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Alkylating substances – Determination of haemoglobin adducts as *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline, *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl) valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine in the erythrocyte fraction of whole blood by GC-MS/MS

Biomonitoring Method – Translation of the German version from 2023

T. Christ[1]
D. Ellrich[1]
G. Leng[1]
C. Schmidtkunz[1]
W. Gries[1]
F. Pilz[2]
G. Scherer[2]
T. Göen[3,*]
A. Hartwig[4,*]
MAK Commission[5,*]

1 *Method development, Currenta GmbH & Co. OHG, CUR-SIT-SER-GS-BLM – Institute for Biomonitoring, Chempark Building Q 18, 51368 Leverkusen, Germany*

2 *External verification, ABF – Analytisch-biologisches Forschungslabor GmbH, Semmelweisstraße 5, 82152 Planegg, Germany*

3 *Head of 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, Deutsche Forschungsgemeinschaft, Friedrich-Alexander-Universität Erlangen-Nürnberg, Institute and Outpatient Clinic of Occupational, Social, and Environmental Medicine, Henkestraße 9–11, 91054 Erlangen, Germany*

4 *Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany*

5 *Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany*

* *email: T. Göen (thomas.goeen@fau.de), A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)*

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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. Alkylating substances play an important role in many industrial processes and include *n*-alkyl and benzyl halides, α,β-unsaturated carbonyl compounds, epoxides, alkylnitrosamines, chloroalkyl ethers, dialkyl sulfates as well as alkyl alkane sulfonates. As alkylating substances are mainly used in industry, most exposure occurs in the workplace. The general population is primarily exposed through cigarette smoke, which contains a variety of alkylating substances. In addition, certain foods, especially highly heated, starchy foods, are a source of exposure to acrylamide, another alkylating substance. The aim of this work was to develop a selective method to simultaneously determine the adducts of different alkylating substances with the *N*-terminal

valine of haemoglobin, namely *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline, *N*-butylvaline, *N*-(2-hydroxypropyl) valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine. The method has been comprehensively validated, and the reliability data have been confirmed by replication and verification of the procedure in a second, independent laboratory. For the determination of the adducts at the *N*-terminal valine of haemoglobin, erythrocytes are isolated from whole blood and subjected to lysis. Globin is precipitated from the haemoglobin solution and, after the addition of *N*-(2-ethoxyethyl)valine-alanine-anilide as internal standard, the alkylated *N*-terminal valines are derivatised and cleaved off by a modified Edman degradation using pentafluorophenyl isothiocyanate. The corresponding pentafluorophenyl thiohydantoin derivates are extracted with *tert*-butyl methyl ether, washed, and analysed by GC-MS/MS. The good precision and accuracy data show that the method is reliable and accurate. The method is both selective and sensitive, and the limits of detection between 10 and 70 pmol/g globin are sufficient to determine occupational exposure and, for some analytes, exposure of the general population to the respective alkylating substances.

1 Characteristics of the method

Matrix	Erythrocyte fraction of whole blood
Analytical principle	Gas chromatography with tandem mass spectrometry (GC-MS/MS)

Parameters and corresponding hazardous substances

Hazardous substance[a)]	**CAS No.**	**Parameter**	**CAS No.**
Diazomethane	334-88-3		
Dimethyl sulfate	77-78-1		
Methyl chloride (chloromethane)	74-87-3		
Methyl bromide (bromomethane)	74-83-9		
Methyl iodide (iodomethane)	74-88-4	*N*-Methylvaline	2566-32-7
Bis(chloromethyl) ether	542-88-1		
Monochlorodimethyl ether	107-30-2		
N-Nitrosodimethylamine (NDMA)	62-75-9		
N-Nitrosomethylethylamine (NMEA)	10595-95-6		
Diethyl sulfate	64-67-5		
Ethyl bromide (bromoethane)	74-96-4		
Ethyl iodide (iodoethane)	75-03-6		
Ethyl methanesulfonate	62-50-0	*N*-Ethylvaline	64991-31-7
Acrylonitrile	107-13-1		
N-Nitrosodiethylamine (NDEA)	55-18-5		
N-Nitrosomethylethylamine (NMEA)	10595-95-6		
Dipropyl sulfate	598-05-0		
Propyl bromide (1-bromopropane)	106-94-5	*N*-Propylvaline	90600-07-0
Propyl iodide (1-iodopropane)	107-08-4		
N-Nitrosodi-*n*-propylamine (NDPA)	621-64-7		

Dibutyl sulfate	625-22-9	*N*-Butylvaline	62765-47-3
Butyl bromide (1-bromobutane)	109-65-9		
Butyl iodide (1-iodobutane)	542-69-8		
N-Nitrosodi-*n*-butylamine (NDBA)	924-16-3		
Propylene oxide (1,2-epoxypropane)	75-56-9	*N*-(2-Hydroxypropyl)valine	91147-54-5
Propylene	115-07-1		
Ethylene oxide	75-21-8	*N*-(2-Hydroxyethyl)valine	21768-51-4
Ethylene	74-85-1		
Acrylonitrile	107-13-1	*N*-(2-Cyanoethyl)valine	51078-49-0
Benzyl chloride	100-44-7	*N*-Benzylvaline	15363-84-5
Benzyl bromide	100-39-0		
Benzyl iodide	620-05-3		
Acrylamide	79-06-1	*N*-(2-Carbamoylethyl)valine	51078-53-6

[a] Other hazardous substances not listed may lead to the haemoglobin adducts that can be determined with this method

Reliability criteria

N-Methylvaline

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 625 pmol or 2499 pmol *N*-methylvaline per gram of globin and n = 6 determinations	s_w = 5.2% or 4.3% u = 13.4% or 11.1%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 625 pmol or 2499 pmol *N*-methylvaline per gram of globin and n = 6 determinations	s_w = 2.4% or 3.6% u = 6.2% or 9.3%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 625 pmol or 2499 pmol *N*-methylvaline per gram of globin and n = 6 determinations	r = 101% or 105%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 625 pmol or 2499 pmol *N*-methylvaline per gram of globin and n = 6 determinations	r = 81% or 106%
Detection limit:	50 pmol *N*-methylvaline per gram of globin	
Quantitation limit:	170 pmol *N*-methylvaline per gram of globin	

N-Ethylvaline

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 564 pmol or 2257 pmol *N*-ethylvaline per gram of globin and n = 6 determinations	s_w = 8.1% or 4.6% u = 20.8% or 11.8%

Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 564 pmol or 2257 pmol *N*-ethylvaline per gram of globin and n = 6 determinations	s_w = 10.8% or 6.4% u = 27.8% or 16.5%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 564 pmol or 2257 pmol *N*-ethylvaline per gram of globin and n = 6 determinations	r = 102% or 103%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 564 pmol or 2257 pmol *N*-ethylvaline per gram of globin and n = 6 determinations	r = 108% or 113%
Detection limit:	15 pmol *N*-ethylvaline per gram of globin	
Quantitation limit:	50 pmol *N*-ethylvaline per gram of globin	

N-Propylvaline

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 515 pmol or 2058 pmol *N*-propylvaline per gram of globin and n = 10 determinations	s_w = 1.8% or 1.2% u = 4.1% or 2.7%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 515 pmol or 2058 pmol *N*-propylvaline per gram of globin and n = 7 determinations	s_w = 3.1% or 2.9% u = 7.6% or 7.1%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 515 pmol or 2058 pmol *N*-propylvaline per gram of globin and n = 10 determinations	r = 99% or 101%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 515 pmol or 2058 pmol *N*-propylvaline per gram of globin and n = 7 determinations	r = 101% or 99%
Detection limit:	15 pmol *N*-propylvaline per gram of globin	
Quantitation limit:	50 pmol *N*-propylvaline per gram of globin	

N-Butylvaline

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 473 pmol or 1892 pmol *N*-butylvaline per gram of globin and n = 6 determinations	s_w = 2.4% or 2.8% u = 6.2% or 7.2%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 473 pmol or 1892 pmol *N*-butylvaline per gram of globin and n = 6 determinations	s_w = 0.8% or 3.8% u = 2.1% or 9.8%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 473 pmol or 1892 pmol *N*-butylvaline per gram of globin and n = 6 determinations	r = 100% or 98%

Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 473 pmol or 1892 pmol *N*-butylvaline per gram of globin and n = 6 determinations	r = 93% or 110%
Detection limit:	15 pmol *N*-butylvaline per gram of globin	
Quantitation limit:	50 pmol *N*-butylvaline per gram of globin	

N-(2-Hydroxypropyl)valine

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 468 pmol or 1871 pmol *N*-(2-hydroxypropyl)valine per gram of globin and n = 6 determinations	s_w = 4.9% or 5.5% u = 12.6% or 14.1%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 468 pmol or 1871 pmol *N*-(2-hydroxypropyl)valine per gram of globin and n = 6 determinations	s_w = 2.4% or 5.7% u = 6.2% or 14.7%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 468 pmol or 1871 pmol *N*-(2-hydroxypropyl)valine per gram of globin and n = 6 determinations	r = 96% or 92%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 468 pmol or 1871 pmol *N*-(2-hydroxypropyl)valine per gram of globin and n = 6 determinations	r = 90% or 102%
Detection limit:	10 pmol *N*-(2-hydroxypropyl)valine per gram of globin	
Quantitation limit:	30 pmol *N*-(2-hydroxypropyl)valine per gram of globin	

N-(2-Hydroxyethyl)valine

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 508 pmol or 2034 pmol *N*-(2-hydroxyethyl)valine per gram of globin and n = 6 determinations	s_w = 5.2% or 4.3% u = 13.4% or 11.1%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 508 pmol or 2034 pmol *N*-(2-hydroxyethyl)valine per gram of globin and n = 6 determinations	s_w = 4.9% or 6.0% u = 12.6% or 15.4%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 508 pmol or 2034 pmol *N*-(2-hydroxyethyl)valine per gram of globin and n = 6 determinations	r = 100% or 95%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 508 pmol or 2034 pmol *N*-(2-hydroxyethyl)valine per gram of globin and n = 6 determinations	r = 103% or 111%
Detection limit:	20 pmol *N*-(2-hydroxyethyl)valine per gram of globin	
Quantitation limit:	70 pmol *N*-(2-hydroxyethyl)valine per gram of globin	

N-(2-Cyanoethyl)valine

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 481 pmol or 1926 pmol *N*-(2-cyanoethyl)valine per gram of globin and n = 6 determinations	s_w = 2.5% or 5.4% u = 6.4% or 13.9%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 481 pmol or 1926 pmol *N*-(2-cyanoethyl)valine per gram of globin and n = 6 determinations	s_w = 6.9% or 2.7% u = 17.7% or 6.9%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 481 pmol or 1926 pmol *N*-(2-cyanoethyl)valine per gram of globin and n = 6 determinations	r = 103% or 103%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 481 pmol or 1926 pmol *N*-(2-cyanoethyl)valine per gram of globin and n = 6 determinations	r = 96% or 114%
Detection limit:	15 pmol *N*-(2-cyanoethyl)valine per gram of globin	
Quantitation limit:	60 pmol *N*-(2-cyanoethyl)valine per gram of globin	

N-Benzylvaline

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 395 pmol or 1581 pmol *N*-benzylvaline per gram of globin and n = 6 determinations	s_w = 2.0% or 1.5% u = 5.1% or 3.9%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 395 pmol or 1581 pmol *N*-benzylvaline per gram of globin and n = 6 determinations	s_w = 6.6% or 2.6% u = 17.0% or 6.7%
Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 395 pmol or 1581 pmol *N*-benzylvaline per gram of globin and n = 6 determinations	r = 99% or 96%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 395 pmol or 1581 pmol *N*-benzylvaline per gram of globin and n = 6 determinations	r = 79% or 100%
Detection limit:	10 pmol *N*-benzylvaline per gram of globin	
Quantitation limit:	30 pmol *N*-benzylvaline per gram of globin	

N-(2-Carbamoylethyl)valine

Within-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 435 pmol or 1741 pmol *N*-(2-carbamoylethyl)valine per gram of globin and n = 6 determinations	s_w = 7.8% or 5.1% u = 20.1% or 13.1%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at an adduct level of 435 pmol or 1741 pmol *N*-(2-carbamoylethyl)valine per gram of globin and n = 6 determinations	s_w = 5.8% or 4.6% u = 14.9% or 11.8%

Accuracy (within-day precision):	Recovery (rel.) at an adduct level of 435 pmol or 1741 pmol *N*-(2-carbamoylethyl)valine per gram of globin and n = 6 determinations	*r* = 99% or 99%
Accuracy (day-to-day precision):	Recovery (rel.) at an adduct level of 435 pmol or 1741 pmol *N*-(2-carbamoylethyl)valine per gram of globin and n = 6 determinations	*r* = 82% or 100%
Detection limit:	70 pmol *N*-(2-carbamoylethyl)valine per gram of globin	
Quantitation limit:	200 pmol *N*-(2-carbamoylethyl)valine per gram of globin	

2 General information on the alkylating substances

Alkylating substances play an important role in many industrial processes and include *n*-alkyl halides, benzyl halides, epoxides, chloroalkyl ethers, dialkyl sulfates and alkyl alkane sulfonates, alkylnitrosamides and alkylnitrosamines, as well as α,β-unsaturated carbonyl compounds (such as acrylamide) (Törnqvist et al. 2002). They may be used as solvents (e.g. alkyl halides) and insecticides (e.g. methyl bromide) or in the production of certain organic chemicals (e.g. acrylonitrile, benzyl chloride, diethyl and dimethyl sulfate, ethylene oxide, propylene oxide) (IARC 1999) as well as in the synthesis of various flavourings and fragrances (e.g. 1-bromopropane) (NTP 2003). 1-Bromopropane is also used in precision cleaning and as a degreasing agent (NTP 2003). Despite its prohibition by the Montreal Protocol, in many countries ozone-depleting methyl bromide is still used in greenhouses, in the storage and transport of goods, and as a sterilising agent on soils assigned to agricultural activities (Barry et al. 2012; Budnik et al. 2012). Ethylene oxide is employed for the sterilisation of medical instruments and materials. Acrylamide is used for the production of polyacrylamides which may be applied as flocculants in water treatment or may be used in dispersion paints and lacquers (Herth et al. 2015). Acrylonitrile serves as a raw material for the manufacture of synthetic materials and fibres and is also used as an insecticide (Brazdil 2012).

1,2-Unsaturated alkenes and their epoxides 1,2-Unsaturated alkenes and their epoxides occur in gaseous (ethene, ethylene oxide, propene) or liquid (propylene oxide) forms (IFA 2023) and are either produced in or imported into the European Economic Area in large amounts (ethylene and propylene > 10 000 000 t/a, ethylene oxide and propylene oxide > 1 000 000 t/a) (ECHA 2023 e, f, g, h). The substances are used as laboratory chemicals and as starting materials for the industrial manufacture of polymers and other chemicals.

Ethylene is a flammable, ubiquitous gas which arises from the incineration of organic material. It is a ripening hormone in plants and is endogenously formed in mammals, including humans. Ethylene is a petrochemical which is produced on a large scale and one of the most important synthesis components of the chemicals industry. More than 80% of ethylene is used for the production of ethylene oxide, ethylene dichloride, and polyethylene (Zimmermann and Walzl 2009).

Ethylene oxide is utilised as a sterilising agent in medical technology and for the fumigation of silos, storage facilities, or shipping containers, whereby these applications are strictly regulated. The substance is furthermore converted into ethylene glycol and ethylene glycol ethers and used for the production of polyester fibres (Rebsdat and Mayer 2001). In human and animal organisms, ethylene oxide can be formed from endogenous ethylene (Filser et al. 1992). Moreover, it occurs naturally in natural gas and has been detected in cigarette smoke as well as in diesel exhaust fumes (Rebsdat and Mayer 2001).

Like ethylene, propylene is an important basic chemical for the chemicals industry and is produced on a large scale. Propylene is used as a fuel gas for heat generation, as a gas for flame cutting, and in the manufacture of numerous chemical products. Most produced propylene is converted into polypropylene, but also into propylene oxide, acrylic acid, acrylonitrile, and cumene (Zimmermann 2013).

Propylene oxide is mainly processed into polyether polyols, from which polyurethanes are manufactured. It is furthermore used for the production of propylene glycols and propylene glycol ethers. To a lesser extent, it is applied directly as a fumigant for agricultural products. For this reason, occupational exposure is a concern primarily for workers of the chemicals industry (Baer et al. 2012).

In the workplace, the 1,2-unsaturated alkenes and corresponding epoxides described herein are primarily inhaled; ethylene oxide may also be absorbed dermally from aqueous solutions or the gaseous phase. Of the alkenes absorbed by inhalation, one part is exhaled unchanged, another part is stored in fatty tissue, and a third part is metabolised to 1,2-epoxides in the liver. Catalysed by epoxide hydrolase, these metabolites are transferred into the corresponding diols, which may be further oxidised or excreted in a conjugated form. Furthermore, the detoxification of the epoxides via reaction with glutathione is possible (IFA 2023). Only a small portion of the absorbed or formed epoxides reacts with endogenous macromolecules to form adducts; in this case, the reactivity of 1,2-epoxypropane is about four times lower than that of ethylene oxide (Pauwels and Veulemans 1998).

In the non-occupationally exposed general population, exposure to 1,2-unsaturated alkenes and their epoxides takes place primarily via smoking and second-hand smoking (Scherer et al. 2022).

Alkyl sulfates Alkyl sulfates are colourless liquids which are only produced as an intermediate (dimethyl sulfate) in the European Economic Area or are produced in or imported into the European Economic Area in amounts of > 1 t/a to < 10 t/a (diethyl sulfate, dipropyl sulfate, and dibutyl sulfate). The alkyl sulfates are used both in industrial processes and in the laboratory as alkylating reagents (ECHA 2021 b, c, 2022 b, 2023 d; RÖMPP-Redaktion 2023). In Germany, however, dimethyl sulfate and diethyl sulfate may only be manufactured or handled in closed systems for industrial production as they are especially hazardous carcinogens (Bundesregierung Deutschland 2010).

In cases of exposure, dimethyl sulfate and diethyl sulfate may be taken up via the respiratory tract or through the skin. For dimethyl sulfate, it can be assumed that a rapid hydrolysis will take place in the blood or in the primarily affected organ systems; the substance is possibly even hydrolysed before resorption. Aside from hydrolysis to monomethyl sulfate, methanol, and sulfuric acid, the reaction with glutathione and the addition to endogenous macromolecules are of particular importance (IFA 2023). Compared to dimethyl sulfate, diethyl sulfate exhibits a higher hydrolysis stability (Kolesnikov et al. 1977). In rats, diethyl sulfate is metabolised to ethyl mercapturic acid regardless of the route of administration (Kaye 1974). The ethylation of endogenous macromolecules can be assumed.

Inhalation and dermal absorption after exposure can likewise be assumed for dipropyl sulfate and dibutyl sulfate. Since the hydrolysis rate in aqueous medium decreases from dimethyl sulfate to dibutyl sulfate (Kolesnikov et al. 1977) while lipid solubility increases, uptake of the intact molecule is assumed to increase with molecular mass. Dipropyl sulfate and dibutyl sulfate should similarly react with glutathione to the corresponding mercapturic acids or alkylate macromolecules. Exposure to alkyl sulfates is not expected for the general population.

Benzyl halides Benzyl chloride and benzyl bromide are colourless liquids, and, at room temperature, benzyl iodide presents as colourless to yellow-coloured needles which melt at 24.5 °C. Benzyl chloride is either manufactured in or imported into the European Economic Area in amounts of ≥ 10 t/a to < 100 t/a, whereas benzyl bromide is only produced as an intermediate. The website of the European Chemicals Agency does not provide any information on the manufacture or import of benzyl iodide. Benzyl halides are used as benzylating agents both in industrial processes and in the laboratory (ECHA 2020 b, 2021 e, 2022 c; RÖMPP-Redaktion 2023), and thereby represent starting materials or intermediates for the production of other chemicals.

With regard to applications, benzyl chloride is used as a basic chemical and intermediate and is further processed, for example, to benzyl alcohol, phthalic acid benzyl esters (e.g. benzyl butyl phthalates as plasticisers), phenylacetic acid via benzyl cyanide (synthetic penicillin), and quaternary ammonium salts (disinfectants and phase-transfer catalysts) as well as to other intermediate products (e.g. for flavourings and dyes). Any direct usage of benzyl chloride as a final product is not known (Lipper et al. 2017).

Benzyl chloride is effectively resorbed via the respiratory tract and through the skin. There are no current data on the metabolism of benzyl chloride in humans. Animal studies conducted in mice and primates confirmed the rapid

metabolism of benzyl chloride to benzyl mercapturic acid, benzyl alcohol, benzaldehyde, and hippuric acid as well as the excretion of these metabolites with the urine (Hall and James 1986). As an alkylating compound, benzyl chloride additionally reacts with macromolecules, forming adducts. The alkylating effect on DNA was proven in an *in vivo* study in mice (Walles 1981).

An analogous metabolism and alkylation can likely be expected for benzyl bromide (IFA 2023) and is also probable for benzyl iodide, for which no data have yet been published.

Alkyl halides Monosubstituted *n*-alkyl halides are polar but non-hydrophilic substances whose boiling points increase with the chain length and the atomic weight of the halogen substituent. Accordingly, at room temperature, methyl chloride, methyl bromide, and methyl iodide are gaseous, whereas ethyl bromide, ethyl iodide, propyl bromide, propyl iodide, butyl bromide, and butyl iodide are liquids, most of them highly volatile.

Methyl chloride is either produced in or imported into the European Economic Area in amounts of ≥1 000 000 t/a; methyl iodide in amounts of ≥100 t/a to <1000 t/a; butyl bromide in amounts of ≥10 t/a to <100 t/a; and propyl bromide in amounts of ≥1 t/a to <10 t/a (ECHA 2018 a, 2021 d, 2022 a, 2023 c). Methyl bromide, ethyl bromide, ethyl iodide, and propyl iodide are only manufactured as intermediates in the European Economic Area, and the website of the European Chemicals Agency does not provide information on produced or used quantities (ECHA 2018 b, 2019, 2020 a, 2021 a).

Methyl chloride is an industrial raw material for the production of di- and trichloromethane, silicones, cellulose ethers (such as methyl cellulose), cationic polymers, and quaternary ammonium compounds. Methyl chloride is also employed for the synthesis of organometallic compounds such as methyllithium, trimethylaluminium, or methyltin compounds, which then serve as intermediates in the production of fine chemicals and as catalysts in polymer production. Methyl chloride is further used as a laboratory chemical (Ohligschläger et al. 2019). Methyl bromide is released both naturally (oceanic emissions, vegetation) as well as anthropogenically (burning of biomass and biofuels, use as a fumigant, use as an industrial and laboratory chemical) (Saltzman et al. 2022; Yoffe et al. 2013). In industry, methyl bromide serves primarily as an alkylating agent, especially for the production of pharmaceuticals (Yoffe et al. 2013). Due to restrictions on use, the applications of the substance as a pesticide for the fumigation of shipping containers, to fight against animal wood pests in the construction sector, and as a soil sterilant have declined (ECHA 2021 a). Methyl iodide is used as an intermediate in the production of pharmaceuticals and pesticides and generally as a methylating agent in organic synthesis. Methyl iodide is additionally formed from marine algae and released from oceans, and may be further detected in combustion gases (IARC 1977).

Ethyl bromide and ethyl iodide are primarily used as alkylating agents in chemical synthesis, both in the laboratory and for the production of pharmaceuticals and other substances (ECHA 2019, 2020 a; RÖMPP-Redaktion 2023).

Propyl bromide is used as a solvent for greases, waxes, and resins or as an intermediate in the synthesis of pharmaceuticals, insecticides, quaternary ammonium compounds, flavourings, or fragrances (Hartwig 2011). Propyl iodide is likewise marketed as a laboratory chemical and is registered as a substance for the production of pharmaceutical products (ECHA 2018 b).

Butyl bromide is used as an alkylating agent in the production of medications and fragrances (RÖMPP-Redaktion 2023). Butyl iodide is applied for the introduction of butyl groups in organic synthesis (Pattenden 1991).

The respiratory tract and the skin are the main routes of absorption for methyl chloride, methyl bromide, methyl iodide, ethyl bromide, and propyl bromide. Butyl bromide is primarily absorbed via the respiratory tract (IFA 2023). A small proportion of the substances is excreted unchanged via exhaled air, another proportion is oxidatively metabolised. The main metabolic pathway takes place via glutathione-*S*-transferase (GSTT1-1)-mediated conjugation of the alkyl group with glutathione. Due to the genetic polymorphism of GSTT1-1, there are rapid and slow metabolisers as well as non-metabolisers. The alkyl cysteine formed is subsequently excreted with the urine (IFA 2023).

Chloroalkyl ethers Bis(chloromethyl) ether and monochlorodimethyl ether are colourless, highly volatile liquids used in organic synthesis, mainly for alkylations (RÖMPP-Redaktion 2023). Bis(chloromethyl) ether is employed as a

reagent in the production of plastics, ion-exchange resins, and polymers. Monochlorodimethyl ether is used industrially as an alkylating agent and solvent in the production of dodecylbenzyl chloride, water repellents, ion-exchange resins and polymers, and as a reagent for chloromethylation (NTP 2021).

In the German chemical industry, these especially hazardous carcinogens may only be produced or used in closed systems. For research and analysis, both substances may be employed openly in the quantities required for these purposes (Bundesregierung Deutschland 2010). Bis(chloromethyl) ether and monochlorodimethyl ether are mainly absorbed via the respiratory tract. Dermal bioavailability is not expected as the substances immediately react upon contact with the skin (hydrolysis and/or alkylation of tissue components) prior to or during skin penetration. It can be assumed that, at the point of entry into the body, the majority of absorbed bis(chloromethyl) ether or monochlorodimethyl ether is virtually immediately hydrolysed to formaldehyde and hydrochloric acid (bis(chloromethyl) ether) or methanol, formaldehyde, and hydrochloric acid (monochlorodimethyl ether). The inhaled portion of chloroalkyl ethers which is not hydrolysed can be taken up systemically, which may lead to the formation of haemoglobin adducts (Hb adducts), among other effects.

***N*-Nitrosodialkylamines** *N*-Nitrosodialkylamines (*N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosomethylethylamine, *N*-nitrosodi-*n*-propylamine, *N*-nitrosodi-*n*-butylamine), according to the German Hazardous Substances Ordinance (*Gefahrstoffverordnung*), are among the most dangerous and highly potent carcinogenic substances (AGS 2018). For this reason, they are of no technical or industrial significance. The aforementioned *N*-nitrosodialkylamines are produced or used in their pure forms only for scientific purposes.

N-nitrosodialkylamines occuring at workplaces form under certain reaction conditions from processes involving secondary amines and nitrosating agents, such as in the metal and chemical industries, rubber and leather industries as well as in foundries, among other areas of application (AGS 2018).

The *N*-nitrosodialkylamines thus formed can be taken up via the respiratory tract or dermally in cases of skin contact. The uptake of precursors with subsequent *in vivo* nitrosation may additionally contribute to occupational exposure. *N*-Nitrosodialkylamines are completely absorbed. During metabolism, among other substances, highly reactive alkyldiazonium ions are formed which alkylate biological macromolecules (such as DNA, RNA, and proteins) (IFA 2023).

The general population may be exposed to the aforementioned *N*-nitrosodialkylamines or their precursors via the consumption of food or alcoholic beverages as well as by smoking.

Alkyl alkane sulfonates Ethyl methanesulfonate, an alkyl alkane sulfonate, is a colourless liquid which decomposes in water, forming ethanol and methanesulfonic acid (Cumming and Walton 1970). Ethyl methanesulfonic acid is primarily used in molecular biology to generate point mutations. Furthermore, it may arise as a production-related contaminant in the antiviral drug Virazept® and may therefore lead to exposure in patients. In investigations studying mice, rats, and primates, Lavé et al. (2009) found that ethyl methanesulfonate was easily absorbed after oral ingestion and that the levels of formed Hb adducts increased proportionally to the administered dose.

Acrylonitrile is a colourless, highly volatile liquid which tends towards spontaneous polymerisation (IFA 2023). Between 1 000 000 t/a and 10 000 000 t/a are produced in or imported into the European Economic Area (ECHA 2023 b). More than half of acrylonitrile is used in the production of polyacrylonitrile fibres for the textile industry, and 15% are employed in the production of acrylonitrile butadiene styrene and styrene acrylonitrile copolymers for the automotive industry. Another 15% of acrylonitrile are used to produce acrylamide and adiponitrile, while about 18% are used in the rubber industry as well as in the production of further polymers (EU 2004).

The general population is mainly exposed to acrylonitrile through smoking (Scherer et al. 2022), whereby 3–15 µg of acrylonitrile have been detected in the smoke of a single cigarette (Hoffmann et al. 2001).

In the workplace, acrylonitrile is mainly absorbed via inhalation and dermal routes. Absorbed acrylonitrile can react with glutathione directly, after epoxidation, or after cleavage of the cyano group and is then excreted with the urine. The cyanoethylene oxide formed by epoxidation also reacts as an electrophilic agent with endogenous macromolecules, forming adducts (IFA 2023).

Acrylamide is a colourless solid which tends towards spontaneous polymerisation (IFA 2023). Between 100 000 t/a and 1 000 000 t/a of acrylamide are produced in or imported into the European Economic Area (ECHA 2023 a). Acrylamide is primarily used for the production of polyacrylamides, which, among other applications, are used as dispersion and flocculant agents in the treatment of drinking water. Furthermore, polyacrylamides with high molecular masses are chemically modified for various purposes and are thereafter used as ion exchangers, thickening agents, or auxiliary substances in the paper industry. In addition, acrylamide is used in the synthesis of paints, as a copolymer for various plastics, and as a sealing compound in the construction sector. In research laboratories, acrylamide is used for the preparation of polyacrylamide gels for electrophoresis (Schettgen 2006).

Acrylamide is also formed by heating starchy foods with low water contents (Tareke et al. 2002), such that the general population may be exposed via the diet. Additional exposure to acrylamide may occur from smoking (Scherer et al. 2022), as 0.24–0.90 µg of acrylamide have been detected in the smoke of a single cigarette (Esposito et al. 2022).

Acrylamide is quickly absorbed via oral, dermal, and inhalation routes and, due to its high water solubility, is rapidly distributed throughout the body. Generally, metabolism is glutathione-dependent and occurs either directly with acrylamide (forming *N*-acetyl-*S*-(2-carbamoylethyl)cysteine) or after epoxidation to glycidamide. With glutathione, this epoxide forms *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)cysteine and *N*-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)-cysteine. Moreover, both acrylamide and its epoxide may react with haemoglobin, forming adducts (IFA 2023).

Diazomethane is a yellow, chemically unstable gas which, upon contact with water, slowly degrades into methanol and nitrogen (IFA 2023). Its industrial applications are limited due to its high reactivity and toxicity. For this reason, diazomethane is primarily applied as a methylating agent at the laboratory level (Greim 1999). In *invitro* studies, methylation of DNA could be proven after direct exposure to diazomethane (Friedman et al. 1965; Kriek and Emmelot 1964); the methylation of further macromolecules cannot be ruled out (Greim 1999).

The Commission has evaluated various alkylating substances. Monochlorodimethyl ether and bis(chloromethyl) ether have been classified by the Commission as Category 1 carcinogens. Acrylamide, acrylonitrile, bromoethane, 1-bromo-propane, benzyl chloride, diazomethane, dimethyl sulfate, diethyl sulfate, ethylene oxide, iodomethane, *N*-nitrosodi-*n*-butylamine, *N*-nitrosodiethylamine, *N*-nitrosodimethylamine, *N*-nitrosodi-*n*-propylamine, and *N*-nitrosomethyl-ethylamine are Category 2 carcinogens, whereas bromomethane, chloroethane, and ethylene are Category 3 carcinogens (DFG 2023).

Table 1 provides an overview of the Commission's classifications of the alkylating substances considered in this method, the details of which can be found in the corresponding toxicological and occupational-health documentations (https://onlinelibrary.wiley.com/doi/book/10.1002/3527600418, https://series.publisso.de/en/pgseries/overview/mak/dam).

Tab. 1 Classification of alkylating substances by the Commission

Substance	MAK value	H/S designation	Carc cat	Preg gr	Muta cat	BV
Acrylamide		H; Sh	2		2	EKA
Acrylonitrile		H; Sh	2			EKA
Benzyl chloride		H	2			
Bis(chloromethyl) ether			1			
Bromoethane		H	2			
Bromomethane	1 ml/m³ Peak lim: I(2)		3	C		
1-Bromopropane		H	2			
Chloromethane	10 ml/m³ Peak lim: II(1)			D		
Diazomethane			2			

Tab. 1 *(continued)*

Substance	MAK value	H/S designation	Carc cat	Preg gr	Muta cat	BV
Diethyl sulfate		H	2		2	
Dimethyl sulfate		H	2			EKA
1,2-Epoxypropane	2 ml/m³ Peak lim: I(2)	Sh	4	C		EKA
Ethylene			3			EKA not established
Ethylene oxide		H	2		2	EKA
Iodomethane		H	2			
Monochlorodimethyl ether			1			
N-Nitrosodi-*n*-butylamine		H	2			
N-Nitrosodiethylamine		H	2			
N-Nitrosodimethylamine		H	2			
N-Nitrosodi-*n*-propylamine		H	2			
N-Nitrosomethylethylamine		H	2			

BV: assessment values in biological material (BAT/EKA/BLW/BAR); Carc cat: carcinogen category (see DFG 2023, Section III); EKA: exposure equivalents for carcinogenic substances; H: danger from percutaneous absorption, MAK: maximum workplace concentration (*maximale Arbeitsplatzkonzentration*); Muta cat: germ cell mutagen category (see DFG 2023, Section IX); Peak lim: peak limitation category (excursion factor); Preg gr: pregnancy risk group (see DFG 2023, Section VIII); Sh: danger of sensitization of the skin

Due to their high reactivity, alkylating compounds are important hazardous substances. For *n*-alkyl halides and benzyl halides, the stability of the halogen-carbon bond increases considerably in the series I < Br < Cl < F , such that aliphatic fluorine compounds do not exhibit any alkylating activity (van Sittert et al. 1997). In the body, alkylating compounds react as electrophiles with the *N*-terminal amino acids of peptides and proteins, among other substances. The adducts are formed either by nucleophilic substitution (e.g. alkyl halides, epoxides, dialkyl sulfates) or by 1,4-addition (e.g. α,β-unsaturated carbonyl compounds) (Törnqvist et al. 2002). Figure 1 provides schematic depicts of the nucleophilic substitution reaction using propylene as an example and the addition reaction using acrylamide as an example.

Hb adducts of alkylating substances are commonly used in biomonitoring to evaluate exposure. The adducts formed at the *N*-terminal valine of globin or haemoglobin are considered, whereby the *N*-terminal valine of the β chain is, due to its low pK_a value ($pK_a = 6.8$), somewhat more reactive than that of the α chain ($pK_a = 7.8$) (Törnqvist et al. 2002).

The stability of haemoglobin is not affected by adduct formation (Neumann et al. 1993), and since neither of the formed Hb adducts are repaired (Törnqvist and Landin 1995), their elimination is determined only by the lifetime of the erythrocytes (about 120 days). Following accidental events, it is also possible, due to first-order elimination kinetics, to deduce the level of original exposure to the hazardous substances during this period (Bader et al. 2012; Bader and Wrbitzky 2006; Leng and Gries 2014). Hb adducts, however, are primarily long-term biomarkers which reflect the cumulative internal exposure to a hazardous substance over a period of about four months and therefore represent an important and effective instrument for risk assessment (Boogaard et al. 1999; Sabbioni and Day 2022; Törnqvist and Landin 1995). Figure 2 shows the structures of the valine adducts which can be determined with this method. Table 2 provides representative levels of these adducts in occupationally exposed persons as well as in the non-occupationally exposed general population.

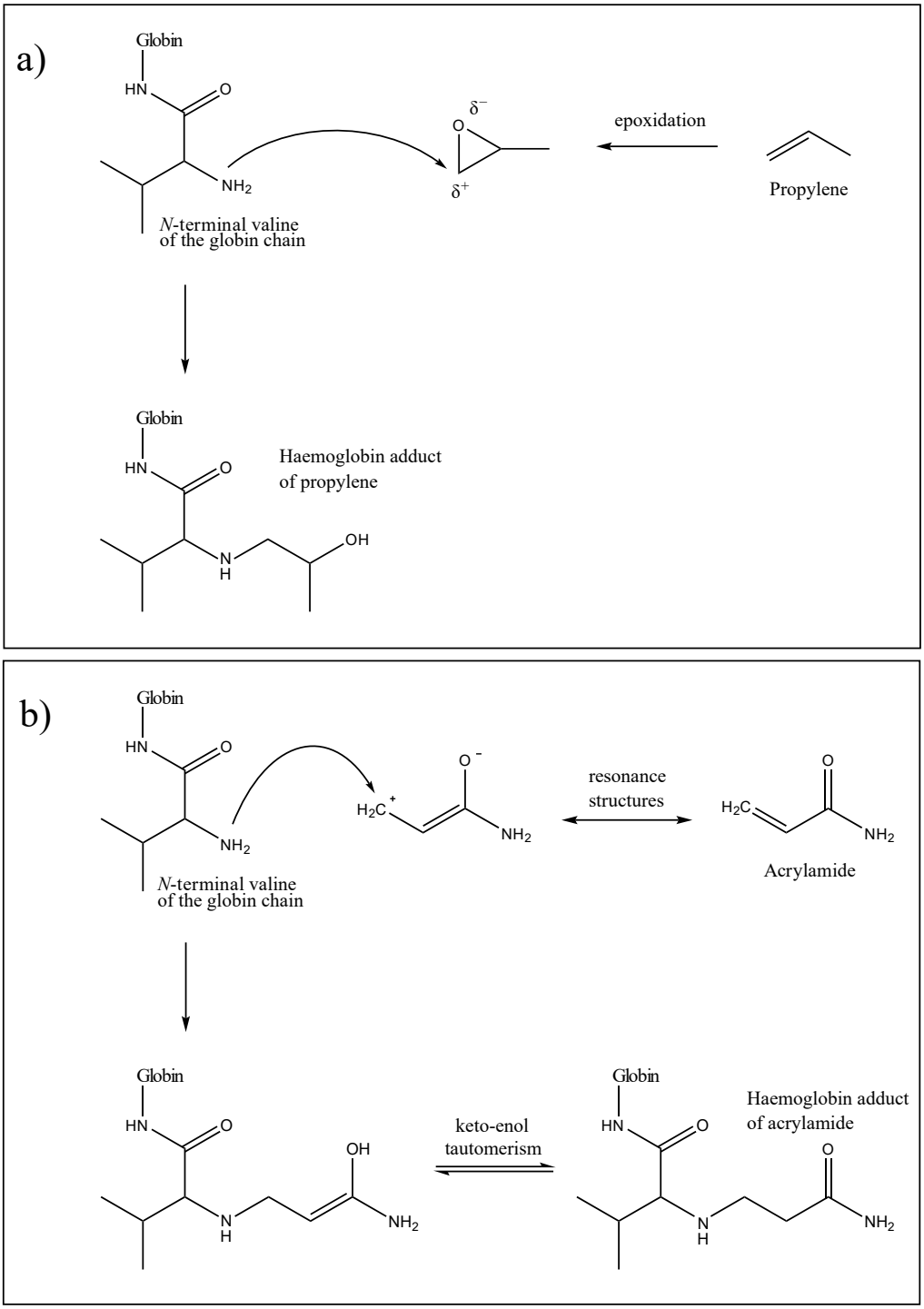


Fig. 1 Mechanisms of adduct formation at the *N*-terminal valine of haemoglobin: a) nucleophilic substitution using propylene as an example, and b) Michael addition (1,4-addition) using acrylamide as an example

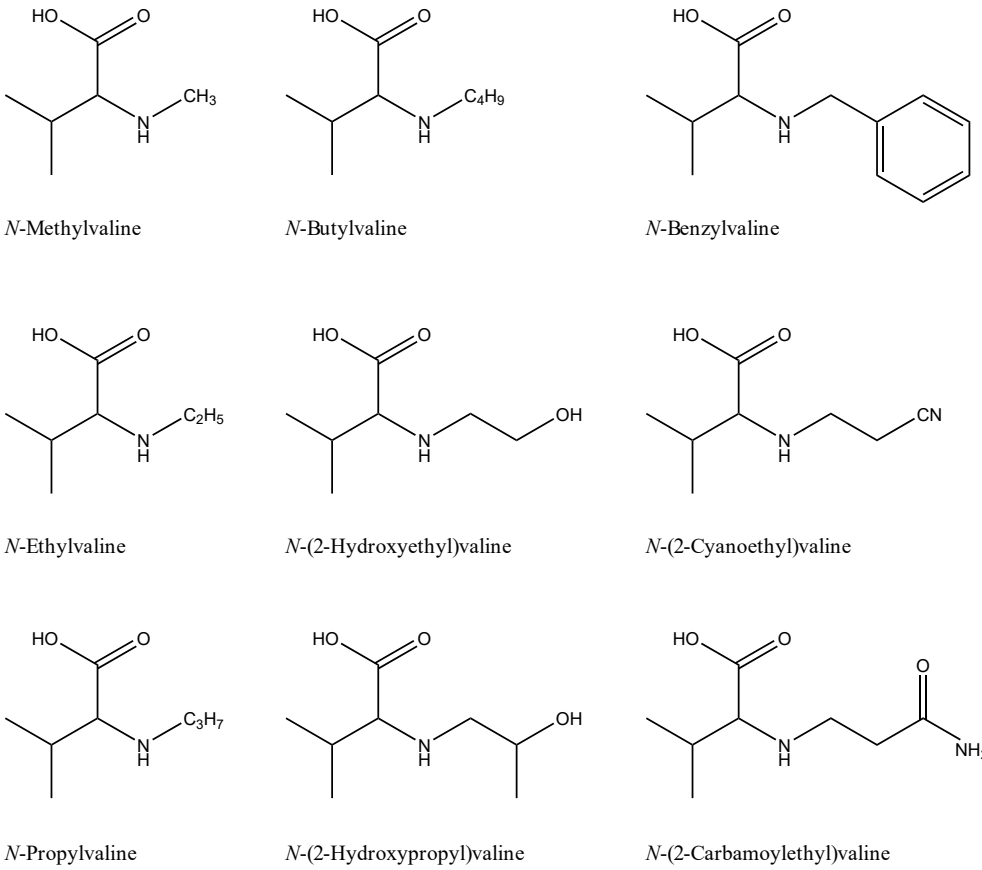


Fig. 2 Structural formulas of the adducts that can be determined with this method

Tab. 2 Hb adduct levels in occupationally exposed persons and in persons of the non-occupationally exposed general population

Study collective	Number of persons and smoker status	Substance	Analyte	Adduct level [pmol/g globin]		Reference
				Mean (±SD)	Range	
Manufacture of surfactants for the textile industry	Workers (38 S; 24 N)	Dimethyl sulfate	*N*-Methylvaline	609[a)]	n. a.–9697	Schettgen et al. 2004
	Controls (2 S; 8 N)	–		509[a)]	n. a.–677	
General population	45 S	–	*N*-Methylvaline	997 ± 203	–	Carmella et al. 2002
	29 N			904 ± 149	–	
General population	39 S	–	*N*-Ethylvaline	3.76 ± 2.77	–	
	28 N			2.50 ± 1.65	–	
Workplaces with potential exposure to propylene oxide	Workers (18)	Propylene oxide	*N*-(2-Hydroxypropyl)-valine	10[a)]	0–18	Ball et al. 2005

Tab. 2 *(continued)*

Study collective	Number of persons and smoker status	Substance	Analyte	Adduct level [pmol/g globin]		Reference
				Mean (±SD)	Range	
Styrene manufacture (SMPO process)	Workers prior to facility maintenance (27)	Propylene oxide	*N*-(2-Hydroxypropyl)-valine	40.2 ± 8.0	–	Boogaard et al. 1999
	Workers after facility maintenance (19)			45.3 ± 8.0	–	
Manufacture of glycol and glycol ethers	Workers (20)	Ethylene oxide	*N*-(2-Hydroxyethyl)-valine	92 ± 25	12–320	
	Controls (23 N)	–		22 ± 5	6–49	
	Controls (13 S)			Increase of 9.4 for each cigarette smoked per day		
General population	26 S	–	*N*-(2-Hydroxyethyl)-valine	200 ± 113	38–501	Bailey et al. 1988
	23 N			52.1 ± 20.5	22–106	
Accidental exposure to ethylene oxide	Workers (5 S; 1 N)	Ethylene oxide	*N*-(2-Hydroxyethyl)-valine	1210 ± 777[c)]	522–2396[c)]	Bader et al. 2012
				177 ± 85[d)]	30–276[d)]	
Accidental exposure to ethylene	Workers and emergency personnel (863)	Ethylene	*N*-(2-Hydroxyethyl)-valine	99[b)]	< LOQ[b)]–949	Leng and Gries 2014
Accidental exposure to acrylonitrile	Workers and emergency personnel (863)	Acrylonitrile	*N*-(2-Cyanoethyl)-valine	98[b)]	< LOQ[b)]–1924	
Polymerisation plant	Maintenance workers (9 N)	Acrylonitrile	*N*-(2-Cyanoethyl)-valine	1984 ± 2066	93.9–5746	Tavares et al. 1996
	Workers performing continuous polymerisation (7 N)			2276 ± 1338	635–4604	
	Controls (office) (11 N)	–		31.1 ± 18.5	8.5–70.5	
General population, vegans	6 N	–	*N*-Benzylvaline	0.22[a)]	0.06–0.37	Gauch et al. 2022
	6 S			0.09[a)]	0.05–0.14	
General population, vegans	6 N	–	*N*-(2-Carbamoylethyl)-valine	25.9[a)]	16.4–41.9	
	6 S			69.0[a)]	22.0–770	
Tunnel construction, chemical grouting	Workers (210)	Acrylamide, *N*-methylol acrylamide	*N*-(2-Carbamoylethyl)-valine	–	< 80–17700	Hagmar et al. 2001
	Controls (18 N)	–		–	20–70	

LOQ: limit of quantitation; N: non-smokers; S: smokers; SMPO: styrene monomer and propylene oxide

[a)] Median

[b)] Values < LOQ (for *N*-(2-hydroxyethyl)valine: 86 pmol/g globin; for *N*-(2-cyanoethyl)valine: 41 pmol/g globin) were included in the calculation as LOQ/2

[c)] Sampling took place days 1–4 following exposure

[d)] Sampling took place days 162–166 following exposure

3 General principles

The procedure described herein is based on the method by van Sittert (1997) and allows for the simultaneous measurement of the adduct levels of the following *N*-terminal Hb adducts in the erythrocyte fraction of whole blood: *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline, *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine.

For the determination of the adducts of *N*-terminal valines in haemoglobin, erythrocytes are separated from whole blood and lysed. The globin is precipitated from the haemoglobin solution and the alkylated *N*-terminal valines are first derivatised with pentafluorophenyl isothiocyanate under the addition of *N*-(2-ethoxyethyl)valine-alanine-anilide as the internal standard and then cleaved via a modified Edman degradation. The thereby resulting pentafluorophenyl thiohydantoin derivates are extracted with *tert*-butyl methyl ether and washed, and the processed samples are determined by GC-EI-MS/MS. For calibration, pooled globin from non-smokers not occupationally exposed to the hazardous substances is used; this pooled globin is mixed with solutions of dipeptide standards which simulate the last two adduct-bearing *N*-terminal amino acids of the haemoglobin chain. The calibration standards are treated analogously to the samples.

4 Equipment, chemicals, and solutions

4.1 Equipment

- GC-MS/MS system (e.g. Bruker 456) with a CTC PAL autosampler and Bruker EVOQ TQ-MS with control and evaluation software (Bruker Corporation, Billerica, USA)
- Capillary separation column (e.g. Rxi-5Sil MS, 30 m × 0.25 mm × 0.25 µm (No. 13623, Restek GmbH, Bad Homburg vor der Höhe, Germany) or DB-5ms Ultra Inert, 30 m × 0.25 mm × 0.25 µm (No. 122-5532UI, Agilent Technologies Deutschland GmbH, Waldbronn, Germany) or Optima 5 HT, 30 m × 0.25 mm × 0.25 µm (No. REF 726106.30, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany))
- EDTA Monovettes® with cannulas (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Orbital shaker (e.g. Multi Reax, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany)
- Laboratory centrifuge (e.g. ROTANTA 460 R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Laboratory freezer (≤ –18 °C)
- Shaking water bath (e.g. JULABO GmbH, Seelbach, Germany)
- Laboratory shaker (e.g. VXR basic Vibrax®, IKA-Werke GmbH & CO. KG, Staufen, Germany) with an adapter for test tubes (e.g. VX 2, IKA-Werke GmbH & CO. KG, Staufen, Germany)
- Nitrogen evaporator (e.g. No. C103199, TurboVap® LV, Biotage Sweden AB, Uppsala, Sweden)
- Water-purification system (e.g. Milli-Q® IQ 7000 water-purification system, Merck KGaA, Darmstadt, Germany)
- Dispensette® III, 2.5–25 ml (e.g. No. 4700 150, BRAND GMBH + CO KG, Wertheim, Germany)
- Dispensette® III, 1–10 ml (e.g. No. 4700 140, BRAND GMBH + CO KG, Wertheim, Germany)
- Dispensette® III, 0.2–2 ml (e.g. No. 4700 120, BRAND GMBH + CO KG, Wertheim, Germany)
- Multipette® with CombiTips® (e.g. Eppendorf AG, Hamburg, Germany)
- Variable pipette, 1–100 µl, with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Transfer pipettes, 3.5 ml (e.g. No. 86.1171, Sarstedt AG & Co. KG, Nümbrecht, Germany)
- 10-ml, 100-ml, and 1000-ml volumetric flasks (e.g. witeg Labortechnik GmbH, Wertheim, Germany)
- Evaporation tubes, 5 ml (e.g. No. 55.526, Sarstedt AG & Co. KG, Nümbrecht, Germany)
- 15-ml threaded test tubes (e.g. No. 3561103, schuett-biotec GmbH, Göttingen, Germany)
- Teflon-lined screw caps, 18 mm (e.g. No. 2924011, Duran®, Schott AG, Mainz, Germany)
- 50-ml centrifuge tubes (e.g. No. 62.548.004, Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Test tubes, 10 ml (e.g. Fisher Scientific GmbH, Schwerte, Germany)
- Silopren™ plugs, 12.5 mm × 16.5 mm × 20 mm (e.g. A. Vogt GmbH & Co. KG, Arnsberg, Germany)

- Crimp-top vials, N 8, 200 µl (e.g. No. 70286, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- Crimp caps, N 8 (e.g. No. 702025, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)

4.2 Chemicals

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

Reference materials

- *N*-Methylvaline-leucine-anilide (e.g. No. 4019919, Bachem Biochemica, Heidelberg, Germany)
- *N*-Ethylvaline-leucine-anilide (e.g. custom synthesis, Bachem Biochemica, Heidelberg, Germany)
- *N*-*n*-Propylvaline-leucine-anilide (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- *N*-*n*-Butylvaline-leucine-anilide (e.g. custom synthesis, Bachem Biochemica, Heidelberg, Germany)
- *N*-((*R,S*)-2-Hydroxypropyl)valine-leucine-anilide (e.g. No. 4025561, Bachem Biochemica, Heidelberg, Germany)
- *N*-(2-Hydroxyethyl)valine-leucine-anilide (e.g. No. 4025019, Bachem Biochemica, Heidelberg, Germany)
- *N*-(2-Cyanoethyl)valine-leucine-anilide (e.g. No. 4019925, Bachem Biochemica, Heidelberg, Germany)
- *N*-Benzylvaline-leucine-anilide (e.g. custom synthesis, Bachem Biochemica, Heidelberg, Germany)
- *N*-(2-Carbamoylethyl)valine-leucine-anilide (e.g. No. 4025471, Bachem Biochemica, Heidelberg, Germany)
- *N*-(2-Ethoxyethyl)valine-alanine-anilide (e.g. custom synthesis, Bachem Biochemica, Heidelberg, Germany)

Other chemicals

- *tert*-Butyl methyl ether (e.g. No. 177040010, Fisher Scientific GmbH, Schwerte, Germany)
- Ethanol (e.g. No. 100983, Merck KGaA, Darmstadt, Germany)
- Ethyl acetate (e.g. No. 07137, Bernd Kraft GmbH, Duisburg, Germany)
- Formamide (e.g. No. F-7503, Merck KGaA, Darmstadt, Germany)
- Sodium carbonate (e.g. No. 71351, Fluka™ by Honeywell Deutschland Holding GmbH, Offenbach, Germany)
- Sodium chloride (e.g. No. 106404, Merck KGaA, Darmstadt, Germany)
- Sodium hydroxide solution, 1 mol/l (e.g. No. 109137, Merck KGaA, Darmstadt, Germany)
- Pentafluorophenyl isothiocyanate (e.g. No. 76755, Fluka™ by Honeywell Deutschland Holding GmbH, Offenbach, Germany)
- 2-Propanol, ≥ 99.8 % (e.g. No. 33539-M, Merck KGaA, Darmstadt, Germany)
- Hydrochloric acid, 1 mol/l (e.g. No. 109057, Merck KGaA, Darmstadt, Germany)
- Toluene (e.g. No. 108389, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Milli-Q®, Merck KGaA, Darmstadt, Germany)
- Helium 5.0 (e.g. Linde GmbH, Pullach, Germany)
- Argon 5.3 (e.g. Linde GmbH, Pullach, Germany)
- Nitrogen (e.g. Linde GmbH, Pullach, Germany)

4.3 Solutions

- Sodium chloride solution (0.9%)
  9 g of sodium chloride are weighed into a 1000-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.

The solution is stored at room temperature and is stable for three months.

- Sodium carbonate solution (0.1 mol/l)
  1.06 g of sodium carbonate are weighed into a 100-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.

The solution is stored at room temperature and is stable for three months.

- Hydrochloric 2-propanol (50 mmol/l)
  About 500 ml of 2-propanol are placed in a 1000-ml volumetric flask and 50 ml of hydrochloric acid (1 mol/l) are added. The volumetric flask is then made up to the mark with 2-propanol.

The solution is stored at room temperature and is stable for one year.

- Ammonia-free formamide (pH ≈ 7)
  If necessary, volatile amines and free ammonia are stripped of formamide using nitrogen. To this end, nitrogen is introduced via metal frit for about one hour. No volatile alkaline compounds should be observed in the vapour space when tested with dampened litmus paper.

4.4 Comparative standards

- Stock solution of *N*-methylvaline-leucine-anilide (0.6 mmol *N*-methylvaline/l)
  Exactly 19.2 mg of *N*-methylvaline-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Stock solution of *N*-ethylvaline-leucine-anilide (0.6 mmol *N*-ethylvaline/l)
  Exactly 20.0 mg of *N*-ethylvaline-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Stock solution of *N*-propylvaline-leucine-anilide (0.6 mmol *N*-propylvaline/l)
  Exactly 20.9 mg of *N*-propylvaline-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Stock solution of *N*-butylvaline-leucine-anilide (0.6 mmol *N*-butylvaline/l)
  Exactly 21.7 mg of *N*-butylvaline-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Stock solution of *N*-((*R*,*S*)-2-hydroxypropyl)valine-leucine-anilide (0.6 mmol *N*-((*R*,*S*)-2-hydroxypropyl)valine/l)
  Exactly 21.8 mg of *N*-((*R*,*S*)-2-hydroxypropyl)valine-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Stock solution of *N*-(2-hydroxyethyl)valine-leucine-anilide (0.6 mmol *N*-(2-hydroxyethyl)valine/l)
  Exactly 21.0 mg of *N*-(2-hydroxyethyl)valine-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Stock solution of *N*-(2-cyanoethyl)valine-leucine-anilide (0.6 mmol *N*-(2-cyanoethyl)valine/l)
  Exactly 21.5 mg of *N*-(2-cyanoethyl)valine-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.

- Stock solution of *N*-benzylvaline-leucine-anilide (0.6 mmol *N*-benzylvaline/l)
  Exactly 23.7 mg of *N*-benzylvaline-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Stock solution of *N*-(2-carbamoylethyl)valine-leucine-anilide (0.6 mmol *N*-(2-carbamoylethyl)valine/l)
  Exactly 22.6 mg of *N*-(2-carbamoylethyl)valine-leucine-anilide are weighed into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Spiking solution I (6 µmol/l)
  Exactly 0.1 ml of each of the nine stock solutions are pipetted into a 10-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Spiking solution II (0.6 µmol/l)
  Exactly 1 ml of Spiking solution I is pipetted into a 10-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- Spiking solution III (0.06 µmol/l)
  Exactly 0.1 ml of Spiking solution I are pipetted into a 10-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.

The ethanolic solutions of the reference substances are stable for at least one year at –18 °C (Schettgen et al. 2016).

4.5 Internal standards (ISTDs)

- Stock solution of *N*-(2-ethoxyethyl)valine-alanine-anilide (0.6 mmol *N*-(2-ethoxyethyl)valine/l)
  Exactly 20.1 mg of *N*-(2-ethoxyethyl)valine-alanine-anilide are weighed into in a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.
- ISTD spiking solution (6 µmol *N*-(2-ethoxyethyl)valine/l)
  Exactly 1 ml of the *N*-(2-ethoxyethyl)valine-alanine-anilide stock solution is pipetted into a 100-ml volumetric flask. The volumetric flask is made up to the mark with ethanol and the solution is thoroughly mixed.

The ethanolic solutions of the internal standard are stable for at least one year at –18 °C (Schettgen et al. 2016).

4.6 Calibration standards

Each 100-mg (± 5 mg) sample of pooled globin from non-occupationally exposed non-smokers is weighed into 50-ml tubes and dissolved in 1.5 ml of ammonia-free formamide. These solutions are supplemented with the individual spiking solutions by pipetting according to the scheme given in Table 3. The calibration standards are subsequently processed analogously to the samples, starting with the addition of sodium hydroxide and the derivatisation reagent (see Section 5.2). After further workup, they are analysed according to Section 6.

Calibration must not take place every workday as long as the quality-control samples included in each analytical run appear normal and nothing on the measuring instrumentation has been changed (such as shortening the column).

Tab. 3 Pipetting scheme for the preparation of calibration standards for the determination of *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline, *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine in the erythrocyte fraction of whole blood

Calibration standard	Spiking solution I [µl]	Spiking solution II [µl]	Spiking solution III [µl]	ISTD spiking solution [µl]	Adduct level [pmol/g globin]
0	–	–	–	100	0
1	–	–	25	100	15
2	–	–	50	100	30

Tab. 3 *(continued)*

Calibration standard	Spiking solution I [µl]	Spiking solution II [µl]	Spiking solution III [µl]	ISTD spiking solution [µl]	Adduct level [pmol/g globin]
3	–	–	100	100	60
4	–	20	–	100	120
5	–	30	–	100	180
6	–	75	–	100	450
7	12.5	–	–	100	750
8	30	–	–	100	1800
9	75	–	–	100	4500

5 Specimen collection and sample preparation

5.1 Specimen collection

After disinfecting the puncture site, an EDTA Monovette® is used to extract 5 ml of venous whole blood. The extraction tube is swivelled several times directly after blood collection. The blood sample is subsequently centrifuged for 10 min at 800 × *g* in order to separate the erythrocytes from the blood plasma. The plasma supernatant is carefully removed with a pipette. The erythrocyte fraction is mixed with 5 ml of a 0.9% saline solution, swivelled several times, and again centrifuged for 10 min at 800 × *g*. The supernatant is again removed with a pipette and discarded. This washing step is repeated until the supernatant is clear and colourless. Based on experience, fresh blood samples must undergo this process three times.

For haemolysis, the erythrocytes are subsequently resuspended in 2.5 ml of ultra-pure water and frozen for at least 60 min at –18 °C.

5.2 Sample preparation

Globin isolation

About 2.5 ml of the erythrocyte lysate are placed in a 50-ml centrifuge tube and 15 ml of hydrochloric 2-propanol are added. After brief shaking, the sample is centrifuged for 10 min at 3300 × *g* and 10 °C. The supernatant is transferred into a new 50-ml tube and the globin is precipitated by adding 10 ml of ethyl acetate.

In order to ensure complete precipitation, after waiting for at least 15 min, the suspension is briefly shaken up. The verifiers of the method recommend refrigerating the sample for at least one hour before vigorous shaking in order to achieve complete globin precipitation. The sample is then centrifuged for 5 min at 2500 × *g* and 10 °C, and the supernatant is removed and discarded. The globin pellet is washed twice (three to four times, if necessary) with 10 ml of ethyl acetate each time, until the supernatant is colourless. After washing with ethyl acetate, the verifiers of the method added another washing step using 5 ml of *n*-hexane. Care must be taken to ensure the pellet is properly resuspended on the orbital shaker before the sample is centrifuged for 5 min at 2500 × *g* and 10 °C. After each washing step, the supernatant is removed. Finally, the pellet is left to dry overnight in a fume hood.

Globin derivatisation

100 mg (± 5 mg) of globin are weighed into a threaded test tube and mixed with 1.5 ml of ammonia-free formamide. To obtain a homogenous solution, the sample is shaken for 15 min on a laboratory shaker. Subsequently, 100 µl of the ISTD spiking solution, 40 µl of the sodium hydroxide solution (1 mol/l), as well as 20 µl of pentafluorophenyl isothiocyanate

are pipetted into the solution. The sample is thoroughly mixed and subsequently kept at 80 °C in a shaking water bath for one hour. In this incubation step, the terminal amino acid of globin is cleaved by modified Edman degradation. As part of this process, the adduct-bearing *N*-terminal valine reacts with pentafluorophenyl isothiocyanate, forming a thiocarbamoyl derivative. Cleaving the globin chain leads to the cyclisation of the thiocarbamoyl derivative to a thiazolinone derivative, which is finally rearranged to a more stable pentafluorophenyl thiohydantoin by heating (Figure 3), which serves as the analyte.

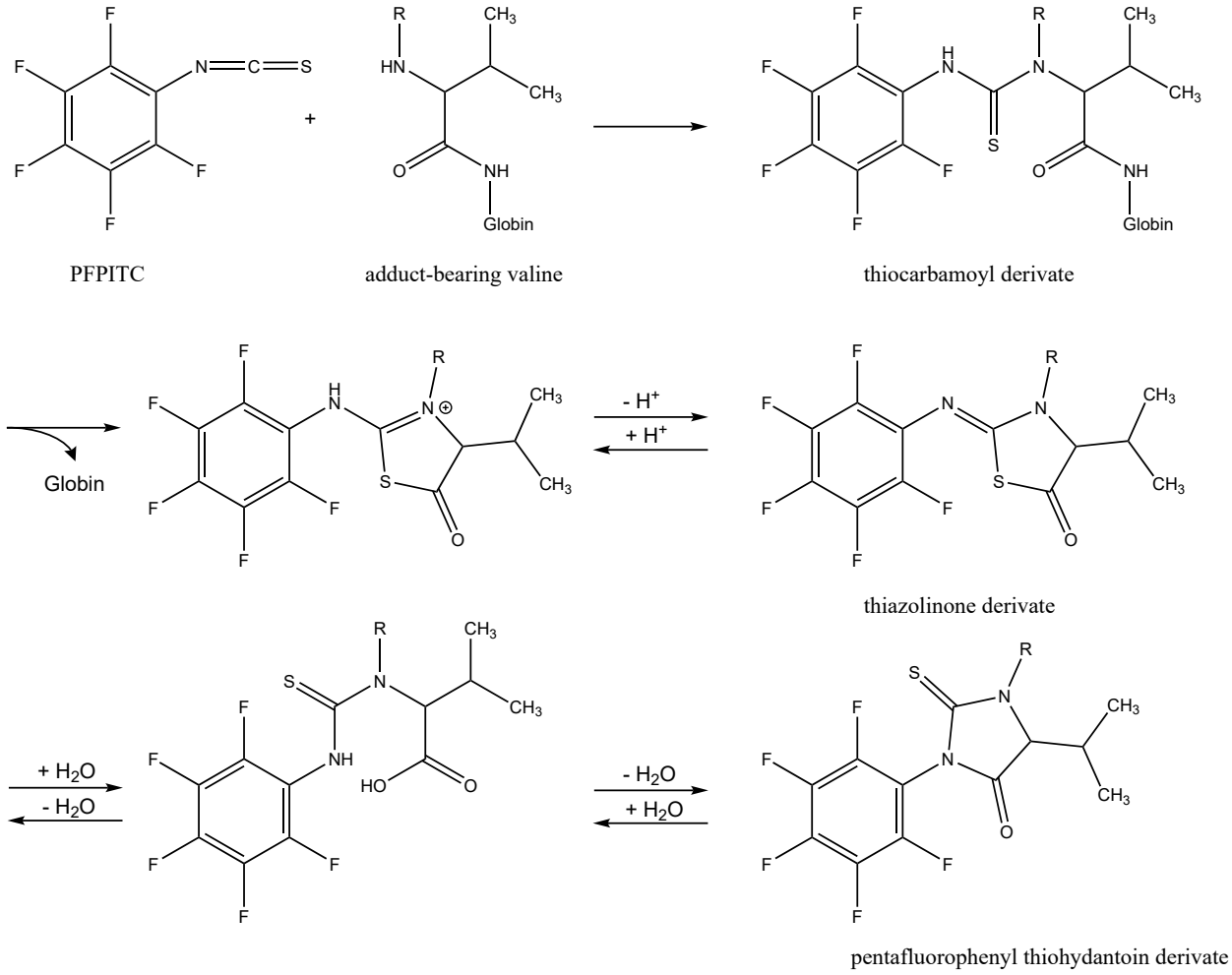


Fig. 3 Mechanism of the modified Edman degradation

Extraction and purification

After cooling to room temperature, the sample is saturated with sodium chloride and extracted twice with 3 ml of *tert*-butyl methyl ether each time. For improved phase mixing during the extraction step, the sample is shaken for 10 min on a laboratory shaker (2500 rpm). Phase separation is carried out by centrifugation of the sample for 5 min at 2500 × *g* and 10 °C. The ether phases are combined in a test tube and evaporated to dryness under a stream of nitrogen. With a gas flow of 1.6 l/min at 35 °C, this process takes about 20 min.

The light-brown residue is then dissolved in 500 µl of toluene and washed first with 2 ml of ultra-pure water (Milli-Q®) and then with 2 ml of the sodium carbonate solution (0.1 mol/l). For this process, each sample is thoroughly mixed on

a laboratory shaker for 10 min and, after each washing step, centrifuged for 5 min at 2500 × *g* and 10 °C. The aqueous layer of the first washing step is discarded. After the second washing step, the toluene layer is carefully pipetted off and transferred into a 5-ml evaporation tube, avoiding the uptake of water.

The solution is evaporated to dryness under a stream of nitrogen (about 20 min at 1.6 l/min and 45 °C). The light-brown residue is completely dissolved in 100 µl of toluene and the solution is transferred into a 200-ml crimp-top vial. Of the sample thus processed, 1 µl is injected into the GC-MS/MS system.

5.3 Sample storage

The erythrocytes must be separated from blood plasma as soon as possible following blood extraction, as only the separation of intact erythrocytes ensures the applicability of the method. The erythrocyte lysates should be used directly for globin isolation. Even at storage temperatures < –18 °C, the storage of erythrocyte lysates can lead to the formation of artefacts (see Section 11.4). In contrast, the isolated, dry globin can be stored for at least three months at –18 °C.

6 Operational parameters

Analytical determination was carried out using a device configuration comprised of a CTC PAL autosampler and a Bruker 456 gas chromatograph coupled with a Bruker EVOQ TQ-MS.

6.1 Gas chromatography

Capillary column:	Stationary phase:	Rxi-5Sil MS (5% diphenyl/95% dimethylpolysiloxane)
	Length:	30 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
Temperatures:	Column:	Initial temperature of 80 °C, 1 min isothermal, increase at a rate of 15 °C/min to 220 °C, increase at a rate of 5 °C/min to 265 °C, increase at a rate of 20 °C/min to 280 °C, 2 min at final temperature
	Injector:	280 °C
	Transfer line:	280 °C
Carrier gas:	Helium 5.0	Flow rate: 1.4 ml/min, constant
Injection:	Injection volume:	1 µl (pulsed, 16 psi for 60 s), 1 min splitless; then 4 min 1 : 100 split, followed by 1 : 25 split
Septum flush:		3 ml/min
Purge time:		1 min, off

6.2 Tandem mass spectrometry

Ionisation:	EI+
Filament:	40 µA
Electron energy:	70 eV
Collision gas:	Argon 5.3 (2 mTorr)
Source temperature:	250 °C
Electron multiplier:	1200 V–2000 V

Operating mode: Single Reaction Monitoring (SRM)

Calibration gas: PFTBA (FC-43)

The instrument-specific parameters must be determined and adjusted by the user for the individual system used. The device-specific parameters indicated in this section have been ascertained and optimised for the system used here.

The retention times and SRM transitions for the individual analytes are given in Table 4. The retention times are intended as reference values and may change as a result of column shortening or charge.

Tab. 4 Retention times and SRM parameters for the investigated analytes and the internal standard

Analyte / ISTD	Retention time [min]	Mass transition [*m/z*]		Collision energy (CE) [V]
		Q1	Q3	
N-Methylvaline	10.70	338	295.8	12
N-Ethylvaline	10.81	352	309.8	12
N-Propylvaline	11.32	366	351.6	10
N-Butylvaline	11.93	380	364.9	15
N-(2-Ethoxyethyl)valine (ISTD)	12.20	396	334.9	18
N-(2-Hydroxypropyl)valine	12.66	364	321.9	12
N-(2-Hydroxyethyl)valine	12.99	308	193.9	22
N-(2-Cyanoethyl)valine	13.11	377	281.9	18
N-Benzylvaline	15.31	414	91.0	18
N-(2-Carbamoylethyl)valine	16.02	378	362.9	12

The method verifiers examined additional fragmentations as qualifiers. Since the GC-MS/MS system used for external verification was comparable but not identical to the system used for method development (Shimadzu AOC-20i/s gas chromatograph with an Rxi-5Sil MS column (30 m × 0.25 mm × 0.25 µm, Restek GmbH, Bad Homburg vor der Höhe, Germany) and a triple-quadrupole mass spectrometer (Shimadzu TQ-8050, Shimadzu Deutschland GmbH, Duisburg, Germany)), the collision energies and dwell times were optimised separately for the GC-MS/MS system used for external method verification (see Table 5).

Tab. 5 Retention times and MRM parameters for the investigated analytes and the internal standards (data from external method verification)

Analyte / ISTD	Retention time [min]	Mass transition [*m/z*]		Status	Collision energy (CE) [V]	Dwell time [ms]
		Q1	Q3			
N-Methylvaline	7.006	338.0	296.1	Quantifier	9	48.3
			310.0	Qualifier	9	
			277.0	Qualifier	21	
N-Ethylvaline	7.118	352.0	310.2	Quantifier	9	48.7
			56.2	Qualifier	21	
			324.0	Qualifier	6	
N-Propyl-$^{13}C_5$,^{15}N-Valine (ISTD)[a)]	7.609	372.0	356.1	Quantifier	12	48.7
			314.3	Qualifier	21	
			326.7	Qualifier	6	

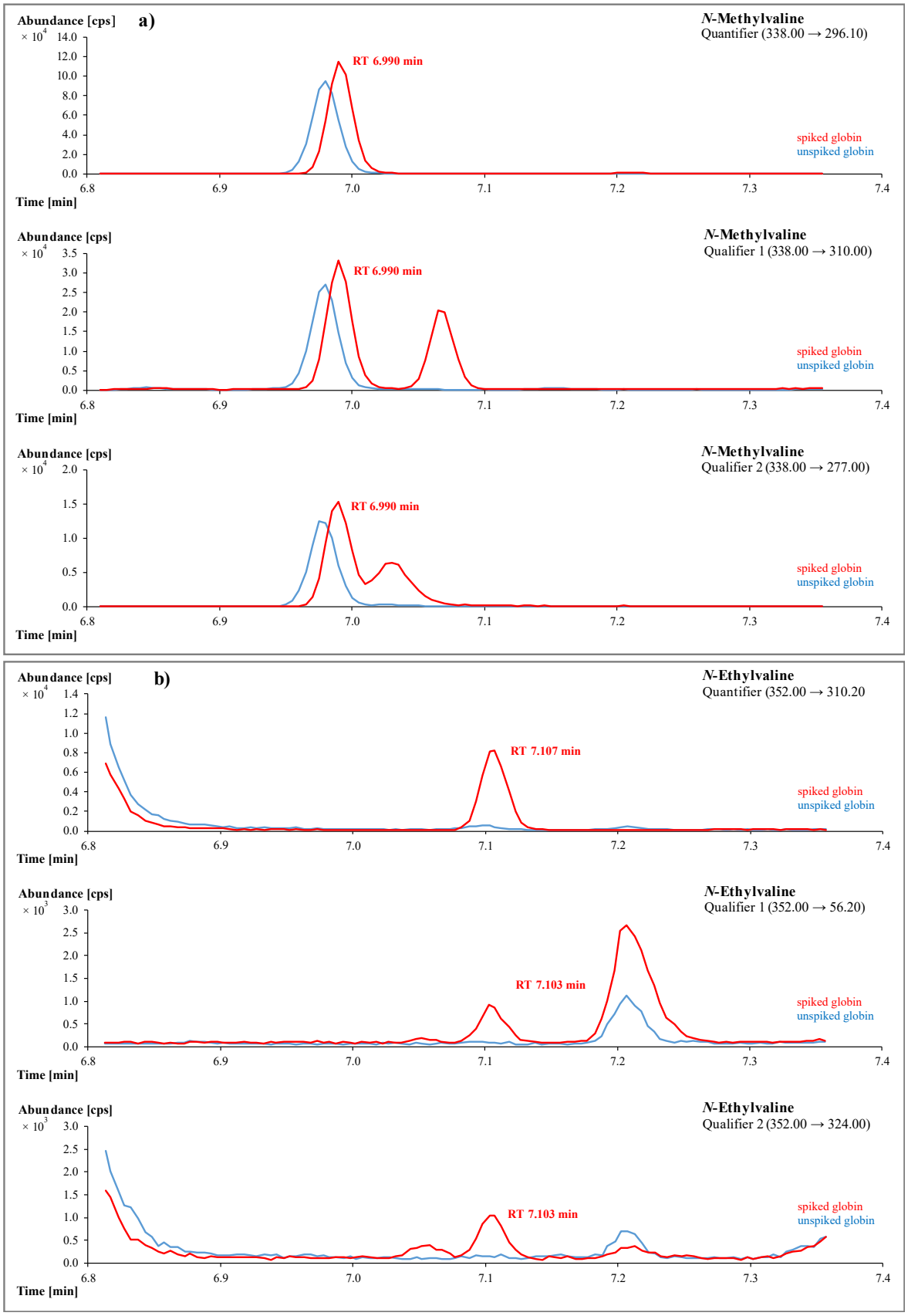
Tab. 5 *(continued)*

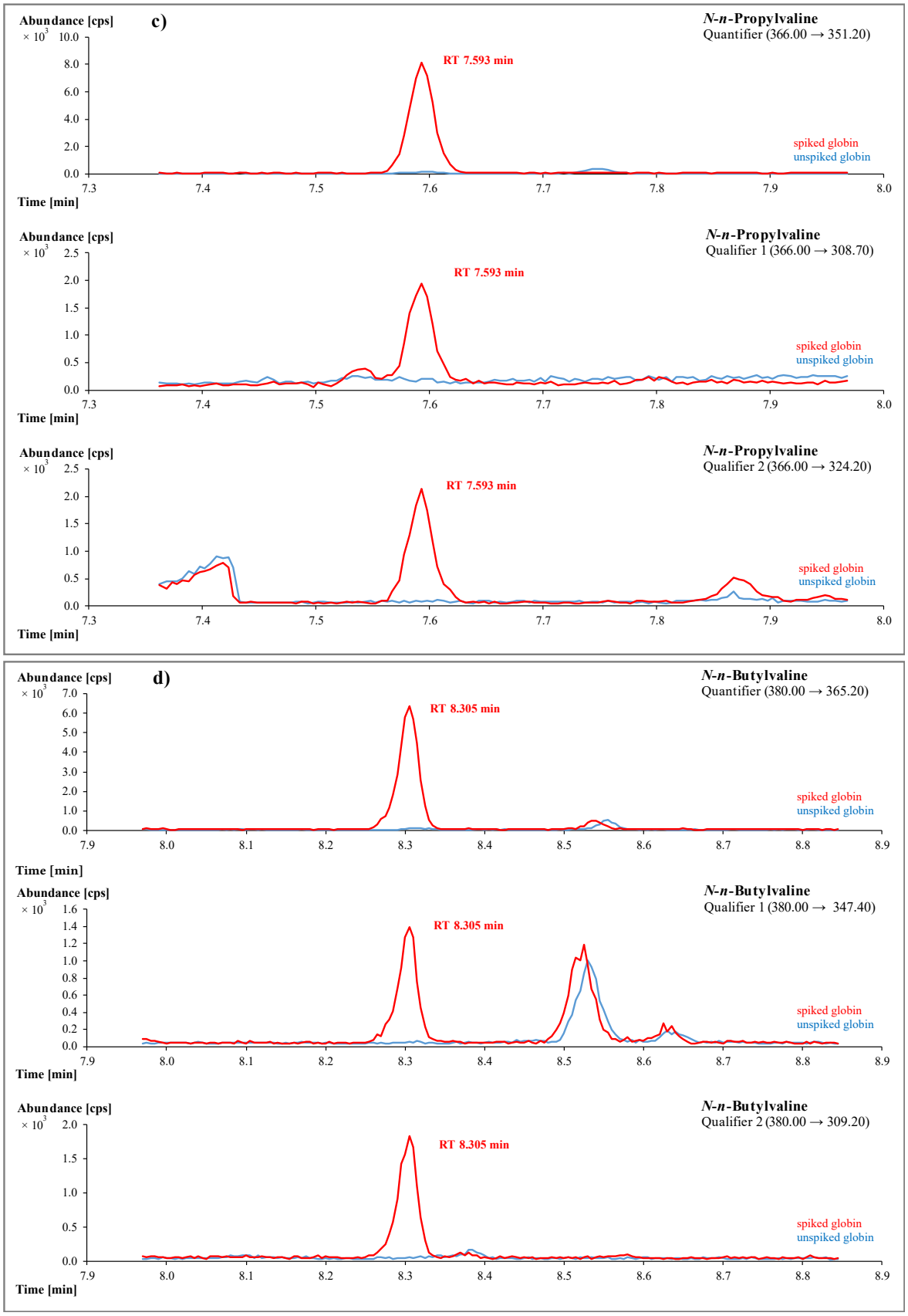
Analyte / ISTD	Retention time [min]	Mass transition [*m/z*]		Status	Collision energy (CE) [V]	Dwell time [ms]
		Q1	Q3			
N-Propylvaline	7.612	366.0	351.2	Quantifier	6	48.7
			308.7	Qualifier	21	
			324.2	Qualifier	6	
N-Butylvaline	8.331	380.0	365.2	Quantifier	15	48.7
			347.4	Qualifier	6	
			309.2	Qualifier	24	
N-(2-Ethoxyethyl)valine (ISTD)	8.572	396.0	334.7	Quantifier	15	48.7
			73.1	Qualifier	6	
			307.8	Qualifier	12	
N-(2-Hydroxypropyl)valine-1	9.136	364.0	322.1	Quantifier	9	32.0
			349.0	Qualifier	18	
			274.9	Qualifier	21	
N-(2-Hydroxypropyl)valine-2	9.171	364.0	322.1	Quantifier	9	32.0
			349.0	Qualifier	18	
			274.9	Qualifier	21	
N-(2-Hydroxyethyl)valine	9.474	308.0	194.0	Quantifier	24	32.0
			86.9	Qualifier	24	
			60.0	Qualifier	24	
N-(2-Cyanoethyl)valine	9.663	377.0	335.1	Quantifier	18	32.0
			54.1	Qualifier	15	
			282.3	Qualifier	6	
N-Benzylvaline	12.017	414.0	91.1	Quantifier	21	98.7
			372.1	Qualifier	9[b)]	
			173.2	Qualifier	18[b)]	
N-(2-Carbamoylethyl)valine	12.648	378.0	363.1	Quantifier	9	98.7
			55.1	Qualifier	21	
			309.0	Qualifier	21	

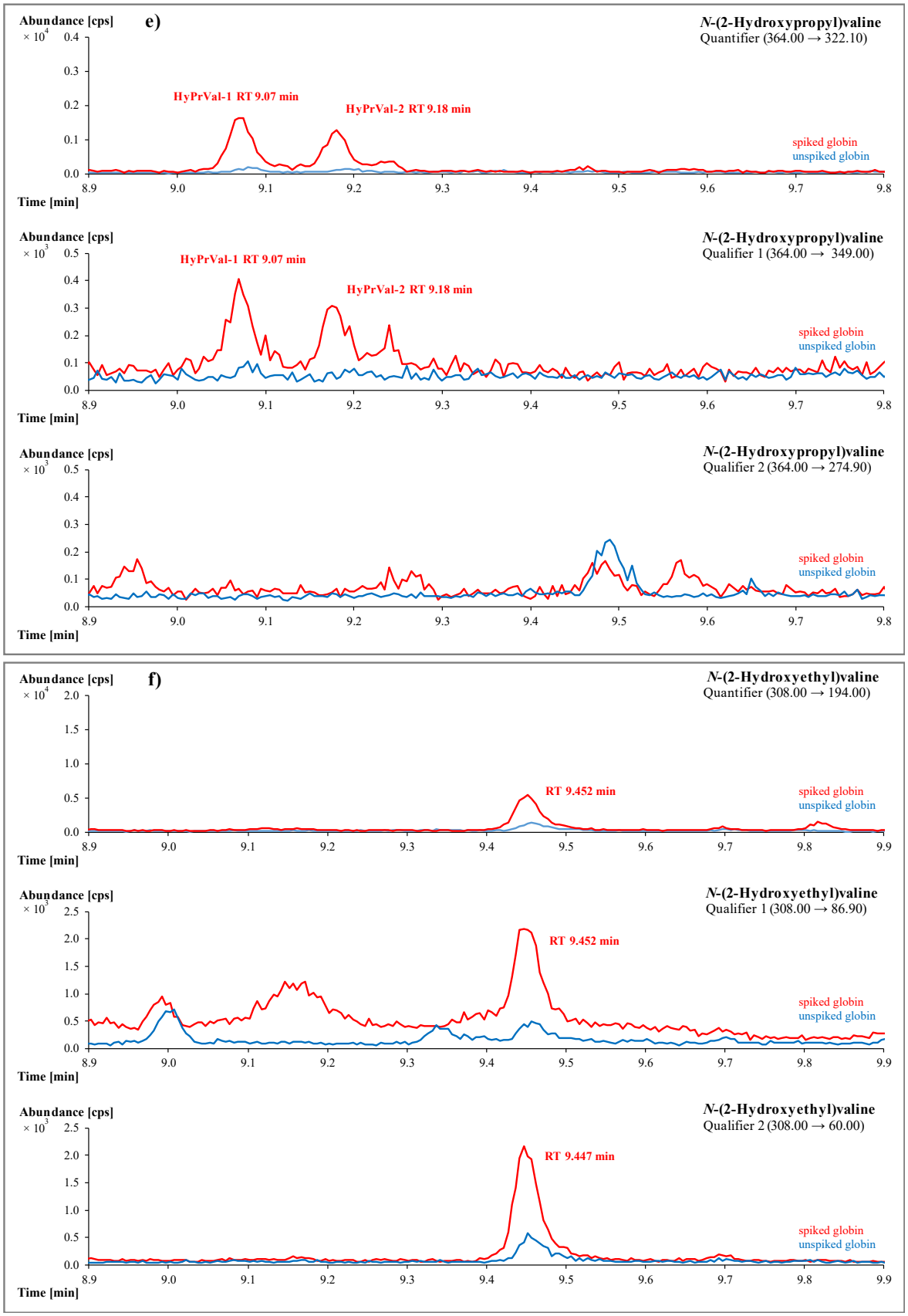
[a)] The verifiers of the method additionally applied this ISTD (see Section 12).
[b)] Neither qualifier could be properly integrated at low adduct levels.

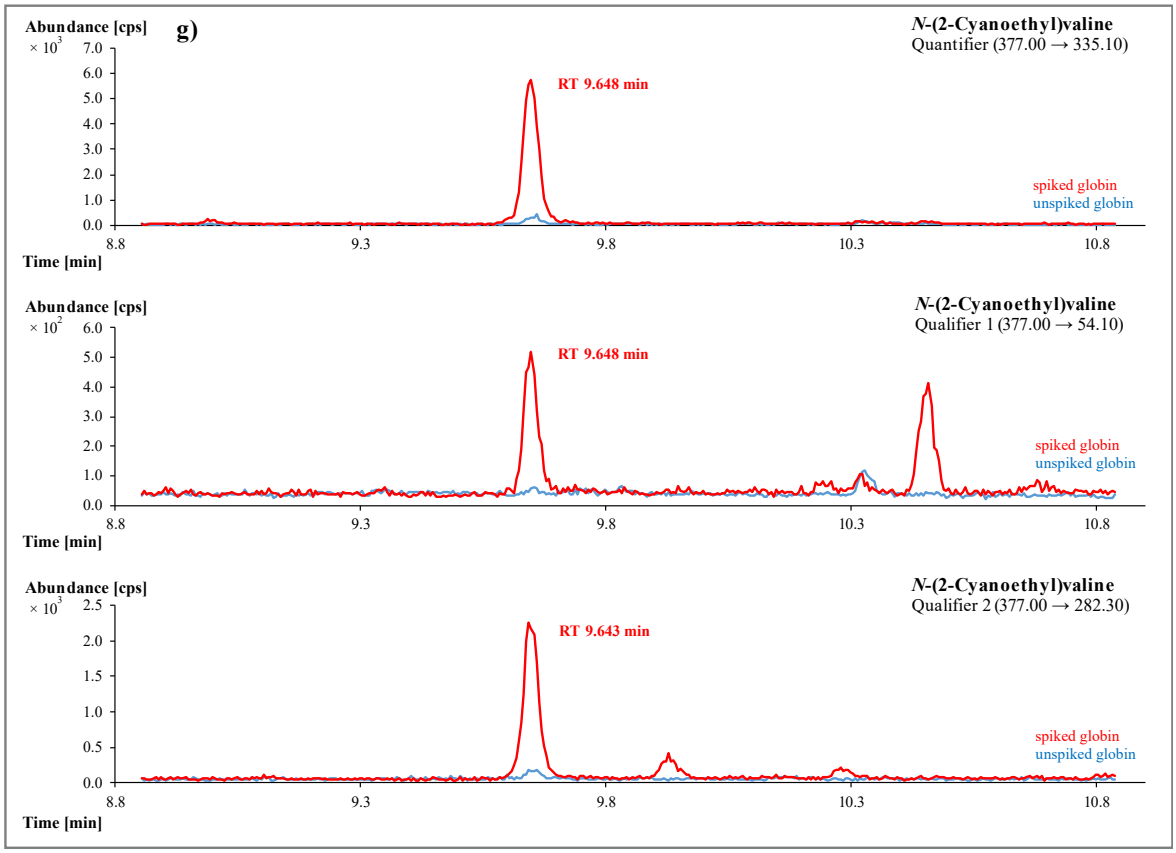
7 Analytical determination

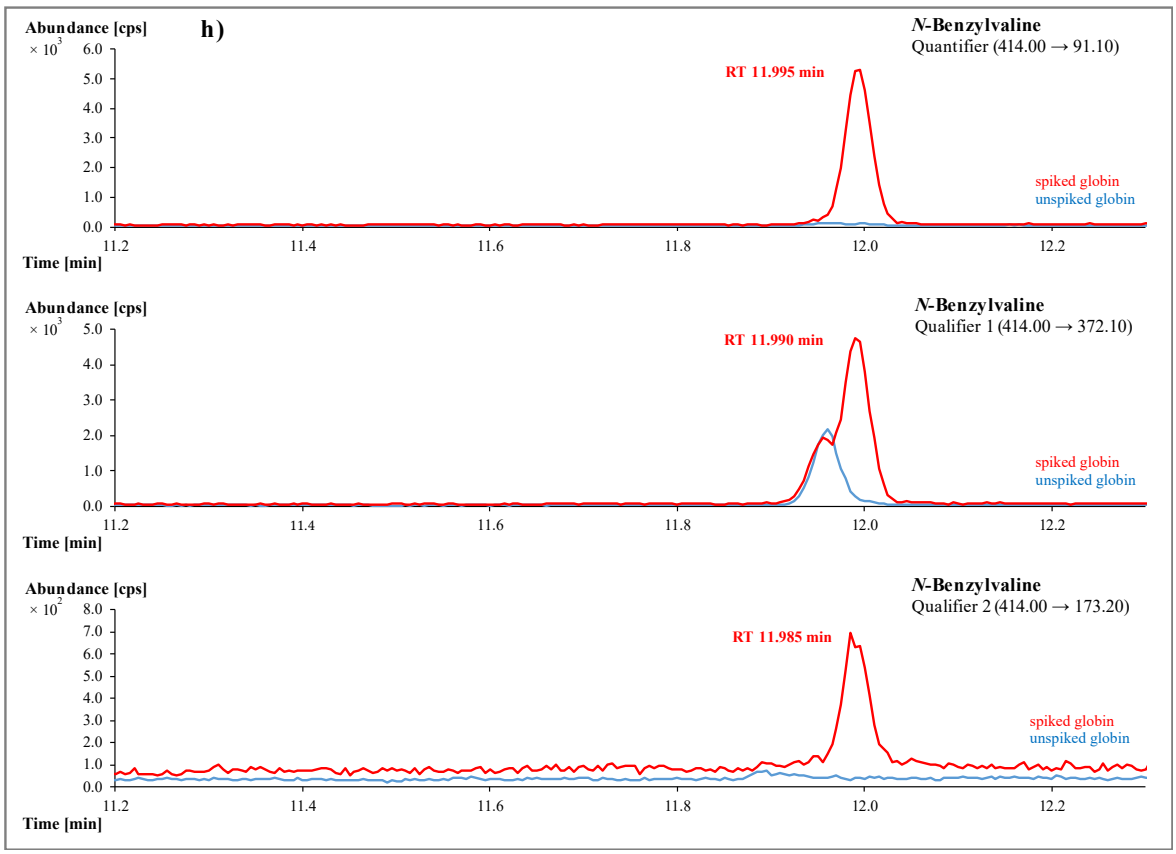
The device parameters are adjusted as indicated and 1 µl of the sample is injected into the GC-MS/MS system. Representative chromatograms of the individual analytes are depicted in Figure 4 (chromatograms provided by external method verifiers). The adduct levels of the spiked globins are 30 pmol/g globin (*N*-(2-hydroxypropyl)-valine and *N*-benzylvaline); 50 pmol/g globin (*N*-ethylvaline, *N*-propylvaline, and *N*-butylvaline); 60 pmol/g globin (*N*-(2-cyanoethyl)valine); 70 pmol/g globin (*N*-(2-hydroxyethyl)valine); 100 pmol/g globin (*N*-(2-carbamoylethyl)valine); and 170 pmol/g globin (*N*-methylvaline).

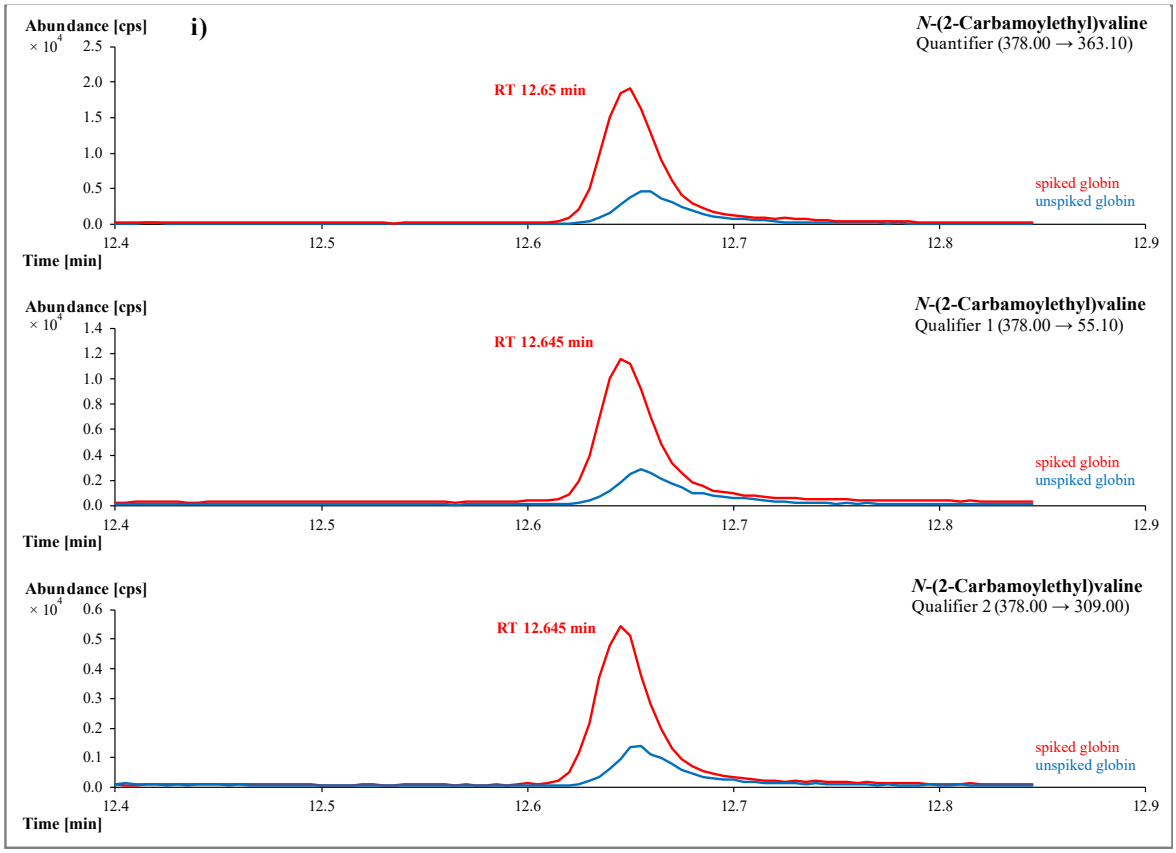

a)
Abundance [cps]
× 10^4
N-Methylvaline
Quantifier (338.00 → 296.10)
RT 6.990 min
spiked globin
unspiked globin
Time [min]
N-Methylvaline
Qualifier 1 (338.00 → 310.00)
RT 6.990 min
N-Methylvaline
Qualifier 2 (338.00 → 277.00)
RT 6.990 min
b)
N-Ethylvaline
Quantifier (352.00 → 310.20
RT 7.107 min
× 10^3
N-Ethylvaline
Qualifier 1 (352.00 → 56.20)
RT 7.103 min
N-Ethylvaline
Qualifier 2 (352.00 → 324.00)
RT 7.103 min

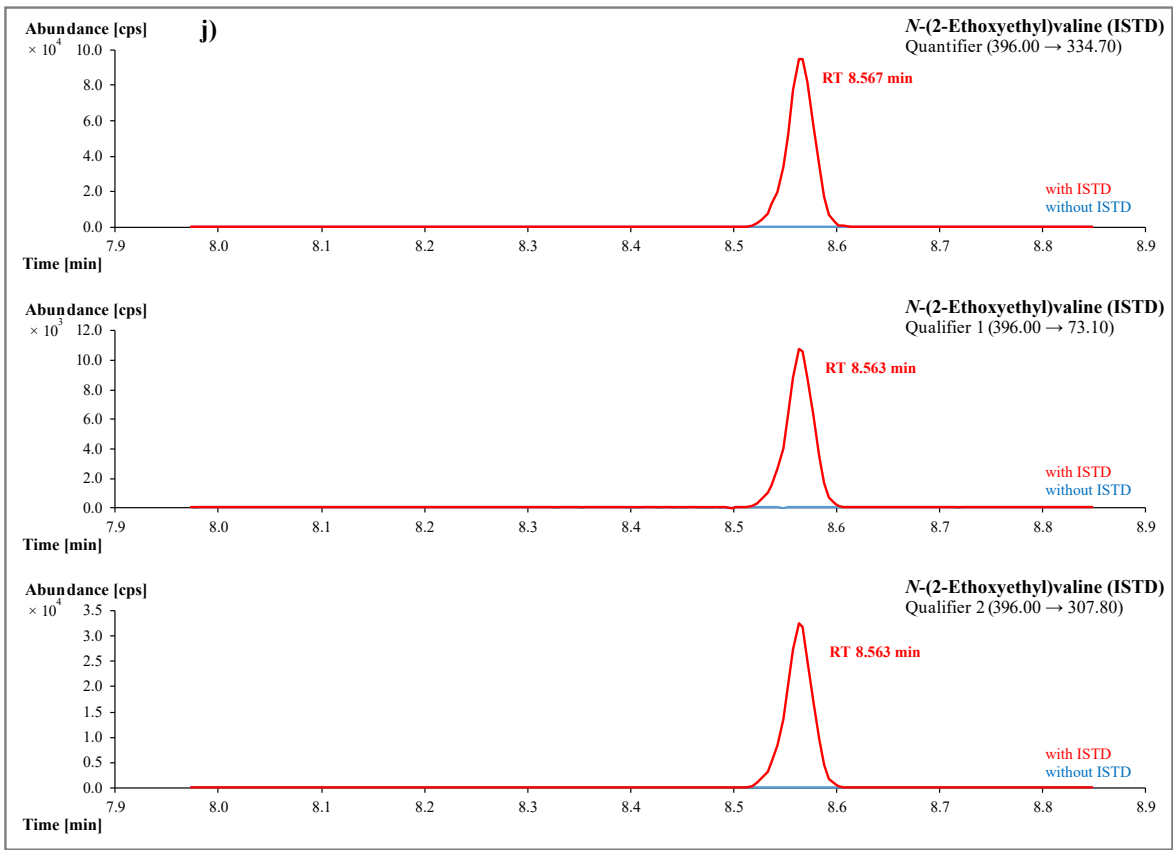

c)
Abundance [cps]
× 10^3
N-n-Propylvaline
Quantifier (366.00 → 351.20)
RT 7.593 min
spiked globin
unspiked globin
Time [min]
N-n-Propylvaline
Qualifier 1 (366.00 → 308.70)
RT 7.593 min
N-n-Propylvaline
Qualifier 2 (366.00 → 324.20)
RT 7.593 min
d)
N-n-Butylvaline
Quantifier (380.00 → 365.20)
RT 8.305 min
N-n-Butylvaline
Qualifier 1 (380.00 → 347.40)
RT 8.305 min
N-n-Butylvaline
Qualifier 2 (380.00 → 309.20)
RT 8.305 min


e)
Abundance [cps]
× 10^4
N-(2-Hydroxypropyl)valine
Quantifier (364.00 → 322.10)
HyPrVal-1 RT 9.07 min
HyPrVal-2 RT 9.18 min
spiked globin
unspiked globin
Time [min]
Abundance [cps]
× 10^3
N-(2-Hydroxypropyl)valine
Qualifier 1 (364.00 → 349.00)
HyPrVal-1 RT 9.07 min
HyPrVal-2 RT 9.18 min
spiked globin
unspiked globin
Time [min]
Abundance [cps]
× 10^3
N-(2-Hydroxypropyl)valine
Qualifier 2 (364.00 → 274.90)
spiked globin
unspiked globin
Time [min]
f)
Abundance [cps]
× 10^4
N-(2-Hydroxyethyl)valine
Quantifier (308.00 → 194.00)
RT 9.452 min
spiked globin
unspiked globin
Time [min]
Abundance [cps]
× 10^3
N-(2-Hydroxyethyl)valine
Qualifier 1 (308.00 → 86.90)
RT 9.452 min
spiked globin
unspiked globin
Time [min]
Abundance [cps]
× 10^3
N-(2-Hydroxyethyl)valine
Qualifier 2 (308.00 → 60.00)
RT 9.447 min
spiked globin
unspiked globin
Time [min]


g)
Abundance [cps]
× 10³
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
RT 9.648 min
spiked globin
unspiked globin
8.8
9.3
9.8
10.3
10.8
Time [min]
N-(2-Cyanoethyl)valine
Quantifier (377.00 → 335.10)
Abundance [cps]
× 10²
6.0
5.0
4.0
3.0
2.0
1.0
0.0
RT 9.648 min
spiked globin
unspiked globin
Time [min]
N-(2-Cyanoethyl)valine
Qualifier 1 (377.00 → 54.10)
Abundance [cps]
× 10³
2.5
2.0
1.5
1.0
0.5
0.0
RT 9.643 min
spiked globin
unspiked globin
Time [min]
N-(2-Cyanoethyl)valine
Qualifier 2 (377.00 → 282.30)


h)
Abundance [cps]
× 10³
6.0
5.0
4.0
3.0
2.0
1.0
0.0
RT 11.995 min
spiked globin
unspiked globin
11.2
11.4
11.6
11.8
12.0
12.2
Time [min]
N-Benzylvaline
Quantifier (414.00 → 91.10)
Abundance [cps]
× 10³
5.0
4.0
3.0
2.0
1.0
0.0
RT 11.990 min
spiked globin
unspiked globin
Time [min]
N-Benzylvaline
Qualifier 1 (414.00 → 372.10)
Abundance [cps]
× 10²
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
RT 11.985 min
spiked globin
unspiked globin
Time [min]
N-Benzylvaline
Qualifier 2 (414.00 → 173.20)


i)
Abundance [cps]
× 10^4
N-(2-Carbamoylethyl)valine
Quantifier (378.00 → 363.10)
RT 12.65 min
spiked globin
unspiked globin
Time [min]
N-(2-Carbamoylethyl)valine
Qualifier 1 (378.00 → 55.10)
RT 12.645 min
N-(2-Carbamoylethyl)valine
Qualifier 2 (378.00 → 309.00)
RT 12.645 min


j)
Abundance [cps]
× 10^4
× 10^3
N-(2-Ethoxyethyl)valine (ISTD)
Quantifier (396.00 → 334.70)
RT 8.567 min
with ISTD
without ISTD
Time [min]
N-(2-Ethoxyethyl)valine (ISTD)
Qualifier 1 (396.00 → 73.10)
RT 8.563 min
N-(2-Ethoxyethyl)valine (ISTD)
Qualifier 2 (396.00 → 307.80)
RT 8.563 min

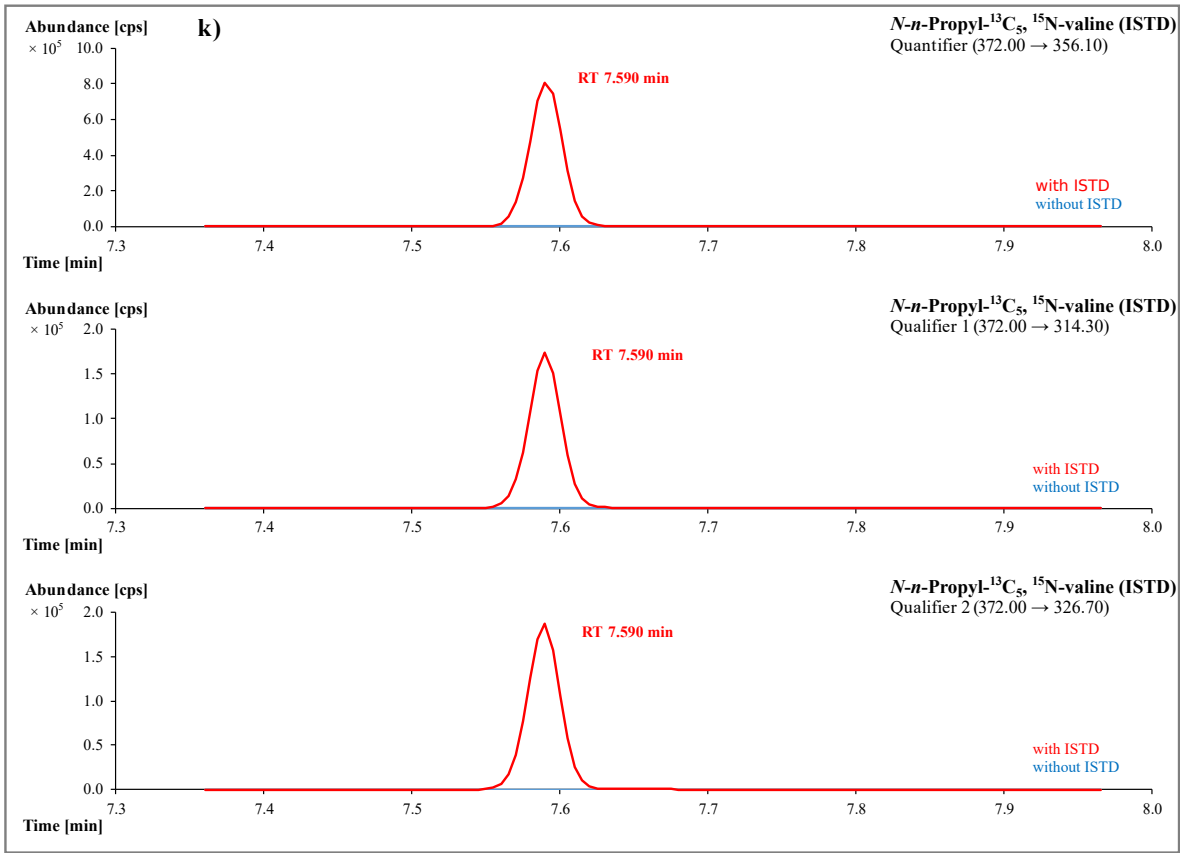


Fig. 4 Chromatograms for a) *N*-methylvaline, b) *N*-ethylvaline, c) *N*-propylvaline, d) *N*-butylvaline, e) *N*-(2-hydroxypropyl)valine, f) *N*-(2-hydroxyethyl)valine, g) *N*-(2-cyanoethyl)valine, h) *N*-benzylvaline, i) *N*-(2-carbamoylethyl)valine, j) *N*-(2-ethoxyethyl)valine (ISTD), and k) *N*-propyl-$^{13}C_5$,^{15}N-valine (ISTD) in the erythrocyte fraction of whole blood

8 Calibration

Calibration is carried out using reference standards obtained by spiking human globin (see Section 4). For globin derivatisation, sodium hydroxide solution and the derivatisation reagent are added to the calibration standards (see Section 5.2). After further workup, the calibration standards are analysed according to Section 6. The calibration curves are generated by plotting the quotients of the peak areas of the individual valine adducts and of the ISTD against the spiked adduct levels.

The linearity of the analytical method was tested, depending on the adduct, between a lower level of 12 to 19 pmol/g globin and an upper level of 3600 to 5700 pmol/g globin. The correlation coefficients thereby achieved were > 0.99. Blank values must be accounted for by subtraction. Especially for *N*-methylvaline, high endogenous background levels are found in human globin.

Representative calibration curves for the individual analytes are depicted in Figure 5. The lower adduct-level ranges (up to about 30–40 pmol/g globin) are additionally provided for each analyte.

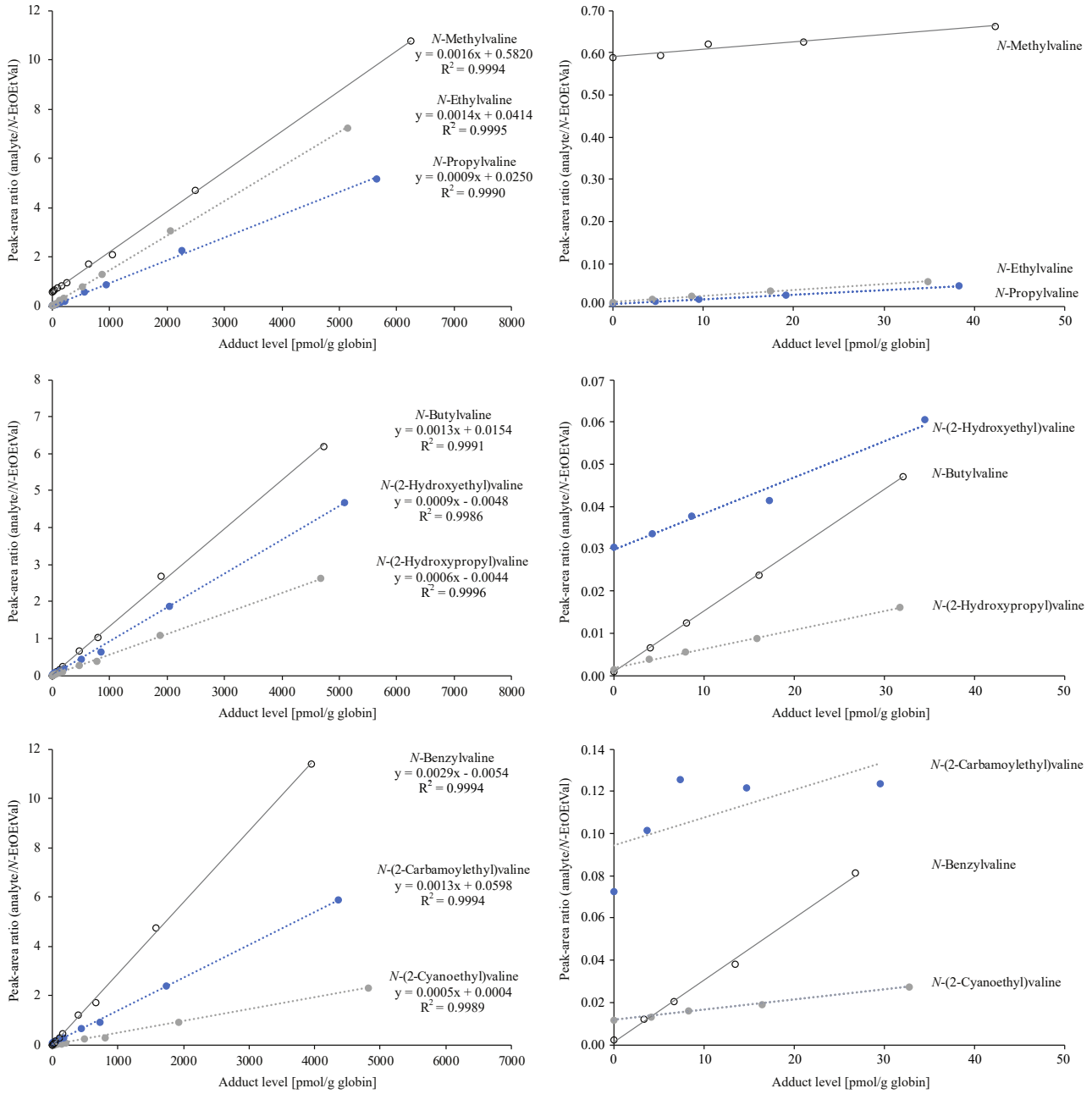


Fig. 5 Representative calibration curves for the determination of *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline, *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl) valine in the erythrocyte fraction of whole blood

9 Calculation of the analytical results

To calculate the analytical results, the peak area of the analyte is divided by the peak area of the internal standard. The quotient thus obtained is inserted into the calibration function corresponding to the analytical run in question and the adduct level is given in pmol/g globin. For routine analysis, the analytical results are calculated using the appropriate device software.

The analytical result may also be related to the blood volume. An approximate average globin concentration of 144 g/l blood can be assumed (Brunn 1992). The factor 10^{-6} converts pmol to µmol, so that the final result is obtained in µg/l.

Adduct level [pmol/g globin] × mean globin concentration [g globin/l blood] × molar mass [g/mol] × 10^{-6} =
adduct level [pmol/g globin] × conversion factor [g globin × µg/(pmol × l blood)] = adduct concentration [µg/l blood]

The molar masses of the valine adducts as well as the resulting conversion factors are given in Table 6. The conversion of an *N*-methylvaline level (150 pmol/g globin) is given as an example:

150 pmol *N*-methylvaline/g globin × 144 g globin/l blood × 131.18 g/mol × 10^{-6} =
150 pmol *N*-methylvaline/g globin × 0.01889 g globin × µg/(pmol × l blood) = 2.83 µg *N*-methylvaline/l blood

Tab. 6 Molar masses of the valine adducts and conversion factors for conversion from [pmol/g globin] to [µg/l blood]

Adduct	Molar mass [g/mol]	Conversion factor [g globin × µg/(pmol × l)]
N-Methylvaline	131.18	0.01889
N-Ethylvaline	145.20	0.02091
N-Propylvaline	159.23	0.02293
N-Butylvaline	173.26	0.02495
N-(2-Hydroxypropyl)valine	175.23	0.02523
N-(2-Hydroxyethyl)valine	161.20	0.02321
N-(2-Cyanoethyl)valine	170.21	0.02451
N-Benzylvaline	207.27	0.02985
N-(2-Carbamoylethyl)valine	188.23	0.02711

10 Standardisation and quality control

Quality assurance 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 b; Bundesärztekammer 2014).

For quality control, a globin control sample exhibiting constant adduct levels (e.g. 1800 pmol/g globin) is included as part of each analytical run. This sample is analysed at the beginning, middle, and end of each analytical run. Since commercial material is not available, the control material must be prepared in the in-house laboratory by spiking pooled globin. The isolated globin can be stored for at least one year at –20 °C. The nominal value and tolerance range (mean ± three standard deviations) of the quality-control material is determined in a pre-analytical period.

For external quality assurance, it is possible to participate in interlaboratory-comparison programs. The program G-EQUAS (German External Quality Assessment Scheme, https://app.g-equas.de/web/) of the German Society of Occupational and Environmental Medicine (*Deutsche Gesellschaft für Arbeitsmedizin und Umweltmedizin*) currently contains four parameters which can be determined with this method (*N*-methylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, and *N*-(2-cyanoethyl)valine).

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

Within-day precision was determined by spiking 100 mg of pooled globin with the reference substances at two concentrations.

Each sample was processed and analysed six times. *N*-propylvaline was processed and analysed ten times, as this analyte was later introduced into the method. The mean, coefficient of variation, and prognostic range (95%) were ascertained from the measurement results for each of the individual analytes. The precision data thus obtained are given in Table 7.

Tab. 7 Within-day precision for the determination of *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline (n = 10), *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine in the erythrocyte fraction of whole blood (n = 6)

Analyte	Spiked adduct level [pmol/g globin]	Measured adduct level [pmol/g globin]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
N-Methylvaline	625	625	5.2	13.4
	2499	2361	4.3	11.1
N-Ethylvaline	564	579	8.1	20.8
	2257	2329	4.6	11.8
N-Propylvaline	515	510	1.8	4.1
	2058	2068	1.2	2.7
N-Butylvaline	473	473	2.4	6.2
	1892	1856	2.8	7.2
N-(2-Hydroxypropyl)valine	468	452	4.9	12.6
	1871	1732	5.5	14.1
N-(2-Hydroxyethyl)valine	508	508	5.2	13.4
	2034	1922	4.3	11.1
N-(2-Cyanoethyl)valine	481	494	2.5	6.4
	1926	1983	5.4	13.9
N-Benzylvaline	395	389	2.0	5.1
	1581	1524	1.5	3.9
N-(2-Carbamoylethyl)valine	435	435	7.8	20.1
	1741	1723	5.1	13.1

Day-to-day precision

Day-to-day precision was determined by spiking 100 mg of pooled globin with the reference substances at two concentrations.

The samples were processed and analysed on six different days. *N*-propylvaline was processed and analysed on seven different days, as this analyte was introduced into the method at a later date. The mean, coefficient of variation, and prognostic range (95%) were calculated from the measurement results for each of the individual analytes. The values thus obtained are given in Table 8.

Tab. 8 Day-to-day precision for the determination of *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline (n = 7), *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine in the erythrocyte fraction of whole blood (n = 6)

Analyte	Spiked adduct level [pmol/g globin]	Measured adduct level [pmol/g globin]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
N-Methylvaline	625	508	2.4	6.2
	2499	2642	3.6	9.3
N-Ethylvaline	564	612	10.8	27.8
	2257	2544	6.4	16.5
N-Propylvaline	515	520	3.1	7.6
	2058	2018	2.9	7.1
N-Butylvaline	473	473	0.8	2.1
	1892	1856	3.8	9.8
N-(2-Hydroxypropyl)valine	468	420	2.4	6.2
	1871	1899	5.7	14.7
N-(2-Hydroxyethyl)valine	508	526	4.9	12.6
	2034	2266	6.0	15.4
N-(2-Cyanoethyl)valine	481	465	6.9	17.7
	1926	2199	2.7	6.9
N-Benzylvaline	395	312	6.6	17.0
	1581	1581	2.6	6.7
N-(2-Carbamoylethyl)-valine	435	358	5.8	14.9
	1741	1741	4.6	11.8

11.2 Accuracy

The accuracy of the analyses was ascertained as mean relative recoveries from the determinations of within-day and day-to-day precision. The recoveries thus obtained are given in Table 9.

Tab. 9 Accuracy for the determination of *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline (n = 10 and n = 7), *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine in the erythrocyte fraction of whole blood (n = 6 and n = 6)

Analyte	Adduct level [pmol/g globin]	Recovery (rel.) r [%]	
		Within-day precision	Day-to-day precision
N-Methylvaline	625	101	81
	2499	105	106
N-Ethylvaline	564	102	108
	2257	103	113
N-Propylvaline	515	99.1	101
	2058	101	98.0
N-Butylvaline	473	100	93
	1892	98	110
N-(2-Hydroxypropyl)valine	468	96	90
	1871	92	102

Tab. 9 *(continued)*

Analyte	Adduct level [pmol/g globin]	Recovery (rel.) *r* [%]	
		Within-day precision	Day-to-day precision
N-(2-Hydroxyethyl)valine	508	100	103
	2034	95	111
N-(2-Cyanoethyl)valine	481	103	96
	1926	103	114
N-Benzylvaline	395	99	79
	1581	96	100
N-(2-Carbamoylethyl)valine	435	99	82
	1741	99	100

11.3 Limits of detection and quantitation

The limits of detection and quantitation were ascertained following the calibration-curve method per DIN 32645 (DIN 2008).

To this end, a non-equidistant 5- or 6-point calibration (with an adduct-level range, depending on the adduct, of 3.35 to 5.29 pmol/g globin (lowest point) as well as 157 to 249 pmol/g globin (highest point)) was generated and then processed and analysed in conjunction with a blank value.

Per DIN 32645, the detection and quantitation limits are calculated from the standard deviation at the blank value of the generated calibration function. The limits of detection and quantitation thus obtained are given in Table 10.

It is important to note that the quantitation limits were ascertained using normal calibrations from routine work, rather than the equidistant calibrations actually required by DIN 32645. This approach was taken due to its efficiency and because, at the time of external method validation, only a broad estimation of quantitation limits was requested in order to ensure the applicability of the method for occupational medicine. During external method verification, the verifying laboratory was able to confirm the given quantitation limits for all analytes except *N*-ethylvaline by fullfilling the criteria of the FDA guideline for the lower limit of quantitation (FDA 2018). Furthermore, both the developing and verifying laboratories showed that considerably lower quantitation limits resulted from the signal-to-noise ratios, although the latter calculation method disregards the precision and accuracy actually achieved for the analytical procedure as a whole. If any particular challenges arise regarding the robustness of the given quantitation limits, the user of the presented analytical method should ascertain the actual quantitation limits using the available instrumentation (see Section 12 for further optimisation possibilities).

The relatively high quantitation limit of the acrylamide marker *N*-(2-carbamoylethyl)valine may be caused by background levels in the pooled globin used for calibration. The same finding is true for the methylation marker methylvaline, whereby the relatively high background values are likely due primarily to endogenous methylation.

Tab. 10 Limits of detection and quantitation for the determination of *N*-methylvaline, *N*-ethylvaline, *N*-propylvaline, *N*-butylvaline, *N*-(2-hydroxypropyl)valine, *N*-(2-hydroxyethyl)valine, *N*-(2-cyanoethyl)valine, *N*-benzylvaline, and *N*-(2-carbamoylethyl)valine in the erythrocyte fraction of whole blood (n = 2)

Analyte	Detection limit [pmol/g globin]	Quantitation limit [pmol/g globin]
N-Methylvaline	50	170
N-Ethylvaline	15	50
N-Propylvaline	15	50
N-Butylvaline	15	50

Tab. 10 *(continued)*

Analyte	Detection limit [pmol/g globin]	Quantitation limit [pmol/g globin]
N-(2-Hydroxypropyl)valine	10	30
N-(2-Hydroxyethyl)valine	20	70
N-(2-Cyanoethyl)valine	15	60
N-Benzylvaline	10	30
N-(2-Carbamoylethyl)valine	70	200

11.4 Sources of error

The quality of the isolated globin directly affects the reliability of the subsequent adduct determination. As a result, only fresh, non-haemolysed EDTA blood samples should be used for globin isolation, since adduct levels up to 80% lower than those of non-haemolysed samples were found in the determination of *N*-methylvaline from the globin of haemolysed samples (Bader 1996). In this respect, it is of utmost importance that the separation of intact erythrocytes takes place as soon as possible following blood extraction. Moreover, the separated erythrocytes must be sufficiently washed with physiological saline solution in order to remove plasma proteins such as serum albumin; these proteins precipitate alongside globin and lead to falsely low results in the determination of globin-related adduct levels.

Additional factors may negatively affect the correct determination of adduct levels: during sample workup, globin isolation must be completely finished (no freezing of intermediate stages) in order to avoid the potential formation of artefacts. Törnqvist (1990) showed that the adduct levels of *N*-(2-hydroxyethyl)valine in frozen erythrocyte lysates may increase up to eightfold within six months in individual cases.

During the derivatisation step, it is important to use sufficiently wide-mouthed threaded test tubes in order to facilitate the suspension and dissolution of globin in formamide. Because the Edman cyclisation reaction is highly pH-dependent, it is of central importance to maintain an optimal pH range of pH 6–7. For this reason, contamination of the applied formamide with volatile amines or ammonia may considerably reduce reaction yields and, in turn, the sensitivity of the method. As such, it is preferable to use either ultra-pure formamide stored at –20 °C or formamide of *pro analysi* grade, adjusted to a pH value of ≈ 7 by nitrogen stripping. It is thereby important to account for the fact that the presence of other free amines can nevertheless lead to unintended side reactions (van Sittert et al. 1997).

The verifiers of the method recommended reconstituting the residue of the ethyl acetate extracts with a larger volume of toluene for easier separation of the organic layer after washing. It is, however, only expedient to use more than 500 µl of toluene in laboratories with a high-speed vacuum concentrator.

Regarding chromatography, peak-splitting was observed in individual chromatograms, which indicates matrix effects that disturb the focussing during the splitless-split injection. In these cases, the sum of both peaks should be evaluated.

It is furthermore important to mention that the stock solution of the *N*-((*R*,*S*)-2-hydroxypropyl)valine-leucine-anilide is a diastereomeric mixture. In chromatograms, both diastereomers are well-separated and exhibit the same response. In the method presented herein, only the first peak was used for quantification; in contrast, the method of Schettgen et al. (2016), published by the Commission, used both peaks. Since adduct formation in humans is not diastereoselective, both approaches are valid.

With regard to instrumentation, it is important to exchange the insert after 150–200 injections. *N*-Ethylvaline may be used to indicate whether a change of insert is necessary, as this substance adheres to the walls of the insert and will slowly become visible in the chromatogram due to memory effects. This tendency is especially important to consider for samples in which *N*-ethylvaline is to be determined; in this case the liner should be replaced in advance of the analytical run.

Outliers in quality-control samples indicate a diminishing separation performance of the column due to adsorption. If the performance is not improved after shortening and baking out the column or after changing the insert, the column must be replaced.

12 Discussion of the method

Previous methods published by the Commission on the determination of Hb adducts are all based on the same principles: the isolation of erythrocytes from whole blood, the isolation of globin from those erythrocytes, the release of globin adducts via modified Edman degradation, and the measurement of the final analyte solutions by gas chromatography-mass spectrometry (GC-MS) (Bader et al. 2010 a; Lewalter et al. 2003; Müller et al. 2013; Schettgen et al. 2016; van Sittert et al. 1997).

The presented method is based on the procedure by van Sittert et al. (1997) and only a few details of sample workup differ from the previous method. Regarding measurement, the method was adapted from the formerly established GC-MS principle to a more selective GC-MS/MS system. Moreover, the method was expanded to include several biomarkers which were not included in the original procedure. Even though the Commission has already published analytical methods for some of these markers (Table 11), the method described herein allows for the quantification of nine Hb adducts of alkylating substances in a single chromatographic run (methyl-, ethyl-, propyl-, butyl-, hydroxypropyl-, hydroxyethyl-, cyanoethyl-, benzyl-, and carbamoylethylvaline). For the first time, the entire homologous series of *n*-alkylvalines from C1 to C4 can be analysed as potential long-term markers of exposure to such substances as alkyl halides or dialkyl sulfates. As such, this method also supplements the Commission's published procedure for the determination of corresponding mercapturic acids as short-term markers in urine (Eckert et al. 2016).

Tab. 11 Hb adducts of alkylating compounds for which the Commission has published methods

Biomarkers in the present method	Methods previously published by the Commission[a)]
N-Methylvaline	van Sittert et al. (1997)
N-Ethylvaline	–
N-Propylvaline	–
N-Butylvaline	–
N-(2-Hydroxypropyl)valine	Schettgen et al. (2016)
N-(2-Hydroxyethyl)valine	van Sittert et al. (1997); Schettgen et al. (2016)
N-(2-Cyanoethyl)valine	van Sittert et al. (1997); Schettgen et al. (2016)
N-Benzylvaline	Lewalter et al. (2003)
N-(2-Carbamoylethyl)valine	Bader et al. (2010 a); Schettgen et al. (2016)

[a)] The method by Schettgen et al. (2016) additionally allows for the quantification of *N*-(2-hydroxy-2-carbamoylethyl)valine as an adduct of the acrylamide metabolite glycidamide. Moreover, a method by Müller et al. (2013) allows for the determination of *N*-(2,3-dihydroxypropyl) valine as an Hb adduct of glycidol.

The reliability criteria of the method are excellent for use in occupational medicine. By using tandem mass spectrometry, a considerable increase in the reliability of results is achieved compared with GC-MS technology; as a result, the precision achieved with this method is much higher than in the original method. Furthermore, the GC-MS/MS chromatograms exhibit significantly less interference and can thereby be evaluated more quickly.

The quantitation limits of this method are sufficient for application in occupational medicine and are partially sufficient for use in environmental medicine as well. The detection limits were reported by van Sittert et al. (1997) to be 12 pmol/g globin (*N*-methylvaline and *N*-(2-cyanoethyl)valine) or 19 pmol/g globin (*N*-(2-hydroxyethyl)valine), but were calculated based on a threefold signal-to-noise ratio and not following DIN 32645 (DIN 2008) or according to the criteria of the FDA (2018) for bioanalysis, as is the case with the method presented herein.

Using *N*-(2-ethoxyethyl)valine-aniline-anilide as ISTD for all analytes presents a practical and cost-effective solution for the field of occupational medicine for which this method was developed. In any case, a single ISTD cannot compensate for all fluctuations associated with the method. It is assumed that the validation data of the method may be further improved by the use of structurally identical, isotope-labelled ISTDs for all analytes; particularly, such changes may enable lower detection and quantitation limits. This concern would be especially relevant for the field of environmental medicine; the signal-to-noise ratios that can be interpreted from the chromatograms are sufficiently low. The developers of the method have tested the use of a synthesised *N*-*n*-propyl-$^{13}C_5$,^{15}N-valine-leucine-anilide as the ISTD for *N*-propylvaline (both obtained by custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) and could reduce the quantitation limit for *N*-propylvaline by a factor of 10 to less than 5 pmol/g globin.

In general, the use of isotope-labelled ISTDs would have the advantage of compensating for specific matrix effects due to varying globin samples. As described by Schettgen et al. (2016), the isotope-labelled ISTDs can be prepared by incubating erythrocyte lysates with isotope-labelled reactive substances.

If appropriate dipeptide standards are available, the method can certainly be expanded to include adducts of further substances which alkylate the *N*-terminal valine of haemoglobin. As such, this method offers a good basis for future developments in the field of haemoglobin-adduct analysis.

Table 12 shows the levels of Hb adducts which were measured with this method in occupationally non-exposed persons. The adduct levels of occupationally exposed persons are presented in Table 13. These data encompass several years and a multitude of businesses, occupations, exposure scenarios, and protective measures. In contrast to *N*-methylvaline, levels of the alkyl homologues *N*-ethylvaline, *N*-*n*-propylvaline, and *N*-*n*-butylvaline are very rarely above the quantitation limit. Especially with respect to *N*-ethylvaline, this finding is consistent with the results of Scherer et al. (2010), who analysed ethyl mercapturic acid, the corresponding urinary biomarker, in smokers. The concentrations thus determined were about fifty times lower than those of methyl mercapturic acid in the same samples. No comparative data have been published for *N*-*n*-propylvaline and *N*-*n*-butylvaline.

Tab. 12 Adduct levels of persons with no occupational exposure determined with the presented method. Values below the LOQ were included as LOQ/2 to calculate mean adduct levels.

Adduct	Smoker status	Number of persons	Adduct level [pmol/g globin]	
			Mean	Range
N-Methylvaline	S	100	450	270–603
	N		371	185–551
N-Ethylvaline	S/N	100	< LOQ	< LOQ–86
N-(2-Hydroxyethyl)valine	S	100	172	< LOQ–603
	N		< LOQ	< LOQ
N-(2-Cyanoethyl)valine	S	100	163	< LOQ–375
	N		< LOQ	< LOQ
N-Benzylvaline	S/N	100	< LOQ	< LOQ
N-(2-Carbamoylethyl)valine	S	83	< LOQ	< LOQ–346
	N	64	< LOQ	< LOQ

LOQ: limit of quantitation; N: non-smokers; S: smokers

Tab. 13 Adduct levels of occupationally exposed persons as determined with the presented method. Values below the LOQ were included as LOQ/2 to calculate mean adduct levels.

Adduct	Sample number n	Adduct level [pmol/g globin]			
		Mean	Median	95th percentile	Range
N-Methylvaline	1906	357	326	571	< LOQ–1336
N-Ethylvaline	851	< LOQ	< LOQ	< LOQ	< LOQ–202
N-*n*-Butylvaline	700	< LOQ	< LOQ	< LOQ	< LOQ
N-(2-Hydroxypropyl)valine	1643	< LOQ	< LOQ	< LOQ	< LOQ–82
N-(2-Hydroxyethylvaline)	1388	131	< LOQ	444	< LOQ–8309
N-(2-Cyanoethyl)valine	2245	93	< LOQ	321	< LOQ–2658
N-Benzylvaline	931	< LOQ	< LOQ	< LOQ	< LOQ–33
N-(2-Carbamoylethyl)valine	645	< LOQ	< LOQ	< LOQ	< LOQ–484

LOQ: limit of quantitation

Instruments used GC-MS/MS system (Bruker 456 with a CTC PAL autosampler and Bruker EVOQ TQ-MS with control and evaluation software, Bruker Corporation, Billerica, USA) as well as capillary separation columns (No. 13623, 30 m × 0.25 mm × 0.25 µm, Rxi-5Sil MS, Restek GmbH, Bad Homburg vor der Höhe, Germany or No. 122-5532UI, 30 m × 0.25 mm × 0.5 µm, DB-5ms Ultra Inert, Agilent Technologies Deutschland GmbH, Waldbronn, 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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