Stereodifferentiation of Chiral Compounds Using Reversed-Phase Liquid Chromatography Coupled with Capillary Gas Chromatography

María Luisa Ruiz del Castillo*, Esther Gómez Caballero, and **Marta Herraiz**
Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

Abstract

A method is described for the enantiomeric quantitation of some chiral compounds via online coupling of reversed-phase liquid chromatography–gas chromatography. The evaluation of some variables affecting the experimentation (i.e., the packing material used in the interface, volume of the transferred fraction, desorption time, initial temperature of the interface, and purge time) makes it possible to optimize the recoveries obtained for some chiral terpenes and lactones using a capillary column of β-cyclodextrin dissolved in OV-1701. The proposed method allows the enantiomeric analysis of aqueous matrices obtaining relative standard deviations lower than 9% and detection limits ranging from 0.26–0.93 ppm for the investigated compounds.

Introduction

The growing interest in stereodifferentiation of chiral compounds in complex matrices is attributed to the usefulness of enantiomeric composition studies in many scientific disciplines. In this respect, the use of new chiral stationary phases as well as the development of new methods for the chromatographic separations of enantiomers have been reported by different authors (1,2).

However, performing enantiomer selective separations in complex matrices may be a difficult task because it typically involves the analysis of trace levels in complex mixtures of hundreds of compounds of different functionalities. For that reason enantiomeric composition studies are usually neglected even though it is already known that stereoisomeric components may differ in activity, toxicology, and metabolism.

The problems encountered when analyzing the enantiomeric composition of complex mixtures may be efficiently addressed by using multidimensional techniques (3,4). Thus, enantiomers of certain components may be finally separated by transferring selected cuts from the first to the second column, where a further separation can be carried out that takes advantage of the high efficiency of capillary columns. In fact, recent progress in the analytical authentication of complex matrices on the basis of chirality evaluation has mainly focused on the latest analytical advances in multidimensional techniques. Specifically, the use of multidimensional gas chromatography (GC) has been widely reported for the determination of enantiomeric compositions of chiral compounds (5,6).

More recently, the combination of high-performance liquid chromatography (LC) and high-resolution GC has been demonstrated as a viable means for addressing complex separation problems (4,7–10).

Most published reports on LC–GC focus on the use of normal phase in the preseparation step because of the ease of handling eluents that produce a relatively low volume of vapor per unit volume of liquid and also show good wettability of retention gaps. However, a very high percentage of LC separations are performed by reversed-phase LC (RPLC) because the use of hydrophobic packing materials (e.g., alkylbonded silica) and aqueous or partly aqueous mobile phases enlarge the range of compounds that can be analyzed. In fact, different approaches for the use of typical RP eluents for LC–GC systems have already been described in the literature (11). Furthermore, the stereodifferentiation of chiral compounds by using coupled RPLC–GC has also been accomplished using a programmed temperature vaporizer (PTV) as interface (12–14), although the applications reported so far mainly refer to the study of oil adulteration or nonaqueous samples. In this respect, our earlier work in RPLC–GC via PTV clearly shows that the rules for the transfer by this technique must be carefully established to ensure the reliability of the analysis. Consequently, further studies aimed to enlarge the field of application of RPLC–GC for enantiomeric composition analysis are highly demanded.

The goal of this work was to evaluate the potential of coupled RPLC–GC for the stereodifferentiation of some chiral compounds occurring in complex matrices. An important aspect was the development of new, rapid, and reliable methods suitable for introducing aqueous samples and aqueous sample extracts into the GC, even at large volumes.

* Author to whom correspondence should be addressed: email ifihc23@ifi.csic.es.

Experimental

Materials

A standard solution containing racemic terpenes (i.e., α-pinene, limonene, and α-terpineol) and racemic γ-lactones (i.e., C_8, C_9, C_{10}, and C_{11}) in methanol (50 ppm of each compound) was used for identification purposes. All of the standards were acquired from Aldrich (Milwaukee, WI) although methanol (HPLC grade) was purchased from Scharlau Chemie, S.A. (Barcelona, Spain), and the water used was obtained from a Milli-Q water purification system (Millipore, Milford, MA). A 5-cm plug length of the packing material was placed in the silylated glass liner (75- × 1-mm i.d. × 2-mm o.d.) of the GC injector, as detailed below. Tenax TA (80-100 mesh) and Gaschrom were achieved from Chrompack (Middelburg, The Netherlands) and Alltech (Deerfield, IL), respectively. Prior to its use, Tenax TA was conditioned under a helium stream by increasing the temperature from 50ºC to 350ºC within 10 min and kept there for 110 min. Gaschrom was also conditioned under a helium stream by maintaining the temperature at 50ºC for 10 min and then by successively increasing to 100, 150, and 200ºC, with each of them kept for 10 min. The final temperature (250ºC) was established for 80 min. Experimentation was carried out by considering different values for some of the variables involved in the LC–GC analysis (i.e., packing materials in the PTV, volume of the transferred fraction, desorption time, initial PTV temperature, and purge time). The obtained results were evaluated in terms of the recoveries estimated for the investigated compounds under the experimental conditions used in each case.

Instrumentation

The RPLC–GC analysis of the investigated samples was performed using Model 1050 HPLC system (Hewlett-Packard, Wilmington, DE) coupled to a Model 8500 GC (PerkinElmer, Norwalk, CT) fitted with a PTV, which acted as the interface of the coupled system.

LC analysis

LC preseparation was carried out with a Model 7125 manual injection valve (Rheodyne, Cotati, CA) with a 20-mL loop, a 5-cm × 4.6-mm i.d. Kromasil column (100-10C_4; Symta, Madrid, Spain) and ultraviolet detection (205 nm). Methanol–water was used as the mobile phase, 2000 µL/min being the flow rate (equal to the speed of sample transfer from LC into GC). The eluent composition (methanol–water, 35:65, v/v) was initially maintained for 18 s; then a linear gradient was applied within 3 s up to 90% methanol, which was kept until 10 min; and subsequently the methanol percentage was increased up to 100% (within 5 min) and maintained during the analysis.

LC–GC transfer

Upon elution of the fraction of interest, transfer was performed (through a 60-cm × 0.32-mm-i.d. fused-silica tube inserted into the septum of the PTV injector) by switching a multiport valve (Model 7060, Rheodyne). According to our previous experience, the injector was maintained at a fixed temperature while passing a helium flow transfer (1000 mL/min). As already reported (12), solvent elimination was promoted during transfer by removing the column end from the injector body before starting the transfer step. After the purge time is established for each analysis, the column is reconnected and the solutes retained in the packing material of the PTV are transferred to the GC column by increasing the interface temperature at 14ºC/s up to either 300ºC (held for 11 min) or 225ºC, depending on whether Tenax TA or Gaschrom, respectively, was used.

GC analysis

The enantiomeric analysis of the transferred fraction from the LC preseparation was accomplished on a 25-m × 0.25-mm-i.d. home-made fused-silica column containing 10% of β-cyclodextrin in OV-1701, 4 mg and 0.25 µm being the amount of β-cyclodextrin in the solution used to coat the column and the film thickness, respectively. Separation efficiency was 4300 theoretical plates/m for a solute with a retention factor (k) equal to 9. The oven temperature was held at 65ºC (5 min) and then successively increased to 70ºC (2ºC/min); 105ºC (5ºC/min); 165ºC (2ºC/min); and 180ºC (5ºC/min). The system was fitted with a flame ionization detector operated at 320ºC.

Results and Discussion

Assay optimization was carried out with a standard solution containing the compounds detailed under the "Experimental" section. Several chiral terpenes [of particular interest in recent years because of their biological activity (15,16)] and lactones [important aroma-active constituents of foods (5)] were studied because they are relevant components of a number of real-life aqueous samples whose complexity may require analysis by RPLC–GC.

As the sorbent used in the PTV body to retain the compounds transferred from LC into GC is a critical parameter to guarantee the effectiveness of the overall procedure, different materials (i.e., Tenax TA, 80/100 and 35/60 mesh, Gaschrom, Thermotrap, and

Table I. Recoveries Obtained from a Standard Solution when Performing the RPLC–GC Analysis with Different Packing Materials under Optimized and Initial Conditions

	Recovery (%)*	
	Tenax TA 80/100 mesh	Gaschrom
α-Pinene	1.95 (0.12)†	21.00 (17.98)
Limonene	31.07 (2.21)	45.83 (38.59)
α-Terpineol	49.69 (0.95)	19.60 (3.10)
γ-Octalactone	87.30 (1.91)	33.90 (5.30)
γ-Nonalactone	64.30 (2.65)	41.60 (7.02)
γ-Decalactone	73.77 (2.42)	58.58 (9.59)
γ-Undecalactone	81.60 (7.57)	30.18 (22.42)

* Estimated from the ratio between the amount obtained from the RPLC–GC analysis under the optimized conditions and the amount in the spiked sample established from GC analysis in the splitless mode.

† Recoveries obtained from a standard solution when performing the RPLC–GC analysis with different packing materials under the initial conditions.

Chromosorb G) were first considered for the evaluation of their usefulness. These materials and the experimental conditions for the RPLC–GC analysis were initially chosen on the basis of our previous experience concerning both sampling of large volumes onto a PTV operated in the solvent split mode and transfer from LC into GC of selected cuts.

For each set of experimental conditions, the obtained results were evaluated (as previously mentioned) in terms of the recoveries estimated from the ratio between the amount obtained for each compound when performing its analysis by RPLC–GC and that resulting from the GC analysis in the splitless mode. Table I shows the values collected under the optimized conditions from the two adsorbents giving the best results (i.e., Tenax TA, 80/100 mesh, and Gaschrom). For the sake of comparison, Table I also includes (in parenthesis) the recoveries obtained under the experimental conditions initially considered. The use of the other sorbents mentioned in the "Materials" section was discarded because either they did not allow the desorption of the retained compounds or they yielded unacceptable recoveries (lower than 3.5%) for all compounds.

From data given in Table I for the inital conditions, it seems evident that the use of Gaschrom would be preferable because higher recovery values were obtained for all of the investigated compounds. But it is also clear that even the use of the two selected materials did not render satisfactory recoveries and, consequently, further optimization was required. As a first step, the influence of the transferred volume on the peak areas obtained from RPLC–GC analysis of the compounds of interest was considered. The best recoveries were mostly observed when transferring into GC a 0.2-min fraction, which corresponds to a 400-µL volume. In this regard, it is convenient to emphasize that the transfer of larger volumes (e.g., 600-µL that corresponds to a 0.3-min fraction) resulted in considerably lower recoveries for the solutes eluted at the beginning of the transferred LC fraction (e.g., those solutes that are first deposited in the packing material placed into the PTV). This is most likely attributable to the fact that large volumes of liquid coming from the LC-preseparation carry solute material towards the bottom of the packed bed and, consequently, the advanced solute material runs out earlier than the solutes retained in the rear zone.

Subsequently, the effect of increasing the desorption time (up to 15 min) was also considered, although a significant improvement of the obtained recoveries was not observed. Similarly, the fact of lowering the initial PTV temperature up to 10ºC did not result in higher recoveries for the more volatile compounds.

Concerning the purge time (i.e., the time during which the passage of a helium flow through the interface is maintained, once finished in the transfer step, to facilitate the complete elimination of the eluent), different values were considered, namely 2, 4, and 6 min. Data obtained showed that the increase of the purge time did not yield higher recoveries, which was probably caused by the fact that a value as low as 2 min was enough to promote the effective elimination of the remaining solvent from the glass liner of the PTV injector when a 400-µL volume fraction was transferred. The successful solvent elimination represents, particularly in the case of aqueous samples, one of the most outstanding advantages of the proposed method because it avoids the possible overlapping of the solvent with the most volatile components and, in turn, minimizes the progressive damage of the GC column. This is an aspect of special significance when handling chiral stationary phases.

Therefore, the performed study allowed the experimental conditions for the RPLC–GC enantiomeric analysis of terpenes and γ-lactones to be established as follows: a transferred volume of 400 µL, initial PTV temperature of 21ºC, desorption time of 11 min, and purge time of 2 min. As can be seen in Table I, the recoveries obtained under these optimized conditions show that higher values are, in general, reached when using Tenax TA as the packing material placed in the PTV. A clear exception is observed for α-pinene; its determination by use of the proposed method is not feasible because of its extremely low recovery. Actually, it is evident that the described optimization enabled the α-pinene recovery to be increased up to 21% when using Gaschrom. But it also seems clear that either Tenax TA did not provide enough

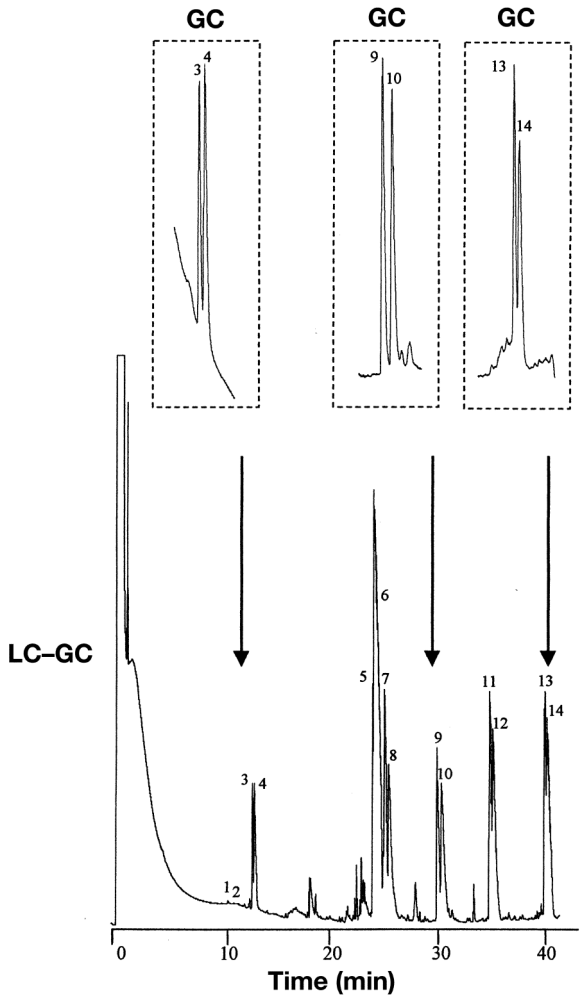

Figure 1. Chromatogram obtained from a standard solution of chiral terpenes and lactones by RPLC–GC analysis under the optimized conditions: packing material in the PTV, Tenax TA 80-100 mesh; transferred volume, 400 µL; initial PTV temperature, 21°C; desorption time, 11 min; purge time, 2 min; and final PTV temperature, 300°C (11 min). The identification peak numbers are: (1) S-α-pinene; (2) R-α-pinene; (3) S-limonene; (4) R-limonene; (5) S-α-terpineol; (6) R-α-terpineol; (7) R-γ-octalactone; (8) S-γ-octalactone; (9) R-γ-nonalactone; (10) S-γ-nonalactone; (11) R-γ-undecalactone; (12) S-γ-undecalactone; (13) R-γ-dodecalactone; and (14) S-γ-dodecalactone.

retention power to efficiently retain the solute during LC–GC transfer or, most likely, the cut time was not adequate to select the whole eluent portion containing α-pinene. This fact makes a slight shift of the transferred LC-fraction essential in case of the specific requirement of the determination of α-pinene by RPLC–GC using the proposed method.

Figure 1 shows the stereodifferentiation achieved under the optimized experimental conditions for the online RPLC–GC analysis. Also included in Figure 1 are the enantioseparations observed for some of the chiral compounds when the GC step is performed exclusively (i.e., when the LC preseparation and the subsequent transfer into GC of the selected fraction are omitted).

As can be seen, the solution of the chiral terpenes and lactones considered in this study could be transferred from LC into GC and separated into their corresponding enantiomers on the chiral column, except (as previously mentioned) α-pinene, which had a level that appeared to be practically undetectable under the optimized conditions. However, from Figure 1 it is also clear that losses of chromatographic resolutions (R_S) are observed for given pairs of enantiomers when comparing values obtained from the GC analysis of the chiral mixture with those corresponding to the online RPLC–GC analysis. Concretely, the R_S values achieved for the pairs of enantiomers (3–4, 9–10, and 13–14) decreased from 1.00, 1.72, and 0.89 (GC analysis) to 0.72, 0.82, and 0.65 (LC–GC analysis), respectively.

Such losses of resolution may be initially acceptable (at least for specific analysis) because of the advantage that involves the use of online RPLC–GC because it avoids the sample preparation step. But it seems evident that in those real samples in which the ratio of the two enantiomers' heights becomes very different from unity, a proper quantitation of the smaller peak can be very difficult to achieve. For that reason, the final step of the optimization must be performed while considering the specific matrix to be analyzed in each case as well as the compounds of interest.

In this respect, it is also interesting to emphasize that the developed method allows the transfer of very small fractions of eluent from LC into GC, which finally results in simpler chromatograms in which peak overlapping of a given pair of enantiomers may be avoided. Moreover, the use of an adequate software may contribute to the accurate determination of peak areas and, consequently, of the enantiomeric excesses.

Table II. RSD Values and Detection Limits For the Investigated Compounds

	RSD (%)*		DL (ppm)†	
	R	S	R	S
α-Pinene	8.5	8.3	28.0	70.2
Limonene	7.8	7.9	0.93	0.93
α-Terpineol	4.1	3.8	0.26	0.46
γ-Octalactone	2.5	2.1	0.56	0.70
γ-Nonalactone	0.8	0.6	0.62	0.70
γ-Decalactone	1.2	1.0	0.46	0.56
γ-Undecalactone	0.9	0.9	0.46	0.51

* RSD values calculated from absolute peak areas and a minimum of three replicates.
† Detection limit estimated from a signal equal to 5 times the base-line noise.

Table II gives the relative standard deviation values (RSD) and the detection limits calculated from the RPLC–GC analysis of a minimum of three replicates of the standard solution described in the "Materials" section. In all cases, good repeatability of the enantiomeric analysis was observed because the RSD values ranging between 0.6 and 8.5% were obtained while the detection limits (estimated from a signal equal to 5 times the base-line noise) varied from 0.26 ppm (for R-α-terpineol) to 0.93 ppm (for R/S-limonene). Obviously, α-pinene was found to be a clear exception, exhibiting an unacceptable detection limit.

Regarding the use of the two selected adsorbents, it is interesting to note the experimental difficulty that involves the use of Gaschrom in RPLC–GC with respect to Tenax TA. This is because the characteristics of the first material make it more difficult to efficiently eliminate the water in the interface and, consequently, this may cause irreversible damage to the GC column. Therefore, when analyzing a specific compound, the selection of packing material must be accomplished on the basis of not only the obtainment of high recoveries but also the volume to be transferred (i.e, it must be taken into account that when large volume fractions are to be transferred, larger volumes of aqueous eluents passing through the interface must be efficiently eliminated).

Conclusion

Stereodifferentiation of chiral compounds occurring in aqueous matrices can be performed by direct RPLC–GC analysis. However, previous optimization of the experimental variables affecting the transfer of the selected cut (as well as the transfer time for each fraction) must be carefully performed in order to achieve both high sensitivity and efficient separation in the GC column while avoiding losses of some of the solutes of interest.

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