

Mass spectrometric identification of isocyanate-induced modifications of keratins in human skin



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ABSTRACT

In the current paper we show that exposure of human callus to isocyanates leads to covalent modifications within keratin proteins. Mass spectrometric analyses of pronase digests of keratin isolated from exposed callus show that both mono- and di-adducts (for di-isocyanates) are predominantly formed on the ϵ -amino group of lysine. In addition, numerous modified tryptic keratin fragments were identified, demonstrating rather random lysine modification. Interestingly, preliminary experiments demonstrate that in case of MDI a similar lysine di-adduct was formed with lung elastin. Our data support the hypothesis that skin sensitization through antigenic modifications of skin proteins by isocyanates could play a role in occupational isocyanate-induced asthma. It is further envisaged that the elucidated adducts will also have great potential for use as biomarkers to assess skin exposure to isocyanates. Advantageously, the various lysine adducts display the presence of a characteristic daughter fragment at m/z 173.1 [lysine-NCO]⁺, enabling generic and rapid screening for exposure to isocyanates.

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1. Introduction

Isocyanates are a group of chemicals which are widely used in chemical industry, e.g. in the production of polyurethanes and as hardener in coatings and paints. Isocyanates have also been implicated in causing adverse health effects. Probably the most notorious representative of the isocyanate family is methyl isocyanate (MIC). This highly toxic chemical accidentally escaped from a chemical plant during the Bhopal disaster in 1984, resulting in thousands of (fatal) casualties [1,2]. Occupational exposure to isocyanates is known to be an important cause of asthma [3]. In addition, alleged health problems after exposure to di-isocyanates like toluene di-isocyanate (TDI) and methylene diphenyl di-isocyanate (MDI) after application of home insulation have also received much attention [4,5].

It has been hypothesized [6–8] that skin exposure to isocyanates can lead to systemic immune sensitization, eventually causing the typical isocyanate-induced asthma-like inflammatory responses. Indeed, the skin is a highly immunogenic organ, able to elicit serious systemic immune responses such as contact allergy, inter alia caused by skin protein modification [9,10].

Within this framework we reasoned that isocyanate modification of particular skin keratins could play a relevant role in this process, because keratins are so abundant in skin tissue. We here report the detailed mass spectrometric elucidation of adducts of a number of isocyanates with keratins present in the stratum corneum of the human skin. It is envisaged that the present work will also lead to novel biomarkers of exposure to isocyanates, as a useful addition to the already existing biomarkers based on adducts to blood proteins [11,12] and urinary metabolites [13–15]. Various studies dealing with keratin adducts of other xenobiotics have been published, demonstrating the potential utility of keratin adducts as biomarker of exposure [16,17].

2. Materials and methods

2.1. Chemicals

2,6-Toluene diisocyanate (2,6-TDI), methylene diphenyl diisocyanate (MDI), 4-bromophenylisocyanate (4-BrPIC), 2-nitrophenylisocyanate (2-NPIC), phenethylisocyanate (PEIC), 2-methacryloyloxyethyl isocyanate (MOI), allylisocyanate (AIC), dithiothreitol (DTT), sodium iodoacetate and TPCK-treated trypsin were purchased from Sigma (St. Louis, MO, USA). Protease mixture of exo- and endonucleases from *Streptomyces Griseus* (pronase) was

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purchased from Fluka (Buchs, Switzerland). For the present study, human callus obtained from a local podotherapist, was used as the source of human keratin. All other chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). All buffers were prepared with Millipore grade water. PD MiniTrap G-25 columns were purchased from GE Healthcare (Buckinghamshire, UK).

2.2. Incubation of human callus with isocyanates

Human callus was mechanically homogenized to a fine powder using a MM 400 ball mixer mill (Retsch, Haan, Germany) to increase the surface area for subsequent exposure to isocyanate compounds. Two different incubation procedures were used for the IC reaction with human callus. Procedure (1) (water-free incubation): the callus (100 mg) was washed twice with DMSO. After centrifugation (30 min, 400 rpm) the supernatant was discarded and the pellet of callus was resuspended in 200 μ L DMSO. Subsequently, 4 μ L of a stock-solution of IC compound in DMSO was added to a 10 mM final concentration, and the mixture was incubated overnight at 37 °C in a temperature-controlled incubator under continuous shaking. Procedure (2) (incubation in aqueous solution): the callus (100 mg) was washed twice with Tris-HCl buffer (20 mM, pH 7.4) and then allowed to hydrate in a 0.9% NaCl solution. After centrifugation (30 min, 400 rpm) the supernatant was discarded and the pellet of hydrated callus was resuspended in 200 μ L 0.9% NaCl. Subsequently, 4 μ L of a stock-solution of IC compound in 2-propanol was added to a 10 mM final concentration, and the mixture was incubated overnight at 37 °C in a temperature-controlled incubator under continuous shaking. Subsequent work-up after incubation was similar for both incubation methods, i.e. the suspension was centrifuged briefly and the supernatant containing the excess IC compound was discarded. Next, the callus was resuspended in Millipore water (2 mL), centrifuged briefly (15 min, 400 rpm) and allowed to incubate for some time (2 h) to allow any unreacted IC to hydrolyze. The supernatant was then discarded. This wash step was repeated three times to remove all excess IC compound.

2.3. Isolation of keratins from human callus

2.3.1. Extraction of keratins

Extraction of keratins was performed following a standard protocol adapted from Van der Schans et al. [18]. Briefly, the exposed callus was soaked in Tris-HCl buffer (5 mL, 20 mM, pH 7.4) overnight, under continuous stirring. After centrifugation (30 min, 400 rpm) the supernatant was discarded and the pellet was stirred in a buffer (5 mL, pH 7.4) containing Tris-HCl (20 mM) and urea (8 M) overnight. After centrifugation (30 min, 400 rpm) the supernatant was again discarded and the pellet was resuspended in extraction buffer (5 mL, pH 7.4) containing Tris-HCl (20 mM), urea (7 M), thiourea (2 M) and bis(2-mercaptoethyl)sulfone (BMS; 0.025 M) overnight, under continuous stirring.

2.4. Enzymatic digestion of keratins

2.4.1. Reduction and carboxymethylation

To an aliquot of keratin in extraction buffer (0.5 mL, roughly 5 mg/mL protein), containing Tris-HCl (20 mM), urea (7 M), thiourea (2 M) and BMS (0.02 M), 2.2 mg DTT was added to reduce all cysteine bridges. The mixture was incubated for 60 min at 50 °C in a heating block. Next, sodium iodoacetate (5 mg) was added for carboxymethylation of free cysteine residues and the mixture was incubated for another 60 min at 50 °C in a heating block. Subsequently, the proteins were isolated from the reaction mixture by filtration over a PD MiniTrap G-25 column, pre-equilibrated

with aqueous NH_4HCO_3 (50 mM), to remove salts and unreacted reagents.

2.4.2. Pronase digestion

A solution of pretreated keratin (reduced and carboxymethylated; 5 mg/mL) in aqueous NH_4HCO_3 (50 mM; 0.5 mL) was incubated with pronase (5%, w/w) at 37 °C overnight to digest the protein material to individual amino acids. Samples were analyzed with LC-Electrospray (Tandem) MS for identification of modified amino acids.

2.4.3. Tryptic digestion and mass spectrometric analysis for keratin identification

A solution of pretreated keratin (reduced and carboxymethylated; 5 mg/mL) in aqueous NH_4HCO_3 (50 mM; 0.5 mL) was incubated with TPCK treated trypsin (5%, w/w) at 37 °C overnight, to ensure maximum tryptic digestion.

A number of samples were analyzed using the data dependent LC-autoMS/MS mode. The ten most abundant ions (charge states 2⁺, 3⁺ and 4⁺) in the MS spectrum (300–1300 m/z , 2 Hz) of eluting peptides were selected for data dependent MS/MS analysis by collision-induced dissociation using nitrogen as the collision gas. MS/MS scans were acquired over the mass range 100–2000 m/z (2–10 Hz, depending on signal intensities).

Peak lists were generated using DataAnalysis 4.1 software (Bruker) and exported as Mascot Generic (MGF) files. These files were searched against the NCBI database with the Mascot search algorithm (Mascot 2.2.04, Matrix Science, London, UK). An MS tolerance of 50 mmu and a MS/MS tolerance of 50 mmu were used. Trypsin was designated as the enzyme and one missed cleavage was allowed. Carboxymethylcysteine was selected as a fixed modification and oxidation of methionine as a variable modification. The samples contained approx. 80 (exposure in water) and approx. 120 (exposure in DMSO) abundant peptides (MW 600–5000) of keratin proteins and except trypsin no other proteins with more than 2 unique tryptic fragments per protein proved to be present.

2.5. LC-electrospray tandem MS analysis of pronase and trypsin digests for adduct elucidation

LC-ES MS(/MS) experiments were conducted on a Q-TOF™ hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, U.K.) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a precolumn splitter (type Accurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland) with a 10 μ L injection loop mounted and a PepMap C₁₈ column (150 $\times$ 1 mm, 3 μ m; LC Packings). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (CH_3CN with 0.2% (v/v) formic acid) was used to achieve separation, following: 100% A (v/v) to 80% (v/v) B in 45 min. (LC-system I) or in 60 min. (LC-system II). The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approx. 40 μ L/min through the column. Mass spectra were recorded at a speed of 1–2.4 s. with nitrogen as the nebulizer and desolvation gas (at a flow of 20 and 400 L/h, respectively). MS/MS product ion spectra were recorded using a collision energy of 10–30 eV, with argon as the collision gas.

Alternatively, experiments were conducted on a Bruker maXis Impact Q-ToF instrument (Bruker Daltonics, Bremen, Germany) equipped with an Ultimate 3000 UPLC (Thermo Fisher Scientific, San Jose, USA). The system was provided with a PepMap C₁₈ column (150 $\times$ 1 mm, 3 μ m; Thermo Fischer Scientific). A gradient of eluents A and B (see above) was used: 100% A to 70% B in 30 min. at a flow rate of 80 μ L/min (LC-system III) or 100% A to 50% B in 20 min. at a flow rate of 50 μ L/min (LC-system IV). As

Table 1

Chemical structures of the elucidated lysine-adducts in pronase digests of callus exposed to isocyanates. The modified lysines were identified by LC–ES MS/MS. All experiments were performed in DMSO and in water.

Chemical structures of the lysine-isocyanate adducts R =	Isocyanate compound	MW monoisot.
	4-BrPIC	343.053
	MDI	370.200
	MDI	413.206
	MDI	542.285
	2-NPIC	310.128
	PEIC	293.174
	AIC	229.143
	MOI	301.164
	2,6-TDI	294.169
	2,6-TDI	466.254

alternative a second column was used, the Acquity UPLC HSS T3 (100 × 2.1 mm, 1.8 μm; Waters) with a gradient of 100% A to 50% B in 50 min. at a flow rate of 200 μl/min (LC-system V). Mass spectra were recorded at a speed of 0.5–4 Hz with nitrogen as the nebulizer and dry gas (at 0.8 Bar and 8 l/min., respectively). The dry temperature was set to approx. 200 °C. Product ion spectra were recorded using a collision energy of 10–15 eV, with nitrogen as the collision gas.

2.6. Synthesis of reference compounds

For a small number of isocyanates we synthesized the ε-lysine derivative, i.e. the putative lysine adducts analysed after pronase digestion, using the method of Kumar et al. [19] with some slight modifications. Briefly, N-α-(*tert*-butoxycarbonyl)-L-lysine (N-Boc-Lys) (250 mg, 1.0 mmol) was dissolved in 0.25 M sodium bicarbonate (10 mL, pH 8.3). The isocyanate (1.1 mmol) in 1,4-dioxane (5 mL) was added dropwise and the mixture was stirred overnight. The reaction mixture was concentrated in vacuo to approximately 10 mL. The reaction mixture was washed

successively with n-hexane (3 × 10 mL) and ethyl acetate (6 × 10 mL). The water layer was acidified (pH 3–4) and extracted with ethyl acetate (3 × 20 mL). These extracts were combined and dried on anhydrous magnesium sulfate, filtered, and evaporated in vacuo. NMR and MS data were in agreement with the expected structures. Subsequently, Boc-Lys-AIC/Boc-Lys-PEIC was dissolved in dichloromethane (5 mL), and trifluoroacetic acid (5 mL) was added. The reaction mixture was allowed to stir for 2 h at room temperature. The solvents were evaporated under reduced pressure and the residue was precipitated through the addition of diethyl ether (20 mL). The precipitate was collected by filtration, washed with diethyl ether (20 mL) and dried *in vacuo*. Yields ranged between 40% and 45%. Mass spectrometric data and retention times matched with the lysine adducts in the respective pronase digests. The structures of the synthesis products were further corroborated by using NMR spectroscopy:

2.6.1. N-ε-Allyl isocyanate lysine adduct

¹H NMR (400 MHz, methanol-*d*₄) δ 5.87 (m, *J* = 15.9, 10.5, 5.3 Hz, 1H), 5.13 (dd, *J* = 38.5, 13.8 Hz, 2H), 3.95 (t, *J* = 6.3 Hz, 1H), 3.75 (d, *J* = 5.2 Hz, 2H), 3.17 (t, *J* = 6.4 Hz, 2H), 2.05–1.85 (m, *J* = 25.8, 20.5, 13.9, 12.7, 6.0 Hz, 2H), 1.65–1.36 (m, 4H).

¹³C NMR (101 MHz, methanol-*d*₄) δ 170.74, 159.82, 135.54, 113.94, 52.58, 41.96, 39.04, 29.82, 29.52, 21.83.

2.6.2. N-ε-Phenylethyl isocyanate lysine adduct

¹H NMR (400 MHz, methanol-*d*₄) δ 7.34–7.16 (m, 5H), 3.83 (t, *J* = 6.2 Hz, 1H), 3.37 (d, *J* = 7.2 Hz, 2H), 3.14 (t, *J* = 6.8 Hz, 2H), 2.78 (t, *J* = 7.2 Hz, 2H), 2.00–1.80 (m, *J* = 30.1, 15.1, 6.5 Hz, 2H), 1.57–1.41 (m, 4H).

¹³C NMR (101 MHz, methanol-*d*₄) δ 171.33, 159.83, 139.35, 128.42, 128.06, 125.86, 53.21, 41.24, 38.99, 36.15, 30.04, 29.63, 21.91.

3. Results and discussion

This paper deals with the elucidation of adducts of a number of isocyanates with human keratins. To this end, human callus was exposed to several (di)isocyanates with the aim of identifying (specific) adducts to keratin. Callus is a readily accessible, rich source of human keratins. For the purpose of this particular study the use of callus can be regarded as a first step towards experiments with human skin. Experiments were performed with powdered callus in order to guarantee that all keratins were still in their original tertiary form and that only those sites which are accessible within the skin will be modified. Incubations were carried out in both H₂O-free DMSO, as well as in aqueous environment. There were several reasons for this. First, we were concerned that the isocyanates were too labile in aqueous environment in order to allow for adduct formation. Second, we believe that the exposures in DMSO mimic the situation of a skin exposure to a neat isocyanate, in which DMSO functions as a non-reactive carrier of the reactive agent towards the insoluble keratin protein. The exposures in water represent a more realistic *in vivo* scenario in which it is assumed that normal human skin contains a considerable amount of water. Since the studies were carried out with human callus containing insoluble keratin, we used rather high concentrations of isocyanates, facilitating adduct elucidation. We were not worried about side reactions at the protein, since the protein was in insoluble form. Self-evidently, experiments with lower concentrations of IC will be carried out in future work, in order to determine lowest detectable exposure levels. After isocyanate exposure of the callus, keratin was isolated according to standard protocols, enzymatically digested and analysed by LC-(tandem) mass spectrometry.

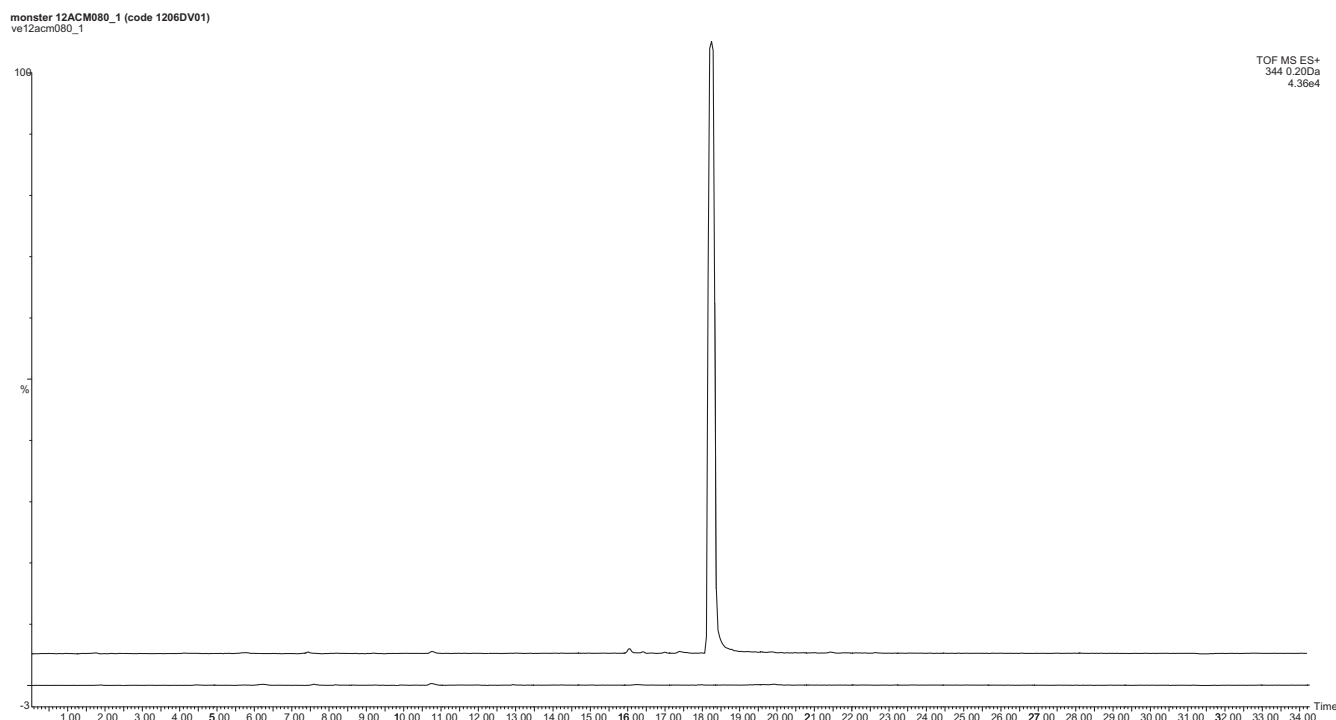


Fig. 1. LC-ES MS analysis (LC-system I). Extracted ion chromatograms of m/z 344, the protonated molecular ion $[M + H]^+$ of the lysine-4BrPIC adduct (see Table 1 for structure) in pronase digests of callus. The lower trace shows the procedure blank (0 mM) and the upper trace shows callus exposed to 4-BrPIC (10 mM).

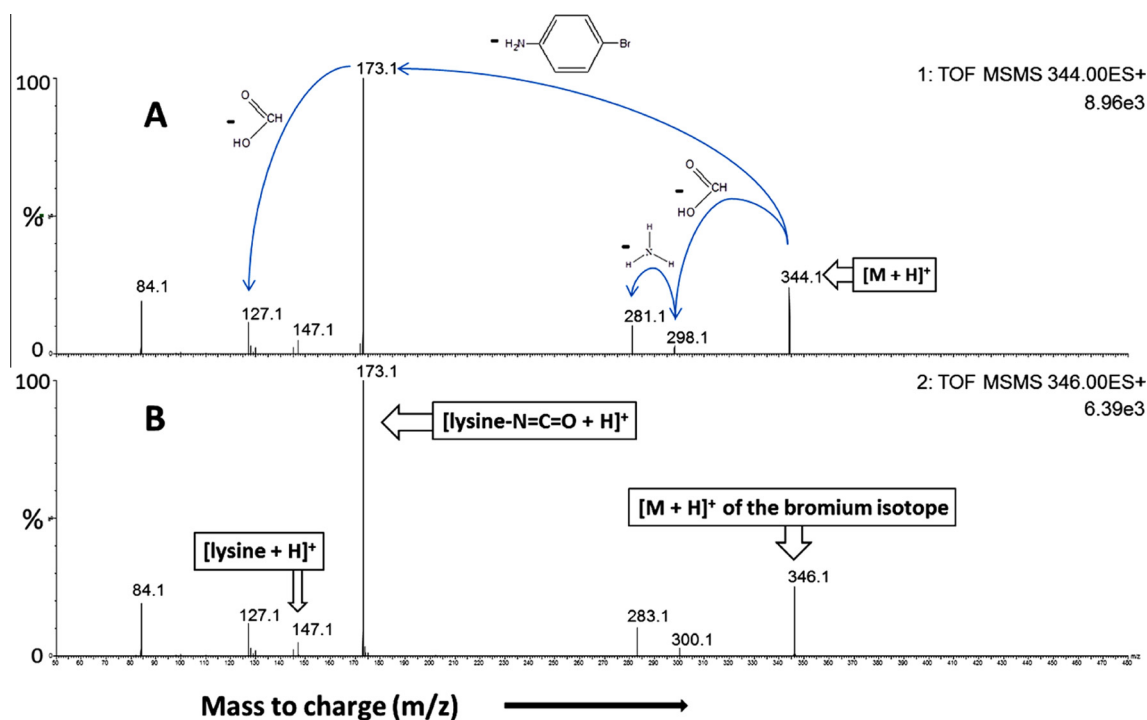


Fig. 2. Product ion mass spectra of the protonated molecular ions $[M + H]^+$ 344/346 of the lysine-(4-BrPIC) adduct in callus exposed to 4-BrPIC. Loss of the 4-bromoaniline from the adduct results in m/z 173.1, the $[lysine-NCO]^+$ mass fragment typical for isocyanate labeling.

We used 4-bromophenyl isocyanate (4-BrPIC) as a screening tool in our experiments to facilitate the mass spectrometric elucidation of the respective binding sites. Bromium atoms produce a specific, easily identifiable isotope pattern in the MS spectrum. We started with pronase digestion of exposed and extracted keratin because this results in the formation of (predominantly)

individual amino acids, with a particular type of modification of an amino acid accumulating in one signal, independent of the specific modification site within the protein.

LC-tandem mass spectrometric analysis of the pronase digests of 4-BrPIC-exposed keratins revealed that the adduct with lysine was predominant, with the modification on the ϵ -amino function

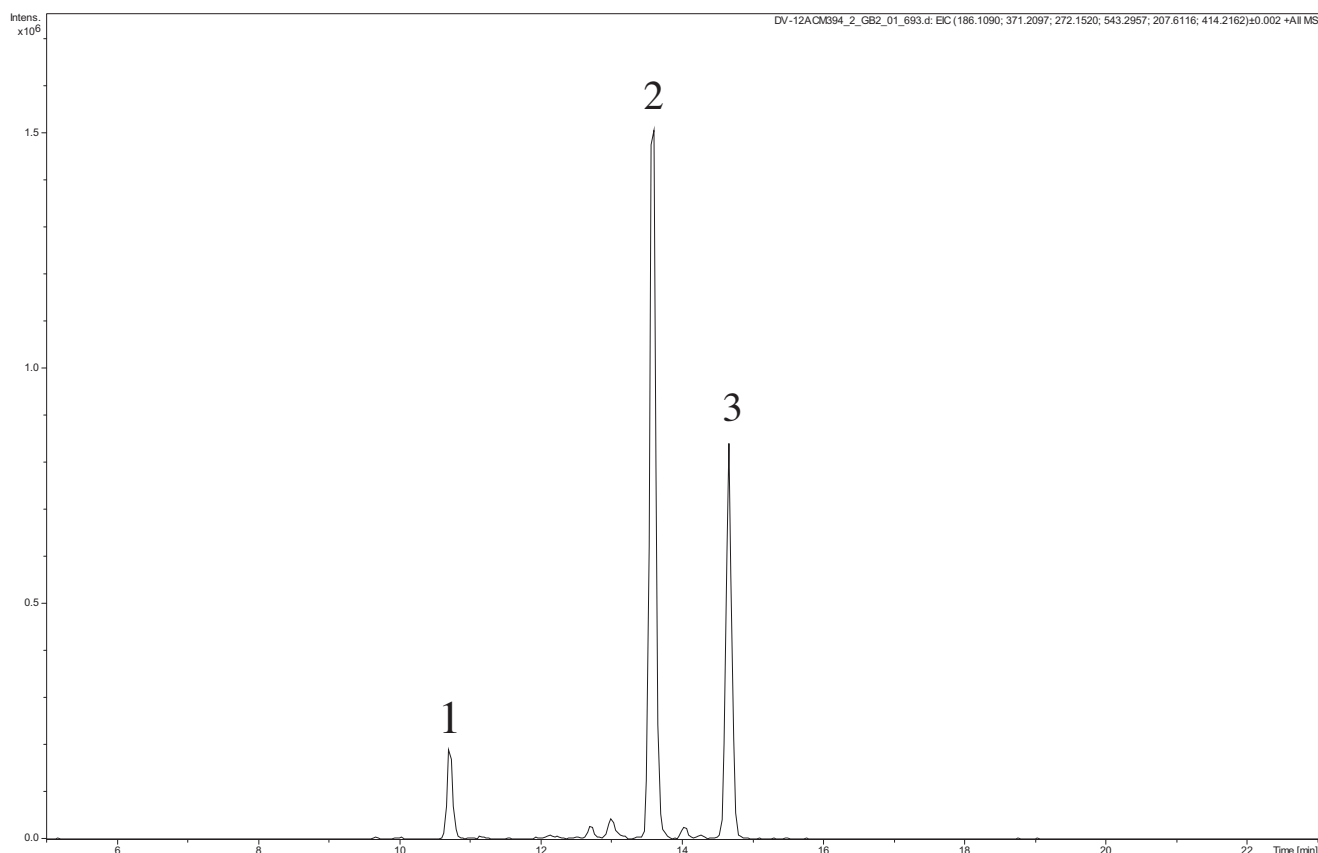


Fig. 3. LC-ES MS analysis (LC-system III). Extracted ion chromatograms of the sum of the doubly protonated molecular ion $[M + 2H]^{2+}$ and the protonated molecular ion $[M + H]^+$ of the lysine-MDI-NH₂-adduct (peak 1, $186.1090^{2+} + 371.2097^+$), of the lysine-MDI-di-adduct (peak 2, $272.1520^{2+} + 543.2957^+$) and of the lysine-MDI-NHCONH₂-adduct (peak 3, $207.6116^{2+} + 414.2162^+$) in pronase digests of callus exposed to MDI in water (10 mM). See Table 1 for the structures.

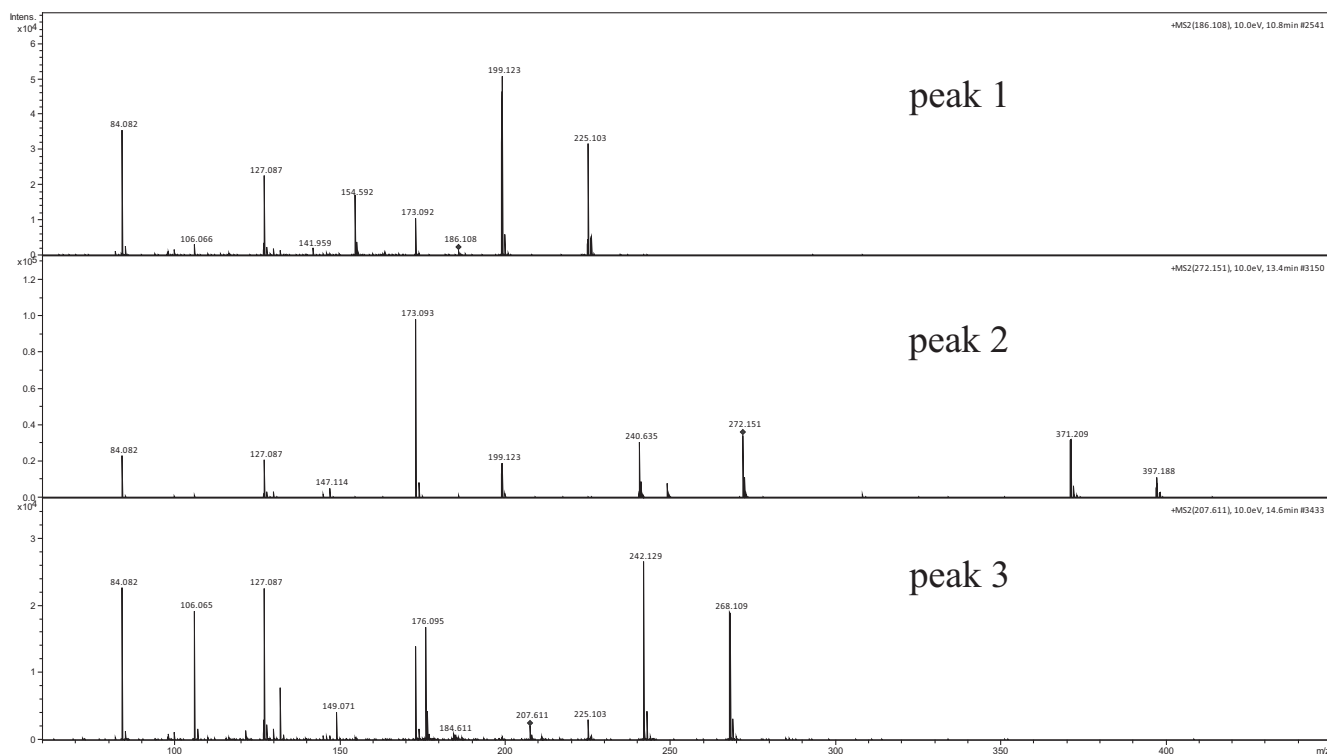


Fig. 4. Product ion mass spectra of $[M + 2H]^{2+}$ 186 (peak 1, lysine-MDI-NH₂-adduct), of $[M + 2H]^{2+}$ 272.2 (peak 2, lysine-MDI-di-adduct) and of $[M + 2H]^{2+}$ 207.6 (peak 3, lysine-MDI-NHCONH₂-adduct); see Fig. 3. Elucidation of the ions; m/z 84.082 ($C_5H_{10}N^+$), m/z 106.065 ($C_7H_8N^+$), m/z 127.087 ($C_6H_{11}N_2O^+$), m/z 154.592 ($C_{19}H_{23}N_3O_2^+$), m/z 173.093 ($C_7H_{13}N_2O_3^+$), m/z 176.095 ($C_{20}H_{24}N_4O_2^+$), m/z 199.123 ($C_{13}H_{15}N_2^+$), m/z 225.103 ($C_{14}H_{13}N_2O^+$), m/z 240.635 ($C_{26}H_{35}N_5O_4^+$), m/z 242.129 ($C_{14}H_{16}N_3O^+$), m/z 268.109 ($C_{15}H_{14}N_3O_2^+$) and m/z 371.209 ($C_{20}H_{27}N_4O_3^+$). Collision energy 10 eV.

(see Table 1 for its chemical structure). Fig. 1 shows the extracted ion chromatogram (EIC) of the 4-BrPIC adducted lysine residue, with the MS/MS spectrum of the adduct (Fig. 2) clearly showing the unique bromium isotope pattern.

For all other isocyanates used in this study, the lysine adducts were the most prominent (see Table 1 for an overview of the most significant adduct structures and corresponding monoisotopic mass). In case of the di-isocyanate MDI, a significant amount of the di-adduct was formed, in which two lysine residues are bridged through the di-isocyanate entity. This is in line with the results obtained by Hettick et al. [20]. The other prominent adduct for MDI was the mono-lysine adduct with the second isocyanate function hydrolysed to an NH_2 . Interestingly, in case of MDI also a urea type ($\text{R}-\text{NH}(\text{C}=\text{O})-\text{NH}_2$) adduct was observed, which is probably formed through reaction of the intact second isocyanate moiety with ammonia present in the NH_4HCO_3 buffer. See Figs. 3 and 4 for mass spectrometric analysis of the pronase digest of the MDI-exposed keratin sample. Remarkably, the urea-type adduct was not observed in the case of 2,6-TDI, though the other two adducts (di- and mono) were similar. As yet, we have no explanation for this. The prominent occurrence of the lysine di-adduct in case of MDI and TDI has also been observed for other proteins, e.g. albumin [19,20]. All daughter ion MS/MS spectra of elucidated lysine-isocyanate adducts from Table 1 show an abundant fragment ion at m/z 173 which can be attributed to $[\text{lysine}-\text{NCO}]^+$,

similar to the $[\text{b}1 + \text{NCO}]^+$ fragments reported by Kumar et al. [19]. This “diagnostic” ion can be used to determine the mass of the isocyanate moiety in unknown lysine-isocyanate adducts in parent scanning mode with triple quadrupole instruments. In high resolution mass spectrometers (Qtof and Orbitrap) the accurate mass of this diagnostic ion m/z 173.0921 ($\text{C}_7\text{H}_{13}\text{N}_2\text{O}_3^+$) can be used for specific and sensitive determination of lysine isocyanate adducts if the instrument is optimized for “in source” fragmentation. In this generic way of measurement we expect to detect levels of isocyanate exposures of 10–100 μM . In case of exposure to known isocyanates the lowest detectable levels for targeted analyses are expected to be reduced to 1–10 μM by using triple quadrupole mass spectrometers in the MRM (Multi Reaction Monitoring) mode. Especially in case of the less volatile isocyanates, percutaneous exposure can be prominent, giving rise to high local concentrations of chemical on the skin. We therefore envisage that the reported adducts can have potential value as biomarkers of (occupational) exposure to isocyanates.

In order to unequivocally assess that modification had indeed occurred at the ϵ -amino function of lysine (and not at the α -amino), we prepared synthetic standards for two of the lysine-isocyanate adducts (i.e., the lysine adducts resulting after modification by PEIC and AIC) according to the method reported by Kumar et al. [19] with some slight modifications. So, reaction of PEIC/AIC with $\text{N}-\alpha$ -(tert-butyloxycarbonyl)-L-lysine (Boc-L-Lys),

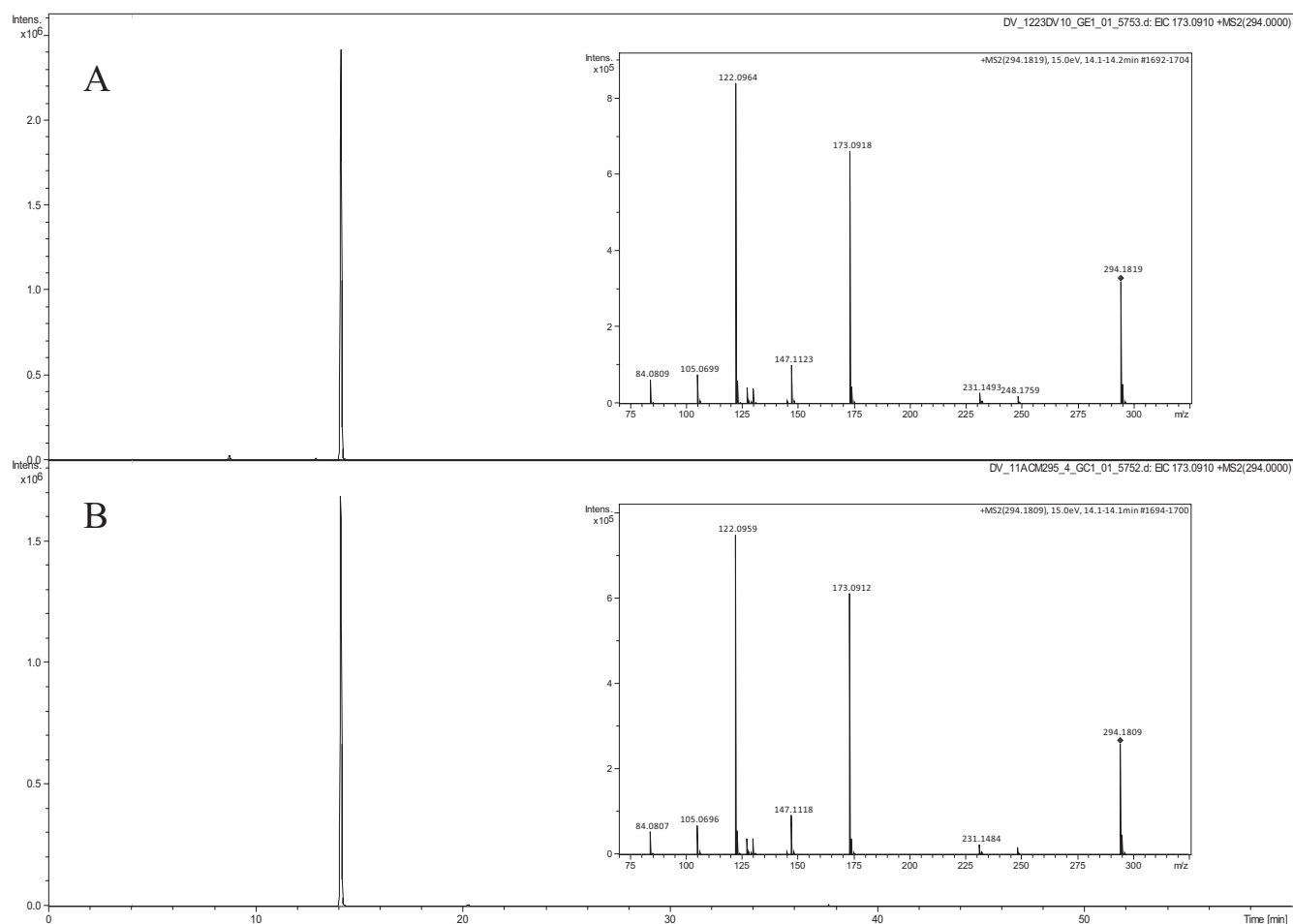


Fig. 5. LC-ES MS/MS analysis (LC-system V). Extracted ion chromatograms of m/z 173.091, the abundant fragment ion in the product ion mass spectrum (MS/MS) of $[\text{M} + \text{H}]^+$ 294.18 of the lysine-PEIC adduct (see Table 1 for structure). Trace A shows callus exposed to PEIC (10 mM) and trace B shows the synthesized standard lysine-PEIC (ϵ -isomer). Both compounds elute after 14.1 min. and the MS/MS spectrum (see inserts) are identical. Elucidation of the ions; m/z 84.0807 ($\text{C}_5\text{H}_{10}\text{N}^+$), m/z 105.0696 (C_8H_9^+), m/z 122.0960 ($\text{C}_8\text{H}_{12}\text{N}^+$), m/z 147.1118 ($\text{C}_6\text{H}_{15}\text{N}_2\text{O}_2^+$), m/z 173.0912 ($\text{C}_7\text{H}_{13}\text{N}_2\text{O}_3^+$), m/z 231.1484 ($\text{C}_{14}\text{H}_{19}\text{N}_2\text{O}^+$), m/z 248.1748 ($\text{C}_{14}\text{H}_{22}\text{N}_3\text{O}^+$) and $[\text{M} + \text{H}]^+$ 294.1809 ($\text{C}_{15}\text{H}_{24}\text{N}_3\text{O}_3^+$, theoretical value 294.1812). Collision energy 15 eV.

Table 2

Tryptic fragments of callus, exposed to 4-bromophenylisocyanate (4-BrPIC) in DMSO and in H₂O. See Table 1 for the structure of lysine-4-BrPIC adduct (MW343.053). All modified peptides, found in DMSO exposure were identified by LC-MS/MS. Almost no cleavage occurred at the C-terminal site of modified lysines.

Tryptic peptide	MW _{monoisot}	Type of keratin (<i>Homo sapiens</i>)	DMSO/H ₂ O exp.
VDQLK(mod)SDQSR*	1371.541	1	DMSO
LRSEIDNVK(mod)K	1397.630	1	DMSO/H ₂ O
NSK(mod)IEISELNR	1498.641	1	DMSO/H ₂ O
RVDQLK(mod)SDQSR*	1527.642	1	DMSO
TNAENEFVTIK(mod)K	1589.672	1	DMSO
NK(mod)LNDLEDALQQA	1795.774	1	DMSO
SLNNQFASFDK(mod)VR*	1834.800	1	DMSO
NVEIDPEIQK(mod)VK(mod)SR	2047.800	1	DMSO
LDSELK(mod)NMQDMVEDYR	2181.834	1	DMSO
SKAEAESLYQSK(mod)YEELQITAGR	2697.191	1	DMSO
NTK(mod)HEISEMNR	1554.588	5	DMSO/H ₂ O
ATGGGLSSVGGGSSTIK(mod)YTTTSSSSR	2602.114	6B	DMSO
AIGGGLSSVGGGSSTIK(mod)YTTTSSSSR	2614.151	6A, 6C, 6E	DMSO
SRAEAEWYQTK(mod)YEELQVTAGR	2798.193	6A, 6C, 6E	DMSO
GEMALK(mod)DAK*	1158.437	6A, 6B, 6C, 6E	DMSO/H ₂ O
SLYGLGSK(mod)R	1233.514	6A, 6B, 6C, 6E	DMSO
LRSEIDHVK(mod)K	1420.646	6A, 6B, 6C, 6E	DMSO/H ₂ O
NTK(mod)QEIAEINR*	1511.636	6A, 6B, 6C, 6E	DMSO/H ₂ O
K(mod)AALEK	855.349	9	DMSO/H ₂ O
IK(mod)FEMEQLNR	1503.617	9	DMSO/H ₂ O
HGVQLEIELQSLK(mod)K	2162.000	9	DMSO
LASYLDK(mod)VQALEEANNLENK	2573.128	9	DMSO
TK(mod)VMDVHDGK	1325.507	14	DMSO
TK(mod)YEELNLR	1462.620	14	DMSO
TK(mod)YEHELALR*	1455.614	16	DMSO/H ₂ O
K(mod)NHEEMLALR***	1565.629	16	DMSO/H ₂ O
DQYEQMAEK(mod)NR*	1607.567	16	DMSO/H ₂ O
DAETWFLSK(mod)TEELNK*	2006.826	16	DMSO
TEELNK(mod)EVASNSLVQSSR	2315.986	16	DMSO
NK(mod)IIAATIENAPILQIDNAR	2502.223	16	DMSO
WTLLEQEGTK(mod)TVR	1755.794	5, 6A, 6B, 6C, 6E	DMSO/H ₂ O
ASLENSLEETK(mod)GR*	1629.663	14, 16	DMSO
DYSPYFK(mod)TIEDLR	1842.746	14, 16	DMSO
EVATNSLVQSGK(mod)SEISELR	2372.049	14, 17	DMSO
NK(mod)YEDEINKR	1504.594	1, 5, 6A, 6B, 6C, 6E, 8	DMSO/H ₂ O
NKYDEINK(mod)R	1504.594	1, 5, 6A, 6B, 6C, 6E, 8	DMSO/H ₂ O
NK(mod)YEDEINK(mod)R	1701.542	1, 5, 6A, 6B, 6C, 6E, 8	DMSO/H ₂ O
ALEENADLEVK(mod)IR	1766.783	13, 14, 16	DMSO/H ₂ O
ILLDVK(mod)TR*	1153.549	14, 16, 17	DMSO/H ₂ O
LASYLDK(mod)VR***	1260.550	10, 14, 15, 16, 17, 19	DMSO/H ₂ O
TLNNK(mod)FASFDKVR	1848.852	2, 3, 5, 6A, 6B, 6E, 7, 8, 75	DMSO

* These peptides were also present in tryptic digests, with lysine (K) modified with PEIC (exposure in water), according to the structure given in Table 1.

** This peptide was, for instance, also identified in tryptic digests of callus exposed to other isocyanates in DMSO, with lysine modified with MDI-NH₂, PEIC, MOI and AIC.

*** This peptide was also identified in a tryptic digest, with lysine modified with 2,6-TDI-NH₂ (exposure in water) and also in tryptic digests, with lysine modified with MDI-NH₂, PEIC, MOI and AIC (exposure in DMSO).

followed by removal of the protecting group, gave the ϵ -amino modified lysine derivatives. Comparison of the synthetic standards with the PEIC and AIC-lysine adduct found in the pronase digests of keratin from PEIC or AIC-exposed human callus showed identical retention times and mass spectrometric data (see Fig. 5 for the PEIC lysine adduct).

It appeared that the adduct levels in case exposure was performed in H₂O-free DMSO was markedly higher than when performed in aqueous buffer. This result is as expected, since on the one hand the hydrolysis of the IC will be less prominent, and on the other hand the potential binding sites within keratins might be better accessible for the isocyanates because of swelling of the callus in DMSO.

In addition to the ϵ -amino lysine adducts, we identified several other isocyanate-modified amino acids, in line with the results published by Hettick et al. [21] and Sabbioni et al. [22,23]. These modifications are most likely the result of isocyanate binding to the α -amino group of N-terminal amino acids of the various keratins and constituted an almost negligible fraction of the total modified residues. However, we cannot entirely exclude the possibility that these adducts were formed because of trace amounts of intact isocyanate still present during enzymatic digestion.

The next step involved trypsin digestion of the isocyanate-modified keratins and subsequent mass spectrometric identification of modified tryptic peptides in order to provide more definitive proof that indeed keratins had been modified by the isocyanates (and not protein impurities present in the keratin sample), as well as to localize the actual modification sites within the keratins. Since we did not use purified keratin for the exposures, but extracts of human callus which consist of a mixture of homologous keratin proteins, identification of modification sites could not be done by simple database screening for the expected mass shift of the theoretical tryptic fragments. It should also be kept in mind that the elucidation of modified tryptic digests in this case is more complex because the adduct sites (lysine residues) coincide with the actual cleavage site, leading to a different cleavage of modified proteins compared to the native ones. In this case we also successfully employed 4-BrPIC as a screening tool to identify modified tryptic peptides. Mass spectra were screened manually for the presence of bromine isotope patterns, which led to identification of a large number of modified target peptide fragments after applying tandem MS analysis (see Table 2). A number of these tryptic peptide fragments could also be identified in the MDI, 2,6-TDI, PEIC, MOI and AIC-exposed samples. Figs. 6 and 7 show

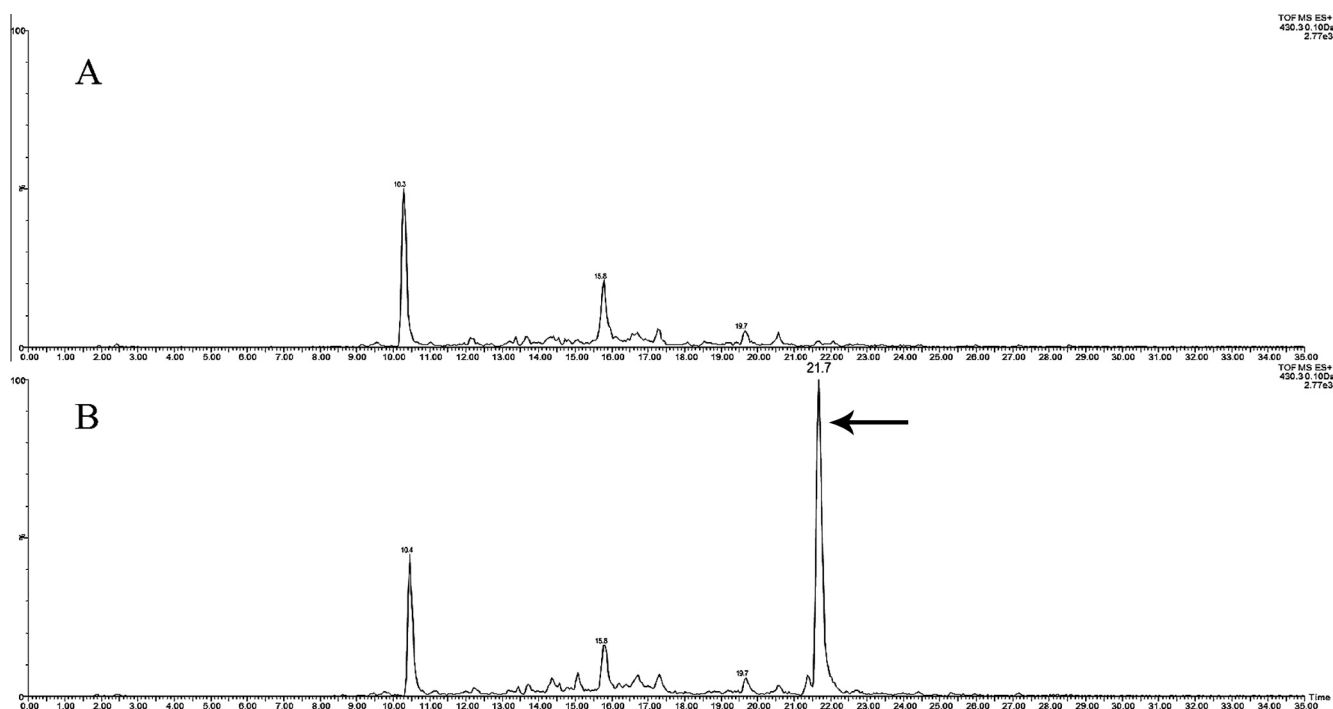


Fig. 6. LC-ES MS analysis (LC-system II). Extracted ion chromatograms of m/z 430.3, $[M + 3H]^{3+}$ of the modified LASYLDK*VR in tryptic digest of callus. The 224 Da mass shift is from the modification at lysine. K* indicates the site of the modification (see Table 1 for the structure). Trace A shows the procedure blank (0 mM) and trace B shows callus exposed to MDI (10 mM).

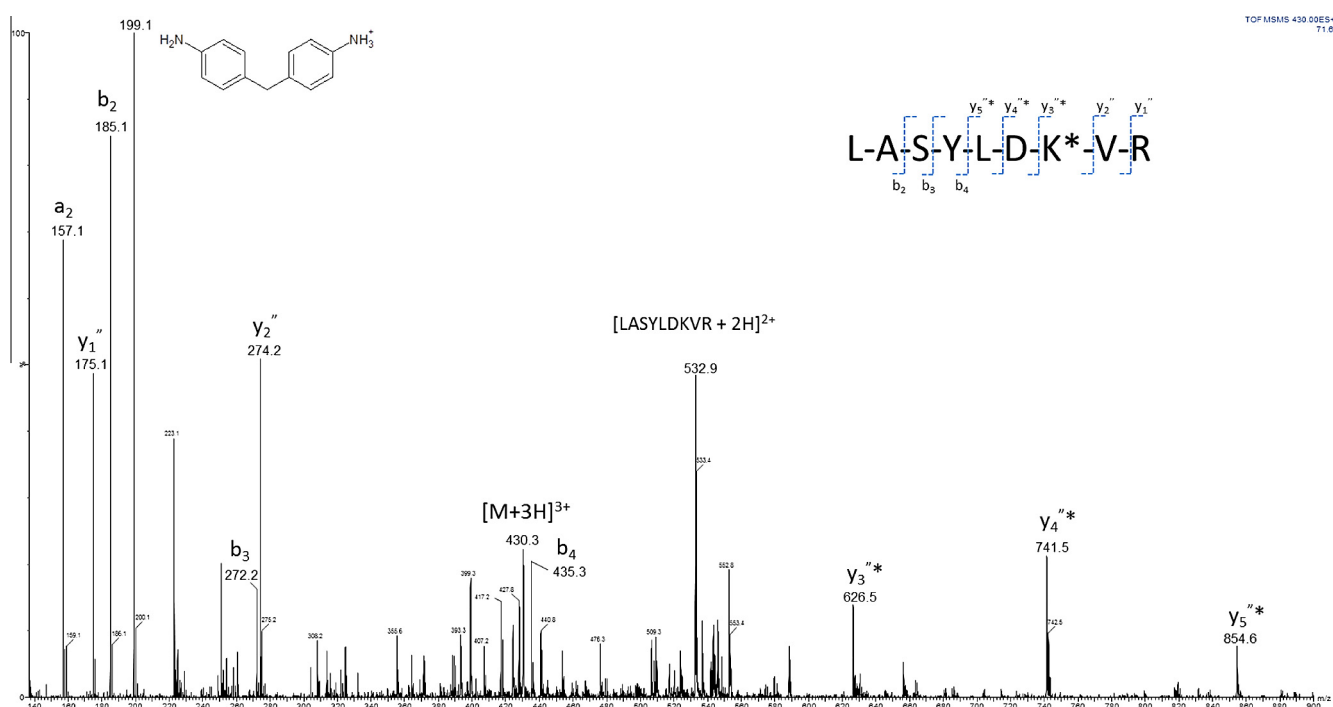


Fig. 7. Product ion mass spectrum of $[M + 3H]^{3+}$ 430.3 of LASYLDK*VR in a tryptic digest of callus exposed to MDI. Characteristic fragments in the spectrum: m/z 157.1 (a_2), m/z 175.1 (y_1''), m/z 185 (b_2), m/z 199.1 (MDI moiety), m/z 272.2 (b_3), m/z 274.2 (y_2''), m/z 435.3 (b_4), m/z 532.9 $^{2+}$ (doubly charged molecular ion of peptide without modification), m/z 626.5 (y_3''), m/z 741.5 (y_4'') and m/z 854.6 (y_5''). Collision energy 16 eV.

relevant EIC and MS/MS spectra of an elucidated modified tryptic peptide (LASYLDK*VR) in case of MDI exposure. Interestingly, this particular peptide was previously reported by us for being modified at the tyrosine (Y) residue by various organophosphates [24]. These peptide sequences are homologous sequences present in

several of the skin keratin subtypes, i.e. in K9, K10, K14, K16, K17 and K5, K6, respectively, and are apparently highly prone to modification by electrophilic chemicals. It should be kept in mind that modification of additional sites within the keratins could have occurred, but that the levels of those modified tryptic fragments

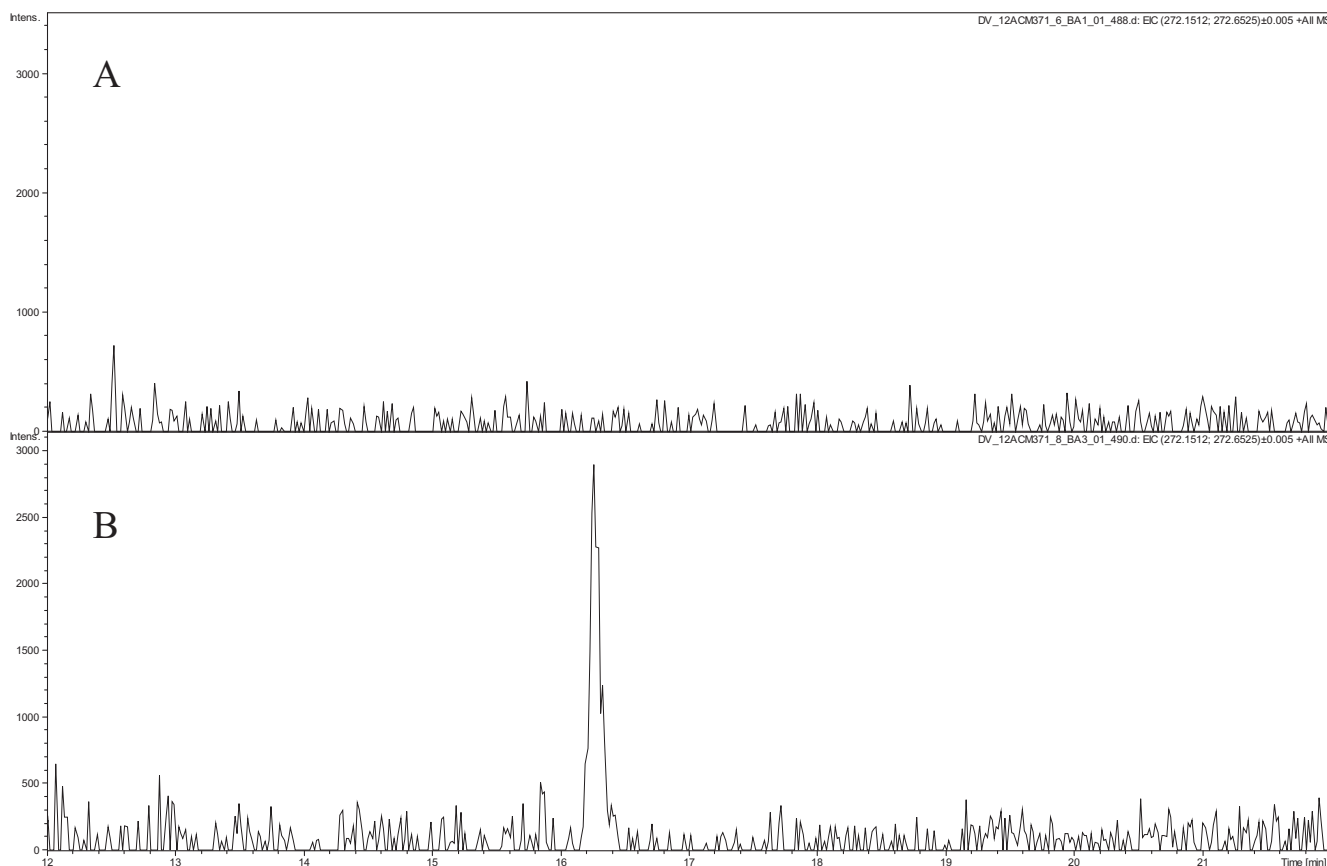


Fig. 8. LC-ES MS analysis (LC-system IV). Extracted ion chromatograms of m/z 272.151 + 272.652 (± 0.005 Da), the doubly charged molecular ion $[M + 2H]^{2+}$, including the isotope, of the lysine-MDI di-adduct (MW 542.285; see Table 1 for structure) in pronase digests of lung elastin. Trace A shows the procedure blank (0 mM) and trace B shows lung elastin after exposure to MDI in water (10 mM).

might be too low for unequivocal detection and elucidation. This might also explain why we did not observe any intra-molecularly crosslinked tryptic peptides, which were to be expected on the basis of the results obtained for the pronase digests (see Table 1).

In conclusion, in this paper we have shown that exposure of human callus to isocyanates leads to covalent modifications within keratin proteins. Although evidence for conjugation of isocyanates with keratins was already presented by Wisniewski et al. [25] and more recently also by Nayak et al. [26], no mass spectrometry data of discrete adducts were reported in these studies. Both mono- and di-adducts are predominantly formed on the ϵ -amino group of lysine residues, in line with results published for other proteins [19,21]. Keratins have highly homologous amino acid sequences and are also abundantly present in the lung epithelium. It can be envisaged that once autoantibodies to skin keratins have formed, they can also respond to the isocyanate-modified keratins present in the lung epithelium. The same may even account for other proteins present in the lung. Thus, in a preliminary experiment a suspension of lung elastin in water was exposed to MDI (10 mM) and after washing the protein was digested with pronase. Mass spectrometric analysis revealed the presence of the similar MDI lysine di-adduct as was formed in skin keratin (see Fig. 8). The elucidated adducts will also have great potential for use as biomarkers in order to assess skin exposure to isocyanates. Advantageously, the various lysine adducts display the presence of a m/z 173.1 daughter fragment $[\text{lysine-NCO}]^+$, enabling generic and rapid screening for exposure to isocyanates when used as diagnostic ion. In this respect, it has been reported that under negative ESI the major fragment of such adducts is 145.1 [19], which would also be useful to apply in mass spectrometry-based biomonitoring studies. Such a

generic screening could easily be integrated in future biomonitoring procedures, e.g. by employing tape-stripping of skin [27].

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The [Transparency document](#) associated with this article can be found in the online version.

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