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Review

Liquid chromatography–mass spectrometry in occupational toxicology: A novel approach to the study of biotransformation of industrial chemicals

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Abstract

Biological monitoring and biomarkers are used in occupational toxicology for a more accurate risk assessment of occupationally exposed people. Appropriate and validated biomarkers of internal dose, like urinary metabolites, besides to be positively correlated with external exposure, have a predictive value to the risk of adverse effects. The application of liquid chromatography–mass spectrometry (LC–MS) in occupational and environmental toxicology, although relatively recent, has been demonstrated valid in the determination of traditional biomarkers of exposure, as well as in metabolism studies aimed at investigating minor metabolic routes and new more specific biomarkers. This review presents selected applications of LC–MS to the study of the metabolism of industrial chemicals, like *n*-hexane, benzene and other aromatic hydrocarbons, styrene and other monomers employed in plastic industry, as well as to other chemicals used in working environments, like pesticides used by farmers, and antineoplastic agents prepared by hospital personnel. Analytical and pre-analytical factors, which affect quantitative determination of urinary metabolites, i.e. sample preparation, matrix effect, ion suppression, use of internal standards, and calibration, are emphasized.

Keywords: Biomarkers of exposure; Metabolites; Urine; Workers

Contents

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1. Introduction

The objective of occupational toxicology is the prevention of health impairment that may result from exposure to chemicals at workplaces. This implies the definition of permissible levels of exposure, that is, levels that according to the present status of knowledge are estimated to cause no adverse health effects during the workers' lifetime. Threshold limit values for 8-h time weighted average (TLVs-TWA) exposure to airborne concentrations of industrial chemicals at workplaces have been established and are regularly updated by the American Conference of Governmental Industrial Hygienists (ACGIH) [1]. Biomarkers can be used for a more accurate assessment of chemical exposure and to predict the consequences of such exposure in groups at risk. A biomarker is "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [2]. Depending on their use, biomarkers can be classified as markers of exposure, effect and susceptibility and can be applied to get insights on the multi-stage and multi-factor process that is thought to link exposure to long-term outcomes, as shown in Fig. 1 [3]. A biomarker of exposure has been defined as "an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism" [3], whereas a biomarker of effect is "any measurable biochemical, physiological or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease" [3]. Biomarkers

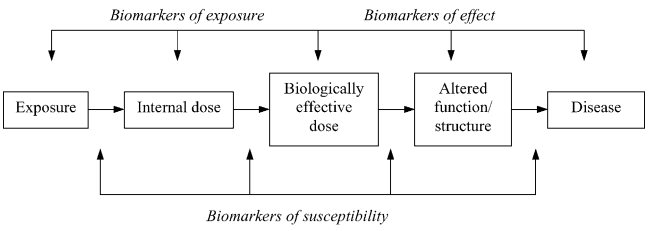


Fig. 1. Use of biomarkers in risk assessment. Adapted from [3].

of susceptibility are effect-modifying factors, which include both genetic (genetic polymorphisms of drug metabolizing and DNA repair enzymes) and acquired conditions. In order to become useful tools in risk assessment, biomarkers must be relevant, e.g. appropriated to provide information on important questions concerning health risks, and valid for both analytical and epidemiological aspects. In particular, external validity is required to obtain results, which can be generalized to other populations.

In biomarker research, the major focus of interest has long been on biomarkers of exposure, with the aim to reduce misclassification deriving from the use of job title alone or, at best, of point estimates of airborne pollution [4]. Moreover, when adsorption mainly occurs through the dermal route or when individual protective devices are used, biomarkers of exposure can provide reliable measurements of internal doses, which are useful to assess dose–response relationships. Several biomarkers of exposure may be available for the same chemical, e.g. the parent compound itself, a metabolite, or a macromolecular adduct (to DNA or protein), and the same biomarker may have different meanings depending on the sampling time. Therefore, the choice of the marker should rely on a number of considerations, but mainly on kinetic parameters and on the knowledge of its toxicological mechanism. An ideal biomarker of exposure should be specific for the exposure of interest, detectable in small quantities, measurable by non-invasive techniques, inexpensive, associated with prior exposure and provided of an excellent positive predictive value to a specific health status. Although less specific than the parent compounds, metabolites have a longer half-life and may reflect day- or week-exposure. When dose-effect and dose–response relationships are known, an appropriate biomarker of dose may be sufficient to assess the risk of adverse effects. For its non-invasiveness, urine collection is widely used to obtain biological samples suitable for the determination of parent compounds and their metabolites, whereas blood drawing is needed for the determination of macromolecular adducts, which are considered biomarkers of biologically effective dose. For a correct interpretation of biological monitoring data, it is important to

identify and take into account all factors, which can influence dose–response relationships, since considerable variability exists in the response of humans to toxic substances. Biological conditions (sex, age, fatty mass, diseases, chronobiological factors), metabolic interferences arising from habits (tobacco and alcohol consumption, diet), and genetic polymorphisms should be carefully considered in order to explain inter- and intra-individual variability [4].

Analytical techniques relying on mass spectrometry, and particularly inductively coupled plasma-mass spectrometry (ICP-MS) for metals and liquid chromatography–*tandem* mass spectrometry (LC–MS–MS) for metabolites, although relatively expensive, seems to have superior and unique characteristics of selectivity and sensitivity needed for the determination of trace and ultra-trace amounts of parent compounds and their metabolites in biological media. The present review will consider the application of LC–MS–(MS) to the determination of biomarkers of exposure and, particularly, to urinary metabolites of organic solvents used in industrial settings; DNA and protein adducts will not be included, since they have been recently reviewed by Koc and Swenberg [5] and Törnqvist et al. [6], respectively.

2. Methods for the analysis of biomarkers of exposure

Xenobiotic metabolic biotransformation generally proceeds via phase I reactions implying chemical modification of the parent compound, e.g., oxidation, reduction, and hydrolysis. During phase II biosynthetic reactions, functional groups (resulting from phase I biotransformation) are conjugated to endogenous substrates to yield polar compounds such as glucuronides, sulfates esters, and mercapturic acids. Most industrial chemicals, like organic solvents and monomers employed in plastic industry, are small lipophilic molecules. As an example, Fig. 2 shows the complete and complex scheme of styrene metabolization in man. Both the parent chemical and its metabolites can be measured as potential biomarkers of exposure. Whereas the parent compound is often apolar, phase I metabolites are polar, and phase II metabolites are very polar ionic compounds.

Analytical methods for routine analysis of biomarkers of exposure are mainly based on chromatographic techniques, both gas chromatography (GC) and liquid chromatography (LC) coupled with different kinds of detectors. GC is used for the determination of unchanged organic solvents present in exhaled air, blood, and urine [7]. Identification of polar metabolites by gas chromatography–mass spectrometry (GC–MS) is difficult, as these products tend to be relatively nonvolatile and thermally unstable. Nevertheless, most of the metabolism studies conducted in the past relied on the use of GC–MS, which was the most advanced technique available in research laboratories. Derivatization of phase I metabolites (carboxylic acids, alcohols) and phase II mercapturic acids is often required prior to GC–MS analysis. In case of sulfate and glucuronide phase II conjugates, enzymatic or acidic hydrolysis followed by extraction and derivatization of the resulting aglycones is needed before injection. All these sample manipulations made the procedures difficult and time-consuming, and in some cases led to the generation of analytical artifacts. Polar metabolites are molecules suitable for determination by LC. Efficient chromatography of acidic metabolites is obtained by using phosphate buffers [8], whereas retention of conjugated metabolites was obtained by ion-pair reversed phase (RP) chromatography [9]. Limitations due to the scarce sensitivity and selectivity of UV detection have been overcome by proper sample preparation: extensive sample clean up to limit interferences from the matrix, sample pre-concentration, derivatization with UV-chromophores or fluorescent reagents have been proposed to improve the characteristics of routinary used LC methods. On the other hand, all these sample handlings make the analytical procedures more complex and lengthy.

3. Liquid chromatography–mass spectrometry

The application of LC–MS in the field of occupational toxicology is relatively recent [10,11] and the number of applications is still limited when compared to other related fields, like pharmacokinetics, clinical chemistry and forensic toxicology [12]. Nevertheless, some recent studies based on the use of LC–MS–(MS) clearly showed the potential of this technique in the determination of traditional biomarkers of exposure, as well as in metabolism studies aimed at investigating minor metabolic routes and new more specific biomarkers. This review will present selected applications of LC–MS to the study of the metabolism of industrial chemicals, like *n*-hexane [13–15], benzene [16–21] and other aromatic hydrocarbons [22–26], styrene [27–33] and other monomers employed in plastic industry [34–38], as well as to other chemicals used in working environments, like pesticides used by farmers [39–41], and antineoplastic agents prepared by hospital personnel [42–47]. Prior to a detailed discussion of these applications, some general issues concerning chromatographic mechanisms, ionization and fragmentation pathways, and quantitative aspects will be addressed in this section.

3.1. Chromatographic separation

The on-line coupling of LC to MS requires the use of proper stationary and mobile phases composed of volatile buffers, acids, and modifiers. Most of the mobile phases previously used were adapted, by substituting phosphate buffers with formate or acetate buffers and by using volatile ion-pairing agents. In one of the first papers dealing with metabolism of an industrial chemical [10], the use of simultaneous anion exchange and RP chromatography on a methylstyrene–divinylbenzene columns was found to be effective for the separation of benzene and five of

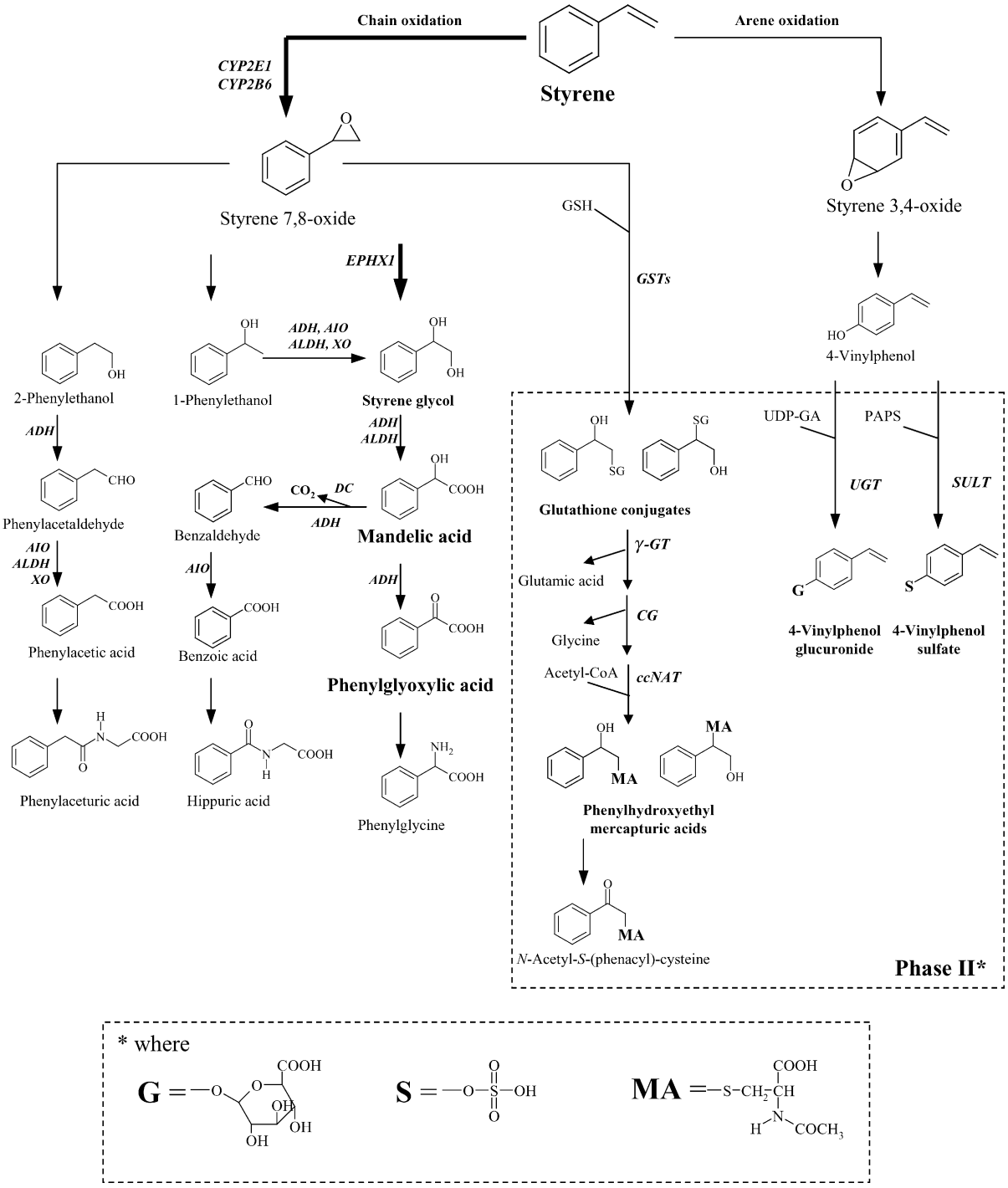
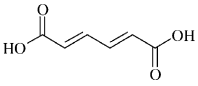
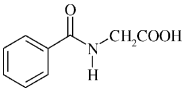
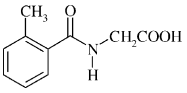
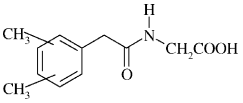
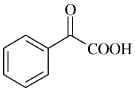
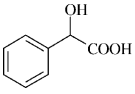


Fig. 2. Scheme of styrene metabolism in man. Adapted from [32]. CYP2E1 and CYP2B6: cytochrome P-450 monooxygenase; EPHX1: microsomal epoxide hydrolase; ADH: alcohol dehydrogenase; AIO: aldehyde oxidase; ALDH: aldehyde dehydrogenase; XO: xanthine oxidase; DC: decarboxylase; GSH: glutathione; GSTs: glutathione *S*-transferases; γ-GT: gammaglutamyl transpeptidase; CG: cysteinyl glycinase; ccNAT: *N*-acetyltransferase; UDP-GA: uridine-5′-diphospho-α-D-glucuronic acid; UGT: UDP-glucuronyl transferase; PAPS: 3′-phosphoadenosine-5′-phosphosulfate; SULT: sulfotransferase.

its phase I and phase II metabolites, phenol, phenyl-β-D-glucuronide, phenylsulfate, phenylmercapturate, and *t,t*-muconic acid. These separations were compatible with both particle beam (PB) and thermospray (TSP) LC–MS interfaces and represented a valid alternative to the use of ion-pairing agents. Glucuronides of molecules of toxicological interest, (phenyl-, naphthyl-, aminophenyl-, and *p*-nitrophenyl-glucuronide) were retained on partially endcapped C18 *high-speed* columns under ion-suppressed RP chromatography conditions and detected by negative-ion atmospheric pressure chemical ionization (APCI)-MS [11]. The use of 2 mM formic acid in the mobile phase was a good

Table 1
Structures and mass spectrometric behavior of phase I metabolites of some important industrial chemicals

Parent compound	Metabolite(s)	Structure	Ion mode	Ion[a], *m/z*	Fragment[b], *m/z*	References
Benzene	*t,t*-MA		NI	141	97	[17]
Toluene	HA		NI	178	134	[22]
Xylene	MHA		NI	192	148	[22]
Trimethylbenzene	DMHs		PI	208	–	[24]
Styrene	PGA		NI	149	105	[22,27]
Styrene	MA		NI	151	107	[27]

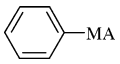
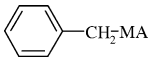
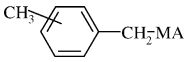
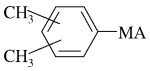
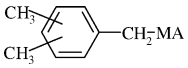
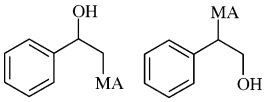
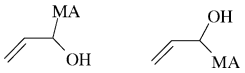
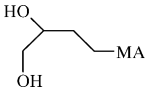
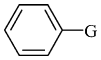
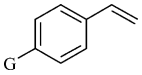
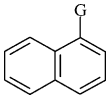
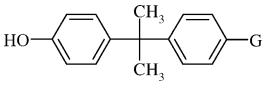
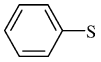
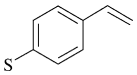
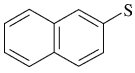
t,t-MA: *t,t*-muconic acid; HA: hippuric acid; MHA: methylhippuric acid; DMHs: dimethylhippuric acids; PGA: phenylglyoxylic acid; MA: mandelic acid.
[a] $[M-H]^-$ for NI, $[M+H]^+$ for PI.
[b] $[M-H-CO_2]^-$.

compromise between the need to prevent deprotonation of the molecules during chromatography and that of avoiding ionization suppression. The same chromatographic mechanism was successfully used also for mercapturates, sulfates, and phase I metabolites. Several kinds of RP C18 stationary phase from the most popular manufacturers have been successfully used; only in two cases, C30 [23,24] and C8 [40] stationary phases have been proposed. Mobile phases made up of (a) water acidified with 0.5–1% of acetic acid [17,25] or 2–100 mM formic acid [21,22,26,27,32] or buffered at pH of about 4 with ammonium acetate (1–50 mM) [23,24,34,35,42–44] and (b) methanol or acetonitrile as organic modifier have been proposed. Except for few cases where the use of isocratic [13,35,42–44] or backflush isocratic elution [20,25,36] was reported, the gradient mode was the preferred solution. The use of a volatile ion-pairing agent, tetrabutylammonium acetate, was used for the chromatography of alkyl phosphates [41].

3.2. Ionization and fragmentation

Both electrospray (ESI) and APCI have been found to be effective in the ionization of metabolites. The structures of metabolites of the most important industrial chemicals discussed in this review, together with their precursor and fragment ion are summarized in Table 1 (phase I) and 2 (phase II metabolites). Pesticides and antineoplastic agents were not included, since they are molecules belonging to different chemical classes and their structures are not generalizable. As shown in Table 1, most of the acidic metabolites were ionized in negative-ion (NI) mode, and $[M-H]^-$ ions were used for selected ion monitoring (SIM) detection in LC–MS or as precursor ions in selected reaction monitoring (SRM) determinations; only in the case of metabolites of trimethylbenzenes, positive ionization (PI) was reported [24]. Fragmentation of the $[M-H]^-$ ions of carboxylic acids generally occurred by loss of CO_2. All NI product ion spectra of mercapturic acids of aromatic compounds (Table 2) were characterized by a fragment ion arising from the loss of CO_2 and $CH_2{=}CH{-}NHCOCH_3$, which was chosen for SRM in LC–MS–MS determinations. A similar fragmentation pathway was observed in the case of mercapturic acids arising from 1,3-butadiene [34], as shown in Fig. 3. PI product-ion spectra were described only for mercapturic acids of trimethylbenzene [24] and styrene [27]; in both cases, the cleavage of the C–S bond and the loss of *N*-acetylcysteine residue with retention of the charge on the aromatic moiety were observed. In case of glucuronides, two complementary fragments at *m/z* 175 and $[M-H-176]^-$ are formed due to the cleavage of the glucuronic bond with retention of the charge on the glucuronic acid and on the aglycone moiety, respectively [11,33,37]. The ion at *m/z* 113 derived from further dissociation of 175 (loss of CO_2 and H_2O) is common to the NI spectra of all glucuronides. Finally, the NI product-ion mass spectrum of sulfate conjugates [33] was characterized by the neutral loss of SO_3 $[M-H-80]^-$. Alkyl phosphates [41] and metabolites of organophosphorous pesticides [39] were ionized in NI mode, whereas ethylenethiourea [40] and antineoplastic agents [43–45] were ionized in PI mode. Specific fragmentation pathways are described in the original papers.

Table 2
Structures and fragmentations of phase II metabolites of industrial chemicals: mercapturic acids, glucuronides and sulfates

Parent compound	Metabolite(s)	R-MA	Ion[a], *m/z*	Fragment[b], *m/z*	References
Mercapturic acids					
Benzene	*S*-PMA		238	109	[17,20]
Toluene	BMA		252	–	[23]
Xylene	MBAs		266	–	[23]
Xylene	DPMAs		266	137	[25]
Trimethylbenzene	DMBs or DMMs		280	–	[23]
			282 (PI)	119 (PI)	[24]
Styrene	PHEMAs		282	153	[27]
Butadiene	MHBMAs		232	103	[34]
Butadiene	DHBMA		250	121	[34]
Parent compound	Metabolite	R-G	$[M-H]^-$, *m/z*	Fragments[c], *m/z*	References
Glucuronides					
Benzene	Ph-G		269	175, 113, 93	[10,11]
Styrene	4-VP-G		295	175, 113	[33]
Naphthalene	α-N-G		319	175, 113, 143	[26]
Bisphenol A	BPA-G		417	175, 113, 241	[37]
Parent compound	Metabolite	R-S	$[M-H]^-$, *m/z*	Fragment[d], *m/z*	References
Sulfates					
Benzene	Ph-S		173	93	[10]
Styrene	4-VP-S		199	119	[33]
Naphthalene	β-N-S		223	143	[26]

S-PMA: *S*-phenylmercapturic acid; BMA: benzylmercapturic acid; MBMs: methylbenzylmercapturic acids; DPMAs: dimethylphenylmercapturic acids; DMBs (or DMMs): dimethylbenzylmercapturic acids; PHEMAs: phenylhydroxyethylmercapturic acids; MHBMAs: monohydroxybutenylmercapturic acids; DHBMA: dihydroxybutylmercapturic acid; Ph-G: phenyl-glucuronide; 4-VP-G: 4-vinylphenol-glucuronide; α-N-G: α-naphthyl-glucuronide; BPA-G: bisphenol A-glucuronide; Ph-S: phenyl-sulfate; 4-VP-S: 4-vinylphenol-sulfate; β-N-S: β-naphthyl-sulfate.

[a] $[M-H]^-$ for NI, $[M+H]^+$ for PI in [24].

[b] $[RS]^-$ for NI, $[R]^+$ for PI in [24].

[c] $[M-H-R]^-$, *m/z* 175; $[M-H-R-CO_2-H_2O]^-$, *m/z* 113; $[M-H-176]^-$.

[d] $[M-H-SO_3]^-$.

3.3. Sample preparation and matrix effect

Sample preparation probably represents the most critical step in quantitative MS. One of the main advantages of LC–MS is the compatibility with aqueous matrices and the possibility to minimize or even eliminate sample preparation prior to analysis. Simplification of pre-analytical work would reduce both the time and the cost of analysis, thus increasing

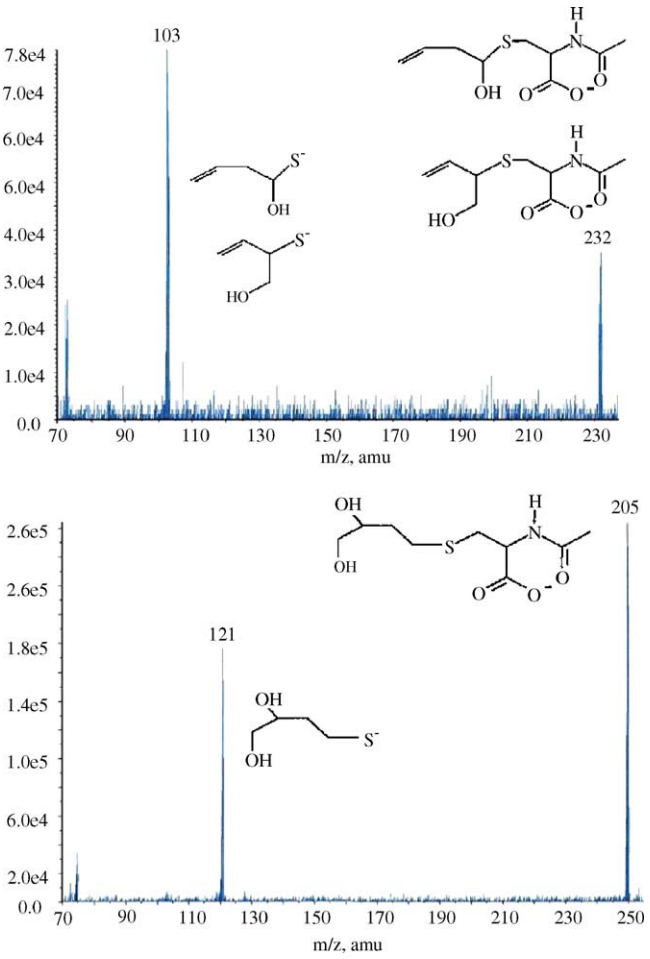


Fig. 3. Product ion spectra and structures of mercapturic acids of 1,3-butadiene, monohydroxybutenylmercapturic acid (MHBMA, top) and dihydroxybutenylmercapturic acid (DHBMA, bottom) obtained in NI-ESI. Reprinted with permission from [34].

the sample throughput. The earlier publications in the field of occupational toxicology emphasized these aspects, since at that time it was still not completely clear that the main limitation in this simplified approach was due to LC–MS susceptibility to matrix effect. Although the mechanism and the origin of the matrix effect are not fully understood, signal suppression is believed to result from competition between matrix components and analyte ions in the sprayed solution for access to droplet surface for gas-phase emission [48]. Depending on the environment in which ionization and ion-evaporation take place, this competition may effectively decrease (ion suppression) or increase (ion enhancement) the efficiency of formation of the desired analyte ion. As recently described by Mallet et al. [49] for a number of model components, all of the most common mobile-phase additives showed a concentration- and compound-dependent ion suppression/enhancement effect in both PI- and NI-ESI. Some recent studies on drug metabolism reported the evidence that the presence of residual matrix components could suppress or enhance the analyte response, resulting in diminished precision and accuracy of quantitative determinations in biological samples, especially at very low concentration levels. Different experimental set-ups have been developed to evaluate ion suppression/enhancement of the signal caused by the matrix [50] or mobile phase additives [49]. Suppression is compound dependent and is mainly observed for the early eluting compounds, caused by polar and unretained matrix components or by overloading of the LC column [51], although losses of the ESI response were observed also later in the chromatograms [50]. The presence of nonvolatile solutes causing changes in the droplet solution properties, rather than gas phase reactions has been identified as the main cause of ionization suppression in ESI ionization of biological extracts [52]. With respect to matrix effect, APCI seemed to be less susceptible and more robust than ESI, and suppression was only observed in the beginning of the chromatogram [52–55]. Large differences in matrix effect were observed between different bio-fluids (urine, oral fluid, and plasma) [55] and between different sample preparation techniques, including direct injection, dilution, protein precipitation, solid-phase extraction (SPE), and liquid–liquid extraction (LLE) [49,50,52,55,56]. Matrix effect was often observed in the case of plasma/serum samples, even after extraction and/or protein precipitation [52,56]. In the case of urine, SPE is often chosen as the suitable solution for matrix effect, although in some cases it was found to magnify the matrix effect by pre-concentrating the interfering substance, whereas direct injection and dilution showed less suppression in LC–ESI–MS–MS [55]. Several approaches have been proposed to compensate for such effect, i.e. an efficient sample clean up [49,52,56] obtained by valve switches or on-line SPE [57], the use of ballistic gradients [58], the application of isotopically labeled internal standards (when available at the adequate isotopic purity) rather than analogue internal standards (ISs) [54,59], a signal enhancement by introduction of additives into the mobile phase [51], the application of the standard addition method [60]. The use of RP-HPLC with rather high retention [54,56], or better the use of 2D LC (LC–LC) [51] was found to be effective to compensate signal suppression. Due to the complex nature of matrix effect, the most effective way to correct seems to be a combination of several factors: an efficient and selective extraction, an efficient chromatographic separation, and eventually the change of the LC–MS interface. Practical, experimental approaches for studying, identifying, and eliminating the effect of matrix on the results of quantitative analyses by LC–MS–MS need to be defined, as recently proposed by Matuszewski et al. [61]. In the light of these findings, a careful evaluation of the matrix effect phenomenon should be recommended in all future studies performed in the field of occupational toxicology.

3.4. Quantitative aspects

The methods discussed in this review deal with quantitation of biomarkers of exposure in a complex biological fluid, like urine. Table 3 summarizes the main characteristics of

Table 3
Summary of application of LC–MS to biomarkers of exposure

Parent compound	Biomarker(s)	Method	Ion mode	Sample preparation	Internal standard	LOD (μg/l)	Reference
n-Hexane	2,5-HD	LC–APCI–MS	PI	Direct injection	None	20	[13]
Benzene	*S*-PMA, *t,t*-MA	LC–ESI–MS–MS	NI	SPE (SAX) + LLE	ID	0.1, 1.0	[17]
	S-PMA	LC–ESI–MS–MS	NI	C18 trap cartridge	Analog	0.04	[20]
	S-PMA	LC–ESI–MS–MS	NI	HPLC purification	Analog	5	[21]
Benzene, toluene, xylene, styrene	*t,t*-MA, HA, MHA, PGA	LC–MS–MS (QqTOF)	NI	SPE (C18, Oasis)	None	1–45	[22]
Toluene, *o*-xylene, trimethylbenzenes	BMA, *o*-MBM, 2,3-, 2,6-, 3,4-DMB	LC–ESI–MS	NI	SPE (C18, Sep Pak)	None	2.4–3.2	[23]
Trimethylbenzenes	DMM (3 isomers), DMH (6 isomers)	LC–ESI–MS–MS	PI	SPE (C18, Oasis)	^{13}C atrazine	0.26–0.42, 0.63–2.0	[24]
Xylenes	DPMA (5 isomers)	LC–ESI–MS–MS	NI	On-line RAM (C18)	None	0.1	[25]
Naphtalene	α-NOH, α-N-G, β-NS	LC–ESI–MS	NI	Direct injection	None	10–100	[26]
Styrene	PHEMA (4 isomers)	LC–ESI–MS–MS	NI	Direct injection	None	0.7–1.0	[27]
	MA, PGA, PGLY, 4-VP	LC–ESI–MS–MS	PI/NI	Direct injection, dilution	None	10–100	[32]
1,3-Butadiene	MHBMA, DHBMA	LC–APCI–MS–MS	NI	SPE (StrataX, polymeric)	ID	0.9, 23	[34]
Toluenediisocyanate	2,4-, 2,6-Toluenediamine	LC–APCI–MS	PI	Hydrolysis + LLE	None	1	[35]
Phtalates	Monoestherphtalates	LC–ESI–MS–MS	NI	On-line RAM (C8)	ID	0.5–2.0	[36]
Bisphenol A	Bisphenol A glucuronide	LC–ESI–MS–MS	NI	Direct injection, dilution	ID	–	[37]
Parathion, parathion-methyl, fenitrothion	4-Nitrophenol, 3-methyl-4-nitrophenol	LC–ESI–MS–MS	NI	LC–LC	ID, analog, none	<1	[39]
Ethylenebisdithiocarbamates	Ethylenethiourea	LC–ESI–MS–MS	PI	Fluorosil + LLE	None	0.5	[40]
Organo phosphorous	Alkyl phosphates	LC–ESI–MS–MS	NI	Direct injection	None	1–2	[41]
Cyclophosphamide	Cyclophosphamide	LC–ESI–MS–MS	PI	LLE	Analog	0.05	[43]
Ifophosphamide	Ifophosphamide	LC–ESI–MS–MS	PI	LLE	None	0.05	[44]
Methotrexate	Methotrexate	LC–ESI–MS–MS	PI	SPE (C18)	Analog	0.2	[45]

2,5-HD: 2,5 hexanedione, *S*-PMA: *S*-phenylmercapturic acid; *t,t*-MA: *t,t*-muconic acid; HA: hippuric acid; MHA: methylhippuric acid; PGA: phenylglyoxylic acid; BMA: *S*-benzylmercapturic acid; *o*-MBM: *o*-methylbenzylmercapturic acid; 2,3-, 2,6-, 3,4-DMB: 2,3-, 2,6-, 3,4-dimethylbenzylmercapturic acid; DMM: dimethylbenzylmercapturic acid; DMH: dimethylhippuric acid; DPMA: dimethylphenylmercapturic acid; α-NOH: α-naphthol; α-N-G: α-naphthylglucuronide; β-N-S: β-naphthyl-sulfate; PHEMA: phenylhydroxyethylmercapturic acid; MA: mandelic acid; PGLY: phenylglycine; 4-VP: 4-vinylphenol; MHBMA: monohydroxybutenylmercapturic acid; DHBMA: dihydroxybutylmercapturic acid; ESI: electrospray; APCI: atmospheric pressure chemical ionisation; PI: positive-ion; NI: negative-ion; SPE: solid-phase extraction; LLE: liquid–liquid extraction; RAM: restricted access material; ID: isotope dilution.

the LC–MS–(MS) methods applied to the determination of biomarkers of exposure, urinary metabolites and unchanged substances, described in the following section, where attention will be paid to sample preparation strategies used to overcome the matrix effect. It should be noted that the lack of commercial isotopically labelled ISs and, in the case of minor conjugated metabolites, the lack of standards themselves is probably the main limitation to the application of quantitative MS in occupational toxicology. Home-made synthesis of standards and ISs [17,21,23,25,27,36], or their biosynthesis [17,37] obtained by administering the (labelled) parent compound to rats and successive HPLC purification have been proposed to overcome this limit. As shown in Table 3, only few methods applied isotope dilution (ID)-MS, whereas in the case of direct sample injection no IS was used. In the absence of authentic standards of conjugated metabolites, a semi-quantitative analysis was proposed to estimate concentrations of 4-vinylphenol-conjugates in the urine of styrene-exposed workers [33].

All the methods described in the following section performed quantitative analyses by means of in-matrix calibrations, except for few cases where sample clean up allowed complete removal of interfering matrix components and calibrating standards were prepared in methanol [17] or water [22,36]. Since the degree of ion suppression may vary in different lots of the same biofluid originating from different subjects or from the same subject over different time periods, application of the standard addition method would be the best choice, but in case of large number of samples, this will result in an unacceptable number of analyses. The use of pooled urine samples from unexposed subjects represents the most widely used alternative to construct external calibration

curves useful to calculate relatively high concentrations of metabolites in the urine of occupationally exposed workers. In some cases, dilution of samples was applied [20,22–24,31] to reduce the matrix effect. The situation is more complicated when low metabolite concentrations, similar to those of the unexposed general population needs to be measured (e.g., policemen, taxi and bus drivers occupationally exposed to urban traffic). In fact, as pointed out by Liao et al. [20], it is extremely difficult to acquire a "truly" blank urine because some organic chemicals are also environmental pollutants. In the case of benzene exposure, urine samples were screened to find out the urine batch with the lowest *S*-phenylmercapturic acid (*S*-PMA) concentration, which was used to construct the calibration curve. The content of *S*-PMA in that urine sample was determined by applying the standard addition methods and the resulting calibration equation was used for the calculation of *S*-PMA concentration in other urine samples, assuming that the variation among sample matrices would not affect significantly the slope and the intercept of the equation [20]. Centrifugation [13,14,21,23–25,34,36,38,41] or filtration [20,26,27,29–33,40,43–45] of urine samples was often applied prior to sample extraction and/or injection.

As there are no certified reference materials commercially available for most of the analytes, quality control samples were often prepared at two or more concentration levels to evaluate the precision and the accuracy of most LC–MS–(MS) methods [17,21,23–25,34,36,40,43]. In method accuracy evaluation, Koch et al. checked for a possible influence of the matrix on the analytical recovery, by performing experiments on eight different urine specimens characterized by a composition as different as possible, i.e., with creatinine concentration ranging from 0.24 to 2.12 g/l [36]. Expression of data as a function of urinary creatinine concentration is widely accepted procedure to take into account for inter-individual differences in metabolite excretion.

4. Application of LC–MS to the human biomonitoring of occupational exposures

4.1. Aliphatic hydrocarbons

4.1.1. n-Hexane

n-Hexane has been mainly used as solvent in glues, printing inks, and varnishes. Chronic exposure to *n*-hexane is known to induce peripheral neuropathy in humans and laboratory animals. The neurotoxicity of *n*-hexane is due to its oxidized metabolite, 2,5-hexanedione (2,5-HD), which has been chosen as a suitable biomarker of exposure to *n*-hexane. Although the analytical determination of "total" 2,5-HD was mainly performed in the past on hydrolyzed urine samples by GC and HPLC, other authors suggested that the determination of "free" 2,5-HD would have been more appropriated, since the free but not the conjugated fraction is responsible for the neurotoxic effects. Both "total" and "free" 2,5-HD was found to be correlated with exposure, "free" 2,5-HD representing about 10% of "total" 2,5-HD. Since 2,5-HD itself does not form any conjugate, to answer the question about the origin of "total" 2,5-HD and other analytical artifacts, i.e. γ-valerolactone, our laboratory has developed LC–MS methods for the characterization of phase I and phase II metabolites in untreated and hydrolyzed urine samples, by using APCI and ESI ionization, respectively [13,14]. Besides 2,5-HD, for which the LC–MS method has been validated, other phase I metabolites of *n*-hexane, i.e. 4,5-dihydroxy-2-hexanone, 5-hydroxy-2-hexanone and 2,5-hexanediol were identified by LC–APCI–MS [13]. Four glucuronides were identified by LC–ESI–MS and confirmed by LC–MS–MS in the urine of rats exposed to *n*-hexane: 2-hexanol-glucuronide, 2,5-hexanediol-glucuronide, 5-hydroxy-2-hexanone-glucuronide, and 4,5-dihydroxy-2-hexanone-glucuronide [14]. Once isolated by SPE and hydrolized, the two latter glucuronides were found to lead to the artifactual formation of both 2,5-HD and γ-valerolactone. The revisitation of *n*-hexane metabolism, possible owing to LC–MS, allowed to draw important conclusions in occupational field: "free" 2,5-HD seems to be both suitable from an analytical point of view and meaningful for biological monitoring purposes, provided that conjugated metabolites are rapidly removed by the body leading to a negligible neurotoxic risk [15]. In year 2001, ACGIH changed the biological exposure index (BEI) for 2,5-HD, recommending the determination of "free" instead of "total" 2,5 HD with a BEI value of 0.4 mg/g creatinine (corresponding to a TWA of 50 ppm) [1]. The accuracy of 2,5-HD LC–MS determination was verified by analyzing samples from interlaboratory quality assurance programme for organic solvent metabolites [13].

4.2. Aromatic hydrocarbons

4.2.1. Benzene

Benzene is an important industrial chemical as well as a ubiquitous environmental pollutant arising from motor vehicle emissions. A relevant source of indoor benzene concentrations could be identified in tobacco smoke. Since at high exposure levels, benzene causes progressive degeneration of bone marrow, aplastic anemia, and leukemia, it has been classified in group A1 (carcinogen to human) by the International Agency for Research on Cancer (IARC). LC–MS was chosen as suitable analytical technique to support the development of more sensitive methods for accurately measuring the existing urinary biomarkers of benzene, i.e. *S*-phenylmercapturic acid (*S*-PMA), *trans*,*trans*-muconic acid (*t*,*t*-MA), hydroquinone (HQ), catechol (CAT), 1,2,4-trihydroxybenzene (benzene triol, BT) in a project funded by the American Health Effect Institute (NIESH). In a comprehensive paper on benzene urinary metabolites, Qu et al. discussed the applicability of these metabolites as possible biomarkers of benzene exposure in human population [16].

Whereas phenolic metabolites were not specific enough to distinguish unexposed subjects from workers exposed at low ambient levels, *S*-PMA and *t,t*-MA were recognized as the most sensitive markers for low level benzene exposure. The actually accepted BEI values for *t,t*-MA and *S*-PMA are 500 and 25 μg/g creatinine, respectively (TWA 0.5 ppm) [1]. The validation of a LC–ESI–MS–MS method for the simultaneous quantitation of *S*-PMA and *t,t*-MA in human urine samples was previously reported by the same author group [17]. The ionization of both the analytes was obtained in NI. Before analyses, samples were spiked with isotopically labelled [$^{13}C_6$]*S*-PMA and [$^{13}C_6$]*t,t*-MA obtained by synthesis and biosynthesis, cleaned by SAX and then extracted with ethyl acetate to eliminate the interfering matrix components. The total recoveries of spiked [$^{13}C_6$]*S*-PMA and [$^{13}C_6$]*t,t*-MA in samples analyzed by LC–MS–MS (n = 28) were 43% (range: 27–55%) and 65% (40–107%), respectively. The limit of detection (LOD) for *S*-PMA was 0.1 μg/l and that for *t,t*-MA was 1 μg/l. The mean levels of urinary *S*-PMA and *t,t*-MA in smokers were, respectively, 1.9-fold (P = 0.02) and 2.1-fold (P = 0.03) higher than those in non-smokers and a correlation was found between *S*-PMA and *t,t*-MA after logarithmic transformation (r = 0.41, P = 0.005, n = 46). Using this method, urinary levels of *S*-PMA and *t,t*-MA were measured in 130 exposed Chinese workers from glue-making and shoe-making plants, as well as in 51 unexposed subjects from nearby food factories [18,19]. Both the metabolites, but specifically *S*-PMA, correlated well with personal benzene exposure over a broad range of exposure (0.06–122 ppm).

An increased sensitivity in the determination of *S*-PMA was obtained by Liao et al. by applying an on-line automatic sample clean up system, made up of an autosampler, a RP C18 trap cartridge, and a two-position switching valve, which was connected directly to the ESI source of a triple quadrupole MS [20]. A commercially available homologous molecule, *N*-*t*-BOC-*S*-(*p*-methylbenzyl)-L-cysteine, was used as IS. Once urine samples added with IS (200 μl) were loaded on the trap cartridge, a washing step of at least 12 min with 100% water before backflush elution with methanol was found to be effective in removing the excessive salt content of urine and to avoid ESI signal suppression. The LOD for a *S*-PMA standard solution was 0.04 μg/l, whereas the detection limit was estimated to be lower than 0.35 μg/l for the determination of trace amounts of *S*-PMA in the matrix. Mean *S*-PMA concentrations (±S.D.) determined in samples from neonates (n = 6) and non-smoking adults (n = 6) were 0.331 ± 0.043 and 1.614 ± 1.300 μg/l, respectively.

Very recently, Pieri et al. proposed an alternative method for *S*-PMA quantitation by using an automatizable HPLC purification followed by LC–ESI–NI-MS–MS analysis [21]. The use of *p*-bromo-*S*-phenylmercapturic acid as IS instead of an isotopically labeled standard allowed reduction of the analysis cost. The ion suppression effect, evaluated at three concentration levels (100, 50, and 25 μg/l) by comparing the peak areas from purified urinary samples with those from methanolic solution containing the corresponding amount of pure *S*-PMA, was about 40%. The LOD of the method, related to 1 ml of urine was 5 μg/l. This sensitivity allowed the detection of *S*-PMA in the urine samples from 12 smokers (range: from 13.6 to >200 μg/l, with three subjects — taxi drivers — exceeding 200 μg/l) but not in the general non-smoking population, for which the purification of an aliquot of at least 2 ml would have been necessary.

4.2.2. Toluene and xylenes

Similarly to benzene, toluene and xylenes are both industial chemicals and environmental pollutants. The main toluene and xylene metabolites, namely hippuric acid and *o*-, *m*-, and *p*-methylhippuric acid, together with *t,t*-MA and the styrene metabolite, phenylglyoxylic acid, have been simultaneously separated using a narrow-bore 1-mm i.d. RP C18 column and detected by a hydrid quadrupole/time-of-flight (QqTOF) mass spectrometer in NI mode [22]. Prior to injection, samples were diluted 100-fold with water and adjusted to pH ∼2 with hydrochloric acid and passed on SPE Oasis cartridges using an automated system (recovery: 88–110%). SPE sample clean up was sufficiently effective to remove interfering matrix components. Although the QqTOF system in the MS–MS mode was not as sensitive as triple-quadrupole in SRM mode (LODs were between 1 and 45 μg/l), its use allowed a secure identification of the analytes in biological matrices. In fact, the high resolution of QqTOF was useful to provide a check that no-coeluting species were interfering. The method was applied to the determination of *t,t*-MA in urine five smokers (range: 65–135 μg/g creatinine) and three workers (165–216 μg/g creatinine) involved in the extraction of plant constituents with organic solvent containing benzene.

A LC–ESI–NI-MS method for the determination of the mercapturic acids of toluene, xylene and trimethylbenzenes, namely benzylmercapturic acid (BMA), *o*-methylbenzylmercapturic acid (*o*-MBA), 2,3- 2,6- and 3,4-dimethylbenzylmercapturic acid (2,3- 2,6- and 3,4-DMB) in human urine has been developed by Moriwaki et al. [23]. Trimethylbenzenes are widely used as organic solvents and raw materials for dyes. Standards of mercapturic acids arising from the conjugation of glutathione (GSH) with the alkyl substituent of the aromatic ring were synthesized by reaction of the corresponding bromoalkylbenzenes with *N*-acetyl-L-cysteine. Separation of analytes was obtained using a RP C30 stationary phase, which allowed the separation of all analytes but not 2,3- and 2,6-DMB. Prior to injection, urine samples were centrifuged and cleaned up by SPE C18 (mean recoveries: 93.2–107.2% for all the compounds). The LODs of the method were 2.4–3.2 μg/l. The peaks of these mercapturic acids were not observed in human urine samples without spiking the standard solutions. An improvement in sensitivity (about 15-fold) was obtained by operating with LC–MS–MS in PI mode, and basal levels of dimethylbenzylmercapturic acids (DMMs) and dimethylhippuric acids (DMHs) were detected for the first time in the urine of an individual not occu-

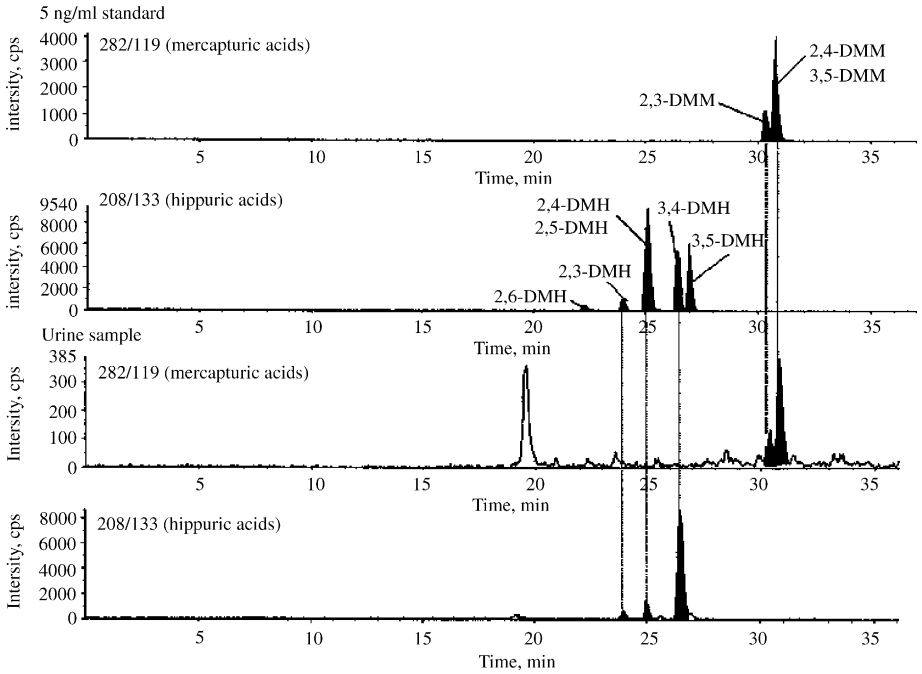


Fig. 4. LC–PI–ESI–MS–MS chromatograms obtained in the SRM mode of a standard solution (5 μg/l) of dimethylbenzylmercapturic acids (DMMs, SRM transition 282 → 119) and dimethylhippuric acids (DMHs, SRM transition 208 → 133) and of an authentic urine sample. Reprinted with permission from [24].

pationally exposed to trimethylbenzenes [24]. The SPE sample clean up was performed using Oasis cartridges and limits of detection were 0.26–0.41 and 0.63–2.0 μg/l for DMMs and DMHs, respectively. Among the six possible regioisomers of DMM, only three (2,3-, 2,4- and 3,5-DMM) were effectively formed because of steric factors; conversely all six regioisomers DMHs were observed, as shown in Fig. 4.

The formation of phenylmercapturic acids via aromatic epoxides in the human metabolism of xylenes has been investigated by Gonzalez-Reche et al. using LC–MS–MS [25]. Similarly to benzene epoxide, these intermediates are thought to be responsible for carcinogenic effects. The five isomers of dimethylphenylmercapturic acid (DPMA), namely 2,3-, 2,4-, 2,5-, 3,4- and 3,5-DPMA were obtained by synthesis and were used for unequivocal identification of metabolites in urine samples of workers exposed to xylenes. An on-line sample enrichment and clean up procedure was developed using a restricted access material (RAM) phase to separate the analytes from the matrix components, especially from the high molecular mass compounds, proteins and lipids. The analytes were then carried over the LC column (Aqua C18) in backflush mode and were eluted without baseline separation between different DMPA isomers. DMPAs were detected in urine samples of 8 of the 25 workers (36%), with concentrations ranging from LOD (0.1 μg/l) to 5.8 μg/l urine. GC–MS analysis performed on the HPLC fraction of the samples with the highest DMPA amounts qualitatively and quantitatively confirmed the formation of DMPAs. By GC–MS it was also possible to separate isomers and to calculate the amount of each isomer as proportion of the sum. The formation of DMPAs was about 0.0003% of that of the xylene main metabolite, methylhippuric acid, thus indicating that the formation of aromatic epoxides is not favoured by any of three xylene isomers.

4.2.3. Naphthalene

Naphthalene, a polycyclic aromatic hydrocarbon present in coal tar and mineral oils, is used as chemical intermediate in the synthesis of insecticides and plastics. Urinary α-naphthol has been proposed as biomarker of exposure to naphthalene, creosote and the insecticide carbaryl; moreover, α-naphthol in combination with 1-hydroxypyrene has been proposed for the biological monitoring of exposure to complex mixtures of polycyclic aromatic hydrocarbons (PAHs). The determination of free and conjugated α-naphthol in untreated urine samples from workers of a naphthalene producing plant (n = 15) was performed by using LC–ESI–MS [26]. Commercial standards of α-naphthol, α-naphthyl-glucuronide (α-N-G) and β-naphthyl-sulfate (β-N-S) were used and were separated using *high speed* (3 cm × 4.6 mm i.d., 3 μm) columns with steep gradients. Due to the relative high concentrations of urinary naphthalene metabolites, the sensitivity of LC–MS (LODs were 0.1, 0.02 and 0.01 mg/l for α-N, α-N-G and β-N-S, respectively) was adequate for biological monitoring purposes and LC–MS–MS was only used for confirmation of the peaks of the β-isomers of N-G and naphthol. As compared to other methods, which make use of prior enzymatic hydrolysis, the possibility of detecting intact glucuronide- and sulfate-conjugates allowed a simplified procedure and preserved information about the relative proportions of metabolites. From the reported results, it appeared that naphthol is excreted as conjugated, preferentially as glucuronide.

4.3. Plastic industry

4.3.1. Styrene

Styrene is one of the most important monomers produced by European chemical industries and used in the production of plastics and synthetic rubbers. It has been calculated that more than 5,000,000 t of styrene are produced in the EU and thousands of workers are occupationally exposed to styrene. The sum of the main styrene metabolites, i.e. mandelic acid (MA) and phenylglyoxylic acid (PGA), which alone represent about 90% of the absorbed dose in man, is practically used as biomarker of styrene exposure. The BEI for the sum MA+PGA proposed by the ACGIH is 400 mg/g creatinine in end-of-shift samples, corresponding to the current TLV 8-h TWA of 20 ppm [1]. A LC–ESI–MS–MS method was developed in our laboratory for the direct analysis of urinary mercapturic acids arising from the conjugation of (*R*)- and (*S*)-enantiomers of styrene-7,8-oxide with GSH, i.e. (*R*,*R*)- and (*S*,*R*)-*N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)cysteine (*R*,*R*-M1 and *S*,*R*-M1) and (*R*,*R*)- and (*S*,*R*)-*N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine (*R*,*R*-M2 and *S*,*R*-M2) [27]. Authentic standards of phenylhydroxyethyl mercapturic acids (PHEMAs) were obtained from synthesis. The four diasteroisomers were separated on a C18-DB column and ionized in NI mode. No sample preconcentration or clean up, but simply filtration and acidification were applied to urine samples collected from 56 workers exposed to styrene and 35 controls. ESI signal suppression was excluded because of the excellent degree of agreement found between data obtained by LC–MS–MS in untreated urine samples (n = 40) and those obtained for the same samples by using an independent HPLC method with fluorescence detection in an interlaboratory quality control [28]. This behavior was probably due to the high retention of mercapturic acids on C18 stationary phase and to the higher selectivity of NI. PHEMAs represented a minor fraction of styrene metabolites in man, and two subgroups of workers were identified, for which the ratio mercapturates/main metabolites was 0.2 and 1.0%, respectively. Among PHEMA diastereoisomers, *R*,*R*-M1 and *S*,*R*-M2 accounted respectively for 50 and 40% of total mercapturates, whereas *R*,*R*-M2 was 7% and only minor amounts of *S*,*R*-M1 were detectable. The resolution of PHEMA diastereoisomers allowed us to study the interference of genetic polymorphisms on the excretion of mercapturic acids and to study the stereoselectivity of glutathione-*S*-transferase-M1 (GSTM1) conjugation in workers [29] and volunteers [30] exposed to styrene, as well as in a case of acute accidental exposure to a solvent mixture containing styrene [31]. The study of the metabolism of styrene has been completed by investigating other major (MA and PGA) and minor styrene metabolites, including phenylglycine (PGLY), 4-vinylphenol (4-VP), and styreneglycol conjugates, glucuronides and sulfates (–G and –S) [32]. To improve identification, rats were co-exposed to styrene and d_8-styrene. A method for the simultaneous determination of MA, PGA, PGLY and the four PHEMAs has been validated with the use of authentic standards. Due to the impossibility of obtaining synthetic standards of 4-VP-conjugates or purifying those biosynthesized by rats, a semiquantitative approach, based on the use of structurally similar compounds as standards, has been developed. When eluted with the same mobile phase composition, glucuronides and sulfates of commercially available compounds, namely phenyl- and naphthyl-derivatives, showed similar ESI–MS–MS response despite the different structure of the aglycone; therefore, calibration plots of α-N-G and β-N-S were used to estimate concentration of the coeluting 4-VP-G and 4-VP-S, respectively. Fig. 5 shows the identification of 4-VP conjugates in the urine of an authentic urine sample from a worker exposed to styrene obtained by LC–ESI–MS–MS. The semi-quantitative method has been extensively applied to large numbers of workers, with the aim of validating 4-VP as a specific styrene biomarker arising from arene oxidation [33].

4.3.2. Butadiene

1,3-Butadiene (1,3-BD) is mainly used for the production of synthetic rubber alone or as copolymer with styrene. Moreover, 1,3-BD is a constituent of environmental tobacco smoke, with concentrations ranging from 3 to 9 μg/m^3. The IARC has classified 1,3-BD as “probable carcinogenic to humans” (Group 2A). The two major urinary metabolites of 1,3-BD are monohydroxybutenylmercapturic acid (MHBMA) and dihydroxybutylmercapturic acid (DHBMA), the former existing in two isomeric forms, i.e. *R*,*S*-1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene and *R*,*S*-2-hydroxy-1-(*N*-acetylcysteinyl)-3-butene (see Fig. 3 for structures and spectra). MHBMA is supposed to be directly formed by reaction of the 1,2-epoxy-3-butene with GSH, whereas DHBMA is formed by reaction of the hydrolyzed 1,2-dihydroxy-3-butene with GSH. These mercapturic acids have been used as biomarkers of internal dose in the biological monitoring of occupational exposure to 1,3-BD. Recently, MHBMA and DHBMA were determined in human and rat urine using LC–APCI–MS–MS [34]. Prior to injection, 5 ml of human urine was purified on SPE-SAX cartridges. Isotopically labeled compounds, i.e. d_6-MHBMA and d_7-DHBMA were used as ISs for calibration and in the calculation of recovery of the method (approximately 100%). For human urine, the precision was <11.2 and <7.2% for MHBMA and DHBMA, respectively. The corresponding LODs were 0.9 and 23 μg/l. When the method was applied to authentic human urine samples from both nonsmokers (n = 10) and smokers (n = 10), mean concentrations (±S.D.) of MHBMA were, respectively, 12.5 ± 1.0 and 86.4 ± 14.0 μg/24h (P < 0.001), and those of DHBMA were 459 ± 72 and 644 ± 90 μg/24 h, respectively. The levels of DHBMA in human urine determined in this study were found to be consistent with those of unexposed subjects reported in earlier studies, whereas in the case of MHBMA some differences were observed. Similarly to 1,3-BD-exposed workers, smokers showed a decrease in the metabolic ratio DHBMA/(MHBMA + DHBMA), thus

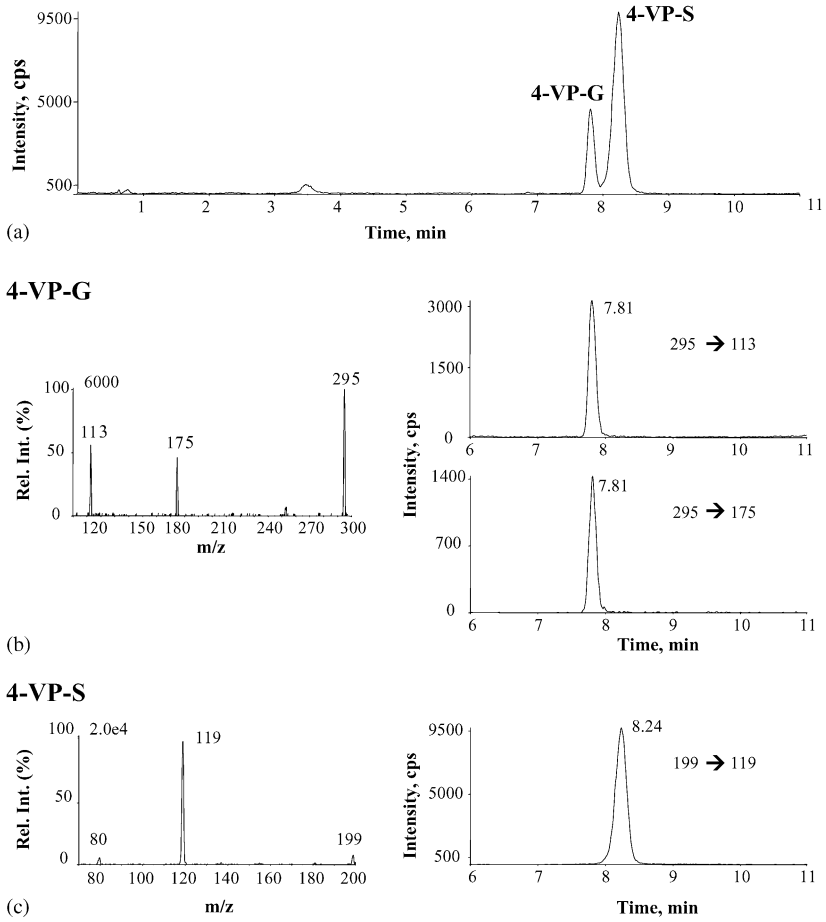


Fig. 5. LC–NI–ESI–MS–MS chromatogram obtained in SRM mode of an authentic urine sample from a worker exposed to styrene (a). LC–NI–ESI–MS–MS product-ion mass spectra (left) and SRM analysis (right) of 4-vinylphenol-glucuronide, 4-VP-G (b) and 4-vinylphenol-sulfate, 4-VP-S (c). Reprinted with permission from [33].

indicating an induction of the GSH-related pathway of the detoxification of 1,2-epoxy-3-butene.

4.3.3. Toluenediisocyanate

Exposure to toluenediisocyanate (TDI), and particularly to 2,6- and 2,4-TDI, occurs in the production of polyurethane. The determination of the corresponding toluenediamines (TDAs), 2,6- and 2,4-TDA, in hydrolyzed urine samples has been proposed for the biological monitoring of workers exposed to TDI. As reported by Sakai et al. [35], the use of LC–APCI–MS instead of GC–MS allowed the analysis of urinary TDAs without derivatization, reducing also the hydrolysis time and chromatographic separation time. Urine samples, hydrolyzed with sulfuric acid (by boiling for 1.5 h) were subjected to LLE with different organic solvents. The use of dichloromethane instead of toluene was found to increase the extraction efficiency from 27 % up to about 75%. A good correlation (r = 0.988) was found between 2,6- and 2,4-TDA concentrations determined by LC–MS and GC–MS in the urine of occupationally exposed workers, which showed urinary levels ranging from LOD (1 μg/l) up to 250 and 63 mg/l for 2,6- and 2,4-TDA, respectively.

4.3.4. Phthalates and bisphenol A

Besides to styrene [33] and butadiene [34] metabolites, detectable urinary levels of phthalate and bisphenol A (BPA) metabolites have been measured by LC–MS–MS in the general population. Despite these methods were not applied to workers, they deal with largely employed industrial chemicals and are therefore worthy of discussion. Phthalates, and particularly diethylhexylphthalate (DEHP), are used as plasticizers, whereas BPA is a component of polycarbonate plastics, used also in dental composite fillings and food-can linings. Both phthalates and BPA are considered as “endocrine disruptors”, with suspected effects of reduced fertility, altered development, and cancer in estrogen-sensitive tissues.

Diesterphthalates are hydrolyzed to form monoesters, which are then excreted as glucuronide conjugates. The method proposed by Koch et al. [36] includes the quantitative determination of several monoester phthalates of DEHP, dioctylphthalate, dibutylphthalate, butylbenzylphthalate, diethylphthalate, as well as of the secondary chain oxidized monoester metabolites of DEHP. The latter compounds are produced by human metabolism by $\omega - 1$ oxidation and are extremely useful to exclude the risk of external contamina-

tion. After enzymatic hydrolysis, the phthalates were stripped from urine by on-line extraction on a RAM precolumn and then transferred in backflush-mode to the RP-LC column. Eluting metabolites were detected by NI–ESI–MS–MS and quantified by isotope dilution, with LODs in the low ppb range (0.5–2.0 μg/l). According to the authors, the method is suited for determining levels of phthalates over a wide range of concentrations and can be applied in the field of environmental medicine for assessing the body burden of the general population but also of occupationally or medically exposed subjects.

Human studies on volunteers exposed to low doses of d_{16}-BPA (to avoid interferences with background concentrations of BPA due to dietary intake or release from plastic materials) showed that d_{16}-BPA glucuronide was the only metabolite detected in urine and blood samples, whereas free BPA was not detectable. Metabolite identification and BPA quantitation in urine and plasma were performed by LC–NI–ESI–MS–MS [37]. In another study, a novel sample preparation was developed, based on a size-exclusion flow extraction of BPA [38]. After enzymatic deconjugation, samples were subjected to the extraction in RP (ODS) and size-exclusion (GPC) modes. The LOD of the LC–NI–ESI–MS was 0.1 μg/l for free BPA. The efficient glucuronidation of BPA and the rapid excretion of the formed glucuronide result in a low body burden of estrogenic BPA in humans at low doses. Application of such sensitive and specific LC–MS methods will be useful for a better risk assessment in plastic industry workers.

4.4. Insecticides and pesticides

The presence of pesticide metabolites in human biological fluids has shown to be an indicator of human exposure to agrochemical compounds. Different approaches for the correct quantification of 4-nitrophenol (metabolite of parathion and parathion-methyl) and 3-methyl-4-nitrophenol (metabolite of fenitrothion) in human urine using LC–MS–MS have been compared by Sancho et al. [39]. These metabolites were taken as model examples, since an isotope-labeled IS (4-nitrophenol-d_4) was commercially available, whereas for most pesticide classes labeled standards are not available and alternative strategies have to be developed. External calibration and the use of two ISs, an isotope-labeled and an analogue compound, as well as the use of LC–LC were compared in terms of matrix interference, i.e. by monitoring the time profile of endogenous interferences of urine by full-scan LC–MS. The use of labeled IS (if available) allowed accurate results, even in the case of high signal suppression, whereas the use of analogue IS required a previous purification step to decrease the amount of interferences. When on-line LC–LC was set up in order to extensively remove interferences suppressing ESI ionization, ESI–MS–MS allowed the correct quantification of analytes in urine also applying an external calibration without the use of any IS.

Ethylenethiourea (ETU) is the main degradation product of ethylenebisdithiocarbamates (EBDCs), a class of agricultural fungucides such as mancozeb, zineb, and metiram. An efficient sample clean up obtained using a Fluorosil phase followed by LLE with dichloromethane allowed the accurate quantification of ETU in human urine by LC–ESI–MS–MS without the use of any IS but with matrix-matched calibrators [40]. The recovery of the extraction procedure was always higher than 85%, and the accuracy of the method was in the range 97–118%. The sensitivity of the method [LOD 0.5 μg/l, limit of quantitation (LOQ) 1.5 μg/l] was adequate to monitor ETU concentrations in the urine from growers exposed to EBDCs ($n = 20$). ETU levels determined at end of the working day (median 3.6 μg/l, range: 1.9–8.2 μg/l) were significantly higher than the corresponding values determined in samples collected at the start of the working day (range: LOQ – 2.1 μg/l).

O,O-Dimethyl phosphate (DMP), *O,O*-diethyl phosphate (DEP), and the corresponding thio- (DMTP and DETP), and dithio-phosphates (DMDTP and DEDTP), are the alkyl phosphates frequently analyzed to monitor exposure to organophosphorus insecticides. These metabolites were analyzed using a LC–NI–ESI–MS–MS method by Hernandez et al. [41]. Adequate chromatographic separation between analytes, some of them showing possible MS interferences, was achieved using a volatile ion-pairing agent, tetrabutylammonium (TBA) acetate, in the mobile phase. Occupational exposure to chlorpyrifos ethyl in farmers who applied this organophosphorus insecticide led to the metabolites DETP and DEP, but not DEDTP and DMTP. A good correlation between the levels of these metabolites and those of the chlorpyrifos-specific metabolite (1,3,5-trichloro-2-pyridinol) was obtained.

4.5. Antineoplastic agents

Occupational exposure to cytostatic drugs (CDs) has been recognized as a potential health hazard since the 1970s. The IARC has divided the drugs into four groups according on the evidence of their carcinogenicity in humans. Biological and environmental monitoring have been recognized as essential instruments to identify the main exposure routes (inhalation, skin contact, ingestion) and to quantify potential health risks in hospital personnel preparing and administering antineoplastic drugs. Since the percentages of unmodified drugs in urine could be very high (about 80–90%) for some CDs, the determination of the parent drugs is often preferred over the detection of their metabolites. A comprehensive overview of the analytical methods applied in this field has been recently published by Turci et al. [42]. The same group proposed several analytical methods based on the use of LC–ESI–MS–MS for the determination of cyclophosphamide [43,44], ifophosphamide [44], and methotrexate [44,45] in human urine. Cyclophosphamide (CP), a group 1 (carcinogenic to humans) alkylating agent was first chosen as model compound because it is extensively used in the treatment of many types of cancers [43]. The LC–MS–MS method, which included LLE with ethyl acetate (recovery >85%), required no deriva-

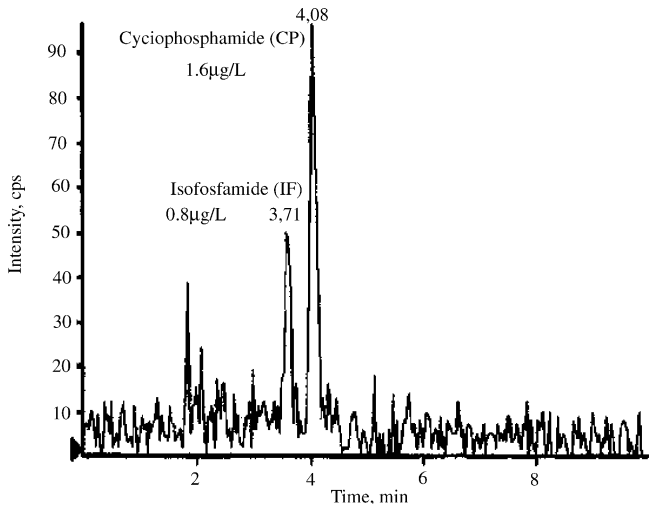


Fig. 6. LC–PI–ESI–MS–MS chromatogram obtained in SRM mode of a blank urine sample spiked with 0.8 μg/l of ifophosphamide (SRM transition 261 → 140) and 1.6 μg/l of cyclophosphamide (SRM transition 261 → 92). Reprinted with permission from [44].

tization, preventing CP from possible thermal and chemical decomposition reactions. Ifophosphamide (IF) was initially used as IS in the validation of the method, which showed high sensitivity (0.2 μg/l) and accuracy in the range 99–103%. It should be noted that the use of IF as IS could not be appropriated, since workers exposed to CP might have been exposed also to IF (leading to overestimation of IS and, consequently, underestimation of the CP concentration). Thereafter, the method was used for the determination of both CP and IF in biological samples without the use of any IS [44]. When applied to authentic human urine samples from hospital personnel, the excretion of unmetabolized CP was detected in 50% of study participants, despite the use of personal protecting devices, with concentrations ranging between 0.1 and 1.9 μg/l. A chromatogram of a blank urine sample spiked with IF and CP is shown in Fig. 6. Due to the insolubility of methotrexate (MTX) in all water-immiscible solvents, SPE rather than LLE was used for sample clean up and concentration (25-fold) in the case of the LC–MS–MS determination of MTX in human urine samples [44,45]. SPE was performed on C18 cartridges and the method was validated by using 7-hydroxymethotrexate as IS. As for other antineoplastic drugs, ionization was obtained in PI mode. All the methods were also applied to environmental samples, such as filters used to collect air samples, wipes used to evaluate contamination of the working area, pads worn by operators, and gloves [46,47].

5. Concluding remarks

For its applicability to biological matrices, LC–MS–(MS) is giving a relevant and unique contribution to the study of biotransformation of industrial chemicals and pollutants: up to now, a number of “novel” minor metabolites, some of them only hypothesized in the past, have been unambiguously identified. Both phase I and intact phase II metabolites (glucuronides, sulfates, and mercapturic acids) can be easily measured avoiding extensive sample manipulation and subsequent artifact generation. As shown by the example of styrene metabolism (Fig. 2), the application of LC–MS–(MS) opened a window on minor metabolic routes, thus making a lot of information available for mechanistic interpretation. Due to the complexity of the metabolic fate of a chemical in the organism, it should be reminded that the accurate analysis of a “novel” metabolite by means of a valid analytical method represents an indispensable, but in no way sufficient step in the more general process of biomarker validation. Such a validation of novel biomarkers is mainly aimed at assessing whether they are useful to address relevant toxicological questions. Otherwise, the use of biomarkers recommended by professional societies, like the ACGIH or the Deusche Forschungsgemeinschaft (DFG), or governmental and international organization is always advisable. Moreover, when a broad spectrum of validated biomarkers of exposure is available for the same substance, the choice of the most appropriated one should rely on a number of factors, including: (i) exposure levels and the relative sensitivity and specificity of individual markers; (ii) toxicokinetics, i.e. biomarker half-life, depending on exposure to be assessed (recent or cumulated); (iii) toxicodynamics, i.e. role of an individual biomarker in the identification of a specific toxicity mechanism occurring at the target organ; (iv) the link of individual biomarkers with a long-term health outcomes or with some relevant intermediate end-point [4].

We believe that LC–MS–MS could play a fundamental role in both environmental and occupational toxicology, in the definition of reference values for metabolites of organic pollutants and for a better assessment of low-level occupational exposures to solvents and organic compounds, respectively. In most West-European countries, exposure levels in working environments are continuously decreasing, and in some cases they are close to outdoor concentrations. One of the main issues of occupational and environmental toxicology is the study of co-exposure to complex mixtures, containing low concentrations of many organic chemicals and pollutants (e.g., urban pollution, environmental tobacco smoke). Biomonitoring of such low and complex exposures requires high sensitivity and selectivity, which can be obtained through the use of LC–MS. On the other hand, the use of very sensitive and selective techniques is revealing the complexity of biomarker research, as we realize that biological and analytical specificity tend to diverge. The ability to determine trace and ultra-trace amounts of parent compounds and their metabolites and adducts in biological media may result in the demonstration that such substances are either ubiquitous or shared with endogenous metabolism. As recently noted, very “specific” metabolites are found among “unexposed people”, raising the question “whether the specificity of exposure biomarkers is a realistic objective, a myth, or even a cult in environmental toxicology” [62].

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