyanate in plasma/serum, urine and saliva (n = 5)

Analyte concentration [mg/l]	Recovery rate r [%]
Plasma/Serum	
0.73	93.5
2.93	92.9
10.6	93.6
Urine	
0.56	94.6
12.2	94.1
38.3	90.5
Saliva	
28.9	97.9
113	89.9
209	88.4

11.3 Matrix effects

During external verification, calibration was also tested in plasma/serum, urine and saliva and was compared to calibration in water. In the different matrices, axially shifted calibration curves were obtained, however with similar slopes.

11.4 Limits of detection and limits of quantitation

The limit of detection was calculated on the basis of the 3-fold and the limit of quantitation on the basis of the 9-fold signal-to-noise ratio, with three different samples being analysed for each matrix. The limits of detection and quantitation for the determination of thiocyanate in plasma/serum, urine and saliva are presented in Table 8.

Tab. 8 Limits of detection and quantitation for the determination of thiocyanate in plasma/serum, urine and saliva (n = 3)

Matrix	Detection limit [mg/l]	Quantitation limit [mg/l]
Plasma/Serum	0.0005	0.0015
Urine	0.0006	0.0017
Saliva	0.0007	0.0020

At the concentration of the lowest calibration standard the calculated concentration deviates from the spiked concentration by less than 5%.

11.5 Sources of error

The extraction of thiocyanate in the presence of a phase transfer catalyst and the simultaneous derivatisation with PFBBr highly depends on the duration of the individual working steps and on other parameters such as temperature and shaking intensity. Therefore, the use of a suitable internal standard (KSCN (^{13}C, ^{15}N)) is essential.

The selectivity of the method was verified during method development by analysing six individual samples with low analyte concentration levels for each matrix. No interfering signals at the retention time of the internal standard were detected in any of the samples, which accounted for more than 5% of the ISTD level. Moreover, the identity of the analyte peak was confirmed by the quantifier/qualifier ratio using six samples per matrix.

During method development, the stability of the analyte was also tested in the different matrices. Stability testing in urine and saliva was performed with fresh matrix samples containing thiocyanate at a low (unspiked) and a high level (spiked urine or saliva). Testing in plasma/serum was performed on serum samples that have been stored for about one year. Short-term stability (20 hours or 24 hours at room temperature) as well as freeze-thaw stability (three and six freeze thaw cycles) and long-term stability (different periods at –20 °C) is given for both concentration levels in the three matrices. The accuracy of this testing ranged between 93.5 and 112% for plasma/serum, between 89.6 and 111% for urine and between 80.3 and 115% for saliva. The data for saliva show, that the analyte stability in salivary samples is not conclusively clarified (see Section 5.1). Thus, saliva samples should be processed and analysed as soon as possible after sampling.

Carry-over effects in the chromatographic system were investigated by multiple injection of processed samples with high thiocyanate levels, alternating with solvent injections. After five injections of a high concentration sample, solvent was injected and analysed. This was done three times per matrix. A minimal carry-over effect was observed in all three matrices, which was at a maximum of 3 µg/l.

12 Discussion of the method

The analytical method presented herein is suitable for the determination of thiocyanate (SCN^-) in both the occupational and environmental concentration range. Thiocyanate in plasma/serum, urine and saliva is a suitable biomarker especially for chronic exposure to low cyanide concentrations, such as those associated with smoking and those found at some workplaces. Unlike the methods already published by the Commission (Riedel et al. 2013; Scherer et al. 2001), this method is also suitable for the quantitation of thiocyanate in urine.

It is a gas chromatographic method with mass-selective detection using a stable isotope-labelled internal standard. It is based on the method published by Toraño and van Kan (2003), which was, however, considerably modified.

The use of a stable isotope-labelled internal standard is essential to compensate for potential variances during sample preparation (extraction, phase transfer, derivatisation). The method permits the rapid, sensitive, specific and reliable determination of thiocyanate in all matrices mentioned and meets all the requirements for an analytical method for human biomonitoring.

Instruments used GC-MS system (e.g. Trace GC Ultra with DSQ mass selective detector and TriPlus, Thermo Fisher Scientific GmbH, Dreieich, Germany), autosampler with cooled sample tray (e.g. Thermo Fisher Scientific GmbH, Dreieich, Germany); peak integration and the calculation of the analyte concentrations was performed using Xcalibur 2.1 software.

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