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Journal of Chromatography B, 774 (2002) 173–185

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Validated method for quantitation of biomarkers for benzene and its alkylated analogues in urine

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Received 30 November 2001; received in revised form 4 April 2002; accepted 4 April 2002

Abstract

A validated gas chromatography–mass spectrometric method for the analysis of the metabolites of benzene and its alkylated analogues in urine is reported. A number of metabolites, as required by authorities for biomonitoring of industrial exposure to aromatic vapour, were analysed simultaneously with preservation of quantitative information concerning positional isomers. The use of this method replaces a combination of analytical methods required for the analysis of all these metabolites. Urine samples were subjected to acidic deconjugation followed by a derivatization step. Phenol, *ortho*-, *meta*-, *para*-cresol, mandelic acid, and *ortho*-, *meta*-, *para*-methylhippuric acid were analysed as their corresponding ethoxycarbonyl derivatives, with single ion monitoring. The mass-to-charge ratios (m/z) of the ions used for quantitation by single ion monitoring of the metabolites were: phenol, 94 m/z; cresols, 108 m/z; mandelic acid, 206 m/z; hippuric acid, 105 m/z; methylhippuric acids, 119 m/z. The mass-to-charge ratios for the internal standards were: [$^2H_6$]phenol, 99 m/z; *p*-chlorophenol, 128 m/z and 3-chloro-4-hydroxyphenyl acetic acid, 214 m/z. The limits of detection for phenol and the cresols were below 0.4 μmol/l and below 0.05 μmol/l for mandelic acid and the hippuric acids. Within-run precision for mandelic acid was 6.2%, for hippuric acid was 7.32% and was below 5% for the rest of the analytes.

Keywords: Benzene; Phenol; Cresol; Methylhippuric acid

1. Introduction

Authorities almost worldwide enforce biomonitoring of persons occupationally exposed to aromatic vapour. Aromatic compounds like benzene and its alkylated analogues originate from industrial processes such as coal gasification and coke manufacturing. Toluene and xylene have various industrial applications like solvents for paints, resins and adhesives. Exposure to these compounds is widespread and occurs quite often in industry.

One of the major toxic effects of benzene is haematopoietic toxicity. Chronic exposure of humans to benzene in the work place leads to bone marrow damage, which may manifest initially as anaemia, leucopoenia, or thrombocytopenia [1]. Continued exposure may result in pancytopenia caused by marrow aplasia. Survivors of aplastic anaemia frequently exhibit a preleucemic state, termed myelodysplasia, which may progress to acute myelogenous leucaemia [2,3].

Benzene is converted to benzene oxide by the

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00206-4

hepatic microsomal mixed function oxidase (CYP2E1) as one of the body's detoxification processes of xenobiotics [4]. The oxide may rearrange non-enzymatically to form phenol. Benzene metabolites that appear in the urine include ethereal sulfates and glucoronides of the phenolic metabolites, muconic acid resulting from ring opening, and mercapturic acids resulting from gluthathione conjugation. The markers for benzene exposure have been reviewed by Ong et al. [5,6]. Fig. 1 illustrates the metabolism of benzene, toluene, xylene and ethyl benzene.

In the case of alkyl benzenes, however, the major metabolic pathways result in metabolites that has a low order of toxicity and which are readily excreted. Toluene is oxidized at a methyl group [7], followed by a series of oxidations leading to benzoic acid, which is conjugated with glycine to form hippuric acid (HA). *o*-, *m*-, and *p*-Xylene follow the same route resulting in and *o*-, *m*-, and *p*-methylhippuric acid (MHA). Although toluene appears to be less aggressive than benzene there is evidence that 1% of inhaled toluene also follows the ''epoxide'' route, which may have the same result as exposure to benzene [8].

Quantitative analyses of the aforementioned metabolites in urine have been performed by colorimetric techniques [9], isotachophoresis [10], high-performance liquid chromatography (HPLC) [11–14], gas chromatography (GC) [15,16] and gas chromatography–mass spectrometry (GC–MS) [17–19]. These methods fall short in that more than one analytical technique has to be employed to analyse the whole spectrum of metabolites as required by the authorities for biomonitoring of industrial exposure to organic solvents. Information on the concentrations of positional isomers is also lost in some of these methods. This is important since quantitative information of specific positional isomer metabolites is required for biomonitoring.

The acidic protons on phenol, cresol and the carboxyl groups of mandelic-, hippuric- and methylhippuric acids necessitate chemical derivatization before gas chromatographic analysis. Silylation [18,20] and esterification procedures [16,17,19,21] are well documented but the former requires anhydrous conditions and the latter is not suitable for phenol and cresol. Alkoxycarbonyl derivatization [22,23], has been shown to simultaneously derivatize the phenolic hydroxyl protons, as well as that of the carboxylic acids. This derivatization procedure also has the advantage that it can be performed directly in the aqueous matrixes since it does not require anhydrous conditions. However, this procedure has not been used before for the derivatization of the biomarkers of aromatic vapour exposure in urine.

This study was undertaken to develop and validate a single gas chromatographic–mass spectrometric method for simultaneous quantitation of a range of metabolites for benzene and its alkylated analogues in human urine. It is based on an acidic deconjugation, extraction and ethoxycarbonyl derivatization. The resulting ethoxyformate derivatives were then subjected to GC–MS quantitation.

2. Experimental

2.1. Materials

Standards for phenol, *o*-, *m*-, *p*-cresol, mandelic acid (MA), hippuric acid (HA) and *o*-, *m*-, and *p*-methylhippuric acid (*o*-MHA, *m*-MHA, *p*-MHA), 3-chloro-4-hydroxyphenyl acetic acid were purchased from Sigma–Aldrich, Steinheim, Germany.

[$^2H_6$]Phenol and chloroform (pesticide grade) were purchased from Fluka, Buchs, Switzerland. *p*-Chlorophenol and ethylchloroformate (ECF) were supplied by Merck, Darmstadt, Germany. The ethylchloroformate was distilled prior to use.

2.2. Solutions

Standards of the metabolites were dissolved in urine–ethanol (70:30), to obtain the highest calibration standard. The concentrations of the analytes in this calibration standard are indicated in Table 1. Urine was obtained from non-exposed healthy volunteers. The internal standards were further diluted in water–ethanol (70:30) to obtain concentrations as indicated in Table 1.

The four lower calibration standards were prepared by dilution of the highest calibration standard with urine–ethanol (70:30). The concentrations of the calibration standards are indicated in Table 2.

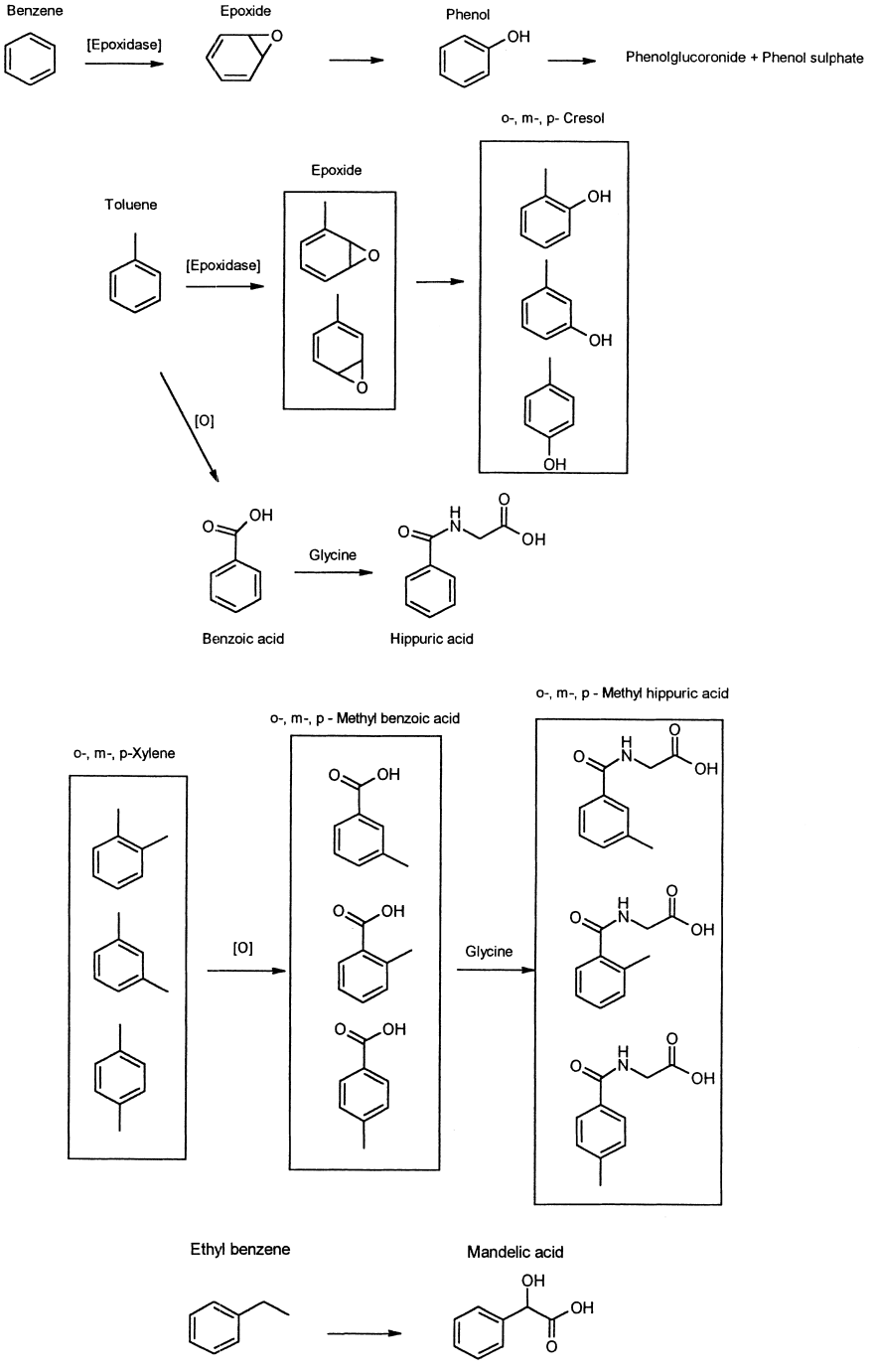

Fig. 1. Scheme of phase 1 and phase 2 metabolism of inhaled benzene, toluene, xylene and ethyl benzene vapour.

Table 1
Concentrations of analytes and internal standard stock solutions

Standard	Concentration (mg/l)	Concentration (mmol/l)
Phenol	80	0.85
o-Cresol	12	0.11
m-Cresol	12	0.11
p-Cresol	12	0.11
Mandelic acid (MA)	2000	13.14
Hippuric acid (HA)	1000	5.58
o-MHA	200	1.04
m-MHA	200	1.04
p-MHA	200	1.04
Internal standards		
[$^2H_6$]Phenol	85	0.85
p-Chlorophenol	76	0.59
3-Chloro-4-hydroxyphenyl acetic acid	4000	21.43

2.3. Acidic deconjugation

An aliquot of calibration standard or urine sample (200 μl) was mixed in a glass tube (125×11 mm) with distilled water (200 μl). The volumes of internal standard solutions added were: [$^2H_6$]phenol, 50 μl; *p*-chlorophenol, 10 μl; 3-chloro-4-hydroxyphenyl acetic acid, 25 μl. Sulfuric acid (2.5 *M*, 100 μl) was added and the tube was capped with a PTFE lined cap. The mixture was heated at 95 °C for 90 min. The tube was allowed to cool before extraction commenced.

2.4. Extraction and derivatization procedure

The hydrolysed sample solution (pH≈2) was extracted twice with peroxide-free diethyl ether (600 μl). The organic phase was transferred into a vial (2 ml) and evaporated under a stream of dry nitrogen at room temperature. The residue was derivatized by the procedure described by Husek et al. [24,25]. Briefly, the procedure is as follows: 200 μl of a mixture of hydrochloric acid (2.5 m*M*)–ethanol–pyridine (60:32:8, v/v), ECF (100 μl) was added to the residue in a vial (2 ml), which was capped with a PTFE lined cap. The solution was shaken gently and allowed to stand for 10 min. A mixture of ECF in chloroform (100 μl, 1%) was added to the mixture and vortexed for 20 s. An aliquot of the organic layer (100 μl) was dried under a stream of dry nitrogen at room temperature and redissolved in chloroform (100 μl). The purpose of the last drying step is to evaporate the ECF and to prevent column deterioration.

Table 2
Concentration of calibration standards

	Standard 1		Standard 2		Standard 3		Standard 4		Standard 5	
	mg/l	mmol/l	mg/l	mmol/l	mg/l	mmol/l	mg/l	mmol/l	mg/l	mmol/l
Phenol	5	0.053	10	0.106	20	0.213	40	0.425	80	0.85
o-Cresol	0.75	0.0068	1.5	0.01375	3	0.0275	6	0.055	12	0.11
m-Cresol	0.75	0.0068	1.5	0.01375	3	0.0275	6	0.055	12	0.11
p-Cresol	0.75	0.0068	1.5	0.01375	3	0.0275	6	0.055	12	0.11
HA	2	0.011	10	0.0558	100	0.558	500	2.79	1000	5.58
MA	10	0.0263	100	0.1314	500	1.314	1000	6.57	2000	13.14
o-MHA	2	0.010	10	0.0517	50	0.258	100	0.517	200	1.035
m-MHA	2	0.010	10	0.0517	50	0.258	100	0.517	200	1.035
p-MHA	2	0.010	10	0.0517	50	0.258	100	0.517	200	1.035

2.5. Equipment

A HP6890 GC system fitted with a HP7683 Auto injector and a HP5973 mass-selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for mass spectrometric analysis. Data collection and integration was performed with HP Chem Station software. A HP-5 (Torrance, CA, USA), capillary column was used (60 m×250 μm; d_f 0.25 μm).

2.6. GC–MS procedure

A 2-μl volume of a derivatized sample was injected in split mode with a split ratio of 1:10. The inlet temperature was set at 250 °C and helium carrier at a constant flow-rate of 1 ml/min (26 cm/s). The oven temperature was ramped from 40 to 240 °C at a ramp rate of 5 °C/min. After 50 min the oven temperature was ramped to 290 °C at a rate of 100 °C/min, with a final isotherm of 10 min. The total chromatographic time was 60.5 min. The mass-selective detector transfer line temperature was set at 280 °C and that of the quadrupole at 106 °C. The source temperature was 230 °C. A solvent delay time of 5 min was used to allow for solvent elution before the source was turned on. All mass spectra were recorded at 70 eV. Chromatograms were recorded in the scan mode first (50–500 m/z) to identify the analytes and ascertain their retention times.

Quantitation was performed in the single ion monitoring (SIM) mode. The mass-to-charge ratios (m/z) of the ions that were used for (SIM) quantitation were as follows: phenol, 94 m/z; cresols, 108 m/z; mandelic acid, 206 m/z; hippuric acid, 105 m/z; methylhippuric acids, 119 m/z; [$^2H_6$]phenol, 99 m/z; *p*-chlorophenol, 128 m/z, and 3-chloro-4-hydroxyphenyl acetic acid, 214 m/z.

2.7. Validation of deconjugation with glucoronide

A standard solution of phenyl glucoronide in distilled water, with concentration equivalent to that of phenol in standard 3 (0.213 mmol/l), was used to ascertain the percentage conversion of the glucoronide to its corresponding aglycone. An aliquot was subjected to the deconjugation and derivatization procedure and the percentage conversion calculated against the internal standard [$^2H_6$]phenol.

2.8. Calibration curves

Five-point calibration curves were set up with the calibration standards as shown in Table 2. The endogenous concentrations of the analytes in the pooled urine were ascertained by back extrapolation of the regression lines. The *x*-intercepts of the calibration curves represent the original endogenous concentration of the metabolite. The sum of the original and added amounts represented the true concentrations of the metabolites in the urine calibration standards. The concentrations and corresponding abundance ratios (analytes relative to the internal standards) were used to set up calibration curves.

2.9. Evaluation of the internal standard characteristics and performance

The characteristics and performance of the internal standards were evaluated by comparing the linear correlation coefficients of the respective calibration curves. Ten samples of a pooled urine sample were analysed to evaluate the precision that resulted from the use of each internal standard.

2.10. Stability of derivatives

The stability of the ethoxycarbonyl derivatives were studied over a period of 22 h by injecting the same sample, once every hour. The response of the analytes were measured relative to the internal standards and the relative standard deviation (RSD) calculated.

3. Results and discussion

Small volumes of high concentration stock solutions are normally added to the matrix by the method of standard addition, to ensure that calibration standards have the same composition as samples. In this case, however, the solubility of some of the analytes (HA and *m*-cresol) in urine hampered this procedure. The highest calibration standard was diluted with urine–ethanol (70:30) to yield the lower calibration

standards. The ethanol contributed to the stability of the calibration standards by preventing the analytes from precipitating from the blank urine. In this way the calibration standards were stable over a much longer period and could also be used as controls for routine analysis.

The concentration of sulfuric acid in the sample to be hydrolysed is critical to the analysis. High concentrations of sulfuric acid resulted in charring of the samples, which led to spurious results. An additional 200 μl of water was added to dilute the acid. A diluted mixture of the internal standards would serve the purpose equally well.

The separation of the positional isomers, especially that of the cresols, was time consuming. The total chromatographic separation time of 60 min can however be reduced by the use of columns with reduced internal diameters or by vacuum outlet chromatography [26].

Chromatograms of calibration standard 3 recorded in both SCAN and SIM modes are shown in Fig. 2. The good chromatographic resolution provides isomeric information. The different sections of the chromatogram had to be enlarged due to the wide concentration differences between the analytes. Note that the acidic proton of the deuterated phenol was replaced by the ethoxycarbonyl substituent during derivatization, which in turn was replaced by a hydrogen atom, via a hydrogen shift during electron impact ionisation [23]. The full-scan mass spectra of the ethoxycarbonyl derivatives of phenol, cresol, hippuric acid, methylhippuric acid and mandelic acid are shown in Fig. 3. The molecular ions as well as the ions used for SIM quantitation are indicated. The molecular ion for the derivatives of methylhippuric acids, m/z 293, could not be detected. The ion used for SIM analysis of mandelic acid (m/z 206) is the result of a McLafferty rearrangement in the molecular ion, followed by a hydride shift.

The ions used for SIM quantitation of the phenol and cresol derivatives (94 and 108 m/z, respectively) are also the molecular ions of the corresponding underivatized compounds, as reported by Kim and Kim [23]. However, chromatographic analysis of an underivatized phenol standard resulted in a broadened chromatographic peak with a retention time different from that of the derivatized analogue. The deteriorated chromatographic properties of the underivatized phenol increased the detection limits to such an extend that this analyte could not be detected in the analytical range of interest.

3.1. Sample preparation

A one-step extractive derivatization reaction reported by Kim and Kim [23] was initially tested. This procedure yielded a mixture of derivatives for the hippuric and methylhippuric acids, resulting in two sets of chromatographic peaks. The first early-eluting set represented reaction products derivatized at the carboxyl functional group only. The second set were those derivatized at both the carboxyl and amide functional groups. The procedure reported by Husek et al. [24,25] however, resulted in the complete formation of ''double derivatized'' hippuric and methylhippuric acids. This derivatization procedure ultimately improved the analytical sensitivity since all the analyte material was converted into double derivatized products. It also resulted in less chromatographic (chemical) interference since the single derivatized products eluted in the same retention window as ethoxycarbonyl derivatives of phenol and the cresols.

3.2. Assay validation

An important factor to consider in method validation is the stability of a derivatised sample during batch analysis. Unstable derivatives will render batch analysis unreliable due to sample deterioration on the autosampler tray. Fig. 4 shows a plot of the results of 10 consecutive analyses of the same sample over a period of 22 h. The y-axis shows the concentration as a percentage relative to the initial concentrations. A calibration standard, with phenol and *o*-cresol concentrations of 80 and 3 mg/l, respectively, was used to study the stability. [$^2H_6$]Phenol was used as internal standard. The RSDs for 10 consecutive analyses of the same sample were 2.0% for phenol and 2.9% for *o*-cresol.

A large variation in the chromatographic peak surface area was observed for phenol, which led to the conclusion that phenol was unstable during the acidic deconjugation procedure. This phenomenon, which might have led to inaccurate and imprecise results, was rectified when the concentrations were

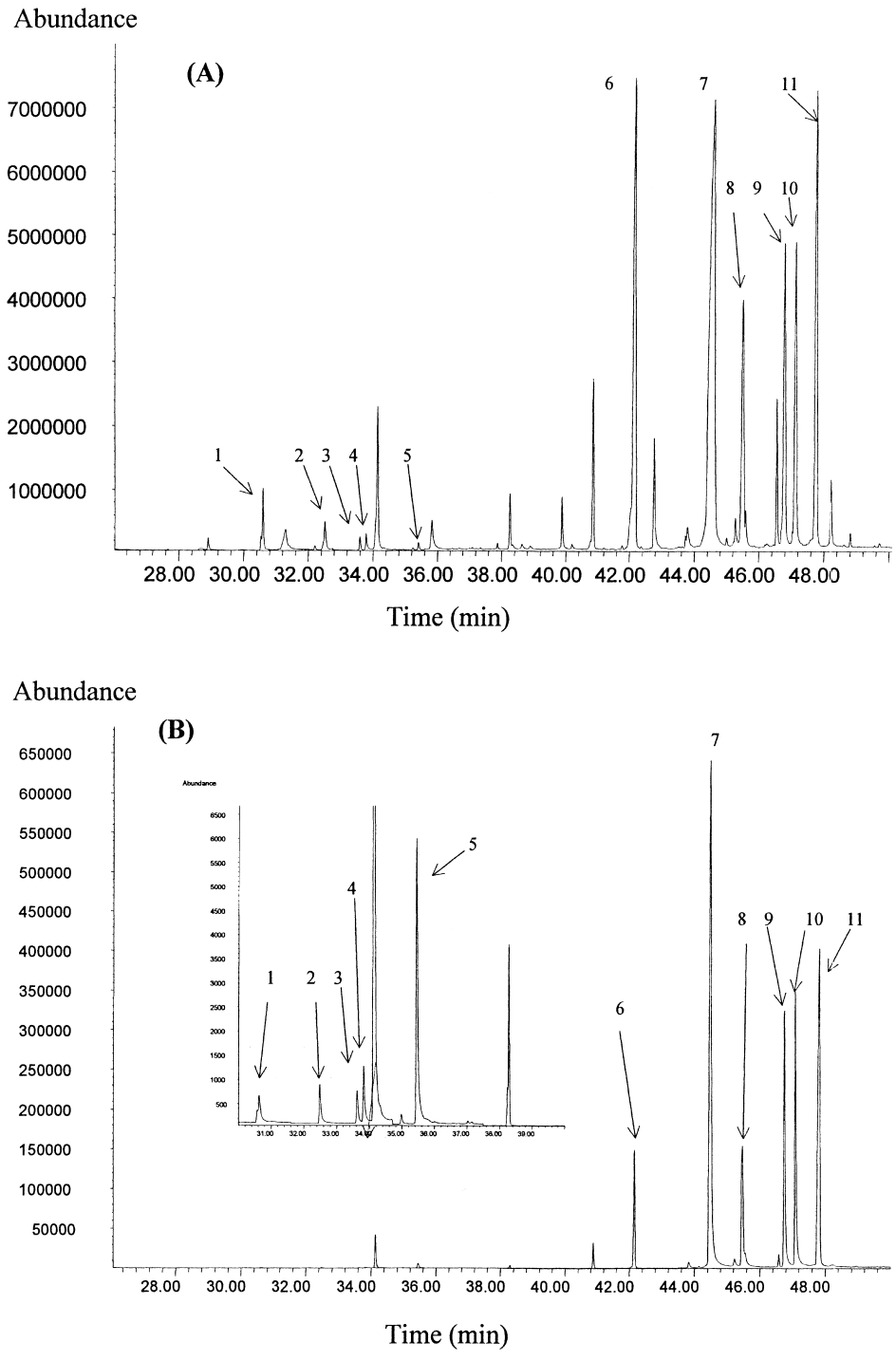

Fig. 2. (A) Chromatogram recorded in SCAN mode (m/z 50–500). (B) The same sample recorded in SIM mode. The first section of the chromatogram had to be enlarged due to the wide concentration differences between the analytes. The elution order is as follows: phenol (1), *o*-cresol (2), *m*-cresol (3), *p*-cresol (4), *p*-chlorophenol (5); mandelic acid (6); hippuric acid (7); *o*-methyl hippuric acid (8); *m*-methylhippuric acid (9), *p*-methylhippuric acid (10), 3-chloro-4-hydroxyphenyl acetic acid (11).

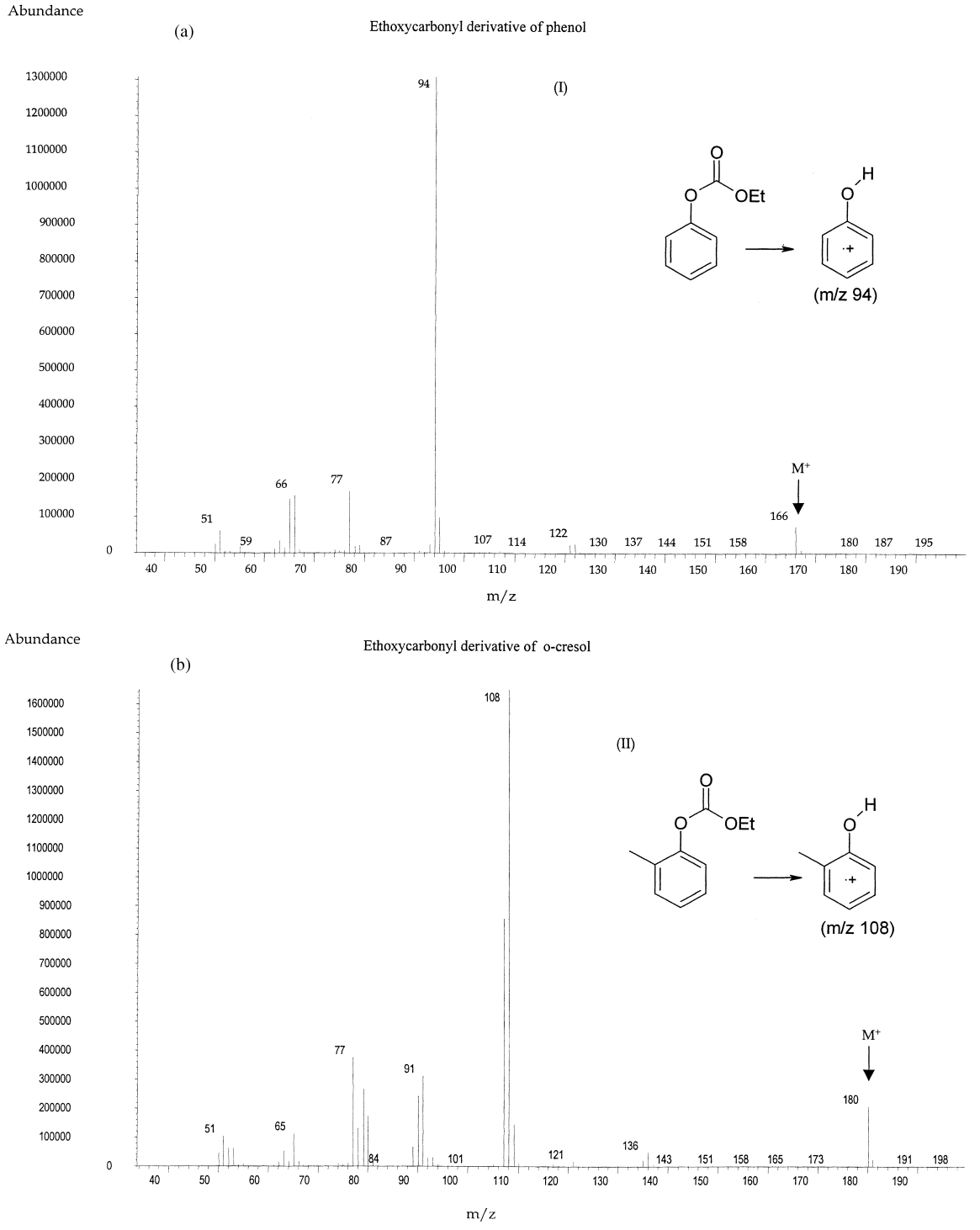

Fig. 3. Full-scan mass spectra of the ethoxycarbonyl derivatives of phenol (I), cresol (II), hippuric acid (III), methyl hippuric acid (IV) and mandelic acid (V).

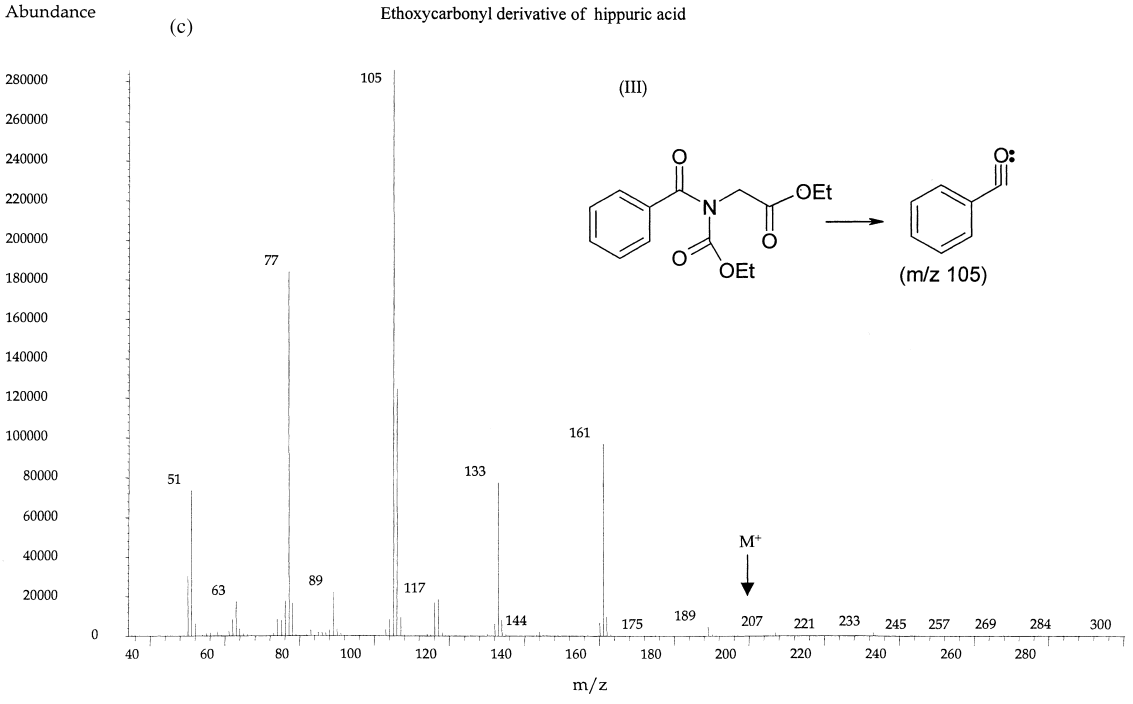

Fig. 3. (*continued*)

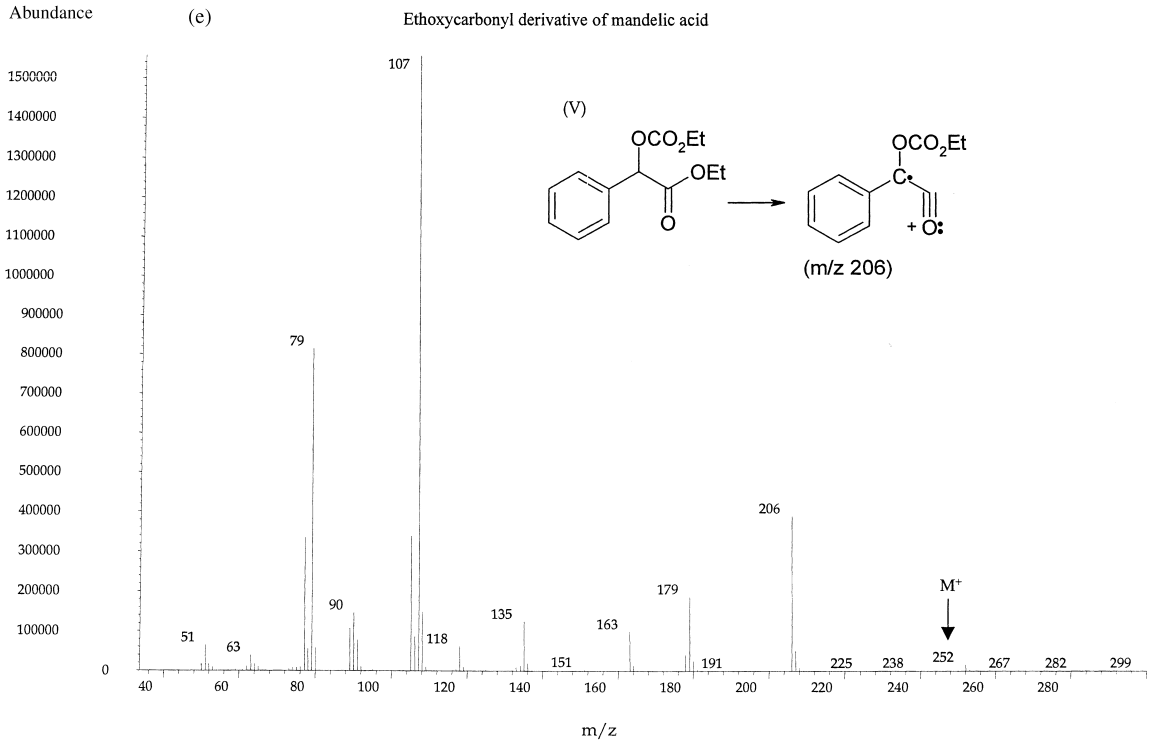

Fig. 3. (*continued*)

calculated relative to a labelled phenol internal standard. In this case, a labelled internal standard was a prerequisite for accurate quantitation of phenol. *p*-Chlorophenol was also considered as an internal standard for phenol but could not sufficiently simulate the chemistry of phenol. It performed satisfactorily as internal standard for the cresols. The

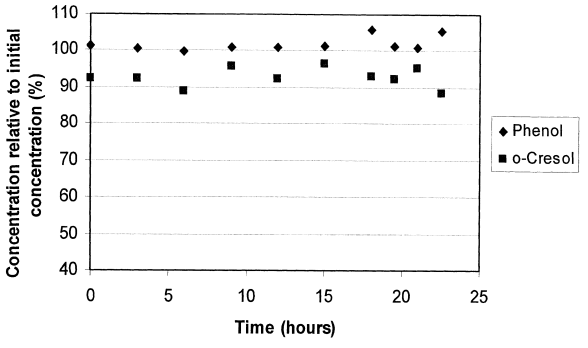

Fig. 4. Plot of the results of 10 consecutive analyses of the same sample, over a period of 22 h. The *y*-axis shows the concentration as a percentage relative to the initial concentrations.

relative standard deviations (within batch) for all the metabolites are indicated in Table 3. Comparison of the linear correlation coefficients showed that 3-chloro-4-hydroxyphenyl acetic acid could be used as internal standard for mandelic and the hippuric acids.

Enzymatic deconjugation by a glucoronidase/sulfatase enzyme [17] may improve the absolute repeatability of the deconjugation step since it can be performed under much milder conditions. This procedure will be more time consuming, typically in the order of 18 h. The phenyl glucoronide was found to be converted 98% complete after 90 min of acidic hydrolysis.

The calibration curves were linear over the concentration range corresponding to the biological exposure index (BEI) [27]. The calibration curves were set up with calibration standards 1 to 5 and the linear regression data are shown in Table 3. The relative standard deviation was determined by analysing 10 samples of calibration standard 3 on the same day.

The maximum permissible amounts of the metabo-

Table 3
Regression analysis parameters of the calibration curves of the metabolites ($n=5$)

Metabolite	Internal standard	r^2	Slope (Relative Response Factor (RRF)) (analyte/I.S.)	y Intercept	RSD (%) (within batch; $n=10$)	Mean recovery (%) at standard 3 concentration level ($n=10$), mean (SD)
Phenol	[$^2H_6$]Phenol	0.998	4.6207	0.6868	4.25	106 (0.009)
o-Cresol	*p*-Chlorophenol	0.982	7.8619	0.0017	3.35	93 (0.0009)
m-Cresol	*p*-Chlorophenol	0.967	13.315	0.0303	3.32	92 (0.0009)
p-Cresol	*p*-Chlorophenol	0.945	17.255	0.0449	4.26	95 (0.012)
Mandelic acid	3-Chloro-4-hydroxyphenyl acetic acid	0.949	0.0595	0.0713	6.2	100 (0.34)
Hippuric acid	3-Chloro-4-hydroxyphenyl acetic acid	0.997	1.3867	0.9565	4.16	102 (0.023)
o-MHA	3-Chloro-4-hydroxyphenyl acetic acid	0.999	1.5986	0.0066	7.32	98 (0.019)
m-MHA	3-Chloro-4-hydroxyphenyl acetic acid	0.996	2.4109	0.0302	4.21	96 (0.011)
p-MHA	3-Chloro-4-hydroxyphenyl acetic acid	0.999	2.9035	0.02	3.45	102 (0.089)

lites in urine are listed in Table 4 as the Biological Exposure Index (BEI) [27–29]. The calculated minimum detection limits (signal-to-noise ratio=3) are also shown. The concentrations of the cresols are relatively low since only 1% of toluene is metabolised via the epoxide route [8]. The sensitivity and selectivity of GC–MS in the SIM mode, however, compliment their analysis.

A chromatogram of the metabolite profiles of an occupationally exposed person and a non-exposed person is shown in Fig. 5. The different sections of the chromatogram were enlarged due to the large variation in metabolite concentrations.

The limits of detection for phenol and the cresols were below 0.4 μmol/l, and for mandelic and the hippuric acids, were below 0.05 μmol/l. Within-run precision for mandelic acid was 6.2%, for *o*-methyl hippuric acid was 7.32% and was below 5% for the rest of the analytes. The assay is linear over a range of 0.053–0.85 μmol/l. This compares favourably with the results of GC–MS methods in the literature, which were designed for the quantitation of urinary phenols [19] and mandelic acid only [16]. Since the proposed method utilizes mass spectrometric detection, the selectivity will be superior to chromatographic methods, which rely only on retention time identification [11,16].

The detection limits of our method are also much lower than was reported previously for HPLC [13] and no special separating conditions are required for the separation of positional isomers [14]. In general the analytical methods for organic solvent exposure

Table 4
Concentration of metabolites in urine at the biological exposure index (BEI) levels

Metabolite	BEI (mmol/mol creatinine)	Minimum detection limit in urine $3\times S/N$ (μmol/l urine)	Minimum detection limit in urine $3\times S/N$ (mmol/mol creatinine)[a]
Phenol	60.1	0.39	0.111
o-Cresol	1.04	0.26	0.074
m-Cresol	1.04	0.17	0.049
p-Cresol	1.04	0.16	0.046
Mandelic acid	1122	0.013	0.004
Hippuric acid	1578	0.045	0.013
o-MHA	882	0.033	0.009
m-MHA	882	0.022	0.006
p-MHA	882	0.023	0.007

[a] A value of 3.5 mmol creatinine/l was used which is based on the assumption that 2 l urine is excreted per day. The normal 95% reference range for creatinine is 7.1–17.7 mmol creatinine/day [30]).

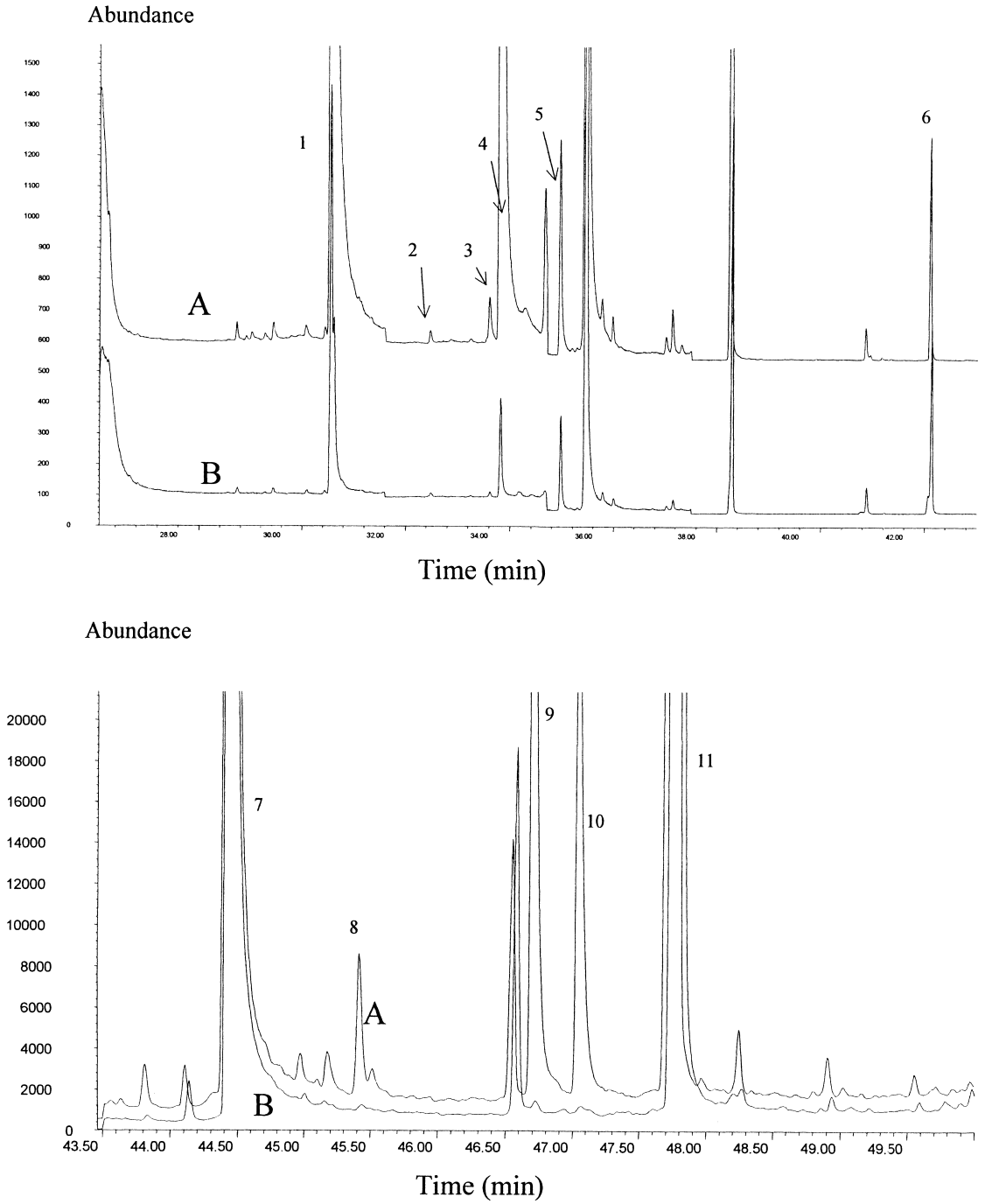

Fig. 5. Metabolite profiles of an occupationally exposed (A) and non-exposed person (B). Phenol (1), *o*-cresol (2), *m*-cresol (3), *p*-cresol (4), *p*-chlorophenol (5), mandelic acid (6), hippuric acid (7), *o*-methylhippuric acid (8), *m*-methylhippuric acid (9), *p*-methylhippuric acid (10), 3-chloro-4-hydroxyphenyl acetic acid (11).

in literature are limited to specific metabolites. Each of these methods are also subjected to their own imprecision and inaccuracy. The proposed method for analysing all the metabolites simultaneously may contribute to the reduction of inter-instrumental and inter-method bias.

4. Conclusion

This sensitive and selective gas chromatographic–mass spectrometric assay is an appropriate method for quantitation of a range of biomarkers for exposure to benzene and its alkylated analogues. This method will replace the use of a combination of analytical methods required to analyse all the metabolites enforced by regulating authorities for organic solvent exposure and still provide information on the concentration of positional isomers.

Acknowledgements

The authors thank the Department of Chemical Pathology, University of Pretoria, Pretoria, South Africa, for supporting this research.

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