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Effect of hippuric acid on the gaschromatographic retention of *S*-phenylmercapturic acid

G. Marrubini *, E. Terulla, G. Brusotti, G. Massolini

Department of Pharmaceutical Chemistry, University of Pavia, Via Taramelli 12, 27100 Pavia, Italy

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Abstract

S-phenylmercapturic acid (PMA) is one specific urinary biomarker of low-level benzene exposure. It is used for biological monitoring of benzene-exposed workers in the petrochemical industry and normally ranges from non-measurable to 10 μg/l levels in non-exposed non-smoking subjects. Benzene-exposure caused by workplace or lifestyle sources is frequently accompanied by toluene exposure, which can cause the occurrence of high levels (from 10 mg/l to more than 2000 mg/l) of hippuric acid (HA) in urine. Both solvents are toxic, and benzene is classified as a human carcinogen. The biological monitoring of benzene and toluene is therefore required for preventive care of exposed workers health.

In this study a GC–MS method was adopted for measuring urinary PMA, which involved liquid–liquid extraction (LLE) with ethyl acetate from acidified urine and esterification with 0.5 N hydrochloric acid in methanol. The method evidenced a GC effect in a conventional HP-5 (30 m × 0.25 mm i.d., 0.25 μm film-thickness) methyl-phenylsilicone capillary column produced by HA on PMA. The results demonstrate that HA at concentrations as low as 250 mg/l can delay the elution of PMA and labelled internal standard from the column. The recognition and discussion of this particular GC phase soaking effect may be of help for those who are occupied in the determination of PMA and of urinary acidic metabolites by GC.

Keywords: Benzene; Toluene; Biological monitoring; *S*-phenylmercapturic acid; Hippuric acid; Phase soaking

1. Introduction

Numerous research works on the monitoring of exposure to volatile organic compounds (VOCs) and their effects on human health are reported every year. In particular, benzene professional exposure in industrially developed countries is still of concern mainly in refineries and fuel deposits, where workers may be exposed to low levels of a number of VOCs. Toluene, along with benzene, is found in settings where oil and fuel are processed, stored, and used [1].

One way to monitor the occupational exposure to benzene is to test urine for specific metabolites. *S*-phenylmercapturic acid (*N*-acetyl-*S*-phenyl-(L)-cysteine, PMA) is considered one of the most specific urinary biomarkers of low-level benzene exposure. It is used for biological monitoring of benzene-exposed workers in the petrochemical industry and as such it is regarded as an important diagnostic tool in occupational medicine on-field practice [2]. The physiologic levels of PMA in urine of people non-exposed to benzene are generally lower than 5 μg/g creatinine (viz broadly about 5 μg/l) in non-smokers, and can exceed the level of 10 μg/g creatinine only exceptionally in heavy smokers [3,4]. Subjects exposed to benzene vapour concentrations as low as 0.5 ppm (viz around 1.5 mg benzene/m^3) during the 8-h workshift may show levels of PMA higher than 20–30 μg/g creatinine in end-shift urine samples. Actually, for a benzene 8-h time-weighted average concentration of 0.5 ppm it is accepted the American Conference of Governmental Industrial Hygienists (ACGIH) Biological Exposure Index (BEI) for urinary PMA which is at 25 μg/g creatinine [3].

* Corresponding author. Tel.: +39 0382 987788; fax: +39 0382 422975.
E-mail address: giorgio.marrubini@unipv.it (G. Marrubini).

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At present, PMA is determined in urine by HPLC methods requiring derivatization and fluorimetric detection [5,6], by LC–MS–MS [1,7–10], and by chemiluminescence-ELISA [11].

All these methods require a purification step. Several validated methods were reported for determining PMA in urine using GC–MS [2,12,13] and GC [14] coupled to electron capture detector. GC is considered a robust and convenient technique for analyzing PMA although it requires extensive sample preparation involving extraction and derivatization. The GC–MS methods, in particular, make use of liquid–liquid extraction with ethyl acetate from acidified urine before derivatization, and further clean-up of the extract, in the case of urinary PMA, is considered not mandatory to obtain good selectivity.

Generally speaking, however, all GC methods can be affected by solvent effects as early demonstrated and reviewed [15]. Solvent trapping and phase soaking, two major solvent effects in GC, are known to be responsible for peak distortion and retardation, respectively [15,16]. Phase soaking, in particular, can take place in the neighbourhood of large peaks of components able to modify the column stationary phase, to act as solvent themselves, and able to migrate with the target analyte thus retardating its elution from the column [17,18]. Despite the early definition of these GC effects and the extension of the concept to all influences due to components different from the injection solvent itself (viz "dirt" coextracted with the target analyte), very few examples have been reported [19,20]. Therefore, it may be difficult to recognize them when they occur in daily GC practice.

This study reports one original example of the mentioned phase soaking effect which to the authors knowledge was not described before in bioanalytical literature dedicated to GC and GC–MS methods.

2. Experimental

2.1. Materials

S-phenylmercapturic acid (purity 99%) was purchased from Tokyo Kasei (Tokyo, Japan), and [$^{13}C_6$]-PMA (purity assayed > 95%) was a kind gift of Dr. Marco Pacenti from the University of Firenze where the molecule was custom-synthesized. *N*-benzoyl-glycine, namely hippuric acid (HA, purity 98%), was obtained by Aldrich (Sigma–Aldrich group, Milan, Italy).

n-Hexadecane and *n*-octacosane standards for GC (purity 99.5%) were purchased from Fluka (Sigma–Aldrich group, Milan, Italy).

Ampoules of 5 ml of hydrochloric acid 0.5 M in methanol (HCl/MeOH) were purchased from Supelco (Sigma–Aldrich group, Milan, Italy). Ethyl acetate, toluene, dichloromethane, and *n*-hexane were analytical-grade reagents obtained from VWR-Merck (Milan, Italy).

2.2. Urine samples

Urine for method testing was collected from three healthy non-smoking volunteers, pooled, and subdivided into aliquots of 10 ml each in polyethylene tubes. Urine from benzene-exposed workers were kindly supplied by the Toxicology Division of the Fondazione Salvatore Maugeri I.R.C.C.S. of Pavia (Pavia, Italy).

All urine samples were frozen at −20 °C immediately after collection and stored in the dark until analysis.

2.3. Sample pretreatment

Sample pretreatment was carried out according to the method of van Sittert et al. [12], with slight modifications. Briefly, aliquots of 3 ml of urine after being thawed were added with the labeled internal standard, [$^{13}C_6$]-*S*-phenylmercapturic acid ([$^{13}C_6$]-PMA, 30 μl of standard solution 20 ng/μl in methanol), in order to obtain a 200 μg/l final concentration. Samples were homogenized by Vortex mixing and were acidified to pH < 2 with 50 μl of concentrated HCl in polythene tubes with screw caps. Ethyl acetate (12 ml) was added for liquid–liquid extraction and the tubes were Vortex-shaken for two minutes. Centrifugation (15 min at 4000 rpm) followed and the supernatant was transferred into glass tubes for evaporation under nitrogen stream at 60 °C.

The dry residue was reconstituted in 0.5 ml of 0.5 M HCl/MeOH, the tube was capped, and the reaction was left at room temperature for 30 min. The red-brownish end-reaction content of the tubes was evaporated to dryness under a gentle nitrogen stream at 60 °C. The residue was reconstituted in 0.1 ml toluene. One microliter of toluene was injected into the GC–MS.

Mass spectra of standard pure PMA methyl ester (PMA-Me) and [$^{13}C_6$]-PMA methyl ester ([$^{13}C_6$]-PMA-Me) were obtained after derivatizing the corresponding free acids dried from methanol standard solutions and extracted from aqueous standard solutions. In the former case aliquots of methanol standard solutions were dried and the residue after derivatization was reconstituted in toluene before injection. Aqueous standard solutions were acidified, extracted, and derivatized as described for urines.

2.4. GC–MS analysis

All analyses were performed on bench-top equipment from Agilent Technologies consisting of model 6890N gas chromatograph connected to a model 5973*Network* mass selective detector, and equipped with a model 7683 Series injector.

Injections were performed at 250 °C in pulsed-splitless mode (injection pulse pressure 20 psi until 1 min, purge flow 50 ml/min at 1 min) in a conventional Agilent split-splitless glass liner (part. No. 5183-4693) containing a plug of deactivated glass-wool at the bottom.

High purity He (99.999% or better) was used as carrier gas at about 10 psi pressure (constant flow of 1 ml/min or around 37 cm/s).

A HP-5 capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness) from Agilent was used under the following conditions: 100 °C for 2.25 min, 10 °C/min to 260 °C, 30 °C/min to 290 °C, 290 °C for 5 min.

The GC–MS interface temperature was 280 °C. The ion source temperature was 230 °C with electron impact ionization energy set at 70 eV. The quadrupole temperature was 150 °C.

Full scan of masses from *m*/*z* 50 to *m*/*z* 300 was carried out to obtain mass spectra of PMA-Me, [$^{13}C_6$]-PMA-Me, and HA methyl ester (HA-Me, Fig. 1). Selected ion monitoring (SIM) was performed on the ions *m*/*z* 253 and *m*/*z* 194 (qualifier and quantifier, molecular mass and base peak of PMA-Me, respectively), and on the ions *m*/*z* 259 and *m*/*z* 200 (qualifier and quantifier, molecular mass and base peak of the internal stdandard [$^{13}C_6$]-PMA-Me, respectively).

3. Results

The effect observed consists of a remarkable delay in the elution of PMA-Me, as illustrated in Fig. 2. What appeared during the routine application of the method was that there is a significative retention time difference of about 9 s between PMA-Me in standard solution and in the urinary extracts. In order to confirm the significance of such difference in the retention time, replicate measurements were carried out on control samples. As summarized in Table 1, after analyzing

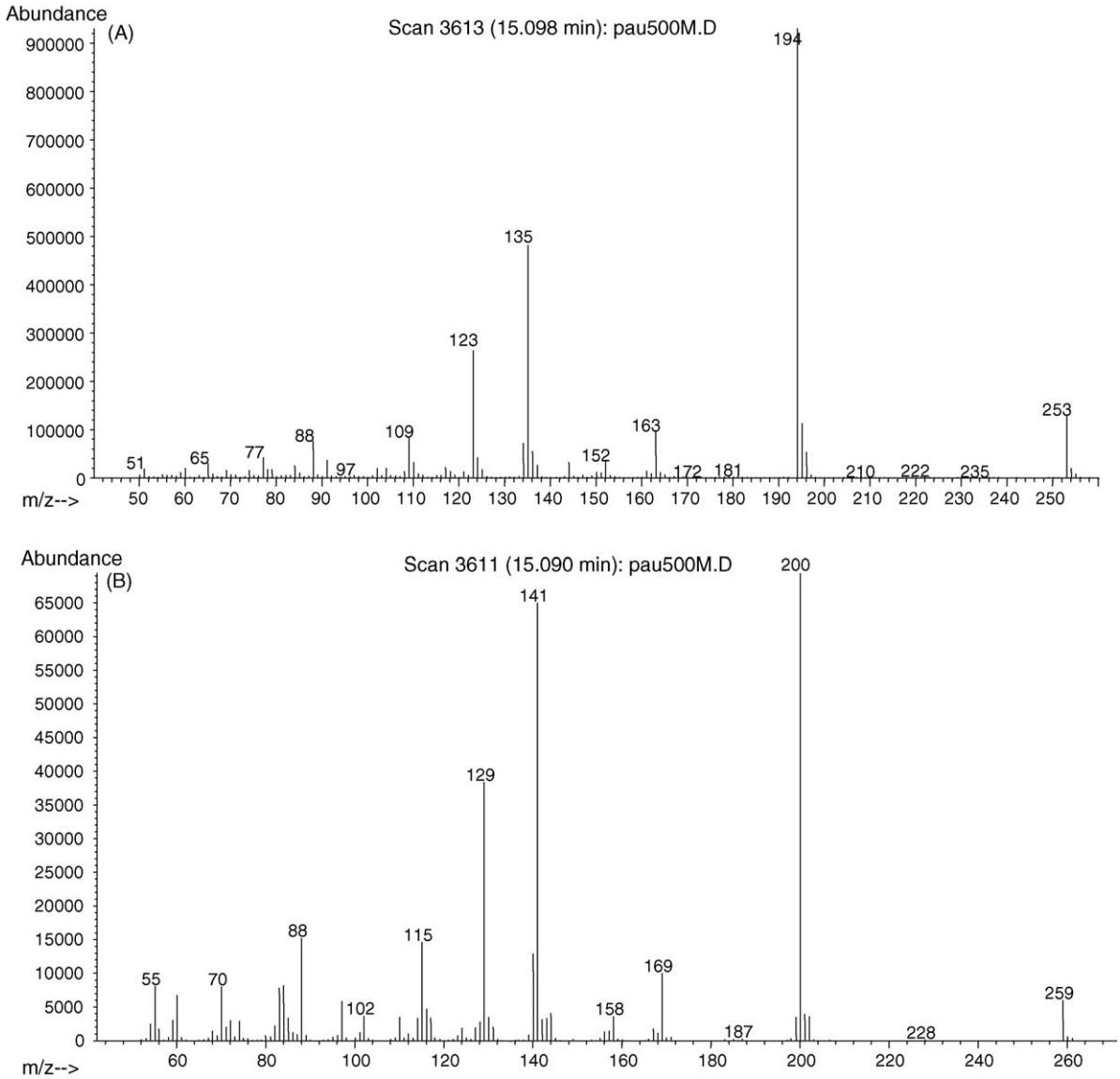

Fig. 1. Mass spectra of *S*-phenylmercapturic acid methyl ester (A, PMA-Me), [$^{13}C_6$]-*S*-phenylmercapturic acid methyl ester (B, [$^{13}C_6$]-PMA-Me), and hippuric acid methyl ester (C, HA-Me).

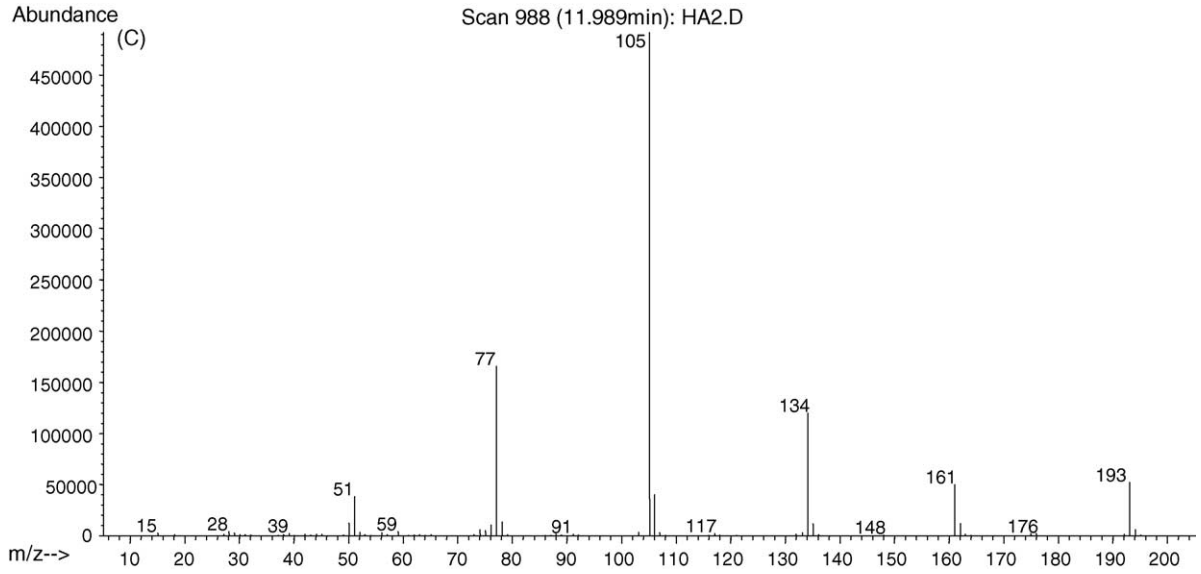

Fig. 1. (*Continued*).

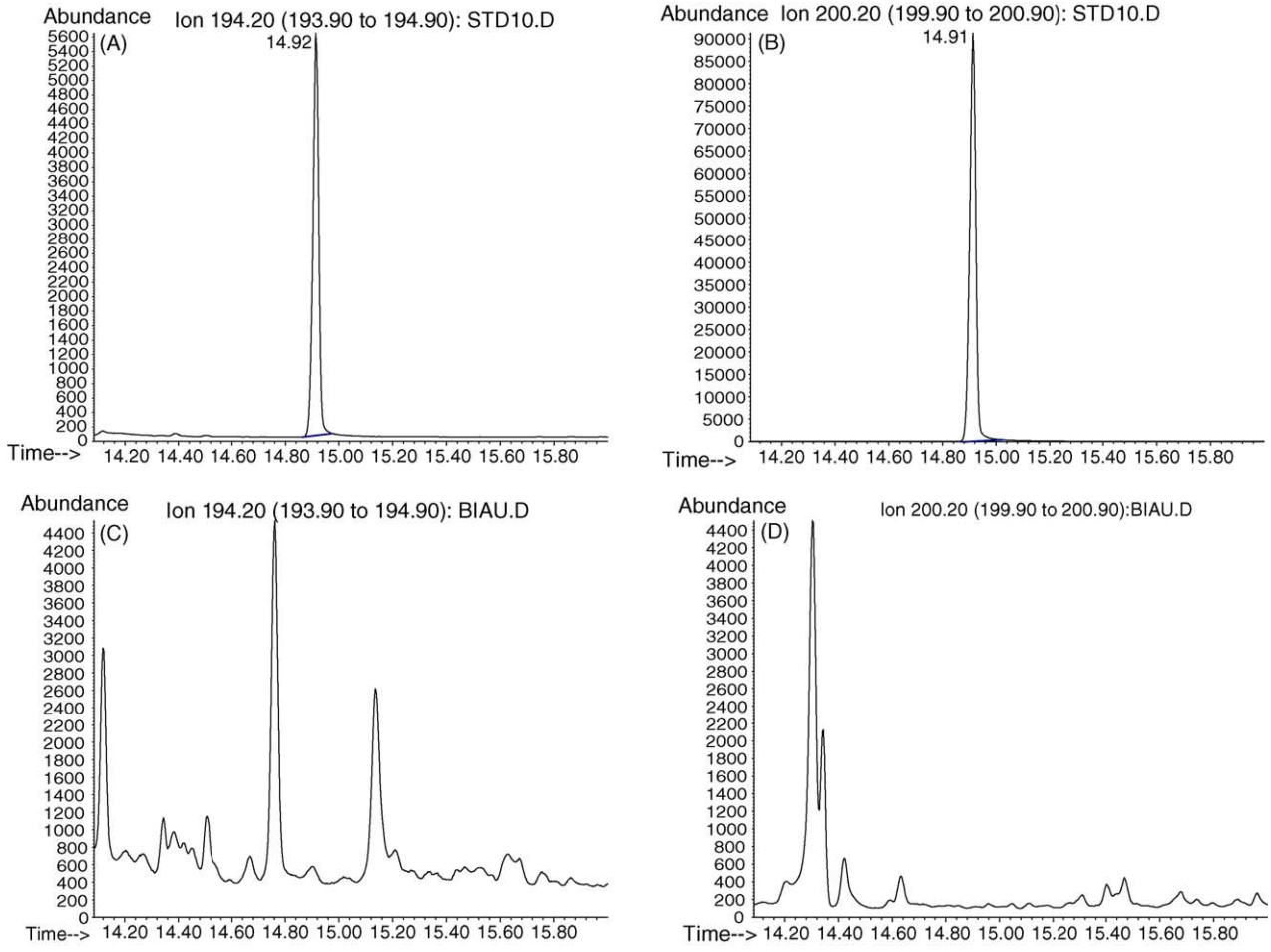

Fig. 2. Delayed elution of PMA-Me and of [$^{13}C_6$]-PMA-Me in urine samples as compared with a standard solution. Chromatograms of ions *m*/*z* 194 and *m*/*z* 200 quantifiers of PMA-Me and [$^{13}C_6$]-PMA-Me, respectively: (A) PMA standard methanol solution at about 10 μg/l; (B) [$^{13}C_6$]-PMA standard methanol solution at about 200 μg/l; (C and D) blank pooled urine of non-exposed subjects; (E) blank pooled urine spiked with 10 μg/l PMA; and (F) 200 μg/l [$^{13}C_6$]-PMA; (G and H) urine sample of a benzene-exposed subject coded NA1195. PMA concentration found was about 20 μg/l, [$^{13}C_6$]-PMA was 200 μg/l.

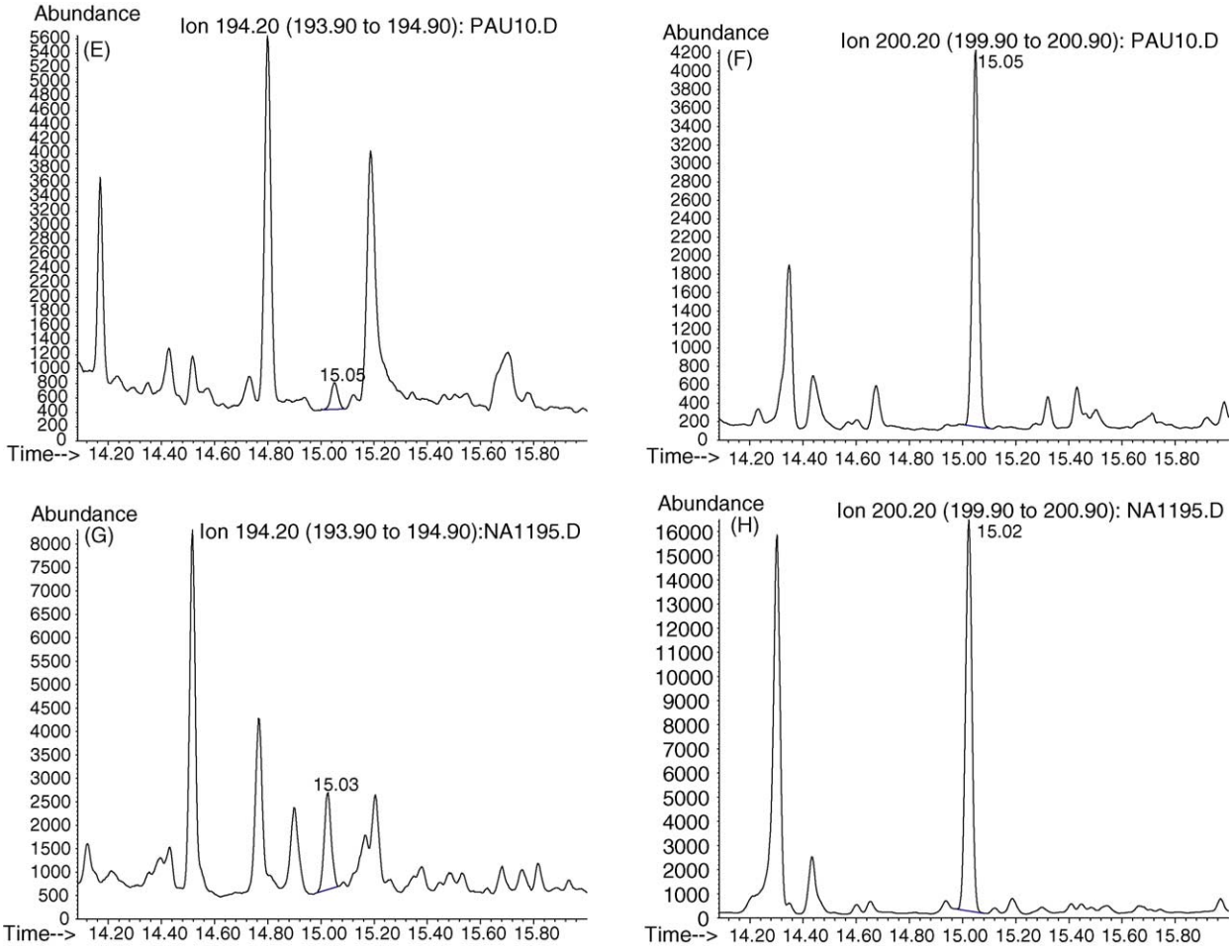

Fig. 2. (*Continued*).

ten different samples prepared in methanol and in blank urine in three different non-consecutive sessions, the *F*-test evidenced a statistical difference at the 99.5% confidence level.

The identity of the peaks attiributed to PMA-Me and to the labeled internal standard was confirmed by full scan mass spectrometry on freshly prepared standard and urine samples. Therefore, it was excluded that the derivatization reaction may have yielded different products in the standard solution and in the urinary extract.

Table 1
Retention time difference between *S*-phenylmercapturic acid methyl ester (PMA-Me) in standard methanol solution and in urinary extracts

PMA-Me	Retention time mean ± S.D. (min)	Variance	*N*
Standard solution	14.831 ± 0.008	0.000057	10
Urine	15.01 ± 0.08	0.0062	10

Data have been collected in three non-consecutive sessions by analyzing ten different samples prepared at 10 μg/l nominal concentration of PMA in methanol and in blank pooled urine; The *F*-test on the variances resulted significant at the 99.5% confidence level; *F* calculated value = 107; *F* critical value = 6.555; 9 degrees of freedom.

The second hypothesis tested was that liquid–liquid extraction (LLE) isolated from urine some compound/s that may alter PMA-Me volatility as compared to the target analyte prepared from standard methanolic solution. Such idea was strongly supported by the observation that the LLE of acidified urine with ethyl acetate is indeed more an enrichment than a purification step.

If this hypothesis was correct, LLE followed by a purification step would reduce the retention time shift. Therefore, 100 μg/l of PMA and 200 μg/l internal standard were added to 3 ml of urine, and the sample was extracted and derivatized. After derivatization, 10 ml of *n*-hexane were added to the 0.5 ml of HCl/MeOH, and the mixture was Vortex-shaken for 2 min. The *n*-hexane supernatant was transferred into a clean glass tube, dried under nitrogen and reconstituted in 0.1 ml toluene before injecting 1 μl of it (Fig. 3, chromatogram C). The methanolic residue was also dried, reconstituted in 0.1 ml toluene, and injected (1 μl) into the GC–MS (Fig. 3, chromatogram B). By comparing the retention times of the three chromatograms of Fig. 3 it appears that *n*-hexane purification brought the PMA-Me peak closer to the expected retention time, while the “methanolic fraction” still shows a PMA-Me

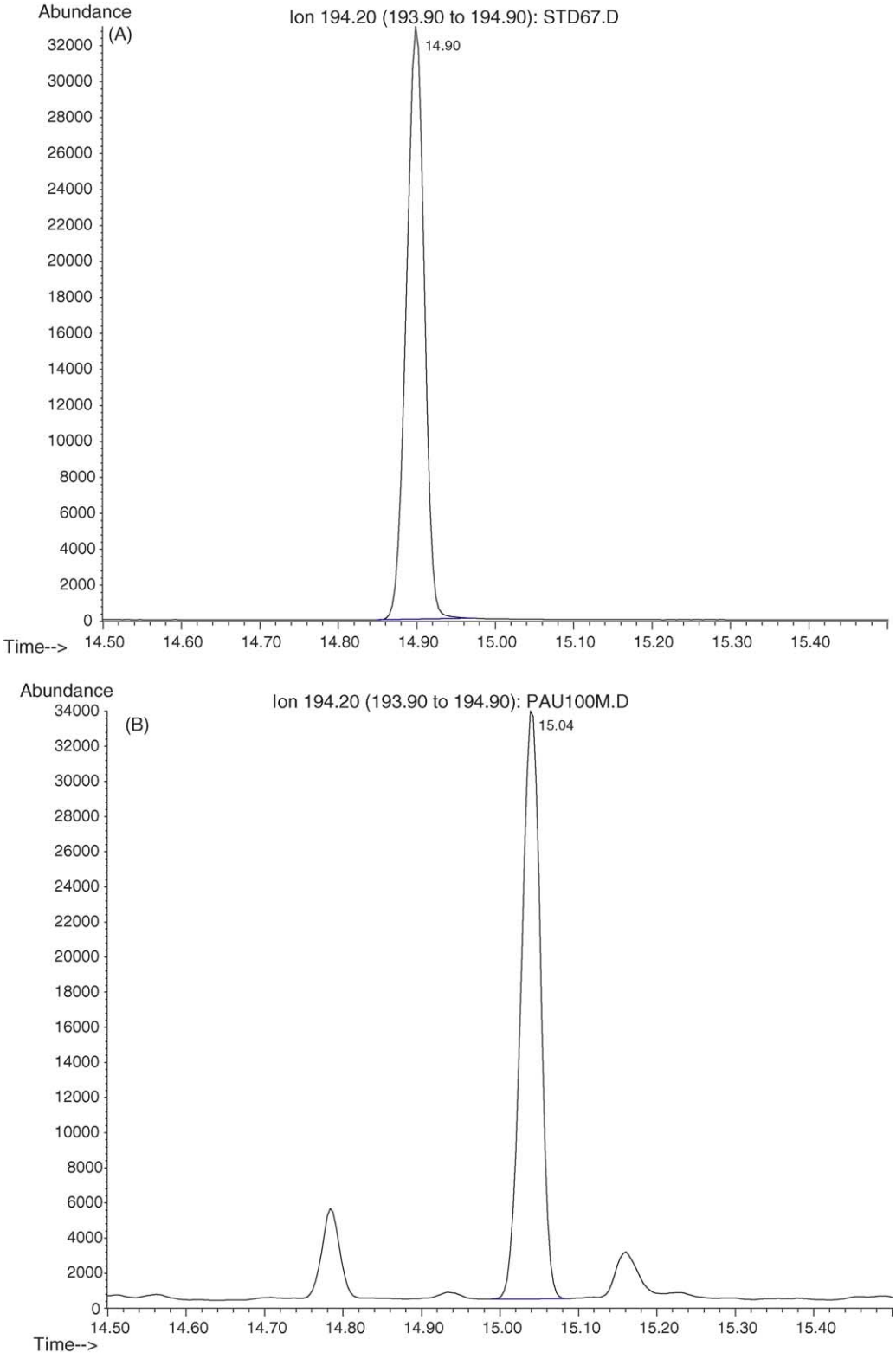

Fig. 3. Further purification of the sample by extraction with *n*-hexane brings PMA-Me peak closer to expected retention time. Chromatograms of ion *m*/*z* 194. (A) Standard PMA solution in methanol, nominal concentration of 67 μg/l, dried and derivatized. (B) Blank urine added with 100 μg/l of PMA, extracted with ethyl acetate, and derivatized. (C) Sample of chromatogram B further purified with *n*-hexane prior to injection.

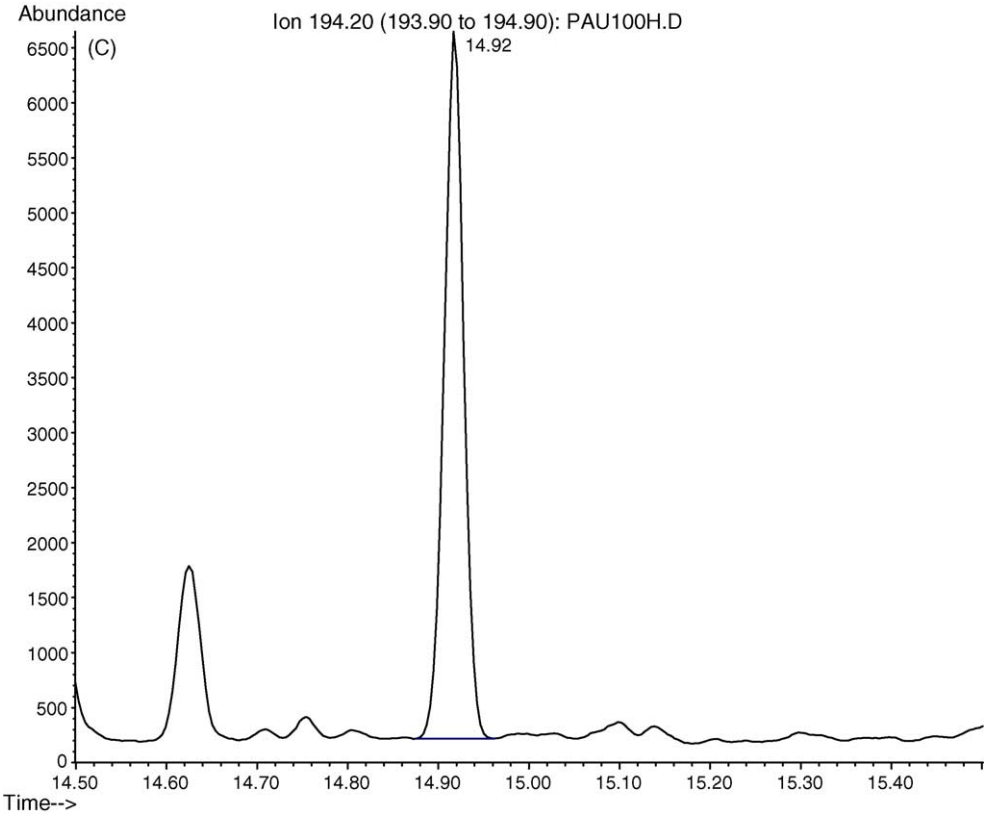

Fig. 3. (*Continued*).

with retention time shifted of about 8 s as compared with the standard.

Additionally, if "dirt" coextracted by LLE with PMA and internal standard was responsible for the retention time shift, then dilution would have had some effect on the retention time shift. This was verified, and Fig. 4 shows chromatograms obtained after derivatizing 67 μg/l of PMA (Fig. 4, chromatogram A), 100 μg/l in urine (Fig. 4, chromatogram C), and a chromatogram of the same spiked urine sample in which 20 μl of standard PMA-Me were added just before injecting it into the GC–MS system (Fig. 4, chromatogram B). The retention time shift due to alleged matrix "dirt" is slightly affected by dilution because of the standard addition.

Systematic full-scan mass spectrometric examination of urine extracts suggested that "dirt" responsible for PMA-Me delay was mainly represented by one overloading peak eluting at about 12 min (Fig. 5). The unknown peak was identified by its mass spectrum as hippuric acid methyl ester (HA-Me), and was demonstrated as follows to be responsible for PMA-Me retention time shift.

Experiments on standard solutions of pure HA in methanol, and of pure PMA and HA in methanol, evidenced that HA-Me could determine a retention time shift on PMA-Me when HA was added to PMA standard methanol solutions at nominal concentrations ranging from 100 to 1000 mg/l (Fig. 6). At concentrations of HA lower than 100 mg/l no delay in the retention of PMA-Me was evident. When HA was present at levels higher than 1000 mg/l only slight erratic differences in the retention time of PMA-Me occurred. It was thus concluded that HA-Me originated from the derivatization reaction on urine extracts in which HA is present. Therefore, the extraction with ethyl acetate afforded HA and PMA, which were converted to methyl esters under the same conditions.

Solvent trapping, early described by Grob Jr. [15,16], was excluded testing both the solvent and the mode used for injecting the samples into the GC. Toluene, dichloromethane and *n*-hexane were tested as injection solvents and all led to the observation of analogous differences between the retention time of PMA-Me in standard solution and in urine extracts. Split, pulsed-split, splitless injection modes showed again no differences as compared to the pulsed-splitless mode adopted in all experiments presented in this paper. The second effect taken into consideration was that named phase soaking [15,17,18]. According to previous work this effect occurs in the presence of abundant components able to modify temporarily the column stationary phase. As shown in Fig. 5 (chromatograms B and C), this could be the case of the present study; the peaks eluting shortly before (e.g. citric acid trimethyl ester) the large peak of HA-Me have retention times almost not altered. Late-eluting peaks (such as di-isoctylphtalate) appear as well with unmodified retention time, witnessing that their elution is not dependent on that of HA-Me. Further evidences of this were accumulated by

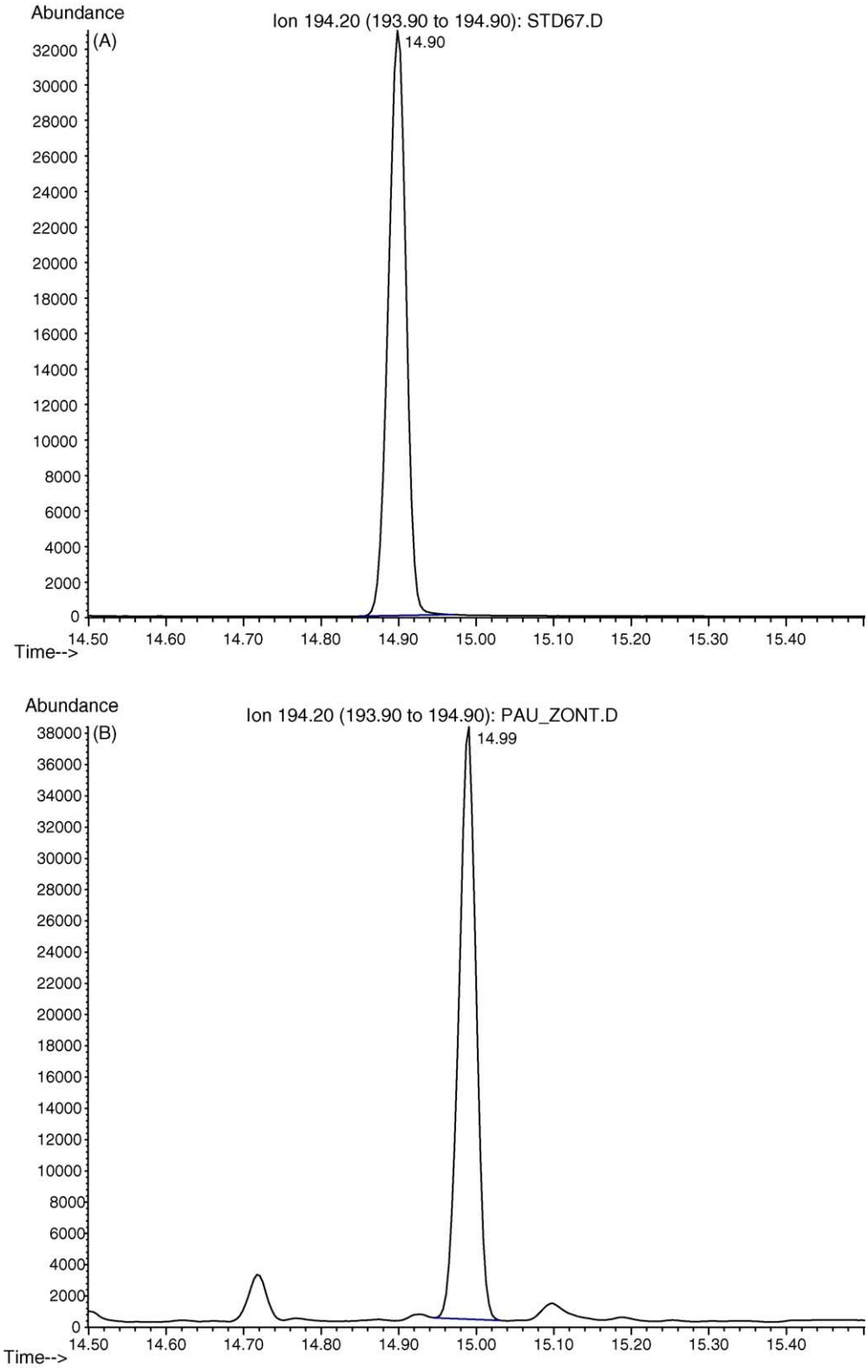

Fig. 4. Dilution affects *S*-phenylmercapturic acid methyl ester (PMA-Me) retention time. Chromatograms of ion *m*/*z* 194. (A) Standard PMA solution in methanol, nominal concentration of 67 μg/l, dried and derivatized. (B) Standard addition of 20 μl of derivatized standard (A) to 100 μl of urinary extract (C) prior to injection into the GC–MS. (C) Urine added with 100 μg/l of PMA extracted with ethyl acetate and derivatized.

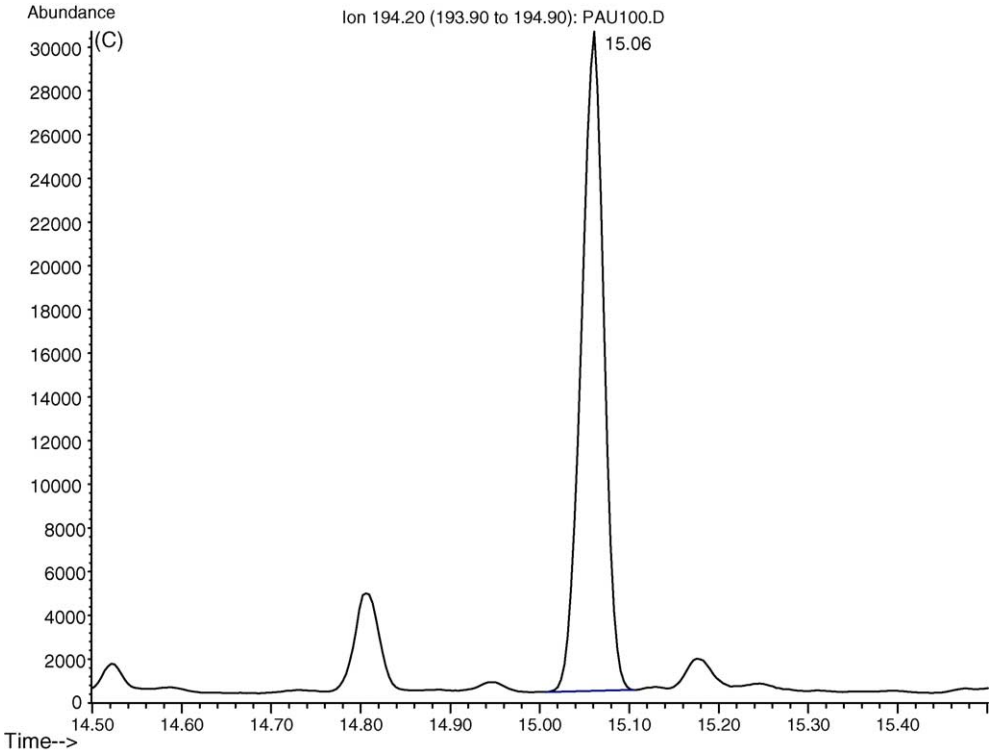

Fig. 4. (*Continued*).

injecting long chain hydrocarbons (60 μg/ml *n*-hexadecane eluting at 10.770 min, and 60 μg/ml *n*-octacosane eluting at 21.045 min). These molecules in either urine or standard solution showed retention times and peak shapes unaffected by the presence of HA-Me in any concentration.

4. Discussion

Hyppuric acid is the major urinary metabolite of toluene, a VOC that accompanies benzene in almost all sources of exposure either on workplace or due to lifestyle (viz smoking, living in urban areas) [1]. The current ACGIH BEI value for urinary HA is 1.6 g/g creatinine in post-shift urine of toluene-exposed workers. However, HA has been demonstrated to be a poor biomarker of exposure to toluene because it is obfuscated by background sources and by wide interindividual variability [21,22]. Actually, HA is also present at remarkable levels (ranging from 10 mg/l to about 2000 mg/l) in urine of people non-exposed to toluene because it is originated by the metabolism of common nutrients existing in all diets [23].

In our study, the procedure of liquid–liquid extraction with ethyl acetate of acidified urine described earlier in validated methods [2,12,13] coextracted large amounts of HA together with PMA. The subsequent derivatization modified equally both molecules owing to their analogous structure, reactivity, and reaction environment, and afforded HA-Me together with PMA-Me.

After injection into the GC–MS system of the derivatized extract, HA-Me delayed the gaschromatographic retention of PMA-Me most likely by migrating with PMA-Me for a certain distance in the column. Evidences of this were that early- and late-eluting components (such as citric acid trimethyl ester and di-isoctylphtalate, respectively) were unaffected by the presence of HA-Me (Fig. 5). In addition, molecules not similar to HA-Me like long-chain aliphatic hydrocarbons had retention times perfectly repeatable in either standard solutions or in spiked urines.

The retardation effect here described is not completely new to expert gaschromatographers. It has been thoroughly studied since the early years of capillary GC but was referred primarily to solvents and modes of injection [15–20]. The occurrence of such effect due to an endogenous metabolite such as HA can cause difficulties in the interpretation of GC–MS SIM data of PMA-Me in urine by shifting to an unpredictable extent (which can range from a few seconds to more than ten seconds) its retention time towards higher retention times. Quantitative analysis based on calibration curves prepared using urinary samples of non-exposed volunteers can also suffer of the presented effect if the amount of HA occurring in the urinary calibrators is much different from that in the samples of benzene-exposed workers. Therefore, it appears that the identification of PMA-Me in urine using GC–MS can not merely rely on GC retention time alone but rather on the mass spectral data acquired with the use of isotope-labeled internal standard.

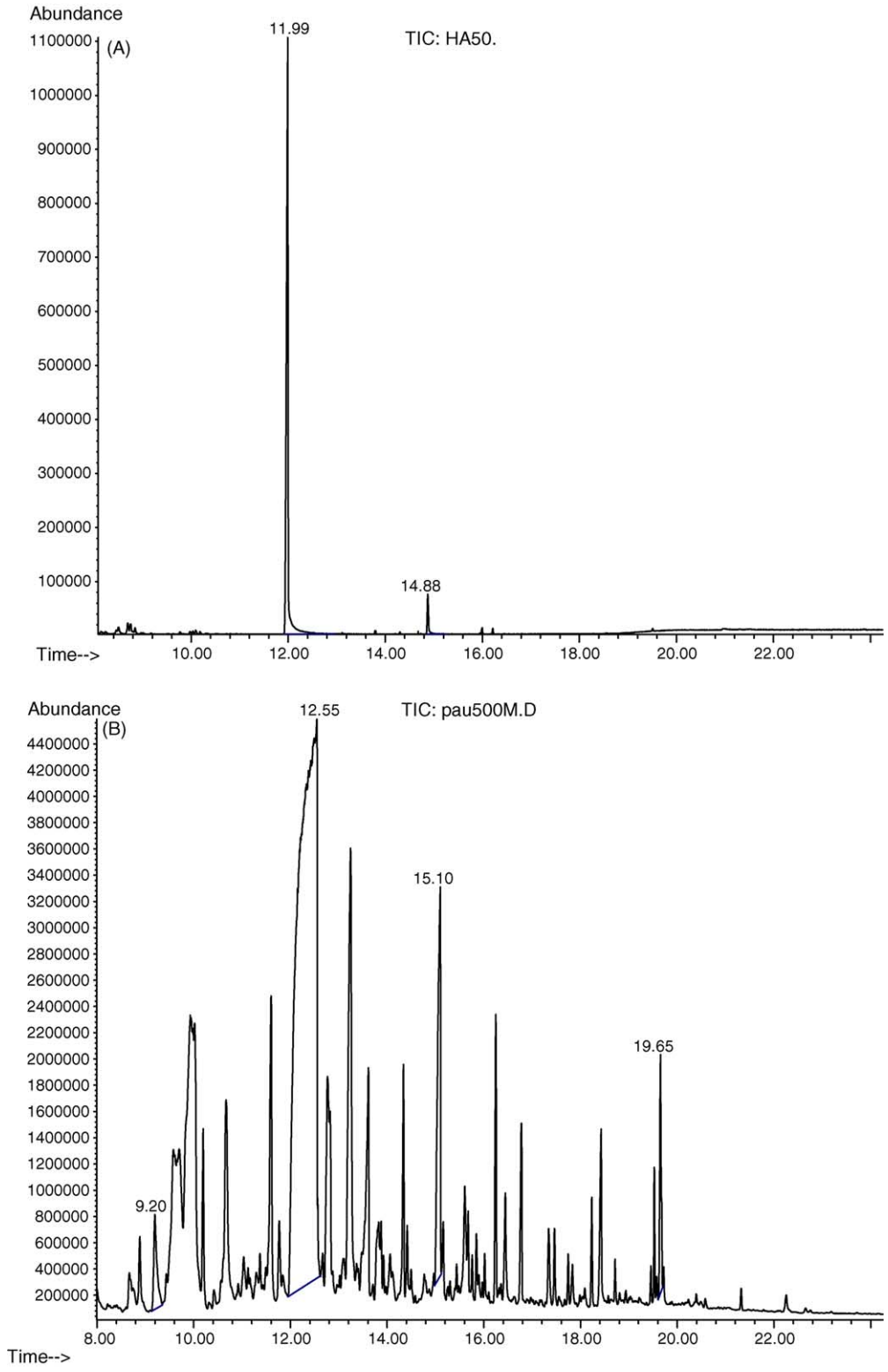

Fig. 5. The retardation effect of HA-Me is specific to PMA-Me, it does not affect neither early-eluting nor late-eluting esters. The effect of sample purification appears by comparing chromatogram (A) with chromatograms (B) and (C). (A) Standard solution of 50 mg/l hippuric acid and 20 μg/l *S*-phenylmercapturic acid in methanol, dried and derivatized (experimental conditions are given in the text). Peak identification is hippuric acid methyl ester (HA-Me) at 11.99 min, and *S*-phenylmercapturic acid methyl ester (PMA-Me) at 14.88 min. (B) Blank urine added with 500 μg/l PMA, extracted in ethyl acetate and derivatized. Peak identification is citric acid trimethyl ester at 9.20 min, HA-Me at 12.55 min, PMA-Me at 15.10 min, and di-isoctyl-phtalate at 19.65 min. (C) Sample of chromatogram B, further purified by extraction in n-hexane prior to injection. Peak identification is citric acid trimethyl ester at 9.25 min, HA-Me at 12.27 min, PMA-Me at 14.97 min, and di-isoctyl-phtalate at 19.66 min.

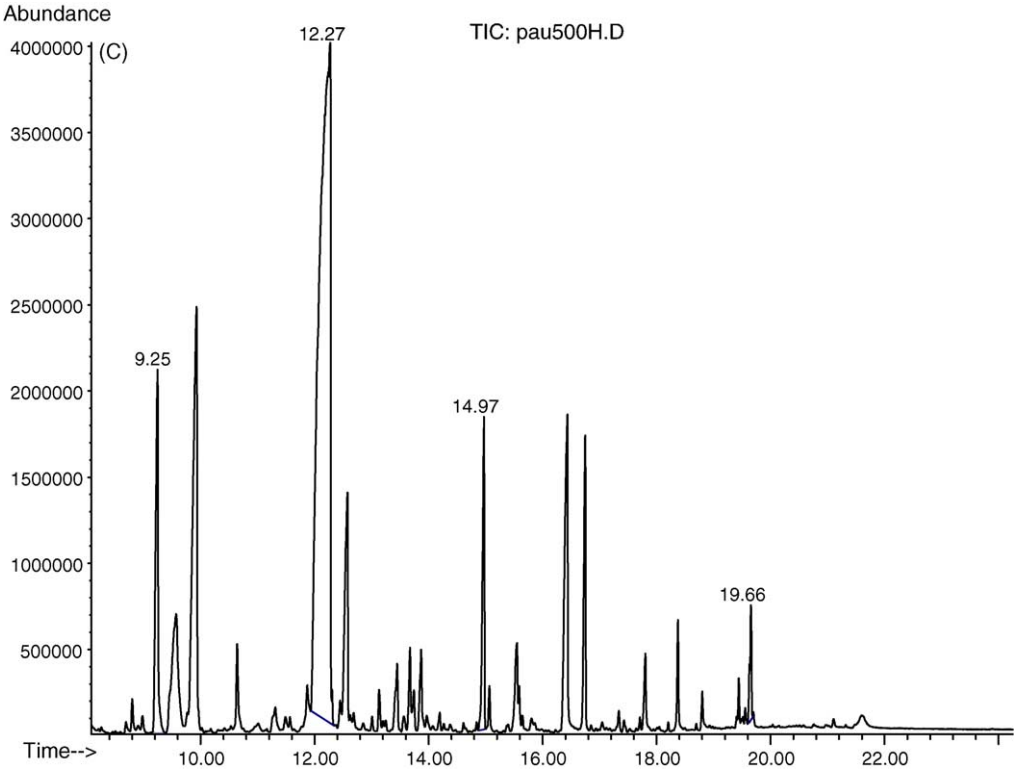

Fig. 5. (*Continued*).

PMA and HA are important biomarkers of exposure to benzene and toluene, respectively. Their biomonitoring was adopted for years in occupational health surveillance and risk assessment. Actually, PMA is hardly measurable in the urine of non-exposed workers and can reach levels of about 25 μg/l exceptionally when prolonged exposure to levels of benzene as low as 0.5 ppm (or about 1.5 mg/m^3) occur [2]. HA, on the contrary, is normally found in human urine at highly variable background levels ranging from 10 to 2000 mg/l. When toluene is absorbed, HA can exceed levels of 2000 mg/l [23]. In this study, it was demonstrated that the concentration at which HA influences PMA gaschromatography is about

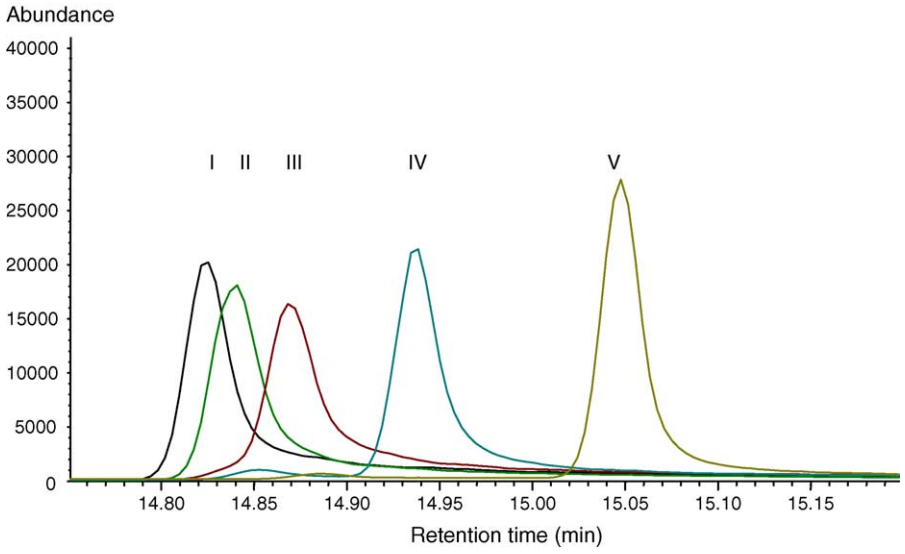

Fig. 6. Chromatograms of selected ion *m*/*z* 194. Retention time shift of *S*-phenylmercapturic acid methyl ester (PMA-Me) reproduced in standard solutions by the addition of hippuric acid (HA). Peaks I to V correspond to standard solutions of PMA at nominal concentration of 10 μg/l derivatized as described in the text. Nominal concentrations of HA were: I, no HA added, II 100, III 250, IV 500, and V 1000 mg/l HA.

250 mg/l (Fig. 6), and thus the described retardation effect is likely to occur in most authentic urine samples.

In conclusion, methods for biomonitoring PMA in urine must meet the requirement to be primarily sensitive in order to be able to determine concentrations on average well below 25 μg/l. However, given the scope of PMA determination (viz biomonitoring and risk assessment) selectivity, and accuracy are also important. To overcome the presented retention time mismatch between standard solutions, spiked blank urine samples, and authentic urines of exposed workers, it is advisable to apply a careful sample preparation (involving clean-up and derivatization) but, more importantly, the use of one isotope-labeled internal standard. Such observation should provide a precaution to all those who interpret GC–MS data derived from urines of workers exposed to benzene and toluene.

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