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# Accurate quantification of the mercapturic acids of acrylonitrile and its genotoxic metabolite cyanoethylene-epoxide in human urine by isotope-dilution LC-ESI/MS/MS

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ABSTRACT

Acrylonitrile is a highly important industrial chemical with a high production volume worldwide, especially in the plastics industry. It is classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC group 2B). During metabolism of acrylonitrile, the genotoxic metabolite cyanoethylene-epoxide is formed. The urinary mercapturic acids of acrylonitrile, namely *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine (CEMA) and cyanoethylene-epoxide, namely *N*-acetyl-*S*-(1-cyano-2-hydroxyethyl)-L-cysteine (CHEMA) are specific biomarkers for the determination of individual internal exposure to acrylonitrile and its highly reactive metabolite.

We have developed and validated a sensitive method for the accurate determination of CEMA and CHEMA in human urine with a multidimensional LC/MS/MS-method using deuterium-labelled analogues for both analytes as internal standards. Analytes were stripped from urinary matrix by online extraction on a restricted access material, transferred to the analytical column and determined by tandem mass spectrometry. The limit of quantification (LOQ) for CEMA and CHEMA was 1 μg/L urine and allowed to quantify the background exposure of the (smoking) general population. Precision within and between series for CHEMA ranged from 2.6 to 8.0% at four concentrations ranging from 8.3 to 86 μg/L urine, mean accuracy was between 94 and 100%. For CEMA, precision within and between series ranged from 2.4 to 14.5% at four concentrations ranging from 15.1 to 196 μg/L urine, mean accuracy was between 91 and 104%. We applied the method to spot urine samples of 83 subjects of the general population with no known occupational exposure to acrylonitrile. Median levels (range) for CEMA and CHEMA in urine samples of non-smokers ($n=47$) were 1.9 μg/L ( < 1–16.4 μg/L) and < 1 μg/L ( < 1–3 μg/L), while in urine samples of smokers ($n=36$), median levels were 184 μg/L (2–907 μg/L) and 29.3 μg/L ( < 1–147 μg/L), respectively. Smokers showed a significantly higher excretion of both acrylonitrile metabolites ($p<0.001$). Due to its automation and high sensitivity, our method is well suited for application in experimental studies on acrylonitrile metabolism or occupational studies.



## 1. Introduction

Acrylonitrile (CAS 107-13-1) is a highly reactive, colorless, volatile liquid that polymerises spontaneously. It is reasonably anticipated to be a human carcinogen based on sufficient evidence in animal experiments [1]. Consequently, IARC has rated acrylonitrile as a group 2B carcinogen, while the Deutsche Forschungsgemeinschaft (DFG) has classified it as a group 2 carcinogen [2,3]. An increased risk for lung cancer or prostate cancer has been linked with high occupational exposure to acrylonitrile of workers of the U.S. textile industry [1], but the results of several epidemiological studies are still inadequate to evaluate the relationship between human cancer and acrylonitrile exposure [1,4].

Acrylonitrile is one of the main basic chemicals in the production of synthetic fibres and plastics as well as rubber [1]. The production of acrylonitrile in the European Union was estimated to be 1.25 Mio t/year between 1994 and 1996 (USA: 1.5 Mio t in 1996) [1,5].

A main source of exposure to acrylonitrile for the general population is tobacco smoke, as it is a constituent of tobacco smoke with concentrations varying from 3 to 15 μg/cigarette [6]. Other exposure sources are negligible for the general population, since residual monomer concentrations of plastics (with potential contact to food) are regulated by law and ambient air concentrations are usually in the range of the LOD [5].

The metabolism of acrylonitrile has been intensively studied in rodents. Within metabolism of incorporated acrylonitrile, the

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highly reactive metabolite cyanoethylen-epoxide (CEO) is formed by liver cytochrome P 4502E1 [7]. The formation of CEO is considered to be responsible for the carcinogenic properties of acrylonitrile in animal experiments. CEO was shown to bind covalently to DNA at much higher rates than acrylonitrile itself and was shown to bind to the phosphate moiety of nucleosides, inducing DNA strand breaks [8,9]. Both acrylonitrile as well as the intermediate metabolite CEO might react with glutathione via enzymatic glutathione-S-tranferases (GSTs) as detoxification reaction. In case of direct reaction with glutathione, *N*-acetyl-*S*-cyanoethyl-cysteine (CEMA) is formed and excreted via urine [10,11]. Reaction of CEO with glutathione might lead to the formation of *N*-acetyl-*S*-(1-cyano)-2-hydroxethyl-cysteine (CHEMA) as well as to the formation of an intermediary cyanohydrin metabolite, which is unstable and finally leads to the excretion of *N*-acetyl-*S*-2-hydroxyethyl-cysteine (HEMA) after elimination of cyanide. The metabolism of acrylonitrile is illustrated in Fig. 1.

As HEMA is also a metabolite of ethylene oxide as well as ethylene with varying urinary background levels [12,13], excretion of HEMA is no longer specific for exposure to acrylonitrile and cyanoethylene-epoxide, respectively. Animal experiments in rats showed that CEMA and HEMA account for 40 and 30% of the dose excreted via urine, while CHEMA makes up for another 13% of the excreted dose [7].

Thus, the excretion of CHEMA is the only specific urinary biomarker of internal exposure to CEO, the cancer initiating metabolite of acrylonitrile. As human biomonitoring should always be aimed to determine the internal dose of the most harmful agent within metabolism of hazardous substances, the quantification of CHEMA in urine would provide valuable data for an accurate risk assessment of human exposures to acrylonitrile. This is especially true as the formation as well as detoxification of CEO is influenced by a number of different (polymorphic) enzymes whose influence is yet unknown [14]. So far, CHEMA has only been identified in urine of highly exposed rats and mice by GC/MS and $^{13}C$ –NMR [7,15]. Most recently, CHEMA as well as CEMA was quantified in human urine [16]. However, a labelled internal standard for CHEMA was lacking in that study and human data on excretion of CHEMA and the fraction of oxidative metabolism in humans are still sparse.

Therefore, the aim of our present study was the development, validation and finally application of an accurate analytical method for the simultaneous determination of the mercapturic acids of acrylonitrile (CEMA) and cyanoethylene-epoxide (CHEMA) in human urine using a previously established column-switching technology [11].

## 2. Experimental

### 2.1. Reagents and standards

All solvents and chemicals used were HPLC or analytical reagent grade. CEMA (*N*-acetyl-*S*-(2-cyanoethyl)cysteine, chemical purity: 98%) and CHEMA (*N*-acetyl-*S*-(1-cyano-2-hydroxyethyl)cysteine dicyclohexylamine salt, chemical purity: 98%) were purchased from TRC (Toronto, Canada). The internal standards $D_3$-CEMA (*N*-acetyl-$D_3$-*S*-(2-cyanoethyl)cysteine, chemical purity: 98%, isotopic purity: > 98%) as well as $D_3$-CHEMA (*N*-acetyl-$D_3$-*S*-(1-cyano-2-hydroxyethyl)cysteine dicyclohexylamine salt, chemical purity: 98%, isotopic purity: > 98%) were also purchased from TRC (Toronto, Canada). Identity and purity of all standards was confirmed by $^1H$ –NMR spectroscopy and mass spectrometric analysis as stated by the supplier (www.trc-canada.com).

Formic acid (100%) was supplied by Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Germany). Ammonium formate was supplied by Fluka (Buchs, Suisse). Standard solutions of both analytes as well as the labelled internal standards were prepared by dissolving 1 mg of CEMA, $D_3$-CEMA and CHEMA (complete amounts available) each in 1 ml of 0.1% aqueous formic acid, resulting in stock solutions of 1 g/L for CEMA and $D_3$-CEMA and 0.56 g/L for CHEMA (due to the molar ratio of the dicyclohexylamine salt to the free acid). In the case of $D_3$-CHEMA, 0.5 mg (complete amount available) was dissolved in 500 µl of 0.1% aqueous formic acid (0.56 g/L). A combined working solution of the internal standards was prepared by dilution with 0.1% aqueous formic acid (concentration 10 mg/L and 5.6 mg/L, respectively). All solutions were kept frozen at –20 °C in small dark brown screw top flasks.

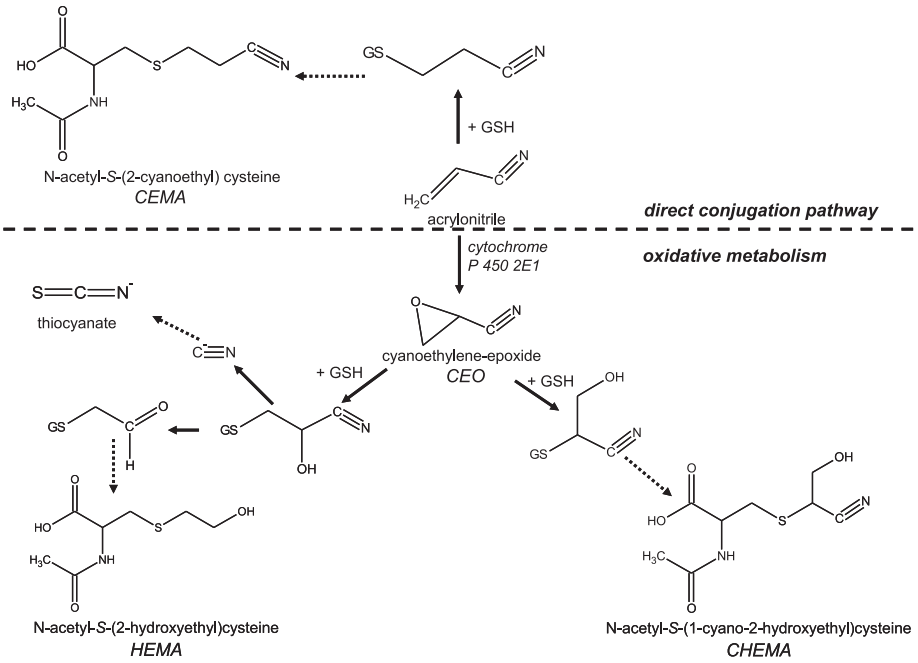


**Fig. 1.** Simplified metabolism of acrylonitrile (GSH = glutathione).

### 2.2. Standard preparation

From the stock solutions of CEMA and CHEMA, two combined working solutions were prepared by dilution with 0.1% aqueous formic acid (concentrations: 10 mg/L CEMA and 5.6 mg/L CHEMA as well as 100 μg/L and 56 μg/L, respectively). From these working solutions of the analytes, 7 calibration standards were prepared by spiking pooled urine obtained from 4 non-smoking persons of the general population (creatinine: 0.63 g/L). These spot urine samples were pooled, frozen at –20 °C, thawed, acidified by adding 1 vol% acetic acid (100%) and filtered by a fluted filter before use. The pooled urine was spiked with concentrations of 5–1.000 μg/L for CEMA and 2.8–562 μg/L for CHEMA. The unspiked pooled urine was used as a blank. Additionally, a blank value consisting of water was included in every analytical series.

### 2.3. Sample preparation

Frozen urine samples were allowed to equilibrate to room temperature. The samples were vigorously shaken and 500-μl aliquots were then transferred to 1.8-ml glass screw-cap vials. Then 500 μl of ammonium formate buffer (50 mmol/L, adjusted to pH 2.5 with formic acid), 20 μl of formic acid (100%) and 10 μl of the working solution of the internal standards were added to the samples. The samples were vortex mixed and centrifuged at 800 *g* for 5 min. When necessary (due to protein precipitation), the supernatant was transferred to a new 1.8-ml glass screw-cap vial. A 50-μl aliquot was then injected into the LC-MS-MS system for quantitative analysis. Urinary creatinine concentrations of each sample were additionally determined photometrically according to Larsen using a 96-well-plate photometer [17].

### 2.4. Instrumentation

The liquid chromatographic system consisted of an Agilent autosampler G1313A equipped with a 50 μl loop, an Agilent G1312A binary pump and an Agilent vacuum degasser G1379A (Agilent, CA, USA) connected to a Sciex API 3000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA). All steps and data processing was controlled by Analyst 1.3.2 Software from Applied Biosystems. For column-switching purposes, a six-port valve (Valco Systems, Houston, TX, USA) controlled by the Analyst software was used and an additional Agilent G 1310A isocratic pump to deliver the eluent for online-enrichment.

The following columns were used in this study: a Luna C 8 (2) 150 × 4.6 mm I.D., 3 μm (Phenomenex, Aschaffenburg, Germany) as analytical column. A precolumn filter (0.5 μm, Supelco) and a guard column Luna C 8 (2), 4 × 3 mm I.D. (Phenomenex, Aschaffenburg, Germany) were placed in front of the analytical column to extend its lifespan. A LiChrocart 25-4 LiChrospher RP-8 ADS 25 × 4 mm I.D., 25 μm particle diameter (Merck, Darmstadt, Germany) was used for online-enrichment and clean up of the samples.

### 2.5. Chromatographic system

The chromatographic set-up has already been used for the quantification of several mercapturic acids in human urine in our laboratory [11,18,19]. For online-enrichment and clean-up, 50 μl of the prepared urine sample (see 2.3.) is injected into the system in position A (loading position) of the six-port valve and transferred to the LiChrospher-column by the isocratic pump using eluent A (water, adjusted to pH 2.5 using formic acid) as mobile phase at a flow rate of 0.3 ml/min. The analytes are retained on the hydrophobic bonded C-8-phase of the column and urinary matrix like proteins and other macromolecules are transferred to waste. After 0.5 min, the six-port-valve is switching to position B (backflush position) and the analytes are transferred to the analytical column in backflush mode using 12% (v/v) of acetonitrile (eluent B) in eluent A (water, adjusted to pH 2.5 using formic acid) delivered by the gradient pump at a flow rate of 0.3 ml/min. One minute later, the six-port-valve is switching to position A again and the separation of the analytes on the analytical columns is performed using the gradient described in Table 1. After 25 min, the system is ready for the next injection.

### 2.6. Tandem mass spectrometry

The tandem mass spectrometric detection was performed on a Sciex API 3000 LC/MS/MS system in ESI-negative mode. The ion-source conditions were identical for all analytes with an electrospray needle voltage of –3000 V in the negative ion mode. Nitrogen as nebulizer and turbo heater gas (425 °C) was set at a pressure of 65 psi. The curtain gas was set to 58 psi. The settings for nebuliser gas, curtain gas and collision gas (nitrogen) for the MS/MS-mode was set to a flow of 8, 8 and 10 instrument units, respectively.

Continuous flow injection (10 μl/min) of standard solutions (100 μg/L) in methanol for the analytes and the internal standards were used to establish the optimum MS/MS-conditions (generated by the Analyst software) for each analyte using a syringe pump (Model '22', Harvard Apparatus, Massachusetts, USA). Optimization of the ion-source conditions (temperature of turbo heater gas, electrospray needle voltage) was performed by triplicate analysis of an aqueous standard (200 μg/L CEMA, 112 μg/L

**Table 1**
Analysis program of the gradient pump.

| Program step | Time (min) | Eluent A (%) | Eluent B (%) | Flow-rate (ml/min) | Position of six-port-valve | Analysis step |
|---|---|---|---|---|---|---|
| 1 | 0 | 88 | 12 | 0.3 | A | Charging |
| 2 | 0.5 | 88 | 12 | 0.3 | B | Transfer |
| 3 | 1.5 | 88 | 12 | 0.3 | A | |
| 4 | 2.0 | 88 | 12 | 0.3 | A | Separation |
| 5 | 8.0 | 76 | 24 | 0.3 | A | |
| 6 | 10.0 | 60 | 40 | 0.3 | A | |
| 7 | 11.5 | 40 | 60 | 0.3 | A | |
| 8 | 17 | 40 | 60 | 0.3 | A | Washing |
| 9 | 21.0 | 88 | 12 | 0.3 | A | |
| 10 | 25 | 88 | 12 | 0.3 | A | Reconditioning |

The second pump (isocratic pump) continuously pumps the mobile phase A (water, adjusted to pH 2.5 using formic acid) for the enrichment step on the LiChrospher C-8-phase at a flow rate of 0.3 ml/min. Solvent A: water, adjusted to pH 2.5 using formic acid; solvent B: acetonitrile.

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

| Analyte | Retention time (min) | Parent ion (Q 1) | Daughter ion (Q 3) | DP | FP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|---|
| CHEMA | 9.29 | 231.0 | 83.9 | −21 | −60 | −10 | −16 | −13 |
| | | 231.0 | 101.9 | −21 | −60 | −10 | −20 | −5 |
| $D_3$-CHEMA | 9.27 | 234.0 | 83.9 | −26 | −60 | −10 | −16 | −13 |
| CEMA | 12.34 | 215 | 161.8 | −61 | −190 | −10 | −12 | −7 |
| $D_3$-CEMA | 12.30 | 218 | 165.0 | −11 | −70 | −10 | −12 | −7 |

DP, declustering potential (V); FP, focusing potential (V); EP, entrance potential (V); CE, collision energy (V); CXP, collision exit potential (V).

CHEMA) under the chromatographic conditions described in Section 2.5., but variations of electrospray voltage in steps of −500 V ranging from −2500 to −4500 V and variations of turbo heater gas temperature in steps of 25 °C ranging from 300 to 450 °C. After that, instrument settings of nebuliser, curtain and collision gas were optimized by triplicate analysis of the same sample under the optimized conditions for electrospray voltage and turbo heater gas temperature.

The operating parameters in the multiple-reaction-mode (MRM) were as follows: resolution of Q1 and Q3 was set to "unit", settling time 5 ms, MR pause 5 ms and scan time 300 ms. Retention times under the described conditions (see Section 2.5.) as well as analyte specific parameters for the mass spectrometric detection are shown in Table 2.

### 2.7. Study subjects

For a pilot study we investigated spot urine samples from 83 persons (79m, 4f) of the general population with no known occupational exposure to acrylonitrile. The age of these persons ranged from 21 to 77 years with a median age of 46 years. All urine samples were stored at −20 °C until analysis.

The subjects were asked about their smoking status. Furthermore, this anamnestic information was additionally verified by specific analysis of the urinary nicotine metabolite cotinine using a slightly modified LC/MS/MS-method developed by Xu et al. with a limit of detection of 1 μg/L urine for cotinine [20]. Forty-seven subjects (44 male, 3 female, with a median age of 48 years, ranging from 21 to 77 years of age) reported to be non-smokers and had urinary cotinine levels ranging from < 1 to 34 μg/L. Thirty-six subjects (35 male, 1 female with a median age of 45 years, ranging from 27 to 66 years of age) reported to be smokers with daily cigarette consumptions of 2–35 cig./day and urinary cotinine levels ranging from 56 to 4940 μg/L. All persons gave written consent about the donation of urine samples for scientific purposes. An approval of the ethics committee of the RWTH Aachen University (EK 206/09) is available for the collection of the urine samples.

### 2.8. Quality control

As there was no quality control material commercially available, one had to be prepared in the laboratory. For the quality control material we spiked a spot urine sample of a non-smoker (creatinine: 0.74 g/L) with concentrations of 150 ($Q_{high}$), 75 ($Q_{medium}$) and 15 μg/L ($Q_{low}$) CEMA as well as 84, 42 and 8.3 μg/L CHEMA. These quality controls were divided into aliquots and stored at −20 °C. For quality assurance, one of each quality control samples (high, medium, low) was included in every analytical series.

Furthermore, a urine sample of a heavy smoker (creatinine: 1.05 g/L, cotinine: 1360 μg/L) was available, divided into aliquots and stored at −20 °C, which was also included in every analytical series.

## 3. Results

### 3.1. Optimization of the method

The chromatographic set-up remained unchanged as compared to our previous publication on the determination of CEMA in human urine [11]. As previously discussed for DHBMA (a mercapturic acid derived from 1,3-butadiene), CHEMA showed only poor retention on the used LiChrospher C-8-column, even when using a completely aqueous solvent, which is mainly due to the high polarity of this analyte. Attempts with an LiChrospher C-4-column did not improve retention of CHEMA under comparable conditions. Despite the rather short time for enrichment (0.5 min), clean-up of the urine samples was satisfactory, allowing separation of the analytes from dissolved urinary proteins and macromolecules. Both analyte peaks as well the peaks of the internal standards are very sharp and show no chromatographic interferences. Fig. 2 shows an exemplary chromatogram of a processed smokers' urine sample that served as quality control.

The source-specific parameters (like electrospray voltage, turbo heater gas temperature) and instrument settings of the gases for tandem-mass spectrometric detection were optimised manually (cf. 2.6.). These parameters turned out to be of significant importance for the performance of the method, resulting in an improvement in sensitivity by a factor of 2-3 as compared to the "standard" setting.

In negative ESI mode, the selected precursor ions at the first quadrupole for both analytes and their labelled analogues were $[M\text{-}H]^-$. The product ion fragments selected were those with the maximum intensities for both analytes ensuring maximum sensitivity. Exemplarily, the product ion mass spectra for CHEMA and $D_3$-CHEMA together with the structures of the fragments for the transition are shown in Fig. 3. To check for a possible D–H isotope exchange interfering with the determination of the mercapturic acids, an aqueous blank value was included in every analytical series. No unlabelled mercapturic acids could be detected in this blank value, proving that the addition of internal standards does not produce any false positive results.

The limit of detection was estimated to be 0.5 μg/L for both analytes (based on a signal-to-noise ratio of three for the registered ion transitions in the range of the analyte peak using pooled urine) and the limit of quantification (LOQ) was 1 μg/L.

### 3.2. Reliability of the method

In order to determine the within-day repeatability, the three quality control samples ($Q_{low}$, $Q_{medium}$, and $Q_{high}$) as well as the smokers' urine sample ($Q_{real}$) were analysed six times in a row. These materials were spiked with 150, 75 and 15 μg/L CEMA and

84, 42 and 8.4 μg/L CHEMA. The relative standard deviations ranged between 2.2 and 8.8% for CEMA and 2.6–3.9% for CHEMA.

The relative standard deviation of the between-day repeatability was determined during ten analytical batches performed between October 2010 and October 2011 and ranged between 5.6 and 14.5% for CEMA and between 3.2 and 8.0% for CHEMA,

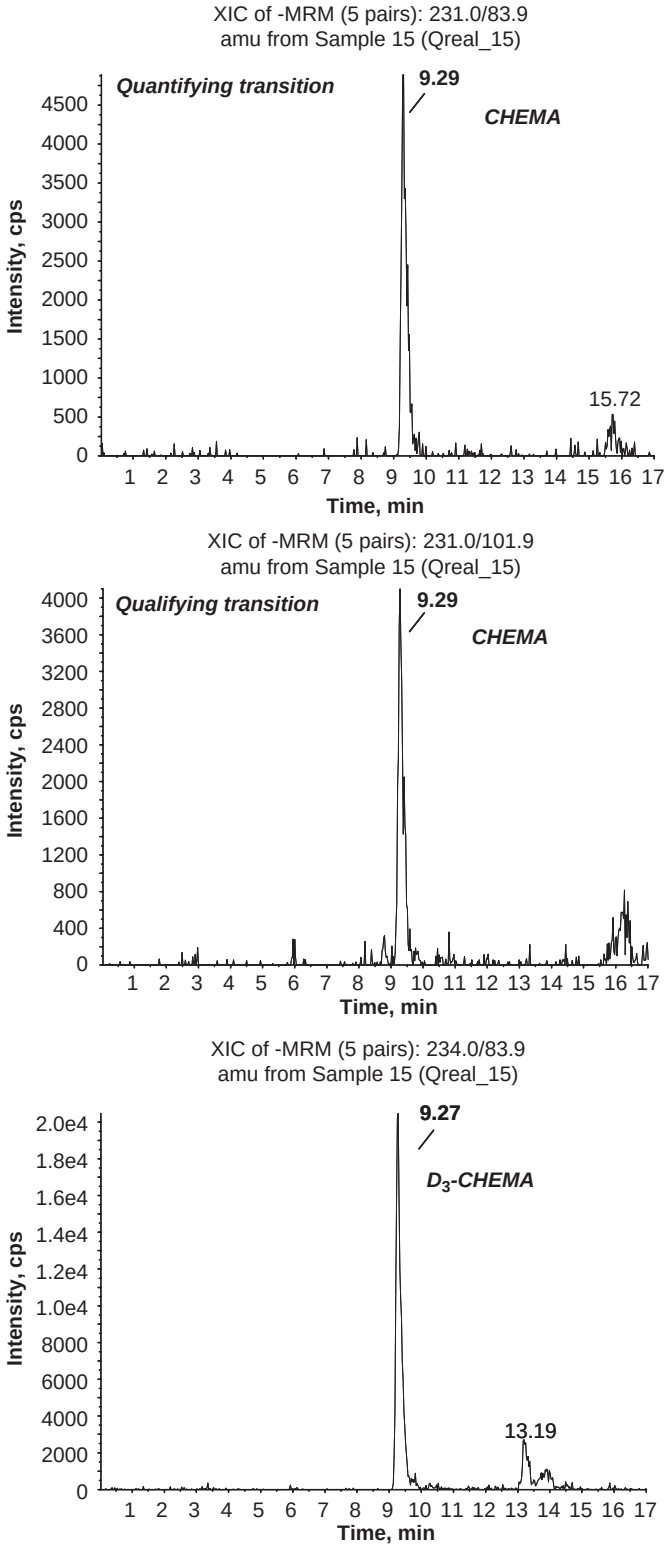


**Fig. 2.** Chromatogram of a processed urine sample of a smoking person not occupationally exposed acrylonitrile (creatinine: 1.05 g/L, cotinine: 1360 μg/L). Concentrations for CHEMA and CEMA were 23.4 μg/L and 187.8 μg/L, respectively.

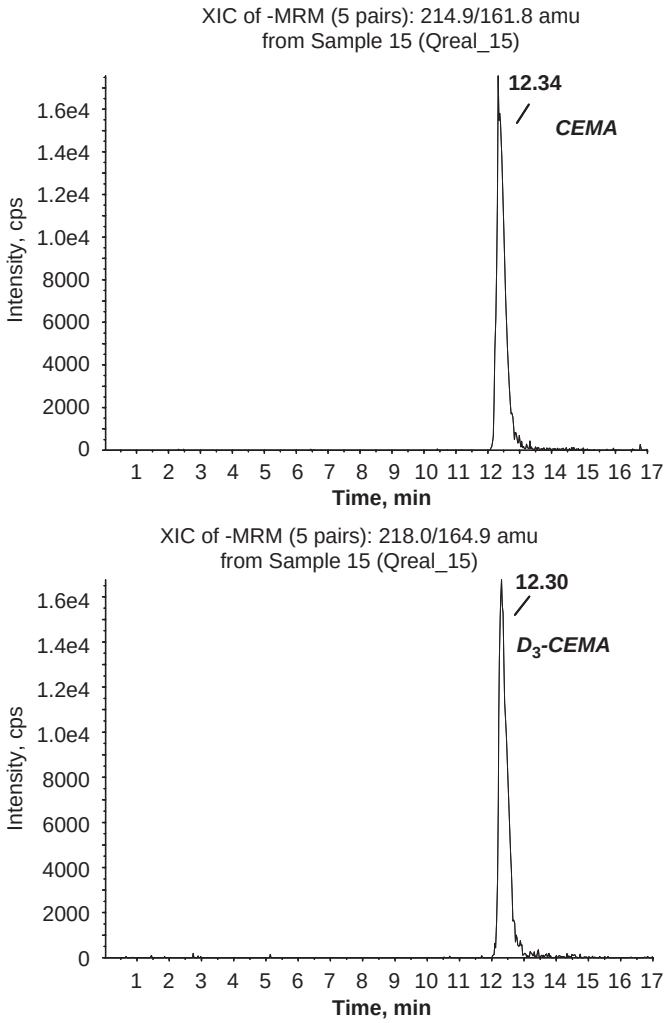


**Fig. 2.** (*continued*)

demonstrating very good repeatability of our method. Accuracy was calculated by comparing the mean results for within-day as well as between-day repeatability with the spiked amounts of the analytes for $Q_{low}$, $Q_{medium}$ and $Q_{high}$ and ranged between 94 and 100% for CHEMA and 91 and 104% for CEMA. These data are summarised in Table 3.

Furthermore, we have checked the accuracy of our method by a special recovery experiment. For this purpose, five different urine specimens were spiked with the analytes at a concentration of 200 μg/L CEMA and 112 μg/L CHEMA. The five urine specimens were selected to reflect a different composition as indicated by urinary creatinine content (range: 0.49–1.63 g/L). Good accuracy results under these conditions prove that the different biological matrix has no influence on the analytical result. For that experiment, mean relative recovery (under consideration of possible background excretions in blank urine samples) for CEMA and CHEMA was determined to be 95% (range: 86–102%) and 97.4% (range: 92–99.4%), respectively. Therefore, accuracy under these conditions can be regarded as excellent, which is mainly due to the use of labelled internal standards for both analytes.

In order to examine the ion suppressing influence of different urinary matrices on the peak signal, peak areas for both analytes of a triplicate analyses of the spiked urinary specimens were compared to peak areas of the triplicate analysis of an aqueous standard of the same concentration. In doing so, the matrix influence of possible co-eluting urinary components can be

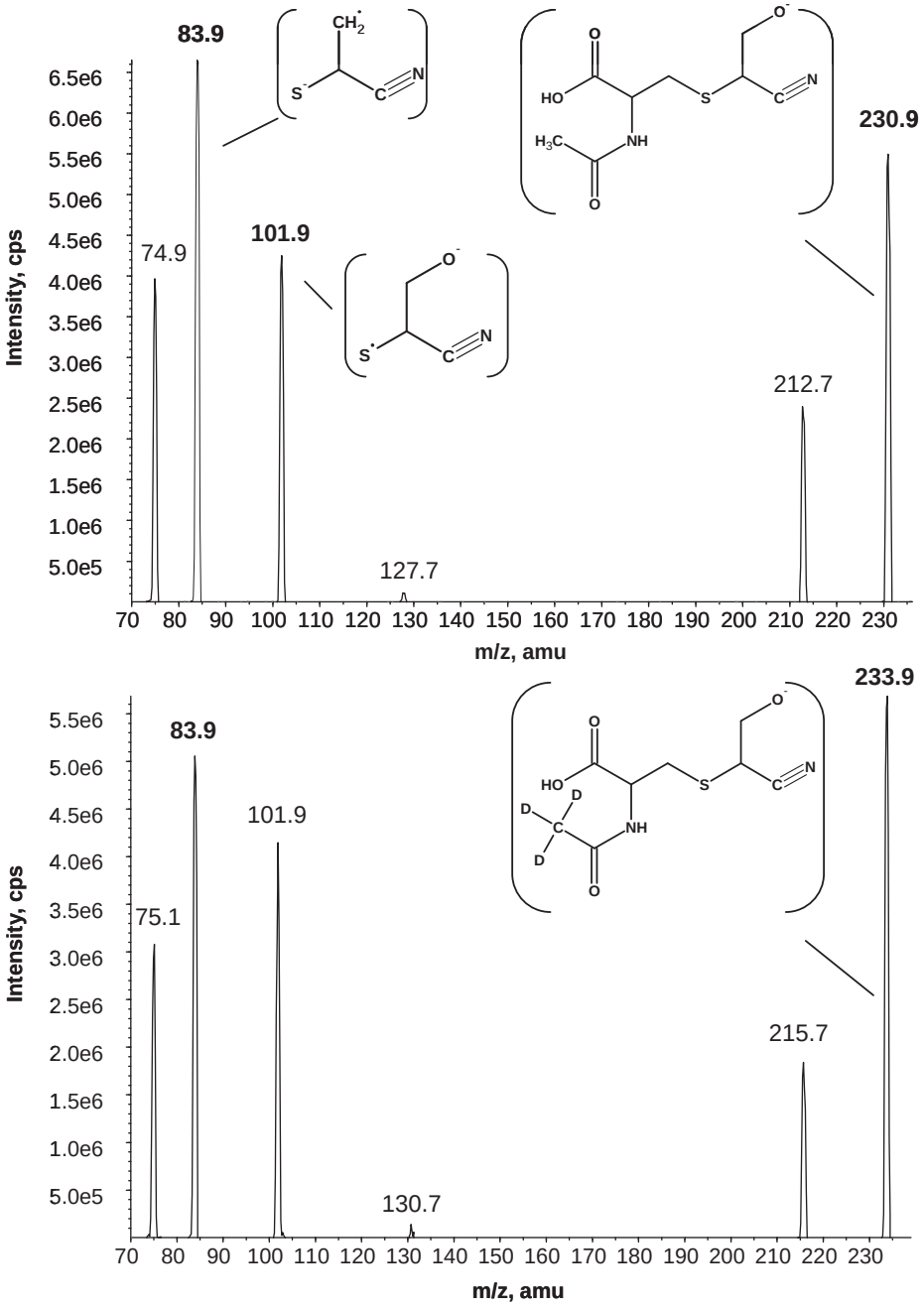


**Fig. 3.** ESI-negative product-ion mass spectra of CHEMA and $D_3$-CHEMA with the predicted structures of the fragments.

**Table 3**
Precision and accuracy data for the analytes.

| Analyte | | $Q_{low}$ | | $Q_{medium}$ | | $Q_{high}$ | | $Q_{real}$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | Within-series ($n=6$) | Between-series ($n=10$) | Within-series ($n=6$) | Between-series ($n=10$) | Within-series ($n=6$) | Between-series ($n=10$) | Within-series ($n=6$) | Between-series ($n=10$) |
| CHEMA | c (µg/L urine) | 8.3 | 8.3 | 41.4 | 42.0 | 82.9 | 85.7 | 23.1 | 23.2 |
| | RSD (%) | 3.9 | 8.0 | 2.6 | 5.9 | 2.9 | 3.2 | 3.5 | 4.7 |
| | Accuracy (%) | 98 | 98 | 98 | 100 | 98 | 94 | – | – |
| CEMA | c (µg/L urine) | 15.5 | 15.1 | 68.1 | 71.8 | 141.5 | 142.9 | 188.6 | 196.4 |
| | RSD (%) | 8.8 | 11.2 | 2.2 | 5.6 | 2.4 | 6.4 | 3.1 | 14.5 |
| | Accuracy (%) | 104 | 101 | 91 | 96 | 94 | 95 | – | – |

**Table 4**
Accuracy in five different urine specimens and effect of urinary matrix on signal intensity.

| Urine | Creatinine (g/L) | Analyte | Blank value (μg/L) | Spiked conc. (μg/L) | Conc. Found (μg/L) | Accuracy (%) | Mean quenching signal suppression effect on signal (%) |
|---|---|---|---|---|---|---|---|
| A | 0.49 | CHEMA | < 0.5 | 112 | 110.9 | 98.2 | 28 |
| | | CEMA | < 0.5 | 200 | 203.4 | 101.4 | 30 |
| B | 0.82 | CHEMA | < 0.5 | 112 | 112.0 | 99.4 | 14 |
| | | CEMA | 1.1 | 200 | 205.0 | 101.9 | 30 |
| C | 1.06 | CHEMA | < 0.5 | 112 | 111.4 | 98.7 | 22 |
| | | CEMA | 1.6 | 200 | 194.3 | 96.3 | 29 |
| D | 1.34 | CHEMA | 1.1 | 112 | 104.6 | 92.0 | 29 |
| | | CEMA | 2.2 | 200 | 174.0 | 85.9 | 40 |
| E | 1.63 | CHEMA | < 0.5 | 112 | 111.2 | 98.5 | 36 |
| | | CEMA | 1.4 | 200 | 180.5 | 89.5 | 44 |

**Table 5**
Results of biological monitoring of the acrylonitrile metabolites CEMA and CHEMA (and cotinine) in urine samples of non-smoking and smoking persons of the general population with no occupational exposure to acrylonitrile. Samples below LOQ (1 μg/L) were set to half the LOQ.

| | | CEMA | | CHEMA | | Cotinine | |
|---|---|---|---|---|---|---|---|
| | | μg/L urine | μg/g creatinine | μg/L urine | μg/g creatinine | μg/L urine | μg/g creatinine |
| Non-smokers ($n$=47) | $n$ > LOQ | 37 (79%) | | 4 (8.5%) | | 46 (98%) | |
| | Median | 1.9 | 1.5 | < 1 | < 1 | 4.6 | 4.0 |
| | 95. Percentile | 6.0 | 5.9 | 1.1 | 0.8 | 21 | 24 |
| | Max. value | 16.4 | 7.8 | 3.0 | 1.8 | 34 | 36 |
| Smokers ($n$=36) | $n$ > LOQ | 36 (100%) | | 34 (94%) | | 36 (100%) | |
| | Median | 184 | 148 | 29.3 | 23.1 | 1488 | 1360 |
| | 95. Percentile | 412 | 393 | 91 | 74 | 3712 | 4360 |
| | Max. value | 907 | 485 | 147 | 98 | 4940 | 4916 |

evaluated. Mean matrix influence for CEMA was determined to be 35% (range: 29–44%) and for CHEMA it was 26% (range: 14–36%). This means that the analyte signal is lower in urine by 35 and 26% as compared to the aqueous standard, respectively. This signal suppression is compensated by the labelled internal standard, however, the determined LOD and LLOQ in concentrated urine samples can be considerably higher by a factor of 2. All data on the accuracy in individual urine samples as well as the signal suppression effect of different urinary matrices are summarised in Table 4.

Finally, a calibration curve prepared in pooled urine (see Section 2.2) and water was analysed in triplicate and slopes for both analytes were compared. As a result, the correlation coefficients were greater than $r$=0.9999 for both calibration curves generated. Mean slopes for CHEMA and CEMA in urine and water were 0.0076 and 0.0077 as well as 0.0020 and 0.0022, respectively. Consequently, the labelled internal standards proved to be very efficient for compensating matrix influences and assuring accurate quantification results.

### 3.3. Stability of CHEMA and CEMA in urine

In order to check for stability of the analytes, three quality control samples ($Q_{low}$, $Q_{medium}$ and $Q_{high}$) were subjected to three freeze-thaw cycles on three different days. After that, the samples were analysed as described and the results were compared to the results obtained for the between-day repeatability. Mean values obtained in these samples were 9.0, 43.2 and 84.8 μg/L for CHEMA and 14.5, 71.5 and 140 μg/L for CEMA. No significant deviation from the mean value of the between-day repeatability was observed. Consequently, a decomposition of the analytes in urine during several freeze-/thaw-cycles could not be confirmed, assuring our previous results for CEMA [11].

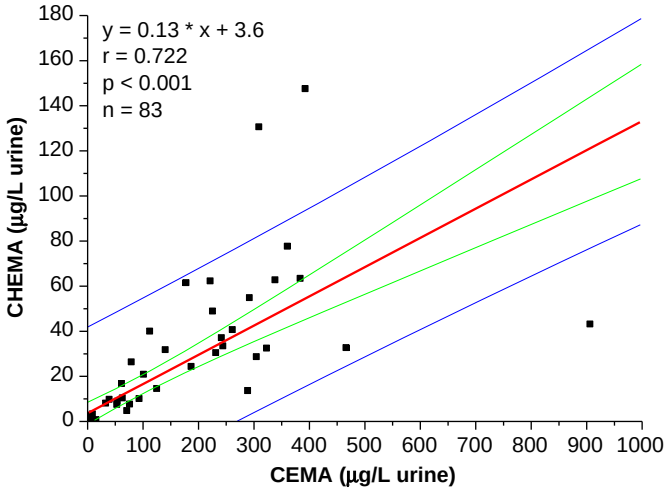


**Fig. 4.** Relationship between urinary levels of the mercapturic acids of acrylonitrile in the examined collective.

### 3.4. Results of biological monitoring

The results of biomonitoring for the acrylonitrile metabolites CEMA and CHEMA in urine samples of the 47 non-smoking and 37 smoking subjects of the general population are summarised in Table 5 (including the results for urinary cotinine). Creatinine levels in these samples ranged from 0.25 to 2.82 g/L.

Urinary CHEMA levels were above the LOD in 8.5% of all non-smoker urine samples and 94% of all smokers' urine samples. In good accordance with our previous study, CEMA levels were detectable in 79 and 100% of the non-smokers' and smokers'

urine samples, respectively. As expected, smokers showed a significantly higher excretion of both acrylonitrile metabolites than non-smokers ($p < 0.001$, Mann–Whitney-*U*-test). The relationship between the urinary levels of the mercapturic acid derived from the direct conjugation of acrylonitrile (CEMA) and the corresponding levels of the mercapturic acid derived from the oxidative metabolite of acrylonitrile (CHEMA) is shown in Fig. 4.

As shown in our previous study [11], urinary CEMA showed a good correlation to urinary cotinine (see Supplementary Material).

## 4. Discussion

We have developed and applied a reliable and accurate procedure for the simultaneous determination of the urinary mercapturates derived from the parent compound (CEMA) as well as the oxidative metabolism (CHEMA) as biomarkers of the individual internal exposure to acrylonitrile and its genotoxic metabolite cyanoethylene-epoxide (CEO).

A recently published method using UPLC-MS/MS reported a similar LOQ (1 μg/L urine) for CHEMA, but an LOQ for CEMA of 0.1 μg/L urine, which is distinctly lower than our LOD of 1 μg/L [16]. However, one of the most important advantages of our procedure compared to this previous method is the use of an isotopically labelled internal standard that guarantees high accuracy of the results. As we have carefully evaluated (see Table 4), the signal for CHEMA in tandem mass-spectrometry might be considerably suppressed in urine samples with different creatinine contents for more than 30%. This effect is only compensated by the use of a labelled internal standard, which is in our view the most effective way to perform reliable quantitative analysis [21]. Thus, our data determined for precision and accuracy were excellent, even in (unspiked) real urine samples. Furthermore, we carefully investigated the effect of the urinary matrix on the result in our method validation and verified that the labelled internal standard is able to compensate all possible interferences.

The LOQ achieved using our method was sufficient to determine a background exposure of the (smoking) general population to acrylonitrile (and cyanoethylene-epoxide) as shown in the application of our method. These data prove that acrylonitrile is metabolised to a certain extent to the genotoxic metabolite cyanoethylene-epoxide in humans. The levels found for urinary CEMA in non-smoking and smoking subjects are in good accordance with our previous investigations [11]. Likewise, Wu et al. reported mean urinary levels of 2.59 μg/g creatinine (range: < 0.05–111.7 μg/g creatinine) for CEMA in a group of mainly non-smoking persons from Taiwan, which is in good congruence with our present results [16].

Concerning urinary CHEMA, Wu et al. reported a geometric mean excretion of 2.41 μg/g creatinine (range: < 0.5–23.09 μg/g creatinine) in this (mainly non-smoking) collective of 36 persons [16]. This level is higher than the levels in our non-smoking group, where CHEMA could only be quantified in 8.5% of all samples. However, the maximum value reported by Wu et al. is within the median range of urinary levels of smokers in our study, pointing to a good comparability of both studies.

The urinary levels for CEMA and CHEMA we determined in our study (see Table 5) are among the first described worldwide with respect to human metabolism of acrylonitrile. Excretion of both metabolites showed a highly significant correlation (see Fig. 4). The ratio between the extent of the oxidative metabolic pathway and direct metabolic conjugation of acrylonitrile is of special importance for the risk assessment of acrylonitrile. The mean ratio of CHEMA/CEMA in this collective is 0.13 (see Fig. 4), with a wide range in ratio of 5–42% (for detectable levels of both metabolites). A possible reason for these large variations might be different activities (e.g., due to genetic polymorphisms) of detoxifying (glutathione-*S*-transferases, GSTs) as well as activating (cytochrome P 450) enzymes involved in the metabolism of acrylonitrile (see Fig. 1). Alternatively, different kinetics of both mercapturates might lead to different ratios of CHEMA/CEMA, depending on the time of sampling since last exposure (cigarette smoking).

Overall these human data on oxidative metabolism of acrylonitrile seem to be somewhat lower than the data reported for rats [7]. However, accurate studies on human metabolism and urinary kinetics of both metabolites are needed.

Compared to human data on the metabolism of the structurally closely related acrylamide, the ratio of the acrylonitrile metabolites CHEMA/CEMA of 0.13 found in this study is in excellent accordance to the levels of the corresponding acrylamide mercapturic acids with a mean GAMA/AAMA-ratio of 0.126 and 0.138 described in two previous studies on spot urine samples of smokers and non-smokers [22,23]. Based on these facts, it can be assumed that oxidative metabolism of acrylonitrile in humans is of similar magnitude compared to its structural analogue acrylamide.

## 5. Conclusion

The method on quantification of urinary mercapturic acids of acrylonitrile presented here is to our knowledge the first method using labelled internal standards for both analytes, assuring accurate quantification regardless of urinary matrix. We have thoroughly validated our method according to international guidelines [24]. Due to its automation and high sensitivity, our method is well suited for the determination of individual exposure to acrylonitrile and its genotoxic metabolite cyanoethylene-epoxide in studies on human or animal metabolism, occupational studies or larger environmental studies.

The application of our method to urine samples of persons with no occupational exposure demonstrated an exposure of the general population to acrylonitrile and cyanoethylene-epoxide, which is higher in smokers. Based on the data obtained in our study, excretion of oxidative metabolites of acrylonitrile in humans is of similar magnitude compared to acrylamide.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.06.074.

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