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Simultaneous quantification of haemoglobin adducts of ethylene oxide, propylene oxide, acrylonitrile, acrylamide and glycidamide in human blood by isotope-dilution GC/NCI-MS/MS☆

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ABSTRACT

Haemoglobin adducts are highly valuable biomarkers of cumulative exposure to carcinogenic substances. We have developed and applied an analytical method for the simultaneous quantification of five haemoglobin adducts of important occupational and environmental carcinogens. The *N*-terminal adducts were determined with gas chromatography as pentafluorophenylthiohydantoine derivatives according to the modified Edman-procedure and subsequent acetonization of the glycidamide adduct *N*-(*R*,*S*)-2-hydroxy-2-carbamoylethylvaline (GAVal). The use of self-synthesized labelled internal standards in combination with tandem mass spectrometry using negative chemical ionisation guarantees both high accuracy and sensitivity of our determination. The limit of detection for *N*-2-hydroxyethylvaline (HEVal), *N*-(*R*,*S*)-2-hydroxypropylvaline (HPVal), *N*-2-carbamoylethylvaline (AAVal) and *N*-(*R*,*S*)-2-hydroxy-2-carbamoylethylvaline (GAVal) was 2 pmol/g globin, for *N*-2-cyanoethylvaline (CEVal) it was determined as 0.5 pmol/g globin, which was sufficient to determine the background levels of these adducts in the non-smoking general population. The between-day-precision for all analytes using a human blood sample as quality control material ranged from 4.7 to 12.3%. We investigated blood samples of a small group (n = 104) of non-smoking persons of the general population for the background levels of these haemoglobin adducts. The median values for HEVal, HPVal, CEVal, AAVal and GAVal in a group of 92 non-smoking persons were 18.1, 4.1, <0.5, 29.9 and 35.2 pmol/g globin, respectively. The adduct levels in 12 persons reporting exposure to passive smoke at home were similar for most adducts with median values of 17.2, 4.1, 1.0, 24.9 and 29.7 pmol/g globin for HEVal, HPVal, CEVal, AAVal and GAVal, respectively. Our results point to an elevated uptake of acrylonitrile caused by passive smoking as indicated by higher levels of the corresponding haemoglobin adduct CEVal.

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1. Introduction

Haemoglobin adducts are defined as covalently bound reaction products of electrophilic (mostly carcinogenic) substances with amino acids of the globin chains of haemoglobin. The extent of the formation of haemoglobin adducts is mainly influenced by the stability of the electrophilic compound under physiological conditions in blood (pH 7.4), its ability to penetrate cell membranes and its reactivity towards nucleophilic centers in the protein chain.

With respect to the long lifetime of erythrocytes in the human body (∼120 days) and the fact that there are no repair mechanisms for haemoglobin adducts (in contrast to DNA-adducts), the quantification of haemoglobin adducts offers the unique possibility to explore even past high (occupational) exposures or chronic low level (environmental) exposures to carcinogenic substances. In addition, multiple (animal) studies could show a close relationship between the binding of several carcinogenic substances (e.g. ethylene oxide, propylene oxide, alkenes, glycidamide) to haemoglobin and DNA in various tissues [1–5]. A human study could even show a close relationship between haemoglobin adducts and DNA-adducts in white blood cells of workers with occupational exposure to propylene oxide [6]. Hence, the determination of haemoglobin adducts is often regarded as a surrogate for the determination of

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Table 1
Classifications of the investigated carcinogenic substances by DFG and IARC.

	DFG	IARC
Ethylene oxide	Group 1	Group 1
Propylene oxide	Group 2	Group 2 B
Acrylonitrile	Group 2	Group 2 B
Acrylamide	Group 2	Group 2 A

DNA-adducts, which is considered to be the initial step of carcinogenesis [7]. With respect to their high specificity in terms of exposure information and the high analytical sensitivity, the determination of haemoglobin adducts probably represents the best measure available up to now in occupational and environmental medicine to estimate human health risks caused by hazardous substances [7].

Since the pioneering works of Ehrenberg et al. [8] and Neumann et al. [9,10] and the analytical breakthrough by the development of the modified Edman-procedure [11], numerous analytical methods for the quantification of different haemoglobin adducts have been published (summarised in Refs. [12,13]). However, most of the methods published so far focussed on the quantification of one or two specific adducts of interest, while methods covering multiple haemoglobin adducts of alkylating agents are scarce [11,14]. Moreover, our previous work failed to simultaneously quantify the background haemoglobin adduct levels of different alkylating substances in the general population [14].

Hence, it was our aim to develop and validate a method for the highly sensitive, simultaneous quantification of the *N*-terminal haemoglobin adducts of the carcinogenic substances ethylene oxide, propylene oxide, acrylonitrile and acrylamide as well as the oxidative, genotoxic metabolite of acrylamide, glycidamide. The classifications of these substances by DFG and IARC regarding carcinogenicity are summarised in Table 1.

The resulting haemoglobin adducts, namely *N*-2-hydroxyethylvaline (HEVal), *N*-(*R*,*S*)-2-hydroxypropylvaline (HPVal), *N*-2-cyanoethylvaline (CEVal), *N*-2-carbamoylethylvaline (AAVal) and *N*-(*R*,*S*)-2-hydroxy-2-carbamoylethylvaline (GAVal) were cleaved from the protein chain according to the modified Edman-procedure and determined by gas chromatography as pentafluorophenylthiohydantoine (PFPTH) derivatives. The glycidamide adduct GAVal is further derivatised to a stable oxazolidone using acidic acetone [15].

The use of modern, state-of-the-art tandem mass spectrometry with negative chemical ionisation in combination with self-prepared isotopically labelled internal standards allowed a highly sensitive and accurate simultaneous quantification of these five haemoglobin adducts. As a first application of this method, we have investigated blood samples of 104 non-smoking persons of the general population with no occupational exposure to any of these alkylating substances.

2. Experimental

2.1. Chemicals and materials

The dipeptide standards for the quantification of the adducts, namely *N*-2-hydroxyethylvaline-leucine-anilide, *N*-(*R*,*S*)-2-hydroxypropylvaline-leucine-anilide, *N*-2-cyanoethylvaline-leucine-anilide and *N*-2-carbamoylethylvaline-leucine-anilide were obtained from Bachem Biochemica (Heidelberg, Germany). The dipeptide standard for the quantification of glycidamide haemoglobin adducts, namely *N*-(*R*,*S*)-2-hydroxy-2-carbamoylethylvaline-leucine-anilide was also custom synthesized by Bachem Biochemica (Heidelberg, Germany).

Isotopically labelled acrylonitrile (acrylonitrile-2,3,3-d_3, isotopic purity 98%) and acrylamide (acrylamide-2,3,3-d_3, isotopic purity 98%) was purchased from Sigma–Aldrich (Munich, Germany) and isotopically labelled propylene oxide (±1,2-propylene-3,3,3-d_3 oxide, isotopic purity 99%) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Formamide ultrapure was obtained from United States Biochemicals (USB) (Cleveland, USA). Acetone, ethyl acetate, diethyl ether, ethanol, 2-propanol, toluene and n-hexane were all of the highest analytical grade available and purchased from Merck (Darmstadt, Germany). Hydrochloric acid (37%) and sulphuric acid (97%) were also obtained from Merck (Darmstadt, Germany). Pentafluorophenylisothiocyanate (PFPITC) was purchased from Fluka (Buchs, Suisse). Helium 5.0, methane 4.5 and argon 5.0 for GC/MS/MS were obtained from Linde (Munich, Germany).

2.2. Blood sampling and isolation of globin

This procedure follows closely the procedure recommended by the Deutsche Forschungsgemeinschaft [16]. Briefly, whole blood was collected from each person by venipuncture using EDTA-containing syringes (e.g. Sarstedt, Nümbrecht, Germany). The erythrocytes were isolated from 5 ml of whole blood by centrifugation (10 min, 800 × *g*). The erythrocytes are then washed three times with 5 ml of 0.9% NaCl solution. Hemolysis was achieved by filling up the isolated erythrocytes to the original blood volume with bidistilled water and subsequent freezing at −18 °C.

For the isolation of globin, 2 ml of hemolysate was added to 12 ml 50 mM hydrochloric acid in 2-propanol. The samples were shaken vigorously and centrifuged at 3500 × *g* for 10 min.

8 ml ethyl acetate was slowly added to the supernatant and the solution was kept at +4 °C for at least 2 h. After centrifugation, the precipitated globin was washed two times with 5 ml ethyl acetate and 5 ml n-hexane and finally dried in a vacuum desiccator overnight. Approximately 200–300 mg of globin can be obtained from 2 ml of erythrocyte lysate.

2.3. Preparation of isotopically labelled internal standard solutions

2.5 ml of erythrocyte lysate of a non-smoking person is transferred to a 5-ml glass vial with screw top and teflon-lined septum. Either 50 μl of d_3-acrylonitrile (0.77 mM), d_3-acrylamide (0.68 mM) or 100 μl of d_3-propylene oxide (1.36 mM) is added to the lysate, the glass vial is closed tightly and the samples are rotated for at least 4 h at room temperature (*Note*: Acrylonitrile, acrylamide and propylene oxide are considered to be carcinogenic! Caution has to be taken in handling these substances!).

2 ml of the treated erythrocyte lysate is then subjected to the globin isolation as described under Section 2.2. These globin samples have a very high content of isotopically labelled *N*-terminal adducts and have to be diluted before they can be used as internal standards. The isotopic purity of these labelled *N*-terminal adducts turned out to be identical to the isotopic purity of the labelled carcinogens used for synthesis (>98%). In our case, the adduct-content of these globins was estimated to be app. 52 μM/g globin of d_3-CEVal, 33 μM/g globin of d_3-AAVal and 45 μM/g globin of d_3-HPVal. Background concentrations of the haemoglobin adducts in the blood sample used for synthesis of the labelled internal standards were 22, 5.3, <0.5, 26, and 19 pmol/g globin for HEVal, HPVal, CEVal, AAVal and GAVal, respectively.

Of course, the concentration of labelled *N*-terminal valine might vary according to the reaction conditions, time of reaction and globin content of the erythrocyte lysate. The following procedure might serve as a clue for the extent of dilution that is necessary.

100 mg of the labelled globin (d_3-CEVal, d_3-AAVal or d_3-HPVal) is solved in 3 ml of formamide to serve as a stock solution of the labelled internal standard. 20 μl of these stock solutions are placed in a 20 ml glass volumetric flask and diluted to the mark with formamide. This solution serves as the working solution 1 of the internal standards and 10 μl of this working solution 1 is added to each sample before processing, corresponding to app. 175 pmol/g globin d_3-CEVal, 110 pmol/g globin d_3-AAVal and 150 pmol/g globin of d_3-HPVal. The stock and working solutions remained stable for at least 1 year stored at −20 °C.

The internal standard for the quantification of GAVal was synthesized by the Department of Food Chemistry and Toxicology of the University of Kaiserslautern, Germany. It is a d-8-labelled, acetonized pentafluorophenylthiohydantoine derivative of the *N*-terminal glycidamide adduct (d_8-acGAVal-PFPTH), namely d_8-N-(2,2-dimethyl-4-oxazolidinoneylmethylvalin)-pentafluorphenylthiohydantoine [17]. A working solution of this internal standard is prepared by dilution in ethanol (c = 50 μg/L). 10 μl of this working solution is added to each sample before processing.

2.4. Sample preparation

The method we applied is based on the method of Paulsson et al. [15], which includes a modified Edman degradation of the alkylated *N*-terminal valine and a subsequent acetonization of the glycidamide-valine-pentafluorophenylthiohydantoine derivative.

100 mg globin was dissolved in 3 ml formamide. 40 μl NaOH (1 M), 15 μl of pentafluorophenylisothiocyanate (PFPITC) and 10 μl each of the working solutions of the internal standards (see Section 2.3.) were added. The samples were rotated overnight at room temperature and finally heated at 45 °C for 90 min in a water bath.

400 μl of saturated aqueous NaCl solution was then added to the formamide phase in order to improve the efficiency of extraction for the very polar PFPTH-derivative of the glycidamide-valine adduct. The PFPTH-derivatives of the *N*-terminal valine adducts were extracted twice with 3 ml diethyl ether by intensively shaking on a vortex mixer (60 s) and subsequent centrifugation at 4500 × g. If necessary due to emulsions, the samples can be knocked on the laboratory table and centrifuged again.

The combined organic phases were evaporated to dryness under a gentle stream of nitrogen. The residue was then dissolved in 1 ml toluene and washed with 2 ml bidistilled water and subsequently with 2 ml of a freshly prepared solution of Na_2CO_3 (0.1 M). Finally, the toluene phase was evaporated to dryness under nitrogen.

Acetonization of the PFPTH-derivative of the glycidamide-valine adduct was then carried out by the addition of 100 μl of a freshly prepared solution of 1% H_2SO_4 in acetone (v/v). The glass vials are sealed and the samples were left at room temperature overnight. The acidic solution was then neutralised by the addition of 150 μl of a freshly prepared solution of $NaHCO_3$ (0.1 M). The derivatives were dissolved in 1 ml of toluene and the solution was washed two times with 2 ml of water. At last, the toluene phase was again evaporated to dryness under nitrogen and the residues were redissolved in 100 μl of toluene. 1 μl of this solution was then analysed by GC/MS/MS in NCI-mode.

2.5. Calibration procedure and quality control

Five calibration standards were prepared by spiking of pooled human globin (obtained from non-smoking laboratory personnel) dissolved in formamide with a solution of the dipeptide standards in ethanol in the range of 0–1500 pmol/g globin (∼40 μg/L blood). Due to the racemic nature of the propylene oxide valine adduct (*N*-(*R*,*S*)-2-hydroxypropylvaline, HPVal) and the glycidamide-valine adduct (*N*-(*R*,*S*)-2-hydroxy-2-carbamoylethylvaline, GAVal), these PFPTH-derivatives are split in two peaks representing the R- and S-form of the adduct (see also Fig. 1). For quantification of these derivatives (and their labelled internal standards d_3-HPVal and d_8-acGAVal), both peaks were integrated and the peak areas were added.

Linear calibration curves were obtained by plotting the quotients of the peak areas of the adducts and the corresponding labelled internal standards as a function of the spiked concentration. These graphs were used to ascertain the unknown concentrations of adducts in globin samples. For HEVal, the labelled propylene oxide adduct (d_3-HPVal) served as internal standard.

For quality control purposes, sufficient amounts of globin were isolated from blood samples of two smoking persons. 100 mg of these quality control globins have been included in each analytical series.

2.6. Gas chromatography

Analysis was carried out on a Varian CP 3800 gas chromatograph equipped with a Varian CP 8400 autosampler and a split/splitless injector operating in splitless mode. The inlet purge off time was 1 min. The operating temperature of the injector was 280 °C.

Chromatographic separation was performed using a DB-17 HT capillary column (crosslinked (50% phenyl)-methylpolysiloxane, 30 m × 0.25 mm I.D., 0.15 μm film thickness) purchased from J & W Scientific (Folsom, CA, USA).

Helium 5.0 was used as the carrier gas at a constant flow of 1.3 ml/min. The initial column temperature of 90 °C was held for 1 min, then raised at a rate of 25 °C/min to 120 °C. It was then raised at a rate of 10 °C/min to 240 °C and finally raised at 25 °C/min to 310 °C, remaining at this temperature for 10 min. The injection volume was 1 μl. The retention times for the PFPTH-derivatives of the adducts and the internal standards under the described conditions are summarised in Table 2.

2.7. Tandem mass spectrometry

A Varian 1200 L tandem mass spectrometer was used in negative chemical ionisation (NCI) mode using methane as reagent gas at a pressure of 6.5 Torr. The temperature of the ion source was kept at 150 °C and the temperature of the MSD transfer line was maintained at 300 °C.

NCI mass spectra of the derivatised analytes were obtained at an energy level of 70 eV. Collision-induced mass spectra of the characteristic ions $[M-HF]^-$ of the PFPTH-derivatives of the adducts were recorded and selective mass transitions were defined and optimised with regard to argon pressure in the collision cell (Q 2) and collision energy using the Varian Mass Spectrometry Workstation software. Argon pressure in the collision cell (Q 2) was optimised at 2 mTorr. For the quantitative analysis of the adducts, multiple reaction monitoring (MRM) was used and the ion transitions listed in Table 2 were monitored.

2.8. Human studies

For a pilot study, we investigated blood samples of 104 white, Caucasian, non-smoking subjects (52 m, 52 f) of the general population living in Bavaria with no known occupational exposure to ethylene oxide, propylene oxide, acrylonitrile or acrylamide in order to evaluate the background levels of the corresponding haemoglobin adducts.

The median age of this group was 35 years, ranging from 6 to 80 years. All subjects were asked about their smoking status and possible exposure to passive smoke in their personal surroundings. 12 persons (5 m, 7 f) reported to be exposed to passive smoke at home with at least 1 person actively smoking in their home.

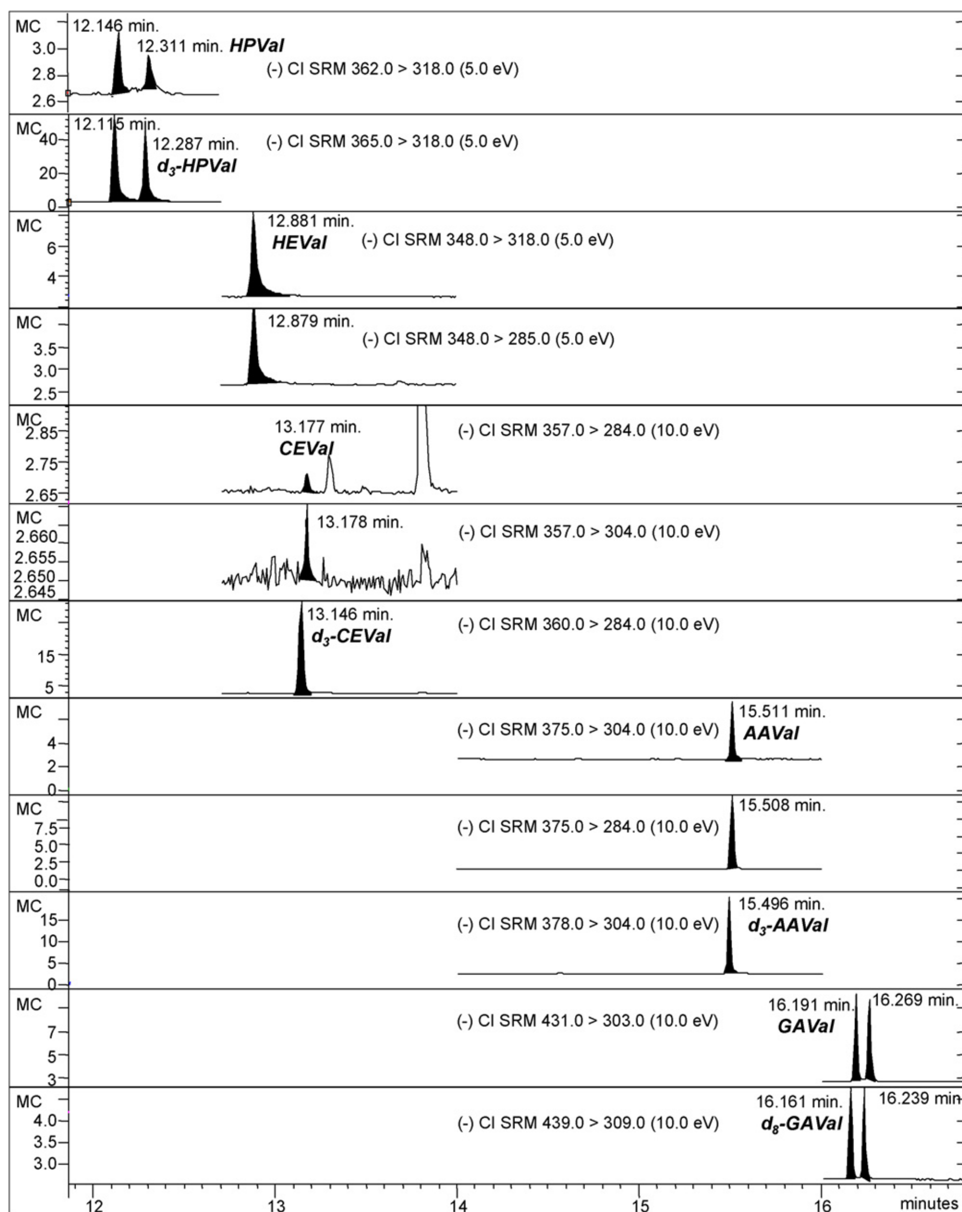

Fig. 1. GC/NCI-MS/MS-chromatogram of the processed blood sample of a child (7 years, f). The concentrations for HPVal, HEVal, CEVal, AAVal and GAVal were 3.3, 33, 0.7, 59 and 52 pmol/g globin.

This subcollective was part of a larger study investigating the background exposure of the general population to acrylamide and aromatic amines in Bavaria [18,19]. All persons were informed in writing about the aims of our study and gave written consent about the donation of blood. This study was approved by the Ethics Committee of the Friedrich-Alexander University Erlangen-Nuremberg.

3. Results and discussion

3.1. General remarks

The procedure described in this manuscript represents a further development of two previously published methods [15,20] that are all based on the original modified Edman-procedure [11]. To our knowledge, it represents the first method that allows the simultaneous quantification of five haemoglobin adducts of occupational and environmental relevance. The use of self-prepared labelled internal standards by incubation of erythrocyte lysate with the labelled electrophiles guarantees high reproducibility and accuracy of our results and can easily be transferred to other haemoglobin adducts in question.

Acetonization of the glycidamide-valine-PFPTH-derivative leads to a stable oxazolidinone derivative with favourable mass spectrometric properties and prevents decay of the initial adduct in the injector of the GC as previously described [15]. As the other valine adducts remain unaffected in this second derivatisation, our procedure allows the simultaneous quantification of haemoglobin adducts of propylene oxide, ethylene oxide, acrylonitrile, acrylamide and glycidamide in one analytical run. However, attempts to include also the adducts of methylating and ethylating agents (*N*-methyl- and *N*-ethylvaline) into our method were unsuccessful. As a full SCAN chromatogram of a processed standard of both *N*-methyl- and *N*-ethylvaline showed, there is a very large, co-eluting peak in this part of the chromatogram, suppressing the ionisation of these analytes in NCI-mode and precluding reproducible tandem mass spectrometric determination.

Table 2
Retention times and MRM-parameters for the selected parent and daughter ion combinations of the analytes.

Analyte	Ret. time [min]	Ion transitions (MS/MS, NCI-mode)		Collision energy
		Q 1	Q 3	Q 2
HPVal	12.13 12.30	362	318	5 eV
d_3-HPVal	12.12 12.29	365	318	5 eV
HEVal	12.87	348	318[a] 285	5 eV
CEVal	13.16	357	304[a] 284	10 eV
d_3-CEVal	13.13	360	284	10 eV
AAVal	15.49	375	304[a] 284	10 eV
d_3-AAVal	15.48	378	304	10 eV
acGAVal	16.19 16.26	431	303	10 eV
d_8-acGAVal	16.16 16.23	439	309	10 eV

[a] This transition was used as quantifier.

The use of modern, highly sensitive and specific tandem mass spectrometry with negative chemical ionisation led to a drastic reduction of LODs compared to previous methods, as recently shown for the haemoglobin adduct of epichlorohydrin [21].

With respect to the propylene oxide adduct (*N*-(*R*,*S*)-2-hydroxypropylvaline, HPVal) and the acrylonitrile adduct (*N*-2-cyanoethylvaline, CEVal), our method allowed the quantification of these adducts also in the non-smoking general population. Although the sample preparation is quite laborious and takes about 3 days, it is well suited for the screening of population exposures to occupational or environmental carcinogens.

3.2. GC/NCI-MS–MS-analysis

The major fragmentation ion in NCI-mode of the PFPTH-derivatives of the analytes is $[M-20]^-$, which corresponds to a loss of HF as a neutral loss. For all analytes, this ion was chosen as precursor ion. NCI-conditions were optimised at maximum intensities of these ions in single-ion-recording mode (optimisation of electron energy, pressure of methane in the ion source and temperature of the ion source) using a processed globin standard (~1500 pmol/g globin). Optimisation of collision-induced fragmentation was then carried out by simultaneous recording of product-ion spectra of these ions at different collision energies in Q 2 with the Mass Spectrometry Workstation software.

For all analytes except HPVal and acGAVal, two specific product ions were recorded in MRM-mode of the tandem mass spectrometer, one of which was used for quantification and the other one as qualifier to guarantee highest specificity. The recorded quantifier ion traces were free of interferences, even at the low levels found in non-smokers' globin (cf. Fig. 1).

3.3. Reliability of the method

3.3.1. Calibration graphs

The calibration graphs were linear for all five haemoglobin adducts in the range of 50–1500 pmol/g globin. In each case, the correlation coefficients of the calibration graphs were higher than 0.998. Calibration was carried out using pooled globin of non-smoking persons and calibration graphs were corrected for background values detected in the pooled globin used.

It has to be emphasized, that a protein matrix is essential for effective performance of the modified Edman-procedure under the conditions described here. Experiments using dipeptide standards without protein (globin) matrix led to poor derivatisation yields.

Dipeptide standards simulating the adduct-bearing last two amino acids of the α-globin chain (Val-Leu-Anilid) were used for the calibration of the adducts, as recommended by the DFG [16]. Earlier experiments revealed that the use of free, adducted valine (e.g. *N*-2-hydroxyethylvaline) instead of the dipeptide standards used here leads to massive overestimations (up to a factor of 8) in the calculated adduct concentrations due to differences in the pK_s-values (and derivatisation yields) of the standards used [22].

3.3.2. Precision of the method

Between-day repeatability of the method was determined using globin samples of two smoking persons. These globin samples were weighed in and processed in every analytical series ($n = 6$), so that the precision data presented here also include the imprecision of weighing in. Smokers were chosen for quality control in order to guarantee detectable amounts of all haemoglobin adducts in question. Especially *N*-2-cyanoethylvaline (CEVal) has not been detectable in non-smoking persons using previous methods.

The relative standard deviation of the between-day repeatability for the different haemoglobin adducts in these native materials

Table 3
Precision between-series for the haemoglobin adducts analysing two native smoker globin samples ($n = 6$).

Analyte	Concentration [pmol/g globin]	Rel. standard deviation [%]
HEVal	223 201	9.6 6.3
HPVal	11.8 8.6	7.3 10.3
CEVal	267 172	8.0 4.7
AAVal	183 149	7.0 6.9
GAVal	86 73	5.5 12.3

Table 4
Results of our study measuring the haemoglobin adducts in 92 non-smoking persons of the general population and 12 non-smoking persons reporting exposure to passive smoke at home.

		HEVal [pmol/g globin]	HPVal [pmol/g globin]	CEVal [pmol/g globin]	AAVal [pmol/g globin]	GAVal [pmol/g globin]
No exposure to passive smoke ($n = 92$)	Median	18.1	4.1	<0.5	29.9	35.2
	Range	7.7–64.6	<2–10.6	<0.5–4.2	14.1–70.9	13.8–65.8
Exposure to passive smoke ($n = 12$)	Median	17.2	4.1	1.0	24.9	29.7
	Range	10.8–38.6	<2–6.7	<0.5–3.4	16.2–50.1	19.7–39.6
Significance (Mann–Whitney)		$p = 0.89$	$p = 0.38$	**$p = 0.07$**	$p = 0.21$	$p = 0.15$

The bold value is the significance of the Mann-Whitney-*U*-test.

range between 4.7 and 12.3%, demonstrating very good repeatability of our method. The data are summarised in Table 3.

3.3.3. Limit of detection

As almost every processed globin sample had detectable levels of at least four haemoglobin adducts (HPVal, HEVal, AAVal, GAVal) due to the environmental uptake of food-borne acrylamide as well as endogenous formation of ethylene or propylene oxide, the limit of detection could only be estimated. The limit of detection, defined as a signal-to-noise ratio of three, for the registered ion transitions listed in Table 1 was considered to be 2 pmol/g globin for HEVal, HPVal, AAVal and GAVal and 0.5 pmol/g globin for CEVal. The limit of quantification was considered to be twice the limit of detection.

3.3.4. Sources of error

The detection of the analytes in NCI/MS/MS-mode is highly sensitive and care has to be taken to avoid carry-over effects in the autosampler. Careful flushing of the syringe in the autosampler using two solvents of different polarity (e.g. toluene and ethanol) is strongly recommended in combination with regular blank solvent injections to monitor possible contaminations.

The vials used for acetonization of the processed globin samples should be sealed tightly. As described in Section 2.4, this derivatisation is usually carried out overnight and leakages might lead to an evaporation of acetone and resulting dryness of the samples. In this case, irreproducible results – especially regarding GAVal – can be expected. Moreover, the samples should be quickly shaken and processed after neutralisation of the acidic acetonization solution with 0.1 M $NaHCO_3$, as the oxazolidinone derivative of GAVal (acGAVal) is not stable in alkaline solution and the formation of "alkaline spots" in the solution should be avoided.

Quantification of CEVal is carried out using the mass transition 357 ≥ 304 despite its lesser intensity compared to the qualifying transition 357 ≥ 284. Experiments processing samples with and without internal standard revealed that this qualifying transition is interfered by the labelled internal standard (d_3-CEVal), especially in the low-concentration range.

3.4. Results of haemoglobin adduct monitoring in the general population

Our method was applied to quantify these haemoglobin adducts in blood of non-occupationally exposed, non-smoking persons of the general population (cf. Section 2.7). The results of the adduct monitoring of 92 non-smoking persons with no exposure to passive smoke (26 females, 20 males) and a group of 12 persons with self-reported exposure to passive smoke at home are summarised in Table 4. Fig. 1 shows the chromatogram of the processed globin of a non-smoking person of the examined collective.

This study confirms a background level of the haemoglobin adducts of ethylene oxide in non-smoking persons due to endogenous formation as previously reported [23–27]. Our results for the haemoglobin adducts of the food-borne toxicant acrylamide and its genotoxic metabolite glycidamide in non-smokers have been described and discussed in our previous paper [28]. In short, haemoglobin adducts of acrylamide and glycidamide are well correlated ($r = 0.54$, $p < 0.001$) and the levels found within our study are within the range of other, previously published studies in Sweden, USA or Germany. It was also found that children (age 5–10) and young adolescents (age 11–18) had higher levels of both acrylamide and glycidamide adducts compared to adults, which is presumably due to their higher intake of food (and consequently also acrylamide) in relation to their body weight [28].

Background levels of the haemoglobin adduct of propylene oxide (HPVal) in the general population have not yet been reported on a broad basis. Czene et al. [6] reported levels of HPVal ranging between 5 and 8 pmol/g globin in 8 control samples, which is in good agreement with the levels reported in our study.

The levels of HPVal in our study are five times lower compared to HEVal and might reflect the lower reactivity of propylene oxide compared to ethylene oxide [29]. Similar to HEVal (and all other haemoglobin adducts), the HPVal-levels are also influenced by personal active smoking as indicated by the two- to three-fold higher levels of HPVal in the smoking quality control globin samples. This is in line with our previous observation regarding the excretion of the urinary mercapturic acid of propylene oxide, 2-HPMA in non-smoking and smoking persons of the general population [30].

The haemoglobin adduct of acrylonitrile (*N*-2-cyanoethylvaline, CEVal) is a highly specific and extremely sensitive parameter for the estimation of individual smoking habits with very good correlations between self-reported daily cigarette consumption and adduct levels [14,26]. Although not statistically significant ($p = 0.07$, Mann–Whitney-*U*), our study could show that self-reported exposure to passive smoke leads to elevated internal exposure of acrylonitrile as determined by haemoglobin adducts (median levels <0.5 pmol/g globin vs. 1 pmol/g globin, see Table 3). The levels of the other adducts investigated were obviously not affected by the exposure to passive smoke, probably due to variations in food patterns (AAVal, GAVal) as well as endogenous metabolism (HEVal, HPVal).

This is in good agreement with the results of Perez et al. [31] who reported CEVal-levels in blood of 3 passive smokers of 1.1 pmol/g globin as compared to 0.76 pmol/g globin in blood of 18 non-smokers. An increased uptake (and excretion) of acrylonitrile in persons exposed to passive smoke could recently also be demonstrated using the excretion of the mercapturic acid of acrylonitrile (CEMA) as biomarker of exposure [32].

4. Conclusion

The method presented here allows for the first time the simultaneous quantification of five different haemoglobin adducts of 4 carcinogenic substances (ethylene oxide, propylene oxide, acrylonitrile and acrylamide). An easy and simple procedure for the preparation of isotopically labelled internal standards is described which enables a highly precise and accurate quantification of the

adducts. The use of tandem mass spectrometry with negative chemical ionisation guarantees high sensitivity and specificity of our method.

The procedure proved to be appropriate for routine analysis of haemoglobin adducts. The limit of detection for all adducts is sufficiently low for the determination of the background levels in the general population. Our study for the first time described the background levels of propylene oxide haemoglobin adducts in a larger group of non-smoking persons of the general population. Moreover, our study points to an elevated effective internal dose of acrylonitrile in persons exposed to passive smoke as indicated by the higher levels of the corresponding haemoglobin adduct. This observation needs to be corroborated in future field studies.

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