



HAL
open science

Simultaneous determination of aromatic acid metabolites of styrene and styrene-oxide in rat urine by gas chromatography - flame ionization detection.

Frédéric Cosnier, Hervé Nunge, Benoît Cossec, Laurent Gaté

► To cite this version:

Frédéric Cosnier, Hervé Nunge, Benoît Cossec, Laurent Gaté. Simultaneous determination of aromatic acid metabolites of styrene and styrene-oxide in rat urine by gas chromatography - flame ionization detection.. *Journal of Analytical Toxicology*, 2012, 36 (5), pp.312-8. 10.1093/jat/bks015. hal-00743297

HAL Id: hal-00743297

<https://hal.science/hal-00743297>

Submitted on 5 Apr 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Simultaneous determination of aromatic acid metabolites of styrene and styrene-oxide in rat urine by gas chromatography - flame ionisation detection

Frédéric COSNIER, Hervé NUNGE, Benoît COSSEC, Laurent GATE

Institut National de Recherche et de Sécurité (INRS)

Rue du Morvan CS 60027

Vandoeuvre, 54519 cedex, France

Tel: 33 3 83 50 20 32

Fax: 33 3 83 50 20 96

frederic.cosnier@inrs.fr

Abstract

A convenient and reliable gas chromatographic method was developed for the simultaneous determination of six aromatic acid metabolites of styrene and styrene-oxide in rat urine: i.e. benzoic (BA), phenylacetic (PAA), mandelic (MA), phenylglyoxylic (PGA), hippuric (HA) and phenylaceturic (PAUA) acids. The method involves a one-pot esterification-extraction procedure, performed directly on urine without prior treatment. Analyses were performed on a RTX-1701 capillary column and the recovered isopropyl esters derivatives were detected by flame ionization detection. The analytical method was validated for: selectivity, linearity, detection and quantification limits, recovery, intra and inter-day precisions. Calibration curves showed linearity in the range of 8 – 800 mg/L except for HA and PAUA (40 – 800 mg/L). Limits of detection were between 0.2 (PPA) and 7.0 (PAUA) mg/L. The intra-day precisions determined at three concentrations levels were less than 5% and 9% for BA, PAA, MA, PGA, on one hand, and HA and PAUA on the other hand respectively. The corresponding mean inter-day precisions for these two groups were 8% and 16% respectively. The method was successfully applied to quantitatively analyze styrene, styrene-oxide, ethylbenzene and toluene metabolites in urines samples from rats exposed by inhalation to these compounds at levels close to the occupational threshold limit values. Provided that this method can be transposed to human urine, it could have applications as part of biological monitoring for workers exposed to styrene or related compounds.

Keywords: Styrene; styrene-oxide; ethylbenzene; aromatic acid metabolites; GC-FID

Introduction

Styrene is an important industrial chemical used in the synthesis and manufacture of polystyrene and many copolymers, as well as numerous other industrial resins (1). Styrene is possibly carcinogenic to humans (2). The mechanisms for the carcinogenicity of styrene are currently incompletely known, but may include both genotoxic and epigenetic elements. Most of the genotoxic effects associated with exposure to styrene are thought to be caused by its major metabolite, styrene-7,8-oxide (SO). This compound is anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in multiple animal species at multiple tissue sites (3). SO itself is used as a chemical intermediate in the production of styrene glycol and its derivatives, in the production of cosmetics, surface coatings, and agricultural and biological chemicals. It is also used as a reactive diluent for epoxy resins and in cross-linked polyesters and polyurethanes (3). Despite its many uses, occupational exposure to SO is primarily linked to styrene oxidation in air (4).

The metabolism of styrene has been extensively studied. Briefly, as shown in figure 1, styrene is mainly metabolized by cytochrome P450 to form SO. SO is transformed by epoxide hydrolase into styrene glycol. This compound is then metabolized into mandelic acid (MA) and further to phenylglyoxylic acid (PGA). In animals, MA is subsequently converted into benzoic acid (BA) and hippuric acid (HA) after conjugation of BA with glycine (5).

Several minor metabolic pathways have also been described (1): conjugation of SO with glutathione to ultimately form phenylhydroxyethylmercapturic acids; ring oxidation resulting in the production of vinylphenols and arylmercapturic acids (6); cytochrome P450 conversion of styrene to 2-phenylethanol; formation of phenylacetaldehyde from 2-phenylethanol or SO. This latter pathway leads to the formation of phenylacetic acid (PAA) and phenaceturic acid (PAUA) once conjugated with glycine.

As part of a larger study on comparative genotoxicity of styrene and styrene oxide, we were interested by the measurement of some of the styrene (and SO) metabolites in urine of rats exposed by inhalation to the corresponding vapors. We chose to measure the following metabolites: MA, PGA, BA, HA, PPA and PAUA. All these acids may also be metabolites of ethylbenzene. BA and HA can be found in the urines of toluene-exposed rats.

Many HPLC methods allowing the simultaneous determination of MA, PGA and HA (and methylhippuric acids (mHAs)) concentrations in urine have been described (7-12). In contrast, few studies describe the determination of BA, PPA and PAUA concentrations (13,

14). The GC methods developed to determine MA, PGA, HA and mHAs systematically involve a derivation step. This may be silylation, using a mixture of hexamethyldisilazane and trimethylchlorosilane (15) or N-methyl-N-trimethylsilyl-trifluoroacetamide (16); or esterification using methanol and hydrochloric acid (17) or trimethyloxonium tetrafluoroborate as a methylating agent (18). With these approaches, mass spectrometry detection is often used rather than flame ionization detection (FID), and the derivation steps are generally preceded by solvent extraction of the urine and evaporation of the extract to dryness. These approaches are both time consuming and complex; we therefore sought to develop a simpler, more rapid method to detect the metabolites in rat urine without the need for an extraction step.

We therefore adapted an analytical method previously developed to measure urinary levels of mandelic acid enantiomers (19, 20) for the determination of MA, PGA, BA, HA, PAA & PAUA in rat urine. This method involved esterification of the six acids using 2-propanol, which could be performed without requiring extraction of compounds from the matrix. The analytical method was validated and then applied for quantitative analysis of styrene, styrene-oxide, ethylbenzene and toluene metabolites in urine samples from rats exposed to these compounds by inhalation.

Experimental

Reagents

Benzoic acid (BA) (>99.5%), hippuric acid (HA) (98%), phenylacetic acid (PAA) (99%), phenylglyoxylic acid (PGA) (>98%), *DL*-3-phenyllactic acid (PhLA) (>97%), phenylacetyl chloride (98%) and hydrogen chloride - 2-propanol solution (1.25 M) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). *DL*-Mandelic acid (MA) (99%), glycine (>99%), anhydrous sodium bicarbonate (>99.8%) and toluene (>99.5%) were obtained from VWR (Fontenay sous Bois, France).

Phenaceturic acid (PAUA) was synthesized using a procedure similar to that used for the production of commercial hippuric acid, i.e. glycine acylation by phenylacetyl chloride (21).

The seven isopropylester derivatives (iPrEDs) of the six acids to be determined and the selected internal standard (I.S. = *DL*-3-phenyllactic acid = PhLA) were synthesized separately in the laboratory and characterized before use.

Preparation of standard solutions

A mixed solution of carboxylic acids (BA, PAA, MA, PGA, HA and PAUA) was prepared in water, with each component at 0.8 g/L i.e., 6.55, 5.88, 5.26, 5.33, 4.47 and 4.14 mM, respectively. An aqueous solution of I.S. (2.0 g/L, i.e. 12.04 mM) was used as an internal standard.

A multi-compound stock solution of esters was also prepared in toluene with each of the iPrEDs at a concentration of 1 g/L. This corresponds approximately to the same level as the previous 0.8 g/L stock solution of acids. Concentrations for this stock solution were thus 6.09, 5.61, 5.15, 5.20, 4.52 and 4.25 mM for BAiPr, PAAiPr, MAiPr, PGaiPr, HAIPr and PAUAIPr, respectively. A stock solution of PhLAIPr (isopropyl ester derivative of I.S.) at 2.5 g/L (12.01 mM) was also prepared in toluene.

All these stock solutions were stored at 4 °C before use.

Sample preparation

Esterification was performed by adding 10 µL of I.S. solution and 500 µL of HCl 1.25 M in 2-propanol to an aliquot of 100 µL urine. The reaction was carried out for 2 h at 100 °C in hermetically closed 10-mL screw-cap glass vials. After cooling to room temperature, 1000 µL of an aqueous solution of NaHCO₃ (0.7 M) and 500 µL of toluene were added to neutralize remaining reagents and extract reaction products. In these conditions, the quaternary mixture (NaHCO₃, water, 2-propanol and toluene) gives two single-phase regions and most of the 2-propanol is recovered in the organic toluene phase (22). The sample was then ready for GC-FID analysis.

GC Conditions

The development and validation of the GC analyses were carried out using a Shimadzu GC-2010 Plus system equipped with a PTV injector, a flame ionization detector and an AOC20i autosampler. GC Solution software (version 2.32) was used for data acquisition and to control the GC system. Samples were separated on a RTX-1701 (with Integra-Guard) capillary column (30 m x 0.25 mm, film thickness 0.25 µm) (Restek, France) with helium as the carrier gas at a constant linear velocity of 40 cm/s. The sample (1 µL) was injected in splitless mode, the split delay and split ratio were set at 0.5 min and 1/50, respectively. The oven temperature was held at 60 °C for 0.5 min, then increased to 160 °C at a rate of 25

°C/min, and further increased to 260 °C at 15 °C/min and held at 260 °C for 2.5 min (total time: 13.67 min). The injector and detector temperatures were set at 240 °C and 260 °C, respectively.

Calibration curves, limits of detection and quantification

For each compound, a calibration curve was established using seven concentrations: 0.8, 0.56, 0.32, 0.16, 0.08, 0.04 and 0.008 g/L, prepared by serial dilution of the stock solution in water. Five replicates were performed at each experimental level and all samples were analyzed in duplicate. Calibration curves were obtained by plotting the target compound/I.S. peak-area ratios as a function of the concentrations of the individual compounds (in g/L). Least-squares linear regression analysis was used to estimate the slopes and intercepts for each calibration curve. The linearity of each calibration function was checked by estimating the error sum of squares and the Fisher ratio (23).

The limits of detection (LOD) and quantification (LOQ) were calculated from the calibration curves, and defined as the concentrations for which the corresponding peak-area ratios would be equal to 3 (LOD) and 10 (LOQ) times the standard deviations ($n = 5$) of the peak-area ratios for the lowest standards.

Recovery studies

The overall procedure recovery reflects the efficiency of both the acid derivatization step and the extraction of the resulting esters.

Global recovery yields were determined at three different concentrations (0.008, 0.08 and 0.8 g/L) by adding known amounts of BA, PAA, MA, PGA, HA and PAUA to urine from a non-exposed rat. Spiked urines were then treated according to the sample preparation procedure described above.

The extraction efficiency for the iPrEDs, was also determined at three different concentrations (0.01, 0.1 and 1 g/L) prepared by serial dilution in toluene. Known amounts of BAiPr, PAAiPr, MAiPr, PGAiPr, HAIiPr and PAUAIiPr were added to a urine sample from a non-exposed rat, or to water. For these experiments, an adjusted sample preparation procedure was used, where 10 μ L water, 100 μ L of the esters solution in toluene, 10 μ L PhLAIiPr in toluene (2.5 g/L), 500 μ L of HCl (1.25 M) - 2-propanol solution, 390 μ L toluene and 1000 μ L of aqueous solution of NaHCO₃ (0.7 M) were added to 100 μ L blank urine (or water). For this experiment, the oven program temperature of the gas chromatograph was slightly different

from the standard program, with the oven temperature held at 60 °C for 0.5 min, increased to 160°C at a rate of 25 °C/min, further increased to 200°C at 10 °C/min, and finally increased to 260 °C at 15 °C/min and held at 260 °C for 2.5 min (total time: 15 min). This did not affect the quality of results.

The recovery yields (procedure recovery and ester extraction efficiency) were calculated for five replicates by concentration level by comparing the amounts of analytes extracted from urine samples with those measured for the corresponding compounds dissolved in toluene.

Assay precision

Quality control (QC) samples were prepared as described above using 0.08, 0.32 and 0.56 g/L solutions. Intra-day precision was assessed by assaying five times each QC sample on the same day. Inter-day precision was determined over three different days. The relative standard deviation (%RSD) was used as an indicator of precision.

Assay of aromatic acids in rat urine after exposure by inhalation

The compounds of interest were measured using the validated method in various urine rat samples collected during independent inhalation experiments carried out in our laboratory for the toxicological assessment of various aromatic compounds (ethylbenzene, styrene, styrene oxide and toluene).

All experiments involving animals were conducted in accordance with the rules provided by the European Union (Directive 2010/63/EU) (24) and were performed in a French Ministry of Agriculture-accredited animal facility.

Before exposure, male Fisher 344 or Sprague-Dawley rats from Charles Rivers Laboratories (L'Arbresle, France) were acclimatized to the experimental laboratory for at least five days with a 12:12 h night/day cycle, and had free access to food and water. Temperature was maintained at 22 ± 2 °C, with a relative humidity of 55 ± 10 %.

Groups of six to eight rats were exposed to ethylbenzene, styrene, styrene oxide or toluene vapors at different concentrations (see Table 1) for 6 hours per day over a period ranging from 1 day to 28 days. Unexposed rats were only exposed to clean air. Details of how pollutants were generated and concentrations controlled were reported elsewhere (25).

Following exposure (9:00 am– 3:00 pm), animals were placed in individual metabolic-type stainless steel cages with free access to food and water from 3:00 pm to 9:00 am the following day (days of urine collection are specified in Table 1). During this 18h-period, their urine was

collected and refrigerated by a cooling system attached to the collection tubes. Urinary samples were frozen immediately after collection and stored at -20 °C . They were thawed just before analysis.

Urinary creatinine concentrations were determined by the Jaffé method using a creatinine test kit (Randox Laboratories, France).

All samples were analyzed in duplicate and a QC sample was added to each series of three samples.

Results and Discussion

Method Validation

Selectivity was evaluated by comparing the chromatograms acquired for extracted blank urine samples (controls from table 1) with those for urine spiked with the iPrED mix solution. Typical chromatograms for the 0.1 g/L iPrED mix solution in toluene show seven peaks corresponding to the six test compounds and the internal standard (Figure 2a). Similar chromatogram profiles were obtained for samples extracted from non-exposed rat urine spiked with 0.1 and 0.001 g/L iPrED mix solution (Figures 2b and 2c). Under the indicated conditions, no interfering peak related to the presence of endogenous matrix components, or to residual reagents hampered detection of the seven iPrED. The two small additional peaks right after the internal standard peak are related to impurities present in the toluene used for the preparation of the mix solution of esters and extraction.

Since BA, PAA, HA and PAUA can be produced endogenously at relatively high concentrations (> 0.2 and 1.0 g/L for PAUA and HA respectively), the calibration curves cannot be prepared in a truly analyte-free urine matrix. The standard addition method was also discarded because it would have required significant extension of the linearity range (nearly doubled for HA). The calibration curves were therefore established by plotting the ratio of peak area for each analyte relative to the peak area for the internal standard vs. the nominal concentrations (g/L) of the calibration standards in water. The method was shown to be linear for BA, PAA, MA and PGA over the full range of concentrations investigated (0.008 – 0.8 g/L), the lower limit of linearity was raised to 0.04 g/L for HA and PAUA. It should be noted that our method is among the few with such a wide linearity range.

The equations for the linear regression curves for BA, PAA, MA, PGA, HA and PAUA were $y = (3.910 \pm 0.014)x - (0.006 \pm 0.003)$, ($r^2 = 0.999$); $y = (10.520 \pm 0.041)x + (0.031 \pm$

0.019), ($r^2 = 0.999$); $y = (6.073 \pm 0.025)x - (0.031 \pm 0.009)$, ($r^2 = 0.999$); $y = (5.390 \pm 0.025)x + (0.010 \pm 0.008)$, ($r^2 = 0.999$); $y = (2.419 \pm 0.017)x + (0.034 \pm 0.012)$, ($r^2 = 0.996$) and $y = (1,768 \pm 0.022)x - (0.028 \pm 0.008)$ ($r^2 = 0.992$, respectively. Slopes and intercepts are given \pm standard error.

The LODs and LOQs for each compound were determined based on these calibration curves (Table 2). These limits are quite comparable to other works, only two publications using GC-MS techniques show lower limits (17, 18) but in these articles, BA, PAA and PAUA are not considered. HA, PAUA, and, to a lesser extent, BA have higher LODs and LOQs than the three other aromatic acids. For HA and PAUA, this is due to their iPrEDs having a lower extraction yield in toluene ($< 60\%$, see Table 3). For BA, the extraction yield of BAiPr is quite high ($> 90\%$ in water), but the esterification yield is low, only allowing about 50% total recovery. This low esterification yield for BA was also observed during BAiPr synthesis.

Extraction efficiency for the all iPrEDs was determined for samples dissolved in water and urine at three concentrations (only two for HAIPr and PAUAIPr). The efficiency of extraction from water or urine was not affected by concentration for any compound. Similarly, overall procedure recoveries are not concentration dependent. Mean (\pm SD) values are presented in Table 3. On average, yields for BAiPr, PAAiPr, MAiPr and PGAIPr were slightly (around 9%) lower for samples dissolved in urine than those dissolved in water. No significant differences were observed for HAIPr, PAUAIPr and PhLAIPr. Finally, the mean (\pm SD) recovery of the internal standard I.S. from urine, calculated from the sample analysis data, was 84.0 ± 12.3 . This is not significantly different from the recovery yield for samples dissolved in water; we can thus assume that a water-based standard solution can be used for calibration and quality controls without introducing bias into the analyses.

Intra-day precision was determined by calculating the %RSD for five replicate analyses of QC samples. Intra-day precision was satisfactory, with %RSD less than 5% for BA, PAA, MA and PGA and 9% for HA and PAUA (Table 4). Concerning the inter-day precision, it is close to an average of 7.9% (± 2.9) for BA, PAA, MA and PGA or 15.5% (± 7.4) for HA and PAUA. This last value is relatively high, but remains acceptable if we consider the urinary concentrations for these two metabolites and the coefficient of variation between exposed subjects, which is around 35%.

Metabolite assay in urine from rats exposed by inhalation

The method was used to assess exposure to a range of compounds based on levels of metabolites present in urine. The content levels of the six aromatic acids were measured in the urine of rats exposed to styrene oxide, styrene, ethylbenzene or toluene vapors, and compared to levels in non-exposed animals. Typical chromatograms obtained for urine samples from exposed rats (Figure 3 a-c) can be compared with a 0.32g/L QC sample (Figure 3d). Results are summarized in Table 5. For all exposure conditions, BA concentrations were low (< 50 mg/g creatinine). However, a trend for increasing concentrations related to increasing atmospheric concentrations of aromatic compounds was observed. Although well resolved chromatographically, the urinary concentrations of PAA could not distinguish exposed rats from control animals for any compound or concentration tested. The background level detected is probably due to the animals' diet. Although less pronounced, a similar problem has been described for HA and PAUA. With these metabolites, exposure to aromatic solvents is identifiable only above a certain threshold. Thus, in the present study, the HA concentration was significantly different from that of control group for animals exposed to SO75, Styr75, Styr250, EtBzn250 and Tol150; while, for PAUA, only the Styr250 and EtBzn250 groups had measurably higher concentrations than control animals. MA and PGA are sensitive markers of exposure to styrene, styrene oxide and ethylbenzene. As expected, they were not detected in either the control or toluene groups. Excretions of these two compounds increased with the level of exposure for a given chemical. Interestingly, the relative levels of these two metabolites were different depending on whether animals were exposed to styrene oxide, styrene or ethylbenzene. After exposure to styrene oxide or styrene, PGA was the major product; whereas MA was the predominant metabolite after ethylbenzene inhalation.

Conclusion

This paper describes the development of a convenient and reliable method for the simultaneous determination of six aromatic carboxylic acids in urine. The method, which involves a one-pot esterification-extraction procedure combined with GC/FID analysis, is robust, selective and sufficiently sensitive to allow detection of these acids in rat urine after exposure by inhalation to aromatic compounds at levels close to the occupational threshold limit values. Provided that this method can be transposed to human urine, it could therefore have applications in biological monitoring, where it could be used on urine samples from workers to measure aromatic acid metabolites.

Acknowledgements

The authors gratefully acknowledge Marie-Josèphe Décret, Sylvie Michaud, Lionel Dussoul and Jean-Claude Gondrexon for their technical assistance.

References

- (1) ATSDR. Toxicological profile for styrene, 2010.
- (2) IARC. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. **82**: 437-550 (2002).
- (3) IARC. Some industrial chemicals. **60**: 321-346 (1994).
- (4) R. Tornero-Velez, S. Waidyanatha, D. Echeverria and S.M. Rappaport. Measurement of styrene-7,8-oxide and other oxidation products of styrene in air. *J. Environ. Monit.* **2**: 111-117 (2000).
- (5) S.J. Sumner and T.R. Fennell. Review of the metabolic-fate of styrene. *Critical Reviews in Toxicology* **24**: S11-S33 (1994).
- (6) I. Linhart, J. Mraz, J. Scharff, J. Krouzelka, S. Duskova, H. Nohova and L. Vodickova. New urinary metabolites formed from ring-oxidized metabolic intermediates of styrene. *Chem Res Toxicol* **23**: 251-257 (2010).
- (7) I. Sperlingova, L. Dabrowska, V. Stransky and M. Tichy. A rapid HPLC method for the determination of carboxylic acids in human urine using a monolithic column. *Anal. Bioanal. Chem.* **378**: 536-543 (2004).
- (8) I.F. Mao, M.L. Chen and E.W. Lo. Simultaneous determination of urinary metabolites of toluene, xylene, styrene and ethyl benzene by solid-phase extraction technique and high-performance liquid chromatographic photo diode array detection. *Int. J. Environ. Anal. Chem.* **64**: 1-9 (1996).
- (9) A. Astier. Simultaneous high-performance liquid-chromatography determination of urinary metabolites of benzene, nitrobenzene, toluene, xylene and styrene. *J. Chromatogr. B.* **573**: 318-322 (1992).
- (10) O. Inoue, K. Seiji, T. Suzuki, T. Watanabe, H. Nakatsuka, H. Satoh and M. Ikeda. Simultaneous determination of hippuric acid, o-, m-, and p-methylhippuric acid, phenylglyoxylic acid, and mandelic acid by HPLC. *Bull. Environ. Contam. Toxicol.* **47**: 204-210 (1991).
- (11) M. Ogata and T. Taguchi. Quantitation of urinary metabolites of toluene, xylene, styrene, ethylbenzene, benzene and phenol by automated high performance liquid chromatography. *Int. Arch. Occup. Environ. Health* **59**: 263-272 (1987).
- (12) G. Poggi, M. Giusiani, U. Palagi, P.L. Paggiaro, A.M. Loi, F. Dazzi, C. Siclari and L. Baschieri. High-performance liquid chromatography for the quantitative determination of the urinary metabolites of toluene, xylene, and styrene. *Int. Arch. Occup. Environ. Health* **50**: 25-31 (1982).
- (13) X.B. Chen, J.H. Pagella, M.L. Bakker and O. Parra. Determination of aromatic metabolites in ruminant urine by high-performance liquid chromatography. *J. Chromatogr. B.* **682**: 201-208 (1996).
- (14) M.J. Arin, M.T. Diez and J.A. Resines. Rapid and simple method for the determination of urinary benzoic and phenylacetic acids and their glycine conjugates in ruminants by reversed-phase high-performance liquid-chromatography. *J. Chromatogr. B.* **582**: 13-18 (1992).

- (15) P.B. Van Roosmalen and I. Drummond. Simultaneous determination by gas-chromatography of the major metabolites in urine of toluene, xylenes and styrene. *Br. J. Ind. Med.* **35**: 56-60 (1978).
- (16) S. Szucs, L. Toth, J. Legoza, A. Sarvary and R. Adany. Simultaneous determination of styrene, toluene, and xylene metabolites in urine by gas chromatography/mass spectrometry. *Arch. Toxicol.* **76**: 560-569 (2002).
- (17) Y. Ohashi, T. Mamiya, K. Mitani, B.L. Wang, T. Takigawa, S. Kira and H. Kataoka. Simultaneous determination of urinary hippuric acid, o-, m- and p-methylhippuric acids, mandelic acid and phenylglyoxylic acid for biomonitoring of volatile organic compounds by gas chromatography-mass spectrometry. *Anal. Chim. Acta* **566**: 167-171 (2006).
- (18) M. Pacenti, S. Dugheri, F. Villanelli, G. Bartolucci, L. Calamai, P. Boccalon, G. Arcangeli, F. Vecchione, P. Alessi, I. Kikic and V. Cupelli. Determination of organic acids in urine by solid-phase microextraction and gas chromatography-ion trap tandem mass spectrometry previous 'in sample' derivatization with trimethyloxonium tetrafluoroborate. *Biomed. Chromatogr.* **22**: 1155-1163 (2008).
- (19) S. Kezic, I. Jakasa and M. Wenker. Determination of mandelic acid enantiomers in urine by gas chromatography and electron-capture or flame ionisation detection. *J. Chromatogr. B.* **738**: 39-46 (2000).
- (20) M. Korn, R. Wodarz, W. Schoknecht, H. Weichardt and E. Bayer. Styrene metabolism in man: gas chromatographic separation of mandelic acid enantiomers in the urine of exposed persons. *Arch. Toxicol.* **55**: 59-63 (1984).
- (21) A.W. Ingersoll and S.H. Babcock. Hippuric acid. *Org. Synth. Coll* **2**: 328 (1943).
- (22) K.Y. Lee. Phase partitioning modeling of ethanol, isopropanol, and methanol with BTEX compounds in water. *Environ. Pollut.* **154**: 320-329 (2008).
- (23) P. Araujo. Key aspects of analytical method validation and linearity evaluation. *J. Chromatogr. B.* **877**: 2224-2234 (2009).
- (24) European Union. Directive 2010/63/EU on the approximation of laws. Regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes, 22 September 2010.
- (25) B. Cossec, F. Cosnier, M. Burgart, H. Nunge and S. Grossmann. Glutathione pathway in ethylbenzene metabolism: Novel biomarkers of exposure in the rat. *Chemosphere* **81**: 1334-1341 (2010).

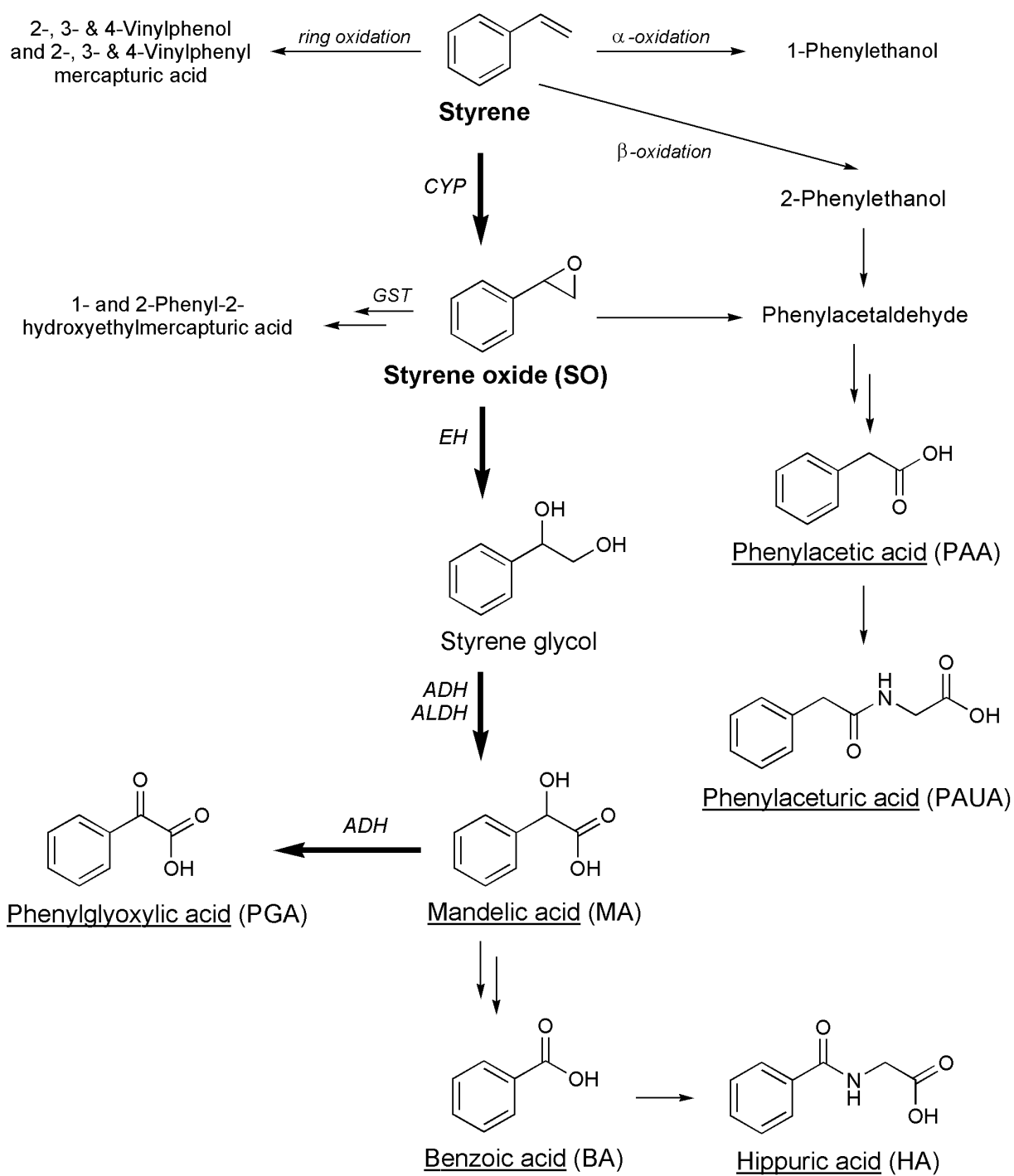


Figure 1. Styrene and styrene oxide metabolism in humans and animals

(ADH: alcohol dehydrogenase, ALDH: aldehyde dehydrogenase, EH: epoxide hydrolase, CYP: cytochrome P450, GST: glutathione-S-transferase)

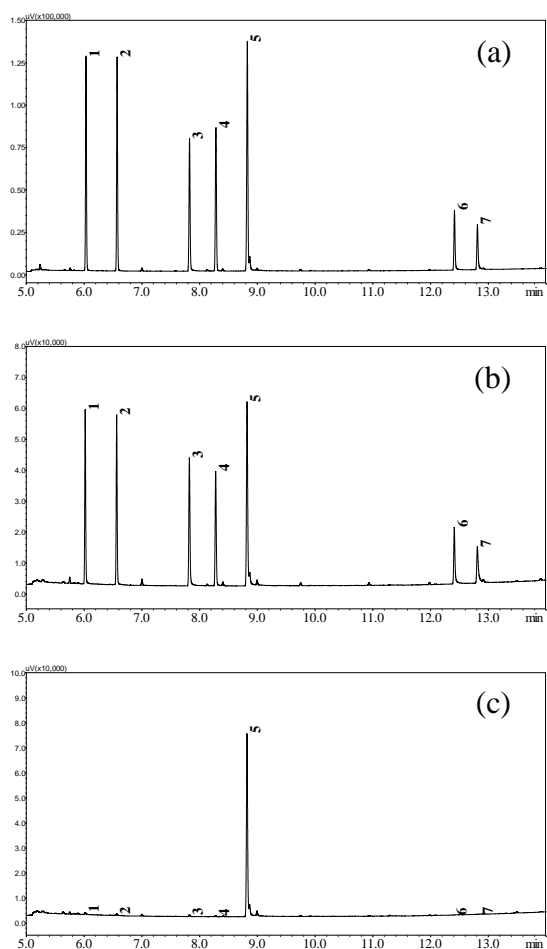


Figure 2. Chromatograms of (a) 0.1 g/L iPrED mix solution in toluene, (b) extracted 0.1 g/L iPrED spiked blank urine and (c) extracted 0.001 g/L iPrED spiked blank urine
1, BAiPr; 2, PAAiPr; 3, MAiPr; 4, PGaiPr; 5, I.S.=PhLaiPr; 6, HAiPr; 7, PAUaiPr

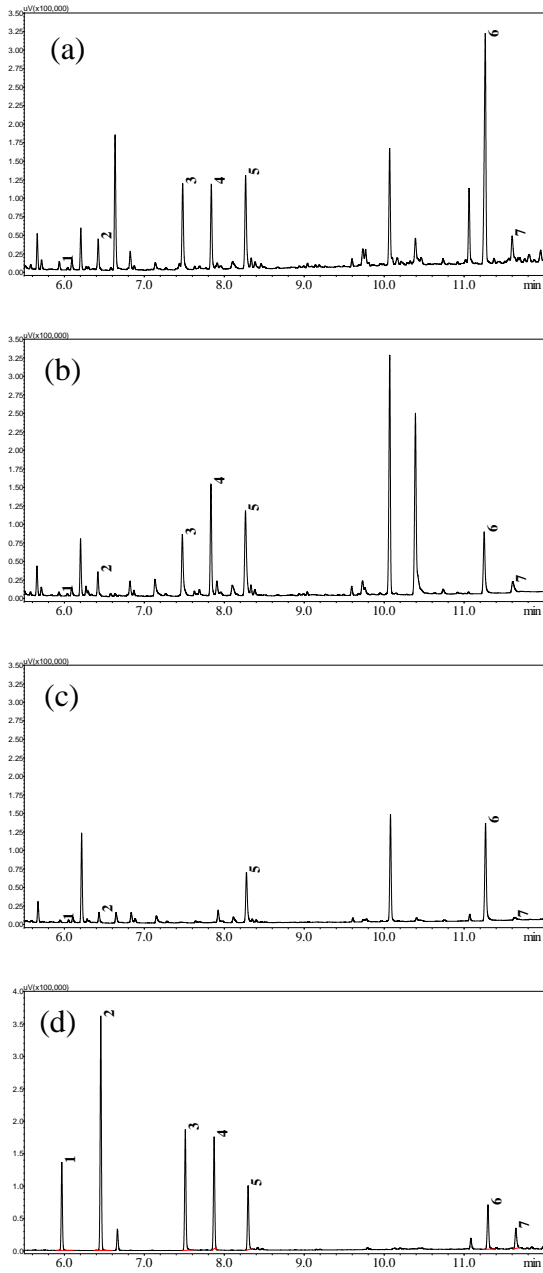


Figure 3. Typical chromatograms of (a) SO75 urine, (b) Sty75 urine, (c) Tol150 urine and (d) 0.32 g/L QC

Table 1. Exposure details

Exposure	Solvent	Atmospheric concentration (ppm)	Rat strain	Animal weight (g)	Number of animals	Day of urine collection
SO25	Styrene oxide	25	F344	150	8	4
SO75	Styrene oxide	75	F344	150	8	4
Styr75	Styrene	75	F344	150	8	4
Styr250	Styrene	250	SD	270	6	1
EtBzn30	Ethylbenzene	30	SD	270	6	1
EtBzn250	Ethylbenzene	250	SD	270	6	1
Tol50	Toluene	50	SD	320	8	5
Control	Unexposed		SD	320	8	5

F344 = Fisher344, SD = Sprague-Dawley

Table 2. Detection and quantification limits

Metabolite	LOD (mg/L)	LOQ (mg/L)
BA	2.1	6.8
PAA	0.2	0.7
MA	0.4	1.3
PGA	0.3	1.0
HA	4.6	15.4
PAUA	7.0	23.2

Table 3. Overall procedure recovery and ester extraction efficiencies in water and urine

Analyte	Recovery ^a (%)	Ester extraction efficiency ^b (%)	
		In water	In urine
BA	50.7 ± 3.2	91.5 ± 5.4	82.6 ± 7.8
PAA	88.0 ± 6.0	89.5 ± 5.3	78.3 ± 7.3
MA	85.5 ± 5.6	88.5 ± 6.3	78.9 ± 5.7
PGA	81.6 ± 5.5	78.6 ± 5.3	71.2 ± 6.7
HA	56.0 ± 14.9	56.8 ± 9.2	60.1 ± 9.4
PAUA	37.0 ± 12.1	44.1 ± 9.0	46.7 ± 9.9
PhLA	82.8 ± 10.7 ^c	81.0 ± 14.9 ^d	77.0 ± 12.9 ^d

^a Mean overall procedure recovery in water expressed as mean ± SD over the three concentrations (0.008, 0.08 and 0.8 g/L) (n = 15) except for HA and PAUA (0.08 and 0.8 g/L) (n = 10)

^b Mean ester extraction efficiency expressed as mean ± SD over the three concentrations (0.01, 0.1 and 1 g/L) (n = 15) except for HA and PAUA (0.1 and 1 g/L) (n = 10)

^c PhLA concentration = 0.2 g/L, as applied throughout the study (n = 15)

^d PhLAIr concentration = 0.25 g/L (n=15)

Table 4. Intra- and inter-day precisions of BA, PAA, MA, PGA, HA, PAUA in water

Analyte	Concentration (mg/L)	RSD (%)	
		Intra ^a	Inter ^b
BA	80	4.5	8.5
	320	4.9	8.8
	560	5.5	11.0
PAA	80	3.4	5.4
	320	5.3	13.1
	560	4.0	8.5
MA	80	4.8	5.5
	320	3.3	3.6
	560	3.8	2.9
PGA	80	4.0	8.7
	320	5.0	8.8
	560	3.7	7.4
HA	80	7.0	24.2
	320	6.7	10.9
	560	8.9	7.9
PAUA	80	7.9	22.7
	320	8.0	19.7
	560	8.6	25.7
PhLA	200	7.3 ^c	6.3

^a n = 5^b n = 3^c Calculated from peak area (n = 15)

Table 5. Urinary concentration (mg/g creatinine) of BA, PAA, MA, PGA, HA and PAUA in rat urine samples (mean \pm SD)

Exposure	BA	PAA	MA	PGA	HA	PAUA
SO25	15 \pm 5	37 \pm 12	103 \pm 18	125 \pm 37	815 \pm 353	249 \pm 88
SO75	34 \pm 10	42 \pm 12	279 \pm 92	294 \pm 118	1619 \pm 525	263 \pm 68
Styr75	31 \pm 14	40 \pm 11	256 \pm 65	419 \pm 104	1714 \pm 1072	200 \pm 38
Styr250	46 \pm 25	67 \pm 16	256 \pm 55	672 \pm 258	1366 \pm 495	485 \pm 152
EtBzn30	12 \pm 3	29 \pm 8	77 \pm 12	47 \pm 6	1015 \pm 353	273 \pm 111
EtBzn250	54 \pm 18	52 \pm 12	405 \pm 57	166 \pm 40	1563 \pm 630	414 \pm 184
Tol50	9 \pm 5	30 \pm 25	/	/	1379 \pm 267	233 \pm 87
Controls	4 \pm 3	58 \pm 18	/	/	996 \pm 426	379 \pm 175