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Review

Mercapturic acids in the biological monitoring of occupational exposure to chemicals

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Abstract

This paper reviews several procedures for determination of mercapturic acids in urine. Special attention was paid to methods useful in relation to human exposure to industrial pollutants, without any description for less sensitive methods used in animal research. Gas chromatographic and liquid chromatographic procedures were considered together with the little information available about thin layer chromatography and immunochemical techniques. After a description of the main industrial pollutants which lead to synthesis of their specific mercapturic acids, the methods for analysing these products are synthetically reported. The comparison among difficulties in sample preparation, complexity of instrumentation and their cost/benefit ratio are discussed.

Keywords: Reviews; Mercapturic acid; Industrial pollutants

Contents

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1. Introduction

Until recent decades, the definition of ''mercapturic acids'' included a rather limited number of organic compounds. In recent years many new mercapturic acids have been identified and the metabolic pathway of their synthesis has been explained.

In theory, an unlimited number of mercapturic acids can be synthesised by the human body. Many products, both endogenous and exogenous, can be partly conjugated with glutathione during their biotransformation processes, producing mercapturic acids.

In 1998 De Rooij at al. [1] presented an extensive review of biochemical mechanisms leading to the synthesis of mercapturic acids, the oxido-reduction involved and the various chemicals that have been proved to contribute to synthesis of these products.

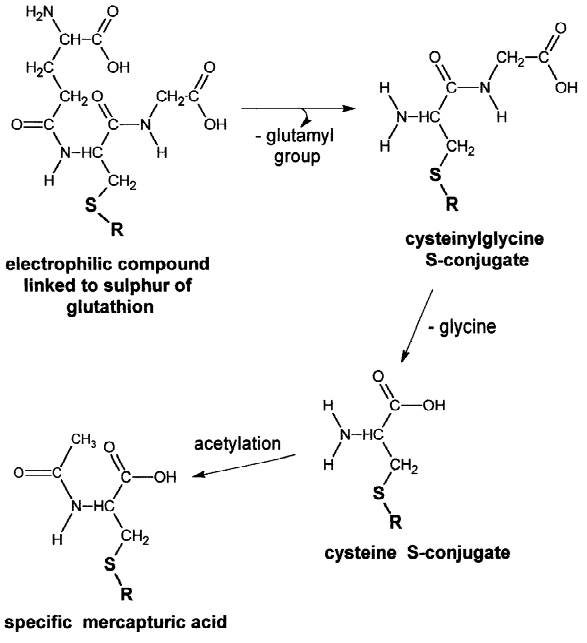

Fig. 1. Main pathways for the synthesis of mercapturic acids.

Fig. 1 shows metabolic pathways more directly involved in synthesis of mercapturic acids. Any electrophilic product present in the body, whether absorbed from the environment or derived from oxido-reduction metabolic processes in the liver or other organs, ''finds'' in the glutathione-SH group a site where conjugation can occur spontaneously or be catalysed by the enzyme glutathione-S-transferase. The ready bond between glutathione and different electrophilic molecules favours the synthesis of innumerable mercapturic acids.

As highlighted in Fig. 1, a radical bonded to glutathione gives rise to an adduct which then tends to be modified only in parts formed by amino-acids. First the glutamic acid radical and then the glycinic radical are divided, these being commonly re-used to synthesise new glutathione. Cysteine-S-conjugates can follow different biotransformation pathways, but that of acetylation into mercapturic acid is often prevalent in man.

This article reports on mercapturic acids of interest in industrial toxicology as biomarkers of exposure to different chemicals. It summarises the main analytical methods used for their measurement in biological media, with a view to discussing prospects for new technical approaches.

2. Mercapturic acids related to occupational exposures to chemicals

In 1879 it was discovered that in laboratory animals some halogenated hydrocarbons lead to synthesis of specific mercapturic acids [2]. However, for many years this information was not used to any extent in industrial toxicology.

Recently, a few mercapturic acids have been considered as biomarkers for professional exposure monitoring. The American Conference of Governmental Industrial Hygienists (ACGIH) [3] and Deutsche Forschungsgemeinschaft (DFG) [4] suggested using phenylmercapturic acid to assess profes-

sional exposure to benzene in 1998 and 1996, respectively.

Mercapturic acid derived from *N*,*N*-dimethylformamide was listed as a marker of this solvent by and ACGIH [3] in 1999.

Limited use of mercapturic acid in professional exposure monitoring is caused by two main problems:

1. limited available knowledge about synthesis of mercapturic acids in humans;
2. specific mercapturic acid analysis generally requires rather complicated methodologies not suited to screening of large groups of workers, since they are costly and slow;
3. few mercapturic acids showed a more useful pharmacokinetic performance than other metabolites of the same parent product.

In Figs. 2 and 3, most of the mercapturic acids coming from the biotransformation of several chemicals used in different industrial facilities are shown. Fig. 2 shows the mercapturic acids related to some aromatic and aliphatic hydrocarbons, while Fig. 3 summarises those synthesised from halogenates and nitrogenous hydrocarbons. In some cases, each chemical gives rise to only one mercapturic acid, but two or more mercapturates were identified in other cases.

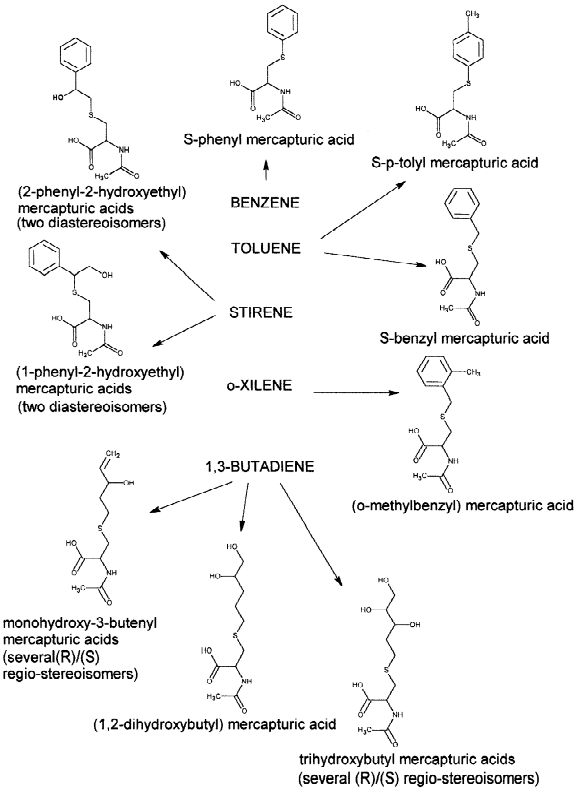

Fig. 2. Mercapturic acids coming from the biotransformation of some aromatic and aliphatic hydrocarbons.

2.1. Phenylmercapturic acid and exposure to benzene

Benzene has become a ubiquitous pollutant, detectable even in desert zone air and at the North Pole.

The main source of pollution is vehicle fuels, in which it is present to a level of almost 1%. Petrol refineries, the chemical industry and wood fires contribute to a more limited extent to environmental pollution.

Smoking involves considerable individual intake of benzene, besides affecting ''indoor'' air quality.

Benzene is a human leukemogen and an animal carcinogen [5].

Phenylmercapturic acid was first detected as a metabolite of benzene in the 1950s, in laboratory animals. This metabolite was produced in smaller quantities than phenol, and for many years has received little attention.

When phenol as a biomarker proved inadequate for low professional exposure to benzene, further studies on biotransformation of this solvent were made, to obtain more information about the carcinogenic risk of benzene exposure and other specific questions.

A research group at Essen University [6,7] made some interesting studies on laboratory animals and workers exposed to the solvent, to ascertain whether phenylmercapturic acid could give information about exposure to benzene and its possible carcinogenic effects.

In rats this metabolite was very specific, because it proved detectable only in animals that were exposed to benzene (detection limit 2 μmol/l). There was a good correlation between urinary excretion of this

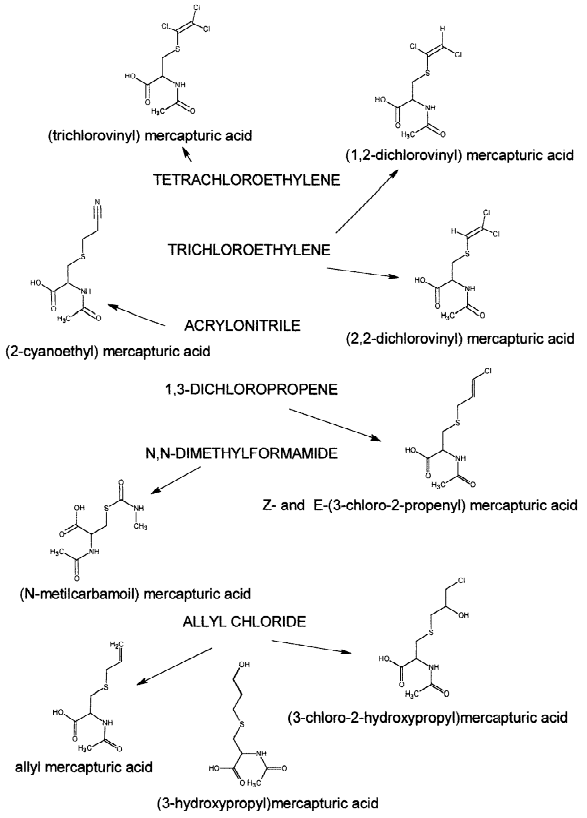

Fig. 3. Mercapturic acids coming from the biotransformation of some halogenates and nitrogenous hydrocarbons.

metabolite and levels of exposure, especially at low levels of exposure.

These promising results found only partial confirmation in humans, particularly in relation to synthesis of phenylmercapturic acid being less marked than in rodents.

In workers exposed to concentrations lower than 1.13 ppm, phenylmercapturic acid showed high sensitivity, starting to increase in urine after 3 h exposure, and rising at the end of the work shift to concentrations 10 times higher than measured in 24 subjects who were not exposed (both smokers and non-smokers). For these subjects, mean urinary concentration of phenylmercapturic acid was 4 μg/g creatinine (SD=4).

In the same period Jongeneelen et al. [8] did a series of studies on animals and humans. They concluded that phenylmercapturic acid was not suitable for biological monitoring of low occupational exposures to benzene (the detection limit of their analytical method was 6 μmol/l).

Subsequent publications from different working groups highlighted the phenylmercapturic acid is a good biomarker for low exposures to benzene. In 2001, ACGIH [3] proposed this metabolite as an index for professional exposure to benzene, with a limit of 25 μg/g creatinine; this value is related to a mean environmental concentration close to 0.5 ppm.

During steady exposure to these benzene concentrations, considering alveolar ventilation of 15 l/min and alveolar retention of 50%, the lung intake is about 3600 μg benzene. A 30 cigarette/day smoker's individual daily uptake of benzene from smoke is 1500–1800 μg. Adding the intake of benzene from indoor and outdoor air, the total benzene that a 30 cigarettes/day smoker absorbs is close to the amount of benzene inhaled by a worker exposed to 1 mg/m^3.

Table 1 summarises the urinary concentrations of phenylmercapturic acid in people exposed only to environmental micro-pollution and not professionally exposed to benzene. Data are shown in relation to smoking habits.

The low concentrations of this metabolite in urine were at first difficult to interpret, because they were often below detection limits for available methods.

The analytic sensitivity of the methods used then made it possible to discriminate smokers from non-smokers, and subjects not professionally exposed to benzene from those exposed even to low concentrations.

Even if with a certain degree of variability, Table 1 highlights that ''physiological'' excretion of phenylmercapturic acid is on average lower in non-smokers than in smokers.

2.2. (1-Phenyl-2-hydroxyethyl) and (2-phenyl-2-hydroxyethyl) mercapturic acids (each with two diastereoisomers) and exposure to styrene

Styrene is one of the most important aromatic hydrocarbons used in preparing polymers and co-polymers, reinforced plastics, synthetic rubber, polyester resins, paints and coatings. In humans, most of the retained styrene is metabolized in the liver by cytochrome P450 isozymes to the chiral *R*/*S* styrene-

Table 1
Urinary concentrations of phenylmercapturic acid in subjects not occupationally exposed to benzene. Data are expressed in μg/g creatinine (NS, non-smokers; S, smokers)

Smoking habit	No. of measurements	Media	Median	Range	Refs.
NS + S	24	4			[6]
NS + S[a]	48		<2	<DL[b]–6	[9]
NS + S	24	4			[6]
NS	38	1.99			[10]
NS	18	4.8	3.6	1–19.6	[11]
NS	8	1.3			[12]
NS	42	1[c]		4.74[d]	[13]
NS	20	1.3	0.3	<DL–18	[14]
S	10	9.2			[12]
S	14	3.61			[10]
S	28	9.1	5.8	<DL–33.4	[11]
S	45	7.8[b]		34.7[c]	[13]
S	19	2.8	0.3	<DL–13	[14]

[a] No statistical difference between non-smokers and smokers.
[b] DL, detection limit.
[c] Geometrical mean.
[d] Upper limit (at 95%).

7,8-exide. Hydrolysis to styrene glycol and further oxidations yield the optical enantiomers mandelic and phenylglyoxylic acids, which are the main urinary metabolites of styrene [15].

In rats treated with styrene, Seuttle-Berlage et al. [16] identified three different mercapturic acids in urine: (1-phenyl-2-hydroxyethyl) mercapturic acid, (2-phenyl-2-hydroxyethyl) mercapturic acid and phenacylmercapturic acid. They accounted for about 10% of the dose, though the proportion of phenacylmercapturic acid was low. The same working group [17] reported that each of the main two mercapturic acids present in urine of rats was a mixture of two diastereoisomers. These results were recently confirmed by NMR spectroscopy [18]. The amount of mercapturic acids in rats dosed with styrene was significantly higher for female (15% of dose) than for male rats (6% of dose), but there was no difference in the diastereoisomer ratio. The regioisomer ratio yielded a 2:1 mixture of (1-phenyl-2-hydroxyethyl) mercapturic acid and (2-phenyl-2-hydroxyethyl) mercapturic acid. The differentiation among the stereochemical structures of the styrene metabolites seems useful in understanding their mutagenic effects: the *R*-enantiomeric forms have higher reactivity toward nucleic acids [19,20].

In humans, the amount of mercapturic acids born from styrene is below 1% of the absorbed dose [21].

Hallier et al. [22] were able to detect these urinary metabolites only in one worker (out of 20). With total mandelic acid ($R + S$) of 1610 mg/l, the level of mercapturic acids was 1 mg/l. With a very sensitive analytical method, Ghittori et al. [23] measured the two diastereoisomers of (1-phenyl-2-hydroxyethyl) mercapturic acid (M1-*S* and M1-*R*) and the sum of diastereoisomers of (2-phenyl-2-hydroxyethyl) mercapturic acid (M2) in 22 workers exposed to styrene (the geometrical mean of the environmental concentrations being 98.6 mg/m^3). In urine collected at the end of the work shift, the geometrical mean of styrene metabolites were as follows: mandelic acid, 472 mg/g creatinine; phenylglyoxylic acid, 156 mg/g creatinine; M1-*S*, 0.65 mg/g creatinine; M1-*R*, 0.032 mg/g creatinine; M2, 1.084 mg/g creatinine. These results confirm that only a very small part of the absorbed styrene is biotransformed to mercapturic acids in humans. Nevertheless, a statistically significant linear correlation was found between styrene exposure and urinary excretion of each of the diastereoisomer mercapturic acids. These results were partly confirmed by Manini et al. [24], who measured all four diastereoisomers

coming from styrene in urine of workers exposed to the solvent within a range of 42.5 to 365 mg/m^3. The geometrical means found in urine samples collected at the end of the shift were 181.6, 12.5, 186.1 and 39.9 μg/g creatinine, respectively, for M1(*R*), M1(*S*), M2(*S*) and M2(*R*). These values in part fit with those reported by Ghittori et al. [23]. On this occasion urinary excretion of mercapturic acids was related to styrene exposure, but also to genetic variability in the synthetic phase of these metabolites. The polymorphism of glutathione-S-transferase is probably involved. These authors suggest that biological monitoring of occupational exposure to styrene by derived mercapturic acid cannot be recommended before studying inter-individual variability.

2.3. S-p-Toluylmercapturic acid and exposure to toluene

Toluene is the aromatic compound predominant in certain industrial solvent mixtures, especially related to the printing industry and facilities where paints, thinners, glues and paintbrush cleaners are prepared or used.

Most of the absorbed toluene leads to biological synthesis of hippuric acid, both in animals and in humans [25]. Other metabolites (cresols, benzoic acid) can be detected in relation to the biotransformation of toluene, including two mercapturic acids.

In 1983 Rietveld et al. [26] studied the formation of mercapturic acids from aromatic aldehydes. Benzaldehyde, via benzylalcohol, which is also the first main oxidisation product of toluene, led to the synthesis of sulphate esters in the form of benzylmercapturic acid. This metabolite was also found in urine of sniffers and printing workers exposed to toluene, but not in unexposed subjects [27].

In 1998, for the first time, *S-p*-toluylmercapturic acid was found in urine of workers exposed to toluene [28]. When the median environmental concentration of the solvent was 237 mg/m^3 (range 49–566), the median concentration of *S-p*-toluylmercapturic acid was 0.020 mg/l (toluene in blood 0.804 mg/l, *o*-cresol 2.3 mg/l and hippuric acid 2300 mg/l). These results show that very little of the absorbed toluene leads to the synthesis of *S-p*-toluylmercapturic acid. Its urinary concentrations were statistically related to the other biological parameters reported above, and associated with toluene exposures. No background excretion of *S-p*-toluylmercapturic acid above the detection limit was observable for unexposed subjects. Environmental micropollution was not detectable through this metabolite. More research about *S-p*-toluylmercapturic acid is needed to ascertain the meaning of this product in occupational and environmental exposure to toluene.

2.4. Mercapturic acids and exposure to other aromatic hydrocarbons

There are few reports about synthesis of mercapturic acid related to exposure to diethylbenzenes and 1,2,4-trimethylbenzene. These reports concern experimental animals (not humans). We summarise the available information.

Linhart et al. [29] studied the biotransformation of 1,2-diethenylbenzene in rats dosed with a single intraperitoneal injection. With other metabolites, [1-(2-ethenylphenyl)-2-hydroxyethyl] mercapturic acid and [2-(2-ethenylphenyl)-2-hydroxyethyl] mercapturic acid were identified. They amounted to 3–5% of the injected dose. The ratio of regioisomeric mercapturic acids was 83:17. Each regioisomer consisted of two diastereomers, with a ratio of 82:28 and 79:21, respectively, for the first and the second reported mercapturic acids.

In urine of rats treated with 1,2,4-trimethylbenzene, Tsujimoto et al. [30] identified three different mercapturic acids, i.e. 2,4-, 2,5-, and 3,4-dimethylbenzyl mercapturic acids. They represented 14–19% of the dose of the injected solvent.

2.5. (N-Methylcarbamoyl) mercapturic acid and exposure to N,N-dimethylformamide

N,*N*-Dimethylformamide is a solvent used in the pharmaceutical and chemical industries, especially for the production of synthetic leather and polyacrylonitrile fibres. In industrial settings, this solvent can be easily absorbed through the lungs and skin.

Its biotransformation in humans is quite complex.

Oxidation of one methyl group results in the formation of *N*-hydroxymethyl-*N*-methylformamide, which is the main urinary metabolite of *N*,*N*-dimethylformamide in both humans and experimental animals [31,32]. This step is followed by the breakage of the hydroxymethyl-radical, leading to the synthesis of *N*-methylformamide. The dehydrogenation of this metabolite gives rise to methylisocyanate which has never been measured in relation to *N*,*N*-dimethylformamide exposure, but is supposed to react rapidly with glutathione and leads to formation of (*N*-methylcarbamoyl) mercapturic acid. This metabolite was also found in urine of rats exposed to methylisocyanate [33]. The identification of mercapturic acid coming from *N*,*N*-dimethylformamide was reported by Mráz and Turecek in 1987 [34] and confirmed by several studies [35–37].

The use of (*N*-methylcarbamoyl) mercapturic acid as a biomarker for human industrial exposure to *N*,*N*-dimethylformamide became official when the American Conference of Governmental Industrial Hygienists [3] and Deutsche Forschungsgemeinschft [4] included it in their annual proposals.

2.6. Mercapturic acids from 1,3-butadiene

In industrial settings 1,3-butadiene is mainly used in the production of different kinds of synthetic rubbers (acrylonytrile–butadiene–styrene, butadiene–styrene, polybutadiene). Phillips et al. [38] measured 1,3-butadiene together with several other volatile organic compounds in six European cities. The median concentrations of the pollutant were 1.2 and 1.37 $\mu g/m^3$, respectively, outside and inside homes across Europe. Vehicle exhausts and cigarette smoke are primary sources of 1,3-butadiene pollution [39].

The biotransformation of 1,3-butadiene in humans and animals is quite complex. Several metabolic routes lead to the synthesis of different compounds, most of which give rise to mercapturic acids. The epoxidation of 1,3-butadiene is an activating step which can yield a mixture of the regio- and stereoisomers, (*R*)/(*S*) (1-hydroxymethyl-2-propenyl) mercapturic acid and (*R*)/(*S*) (2-hydroxy-3-butenyl) mercapturic acid, which will be called monohydroxy-3-butenyl mercapturic acids. Together with these metabolites, butadiene monoepoxide leads to the synthesis of DNA and protein adducts.

Hydrolysis of the epoxide to 1,2-dihydroxy-3-butene is the basic reaction which leads to the formation of 1,2-dihydroxy-3-butenyl mercapturic acid, the main urinary metabolite of 1,3-butadiene in humans.

Part of butadiene monoepoxide is further oxidized to its diepoxide, 1,2:3,4-diepoxybutane, which yields different regio-isomers of 2,3,4-trihydroxybutyl mercapturic acid. Of course the reported parent metabolite too can react with DNA and proteins.

There are remarkable species-related differences in the metabolism of 1,3-butadiene [40,41]. Cigarette smoke can increase uptake in humans [42]. Human exposures to 1,3-butadiene have a very wide range: in control subjects van Sittert et al. [43] reported a median value of 0.007 ppm (range: 0–0.038 ppm). In workers engaged in different jobs, median exposure over a period of 60 days ranged from 0.17 to 0.59 ppm (during a 60-day average level). Individual daily exposure could also be higher than 10 ppm.

In both control subjects and workers, the urinary concentration of trihydroxybutyl mercapturic acids was not detectable.

Monohydroxy-3-butenyl mercapturic acid was always detectable in control subjects, but at low concentrations (always lower than 8.2 $\mu g/l$). In urine samples collected from exposed workers at the end of their work shift, mono-hydroxy-3-butenyl mercapturic acid was significantly higher than in pre-shift urine samples and in those provided by non-exposed subjects.

In workers whose median exposure to 1,3-butadiene was 4.3 ppm (8 h time-weight average), the median urinary concentration of mono-hydroxy-3-butenyl mercapturic acid was 97 $\mu g/l$.

The urinary concentrations of 1,2-dihydroxybutyl mercapturic acid were much higher in urine collected from both control subjects and exposed workers.

In non-exposed people, the concentration of 1,2-dihydroxybutyl mercapturic acid ranged between 197 and 1211 $\mu g/l$. This background is much higher than that just reported.

In workers exposed to 1,3-butadiene (median 4.3 ppm), the median urinary concentration of 1,2-dihydroxybutyl mercapturic acid was 2719 $\mu g/l$ (range 342–20,213 $\mu g/l$).

Urinary concentrations of both mercapturic acids were statistically related to exposure to 1,3-butadiene. Their concentrations were generally higher in urine samples provided at the end of the work shift than at the beginning. This suggests that the average urinary half life of these metabolites is quite short and does not allow their accumulation within the body [43].

2.7. Trichlorovinylmercapturic acid and exposure to perchloroethene

Perchloroethene is extensively used in industry for metal degreasing and as a dry cleaning agent. Its biotransformation is low. In humans, only about 2% of the absorbed dose can be found in urine as trichloroacetic acid, while 97–99% is excreted with alveolar air [44]. Trichlorovinylmercapturic acid was recently found [45] in urine samples obtained from rats and humans exposed to trichloroethene.

At present there is not enough information to confirm that this metabolite is a useful product in biological monitoring of occupational exposure to perchloroethene. Its identification is nevertheless another interesting approach to studying the mercapturic acids.

2.8. 2,2- and 1,2-dichlorovinylmercapturic acids and exposure to trichloroethylene

Trichloroethylene is another organic solvent extensively used in several industrial processes.

Trichloroethylene is biotransformed to several metabolites, both in rodents and in humans, with trichloroethanol and trichloroacetic acids as the main urinary metabolites. In 1986, Dekant et al. [46] identified 1,2-dichlorovinylmercapturic acid in urine of rats treated with trichloroethylene. Bernauer et al. [47], comparing trichloroethylene biotransformation in rats and humans found that only 0.001% of the absorbed solvent was detectable in urine as mercapturic acids. These were excreted at quite a slow rate (they were detectable 48 h after a single exposure to 40 ppm), but showed characteristics of saturation after inhalation of 160 ppm for 6 h. Even if these metabolites could suggest a risk of renal toxicity [48], at present they are not adequate to measure the risks of exposure to trichloroethylene.

2.9. Allyl- and 3-hydroxy-propylmercapturic acids from exposures to allyl chloride (3-chloropene)

Allyl chloride is used as an intermediate in the synthesis of epichlorhydrin, a precursor in the production of epoxy resins. The biotransformation of allyl chloride has been investigated almost exclusively in rats, where allylmercapturic acid was a main metabolite, while 3-hydroxy-propylmercapturic acid was a minor metabolite [49]. These mercapturic acids as metabolites of allyl chloride were confirmed by de Rooij et al. [50]: $30\pm6\%$ of the solvent dose was excreted as allylmercapturic acid, while less than 3% was excreted as 3-hydroxy-propylmercapturic acid, with no clear dose–excretion relationship. 3-Chloro-2-hydroxypropyl mercapturic acid was also identified as a metabolite of allyl chloride, amounting to $0.21\pm0.08\%$ of the injected dose.

In 1997, de Rooij et al. [51] studied exposure to allyl chloride in a group of workers during maintenance and inspection operations in a factory where the allyl chloride was usually produced in a closed system. The rise in urinary concentrations of allylmercapturic acid during the work shift was related to the intensity of occupational exposure to allyl chloride. A small amount of allyl mercapturic acid was also present in urine of people not exposed to the solvent. A few workers, regular consumption of garlic and a number of unknown factors caused high excretion of allylmercapturic acid in urine, without any allyl chloride exposure. Smoking is not associated with high urinary concentration of allylmercapturic acid.

Sanduja et al. [49] found that 3-hydroxy-propylmercapturic acid can be recovered in urine of rats given acrolein (78% of dose), allylalcohol (28%), allylchloride (21%), allylbromide (3%), allylcyanide (3.7%) and cyclophosphamide (2.6%). This unspecific metabolite was suggested as a marker of exposure to allylic and other compounds which lead to the metabolic formation of acrolein.

2.10. (Z- and E-3-chloro-2-propenyl) mercapturic acids from exposures to 1,3-dichloropropene

1,3-Dichloropropene (*cis*/*trans*) is a soil fumigant often used in the flower bulb industry and in starch potato growing. From time to time workers have to use it and exposure can be considerable. The car-

cinogenicity of this product in animals prompted several studies on biological monitoring of occupational exposure to 1,3-dichloropropene.

Osterloch et al. [52] found that exposure to 1,3-dichloropropene, at concentrations ranging between 0.59 and 1.86 mg/m^3 for about 2.5–6 h, gave concentrations of 3-chloro-2-propenyl mercapturic acid of 5.2–27 mg/l in urine collected at the end of the working day. A correlation was found between exposure to the fumigant and urinary excretion of the two specific mercapturic acids (mg/24 h), which were not analysed separately. These metabolites were quickly eliminated, the highest urinary excretion occurring during the application period. These results were confirmed by van Welie et al. [53], who measured the specific mercapturic acids related to *Z*- and *E*-1,3-dichloropropene exposure in subjects applying the pesticide and bystanders. This exposure leads to the synthesis of (*Z*- and *E*-chloro-2-propenyl) mercapturic acids, which are excreted in urine quite quickly with first order rate elimination kinetics. Their average half-life in urine was around 5 h. The 8-h threshold weighted average (TWA) exposures of the professionally exposed subjects were in the ranges 0.15–10.78 and 0.11–7.88 mg/m^3. Equal respiratory exposure to *Z*- and *E*-1,3-dichloropropene resulted in significantly higher urinary excretion of (*Z*-chloro-2-propenyl) mercapturic acid than (*E*-chloro-2-propenyl) mercapturic acid, with an average ratio of 2.3. Both the excretion rate (mg/h) and the concentration based on creatinine excretion (μg/mg creatinine) of the two mercapturic acids were significantly correlated with the 8-h TWA exposure.

Brouwer et al. [54] studied exposure to *cis*-1,3-dichloropropene in some workers engaged in starch potato growing. Levels were similar to those reported by van Wilie et al. [55] (range 0.1–9.5 mg/m^3, with a geometrical mean of 2.7 mg/m^3). These authors confirmed a statistically significant relationship between the 8-h TWA air concentrations of *cis*-dichloropropene and the urinary concentrations of (*cis*-dichloropropene) mercapturic acids. Data were consistent with those reported above.

2.11. Mercapturic acids from exposure to acrylonitrile

Acrylonitrile is an important chemical used in the production of acrylic fibers, plastics, rubber and polymers for coating. Its carcinogenic, reproductive or neurotoxic effects in laboratory animals make it necessary to carry out careful controls in workers exposed to acrylonitrile.

The biotransformation of this chemical gives rise to several mercapturic acids, which have to be considered its main metabolites. In rats and mice dosed with [2,3-^{14}C]acrylonitrile, they accounted for 75–100% of the total urinary metabolites [56]. Among these, 2-cyanoethylmercapturic acid was the major component in urine of rodents treated with acrylonitrile; 2-hydroxyethylmercapturic acid, carboxy-methylmercapturic acid and 1-cyano-2-hydroxyethylmercapturic acid were also identified in biological samples of the same rodents, but to a far lesser extent.

In humans, only 2-cyanoethylmercapturic acid was found in subjects experimentally exposed to 5–10 mg/m^3 of acrylonitrile [57]. Its highest concentrations in urine were measured at the end of exposure, with a calculated half-life ranging between 7 and 9 h. No research on urinary 2-cyanoethylmercapturic acid has been reported in exposed subjects.

3. Analysis of urinary mercapturic acids

Analysis of mercapturic acids in urine is not an easy task, because there are many of them and their structure is to a certain extent similar. N-Acetylated cysteine is common to all mercapturic acids. The S-bound groups have different structures, sometimes lipophilic sometimes not. The resulting mercapturic acids are always sufficiently hydrophilic to be readily excreted in urine, where they are mixed with thousands of other organic products from which analytical separation is often difficult. Several GC or HPLC separation techniques have been developed, but most of these methods are quite expensive and time-consuming.

An important consideration is that most methods for analysis of mercapturic acids were developed for kinetic studies in laboratory animals treated with different amounts of the parent products. Since derived mercapturic acids were present in urine in quite high concentrations, analyses were relatively easy. The situation is much more complex when analysing mercapturic acids in urine samples obtained from workers exposed to pollutant concen-

trations 100–1000 times lower than in animals. Methods used for experimental studies with animals are often not suitable for the analysis in humans.

Most of the chromatographic methods indicated in Table 2 can give satisfactory results, but often they involve complex and time-consuming preparation and derivatization phases. This is why mercapturic acids have been rarely used as biomarkers of occupational exposure to pollutants.

3.1. Gas chromatographic methods

Among the gas chromatographic methods summarised in Table 2, the one reported by Einig et al. [58] theoretically allows analysis of all the mercapturic acids. Linking pentafluorobenzyl bromide to the acid radical of the cysteine makes it possible to transform each mercapturic acid into a product potentially detectable by GC with an ECD detector.

The authors applied this chromatographic technique to analysis of *S*-phenylmercapturic and benzylmercapturic acids.

The sample preparation started with acidification of 5 ml of urine by 0.2 ml of concentrated HCl. Three different internal standards (512 ng of 4-fluorophenyl-mercapturic acid, 504 ng of 4-methylphenyl-mercapturic acid and 540 ng of 4-fluorobenzyl-mercapturic acid) dissolved in 50 μl of acrylonitrile were added. The mixture was extracted twice, using 5 ml ethyl acetate, which was afterwards evaporated under a stream of nitrogen. An aliquot of 500 μl of acrylonitrile was used to dissolve the dry residue. Methylation was performed by adding 100 μl of diisopropylamina and 100 μl of pentafluorobenzyl bromide. Derivatization was obtained by

Table 2
Main analytical methods used for the analysis of different mercapturic acids in urine samples obtained from people exposed to different industrial pollutants

Parent compound	Mercapturic acid (MA)	Method	Detection limit	Refs.
Benzene	*S*-phenyl MA	GC/ECD	60 ng/l	[58]
Benzene	*S*-phenyl MA	GC–MS	1000 ng/l	[59]
Benzene	*S*-phenyl MA	GC–MS	5000 ng/l	[9]
Benzene	*S*-phenyl MA	HPLC/UV	–	[8]
Benzene	*S*-phenyl MA	HPLC fluorescence	500 ng/l	[12]
Benzene	*S*-phenyl MA	LC–ES-MS–MS	100 ng/l	[11]
Toluene	*S*-benzyl MA	GC/ECD	65 ng/l	[58]
Toluene	*S*-benzyl MA	HPLC–ESI-MS	3200 ng/l	[64]
Styrene	1 (and 2)-Phenyl-2-hydroxyethyl MA	HPLC/UV	7000 ng/l	[65]
Styrene	1 (and 2)-Phenyl-2-hydroxyethyl MA	LC–ESI-MS–MS	700–1000 ng/l	[24]
Styrene	1 (and 2)-Phenyl-2-hydroxyethyl MA	TLC	100 ng/l each	[22]
o-Xylene	*o*-Methylbenzyl MA	HPLC–ESI-MS	3000 ng/l	[64]
Trimethylbenzene	2,3- and 2,6-Dimethylbenzyl MA	HPLC–ESI-MS	5500 ng/l	[64]
Trimethylbenzene	3,4-Dimethylbenzyl MA	HPLC–ESI-MS	2400 ng/l	[64]
Allyl chloride	Allyl MA	GC–MS	10,000 ng/l	[51]
Allyl chloride	3-Hydroxy-propyl MA	GC–MS	–	[51]
1,3-Butadiene	1,2-Dihydroxybutyl MA	LC–NECI-MS–MS	5000 ng/l	[43]
1,3-Butadiene	Mono-hydroxy-3-butenyl MA	LC–NECI-MS–MS	500 ng/l	[43]
Trichloroethylene	Dichlorovinyl MA	GC–MS	10,320 ng/l	[62]
Trichloroethylene	Dichlorovinyl MA	GC–MS	12,900 ng/l	[47]
Tetrachloroethylene	Trichlorovinyl MA	GC–NCI-MS	250,000 ng/l	[45]
cis-1,3-Dichloropropene	*cis*-Chloro-2-propenyl MA	GC–MS	–	[54]
Z- and *E*-1,3-Dichloropropene	*Z*- and *E*-3-Chloro-2-propenyl MA	GC/sulfur detector	100,000 ng/l	[55]
N,*N*-Dimethylformamide	(*N*-Methylcarbamoyl) MA (AMCC)	GC/NPD		[34]
N,*N*-Dimethylformamide	(*N*-Methylcarbamoyl) MA (AMCC)	GC/TSI or MS	30,000 ng/l	[60]
N,*N*-Dimethylformamide	(*N*-Methylcarbamoyl) MA (AMCC)	GC–MS	300,000 ng/l	[61]
N,*N*-Dimethylformamide	(*N*-Methylcarbamoyl) MA (AMCC)	HPLC	1,320,000 ng/l	[66]
N,*N*-Dimethylformamide	(*N*-Methylcarbamoyl) MA (AMCC)	HPLC	1,800,000 ng/l	[67]
Acrylonitrile	2-Cyanoethyl MA	GC/FID	1,000,000 ng/l	[57]

heating to 40 °C for 35 min, and stopped by adding 500 μl of water. This step was followed by a second extraction phase, where twice 500 μl hexane was used. The presence of peak interferences at the gas chromatographic analysis required further purifications of the sample by a HPLC technique. A 100× 2.1 mm I.D. HPLC precolumn and a 20×2.1 mm I.D. column with Hypersil (particle size 5 μm) were used. A 100-μl volume of the extract was injected, and purified by an *n*-hexane–2-propanol gradient from 100% *n*-hexane to 5% 2-propanol in 10 min. The flow-rate was 1 ml/min. The fraction was collected at 6.9–8.6 min, ready for the GC analysis. An Rtx-50 column (30 m, 0.25 mm I.D., film thickness 0.10 μm) was used for the GC separation. The programmed temperature vaporisation of the injector allowed part of the solvent present in the 10 μl injected to be evaporated for 15 s at 55 °C. After that the injector was heated ballistically to 340 °C. The oven temperature was 70 °C for 3 min. It was then increased by 20 °C/min to 240 °C, which was maintained for 35 min (before further analysis, the column was heated at 300 °C for 10 min for cleaning). The ECD was set at 370 °C, with a pulse height of 50 V and pulse duration of 0.1 ms. In Fig. 4 is reported the chromatogram obtained from a urine sample of a subject (non-smoker) exposed only to the environmental micropollution [58].

Theoretically this method could be used to analyse all the mercapturic acids at a low detection limit, but it is neither user friendly nor cheap. The analysis of *S*-phenylmercapturic acid can be performed by the GC technique suggested by Stommel et al. [6], with a few modifications proposed by the DFG [59]. This can be considered the only official method for measurement of a mercapturic acid. It is based on a capillary gas chromatograph coupled with a mass spectrometer.

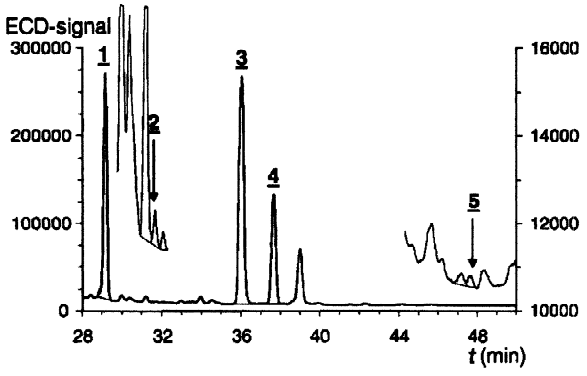

Fig. 4. Chromatogram of a urine sample from a person (non-smoker) exposed to environment micropollution: with the internal standards (peaks 1, 3 and 4) there are the amplified peaks (2 and 5) according to *S*-phenylmercapturic and *S*-benzyl-mercapturic acids, respectively [58].

Urine samples are acidified to a pH value lower than 2 with concentrated HCl (10 μl/1 ml urine). This treatment allows storage of the biological sample at 4 °C for 5 days, or at −20 °C for up to 6 months.

Ten-ml aliquots of urine have 1 ml internal standard added (10 mg/l of *p*-fluorophenyl-mercapturic acid in 1 l of water acidified with 10 ml of 2 *M* HCl). The sample is extracted twice, with 25 ml ethyl acetate in separate funnels, and the combined organic phases are centrifuged at 300 rev./min for 20 min in a rotary evaporator at 40 °C. The removed solvent is then evaporated until dry. The residue is dissolved in 1 ml methanol and 3 ml diazomethane are added. This preparation is then allowed to process at room temperature for 1 h. One μl of the clean supernatant solution (which is well separated from the precipitate salts) is analysed by GC–MS.

A capillary fused-silica DB-WAX column (30 m length, 0.25 mm I.D., 0.25 μm film thickness) is used. Initial temperature is 90 °C for 5 min, raised to 230 °C at 12 °C/min and kept in isothermal conditions for 10 min. The injector port temperature is at 200 °C (with a splitless time of 2 min), while the interface is 250 °C.

The mass spectrometer, set at electronic collision ionisation, used ionisation energy of 70 eV. The recorded fragments were: $m/e = 194.042$ for *S*-phenylmercapturic acid and $m/e = 212.032$ for the internal standard.

This method is accurate and precise, its detection limit being 1 μg/l of the analyte. Possible sources of error are related to: (a) a low resolution mass spectrometer allowing interferences (especially caffeine); (b) the quality of the ethyl acetate, which may contain traces of oxidizing agents; (c) the stability of the mass spectrometer, which is a critical factor.

The use of diazomethane, which is a well-known carcinogen, is a major limit of this method.

Analysis of *S*-phenylmercapturic acid in human

urine has also been performed by van Sittert et al. [9]. These researchers used a modification of the method published by Stommel et al. [6] and the DFG method referred to above [59]. In this case the derivatization of the extracted mercapturic acids was performed using 2 ml methylating agent (methanol/1.25 *M* HCl), after total evaporation of the ethyl acetate. The methylating agent was prepared by passing 46 g HCl gas through 1 l of methanol. The methylation of *S*-phenylmercapturic acid and *S*-benzyl-mercapturic acid (internal standard) was allowed at room temperature for 30 min. After total evaporation of the solution in a gentle stream of nitrogen at 45 °C, the sample was dissolved in dichloromethane (1 ml) and used for the GC–MS analysis. In this case a fused-silica DB-1 capillary column (60 m×0.22 mm I.D., 0.1 μm film thickness) was used. The oven temperature was programmed at 35 °C for 1 min, followed by 10 °C/min steps up to 300 °C. This temperature was held for 7.5 min. The recorded fragments were: *m*/*z* 194 for *S*-phenylmercapturic acid, and *m*/*z* 176 for the internal standard (methylester of benzyl-mercapturic acid).

For analysis of mercapturic acid coming from *N*,*N*-dimethylformamide, *N*-methyl-carbamoyl mercapturic acid, some different methods have been proposed. The first, proposed by Mraz et al. [34], broke down mercapturic acid to ethyl-*N*-methylcarbamate, which was then analysed. This method has been recently improved by Käfferlein and Angerer [60], for application to biological samples obtained from the general population. It is not an easy method and needs a preparatory phase with both liquid and solid-phase extractions.

A volume of 5 ml urine is saturated with 2.5 g sodium chloride and acidified with 500 μl 1 *M* HCl solution. To this are added 100 μl of the internal standard solution (*N*,*N*-dimethylpropionic acid amide at a concentration of 100 mg/l in water), and extracted twice with 5 ml tetrahydrofuran. The combined organic phases are cleaned by adding 1 g cation-exchange resin and shaking the mixture gently for 1 h. The supernatant solution is transferred in a clean vial, to which are added 3 ml tetrahydrofuran (used for washing the resin). The samples are then evaporated in a stream of nitrogen to about 0.5 ml, after which 2 ml ethanol and 1.5 g anhydrous potassium carbonate are added. The immediate shaking of samples favours the conversion of *N*-methylcarbamoyl mercapturic acid to ethyl-*N*-methylcarbamate (yield 60%). This clean-up procedure is adequate for determination of *N*-methylcarbamoyl mercapturic acid in urine samples of workers exposed to *N*,*N*-dimethylformamide.

The analysis could be completed by using a gas chromatograph with a DX-4 (15% dimethylpolysiloxane, 85% polyethylene glycol) capillary column (60 m length, 0.25 mm I.D. and 0.25 μm film thickness), a Thermoionic Sensitive Detector (at 280 °C) and a split/splitless capillary injector (at 280 °C). The oven temperature is programmed at 100 °C for 10 min, then raised to 140 °C at 3 °C/min and subsequently to 240 °C at 25 °C/min. This final temperature is maintained for 15 min.

To increase the detection limit of the method, other preparatory phases are needed. The samples are centrifuged at 4000 rev./min for 10 min. The supernatant is diluted three times with water and extracted twice using 5 ml methylene chloride. The combined organic phases are dried over anhydrous sodium sulphate for 30 min. After transferring the solution into a clean vial, a volume of 3 ml methylene chloride used for washing the sulphate is added. The organic solvent is then evaporated to about 100 μl. After this 50 μl of ethanol are added and the solution is evaporated to a final volume of 50 μl. One μl was used for the injection in GC–MS-EI-MID (electronic impact ionisation-multiple ion detection).

The splitless mode of the injector system is run for 1 min. The injector and detector temperature is 280 °C. A silica capillary HP-Innowax column with a stationary phase of 100% polyethylene glycol (60 m length, 0.32 mm I.D., 0.25 μm film thickness) is used. Helium is the carrier, with a flow-rate of 1.2 ml/min. The oven temperature is programmed at 80 °C for 15 min, then raised at a rate of 20 °C/min to 100 °C and kept at this temperature for 11 min. Ramp rate 2 raises the temperature to 120 °C at 20 °C/min. After 5 min, the ramp rate 3 raises the temperature to 240 °C at 20 °C/min. This final temperature is held for 15 min.

The monitored ions are as follows: *m*/*z* 103 and 75 for ethyl-*N*-methylcarbamate; *m*/*z* 101 and 72 for the internal standard.

The detection limit of the method is 30 μg/l.

The GC–MS method proposed by Casal-Lareo et al. [61] was applied only on urine samples obtained from workers exposed to *N*,*N*-dimethylformamide. Aliquots of urine (1 ml) with 100 μl of concentrated HCl and 30 μl of malonic acid solution (1 g/l) added as the internal standard, were used. After addition of NaCl until saturation, the samples were twice extracted with ethyl ether (3 ml). The combined extraction solvent, dehydrated with 300 mg anhydrous sodium sulphate, was evaporated under vacuum. The resulting dry residue was mixed with 100 μl of silylating reagent (BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide and 1% TMCS, trimethylchlorosilane) and kept at 65 °C for 30 min. Aliquots of 1 μl were injected into a GC–MS, the injector and transfer line being kept at 280 °C. A HP-Ultra2 silica capillary column (50 m length, 0.32 mm I.D. and 0.17 μm film thickness) was used, with the following temperature program: 80 °C for 1 min, increased to 250 °C at a rate of 10 °C/min.

The monitored ions were as follows: *m*/*z* 292 and 233 for silylated *N*-methylcarbamoyl mercapturic acid; *m*/*z* 248 and 233 for the silylated internal standard. The detection limit of the method was 0.5 mg/l.

Measurement of 1,2-dihydroxybutyl- and monohydroxy-3-butenyl mercapturic acids related to human exposure to 1,3-butadiene was recently proposed by van Sittert et al. [43]. To 1-ml urine samples, acidified to pH 2.5, were added 200 mg of NaCl and 100 μl of internal standard solution (deuterated 1,2-dihydroxybutyl- and monohydroxy-3-butenyl mercapturic acids in methanol at a concentration of 100 μg/l). The metabolites were extracted with ethyl acetate containing 20% (v/v) methanol. After evaporation of the organic phase, the mercapturic acids were derivatized by methylation in 1 ml methanol/anhydrous HCl (1/10), for 15 min at room temperature. This was followed by reaction with pentafluorobenzoylchloride (7 μl) in toluene (400 μl) and pyridine (2 μl), for 60 min at 60 °C. After evaporation of the solvents, the methylated and pentafluorobenzoylated metabolites were dissolved in 100 μl of toluene and analysed by gas chromatography with negative electron capture ionisation tandem mass spectrometry (GC–NECI-MS–MS). One μl of the toluene solution was used for on-column injection, the column being a DB-1 (15 m×0.32 mm, 0.25 μm film thickness). The oven was maintained at 80 °C for 1 min, increased at a rate of 10 °C/min to 320 °C and kept at this temperature for 5 min. The following ions were monitored: *m*/*z* 653→167, 221 (for 1,2-dihydroxybutyl mercapturic acid), 441→175, 176 (for monohydroxy-3-butenyl mercapturic acid and 447→175, 176 (for d_6-monohydroxy-3,3-butenyl mercapturic acid).

The detection limit for these metabolites was 0.5 μg/l.

Analysis of the allyl mercapturic acid and 3-hydroxypropyl mercapturic acid in urine was proposed by De Rooij et al. [51], using a GC–MS method. One-ml aliquots of urine samples (acidified with 0.1–0.2 ml 0.2 *M* HCl), to which the internal standard (D5-phenyl mercapturic acid) was added, were extracted with solid-phase extraction columns (RP18, Baker), preactivated with methanol and water. The columns were dried by centrifugation (400 *g* for 10 min) and treated with 3 ml of methanol. The solvent was evaporated in a stream of nitrogen. The dry residue was methylated with 2 ml of a 1.25 *M* solution of HCl gas in methanol at room temperature. After 30 min the solvent was evaporated and the residue dissolved in 0.5 ml ethyl acetate. This solution was analysed by GC–MS, with an injector and inlet line temperature of 250 °C; the column was a fused-silica Cp Sil 5 CB column (50 m×0.25 mm I.D. and 0.2 μm film thickness). The oven temperature was programmed from 50 °C for 1 min to 250 °C at 20 °C/min. *M*/*z* 158 and 217 (ions of the methyl ester of allyl mercapturic acid), together with *m*/*z* 235 and 176 (ions of the methyl ester of 3-hydroxypropyl mercapturic acid), were monitored (with those of the internal standard).

Analysis of trichlorovinyl mercapturic acid was also performed with a GC–MS technique with negative ion detection [45]. Three-ml aliquots of human urine, to which the deuterated metabolite was added as the internal standard, were acidified to pH 1–2 with 1 *M* HCl. After triple extraction with 2 ml of ethyl acetate, the recovered solvent was completely dried and the resulting residues derivatized with 200 μl methanolic BF_3 for 30 min at 60 °C. The solution was extracted with 1 ml of chloroform. After drying over sodium sulphate and evaporation of the solvent, the residues were dissolved in 0.5 ml

of chloroform and analysed by GC-electron-capture MS. A DB-1 fused-silica capillary column (30 m× 0.25 mm I.D. and 1 μm film thickness) was used. The recorded ions were: *m/z* 178, *m/z* 180, *m/z* 181 and *m/z* 183). The initial oven temperature of 55 °C was increased to 300 °C at 20 °C/min. The injector and the transfer line temperature was 280 °C, while the ion source temperature was 150 °C. Fig. 5 shows the gas chromatographic analysis of the (trichlorovinyl) mercapturic acid extracted from a human urine sample collected some hours after exposure to 10 ppm perchloroethene for 6 h [45].

A double extraction was used in the detection of 1,2-dichlorovinyl and 2,2-dichlorovinyl mercapturic acids in urine of subjects exposed to trichloroethylene [47]. Deuterated 1,2-dichlorovinyl mercapturic acid was used as the internal standard, added to 3-ml urine samples. After acidification to pH 1 with HCl (2 *M*), extraction with 2 ml diethylether each was performed four times. The solvent was then removed in a vacuum and the dry residue was dissolved in water (1 ml). This solution was added to a preconditioned C_{18}-cartridge (200 mg/3 ml), which was first washed in a water solution with trifluoroacetic acid (pH 2) and then with water–methanol (8:2, v/v). The mercapturic acids were then eluted with 2 ml methanol. After removing the solvent by evaporation, 0.5 ml methanol was added. The derivatization of the acids was performed by 3 ml of an ethereal solution of diazomethane. The evaporation of the ether was followed by addition of 1 ml of chloroform, part of which was analysed by GC–MS with chemical ionisation and negative ion detection. A DB-1 fused-silica capillary column (30 m, 0.25 mm I.D., 0.1 μm film thickness) was used. A linear temperature program from 40 to 300 °C at 10 °C/min was applied to the column; the injector temperature and that of transfer line were, respectively, 200 and 250 °C, while the ion source temperature was 150 °C. The fragments *m/z* 178, *m/z* 180, *m/z* 182, *m/z* 184, *m/z* 235 and *m/z* 237 were monitored.

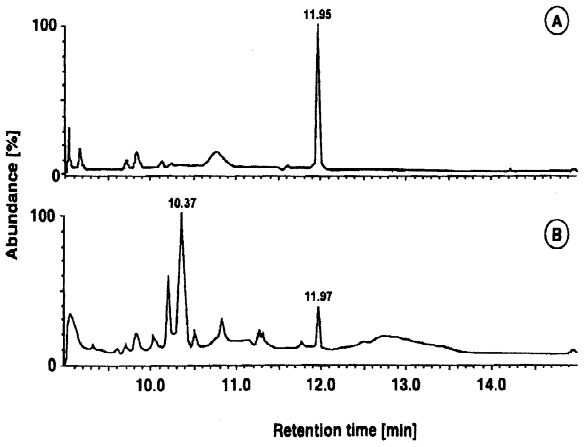

Fig. 5. Gas chromatographic separation of a human urine sample: (A) *m/z* 181 for [$^2H_3$](trichlorovinyl) mercapturic acid methyl ester (t_R 11.95 min); (B) *m/z* 178 for (trichlorovinyl) mercapturic acid methyl ester (t_R 11.97) [45].

A similar method was used by Bloemen et al. [62] for the analysis of the two mercapturic acids coming from trichloroethylene. The main difference was related to sample purification. Previous researchers used an SPE after the diethylether extraction, while these authors proceeded directly to the derivatization and then extracted the acidified sample with two volumes of ethyl acetate. The residues obtained through the evaporation of ethyl acetate were dissolved in methanol (500 μl), and then treated with diazomethane (1 ml) dissolved in ether. After evaporation of the solvent and excess diazomethane, the final residue was treated with 250 μl of ethyl acetate and analysed by GC–MS. The capillary column (CP Sil-SE, 25 m×0.22 mm I.D.) was programmed from 60 to 280 °C at 20 °C/min.The injector and the ion source were kept at 280 °C. The fragments *m/z* 200 and *m/z* 202 were monitored. The detection limit for the mercapturic acids was 0.04 μmol/l.

Urinary analysis of (*Z*- and *E*-3-chloro-2-propenyl) mercapturic acids coming from *Z*- and *E*-1,3-dichloropropene has been performed by van Wilie et al. [63], with a GC/sulfur-selective detector technique, and by Brouwer et al. [54] with a GC–MS technique.

Van Wilie et al. [63] studied a group of workers exposed to a mixture of *Z*- and *E*-1,3-dichloropropene, to measure both mercapturic acids coming from the fumigant mixture. To 5 ml urine the internal standard (benzyl mercapturic acid 6.9 or 69 μg, depending on the expected concentration of

metabolites) and 350 μl of 2 *M* hydrochloric acid were added (the resulting pH was 1–2). Two 2-ml volumes of ethyl acetate were used for extraction, by vortex-mixing for 30 s. After centrifugation at 2500 *g* for 5 min, the recovered ethyl acetate was combined and evaporated under a gentle nitrogen flow at 37 °C. The dry residues were dissolved in 500 μl methanol and methylated by adding 1 ml of an ethereal diazomethane solution. The reaction was allowed to continue for 1 h at room temperature. After evaporation of the methylating agent, the residues were dissolved in ethyl acetate (200 or 500 μl) for the GC analysis. A gas chromatograph with a sulphur-selective detector and a fused-silica WCOT CP Sil 19CB column (25 m×0.25 mm I.D., 0.18 μm film thickness) was used. The temperatures of the injector port, oven and detector were 290, 210 and 250 °C respectively. The helium flow-rate was about 3 ml. In the detector the hydrogen flow-rate was 142 ml/min, the air flow-rates being 55 and 165 ml/min. The detection limits were 107 and 115 ng/ml, respectively, for *Z*- and *E*-(3-chloro-2-propenyl) mercapturic acids.

With a GC–MS technique, Brouwer et al. [54] measured (*cis*-3-chloro-2-propenyl) mercapturic acid in biological samples from workers exposed to the fumigant, commercially available nematocides consisting of more than 95% *cis*-1,3-dichloropropene.

One-ml aliquots of acidified urine (with 20 μl of 6 *M* HCl), to which D5-*S*-phenyl mercapturic acid was added as an internal standard, were extracted with 4 ml ethyl acetate. The organic phase was transferred to a clean tube and gently evaporated with nitrogen. The residues were treated with 2 ml methylating agent (1.25 methanolic HCl prepared by passing 46 g hydrochloric acid gas through 1 l methanol), for 30 min at room temperature. The methanol was then evaporated in a water bath at 45 °C, by nitrogen flow. The dry residue was dissolved in 1 ml dichloromethane and used for the GC–MS analysis. A 60 m×0.25 mm I.D. fused capillary DB-1 column with a film thickness of 0.1 μm was used. The injector and the transfer line were kept at 250 °C. The oven temperature, kept at 35 °C for 1 min, was increased to 200 °C at 10 °C/min, and then to 300 °C at 5 °C/min. The final temperature was maintained for 7.5 min. The ion source temperature was 180 °C. The fragments *m*/*z* 176, *m*/*z* 117, *m*/*z* 199 and *m*/*z* 258 were recorded (two for (*cis*-3-chloro-2-propenyl) mercapturic acid and two for the methylated internal standard).

Urinary analysis of 2-cyanoethyl mercapturic acid was performed by Jakubowski et al. [57] with a GC/FID technique. This method is very similar to that described for *S*-phenylmercapturic acid. Briefly, to 2.5 ml of urine the internal standard (*N*-acetylomethionine) was added. The preparation was acidified with 50 μl of concentrated HCl, saturated with NaCl and extracted with 25 ml of ethyl acetate. The organic layer was evaporated until dry. The residue was dissolved in 250 μl of ethanol and methylated with gaseous diazomethane. One μl was used for injection into the gas chromatograph, equipped with an FID detector. A glass column (1 m×2 mm I.D.), packed with 3% OV-225 on Varaport 30 (100–120 mesh), was used. The oven temperature was increased from 140 to 250 °C, at a rate of 10 °C/min; injector and detector temperatures were 180° and 260 °C, respectively. Argon was used as the carrier gas, at a flow-rate of 40 ml/min.

3.2. HPLC methods

Most HPLC methods have been developed for analysis of specific mercapturic acids. Recently, Moriwaki et al. [64] have reported a method which makes it possible to measure several mercapturic acids by using an LC–ESI-MS technique. The application was intended for five kinds of mercapturic acids, namely benzylmercapturic acid, *o*-methylbenzyl mercapturic acid, 2,3-, 2,6- and 3,4-dimethylbenzyl mercapturic acids, which are metabolites of toluene, *o*-xylene and trimethylbenzene. The urine samples were not obtained from workers exposed to the reported solvents, but spiked with the reported mercapturic acids at a concentration range 1–100 ng/l. The clean-up procedure started from 1 ml of centrifuged urine, to which 0.5 ml of mercapturic standard (for preparing solutions 1–100 μg/l) and 3.5 ml of distilled water were added. The sample was extracted by SPE, using a Sep-Pak Plus tC_{18} cartridge preconditioned with 2.5 ml methanol and 5 ml of 20 m*M* ammonium acetate solution, adjusted to pH 4 by addition of acetic acid. The sample

solution was loaded on a conditioned cartridge, which was rinsed with 10 ml of the ammonium acetate solution and dried by centrifuging for 10 min. After eluting the cartridge with 4 ml of acetonitrile, the solvent was evaporated in a flow of nitrogen and the dry components dissolved in 1 ml of the ammonium acetate solution. A 20-μl aliquot was injected into the LC–ESI-MS. The LC analysis used a Develosil C30-OG column (5 μm particle size, 150×4.6 mm I.D.) at 40 °C, with a mobile phase A (the reported ammonium acetate solution) and a mobile phase B (acetonitrile). The flow-rate was 0.5 ml/min, with a gradient as follows: 0–20 min, a linear increase from 5 to 50% (phase B), which was then held constant for the following 10 min. The operating conditions of ESI-MS were as follows: the drying nitrogen gas temperature was set at 340 °C, at a flow-rate of 12 l/min. The capillary was held at a potential of −3500 V relative to the counter electrode for the negative-ion mode. The monitored ions (SIM) were *m*/*z* 252, 266 and 280.

The advantages of this method are its selectivity and sensitivity. In addition, the procedure for preparing samples is not difficult or time-consuming.

In most cases the measurement of one specific mercapturic acid needs a dedicated method. Urinary analysis of *S*-phenylmercapturic acid with HPLC has been reported on several occasions in samples obtained from animals treated with the solvent, but rarely for study of human exposure.

Jongeneelen et al. [8] proposed a HPLC method for analysis of *S*-phenylmercapturic acid in urine of rats and workers exposed to benzene. The detection limits were not low enough to identify the background level of the metabolite in human urine. Because of this, we offer only a few remarks about this method. The acidified urine samples were extracted with diethyl ether, which was then evaporated. The residues were dissolved in methanol, and used for the HPLC analysis on a reversed-phase column and a UV-detector at 256 nm.

Maestri et al. [12] described a quite complex method with a complicated sample preparation phase. Aliquots of 4 ml of centrifuged urine, acidified to pH 1 with HCl, were applied to Sep-Pak C_{18} cartridges (preconditioned with methanol and 10 m*M* HCl). After washing with 5 ml of water–methanol–acetic acid (89:10:1, v/v) and 0.5 ml of toluene, the cartridges were air-dried for 10 min. Each sample was then eluted with 2.5 ml of chloroform–acetic acid (99:1 v/v) mixture. The elute was dried under nitrogen and reconstituted with 1.1 ml of 10 m*M* phosphate buffer (pH 7). One ml of this solution was applied to a SAX cartridge (preconditioned with 3 ml of methanol and 1 ml of 50 m*M* phosphate buffer (pH 7)). The cartridge was washed with 2 ml of 10 m*M* phosphate buffer and 6 ml of 10 m*M* HCl, before eluting the *S*-phenylmercapturic acid with 2 ml of 1 *M* phosphate buffer (pH 7).

This first cleaning phase was followed by the deacetylation and derivatization of the obtained *S*-phenylcysteine; 0.5 ml of the elute was incubated with acylase (1100 U), for 90 min at 30 °C; the deproteinisation of the sample was performed by ultrafiltration for 30 min at 6000 rev./min, using Millex Ultrafree-MC 10000 NMWL. Derivatization was achieved by adding 5 μl of the derivatization reagent (50 mg of *o*-phthalaldehyde in 300 μl of methanol with 0.2% of 2-mercaptoethanol) to 55 μl of the ultrafiltrate. After 5 min the mixture was ready for injection into the HPLC with a fluorescence monitor (excitation 330 nm, emission 440 nm). The column was a Supelcosil C_{18} 150×4.6 mm, 3 μm. Solvent A was prepared with acetate buffer (0.05 mol, pH 6.5), 0.83 g/l tetraethylammonium chloride and 1% tetrahydrofuran. Solvent B was methanol. The flow-rate was 0.8 ml/min, and the gradient programme ran as follows: for the first 12 min the methanol level was 54%, increasing to 70% in 4 min and held at this proportion for 7 min; in 3 min the methanol was reduced to 54%, and held at this proportion for another 14 min.

Recovery of the *S*-phenylmercapturic acid added to urine was 90%. The detection limit was 0.5 μg/l. An important limit of the method was the short stability of the adducts from the *o*-phthalaldehyde. Strict standardisation of the derivatization using an autosampler was necessary in order to obtain reliable results.

The same method, with few modifications, was used by Kivistö et al. [14] who measured *S*-phenylmercapturic acid in urine of workers at two facilities where benzene was produced or was an important air pollutant.

The long description of the method indicates that it is neither simple nor short. The preparation phase

is very time-consuming, and an auto-sampler is essential for timing the derivatization phase and the subsequent analysis.

In 1999, Melikian et al. [11] developed a sensitive and specific liquid chromatographic–tandem mass spectrometric assay for determination of urinary *S*-phenylmercapturic acid and *trans-trans*-muconic acid, which is another minor metabolite of benzene. The clean-up of urine samples was performed by solid-phase extraction. One-ml urine samples, spiked with 15 ng of [$^{13}C_6$]-*S*-phenylmercapturic (internal standard), were passed through a SAX cartridge conditioned with methanol (5 ml) and water (5 ml). The cartridge was treated with water (3 ml), 5 m*M* phosphate buffer, pH 7 (3 ml), and 1% aqueous acetic acid (3 ml); finally, the metabolites were eluted with 4 ml of 10% aqueous acetic acid. This fraction was extracted three times with 5 ml of ethyl acetate and the recovered solvent was evaporated until dry under nitrogen. The residue was dissolved in 150 μl of methanol mixed with aqueous acetic acid (20:80), and a 30 μl sample was analysed by liquid chromatography–electrospray-tandem mass spectrometry in selected reaction monitoring (LC–ES-MS–MS-SRM). A Phenomenex Ultramex 5 μ C_{18} narrow-bore column (250×2 mm) was used, with a flow of 0.9 ml/min, reduced with a pre-injector splitter to 160 μl/min. A gradient from 80% solvent A (0.5% aqueous acetic acid) and 20% solvent B (methanol) to 100% solvent B over 5 min was used. The mass spectrometer was operated in the negative ion mode. The spray voltage was 4.1 kV, and the capillary temperature 220 °C. The ion was monitored at $m/z=238\rightarrow109$ for *S*-phenylmercapturic acid and $m/z=244\rightarrow115$ for the internal standard. The detection limit was 0.4 μg/l urine (corresponding to 0.02 ng/injection). This method is sensitive enough to measure *S*-phenylmercapturic acid in urine of smokers or non-smokers, not occupationally exposed to benzene.

In humans, the diastereoisomeric forms of mercapturic acids derived from styrene have been analysed by Maestri et al. [65], using a HPLC method with fluorescence detection. The preparation of biological samples is not easy and needs several steps. A 100-μl volume of acidified (to pH 1–2 with H_3PO_4) filtered urine was injected into a HPLC system equipped with a Water Resolve C_{18} precolumn. Water (pH 3 with H_3PO_4) and methanol (82:18, v/v), at a flow-rate of 2 ml/min, were used as the mobile phase. The fraction containing the two diastereoisomeric forms of (1-phenyl-2-hydroxyethyl)- and (2-phenyl-2-hydroxyethyl) mercapturic acids and eluting between 0.7 and 1.15 min, was collected. The purified sample was then deacetylated by adding 200 μl of a solution containing 42,000 U/ml acylase I in a 0.5 *M* phosphate buffer (pH 7.0). After 16 h at 37 °C, the sample was deproteinized by centrifugal ultrafiltration (15 min at 1000 rev./min). The products present in the filtered sample (90 μl) were derivatized for 5 min at room temperature, with 10 μl of the fluorescent reagent (50 mg of *o*-phthaldialdehyde in 300 μl of a 0.2% solution of 2-mercaptoethanol in methanol added with 2 ml of borate buffer at pH 10). The mixture was then ready for injection into a HPLC apparatus, with an ODS Hypersil column (250×4.6 mm, I.D. 3 μm) at 35 °C and spectrofluorometer detector. Separation was performed by gradient elution with acetate buffer (0.05 *M*, pH 6.5) and methanol. The excitation and emission wavelengths of the detector were set at 330 and 440 nm, respectively. As shown in Fig. 6 [65] the retention times of stereoisomers ''*S*'' and ''*R*'' of (1-phenyl-2-hydroxyethyl)-mercapturic acid were 52.8 and 73.7 min, while the two diastereoisomers of (2-phenyl-2-hydroxyethyl)-mercapturic acid were detectable at 70.5 min. The reproducibility and the accuracy of the method are good (with variation coefficients always lower than 6%); the detection limit of each metabolite was about 7 μg/l.

This method is quite time-consuming. The fluorescent derivatives of styrene mercapturic acids show a long-term instability, requiring careful control of the reaction time. The authors who developed this method suggest that it should not be used for routine analyses.

In contrast with this method, Manini et al. [24] described a quite simple and easy automated analysis of the styrene-specific mercapturic acids using an LC–ES-MS–MS. The preparation phase is very short. After filtration and acidification with formic acid (0.1 *M*) of a 250-μl urine sample, 5 μl were injected into the LC column. For the separation of the four diastereoisomers of styrene-specific mercapturic acids, a Supelcosil C_{18} DB column 75×3.0 mm I.D., 3 μm was used. A 20 m*M* aqueous formic acid

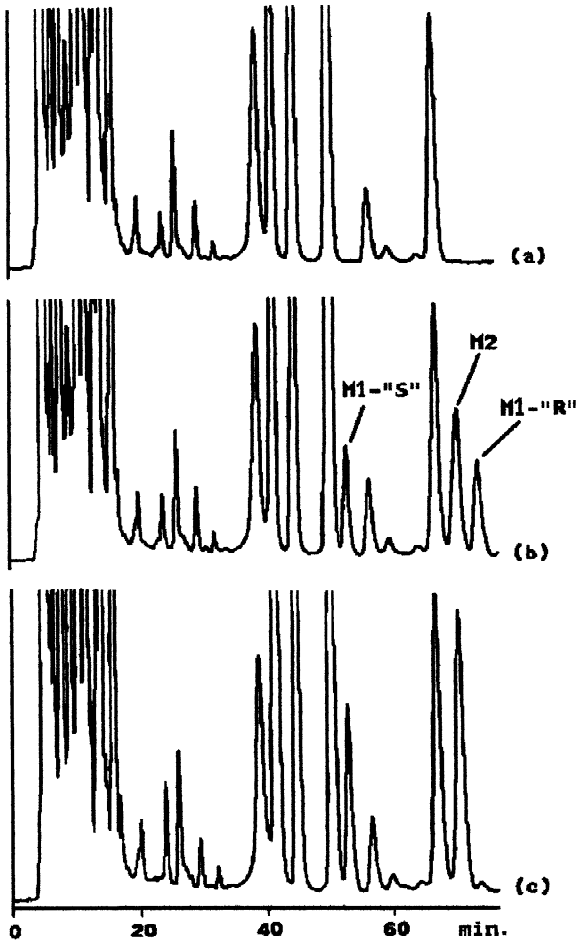

Fig. 6. Chromatograms obtained from urine samples: (a) urine from a non-exposed subject; (b) the same sample spiked with mercapturic acids (diastereoisomers of 1-phenyl-2-hydroxyethyl mercapturic acids, M1-*S* and M1-*R*) and (diastereoisomers of 2-phenyl-2-hydroxyethyl mercapturic acids, M2); (c) urine sample from a worker exposed to styrene [65].

(pH 3) and methanol at a flow-rate of 0.5 ml/min were the eluting solution, with the following program: 5% methanol, held for 7 min; from 5% to 15% methanol in 15 min, held for 1 min, from 15% to 75% methanol in 3 min, held for 2 min. The eluate was split (1:20) before ionisation of the analytes, by electrospray in negative-ion mode. The reactions *m*/*z* 282.1 to 153.0, typical of all these mercapturates, and *m*/*z* 281.1 to 123, characteristic of ''*S*'' and ''*R*'' (1-phenyl-2-hydroxyethyl)-mercapturic acids, were evaluated.

The diastereoisomeric forms of mercapturic acids cannot be distinguished by mass spectrometry if they are not separated by a proper column with suitable gradient elution conditions. Each analysis lasts about 30 min. The detection limits range between 0.7 and 1 μg/l for each stereoisomer.

Apart from the cost of the instrumental apparatus, this method meets the requirements of the biological monitoring of workers exposed to styrene.

Recently, a HPLC method with a UV detector has also been proposed by Negri et al. [66] for the methylcarbamoyl mercapturic acid, coming from *N*,*N*-dimethylformamide. This method involves a time-consuming preparatory phase, but it is suitable for biological monitoring of workers exposed to this solvent. There is a double solid-phase extraction. The first uses a C_{18} (500 mg/3 ml) cartridge, preactivated with methanol and 1 *M* HCl (3 ml of each); 2-ml aliquots of urine, acidified with 200 μl of 2.4 *M* HCl and centrifuged at 4000 rev./min for 15 min, are added to the cartridges. Treatment with 3 ml of H_3PO_4 1% (v/v) is followed by the elution of the mercapturic acid with another 3 ml H_3PO_4 1%. This solution is passed through a second kind of cartridge (Isolute ENV$^+$ 200 mg/3 ml), activated in the same way as the first. Triple washing is performed before the elution of the metabolite: first with 3 ml H_3PO_4 1%, and methanol 20%, then with 2 ml H_3PO_4 1% and finally with 2 ml 0.5 *M* phosphate buffer pH 7. The *N*-methylcarbamoyl mercapturic acid is recovered in vials containing 340 μl 2.4 *M* HCl, by using 4 ml 0.5 *M* phosphate buffer pH 7 mixed with methanol 5%. The solution is then ready for HPLC analysis with an Amines Ion Exclusion HPX-87 H, 300×7.8 mm, 9 μm at 37 °C. The mobile phase is 1 m*M* sulphuric acid, with a flow-rate of 0.85 ml/min; the detector UV is set at 196 nm. The detection limit is 1.32 mg/l. Analysis of the *N*-methylcarbamoyl mercapturic acid was also proposed by Perbellini et al. [67], using a HPLC with a UV detector at 346 nm. The method was very simple: a 10-μl volume of urine was added to 250 μl of carbonate–hydrogen carbonate buffer (pH 9.27) and 250 μl dabsyl chloride solution (6 m*M* in acetonitrile). After gentle shaking, the sample was placed at 70 °C for 10 min and after that centrifuged at 1300 rev./min for 3 min. A 100-μl volume of the supernatant was used for injection in HPLC with a C_{18} column (150×4.6 mm,

5 μm). The mobile phase was acetonitrile and water. Acetonitrile, 30% at the beginning of the chromatograph run, was increased to 80% in 23 min. The flow-rate was set at 1 ml/min. Fig. 7 shows a chromatogram of the dabsylated *N*-methylcarbamoyl mercapturic present in a urine sample provided by a worker exposed to *N,N*-dimethylformamide [67].

This cheap and user-friendly method is certainly useful for evaluation of occupational exposure to *N,N*-dimethylformamide, but it seems unreliable at very low concentrations. In subjects not professionally exposed to the parent solvent, a median concentration of 3.9 mg/l was found. Negri et al. [66] and Kafferlein and Angerer [60] found average concentrations lower than 1.32 mg/l and 0.04 mg/l, respectively.

3.3. Other techniques

A thin layer chromatography using chiral plate material and selective staining with vanadium pentoxide was used by Hallier et al. [22], to measure styrene-derived mercapturic acids. A 1-ml aliquot of acidified urine (with HNO_3 to pH 1) was purified over a 4-ml extraction column packed with Poropak Q (80–100 mesh), previously conditioned with water (20 ml) and a 5-ml 0.1 *M* HNO_3 solution. After washing with 2 ml of the acid solution, the mercapturic acids were extracted with ether, which was then evaporated under nitrogen. The residue was dissolved in 10 μl of methanol and applied to a thin layer plate (DC-plate Sigur 25 UV_{254}), which was developed in a mixture of chloroform, ethyl acetate, acetic acid and *n*-butanol (4:4:1:1). The dried chromatogram was treated with a solution of 0.05 *M* $K_2Cr_2O_7$ in 50% acetic acid and sprayed with a 0.1 *M* aqueous solution of silver nitrate. The optical enantiomers of the styrene-specific mercapturic acids were revealed as yellow spots on a reddish-brown background. The detection limit was about 1 μg per spot; despite the apparent high sensitivity of the method, the styrene-specific mercapturic acids were found in only one worker (out of 20), whose exposure averaged 157 mg/m^3 and whose urinary concentrations of mandelic acid (the main urinary metabolite of styrene) ranged between 80 and 1610 mg/l. The reported results are in evident conflict with those reported by other researchers and summarised in previous paragraphs. It is not clear why.

The application of immunochemical techniques for measuring mercapturic acids in urine is a recent exciting prospect for the analysis of these products in biological samples. Aston [68] proposed this methodology for the analysis of urinary *S*-phenylmercap-

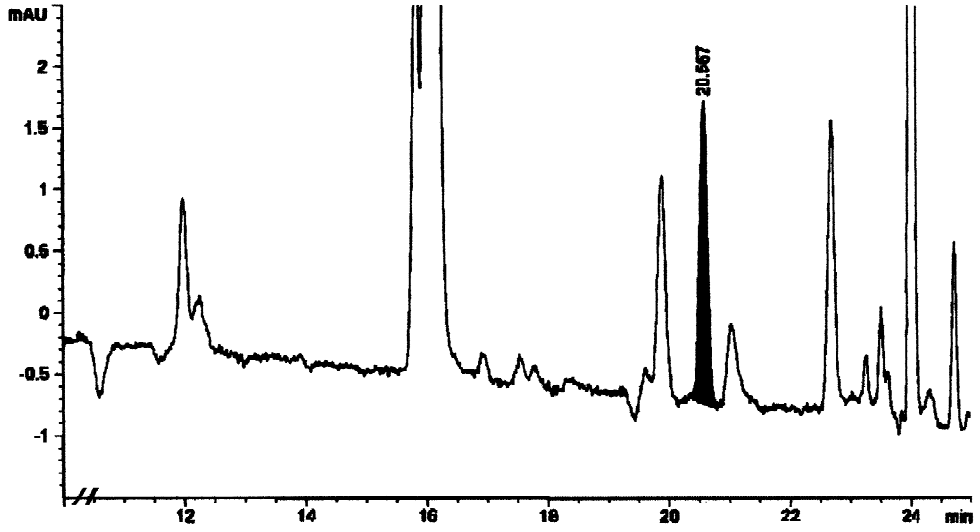

Fig. 7. HPLC chromatogram of a urine sample obtained from a worker exposed to *N,N*-dimethylformamide: at retention time of 20.567 min there is the peak of the dabsylated *N*-methylcarbamoyl mercapturic acid [67].

turic acid. The main elements on which this method is based are: the production of specific antibodies, an immunoenrichment procedure to purify and concentrate the benzene metabolite and an immunoassay which quantifies its urinary concentrations. Some of these aspects give reliable results, but more experiences are needed for the application in industrial settings.

4. Final considerations

Within Occupational Medicine and Industrial Toxicology the biological monitoring of different kinds of exposure to chemicals is often basic for risk assessment and risk management.

As reported above, two mercapturic acids (*S*-phenylmercapturic and *N*-methylcarbamoyl mercapturic acids) have been proposed by ACGIH [3] and/or DFG [4] for purposes of evaluating exposure to benzene and *N*,*N*-dimethylformamide. Bearing in mind the high number of workers who should be checked, these mercapturic acids are still little used as biomarkers of exposure. In our opinion the main reason is related to analytical problems, involving high costs in relation to preparation time or instrumentation.

All the GC methods, as reported above, constantly involve a difficult preparation phase and derivatization phase (sometimes requiring carcinogenic products). There are no easy or cheap GC methods for this kind of analysis.

Among the HPLC methods for the measurement of mercapturic acids, some have a quite simple preparation phase. The method proposed by Manini et al. [24] for analysing mercapturic acids from styrene has a preparation phase with only filtration and acidification of the urine sample before injection into an LC–ES-MS–MS. Probably several other mercapturic acids can be analysed with the same method. The expensive instrumentation used is the negative aspect of the method, because such instruments are available almost exclusively for specific studies, while they are not convenient for routine checking of workers' exposure.

As the intensity of exposure to chemicals in industrialised countries tends to decrease, their biological monitoring depends on sensitive and specific analytical methods. This means that, in the short term, the measurement of most mercapturic acids in urine will be quite expensive, but new instruments are making this kind of analysis easy.

Theoretically, analysis of mercapturic acids for biological monitoring of professional exposure should be useful subject to two main conditions: (a) if mercapturic acid can afford more specific information about occupational exposure than other metabolites; (b) if its kinetics make it possible to evaluate several days of exposure, because of a quite long biological half-life.

Most mercapturic acids have a short biological half-life and their analysis will probably not give different information than other known metabolites. For instance, *S*-phenylmercapturic acid has a very short half-life, but it is more specific than other biotransformation products of benzene like phenol or *trans-trans*-muconic acid (the last can also derive from sorbic acid, a diffuse food additive).

N-Methylcarbamoyl mercapturic acid has a half-life of about 23 h [34–36]. It accumulates during the working week, so that its urinary concentration should explain the amount of *N*,*N*-dimethylformamide uptake during several days of exposure.

In conclusion, the analysis of the mercapturic acids in biological media is considerably increasing information about electrophilic products absorbed or synthesized by the human organism. Besides their convenient use in the industrial field, they can afford physio-pathological interpretation of several biological effects caused by many chemicals widely used in industrial settings.

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