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Determination of mandelic acid enantiomers in urine by gas chromatography and electron-capture or flame ionisation detection

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Abstract

A sensitive and stereospecific GC method was developed for the analysis of *R*- and *S*-enantiomers of mandelic acid (MA) in urine, using a chiral CP Chirasil-Dex-CB column. The enantiomers of MA were derivatised with isopropanol into their corresponding isopropyl esters and determined either directly with flame ionisation detection (FID) or after subsequent derivatisation of a hydroxy group with pentafluoropropionic anhydride with electron-capture detection (ECD). Both derivatisation steps proceeded with negligible inversion of enantiomers (<1%). The limit of detection of the FID determination was 8 and 5 mg/l for *R*-MA and *S*-MA, respectively and of the ECD determination 1 mg/l for both enantiomers. Repeatability (within-day precision) and reproducibility (day-to-day precision) was for both enantiomers below 7.5% for the FID and below 5.8% for the ECD analysis. The method was applied to urine of volunteers exposed to 105 and 420 mg styrene/m^3 air. In the urine of the exposed volunteers, the *S*-enantiomer showed higher excretion compared to that of the *R*-enantiomer, with marked interindividual differences in excretion of both enantiomers.

Keywords: Enantiomer separation; Mandelic acid

1. Introduction

Mandelic acid (MA) is a major metabolite of styrene and it is generally accepted as a biological indicator of occupational exposure to styrene [1–3]. Styrene is an organic solvent used in large quantities in industry. Adverse effects on the central nervous system have been reported to occur in situations of long-term exposure [4]. The International Agency for Research on Cancer classifies styrene as a possible human carcinogen [5]. The occupational exposure limit for styrene ranges, depending on the country, from 42 (Germany) [2] to 210 mg/m^3 (USA) [3], with a tendency of decreasing.

The microsomal cytochrome P-450 dependent liver monooxygenase, and the epoxide hydrolase transform styrene via the chiral styrene epoxide (SO) into the chiral phenylglycol, which is further metabolised to the chiral *R*- and *S*-MA and phenylglyoxilic acid (PGA) [6]. *R*- and *S*-enantiomers of SO showed different mutagenicity in the Ames test [7] and liver

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toxicity in mice [8]. In man, the ratio of enantiomers has been found to vary interindividually, leading to interindividual differences in the urinary excretion of the chiral metabolites *R*- and *S*-MA [6,9]. In consideration of the differences in biological effect between the two enantiomers of SO, the precise enantiomeric composition of MA may therefore be of toxicological importance.

The development of the presented method was part of a larger study on interindividual differences in the metabolism of styrene. In that study, volunteers were exposed for 1 h to 105 and 420 mg styrene/m^3 air. To enable a 24-h follow-up of the excretion of the MA enantiomers in urine, a sensitive gas chromatography (GC) method was needed. In the majority of the published GC methods, racemic MA is determined, based on the esterification of the carboxylic group of the MA using flame ionisation detection (FID) [10–12]. For the determination of the MA enantiomers, Korn et al. described a FID method including esterification with isopropanol [6]. To improve chromatographic separation on a Chirasil-Val column, in the case of very different amounts of the enantiomers, esterification with 3-pentanol instead of isopropanol was used [13]. Bonner at al. [14] developed a three-step derivatisation procedure involving the subsequent conversion of MA to mandelyl chloride, *N*-propylmandelamide and *O*-trimethylsilyl-*N*-propylmandelamide and GC–FID analysis on a *N*-docasanoyl-L-valine *tert*.-butylamide column. In the first instance, we developed an esterification method using FID. During development of the method, esterification with different alcohols and separation on different chiral columns was investigated. Good separation of enantiomers was achieved with isopropanol with no conversion of enantiomers during esterification. However, in some urine samples the concentrations of the enantiomers were below the detection limit. In order to improve the sensitivity, additional derivatization by *O*-acylation of the hydroxylic group with pentafluoropropionic anhydride (PFPA) was performed, enabling more sensitive electron-capture detection (ECD). In the present article we describe the analytical performance of both FID and ECD determination; depending on the concentration level in the samples the choice of the used detection method can be made.

2. Experimental

2.1. Chemicals

R- and *S*-MA (>99%) were purchased from Merk–Schuchardt, L-3-phenyllactic acid (>98%) from Aldrich (Milwaukee, WI, USA), PFPA and pyridine from Fluka (Buchs, Switzerland), isopropanol, hexane, dichloromethane (DCM) and hydrogen chloride (HCl) from Merck (Darmstadt, Germany), and methanol (dry) from J.T. Baker (Deventer, The Netherlands)

2.2. Sample preparation

2.2.1. Urine sample storage

Immediately after collection, 15-ml aliquots of urine were stored in polyethylene bottles at −18°C until analysis. Before analysis, frozen samples were conditioned at 37°C for 15 min, with frequent stirring. To prepare quality control samples (QCs), two pools of 0.5 l of urine were spiked with *R*- and *S*-MA at two concentration levels (0.14 and 0.5 g/l). From these pools, 1 ml of an aliquot was distributed to the 4-ml screw-cap glass vials and stored at −18°C until analysis.

2.2.2. Standard solutions and calibration curves

Standard solutions of *R*- and *S*-MA were prepared in water; the concentrations of the stock solution was 2 g/l. For the calibration line, five working standard solutions in urine in the range 10–600 mg/l of each enantiomer were prepared. For internal standardisation a water solution containing 8 g/l of L-3-phenyllactic acid (I.S.) was used. The concentrations of *R*- and *S*-MA in urine were calculated by internal standardisation using peak heights. All samples were analysed in duplicate. To each series of 20 samples, one QC sample was added.

2.3. Analytical procedure

2.3.1. Esterification with isopropanol (FID determination)

To an aliquot of 1 ml urine 100 μl of I.S., 300 μl water and 1.25 ml of 1 *M* HCl–isopropanol was added. The esterification was carried out for 30 min at 100°C in hermetically closed 4-ml screw-cap glass

vials. After cooling down to room temperature, the isopropyl esters of *R*- and *S*-MA were extracted with 1 ml of hexane. The sample was then ready for GC–FID analysis.

2.3.2. Derivatization with PFPA (ECD determination)

To an aliquot of 100 μl of a hexane extract containing the isopropyl esters of MA enantiomers, 500 μl of 4% pyridine in DCM and 30 μl of PFPA were added. The derivatization was carried out at 60°C for 60 min. After cooling down to room temperature, the samples were evaporated to dryness under an N_2 stream, and the residue was dissolved in 0.5 ml hexane. The sample was then ready for GC–ECD analysis.

2.4. Chromatography

2.4.1. FID analysis

GC separation was carried out using a Carlo Erba HRGC 5300 GC system equipped with a FID instrument (Interscience, The Netherlands). The column was a CP Chirasil-Dex-CB (25 m×0.25 mm I.D., 0.25 μm film thickness; Chrompack, The Netherlands). Initial column temperature was maintained isothermally at 150°C for 9 min; the column temperature was then programmed to 180°C at a rate of 30°/min, holding it for 3.5 min. Helium column carrier gas flow was 2 ml/min and nitrogen make-up gas flow-rate was 30 ml/min. The injector and detector temperature was set at 250°C. The sample (3 μl) was injected with a split injection technique (split ratio 1:15).

2.4.2. ECD analysis

The chromatography was carried out on a Hewlett-Packard 5890 GC system (Hewlett-Packard, USA) equipped with a ^{63}Ni ECD instrument. The column was a CP Chirasil-Dex-CB (25 m×0.25 mm I.D., 0.25 μm film thickness; Chrompack). Initial column temperature was maintained isothermally at 100°C for 7.5 min; the column temperature was then programmed to 130°C at a rate of 10°/min, holding it for 1 min and than to 180°C holding it for 1 min. Helium column carrier gas flow was 2 ml/min and nitrogen make-up gas flow-rate was 30 ml/min. The injector temperature was 250°C and detector temperature 300°C. The sample (1 μl) was injected with a split injection technique (split ratio 1:30).

3. Results and discussion

3.1. Esterification of carboxylic group with alcohols

Enantiomers of α-hydroxy carbonic acids, such as *R*- and *S*-MA have a strong tendency for spontaneous racemization during derivatization [14]. During the development of the method we investigated the esterification of the carboxylic group with methanol, ethanol and isopropanol and chromatography on a CP Cyclodex B 236 M (Chrompack) and a Chirasil-Dex-CB (Chrompack) column. Better separation was obtained on a Chirasil-Dex column and that column was used for further analyses. For the analysis of the isopropyl esters of MA enantiomers, Korn et al. [6] used a Chirasil-Val column, however, they could not achieve a baseline separation in the case of very different amounts of enantiomers. Using a Cyclodex column we achieved a good separation of enantiomers for methyl and isopropyl esters (Fig. 1A). As seen from the chromatograms, the methylester of *R*-MA eluted before *S*-MA in contrast to their corresponding isopropylesters which showed the opposite elution order (Fig. 1A). The esterification with methanol and isopropanol proceeded with negligible inversion (<0.1%) of enantiomers as concluded from experiments with enantiopure standards. Since isopropyl esters gave a higher yield, the method was further optimised for isopropyl esters.

Most of the esterification methods described in the literature are based on the solvent extraction of MA from the urine followed by evaporation of a solvent to dryness and subsequent esterification [6,10,11]. In the present method we performed the esterification directly in the urine without previous extraction of MA into an organic solvent. The esterification was completed at 100°C in 30 min.

3.2. Acylation of the hydroxy group with PFPA

The presence of a hydroxy group in MA offered further possibility for derivatisation. Acylation with PFPA enabled a more sensitive detection with ECD.

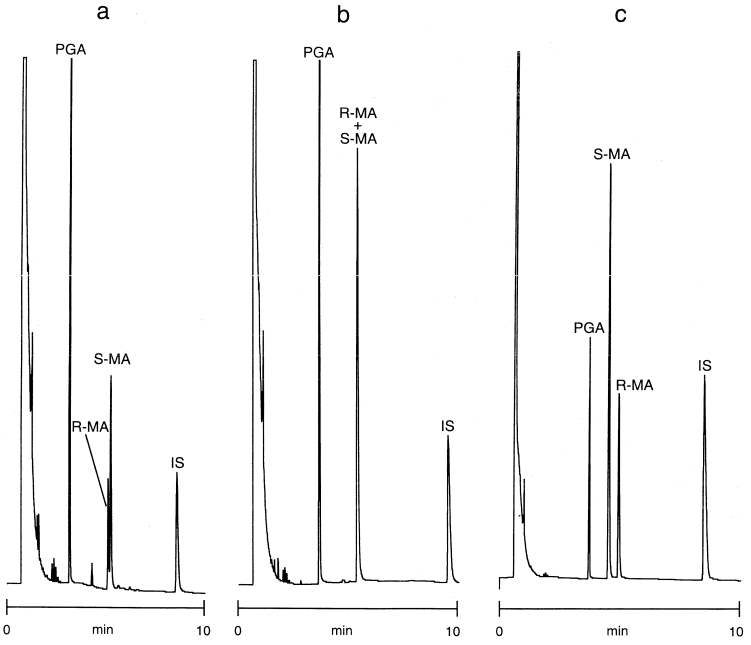

Fig. 1. (A) GC–FID chromatograms of *R*-MA, *S*-MA and PGA standards after esterification with (a) methanol, (b) ethanol and (c) isopropanol. Column: Chirasil-Dex-CB, 13 m×0.25 mm I.D., 0.25 μm film thickness, column temperature, 140°C isothermally; carrier flow (He), 2 ml/min. The concentration of the *S*-enantiomers is approximately two-times higher than the concentration of the *R*-enantiomers. (B) GC–ECD chromatograms of *R*-MA and *S*-MA standards after esterification with (a) methanol, (b) ethanol and (c) isopropanol and subsequent derivatisation with PFPA. Column: Chirasil-Dex-CB, 13 m×0.25 mm I.D., 0.25 μm film thickness, column temperature, 100°C isothermally; carrier flow (He), 3 ml/min. The concentration of the *S*-enantiomers is approximately two-times higher than the concentration of the *R*-enantiomers.

The derivatisation was complete within 60 min with no racemisation (<1%). The addition of pyridine proved to be essential for the derivatisation; without pyridine the reaction yield was about 10-times lower. In Fig. 1B the chromatograms of methyl, ethyl and isopropyl esters of MA after derivatization with PFPA are shown. As seen from the chromatograms, the elution order of the *R*- and *S*-enantiomers after derivatization with PFPA is opposite to that of their corresponding isopropylesters (Fig. 1A).

3.3. Chromatographic separation

3.3.1. FID determination

Fig. 2A shows the FID chromatograms of a blank urine (a), of the same urine enriched with the standards of L-3-phenyllactic acid (I.S.), *R*- and *S*-MA (b), and of urine from a volunteer exposed to 105 mg/m^3 styrene for 1 h (c). In order to exclude the possible interference with an another metabolite of styrene, phenylglyoxylic acid (PGA), a standard of PGA was added as well. As seen from the chromatograms, the MA enantiomers are baseline-resolved and elute as sharp, symmetrical peaks and without interfering peaks. However, during routine analysis of urine samples we noticed gradual deterioration of the column. This was manifested as a peak tailing of MA enantiomers and I.S.; whereas the PGA peak remained sharp and well defined. Analysis of a test mixture provided by the manufacturer (Chrompack), containing alcohols, diols and hydrocarbons, showed a peak tailing for all compounds containing hydroxy groups implying an interaction of free hydroxy groups with the active sites of the fused-silica column. With a new column, the same problem of peak tailing occurred after approximately 200 samples (400 injections). The solvent washing of the column or cutting column ends did not improve the performance of the column so that it had to be replaced by a new one.

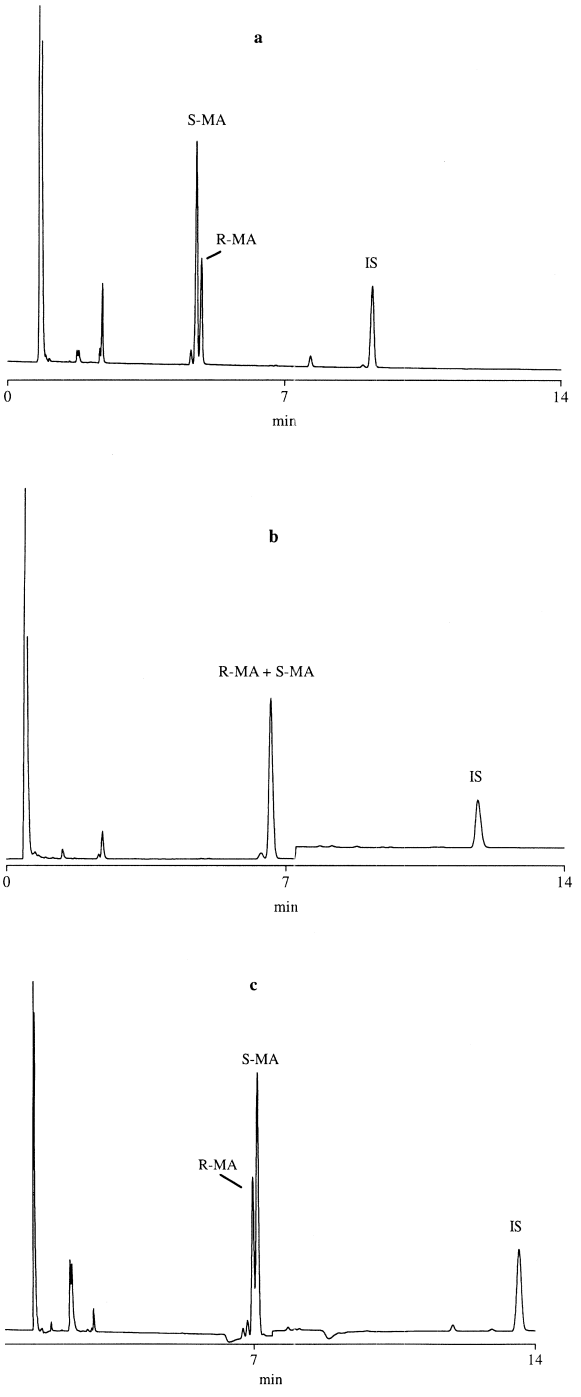

Fig. 1. (*continued*)

3.3.2. ECD determination

A good separation of the enantiomers (the resolution factor of the peaks was 1.4 and the peak asymmetry factor of both enantiomers was 1) was achieved also after derivatization with PFPA (Fig. 2B). Since the analytical procedure includes two derivatization steps, it was important for the I.S. to behave in the same manner as the analytes. Phenyllactic acid has, identically to the MA, a carboxylic and a hydroxy group and therefore undergoes both derivatization steps. As seen from the chromatograms, the I.S. eluted as a sharp peak and did not interfere with the MA enantiomers. In none of the blank urines (40 urines), a peak in the chromatogram in the place of the I.S. appeared.

In contrast to the FID analysis of the isopropylesters, PFPA derivatives showed no peak tailing over months of use (approximately 300 samples were analysed until now). Even the column which was not suitable anymore for FID analysis gave well defined and sharp peaks and the resolution and peak asymmetry factors remained constant. Derivatization of the hydroxy group of MA with PFPA, has therefore two advantages. Besides improving the sensitivity of the determination, the chromatographic column could be used much longer. To our knowledge the presented method is the first GC–ECD method for the analysis of the MA enantiomers. Other published methods concern less sensitive GC–FID determinations [6,13,14] and more time-consuming thin-layer chromatography [16].

3.4. Calibration, reproducibility and limit of detection

Calibration curves of *R*- and *S*-enantiomers were linear in the range of 10–600 mg/l for both, FID and ECD analysis. The limit of detection (LOD) was determined from the calibration curves made in blank urine (four different added concentrations in the range 10–100 mg/l; five determinations). The LOD was calculated from these regression lines by use of the intercept (a) and standard error of its estimate SE(a) as follows: $y=a+3\mathrm{SE}(a)$ [15]. For the FID analysis, the LOD amounted to 8 and 5 mg/l for *R*-MA and *S*-MA, respectively. The LOD of the ECD analysis was 1 mg/l for both enantiomers.

The presented method showed to be reproducible

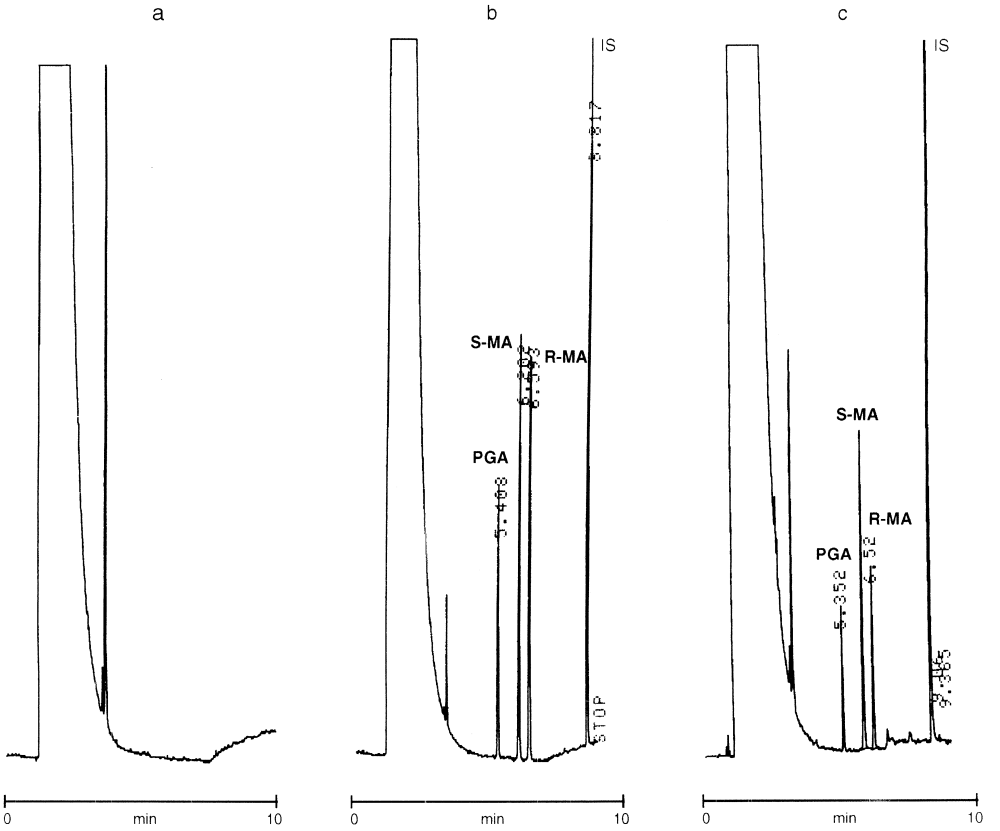

Fig. 2. (A) GC–FID chromatograms after esterification with isopropanol of (a) blank urine, (b) blank urine spiked with standards of *R*-MA, *S*-MA, PGA and PLA (I.S.) and (c) urine from a volunteer exposed to 105 mg/m^3 styrene for 1 h. Chromatographic conditions as described in Section 2.4. (B) GC–ECD chromatograms after esterification with isopropanol and subsequent acylation with PFPA of (a) blank urine, (b) blank urine spiked with standards of *R*-MA, *S*-MA and PLA (I.S.) and (c) urine from a volunteer exposed to 105 mg/m^3 styrene for 1 h. Chromatographic conditions as described in Section 2.4.

within- and between-days. The repeatability (within-day precision) of both methods, determined at two concentration levels (150 and 500 mg/l) by analysis of seven aliquots of a urine sample, ranged from 3.5 to 6.8%, and is shown in Table 1. The reproducibility between different assays (between-day precision) was determined by analysing the QC samples (150 and 500 g/l) during a period of 3 months and ranged from 4 to 7.5% (Table 1).

3.5. Samples

Urine samples were available from the volunteers (n=20) exposed to 105 and 420 mg/m^3 styrene for 1 h. The exposure to styrene was below the time weighted occupational exposure limit in The Netherlands. The Medical Ethics Committee of the Academic Medical Centre, University of Amsterdam approved the experimental protocol. Each subject signed an informed consent form. The exposure experiments were part of a study on the interindividual differences in the metabolism of styrene. The concentrations of the enantiomers of MA were determined in the urine before the onset of exposure and in the urine samples collected 0–2, 2–4, 4–8, 8–12 and 12–24 h after the onset of exposure. The concentrations of the MA enantiomers in urine samples of volunteers ranged from 2 to 600 mg/l, and were in some urines below the LOD of the FID method. The LOD of the ECD method was below

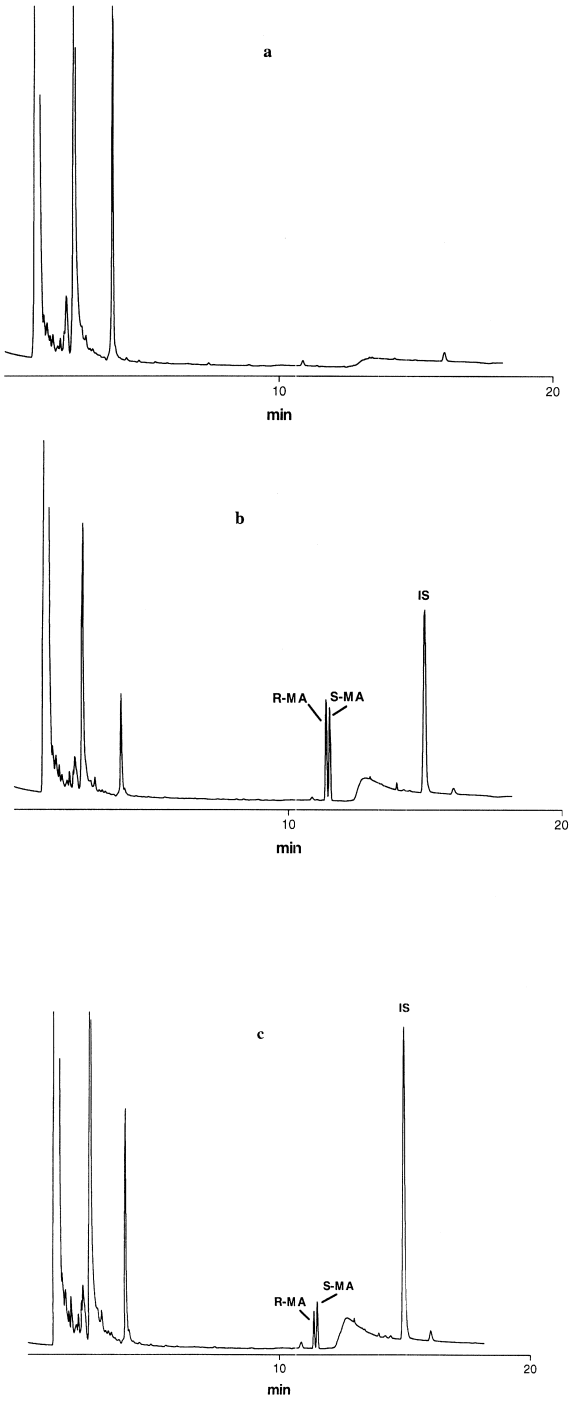

Fig. 2. (*continued*)

the concentration levels in all urine samples and could even easily be improved by taking larger volume of extracts before derivatisation with PFPA (according to the procedure, 100-μl aliquot of hexane extracts was taken for the derivatisation). The correlation between the results obtained with the FID (MA_{FID}) and the ECD (MA_{ECD}) showed that both methods gave results consistent with each other. The slope and intercept of the regression lines representing the relation between the values determined by FID and ECD, approached unity and zero, respectively: $R\text{-}MA_{FID}=1.038$ (± 0.020) $R\text{-}MA_{ECD}+0.001$; $S\text{-}MA_{FID}=0.994$ (± 0.020) $S\text{-}MA_{ECD}+0.000$.

The time courses of *R*- and *S*-MA in urine of one subject exposed for 1 h to 105 and 420 mg/m^3 styrene, determined by ECD are shown in Fig. 3. In the urines of volunteers, the *S*-enantiomer showed higher excretion compared to that of the *R*-enantiomer, with marked interindividual differences in excretion of both enantiomers. The difference between the highest and the lowest amount of MA, excreted during 24 h of collection was for both enantiomers approximately factor three. The result of this metabolic study will published elsewhere. In an occupationally exposed group, Korn et al. [13] reported the ratios of *S*- and *R*-enantiomers in the spontaneous urine after the end of the shift to be between 1:1 and 2:1 which were subject to high individual fluctuations. In a study of Hallier et al. the ratio of the *R*- and *S*-enantiomers ranged from 0.7 to 2.2 [16]. As pointed out in the latter study, this marked interindividual difference in excretion of MA could be related to enzyme polymorphism. Individual metabolic preference for one of the stereochemical forms may be therefore a disposition factor for the genotoxic effects of SO.

To summarise, both FID and ECD methods were shown to be suitable for the determination of MA enantiomers in the urine of persons exposed to styrene. Both derivatization steps proceeded with negligible inversion of enantiomers. The FID method was less sensitive in comparison to the ECD method and that could become an disadvantage when lower exposure levels should be monitored. In addition, in the routine analysis, fast deterioration of the column is an important shortcoming of the FID determination. The ECD method involves one derivatization step more; however, it improves the sensitivity of the

Table 1
Repeatability ($n=7$) and reproducibility ($n=30$) of the FID and ECD analysis of *R*- and *S*-MA (relative standard deviation of the mean; RSD, %)

	Concentration (g/l)	Repeatability (RSD, %)		Reproducibility (RSD, %)	
		FID	ECD	FID	ECD
R-MA	0.14	4.7	5.7	5.4	4.5
R-MA	0.50	6.8	4.5	7.5	4.1
S-MA	0.14	3.5	5.8	6.5	4.5
S-MA	0.50	6.9	4.4	5.1	4.0

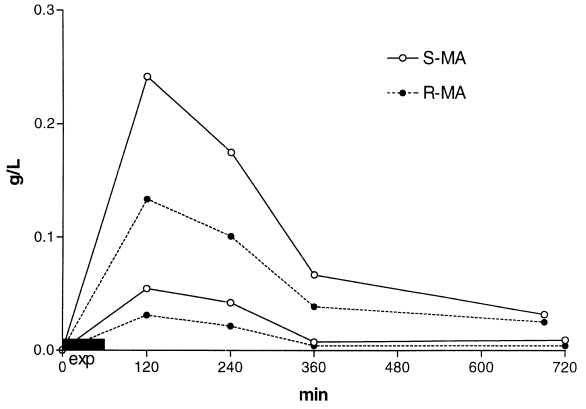

Fig. 3. Time course of *R*- and *S*-MA in urine of a volunteer exposed to 105 (lower curves) and 420 mg/m^3 (upper curves) styrene for 1 h, determined by the ECD method.

method and avoids the problems of peak tailing caused by interaction of a hydroxy group with the active sites of the fused-silica column.

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