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Enantiomeric analysis of limonene and carvone by direct introduction of aromatic plants into multidimensional gas chromatography

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Running title: Stereodifferentiation of terpenes in aromatic plants

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ABSTRACT

Analysis of chiral compounds in complex mixtures is achieved by multidimensional gas chromatography using heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin stationary phase as the main column of the system to separate specific selected cuts containing components unresolved in the first dimension. The proposed procedure allows rapid analysis of both solid and liquid matrices by direct introduction, into the programmed temperature vaporizer (PTV) of a gas chromatograph, of either the plant material or the essential oil, respectively. A comparison between enantiomeric excesses data obtained, from plant leaves (or plant seeds) and the corresponding essential oils, by direct injection (i.e., without sample pretreatment or concentration step) into the multidimensional system is also included. Reported data demonstrate that no racemization occur during analysis as identical enantiomeric excesses are obtained in both cases for specific chiral compounds.

KEYWORDS: Chirality; Direct Introduction; Enantiomers; Essential oils, Multidimensional Gas Chromatography (MDGC); Plant material

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1. Introduction

Reliable analysis of mixtures containing numerous compounds from various chemical classes demand sufficient resolutions of specific pairs of components. However, this can be difficult to accomplish even if stationary phases with adequate selectivities as well as columns with the required separation efficiencies, in terms of theoretical plate numbers, are used. Thus, high-resolution and/or high-performance analyses are to be performed specially in those cases in which maximum resolutions are required (e.g., for the separation of compounds with similar structures and, specifically, for achieving enantiomeric resolution of optical isomers).

Generally speaking, the analysis of complex mixtures cannot be accomplished by a single chromatographic separation (i.e., in a one-dimensional system), even when careful optimization of chromatographic parameters has been performed. Particularly, mixtures of compounds covering a wide range of concentrations usually require successive chromatograms to be run in order to adjust the sample size demanded for each compound of interest while also controlling overloading due to major components occurring in concentrated samples.

As previously reported [1,2], very often selected fractions of the eluate resulting from a pre-separation can be analyzed, without losing relevant information, instead of analyzing the total mixture. Moreover, the fact that the main separation can be carried out undisturbed by peak overlapping is an interesting advantage concerning the reliability of the compound identification. Thus, by combining two columns of different polarities and selectivities, two sets of retention data can be finally obtained from a single sample introduction into the system [3-6].

In this respect, the use of a double column system may allow the selective removal of some disturbing components in such a way that they are prevented from entering the

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main separation. Actually, only a sharp cutting of significant peaks are selected and subsequently allowed to enter the second column in which the chromatographic resolution of the target compounds can be lastly achieved [7-11].

Specifically, multidimensional gas chromatography (MDGC) meets a number of the well-known requirements that involves the analysis of chiral compounds in complex mixtures as demonstrated when analyzing different real-life samples [12-17].

On the other hand, it is widely recognized the interest of developing reliable analysis of natural plant components due to their extensive use as raw materials in the agro-food, pharmaceutical and cosmetic industries. The complexity of the sample very often demands sample preparation and concentration steps to be performed prior to the beginning of the chromatographic analysis itself. To this aim, there has been proposed the use of different techniques such as liquid-liquid extraction, steam distillation, headspace sampling, high-pressure solvent extraction, supercritical fluid extraction as well as other solvent free methods (e.g., solid phase microextraction and stir bar sorptive extraction) [18-21].

Nevertheless, these steps may be source of errors as well as artifacts and, occasionally, the initial composition of the sample can be altered due to rearrangement reactions resulting from reactive components. This aspect is particularly relevant when aiming the enantiomeric resolution of chiral compounds as, under some experimental conditions applied during sample preparation, racemization can be brought about, and consequently, unreliable enantiomeric ratios will be eventually established.

Previous work has shown the possibility of analyzing volatile compounds in solid matrices by direct introduction into the programmed temperature vaporizer (PTV) of a one-dimensional GC system [22] but risks of column overloading and overlapped peaks could not easily be prevented. Also, the use of adsorbent materials to trap (as well as to

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concentrate) the target compounds has been earlier proposed although a preparation step was required prior to the chromatographic separation in order to sweep the analytes from the matrix and, subsequently, to retain them onto a suitable material placed inside the injector [23,24].

The aim of this work was to evaluate the usefulness of on-line coupled MDGC-MS to perform the chiral analysis of both solid and liquid matrices by direct introduction (i.e., without any kind of sample handling), into the PTV of the chromatograph housing the pre-column, of either solid plant material or the essential oils obtained thereof.

2. Materials and methods

2.1. Materials

Plants of *Mentha piperita* L. (*Mentha spicata x Mentha aquatica*, chemotype menthone) and seeds from *Carum carvi* L. (caraway, chemotype estragole) coming from wild plants grown up in Murcia (Spain) were collected and dried. Essential oils were obtained, using a Clevenger-type system, by hydrodistillation for 3 hours of aerial parts from *M. piperita* and seeds from *C. carvi* and subsequently dried with anhydrous sodium sulphate, thoroughly shaking and standing until the supernatant oil had become clear, and kept in amber vials at 4 ºC until starting their chromatographic analysis. *M. piperita* and *C. carvi* were selected because of the availability of both the plant material and the essential oil obtained thereof with the guarantee of knowing for certain their origin and traceability.

For identification purposes, a test solution containing (*R*)-limonene, (*S*)-limonene, (*R*)-carvone and (*S*)-carvone was used. To establish the enantiomeric composition of limonene and carvone in both *M. piperita* and *C. carvi,* two different approaches were

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considered (i.e., direct introduction into the MDGC system described below of a plant material as well as of the corresponding essential oil resulting from the same plant).

2.2. Chromatographic columns

Column 1: 30-m x 0.25-mm i.d. fused-silica capillary column coated with a 0.25-µm layer of 5% phenyl-95% polydimethylsiloxane (ZB-Wax, Micron Analítica, S.A., Madrid, Spain).

Column 2: 30-m x 0.25-mm i.d. fused-silica capillary column having a 0.25-µm film thickness of heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin, (Chirasil-β-Dex, Varian, Middelburg, The Netherlands).

2.3. Direct Introduction of the solid material

A 0.8-mg sample weight of dried and crushed leaves or seeds from the plant material was introduced without any pre-treatment into the glass liner (54 mm x 3.4 mm i.d. x 5 mm o.d.) of the PTV injector between two small plugs of deactivated glass wool. The glass liner was placed into the injector, kept at 40 ºC, after having interrupted carrier gas circulation. Once established again carrier gas flow, the chromatographic analysis was performed by thermal desorption and subsequent transfer of the material to the capillary column by increasing (at approximately 200 ºC/min) the injector temperature up to 250 ºC. The end temperature was maintained for 5 min and the PTV was operated in the split mode, 10:1 being the split ratio. After completing the thermal desorption step, the sample was analyzed using either GC-FID with the ZB-Wax column described in Section 2.2 or MDGC-MS with the ZB-Wax and the heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin as the pre-column and the main column, respectively (see Section 2.2 for further details). In all cases, the flame ionization detector (FID) used was set at 250 ºC.

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2.4. MDGC-MS analysis of plants and essential oils obtained thereof.

Direct introduction of plants as well as of the essential oils obtained from them were performed under the following conditions: The MDGC equipment consisted of two independent gas chromatographs (Varian, model CP-3800, Palo Alto, CA, USA) housing two columns, namely pre-column and main column which were serially coupled through a Deans (Varian) based switching system, and a transfer line kept at 180 °C throughout the experimentation. This pneumatic flow switching with a valveless Deans switch system was used to transfer the selected analytes (contained in the so-called heart-cut) from one column to the other because it eliminates the problems associated with the use of mechanical valves (e.g., dead volumes, sample adsorption and bad resistance to high temperatures). However, pressure adjustments were required before being operational as the flow switching during the transfer time is achieved by pressure-directed changes in flow from an auxiliary electronic pressure control (EPC) module. In this case, flow direction control was obtained through a solenoid valve so that flow eluting from the first column could be directed either to a detector (FID) or to the main column, upon elution of the selected cut containing the target compounds. This Deans switch system could be applied at high temperatures because it uses flow channels with no valves or rotor faces, so that sample components are not in contact with any moving part. Moreover, a system of flows between and in the columns could also be established for the reversal of the eluent direction after peaks of interest were detected. This backflushing protects the colmns from degradation and contamination and also allows column changes and injector maintenance whitout loss of vacuum in the MS detector.

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The pre-separation was performed using the ZB-Wax column mentioned in Section 2.2. The oven program temperature was started at 60 ºC (1 min), increased at 4 ºC/min up to 150 ºC (5 min), and finally raised from 150 to 200 ºC at 5 ºC/min (20 min). The selected cuts were transferred into the main column (i.e., to the second dimension) and analyzed using β– heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin as stationary phase (column 2 in Section 2.2.). The oven temperature was initially set at 50 ºC (15 min) and then successively raised to 70 °C (1 °C/min), to 140 ºC (2 °C/min) and finally to 200 °C (4 ºC/min). In both dimensions, helium served as the carrier gas at an approximate head pressure of 30 psig in the pre-column and 24 psig in the main column. In all cases, a 0.1-µL volume of essential oil (without previous dilution) was injected into the MDGC system.

Separations achieved in the pre-column were monitorised using an FID detector (operated at 250 ºC) while the main column was connected to a Saturn 2000 ion-trap mass spectrometer (Varian). Data acquisition was carried out using a Star Toolbar system (Varian). The target compounds were identified by matching the GC retention times observed in both dimensions with those obtained from standards analyzed under identical conditions. For identification purposes, mass spectra recorded from the standard compounds were also compared with those provided by the US National Institute of Standards and Technology (NIST) library. For the MS, the electron multiplier was set to 1850 V and ionization was performed by electron impact (EI). Temperatures of the transfer line, the manifold and the trap were established at 180 ºC, 120 ºC and 220 ºC, respectively. The recorded spectra covered the range from 40 to 650 m/z and, additionally, the select ion monitoring (SIM) mode was used for specific fragmentations.

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Under the experimental conditions applied in the overall analysis, acceptable blanks (i.e., analyses made with no sample injected to clean out any impurities that might have accumulated in the columns) were obtained for the complete procedure between consecutive runs.

3. Results and discussion

3.1. General considerations

Initially we considered the interest of avoiding the sample preparation step, usually required prior to the chromatographic analysis of plant materials, not only because it is frequently time consuming and laborious but mainly because the handling of the sample may result in risk of losses, artefact formation, contamination and, eventually, in less reliable results. We also kept in mind that sample pretreatment is particularly important when performing the stereodifferentiation of chiral compounds as the risk of racemization (and, consequently, misestimation of enantiomeric compositions) increases when external concentration techniques must be applied. Precisely for these reasons, we decided to eliminate this cause of uncertainty by limiting the manipulation of the sample to its placement into the injector of the gas chromatograph.

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3.2. Direct introduction of solid materials into one-dimensional GC

Fig. 1 includes the chromatograms resulting from direct introduction, using a one-dimensional GC system, of the solid materials detailed in Section 2.1 and the subsequent separation in the ZB-Wax column. When examining the two analyses performed by directly introducing either *C. carvi* seeds or *M. piperita* leaves into the PTV of the gas chromatograph, it is evident the poor resolutions observed in both cases even though ballistic heating applied (to achieve thermal desorption) immediately after

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sample introduction might have contributed to prevent peak broadening. It is also clear that some peak shapes might have been improved by decreasing the initial PTV temperature (to focus the analytes at the head of the column) but, in any case, the severe peak distortion observed for most solutes in both Fig. 1a and 1b, does not allow the acceptance of these results inasmuch as the volatile profile of the sample can not be assessed. Furthermore, in spite of the fact that the resolution obtained in the first column might have been improved by changing experimental conditions, it should be taken into account that when only applying standard GC instrumentation, namely a one-dimensional technique, various difficulties may arise with the separation and identification of complex mixtures (e.g., insufficient separation efficiency, selectivity and peak capacity). That is way some new peak overlappings usually occur while trying to remove others and, hence, peak separation and identification will likely remain a difficult issue. For these reasons, we decided to make use of the fact that peak capacity enhancement achievable by multidimensional chromatography is considerably higher than that resulting from the optimization of the one-dimensional separation.

3.3. One-dimensional GC analysis of essential oils

As can be seen in Fig. 2a and 2b, better resolutions were achieved when using the same column mentioned in the previous section (i.e., the ZB-Wax column) to analyze the essential oils resulting by hydrodistillation of the same solid materials, namely seeds and crushed leaves, considered in Fig. 1. However, the evidence that major constituents of the matrix may disturb the gas chromatographic separation of minor peaks demonstrates that relevant compounds occurring in low concentrations may be chromatographically overlapped by other components, thus decreasing the reliability of

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those analyses for which high efficiencies are strongly required (e.g., the determination of the enantiomeric composition of chiral compounds).

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3.4. Chiral analysis of plant materials and essential oils by MDGC-MS

As previously mentioned, the four chromatograms included in Fig. 1 and 2 were obtained with a one-dimensional GC system by using exclusively the column 1, as detailed in Section 2.2, for the chromatographic separation. To take advantage of the
230 capability of a MDGC system, we serially connected this column, via a Deans based system, to a chiral column, namely, the heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin described under column 2 in Section 2.2. Thus, we tried to achieve the high peak capacity required to perform the following three steps: a) pre-separation in the pre-column, b) cutting and transfer from the first to the second column and c)
235 stereodifferentiation of chiral compounds in the main column.

In fact, by the transfer of the selected cuts from the pre-column to the main column, we could re-separate the target compounds not only at different selectivity but also with higher efficiency. Thus, in this particular case, we decided to combine a selective pre-column (to narrow eluate cuts containing a limited number of components) with a highly
240 efficient and enantioselective main column to reach enantiomeric resolutions.

Fig. 3 shows the fractions (or selected cuts) transferred, from the achiral pre-column to the chiral main column, when either directly introducing into the PTV *C. carvi* seeds or injecting the *C. carvi* essential oil obtained thereof. As can be seen, transfer of fractions eluted from 27.5 to 29.5 min and from 39.5 to 41.5 min in Fig. 3a enabled us
245 to obtain the enantiomeric resolutions for limonene and carvone given in Fig. 3c. In this case, both (*R*)- and (*S*)-limonene were detected, 18 % being the enantiomeric excess calculated for this compound. However, an *ee* value as high as 100 % was established

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for carvone as only one enantiomeric form, specifically the (*S*)-, was identified in the chromatogram recorded in the main column. The absence of the (*R*)-enantiomer was initially excluded from the observation of the obtained chromatograms by using the MS Workstation software (Varian, version 6.6). Moreover, other experimental runs performed by transferring different fractions from those shown in the present work enabled us to ensure the presence of only one of the two possible enantiomeric forms of carvone.

On the other hand, when transferring the cuts indicated in Fig. 3b (i.e., 27.5-29.5 min and 39.5-41.5 min) to the chiral column, relatively clean chromatograms (see Fig. 3d) were obtained for the analysis of the essential oil resulting from the same *C. carvi* seeds. Also in this case, both (*R*)- and (*S*)-enantiomers were detected for limonene while carvone was exclusively found in the (*S*)-form. Furthermore, from the chromatogram shown in Fig. 3d, an *ee* value of 18% was established for the limonene occurring in the *C. carvi* essential oil.

Table 1 gives the enantiomeric excesses (ee), separation factors (α) and enantiomeric resolutions (Rs) obtained from MDGC-MS analysis of solid materials and essential oils resulting thereof. As identical, *ee* value were obtained for limonene in both *C. carvi* seeds and its essential oil, it is clear that hydrodistillation applied for 3 hours does not modify the enantiomeric composition of limonene. Moreover, as carvone occur in both the solid material and the essential oil as an enantiopure compound, it is also evident that the experimental conditions established for the analytical procedure itself does not produce the racemization of the target analytes and, consequently, the reliable stereodifferentiation of chiral compounds may be finally obtained.

In this respect, it is interesting to underline the high sensitivity achievable when directly introducing the plant material into the MDGC system. Precisely for this reason,

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more complex chromatograms can be eventually recorded in the main column in comparison to those resulting from the essential oil (see Fig. 3c and 3d taking into account the different y-scales used for recording the four chromatograms included in Fig. 3). Although higher sensitivity may result in peak overlappings and, consequently, in poorly resolved compounds, it is obvious that it can also be advantageous when analyzing trace compounds. In any case, peak coelutions are irrelevant providing that the target compounds are satisfactorily separated as it is the case for limonene and carvone in Fig. 3c.

Fig. 4 illustrates the cuts transferred when analyzing either the *M. piperita* crushed leaves (27.5-29.5 min and 39.5-41.5 min in chromatogram 4a) or the *M. piperita* essential oil obtained thereof (27.5-29.5 min and 39.5-41.5 min in chromatogram 4b). In this case, the stereodifferentiation achieved in the main column disclosed the occurrence of only one of the possible enantiomeric forms, namely, (*S*-) for limonene and (*R*-) for carvone, in the leaves (Fig. 4c) as well as in the essential oil (Fig. 4d**)**. Thus, results obtained from the analysis of *M. piperita* also confirm that the reported procedure effectively prevents racemization of the target chiral compounds.

From figures 3c, 3d, 4c and 4d it is clear that the limonene/carvone ratios, calculated from peak areas by direct introduction of the plant material, are lower than those obtained from the essentials oils resulting from the same plants. As earlier reported by other authors, the composition of monoterpenes may be altered by hydrodistillation since carvone (more polar) is extracted more efficiently than limonene (apolar) [25]. Moreover, when uncrushed caraway seeds are distilled, carvone is extracted more efficiently (due to its higher polarity) than limonene whereas, from sufficiently crushed seeds, limonene is extracted more efficiently due to its higher volatility [26].

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Apparently, this is contradictory to our results as the chromatograms obtained by directly introducing the crushed plant material into the glass liner show, approximately, the same amount of limonene and carvone whereas higher limonene/carvone ratios were found when analyzing the essential oil extracted from uncrushed material. However, it should be noted that other authors have already reported variations in the composition of limonene during plant storage [27] and, precisely, the solid material used in the present work for direct introduction was stored at room temperature until it was crushed before performing the analysis, while the material used for obtaining the essential oil was distillated immediately after harvest and then it was kept at 4 °C until analysis.

On the other hand, there have been previously reported that small amounts of (*R*)-carvone against a majority presence of (*S*)-carvone has been found in essential oils obtained from caraway seeds [25]. Also, in extracts of caraway seeds and leaves of spearmint (*Mentha spicata*), obtained by supercritical fluid extraction and analyzed by HPLC with simultaneous detection of optical rotation and UV absorption, there have been determined that caraway seeds contain exclusively (*S*)-carvone, while spearmint leaves contain 93% of (*R*)-carvone [28]. Thus, the presence of (*S)*-carvone and (*R*)-carvone in caraway and mint, respectively, seems to be dominant, either as only one enantiomeric form or in much higher amount than the counterpart enantiomer.

In any case, it should be considered that, generally speaking, aromatic plants show a great chemical variability, even at intraspecific level. Moreover, the chemical composition can be affected by both the phenological stage at the harvest time and the extraction method used. Thus, different enantiomeric compositions can be found when performing the chiral analysis.

Other alternative methods can also be adequate for enantiomeric analysis of plant material and/or essential oils such as, among others, use of adsorbents or absorbents as

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packing materials for the introducton of large volumes of liquid samples in GC via PTV, as in reference [29]; use of the on-line coupling PTV-PTV-GC to sweep volatile compounds away from the sample matrix (previously introduced in the first PTV) to be retained in a suitable material placed inside the second PTV injector and, subsequently, thermally desorbed [23] and use of a solid phase microextraction step prior to the multidimensional chromatographic analysis (i.e., SPE-MDGC) [30].

The procedure proposed in the present work is advantageous inasmuch as it does not require a sample preparation step prior to the chomatographic analysis and, consequently, the risk of both artefact formation and racemization reactions during sample handling can be substantially reduced. As a result, this method provides a good alternative to determine (or verify) the enantiomeric composition of target chiral compounds, what is of special interest if differences between biological activities of a pair of enantiomers must be investigated.

However, it is also clear that chromatograms obtained from direct introduction of solid plant material into the PTV and subsequent MDGC analysis show overlapped peaks. Thus, for a complete analysis of chiral and achiral components in plant materials, consecutive fractions, covering the total time required for the analysis in the pre-column, should be transferred in sequential runs and, subsequently, analyzed in the main column.

As far as the repeatability of the proposed method is concerned, relative standard deviation values (RSDs) lower than 10% were obtained (from a minimum of three replicates) for the absolute peak areas of the target compounds (i.e., (*R*)-limonene, (*S*)-limonene, (*R*)-carvone and (*S*)-carvone). It should also be noted that the change of the liner to carrry out the proposed sampling mode can be easily and rapidly performed and it does not require modification/alteration from the standard design.

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4. Conclusions

Direct analysis (i.e, without any kind of sample pretreatment) of solid materials and the essential oils obtained thereof allows the rapid stereodifferentiation of chiral compounds by using multidimensional gas chromatography. The reported procedure does not demand the sample preparation step usually required when analyzing plant materials and also precludes racemization risk during experimentation so that reliable determination of characteristic enantiomeric excesses can be eventually achieved.

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Table 1. Enantiomeric excesses (ee), separation factors (α) and enantiomeric resolutions (Rs) obtained from MDGC-MS analysis of both solid material (*Carum carvi* seeds and *Mentha piperita* leaves) and essential oils obtained thereof.

Plant		Solid material			Essential Oil		
		ee (%)[a]	α[b]	Rs[c]	ee (%)[a]	α[b]	Rs[c]
Carum carvi	(*S*)-limonene (*R*)-limonene[d]	18	1.007	1.070	18	1.008	1.069
	(*S*)-carvone[d] (*R*)-carvone	100	–	–	100	–	–
Mentha piperita	(*S*)-limonene[d] (*R*)-limonene	100	–	–	100	–	–
	(*S*)-carvone (*R*)-carvone[d]	100	–	–	100	–	–

470

[a]Excess of predominant enantiomer expressed as a percentage, that is: [(predominant enantiomer – minor enantiomer)/(predominant enantiomer + minor enantiomer)] × 100.

[b]Separation factor calculated as the ratio of the adjusted retention times of the later to the first eluting enantiomer.

475 [c]Enantiomeric resolution calculated as the ratio between the difference of the retention times of each enantiomeric pair and their average band widths.

[d]Predominant enantiomer.

(–): Non-calculated value due to the presence of only one enantiomeric form.

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FIGURE CAPTIONS

Fig. 1. Chromatograms obtained from the direct introduction, into the PTV injector of a one-dimensional GC system, of either *C. carvi* seeds (1a) or *M. piperita* crushed leaves (1b) using a 30-m x 0.25-mm i.d. fused-silica ZB-Wax column under the experimental conditions detailed in Section 2.3.

Fig. 2. GC analysis of the essential oils resulting by hydrodistillation of the same plant materials as in Fig.1. Essential oils from *C. carvi* seeds (2a) and *M. piperita* (2b) were analyzed using the same column and experimental conditions as in Fig. 1.

Fig. 3. Chromatograms obtained in the pre-column of the MDGC system from either direct introduction of *C. carvi* seeds (3a) or injection of *C. carvi* essential oil (3b) and stereodifferentiations achieved in the main column (3c and 3d) of limonene and carvone by transferring, from the first to the second dimension, the indicated cuts resulting from the pre-column separations.

Fig. 4. Chiral resolution, by MDGC analysis of limonene and carvone, from either direct introduction of *M. piperita* crushed leaves (4c) or injection of *M. piperita* essential oil (4d), resulting from transfer the fractions selected in the separation achieved with the pre-column (chromatograms 4a and 4b).

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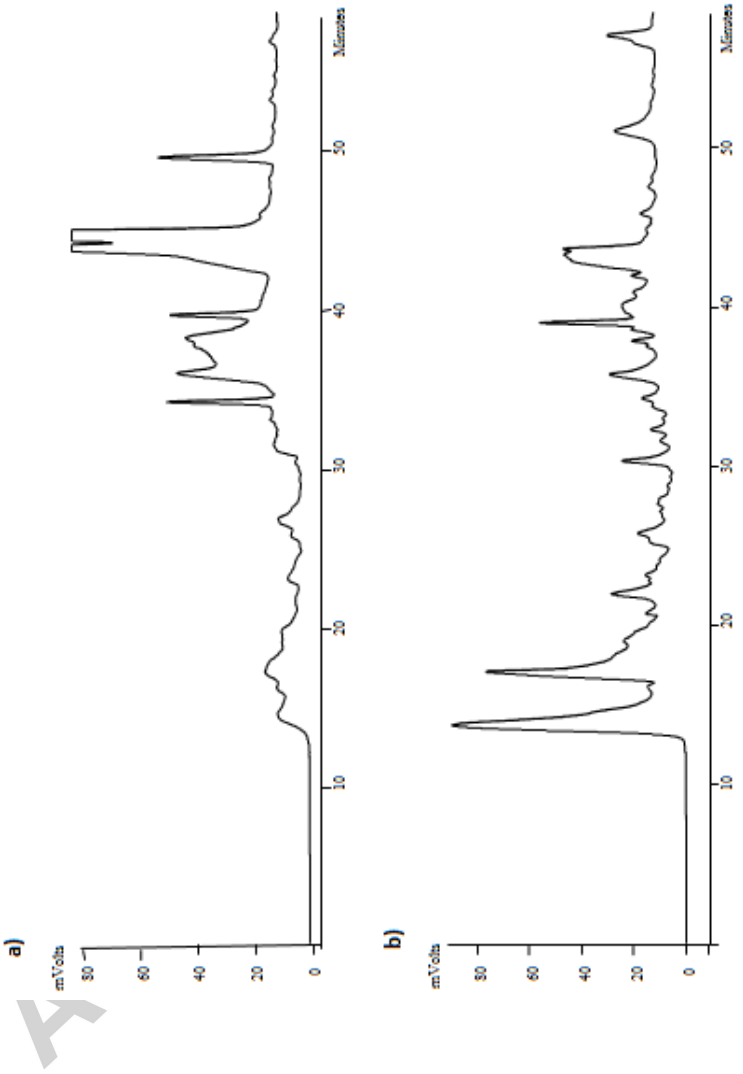

Figure 1

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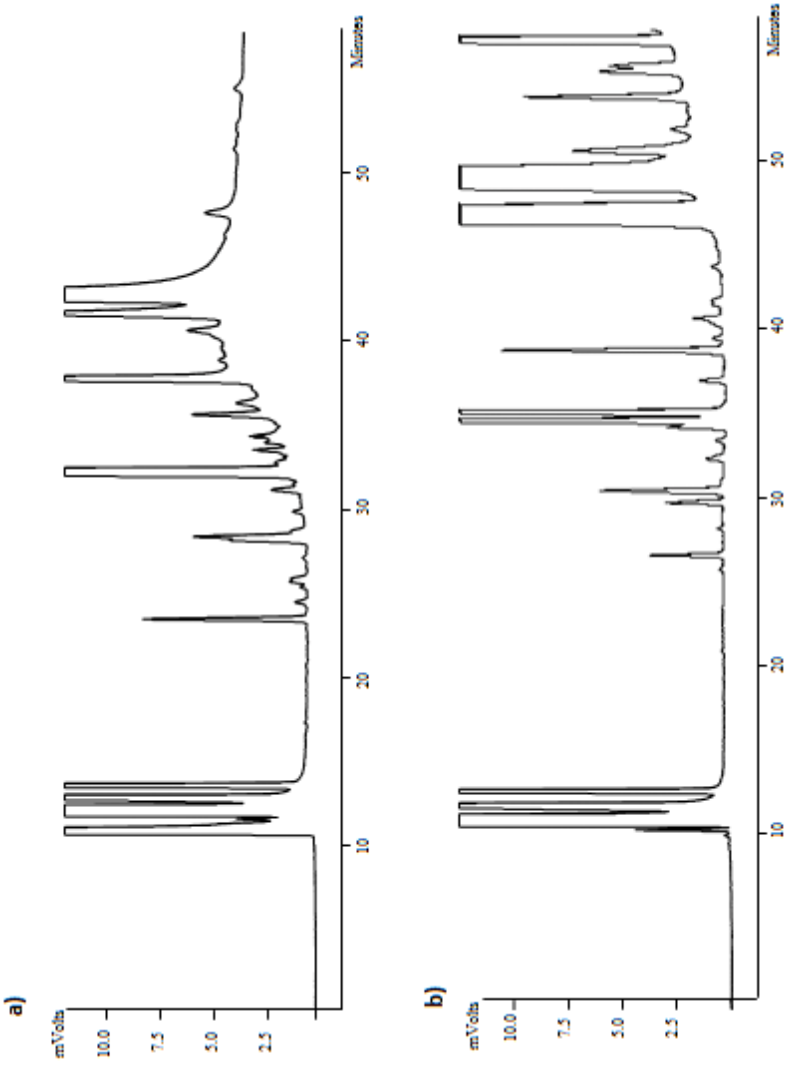

Figure 2

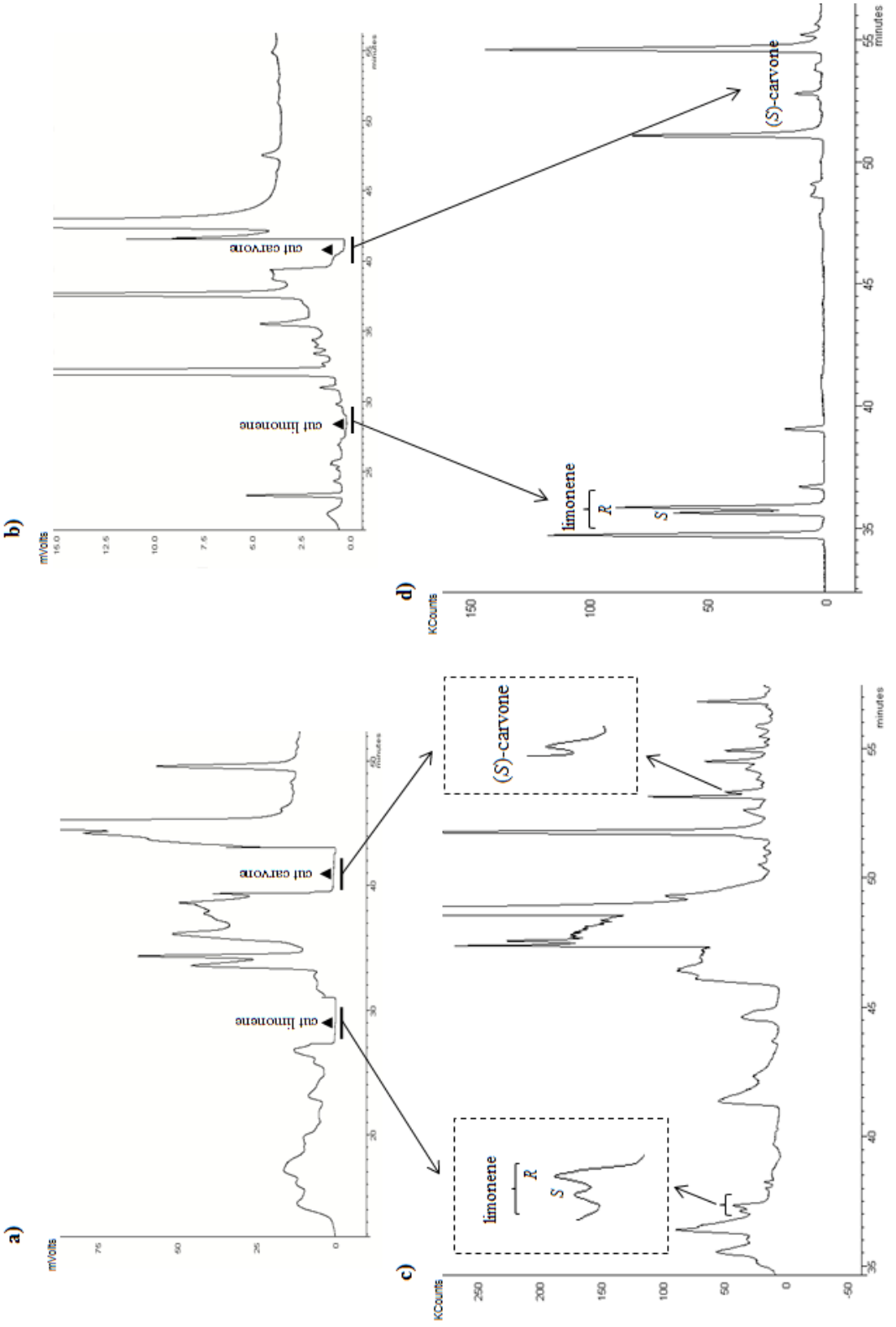
a)
mVolts
cut limonene
cut carvone
minutes
b)
mVolts
cut limonene
cut carvone
minutes
c)
KCounts
limonene
R
S
(S)-carvone
minutes
d)
KCounts
limonene
R
S
(S)-carvone
minutes

Figure 3

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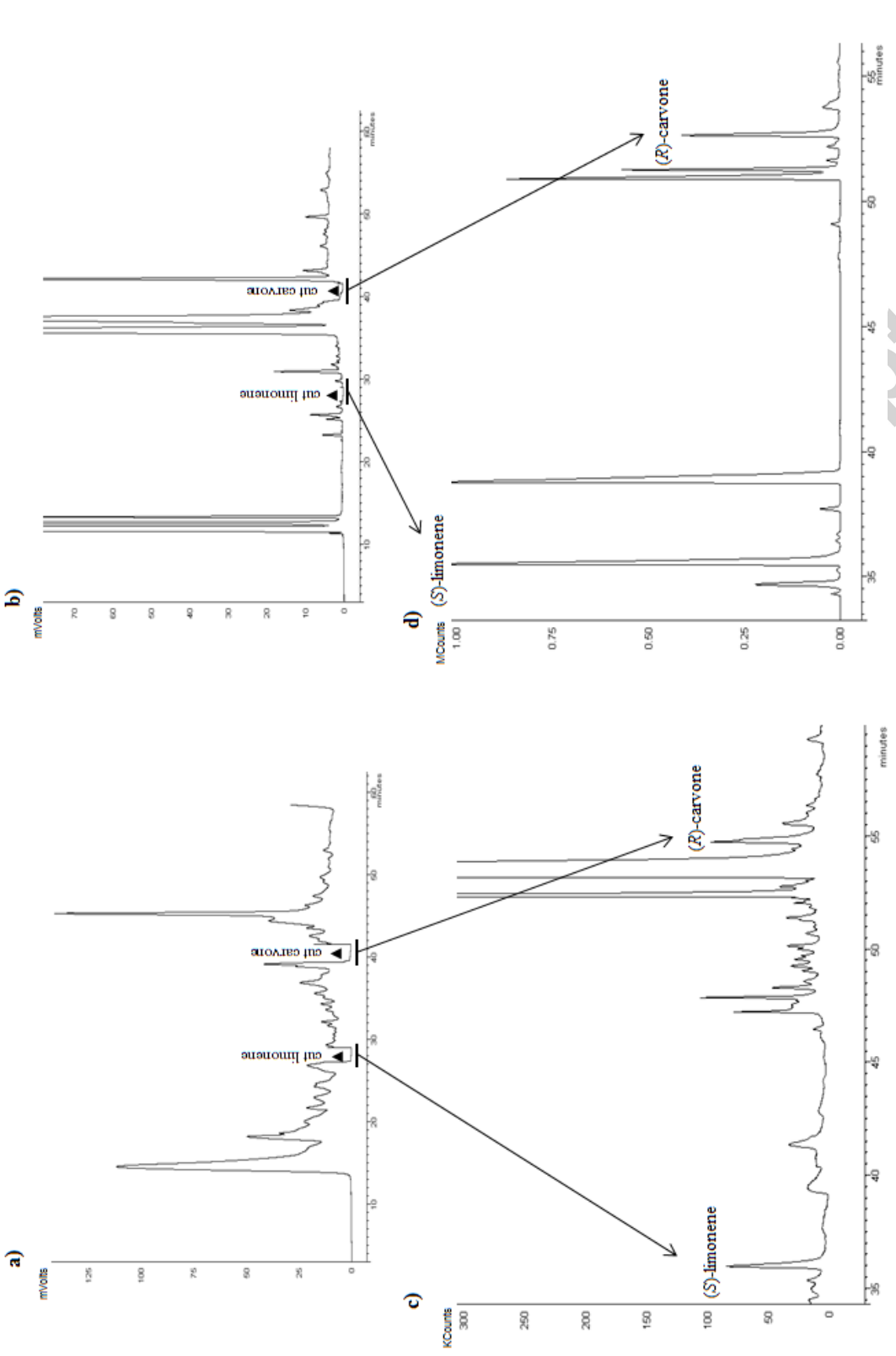

Figure 4

HIGHLIGHTS

- Solid plant material is introduced into multidimensional gas chromatography.
- No sample pretreatment is required and racemization risk is precluded.
- Stereodifferentiation of chiral terpenes is achieved in the main column.
- Identical enantiomeric excesses are obtained for plant materials and essential oils.

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