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Analysis of benzene, toluene, ethylbenzene and *m*-xylene in biological samples from the general population

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Abstract

A method for the determination of benzene, toluene, ethylbenzene and xylene in blood and urine of people not occupationally exposed to solvents is described. The headspace technique combined with gas chromatography with a mass spectrometer detector is used. The sensitivity of recent mass spectrometers is good enough to furnish reliable results also in biological samples collected from the general population. No treatment for concentrating solvents present in the blood or urine is necessary. The main features of the method are easy preparation of biological samples, small volumes (7 ml), good repeatability and linearity in the range of interest. The limits of detection in blood were 16, 43, 22 and 52 ng/l for benzene, toluene, ethylbenzene and *m*-xylene respectively. Slightly greater sensitivity was found for urine samples. The results obtained in biological samples from 25 woodworkers not occupationally exposed to BTEX (15 non-smokers and 10 smokers) are comparable to those obtained by other investigators.

Keywords: Benzene; Toluene; Ethylbenzene; *m*-Xylene

1. Introduction

Benzene, toluene, ethylbenzene and *m*-xylene (BTEX) are ubiquitous pollutants mainly due to engine emissions, tobacco smoke and industrial pollution. They have been measured in indoor and outdoor air samples by several groups of researchers [1–5].

These aromatic hydrocarbons are also detectable in biological samples: Wallace et al. [3] have reported concentrations ranging from 1 and 12 ng/l in alveolar air of people not occupationally exposed to solvents.

Measurement of these solvents in blood and urine samples collected from the general population can be used to calculate the individual 'body burden' and to establish the reference values for comparing data for occupational exposure.

The quantification of BTEX in biological samples is not an easy task when their concentrations are lower than 1 μg/l as usually happens in people not occupationally exposed to solvents.

In a recent article, Fustinoni et al. [6] describe a reliable method for measuring BTEX in urine based on headspace solid-phase microextraction at 40°C. The features of the method are: Linearity in the range of interest (from the detection limits up to 5000 ng/l), good repeatability (coefficient of variation 2–7%), high specificity related to mass spec-

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trometric detection and detection limit in the 12–34 ng/l range.

Previously, Kok and Ong [7] and Kivisto et al. [8] suggested measuring benzene in blood and urine using a method based on headspace gas chromatography and photoionisation detection. The biological samples are incubated at 60°C for 30 min and 0.5 ml of headspace gas is used for the GC analysis. The recovery and reproducibility are over 90%. The detection limits of benzene in blood and urine are 54 and 43 ng/l, respectively [7]. Similar results were obtained by Kivisto et al. [8], apart from the detection limits which were higher than those reported by the previous research group (about 350 ng/l for both blood and urine).

Perbellini et al. [9] and Ghittori et al. [10] have published results for solvents in biological media from the general population using differently applied 'purge and trap' techniques. The detection limits for benzene with these methods range from 15 to 50 ng/l.

This article describes the simultaneous determination of benzene, toluene, ethylbenzene and *m*-xylene in blood and urine. The sensitivity of recent mass spectrometers yields reliable results also in biological samples from the general population.

2. Experimental

2.1. General

2.1.1. Chemical and standard preparation

Benzene, toluene, ethylbenzene, *m*-xylene and methanol (laboratory grade purity) were purchased from Carlo Erba (Milan, Italy). Benzene-d_6 (>99.96 atom % D) was obtained from Sigma–Aldrich (Milan Italy).

A methanol solution containing the 4 aromatic hydrocarbons was prepared: Each product was present at a concentration of 200 mg/l (about 23 μl of each solvent in 100 ml of methanol). This solution was maintained at 4°C.

The preparation of daily calibration curves started with the solution described which was diluted 1000, 10 000 and 100 000 times in water. The water solutions obtained had concentrations of 200, 20 and 2 μg/l, respectively, and were used to spike the calibration samples: For example, the preparation of a urine or blood sample containing 240 ng/l of each hydrocarbon was done by adding 84 μl of the 20 μg/l solution to a vial containing 7 ml of a biological sample.

The internal standard solution containing deuterated benzene in water (50 μg/l) was prepared daily by diluting a methanol solution of 100 mg/l in water (1:2000).

A volume of 30 μl of the solution obtained was added to 7 ml of biological samples.

All adding operations (both standards and internal standard) were performed with a microsyringe whose needle perforated the septa of the closed vials. This technique is less pollutant than the one in which the vials are opened in order to add standards and internal standard.

2.1.2. Equipment

Glass tubes (12.5 ml effective volume) with PTFE septa and screw caps were used for storage of biological samples immediately after collection. All these pieces of equipment were maintained at 80°C for almost 24 h; before use, glass tubes were additionally cleaned by fluxing with ultra-pure air. The same treatment was used for glass vials (11 ml volume), and their PTFE septa and aluminium seals where the biological samples were transferred for analysis. Immediately after introducing the samples, the vials were closed with 20 mm butyl rubber lined with PTFE septa and crimped with perforated aluminium seals.

The biological samples were injected into the gas-chromatograph with an HP 7694E headspace autosampler (Hewlett-Packard), connected via a volatile interface configured in the direct injection mode.

An HP 6890 gas chromatograph (Hewlett-Packard), interfaced with the HP 5973 mass detector operating in the electron impact (EI) mode was used.

The gas chromatograph was equipped with a hybrid column: PoraPLOT Q (5 m length, 0.32 mm I.D., 10 μm film thickness, Chrompack) connected to an HP-5MS (30 m length, 0.25 mm I.D., 0.25 μm film thickness, Hewlett-Packard).

2.1.3. Sample collection

To study the features of the method, blood and urine samples were obtained from a non-smoking volunteer. The urine specimens were collected directly from the donor in 500 ml glass bottles. Three hundred and fifty ml of blood were supplied by two healthy researchers from our laboratory. The samples were stored at 4°C until analysis (2–3 days).

Another 25 urine and blood samples were obtained from a group of 25 woodworkers living in rural areas. Ten of them smoked 3–20 cigarettes a day, while the other 15 were non-smokers. Biological samples, immediately after collection, were placed in glass tubes which were filled to capacity, closed with screw caps and maintained at 4°C until test time (no more than 4 days). Blood samples were added with 2 drops of EDTA as an anticoagulant.

2.1.4. Sample preparation

The urine and blood samples (7 ml) were transferred after gentle mixing, into the open analysis vial just washed with ultra-pure air. Thirty microlitres of internal standard solution were added to the final concentration of 200 ng/l. These operations were performed rapidly.

2.1.5. Quality control (Calibration, detection limit and repeatability)

Urine and blood from non-smoking, non-occupationally-exposed donors were used for calibration and estimation of the repeatability of the assay.

For calibration, the samples were prepared as above.

Eight urine calibration samples spiked with 0, 15, 30, 60, 120, 240, 480 and 960 ng/l of BTEX, and eight blood calibration samples spiked with 0, 15, 30, 60, 120, 240, 480 and 960 ng/l of benzene and toluene and 0, 30, 60, 120, 240, 480, 960 and 1920 ng/l of ethylbenzene and *m*-xylene were used.

Calibration samples were run as described in Section 2.2.

Least-squares linear regression analysis was used to estimate the slopes (*b*) and intercepts (*a*) of the calibration curves $y = bx + a$, where y is the chromatographic area of the analyte and x is the sample concentration of the analyte (ng/l).

The limit of detection (LOD) of the assay for each aromatic hydrocarbon was calculated according to the expression: $\mathrm{LOD} = (3\ Sy - a)/b$ where Sy is the standard error of the estimate, 'a' is the intercept and 'b' the slope.

The repeatability of the assay (as coefficient of variation, C.V.%) was estimated by repeated analysis of urine and blood samples (5 per concentration) spiked with BTEX at the concentrations of 60 and 480 ng/l for urine and spiked with 60 and 480 ng/l (benzene and toluene) or 120 and 960 ng/l (ethylbenzene and *m*-xylene) for blood. Accuracy was calculated after subtracting the background concentration of the biological samples and comparing the results with the spiked amounts: The mean percentage ratio was reported.

2.2. Gas chromatography–mass spectrometry

A headspace auto-sampler using a loop volume of 1 ml was used. After a 60 min equilibration over a rotating shaker at room temperature (22–23°C), urine samples were placed on the auto-sampler. Blood samples were heated at 50°C and shaken for 60 min in the autosampler before the headspace was withdrawn: The loop and transfer line temperatures were both 110°C. The transfer line was connected to the gas-chromatograph via a volatile interface heated at 120°C with a 'split removed' configuration.

The oven temperature of the gas-chromatograph was kept at 100°C during the injection (1 min). The temperature was then increased to 210°C at a rate of 20°C/min and this temperature was maintained for 4 min. Helium was used as the carrier at 2.2 ml/min constant flow.

The mass detector, with the source kept at 250°C, operated in electron impact mode with the selected ion monitoring mode. The solvent delay time was 3 min, and the dwell time 50 ms. The masses detected were m/z 78 and 77 for benzene, 91 and 97 for toluene, 91 and 106 for ethylbenzene and xylene. Benzene-d_6 as internal standard was monitored with m/z 84. The 2 masses recorded for each compound were used to check the isotopic ratio; their quantification was based on the peak areas of the following masses: 78 for benzene, 84 for benzene-d_6 and 91 for the other solvents.

Approximate retention times were as follows: Benzene = 6.02 min, benzene-d_6 = 6.05 min,

toluene = 7.20 min, ethylbenzene = 8.56 min, *m*-xylene = 8.62 min.

Quantification was not based on the ratio of the chromatographic peak area of the analyte to the internal standard because the addition of a very small amount of internal standard gives rise to minor errors. These make for a slightly worse correlation coefficient of the regression lines as compared to data not corrected for the internal standard. When data were processed without the ratio to the internal standard, the calibration curves showed correlation coefficients ranging from 0.9994 to 0.9999, while the data calculated using the internal standard yielded coefficients ranging from 0.9976 to 0.9994.

The internal standard was used to check that the individual injections were good enough, with no problems of injection needle or carrier flow.

3. Results

3.1. Chromatographic separation

Fig. 1A and B shows the single ion mass chromatograms corresponding to a blood sample from a control subject and to the same sample spiked with 240–480 ng/l of the standards. In the first chromatogram the blood concentrations of benzene, toluene, ethylbenzene and *m*-xylene were 45, 208, 43 and 93 ng/l, respectively.

A similar pattern is reported in Fig. 2A and B obtained from a urine sample. The concentrations of benzene and toluene were 53 and 96 ng/l, respectively; the concentrations of ethylbenzene and *m*-xylene were lower than their detection limits.

The analytes are univocally characterised on the basis on their retention time and mass-to-charge ratio.

3.2. Calibration curves

The resulting calibration curves were linear in the range investigated for BTEX in both blood and urine.

The LOD for benzene, toluene, ethylbenzene and *m*-xylene were: 13, 13, 17 and 13 ng/l, respectively, in urine and 16, 43, 22 and 52 ng/l, respectively, in blood.

The repeatability of the assay, estimated by the coefficient of variation (C.V.%) calculated by repeating 5 samples for each concentration, is reported in Tables 1–4.

Fig. 3A shows the calibration curves of the analytes in blood samples. Similar results were obtained with urine samples (Fig. 3B).

Calibration curves obtained on two other working days in different weeks yielded comparable results.

3.3. Quality control

Tables 1–4 present the intra- and inter-day precision (C.V.%) and accuracy (%) calculated by analysis of urine and blood on three different days. The concentrations of BTEX ranged from 60 to 960 ng/l in blood and from 60 to 480 ng/l in urine (each measure is the mean of 5 samples).

3.4. Measurement of BTEX in woodworkers

Tables 5 and 6 summarise the statistical parameters of BTEX in blood and urine samples obtained from 25 woodworkers living in rural areas.

Benzene in urine had a median value of 77 ng/l, but there was a statistical difference between non-smokers (median: 66 ng/l) and smokers (median: 125 ng/l). The comparison was performed using the Mann–Whitney–Wilcoxon test: $W = 106$; $P < 0.05$.

In blood the corresponding median concentrations were 106, 87 and 246 ng/l, respectively; the W test confirmed the difference between non-smokers and smokers ($W = 109$, $P < 0.05$).

Toluene had the highest concentrations among the solvents studied. In smokers the median blood and urine concentrations were 780 and 259 ng/l, respectively, while in non-smokers they were 428 and 416 ng/l. The difference was statistically significant ($W = 113$; $P < 0.05$) only in blood samples.

Ethylbenzene had the lowest urinary concentrations in comparison with the other hydrocarbons studied: In 14 samples (out of 25) they were lower than the detection limit (for the statistical calculation these values were recorded as half the method's detection limit [11]). In blood, ethylbenzene was detectable in 29 out of 30 samples and its median concentrations were more than 10 times higher than in urine, as the blood/water partition coefficient suggests [12]. No statistical difference was found in

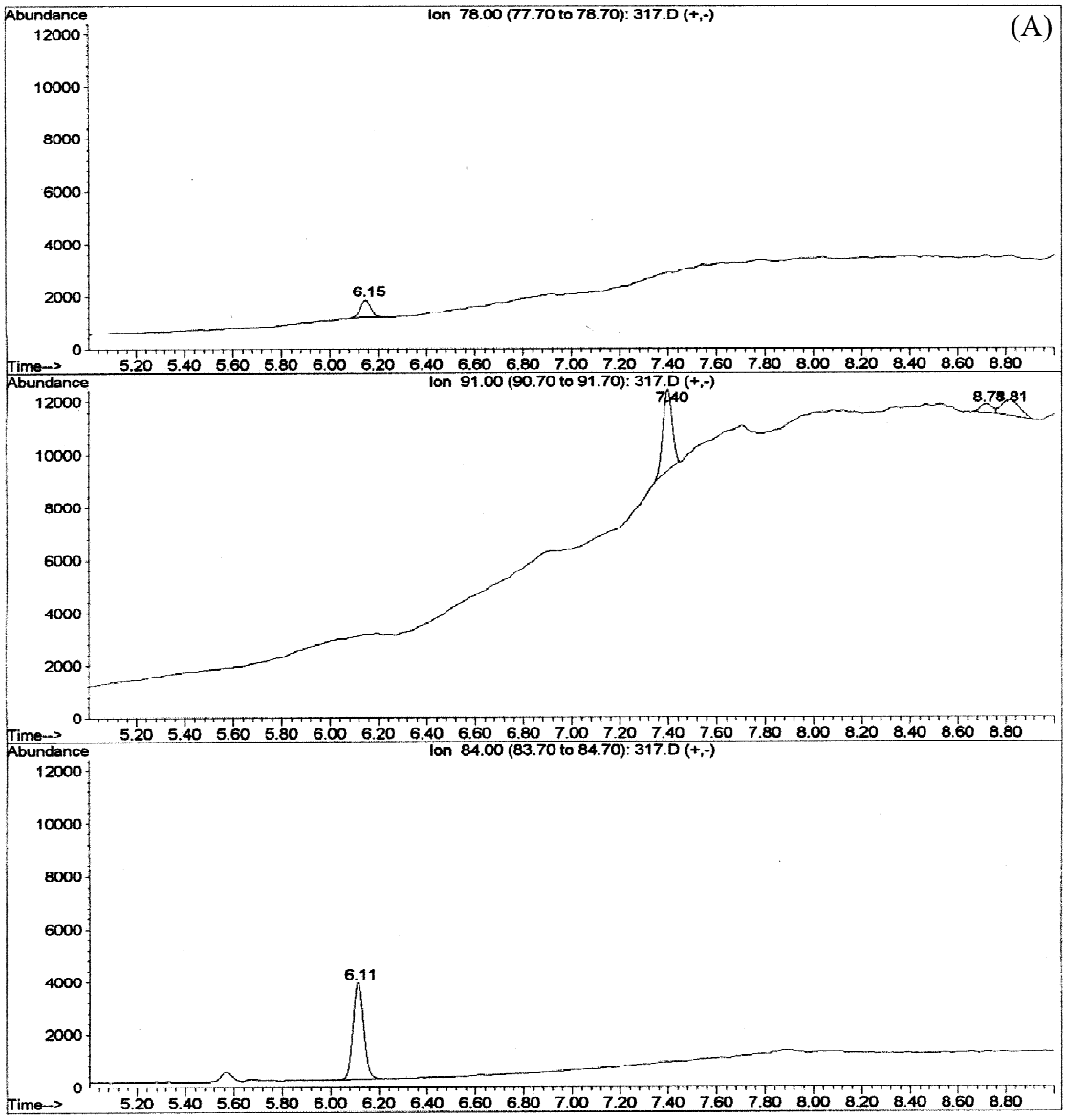


Fig. 1. Single ion mass chromatograms with the retention time (RT) corresponding to a blood sample from a control subject (A) and the same sample added with 240 ng/l of benzene (RT = 6.15) and toluene (RT = 7.4) and 480 ng/l of ethylbenzene (RT = 8.71) and *m*-xylene (RT = 8.83) (B). The internal standard ($m/z = 84$) has a retention time of 6.11.

biological samples obtained from non-smokers and smokers (Tables 5 and 6). *m*-Xylene had median blood concentrations 4 times higher than the urine concentrations. This solvent was always detectable in biological samples; we were unable to find any statistical difference in blood and urine concentrations of *m*-xylene when comparing data from non-smokers and smokers (Tables 5 and 6).

The correlation between blood and urinary concentrations of the solvents studied was statistically significant for benzene, ethylbenzene and *m*-xylene as reported here below:

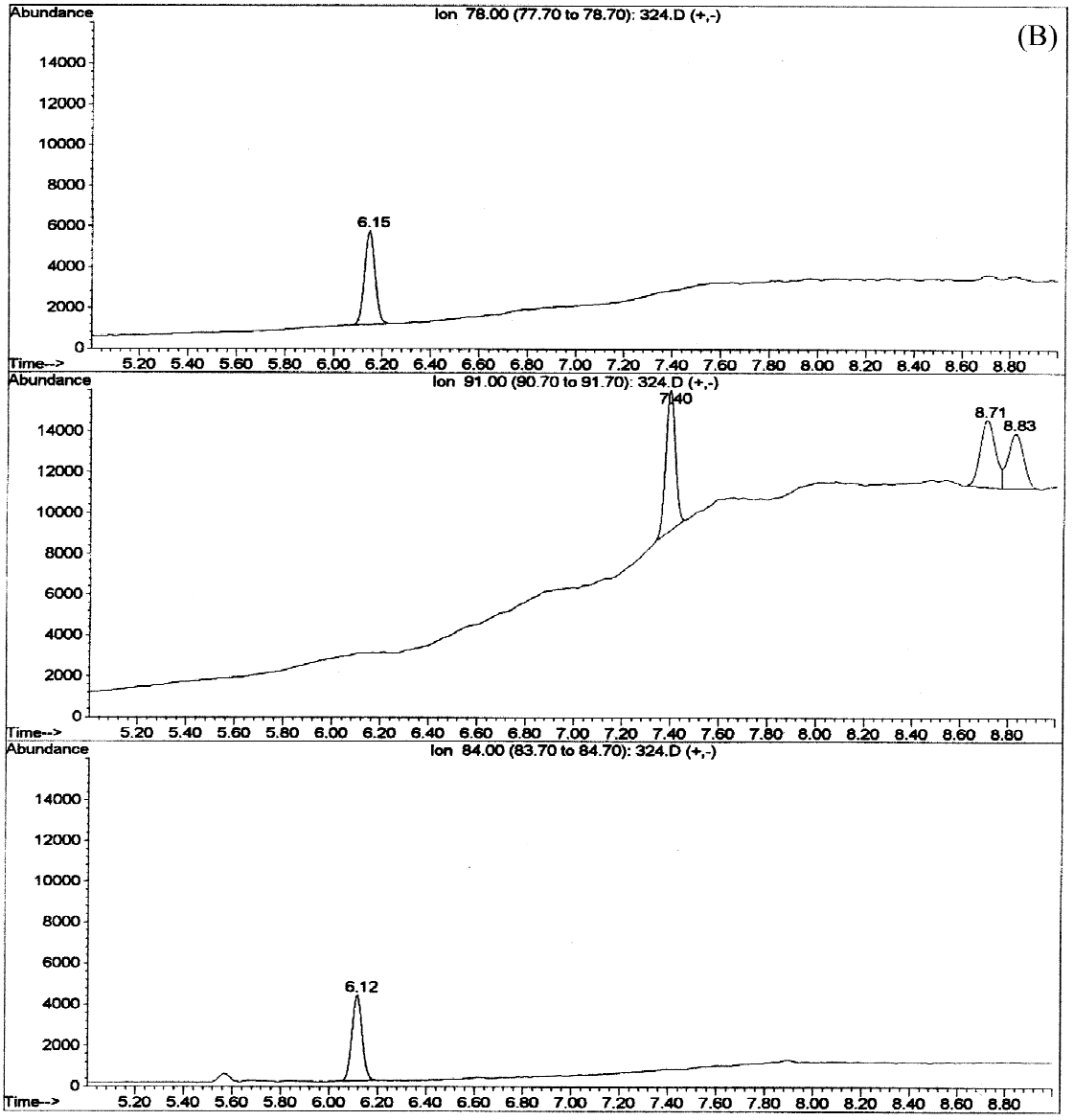


Fig. 1. (*continued*)

Benzene: Cb = 2.71 Cu − 57; $r = 0.8873$; $P < 0.001$.

Ethylbenzene: Cb = 7.13 Cu + 109; $r = 0.4972$; $P < 0.05$.

m-Xylene: Cb = 9,3 Cu − 266; $r = 0.6332$; $P < 0.001$.

where 'Cb' and 'Cu' are blood and urinary concentrations, respectively.

No correlation was found between blood and urinary concentrations of toluene.

4. Discussion

The method reported here is not difficult to implement. Great care should be taken to optimise the instrument working conditions and the calibration curves. The preparation of the biological samples is easy, but the addition of very low concentrations of deuterated benzene as internal standard is a critical phase. We added the internal standard in order to

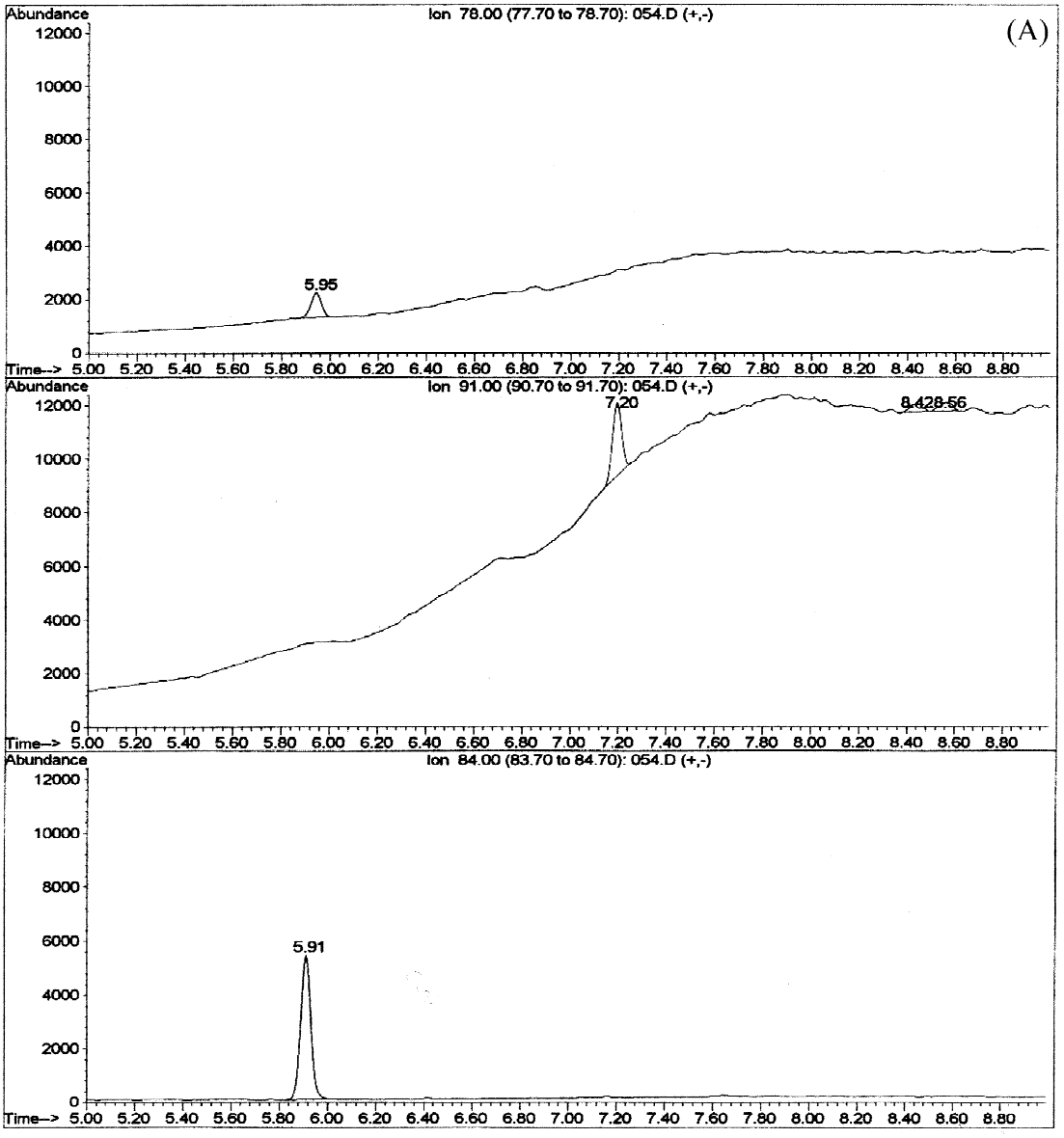
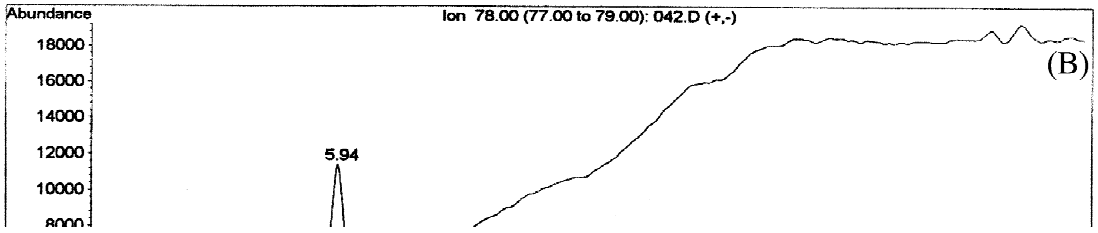


Fig. 2. Single ion mass chromatograms with the retention time (RT) corresponding to a urine sample from a control subject (A) and the same sample added with 240 ng/l of benzene (RT = 5.95), toluene (RT = 7.2), ethylbenzene (RT = 8.42) and *m*-xylene (RT = 8.56) (B). The internal standard ($m/z = 84$) has a retention time of 5.91.

check that the injections were regular; this means that there were no problems with the needle of the autosampler, carrier flow and split or splitless injection. If the internal standard suggested that the single analysis was performed without trouble, the subsequent concentrations of BTEX in biological samples were estimated by peak area (ignoring the ratio to the internal standard). We found that the variations of analyses for calibration curves related to the internal standard were a little worse than those obtained from 'uncorrected' data.

Benzene concentrations in blood samples obtained



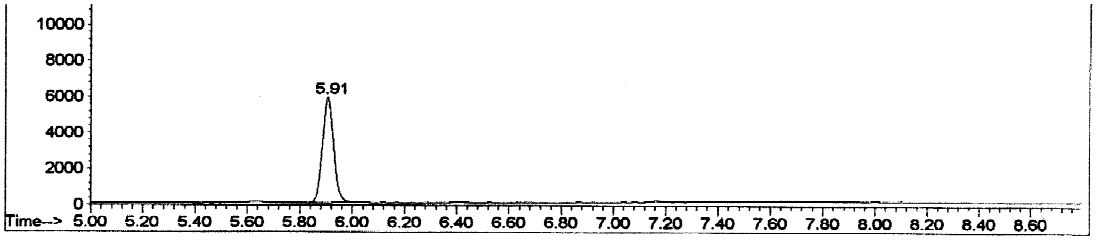


Fig. 2. (*continued*)

from the general population, even when measured by different techniques, have yielded comparable results: In non-smokers the benzene concentration is, on average, lower than in smokers [9,13] and city dwellers have higher concentrations than people living in rural areas [14].

Benzene in urine has also been measured by several research groups and its concentrations present similar ranges to those found in this study [7,15]. In smokers, the median urinary benzene concentration was 2 to 5-fold higher than in non-smokers. As reported above, there was a strong linear correla-

Table 1
Intra- and inter-day precision (C.V.%) and accuracy (%) of the batch calculated on the basis of concentrations ranging from 60 to 120 ng/l of BTEX in blood (each measure is the mean of 5 samples)

	Benzene (60 ng/l)		Toluene (60 ng/l)		Ethylbenzene (120 ng/l)		*m*-Xylene (120 ng/l)	
	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy
Day 1	5.02	96	1.3	97.4	2.9	98.3	3.5	105.6
Day 2	1.52	98.5	1.2	88.9	5	93.6	6.0	102
Day 3	2.33	104.3	2.3	87.2	7.5	110	7.9	107
Overall	9.3	99.3	13.2	91.6	13.1	100.6	13.5	104.8

[a] Rep. = repeatability.

Table 2
Intra- and inter-day precision (C.V.%) and accuracy (%) of the batch calculated on the basis of concentrations of BTEX (60 ng/l) in urine (each measure is the mean of 5 samples)

	Benzene (60 ng/l)		Toluene (60 ng/l)		Ethylbenzene (60 ng/l)		*m*-Xylene (60 ng/l)	
	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy
Day 1	0.8	88.3	3.1	100.3	8.6	93.3	7.3	97.5
Day 2	3.4	93.5	2.1	98.2	13.4	88.4	7.6	95.7
Day 3	8.0	107.3	3.0	99.7	10.7	97.9	6.8	96.5
Overall	13.6	96.3	6.8	99.4	11.1	93.2	7.1	96.6

[a] Rep. = repeatability.

Table 3
Intra- and inter-day precision (C.V.%) and accuracy (%) of the batch calculated on the basis of concentrations ranging from 480 to 960 ng/l of BTEX in blood (each measure is the mean of 5 samples)

	Benzene (480 ng/l)		Toluene (480 ng/l)		Ethylbenzene (960 ng/l)		*m*-Xylene (960 ng/l)	
	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy
Day 1	1.9	98.1	7.4	89.6	2.6	89.3	4.6	91.4
Day 2	3.4	102.4	1.2	92.8	4.7	94.2	2.3	95.9
Day 3	0.6	103.3	2.7	95.4	3.5	100.0	3.4	111.2
Overall	3.1	101.3	7.9	92.6	13.8	94.5	7.9	99.5

[a] Rep. = repeatability.

Table 4
Intra- and inter-day precision (C.V.%) and accuracy (%) of the batch calculated on the basis of concentrations of BTEX (480 ng/l) in urine (each measure is the mean of 5 samples)

	Benzene (480 ng/l)		Toluene (480 ng/l)		Ethylbenzene (480 ng/l)		*m*-Xylene (480 ng/l)	
	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy	Rep.[a]	Accuracy
Day 1	1.8	110.0	2.9	107.6	2.8	106.4	4.0	107.7
Day 2	1.5	102.1	1.5	102.4	2.2	105.3	3.7	104.9
Day 3	1.7	106.5	1.9	108.4	2.0	105.1	3.8	102.3
Overall	3.6	106.2	3.6	105.9	2.3	105.6	4.3	104.8

[a] Rep. = repeatability.

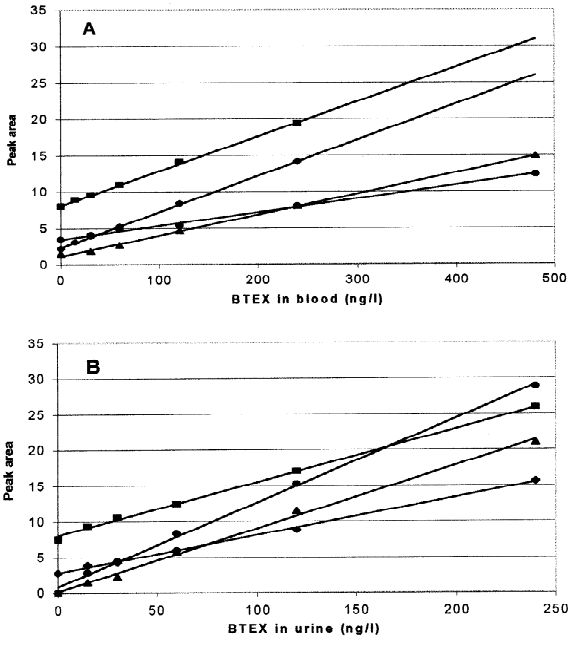


Fig. 3. (A) Calibration curves of benzene (♦), toluene (■), ethylbenzene (▲) and *m*-xylene (●) in blood. (B) Calibration curves of benzene (♦), toluene (■), ethylbenzene (▲) and *m*-xylene (●) in urine.

tion between benzene concentration in blood and urine; from the slope of the regression line, benzene in urine is about 2.7 times lower than in blood.

Kok and Ong [7] also found a similar linear correlation, but their results differed from ours, in that their median benzene concentration was higher in urine than in blood, both in non-smokers and in smokers. This finding is probably related to the heating of the urine at 60°C for 30 min prior to analysis.

In one of our previous papers, markedly different results were found (not only statistically different) depending on the preparation of the urine samples [16]. Acidification or heating of urine samples at 80°C before the analytical phase gives rise to values, on average, 4 times higher than in non-acidified or non-heated samples (in some cases the values were more than 20 times higher).

The explanation of these results is not clear; comparison of urinary benzene concentrations obtained by researchers with different analytical techniques shows widely differing values.

In our present method for measuring benzene and other aromatic hydrocarbons in urine we do not use any treatment of urine samples. Perhaps the detection limits are not the highest, but we can be confident we are measuring free benzene without any interference related to benzene released by heating or acidification.

Our results for toluene concentration in blood showed a median value of 559 ng/l. Similar results were found by Wang et al. [17], by Fustinoni et al. [18] and by Bergamaschi et al. [19]. Different concentrations were obtained by Hajimiragha et al. [13] who found a geometric mean of 1284 ng/l of toluene in non-smokers and 2019 ng/l in smokers. Some groups of researchers have suggested a significant difference in toluene concentration in non-smokers and smokers, while others have not.

Toluene concentrations in urine have been measured on very few occasions: The median values or geometric means range from 204 to 430 ng/l [2,19], as in the case of our results. We were unable to find any correlation between blood and urinary concentrations of toluene. No such correlation has been reported in the literature.

Ethylbenzene was always found in our blood samples and its median concentrations were similar in non-smokers and smokers (Table 5). Ashley et al. [20] and Dunemann et al. [21] also measured ethylbenzene in blood: The average concentrations they reported were 120 and 300 ng/l, respectively. In non-smokers the blood concentrations of this solvent were statistically lower than in smokers. Hajimiragha et al. [13] found median concentrations of 837 ng/l in smokers and 651 ng/l in non-smokers: These values are considerably higher than those reported by other investigators.

Very little ethylbenzene was found in urine: its median concentration was lower than the detection limit. About half the results ranged from 22 to 47 ng/l. Minoia et al. [2] measured ethylbenzene in urine in 3 different groups of school-children with median concentrations of 77, 35 and 33 ng/l. Although there are difficulties in measuring this solvent in urine, a good correlation was found between blood and urine ethylbenzene concentrations. *m*-Xylene concentrations in blood and urine showed a statistically significant linear correlation with values about 9-fold higher in blood (from the

Table 5
Blood concentrations of BTEX (ng/l) in samples obtained from woodworkers, also subdivided by smoking habit (15 non-smokers and 10 smokers)

	Median	Geom. M.	Average	SD	Minimum	Maximum
Benzene						
all data	106	152	266	323	46	1187
non-smokers	87	108	141	126	46	472
smokers	246	253	453	434	51	1187
Toluene						
all data	559	661	1100	1496	120	6040
non-smokers	428	502	844	1454	120	6040
smokers	780	1000	1486	1551	348	5148
Ethylbenzene						
all data	145	162	231	180	<D.L.	596
non-smokers	145	153	222	168	<D.L.	496
smokers	148	175	243	207	63	596
m-Xylene						
all data	457	533	719	531	92	1713
non-smokers	535	552	735	497	92	1451
smokers	411	506	696	605	203	1713

Geom. M. = geometric mean; SD = standard deviation; <D.L. = lower than its detection limit.

Table 6
Urinary concentrations of BTEX (ng/l) in samples obtained from woodworkers, also subdivided by smoking habit (15 non-smokers and 10 smokers)

	Median	Geom. M.	Average	SD	Minimum	Maximum
Benzene						
all data	77	85	119	105	24	409
non-smokers	66	66	86	71	24	248
smokers	125	125	169	132	42	409
Toluene						
all data	284	334	404	281	131	1227
non-smokers	416	356	436	313	143	1227
smokers	259	303	357	232	131	856
Ethylbenzene						
all data	8.5	14	17	13	<D.L.	47
non-smokers	8.5	13	17	13	<D.L.	47
smokers	8.5	14	17	12	<D.L.	37
m-Xylene						
all data	96	101	106	36	63	184
non-smokers	99	107	112	33	72	184
smokers	79	91	97	41	63	171

Geom. M. = geometric mean; SD = standard deviation; <D.L. = lower than its detection limit (17 ng/l).

slope of the regression line). On the basis of the geometric means the *m*-xylene blood:urine concentration ratio was 5:1. Our data agree with those of Fustinoni et al. [18] who found similar concentrations in non-smokers and smokers.

In conclusion, the method for measuring BTEX in biological samples described here is not complex and is sensitive and specific enough for measuring BTEX in biological samples from subjects of the general population, not occupationally exposed to solvents. The results are comparable to those reported by most of the research groups interested in this field and especially with regard to the BTEX concentration in blood. Though theoretically there should always be a close correlation between blood and urinary concentrations, the urinary BTEX concentrations reported by the different groups of investigators tend to vary very considerably. Sometimes this is related to the different methodologies used in the analytical procedures, and sometimes it is not possible to identify the reason for the differences. Further research is needed in order to better understand the meaning of urinary concentrations of BTEX and other organic solvents.

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