Determination Of Mandelic Acid Enantiomers In Urine By Derivatization In Supercritical Carbon Dioxide Prior To Their Determination By Gas Chromatography

M. Valcárcel*, M. Zougagh, L. Arce, A. Ríos[a]
Department of Analytical Chemistry, University of Córdoba, Campus de Rabanales, Edificio Marie Curie, E-14071 Córdoba, Spain, qa1meobj@uco.es
[a] *Department of Analytical Chemistry and Food Technology, University of Castilla–La Mancha, Campus de Ciudad Real, E-13004 Ciudad Real, Spain*

The use of a supercritical carbon dioxide reaction medium for the determination of the (R)- and (S)- enantiomers of mandelic acid (MA) is proposed. The process involves a previous derivatization step under supercritical conditions by which the carboxyl group is esterified with methanol that is followed by acylation of the hydroxyl group in methyl MA with pentafluoropropionic anhydride in the absence of a catalyst. These derivatization steps cause no enantiomeric inversion. The derivatized enantiomers are extracted and quantified by gas chromatography. A BETA DEX 225 capillary column allows the separation of (R)-MA and (S)-MA as pentafluoropropionyl methyl esters with good resolution and precision. The overall method was used to determine both enantiomers in urine samples.

INTRODUCTION

Mandelic acid (MA) is a major metabolite of styrene and widely used as a biological indicator of occupational exposure to styrene [1,2]. The recent need to precisely determine the enantiomeric composition of urine samples containing mixtures of (R)- and S(+)-mandelic acid prompted us to explore the options for the gas chromatographic (GC) resolution of α-hydroxy acid enantiomers.

Recently, Kezic et al. [3] reported a selective liquid–liquid extraction (LLE) method based on the derivatization of mandelic acids in urine samples, followed by gas chromatography (GC) with flame ionization detection (FID) or electron-capture detection (ECD). This method is time-consuming and very expensive owing to the short lifetime of the chromatographic column (400 injections).

Supercritical fluid extraction (SFE) is a fast preparation technique for use prior to the chromatographic analysis of samples, where it simplifies and facilitates automation of the preliminary operations of analytical process [4]. The SFE technique has the advantage that derivatization can performed *in situ* (*viz.* inside the extraction thimble during the static SFE period). Thus, samples are first extracted in the static mode to allow the derivatization reagents to act, and then in the dynamic mode.

The primary aim of this work was to accomplish for the first time the derivatization of mandelic acids using a supercritical fluid. Conducting the SFE reaction *in situ* reduces sample handling, decreases the overall number of preparative steps and provides extracts that are ready for direct analysis by gas chromatography. An

additional aim was to develop an effective method for determining the enantiomeric ratio of (R)-MA to (S)-MA in urine samples.

I- MATERIALS AND METHODS

All chemicals used were analytical reagent grade or better. (R)- and (S)-MA were purchased from Sigma; L-3-phenyllactic acid from Aldrich; pentafluoropropionic anhydride (PFPA) from Fluka; and isopropanol, methanol, ethyl acetate, dichloromethane (DCM), pyridine and hydrogen chloride (HCl) from Panreac. Filter paper was used as a solid support.

Immediately after collection, 25-mL aliquots of urine were spiked with (R)- and (S)-MA at variable concentration levels over the range 0.010–0.250 g L^{-1}. From these pools, 0.5 mL aliquots were distributed to 0.5-mL Ependorf and stored at –18 ºC until analysis.

Standard solutions of (R)- and (S)-MA were prepared in water. The concentration of the stock solution was 2 g L^{-1}. The calibration curve was obtained from six working standard solutions containing a 10–500 μg mL^{-1} concentration of each enantiomer in urine. Internal standardization was done with a 10 g L^{-1} solution of L-3-phenyllactic acid (IS) in ethyl acetate. The concentrations of (R)- and (S)-MA in urine were calculated by internal standardization based on peaks areas. All samples were analysed in triplicate.

The SFE system used included a Hewlett-Packard 7680-T extractor. A Model 1050A quaternary HPLC pump was coupled with the extractor to insert the derivatizing reagent. The trap was filled with octadecylsilica (ODS, C18). All analyses were performed on a Fisons 8000 gas chromatograph interfaced to an MD 800 mass spectrometer. The injection port, transfer line and detector temperatures were kept at 250, 250 and 200ºC, respectively, throughout the experiments. The initial column temperature was held isothermally at 120ºC for 7.5 min, after which it was raised to 150ºC at 7.5ºC min^{-1}, held for 5 min, raised to 180ºC at 25ºC min^{-1} and held for 2 min. A volume of 1 μL of sample was injected in the split mode (1:25 ratio) in all analyses. Helium at flow rate of 1.5 mL min^{-1} regulated by digital pressure and a flow controller was used as carrier gas.

The mandelic acids were derivatized using the method of Kezic *et al.* [3]. To an aliquot of 1 mL of urine fortified with MA, 100 μL of IS, 300 μl of water and 1.25 mL of 1 M HCl in isopropanol or methanol was added. The esterification reaction was conducted in tightly closed 4-mL screw-cap glass vials at 100ºC for 30 min. After cooling to room temperature, the isopropyl esters of (R)- and (S)-MA were extracted with 1 mL of hexane, a 100 μL aliquot of the extract being supplied with 500 μL of 4% pyridine in DCM and 30 μL of PFPA. The derivatization reaction was performed at 60ºC for 60 min. After cooling to room temperature, the samples were evaporated to dryness under an N_2 stream and the residue was dissolved in 0.5 mL of hexane, 1 μL aliquots being analysed on the gas chromatograph as described below.

Mandelic acids were derivatized in 7-mL stainless steel thimbles. The thimble containing the sample was placed in the extraction chamber for extraction of its components. When the chamber was closed, the porous frits contained in the caps at the ends of the vessel became high-pressure seals and allowed the sample to be held in place. The unit included a nozzle/trap assembly that acted as a controllable variable restrictor and allowed depressurization of the supercritical fluid and independent control

of the pressure and flow rate of the supercritical fluid. This variable restrictor kept the extraction vessel under pressure, reduced the typical risk of plugging of fixed restrictors and provided an interface affording operation at atmospheric pressure as a result. The extraction module was furnished with an internal trap filled with solid material and was fully automated. The supercritical fluid containing the sample components entered the trap through the nozzle, where it was depressurized. The sample components were then retained on the trap while the supercritical fluid was evaporated and the trap vented. Subsequently, the material collected on the trap was dissolved in an appropriate rinsing solvent and removed from the trap to a vial or waste through an exit line.

The esterification reaction was performed by adding 250 μL of a 9:1 (v/v) methanol/HCl mixture and 100 μL f IS to a volume of 100 μL of urine sample held on filter paper in the SFE cell. The cell was accommodated in the extraction chamber and allowed to equilibrate at the preset temperature before extraction. Once the target pressure (165 bar) and temperature (70ºC) were reached, the CO_2 bypassed the extraction cell and the sample was esterified in the static mode for 5 min, after which the 9:1 (v/v) methanol/pentafluoropropionic anhydride mixture was pumped at flow rate of 0.02 mL min^{-1} for 5 min. After the extraction cell was filled with a volume of 100 μL, the pump was stopped and the mixture kept at the selected temperature and CO_2 density for 5 min to conduct the acylation reaction.

The pentafluoropropionyl methyl esters of the mandelic acid enantiomers were isolated from the reactor of the SFE module, where derivatization had previously been performed under the following experimental conditions: cell temperature 70ºC; supercritical fluid density 0.65 g mL^{-1}; supercritical CO_2 flow rate 4 mL min^{-1}; dynamic extraction time 5 min; trap temperature 35ºC, nozzle temperature 45ºC. The analytes were collected on an ODS trap. In a subsequent step, the trap was rinsed with 2 mL of an ethyl acetate stream circulated at 2 mL min^{-1} by means of syringe pump. A volume of 1 μL of the extract thus obtained was sampled into the gas chromatograph.

The analytical figures of merit used to characterize the proposed SFE–GC method included the linear dynamic range and sensitivity (expressed as the limit of detection). Individual calibration graphs were run by using volumes of 100 μL of urine spiked with mixtures of (R)- and (S)-MA at identical concentrations over the range 10–500 μg mL^{-1}. Each solution was extracted in triplicate. Table 1 lists the linear range, intercept, slope of the curve, and the regression coefficient, for each individual enantiomer.

Table 1. Analytical figures of merit for the proposed method.

Parameter	**R-MA**	**S-MA**
Working concentration range (μg mL^{-1})	10–500	10-500
Calibration function (6 standards, $n = 3$, C in μg mL^{-1})	$Y = 65053C - 334\,167$	$Y = 71621C - 597\,583$
Correlation coefficient (R^2)	0.9998	0.9998
Standard deviation of residuals ($S_{y/x}$)	136275	154932
Limit of detection (μg mL^{-1})	6.3	6.5
Precision, RSD (%) ($n = 10$)	3.9	3.1

The proposed method was used to analyze urine samples from ten volunteers. The samples were found to contain neither MA enantiomer, so they were spiked with (R)-MA and (S)-MA at variable concentrations. The differences between the concentrations added and those found were small in all cases.

The proposed method was validated by using the mandelic acid derivatization method previously reported by Kezic *et al.* [3] as reference. The latter method uses LLE of the mandelic acid derivatives. As can be seen in Figure 1, the two methods provided similar results.

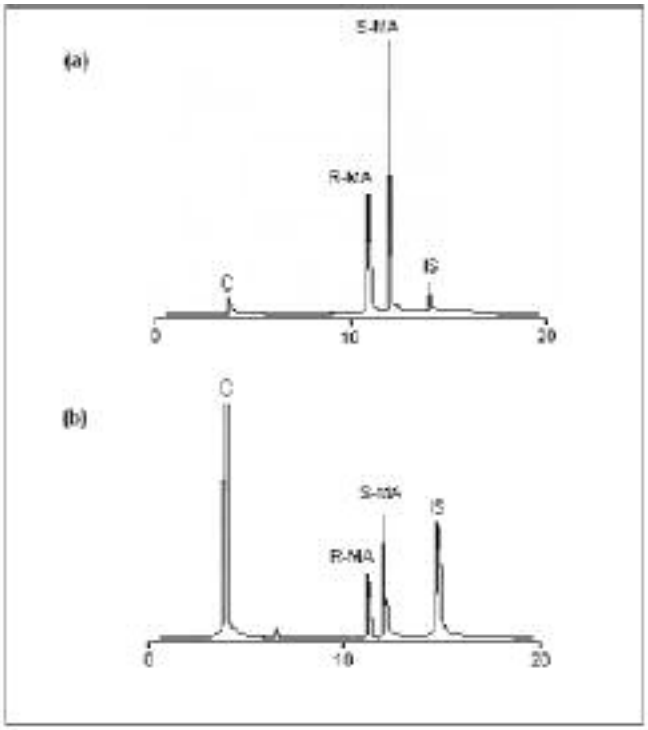

Figure 1. Gas chromatogram for (R)-MA and (S)-MA after esterification and subsequent acylation with PFPA of a urine sample spiked with 100 μg mL^{-1} concentrations of (R)-MA and (S)-MA, using the proposed SFE method (*a*) and an LLE method (*b*). IS internal standard; C unknown peak.

The reaction yield was higher with the SFE method by virtue of losses of the MA analytes during derivatization and extraction being small. The time required to conduct the overall procedure (*viz.*, derivatization, isolation of derivatives and GC analysis) was significantly different (*viz.*, 50 min with the proposed supercritical fluid extraction method *versus* 140 min with the liquid–liquid extraction method). Moreover, because the use of supercritical fluid extraction as proposed in this work allows one to exploit its advantages as an extraction method, the sample preparation step and clean extracts obtained are two highly interesting additional bonuses. In fact, the clean extracts provided by the SFE method (see peak *c* in Figure 1*a*) increase the lifetime of the gas chromatographic column; on the other hand, the non-clean extracts typical of LLE (see peak *c* in Figure 1b) lead to rapid deterioration of the column (after only 400 injections).

CONCLUSIONS

The use of supercritical carbon dioxide as the medium for derivatization reactions seems to be an effective alternative to existing choices. Derivatization improves the solubility of the analytes in supercritical carbon dioxide, increases analyte volatility for gas chromatographic analysis and allows sample preparation steps to be integreated in order to reduce analysis times and costs. The proposed method not only provides efficient separation of MA enantiomers without racemization, but also meets legal regulations regarding the use of contaminating solvents such as the pyridine employed in the LLE method, which is toxic and has a very unpleasant odor.

REFERENCES

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