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Acrylonitrile, 2-bromoethanol, chloroacetaldehyde, 2-chloroethanol, 1,2-dibromoethane, 2,2´-dichlorodiethyl ether, 1,2-dichloroethane, 1,1-dichloroethene, ethylene oxide, monochloroacetic acid, 1,1,2-trichloroethane, vinyl bromide, and vinyl chloride – Determination of thiodiglycolic acid in urine by UPLC-MS/MS

Biomonitoring Method – Translation of the German version from 2022

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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 verified the presented biomonitoring method. With this method, thiodiglycolic acid (thiodiacetic acid, TDAA), one of the main metabolites of vinyl chloride, acrylonitrile, 2-bromoethanol, chloroacetaldehyde, 2-chloroethanol, 1,2-dibromoethane, 2,2′-dichlorodiethyl ether, 1,2-dichloroethane, 1,1-dichloroethene, ethylene oxide, monochloroacetic acid, 1,1,2-trichloroethane, and vinyl bromide is determined in urine. The urine samples are diluted with 0.033% formic acid and mixed with a deuterated internal standard. TDAA is separated from matrix compounds by liquid chromatography and is subsequently detected with tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in ultra-pure water.

1 Characteristics of the method

Matrix	Urine
Analytical principle	Ultra high-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS)

Parameter and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
Acrylonitrile	107-13-1		
2-Bromoethanol	540-51-2		
Chloroacetaldehyde	107-20-0		
2-Chloroethanol	107-07-3		
1,2-Dibromoethane	106-93-4		
2,2′-Dichlorodiethyl ether	111-44-4		
1,2-Dichloroethane	107-06-2	Thiodiglycolic acid	123-93-3
1,1-Dichloroethene	75-35-4		
Ethylene oxide	75-21-8		
Monochloroacetic acid	79-11-8		
1,1,2-Trichloroethane	79-00-5		
Vinyl bromide	593-60-2		
Vinyl chloride	75-01-4		

Reliability criteria

Thiodiglycolic acid (TDAA)

Within-day precision:	Standard deviation (rel.)	s_w = 5.8%, 4.2%, or 2.6%
	Prognostic range	u = 13.1%, 9.4%, or 5.8%
	at a concentration of 0.38 mg, 1.2 mg, or 10.1 mg TDAA per litre of urine and n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 2.7%, 4.1%, or 6.5%
	Prognostic range	u = 6.6%, 10.0%, or 15.9%
	at a concentration of 0.38 mg, 1.2 mg, or 10.1 mg TDAA per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 96%, 102%, or 99%
	at a concentration of 0.2 mg, 1.0 mg, or 10.0 mg TDAA per litre of urine and n = 10 determinations	
Detection limit:	0.03 mg TDAA per litre of urine	
Quantitation limit:	0.09 mg TDAA per litre of urine	

2 General information on vinyl chloride and further TDAA-forming substances

Vinyl chloride, which presents in a gaseous state at room temperature, is a basic chemical compound and is primarily used in the manufacture of polyvinyl chloride (PVC) (IARC 2012 b). Of the manufactured PVC, 75% is used in the construction sector in the form of window frames, panelling, floor coverings, piping, and cable sheaths. In 2019, about 5 million tonnes of PVC were processed in Europe, about 10% of all plastics produced in Europe (PlasticsEurope 2020). The most recent data for Germany are from 2014: in this year, about 1.52 million tonnes of PVC were processed (AGPU 2016).

Occupational exposure to vinyl chloride is most likely to occur in the manufacture of vinyl chloride and PVC as well as in PVC-processing plants. In such cases, vinyl chloride is mainly inhaled; only a very small proportion of vinyl chloride is absorbed dermally (Hartwig and MAK Commission 2019 c). In the European Union in 1999, nearly 40 000 workers were exposed to vinyl chloride in their workplaces (IARC 2012 b).

In principle, the general population may also be exposed to vinyl chloride. Inhalation exposure might occur by inhaling contaminated air; oral exposure might occur by ingesting contaminated drinking water or foodstuffs; and dermal exposure might occur via skin contact to PVC-containing consumer goods. Potential exposure to vinyl chloride is, however, minimal for the majority of the general population (NTP 2021).

Moreover, vinyl chloride can be detected in mainstream smoke from cigarettes (1.3–16 ng/cigarette) and cigars (14–27 ng/cigar) (Hoffmann et al. 1976; IARC 2004), suggesting that smoking may also lead to vinyl chloride exposure.

Vinyl chloride has been designated into Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) (IARC 2012 b) and by the MAK Commission as a Category 1 carcinogen (substances that cause cancer in man and can be assumed to contribute to cancer risk) (DFG 2021; Henschler 1993 b). Details on the toxicological evaluation can be found in the corresponding MAK Value Documentations published by the Commission (Hartwig and MAK Commission 2019 c; Henschler 1993 b) as well as in the corresponding IARC monographs (IARC 1974, 1979, 1987, 2008 b, 2012 b).

Absorbed vinyl chloride is either exhaled unchanged or, after oxidative biotransformation, converted to metabolites which are then excreted renally. A simplified metabolism scheme for vinyl chloride is presented in Figure 1.

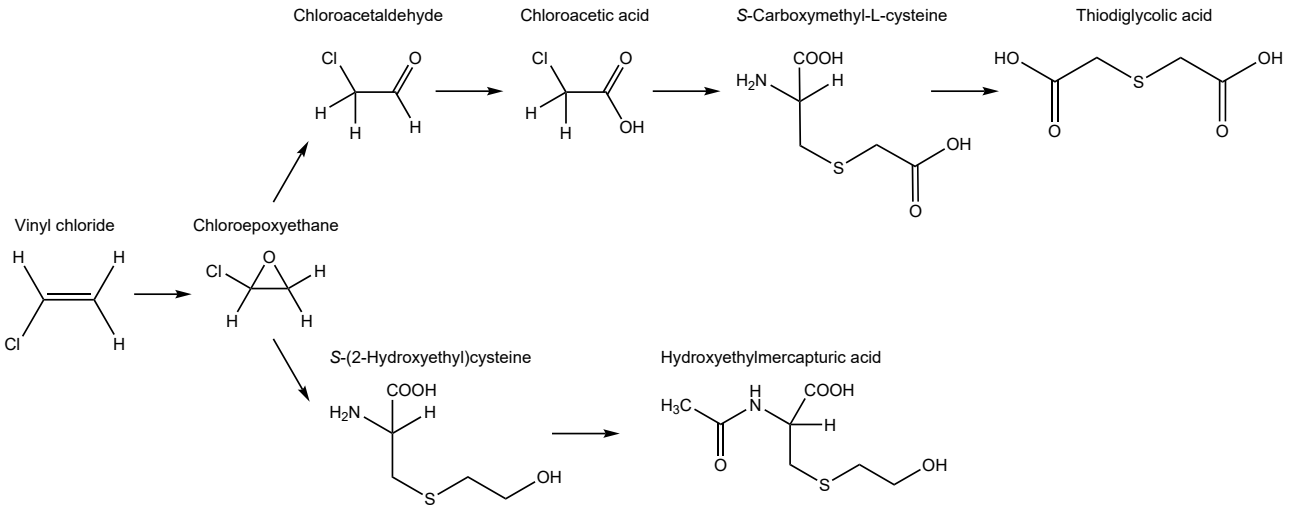

Fig. 1 Simplified metabolism scheme for vinyl chloride

The determination of TDAA in urine serves to measure occupational exposure to vinyl chloride, since *S*-(carboxymethyl)cysteine only arises as an intermediate product, which is then further broken down into TDAA, and since *S*-(2-hydroxyethyl)mercapturic acid (*N*-acetyl-*S*-(2-hydroxyethyl)cysteine) is only formed in very small amounts (Müller and Norpoth 1995). The EKA correlation (*Expositionsäquivalente für krebserzeugende Arbeitsstoffe*, exposure equivalents for carcinogenic substances), which reflected the relationship between vinyl chloride concentration in the air and TDAA excretion over a period of 24 hours following the start of a shift (Kraus and Schettgen 2016; Müller and Norpoth 1995), was withdrawn in 2021. The reason for the withdrawal of the EKA correlation was a study by Cheng et al. (2001), which showed that the correlation between vinyl chloride in workplace air and TDAA in urine is no longer statistically significant at concentrations of < 5 ml/m^3.

The Commission has derived a biological reference value (*Biologischer Arbeitsstoff-Referenzwert*, BAR) for vinyl chloride of 1.5 mg TDAA/l urine (Kraus and Schettgen 2016).

When evaluating the analytical results, it is important to note that TDAA can be formed by hazardous substances other than vinyl chloride, such as acrylonitrile, 2-bromoethanol, chloroacetaldehyde, 2-chloroethanol, 1,2-dibromoethane, 2,2′-dichlorodiethyl ether, 1,2-dichloroethane, 1,1-dichloroethene, ethylene oxide, monochloroacetic acid, 1,1,2-trichloroethane, and vinyl bromide as well as by pharmaceuticals such as cyclophosphamide, ifosfamide, carmustine, lomustine, mannomustine, dibenamine, phenoxybenzamine, and clomethiazole (Müller and Norpoth 1995). Furthermore, TDAA can also be excreted from persons who are exposed neither to vinyl chloride nor to any of the other substances listed above. The formation of reactive metabolites as part of fatty acid metabolism is discussed as a possible cause for this endogenous excretion of TDAA (Müller et al. 1979).

Further information on the principal question of which hazardous substances are also metabolised to TDAA can be found in the addendum to the MAK Value Documentation for 2,2′-dichlorodiethyl ether (Henschler 1978). This document indicates that TDAA arises as a urinary metabolite of three compound groups, specifically (1) vinyl halides, (2) ethyl halides, and (3) ethyl halide-containing compounds, from which ethyl halide groups can be cleaved oxidatively. The metabolism of halogenic TDAA-forming substances seems to proceed consistently via bromo- or chloroacetaldehyde and/or monobromo- or monochloroacetic acid as intermediates (Henschler 1993 a). It can generally be stipulated that the structurally different hazardous substances are metabolised via glutathione conjugation. These conjugates are broken down to *S*-(carboxymethyl)cysteine, from which TDAA is then formed (Müller et al. 1976; Müller and Norpoth 1995; Vermeulen et al. 1989; Yllner 1971).

The following subsections will briefly discuss the other substances which are metabolised to TDAA and excreted with the urine, whereby data on the formation and excretion of TDAA mostly originate from animal studies. The evaluations by the Commission for each of the TDAA-forming substances are summarised in Table 1. An assessment value in biological material, based on TDAA, was only derived for vinyl chloride. Since TDAA detection is not substance-specific, the BAR of 1.5 mg TDAA/l urine can be used for all TDAA-forming substances. With the corresponding mercapturic acid or globin adduct, there are more specific biomarkers for acrylonitrile and ethylene oxide for which assessment values have been derived (DFG 2021; Eckert et al. 2022; Jäger et al. 2021).

Details on the toxicological evaluation can be found in the corresponding documentations published by the Commission referenced for each individual substance. The Commission has not published documentations for 2-bromoethanol and vinyl bromide.

Tab. 1 MAK values, designations, classifications, and assessment values of the Commission for the TDAA-forming substances which can be detected with this method (DFG 2022)

Hazardous substance	Maximum workplace concentrations, designations, classifications	Assessment values in biological material
Acrylonitrile	Perc abs: H; Sens: Sh; Carc cat: 2	BAR: *S*-(2-cyanoethyl)mercapturic acid in urine[a), b)]: 15 µg/g creatinine BAR: *N*-(2-cyanoethyl)valine in the erythrocyte fraction of whole blood[c)]: 12 pmol/g globin EKA: see section XVI.1 *N*-(2-cyanoethyl)valine in the erythrocyte fraction of whole blood[c)]
Chloroacetaldehyde	Perc abs: H; Carc cat: 3	–
2-Chloroethanol	MAK: 2 ml/m³; 6.7 mg/m³; Peak lim: II(1); Preg gr: C; Perc abs: H	–
1,2-Dibromoethane	Perc abs: H; Carc cat: 2	–
2,2′-Dichlorodiethyl ether	MAK: 0.5 ml/m³; 3.0 mg/m³; Peak lim: II(2); Preg gr: D; Perc abs: H	–
1,2-Dichloroethane	Perc abs: H; Carc cat: 2	–
1,1-Dichloroethene	MAK: 2 ml/m³; 8.0 mg/m³; Peak lim: II(2); Preg gr: C; Carc cat: 3	–
Ethylene oxide	Perc abs: H; Carc cat: 2; Muta cat: 2	BAR: *S*-(2-hydroxyethyl)mercapturic acid in urine[a), b)]: 5 µg/g creatinine BAR: *N*-(2-hydroxyethyl)valine in the erythrocyte fraction of whole blood[c)]: 60 pmol/g globin EKA: see section XVI.1 *N*-(2-hydroxyethyl)valine in the erythrocyte fraction of whole blood[c)]
Monochloroacetic acid	MAK: 0.5 ml/m³; 2.0 mg/m³; Peak lim: I(2); Preg gr: C	–
1,1,2-Trichloroethane	MAK: 1 ml/m³; 5.5 mg/m³; Peak lim: I(2); Preg gr: D; Perc abs: H; Carc cat: 3	–
Vinyl chloride	Carc cat: 1	BAR: TDAA in urine[d)]: 1.5 mg/l EKA: not established, see section XVI.2

BAR: biological reference value (*biologischer Arbeitsstoff-Referenzwert*); EKA: exposure equivalents for carcinogenic substances (*Expositionsäquivalente für krebserzeugende Arbeitsstoffe*); Perc abs: danger from percutaneous absorption; Carc cat: carcinogen category; Muta cat: germ cell mutagen category; MAK: maximum workplace concentration (*maximale Arbeitsplatzkonzentration*); Preg gr: pregnancy risk group; Peak lim, peak limitation category (excursion factor)

[a)] sampling time: end of exposure or end of the shift

[b)] sampling time: at the end of the shift, for long-term exposures after several previous shifts

[c)] sampling time: after exposure for at least 3 months

[d)] sampling time: at the beginning of the next shift

Acrylonitrile is used primarily in the manufacture of acrylic fibres, which are produced via the polymerisation of acrylonitrile with additional comonomers. Acrylonitrile is additionally used for the manufacture of resins, nitrile rubber, and elastomers (IARC 1999 c). In animal studies with rats, TDAA was detected as a minor metabolism product in urine following oral application of acrylonitrile (Bolt and Lewalter 1995; Greim 2007)

2-Bromoethanol is used as a solvent and as a raw material for organic synthesis.

Jones and Wells (1981) postulated that 2-bromoethanol is metabolised in rats via two pathways. One pathway involves conversion into ethylene oxide, from which *S*-(2-hydroxyethyl)cysteine and the corresponding mercapturic acid are formed following glutathione conjugation. In the other pathway, 2-bromoethanol is first oxidised to bromoacetaldehyde and monobromoacetic acid and is subsequently excreted in the form of *N*-acetyl-*S*-(carboxymethyl)cysteine.

The further metabolism to TDAA of intermediate bromoacetaldehyde or of monobromoacetic acid, which is formed metabolically, has been shown in rats (Wormhoudt et al. 1997).

Chloroacetaldehyde is mainly used as an organic intermediate product in the manufacture of 2-aminothiazole and further compounds (NRC 2012).

Following intake via inhalation, oral, or dermal routes, chloroacetaldehyde is almost completely eliminated, primarily with the urine, within 24 hours. In rats, after application of 50 mg chloroacetaldehyde/kg body weight by gavage, in addition to *S*-(2-hydroxyethyl)mercapturic acid, 9.2% of the dose was identified as TDAA (Green and Hathway 1977; Greim 1999).

2-Chloroethanol is an intermediate product in the synthesis of ethylene oxide and ethylene glycol, as well as in the manufacture of indigo, dichloroethyl formal, and thiodiethylene glycol. Furthermore, it is used as a solvent (US EPA 2012).

Following intake via inhalation, oral, or dermal routes, 2-chloroethanol is almost completely eliminated, primarily with the urine, within 24 hours. After oral application of 5 mg radiolabelled chloroethanol/kg body weight in rats, TDAA and 2-(carboxymethyl sulfinyl)acetic acid were detected as primary metabolites at about 45% each. After an oral dose of 50 mg/kg body weight, 70% of urinary radioactivity was caused by TDAA (Hartwig and MAK Commission 2019 a).

1,2-Dibromoethane was used as a solvent and in organic synthesis, but also in impregnating agents and as a fumigant, e.g. for grains (IARC 1999 d). Uses today include treatment of logs for termites and beetles, control of moths in beehives, and as a preparation for dyes and waxes (ATSDR 2021).

In a study with rats, 67–76% of 1,2-dibromoethane was exhaled unchanged following oral administration; 34–38% was converted into inorganic bromide (Miller and Haggard 1943). 2-Bromoacetaldehyde, which is conjugated with glutathione and subsequently metabolised to mercapturic acids, represents the main metabolite (Guengerich 1994). Two important urinary metabolites of 1,2-dibromoethane are *N*-acetyl-*S*-(2-hydroxyethyl)cysteine and *S*-(2-hydroxyethyl)-cysteine (Henschler 1977). Moreover, TDAA was also identified as a urinary metabolite in rats (Wormhoudt et al. 1997).

2,2′-Dichlorodiethyl ether is used primarily in the production of pesticides and other chemicals. It is furthermore used as a solvent, as a component of paints and lacquers, and as an antirust agent (ATSDR 2017; IARC 1975).

Following intake, the substance is oxidatively cleaved *in vivo*, whereby chloroethanol and chloroacetaldehyde are presumed to be intermediate products. The metabolites *S*-(carboxymethyl)cysteine and TDAA were detected in rat urine (ATSDR 2017; Henschler 1978; Lingg et al. 1979; Müller et al. 1979).

1,2-Dichloroethane is primarily used in the manufacture of vinyl chloride. It is furthermore used in the production of tri- and tetrachloroethylene, ethylene amines, and trichloroethane; in paints and lacquers; as a component of metal-degreasing agents; and as a solvent (IARC 1999 b).

After intraperitoneal injection in mice, 51–73% of administered 1,2-dichloroethane was excreted with the urine. Three main metabolites were found: monochloroacetic acid (6–23%), *S*-(carboxymethyl)cysteine (44–46% free + 0.5–5% conjugated), and TDAA (33–34%) (Yllner 1971). In rats (Osborne-Mendel), after a single oral dose (150 mg/kg body weight in corn oil) or inhalation exposure (150 ml/m^3, 6 hours), in each case, 85% of the metabolised amount was excreted with the urine. Following oral dosage in rats, TDAA represented one of two main urinary metabolites at 70% (Henschler 1983, 1992).

1,1-Dichloroethene is used as a reactant in organic synthesis, in the production of PVC copolymers, and, rarely, as a solvent (ATSDR 2022).

1,1-Dichloroethene is metabolised by the spontaneous rearrangement of the formed epoxide and the subsequent hydrolysis to monochloroacetic acid, which has been detected in the urine of rats and mice. In the presence of glutathione transferases, monochloroacetic acid is conjugated with glutathione. In further degradation steps, the thereby occurring *S*-(2-carboxymethyl)cysteine forms dithioglycolic acid, thioglycolic acid, and thiodiglycolic acid, all of which could be detected. This degradation represents the main pathway of decomposition in rats (ATSDR 2022; Greim 1997).

Ethylene oxide is primarily used in the production of ethylene glycol. Small amounts are also used for sterilisation, as a fumigant, or as an insecticide (IARC 2012 a). The general population is mainly exposed to ethylene oxide through smoking (7 µg per cigarette) (IARC 2004).

In humans, ethylene oxide can be metabolised via epoxide hydrolase and glutathione-*S*-transferase (Li et al. 2011). Spontaneous hydrolysis and conjugation with glutathione also take place (Filser and Klein 2018). The detoxification of ethylene oxide by glutathione leads to the urinary excretion of the metabolites *S*-(2-hydroxyethyl)mercapturic acid, *S*-(2-hydroxyethyl)-*L*-cysteine, and TDAA (Hartwig and MAK Commission 2019 b; Scheick et al. 1997).

Monochloroacetic acid is used for the manufacture of cellulose ethers, as a herbicide, and in the production of glycine and thioglycolic acid, as well as in the manufacture of various pigments and synthetic caffeine (US EPA 2000).

In mice, monochloroacetic acid is metabolised to *S*-(carboxymethyl)cysteine and TDAA, which are then excreted with the urine (Greim 1998; Hartwig and MAK Commission 2020).

1,1,2-Trichloroethane is, like 1,2-dichloroethane, primarily used in the production of vinyl chloride and for the synthesis of polychlorinated organic compounds. It is furthermore used as a solvent for fats, oils, and waxes (IARC 1999 a).

The metabolites monochloroacetic acid, *S*-(carboxymethyl)cysteine, TDAA, 1,2-dichloroethanol, and oxalic acid were identified in rodent urine. After intraperitoneal application of 0.1 or 0.2 g [1,2-^{14}C]1,1,2-trichloroethane/kg body weight, 73–87% of the applied dose was found in mouse urine. The main metabolites were (percentage of urinary radioactivity): monochloroacetic acid (6–31%), *S*-(carboxymethyl)cysteine (29–46% free and 3–10% conjugated), and TDAA (38–42%) (Henschler 1981; Yllner 1971).

Vinyl bromide is, among other applications, used as an intermediate product in the synthesis of pharmaceutical products, in the manufacture of vinyl magnesium bromide, and as a component of various polymers (IARC 2008 a).

Vinyl bromide is metabolised *in vitro* by cytochrome P450 2E1 to bromoethylene oxide, which can further re-arrange to bromoacetaldehyde (IARC 2008 a). As with vinyl chloride, it must be presumed that vinyl bromide is further metabolised to TDAA (Henschler 1978).

From persons without occupational exposure to vinyl chloride or further TDAA-forming substances, 50 spontaneous urine samples with creatinine concentrations between 0.17 g/l and 4.87 g/l were measured within the framework of method development. TDAA was detected in 44 samples (88%) with a median value of 0.21 mg/l or 0.20 mg/g creatinine (see Table 2). With 0.41 mg/l or 0.43 mg/g creatinine, the 95th percentile lied substantially below the current BAR of 1.5 mg/l. These results confirm earlier investigations from 1997–2002 (data not published). The average TDAA concentration of 38 urine samples from this time period was 0.29 mg/l or 0.20 mg/g creatinine with a maximum value of 0.71 mg/l or 0.63 mg/g creatinine.

Tab. 2 TDAA concentrations in 50 spontaneous urine samples from 50 persons without occupational exposure to vinyl chloride or further TDAA-forming substances

	TDAA [mg/l]	**TDAA [mg/g creatinine]**
Mean ± SD	0.23 ± 0.14	0.22 ± 0.10
Median	0.21	0.20
95th percentile	0.41	0.43
Range	< LOQ–0.84	< LOQ–0.50

LOQ: limit of quantification

An analytical method for the determination of TDAA in urine has already been published by the Commission (Müller 1982). In this procedure, a diazomethane-ether mixture is added to dried urine and the methyl ester of TDAA is subsequently separated by gas chromatography and detected by mass spectrometry. This method is considered laborious, and diazomethane should be substituted as a derivatisation agent due to its carcinogenicity. For this reason, a UPLC-MS/MS method has been developed which exhibits similar reliability data and does not use diazomethane.

3 General principles

The method described herein allows for the selective and sensitive quantification of TDAA in urine in both occupational health and environmental medicine. The urine samples are diluted with 0.033% formic acid and mixed with a deuterated internal standard. The analyte is separated from matrix components by liquid chromatography and subsequently detected by tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in ultra-pure water.

4 Equipment, chemicals, and solutions

4.1 Equipment

- UPLC system (e.g. Waters Acquity UPLC H-Class, Waters GmbH, Eschborn, Germany)
- Tandem mass spectrometer (e.g. Waters Xevo TQS, Waters GmbH, Eschborn, Germany)
- Separation column: Zorbax SB-C8 RRHD, 1.8 µm; 2.1 × 150 mm (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Water purification system (e.g. Merck Milli-Q®, Merck KGaA, Darmstadt, Germany)
- Vortex mixer (e.g. Multi Reax, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany)
- Various pipettes and Multipettes® with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- 10-ml and 5000-ml volumetric flasks (e.g. witeg Labortechnik GmbH, Wertheim, Germany)
- 1.5-ml reaction vials (e.g. Eppendorf AG, Hamburg, Germany)
- 1.8-ml sample vials (e.g. Agilent Technologies Germany & Co. KG, Waldbronn, Germany)
- Urine cups (e.g. Uroboxen®, Diagonal GmbH & Co. KG, Münster, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade.

- Acetonitrile, > 99.9% (e.g. No. 10001334, Fluka™, Honeywell Deutschland Holding GmbH, Offenbach, Germany)
- Formic acid, 98–100% (e.g. No. 05311.3010, Bernd Kraft GmbH, Duisburg, Germany)
- Ultra-pure water (e.g. Milli-Q® Direct Water Purification System, Merck KGaA, Darmstadt, Germany)
- 2,2′-Thiodiacetic acid, 98% (e.g. No. T30007, Merck KGaA, Darmstadt, Germany)
- 2,2′-Thiodiacetic-2,2,2′,2′-d_4 acid, 99% (e.g. No. D-7812, C/D/N Isotopes Inc., Point-Claire, Canada)
- Argon 5.0 (e.g. Linde GmbH, Pullach, Germany)

4.3 Solutions

- 0.033% Formic acid
  1.67 ml of concentrated formic acid are pipetted into a 5000-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water. The solution has a pH value of 2.9.

4.4 Internal standard (ISTD)

- ISTD stock solution (1000 mg/l)
  10 mg of d_4-TDAA are weighed into a 10-ml volumetric flask and dissolved in 5 ml of ultra-pure water. The volumetric flask is then made up to the mark with ultra-pure water.
- ISTD spiking solution (10 mg/l)
  100 µl of the ISTD stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.

The stock and spiking solutions of the internal standard are stored in the refrigerator at 4 °C.

4.5 Calibration standards

- Stock solution (1000 mg/l)
  10 mg of TDAA are weighed into a 10-ml volumetric flask and dissolved in 5 ml of ultra-pure water. The volumetric flask is then made up to the mark with ultra-pure water.
- Dilution solution I (100 mg/l)
  1000 µl of the stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.
- Dilution solution II (10 mg/l)
  100 µl of the stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.

The stock and dilution solutions are stored in the refrigerator at 4 °C.

The calibration standards are prepared by spiking about 1 ml of water with the stock or dilution solutions according to the pipetting scheme given in Table 3. The calibration standards are processed analogously to the urine samples according to Section 5.2.

Tab. 3 Pipetting scheme for the preparation of calibration standards for the determination of TDAA in urine

Calibration standard	Dilution solution II [µl]	Dilution solution I [µl]	Stock solution [µl]	Water [µl]	Analyte concentration [mg/l]
0	–	–	–	1000	0.0
1	10	–	–	990	0.1
2	20	–	–	980	0.2
3	50	–	–	950	0.5
4	–	10	–	990	1.0
5	–	20	–	980	2.0
6	–	50	–	950	5.0
7	–	–	10	990	10.0
8	–	–	20	980	20.0
9	–	–	50	950	50.0

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine samples are collected in urine cups and subsequently stored at 4 °C until sample preparation. For longer term storage (> 3 days), the urine samples should be frozen at –18 °C.

5.2 Sample preparation

The urine samples are brought to room temperature and thoroughly mixed. 890 µl of 0.033% formic acid are placed in a 1.8-ml sample vial, 100 µl of the ISTD spiking solution are added, and finally, 10 µl of the sample are added by pipetting. The vial is sealed and shaken for 10 seconds on a vortex mixer, and the sample is applied for analysis.

6 Operational parameters

Analytical determination is performed using a UPLC system with a tandem mass spectrometer (UPLC-MS/MS).

6.1 Ultra high-performance liquid chromatography

Separation column:	Agilent Zorbax SB-C8 RRHD (1.8 µm; 2.1 × 150 mm)
Separation principle:	Reversed phase
Injection volume:	10 µl
Column temperature:	30 °C
Flow rate:	0.2 ml/min
Eluent:	A: Formic acid (0.033%; pH = 2.9)
	B: Acetonitrile
Runtime:	8 min
Gradient program:	see Table 4

Tab. 4 Gradient program for the determination of TDAA in urine

Time [min]	Eluent A [%]	Eluent B [%]
0.0	95	5
6.0	10	90
7.0	10	90
7.1	95	5
8.0	95	5

6.2 Mass spectrometry

Ionisation:	Electrospray ionisation (ESI–)
Detection mode:	Multiple Reaction Monitoring (MRM)
Capillary:	0.90 kV
Cone:	24 V

Source temperature:	150 °C
Desolvation temperature:	500 °C
Cone gas flow:	150 l/h
Desolvation gas flow:	1000 l/h
Collision gas:	Argon
Collision gas flow:	0.15 ml/h
Parameter-specific settings:	see Table 5

The instrument-specific parameters must be ascertained and adjusted for the MS/MS system utilised. The instrument-specific parameters given in this section have been determined and optimised for the system used here (Waters Acquity UPLC H-Class, Waters Xevo-TQS tandem mass spectrometer).

Two fragment-ion transitions were selected for TDAA. One transition serves the purpose of quantification (quantifier) and the other serves the purpose of confirmation (qualifier). Only one mass transition was used for the ISTD. The selected fragment-ion transitions as well as the retention times are summarised in Table 5.

Tab. 5 Retention times and MRM parameters for the determination of TDAA in urine

Analyte/ISTD	Retention time [min]	Mass transition [*m/z*]	Status	Collision energy [V]	Dwell time [s]
TDAA	3.3	149 → 105	Quantifier	12	0.025
	3.3	149 → 61	Qualifier	8	0.025
d_4-TDAA	3.3	153 → 109	ISTD	8	0.025

7 Analytical determination

10 µl of the diluted urine samples (see Section 5.2) are injected into the UPLC-MS/MS system and separated by reversed-phase chromatography. The analyte is identified by the retention time and the specific mass transitions. The retention time given for TDAA in Table 5 can only serve as a point of reference. The user must ensure the separation performance of the column and the resulting retention behaviour of the analyte. Representative chromatograms for the determination of TDAA in urine are depicted in Figure 2.

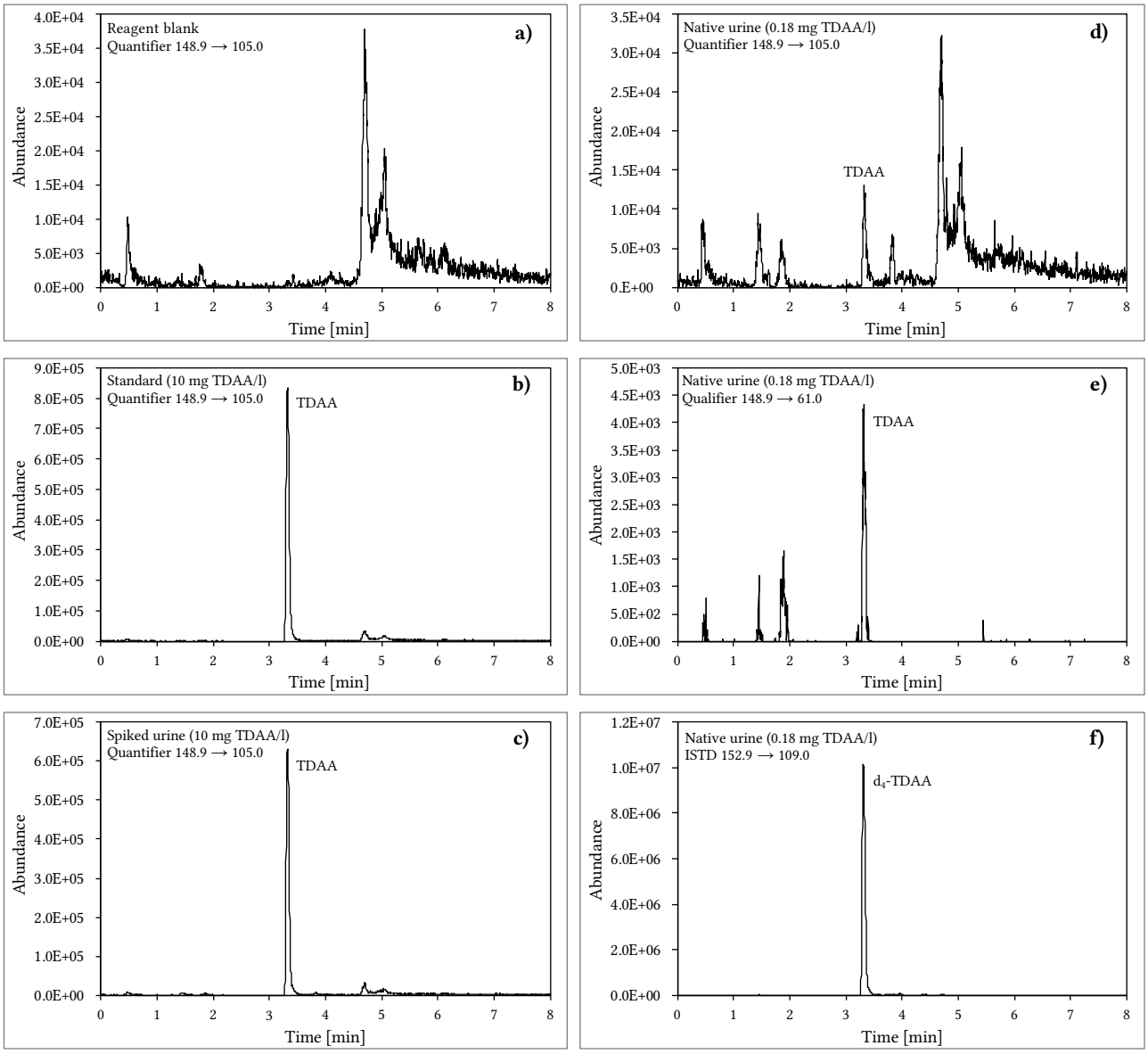

Fig. 2 Chromatograms of a) a reagent blank, b) a calibration standard spiked with 10 mg TDAA/l, c) a urine sample spiked with 10 mg TDAA/l, as well as d)–f) a native urine sample (0.18 mg TDAA/l)

8 Calibration

During method development, it was proven that the slope of the calibration curve in urine does not differ from that of the calibration curve in water (see Figure 3). For this reason, the calibration standards can be prepared in water, so that the TDAA background levels which arise in the urine matrix do not need to be considered for calibration. The calibration standards are prepared as described in Section 4.5, diluted analogously to the urine samples (see Section 5.2), and analysed. The calibration curve is generated by plotting the peak-area ratio of the analyte and the deuterated ISTD against the spiked concentration of the analyte. Using the specified instrumentation, the calibration curve is linear from the limit of quantitation up to 50.0 mg/l. Figure 4 shows a representative calibration curve.

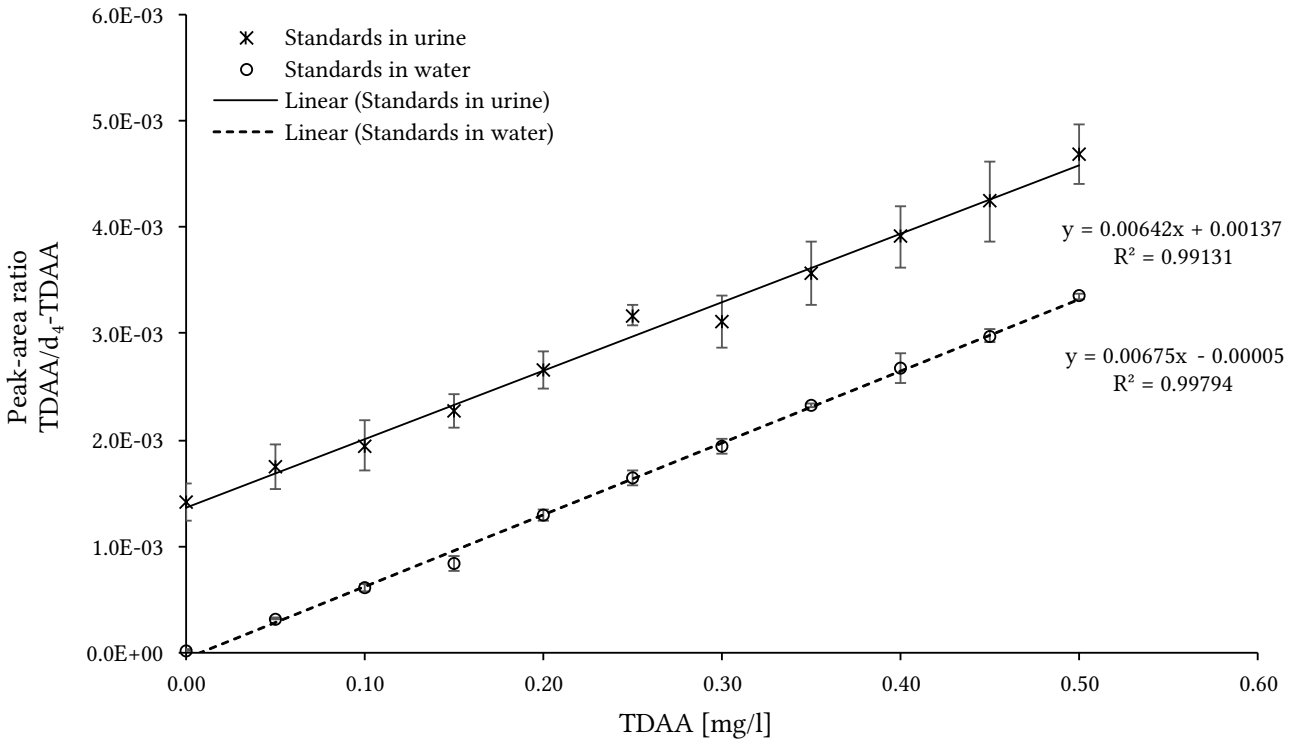

Fig. 3 Calibration curves prepared in water and urine for the determination of the limits of detection and quantitation (mean ± SD; n = 3)

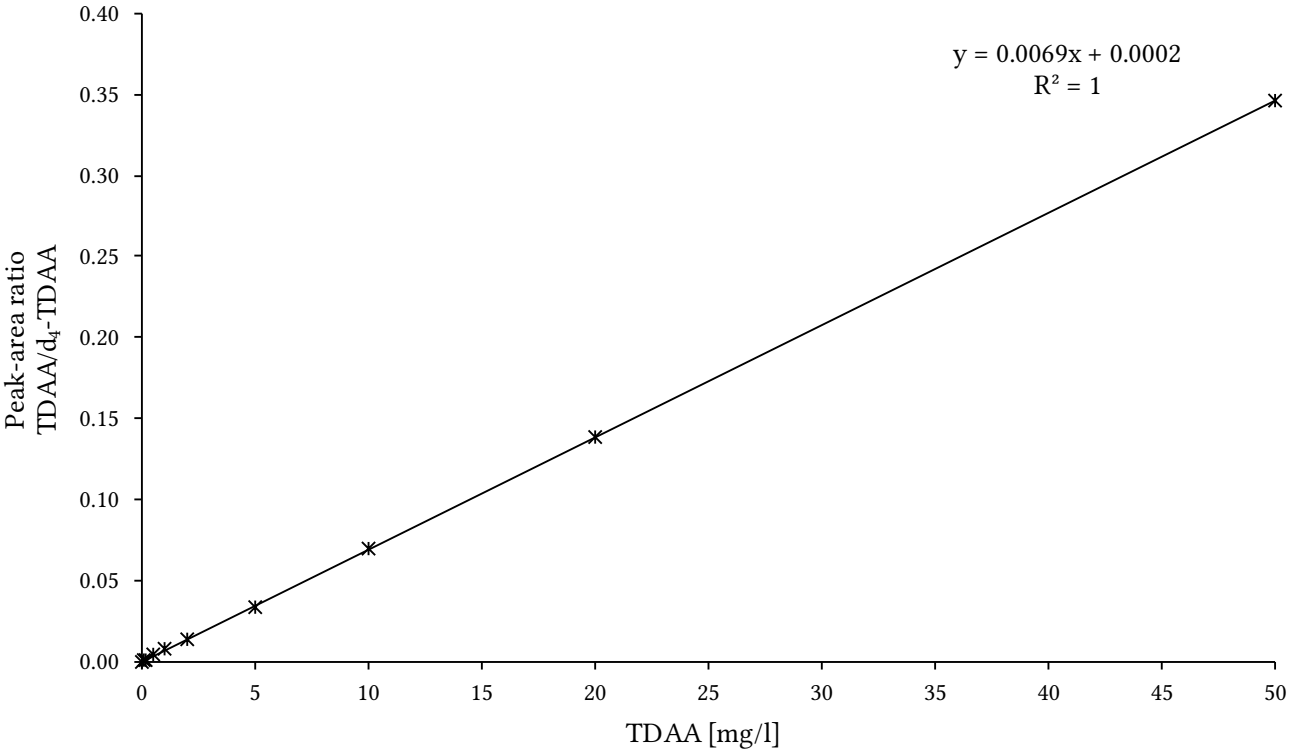

Fig. 4 Calibration curve for the determination of TDAA in urine

9 Calculation of the analytical results

To calculate the analyte concentration in a urine sample, the peak area of the analyte is divided by the peak area of the ISTD. Using the calibration function of the analytical run in question (cf. Section 8), this quotient can be used to calculate the analyte concentration in mg/l urine. If a reagent blank value arises, it must be subtracted from the analytical results.

If the measured value exceeds the calibration range, the sample is diluted with ultra-pure water, reprocessed, and newly analysed.

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 published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

For the quality control of the individual analytical runs, three quality-control samples with known analyte concentrations are processed and analysed parallel to the samples. As there is currently no commercially available control material for TDAA, it must be produced in the in-house laboratory. To this end, pooled urine is spiked with TDAA at concentrations of 0.2 mg/l (low quality control), 1.0 mg/l (medium quality control), or 10.0 mg/l (high quality control), aliquoted, and frozen at –18 °C until use. These quality-control materials are processed and analysed as part of each analytical run. Simultaneously, a reagent blank is included in each analytical run.

The nominal values and tolerance ranges of the quality-control materials are investigated as part of a pre-analytical period (Bader et al. 2010).

11 Evaluation of the method

The reliability of this method was confirmed by comprehensive validation as well as by replication and verification in a second, independent laboratory.

11.1 Precision

Within-day precision

For the determination of within-day precision, urine was spiked with three TDAA concentrations, processed, and analysed ten times each in one day. The results of these measurements are summarised in Table 6.

Tab. 6 Within-day precision for the determination of TDAA in urine (n = 10)

Analyte	Concentration [mg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
TDAA	0.38	5.8	13.1
	1.20	4.2	9.4
	10.1	2.6	5.8

Day-to-day precision

For the determination of day-to-day precision, the same material was used as was applied for the determination of within-day precision. Processing and analysing the three differently spiked urines on six different days yielded the precision data given in Table 7.

Tab. 7 Day-to-day precision for the determination of TDAA in urine (n = 6)

Analyte	Concentration [mg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
TDAA	0.38	2.7	6.6
	1.20	4.1	10.0
	10.1	6.5	15.9

11.2 Accuracy

In order to determine the accuracy of the method, pooled urine samples were spiked with 0.2 mg TDAA/l, 1.0 mg TDAA/l, or 10.0 mg TDAA/l, processed ten times in parallel, and analysed. In addition, the unspiked urines were processed and their TDAA background concentrations were ascertained. The calculation of the relative recovery rates was carried out using the determined concentrations in the spiked urine samples, subtracting any TDAA background levels found in the unspiked urine samples. The relative recovery rates thus obtained are given in Table 8.

Tab. 8 Relative recovery for the determination of TDAA in urine (n = 10)

Analyte	Spiked concentration	Mean rel. recovery r [%]	
	[mg/l]	Mean ± SD	Range
TDAA	0.2	96.0 ± 10.9	78.0–113
	1.0	101.5 ± 5.1	90.6–108
	10.0	99.2 ± 2.6	96.3–104

11.3 Matrix effects

In order to check for matrix effects, ten individual urines (creatinine levels in the range of 0.17–2.61 g/l) were processed and analysed prior to spiking and after spiking with 0.5 mg or 5.0 mg TDAA/l. Mean relative recoveries were found to be 94 ± 8% (80–106%) for the low concentration and 97 ± 3% (92–101%) for the high concentration.

11.4 Limits of detection and quantitation

The limits of detection and quantitation were determined according to DIN 32645 (DIN 2008). To this end, an equidistant 10-point calibration was prepared in pooled urine (concentration range of 0.05–0.5 mg TDAA/l), then processed and analysed in conjunction with a blank value (see Figure 3). Table 9 shows the limits of detection and quantitation thus obtained for the determination of TDAA in urine.

Tab. 9 Limits of detection and quantitation for the determination of TDAA in urine

Analyte	Matrix	Detection limit [mg/l]	Quantitation limit [mg/l]
TDAA	Urine	0.03	0.09

11.5 Sources of error

Experiences to date with the method for the determination of TDAA described herein indicate no particular risk for external contamination. It is, however, advisable to include a reagent blank as part of each analytical run in order to recognise any workup-related contaminations or impurities in the reagents. During method development, the use of the deuterated internal standard was necessary as, without ISTD, falsely low results were found, especially in cases of high TDAA concentrations. Within the framework of external method verification, during which an Acquity UPLC BEH C8 column (1.7 µm; 2.1 × 150 mm) (Waters GmbH, Eschborn, Germany) was used in place of the Agilent Zorbax SB-C8 RRHD column, such falsely low results were not observed, suggesting that, depending on the column used, a deuterated ISTD may be unnecessary.

12 Discussion of the method

The UPLC-MS/MS method described herein allows for the determination of TDAA in urine. The method is fast, selective, and displays both good accuracy and linearity. The quantitation limit is sufficient to measure TDAA background levels in most urine samples. During a survey of 50 urine samples from the non-occupationally exposed general population, only about 12% of measured TDAA values lied below the limit of quantitation.

Compared to the Commission's already published method, which uses diazomethane derivatisation, the validation data of this UPLC-MS/MS method are either similar or improved.

Instruments used Ultra high-performance liquid-chromatography system (Waters Acquity UPLC H-Class, Waters GmbH, Eschborn, Germany) with tandem mass spectrometer (Waters Xevo TQS, Waters GmbH, Eschborn, Germany).

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

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