

## Changes in the Enantiomeric Distribution of Selected Volatile Constituents of *Mentha pulegium* L. Powders Caused by Hot Water Treatment

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The variation, in general, of the composition of the aromatic fraction and, in particular, of the enantiomeric composition of certain chiral volatile compounds of commercial *Mentha pulegium* L. powders caused by boiling water was evaluated. A comparison between the volatile profile of the studied herbs demonstrated that most *M. pulegium* L. samples contained high proportions of *Mentha piperita* L., even when this information was not specified on the label. Likewise, substantial changes in the volatile fraction of the infusions with respect to the composition of the original plant used in their preparation were found. The enantiomeric composition of some chiral compounds of the dried plant material, particularly limonene, was modified by adding hot water, whereas others were kept invariable. The results shown in this work reflect the need for the control of the composition of commercial powders and brews of *M. pulegium* L. to ensure their correct application.

**KEYWORDS:** *Mentha pulegium* L.; infusion; powders; SPME; chiral

### INTRODUCTION

*Mentha pulegium* L., commonly known as pennyroyal, is a herbaceous plant which belongs to the family Lamiaceae. It is principally characterized by its intense aroma and its use as a digestive tonic in the treatment of flatulence and intestinal colic (1). Besides, it is employed in the elaboration of industrial detergents and perfumes and as a natural source of pulegone (2). Although information on the composition of both *M. pulegium* L. herb and the infusion prepared thereof is scarce in the literature, the aromatic fraction of *M. pulegium* L. essential oil has been largely studied by various methods (3–6). As a result, all reports are in accordance with the occurrence of pulegone, menthone, and isomenthone as chief components as well as a number of minor compounds, such as  $\alpha$ -pinene,  $\beta$ -pinene, limonene, and menthol, among others.

On the other hand, it is known that the stereochemistry of the molecules influences notably their properties because of the chiral nature of most biological receptors. This way, the molecule/receptor interaction depends to a great extent on the structure of the compound (7). In this respect, some pairs of enantiomers can exhibit different sensorial properties so that slight variations in the enantiomeric composition may result in modifications of flavor perception. Specifically for the components of *M. pulegium* L., the toxicological effects on liver and lung related to pulegone have been ascribed to the (+)-enantiomer, while only (–)-menthol, of the eight stereoisomers

of menthol (i.e., (±)-menthol, (±)-neoisomenthol, (±)-neomenthol, and (±)-isomenthol), exhibits the typical peppermint odor and the greatest cooling effect (8–11). Equally, it is also known that whereas the (+)-limonene is usually associated with lemon aroma, the (–)-enantiomer gives off orange aroma (12). Precisely for this reason, the consideration of stereochemical aspects is of great importance when the chemical composition is intended to be determined. In this respect, chirality is particularly helpful when one tries to gain insight into the properties of some herbs.

The aim of this work was to study the modification of the aromatic fraction of *M. pulegium* L. powders caused by boiling water with a view to associating certain features of this herb (i.e., sensorial perception) with some of its constituents. For this purpose, particular attention was paid to the enantiomeric composition of chiral compounds because of the relevance of chirality in the biological properties of the molecules.

### MATERIALS AND METHODS

**Samples and Materials.** The chemical standards (i.e.,  $\alpha$ -pinene,  $\beta$ -pinene, limonene, menthol, menthone, neomenthol, and pulegone) used in the identification of the target compounds were obtained from Fluka (Switzerland). For all of them, both enantiomers were individually provided (99% enantiomeric purity). Methanol used to prepare the standard solutions was purchased by Labscan Ltd. (Dublin, Ireland). A standard solution of 0.6 mg approximately of each compound in 10 mL of methanol was employed to optimize the chromatographic separation of all compounds of interest.

*M. pulegium* L. from six different commercial brands (samples 1–6) and a *Mentha piperita* L. sample (sample 7) were used as powders and

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as infusions to perform the analyses. According to the label designation, the composition and expiration dates of these samples were as follows: (sample 1) 95% *M. piperita* L. and 5% *M. pulegium* L., December 2009; (sample 2) no information on composition, May 2008; (sample 3) 88% *M. piperita* L. and 12% *M. pulegium* L., July 2007; (sample 4) 100% *Mentha* (unspecified), March 2007; (sample 5) 100% pure *M. pulegium* L., February 2008; (sample 6) 100% pure *M. pulegium* L., May 2008; (sample 7) 100% pure *M. piperita* L., no information on the expiration date.

All these samples were purchased from the supermarket in bags (approximately 1.3 g of powder in each one), except sample 6, which was obtained as a 40 g bag of dried plant, and sample 7, which was provided as a 2.0 g bag of dried material.

The infusions were simply prepared by pouring 200 mL of boiling water and the contents of each bag (in the case of sample 6, 1.3 g was weighed) into a mug followed by 5 min of settling time. The temperature reached by the brew after this time was approximately 60 °C.

**Extraction.** The isolation of the compounds of interest was carried out by solid-phase microextraction (SPME). A poly(dimethylsiloxane)-coated SPME fiber (film thickness 100  $\mu\text{m}$ ) installed in an SPME fiber for manual use (Supelco, Madrid, Spain) was used to accomplish the extractions. A 0.1 g mass of each herb was placed into a 5.0 mL vial which was sealed with 3 cm of plastic film with characteristics suitable for the SPME (i.e., low water permeability and insensitivity to moisture vapor and the most common reagents). The vial was subsequently heated at 60 °C for a few minutes prior to the extraction to enrich and stabilize the sample headspace in the compounds of interest. Finally, the extraction of the volatiles was performed by exposing the fiber to the headspace of the dried material for 10 min at 60 °C. These conditions were selected as a result of testing several values of extraction time and temperature, as explained in the Results and Discussion.

In the case of the infusions, a 5.0 mL volume of each brew was placed in a 10 mL vial. Afterward, the same extraction procedure described above was followed for all samples. Additionally, the SPME of sample 5 was performed by sinking the fiber into the liquid sample for comparison. In all instances, the samples (powders and infusions) were analyzed as specified below.

**Gas Chromatography–Mass Spectrometry Analysis.** A Hewlett-Packard model 6890 gas chromatograph coupled to an Agilent 5989A quadrupole instrument (Palo Alto, CA) and fitted with both a split/splitless injector and a flame ionization detector (FID) was used. To accomplish the GC separations, two different fused silica columns were used: a 25 m  $\times$  0.25 mm i.d. capillary column coated with a 0.25  $\mu\text{m}$  layer of permethylated  $\beta$ -cyclodextrin (Chirasil- $\beta$ -Dex, Chrompack) and a 30 m  $\times$  0.25 mm i.d. capillary column coated with a 0.25  $\mu\text{m}$  layer of 2,3-diacetoxy-6-*O*-(*tert*-butyldimethylsilyl)- $\gamma$ -cyclodextrin (Restek). The GC column was initially programmed at 4 °C/min from 45 °C (5 min) to 100 °C (3 min), subsequently at 2 °C/min to 125 °C, and finally at 6 °C/min to 180 °C (5 min) for both chromatographic columns. Helium was used as the carrier gas at a constant flow of 1 mL/min. The fiber desorption was carried out at 250 °C for 5 min. The injector was operated in either the splitless mode or the split mode by setting various split ratios (1:10, 1:25, 1:50, and 1:100) according to the sensitivity required. The source and the quadrupole temperatures were set at 230 and 100 °C, respectively. Data acquisition from the mass spectrometer was accomplished with the HP-ChemStation system. In all instances the volatile compounds in the samples were mainly identified by matching the obtained mass spectra with those provided by the Wiley library. Some peak identities were additionally confirmed by comparison with the mass spectrum and retention time data provided by the standards run under the same experimental conditions. The chromatograms resulting from the detection by FID (operated at 250 °C) were also recorded. The extraction and GC analysis of all samples were carried out at least in duplicate.

## RESULTS AND DISCUSSION

As previously commented the experimental conditions used to extract the volatile constituents from both plants and infusions of *M. pulegium* L. were selected as a result of combining distinct

extraction temperatures (50, 60, and 70 °C) and fiber exposure times (2, 5, 10, and 15 min). This combination was accomplished as follows: First, the lowest extraction temperature (50 °C) was tested at all exposure times. As a result, 15 min was directly ruled out since the peak areas obtained were negligible. Second, 60 and 70 °C were combined with the other exposure times (2, 5, and 10 min). The choice of the extraction conditions was always based on those values that provided the highest areas of all peaks by SPME–GC. Finally, 60 °C and 10 min were selected as the best experimental conditions since for most components temperatures higher than 60 °C resulted in the complete loss of the target compounds and extraction times shorter than 10 min did not provide acceptable peak areas. The SPME fiber (PDMS, film thickness 100  $\mu\text{m}$ ) used to accomplish the extraction of the volatile compounds was selected on the basis of data reported in the literature on the extraction of volatile components from various matrixes (13, 14) as well as of our own experience in the isolation of volatile constituents from plants (15). As mentioned in the Materials and Methods, the experimental conditions selected (60 °C, 10 min) were initially applied to the SPME of the headspace corresponding to all powders. Subsequently, these same values were applied to both the headspace and liquid phase of sample 5 infusion for comparison. As a result of this latter experiment, the volatile compositions of the solution and dry plant were extremely similar. For that reason, the results will only refer to the sample headspace hereafter.

The repeatability of the proposed method was estimated by measuring the relative standard deviation (RSD) for all studied compounds from three replicates of all samples using the optimum experimental conditions. In all instances, the values ranged from 0.8% to 14.9% for the dried plant samples and from 5.8% to 18.0% for the infusions, depending on the specific compound considered.

By applying the above-mentioned experimental conditions, a similar qualitative composition was found for all samples studied. Specifically, menthol, pulegone, and menthone were the main components in most samples, whereas  $\alpha$ -pinene,  $\beta$ -pinene, limonene, and neomenthol occurred as minor compounds. Regarding the quantitative composition, **Table 1** represents the relative proportion of each compound with respect to the total sum of the compounds included in this study in *M. pulegium* L. dried plant material and in the infusions obtained thereof. The extraction conditions applied in the analyses were those selected as more advantageous (60 °C during 10 min) while permethylated  $\beta$ -cyclodextrin was initially used as the stationary phase. As can be observed,  $\beta$ -pinene could not be properly determined in any plant under these experimental conditions due to its overlapping with other matrix components. As mentioned above, menthol, pulegone, and menthone, in that order, were, by far, the major components in samples 1–5, whereas pulegone and, to a lesser extent, neomenthol seemed to be the most representative compounds in sample 6. Besides, a particularly remarkable aspect in this last sample was the utter absence of menthol. The occurrence of pulegone as the most characteristic compound indicates that sample 6 was actually the only pure *M. pulegium* L. sample (3–6). On the other hand, from the composition of the samples (**Table 1**), the presence of a considerable proportion of *Mentha* other than *M. pulegium* L. in samples 1–5 was clear. This was not surprising in samples 1 and 3 as the existence of high proportions of *M. piperita* L. was specified on the label designation. However, it was unexpected in samples 2 and 4, in which no information on the composition was mentioned, and, particularly, in sample 5,

**Table 1.** Relative Proportion (%) of Each Compound with Respect to the Total Sum of the Compounds Identified by SPME–GC–MS in *M. pulegium* L. Dried Plant Material and in the Infusions Obtained from Them by Using Permethyated  $\beta$ -Cyclodextrin<sup>a</sup>

compound	sample 1		sample 2		sample 3		sample 4		sample 5		sample 6	
	P <sup>b</sup>	I <sup>c</sup>	P	I	P	I	P	I	P	I	P	I
$\alpha$ -pinene	0.076	nd <sup>d</sup>	0.02	nd	0.03	nd	0.02	nd	0.04	nd	0.14	nd
$\beta$ -pinene	ov <sup>e</sup>	0.2	ov	0.14	ov	0.06	ov	0.14	ov	0.34	ov	nd
limonene	0.27	nd	0.07	nd	0.06	0.06	0.12	0.08	0.15	n.d.	0.43	0.086
menthone	15.51	15.7	14.80	30.0	21.84	24.04	37.59	29.4	10.91	17.4	1.56	1.87
pulegone	21.12	2.3	25.83	3.11	24.02	12.2	14.34	2.57	21.50	2.87	78.91	97.42
neomenthol	2.49	6.5	4.22	2.74	12.66	3.8	2.81	6.11	5.19	5.25	18.94	0.58
menthol	61.45	75.3	55.05	63.97	41.31	59.8	45.12	43.3	62.21	74.09	nd	nd

<sup>a</sup> Values corresponding to the sum of both Enantiomers when present. <sup>b</sup> Sample as dried plant material. <sup>c</sup> Sample as infusion. <sup>d</sup> Not detected. <sup>e</sup> Overlapped with interferences.

**Table 2.** Enantiomeric Excess (%) and Predominant Enantiomer of Chiral Volatile Compounds Identified by SPME–GC–MS in *M. pulegium* L. Dried Plant Material and in the Infusions Obtained from Them by Using Permethyated  $\beta$ -Cyclodextrin as the Stationary Phase

compound	sample 1		sample 2		sample 3		sample 4		sample 5		sample 6	
	P <sup>a</sup>	I <sup>b</sup>	P	I	P	I	P	I	P	I	P	I
(+)- $\alpha$ -pinene <sup>c</sup>	<5	nd <sup>d</sup>	<5	nd	6.0	nd	7.6	nd	9.9	nd	23.3	nd
(-)- $\beta$ -pinene	ov <sup>e</sup>	100	ov	100	ov	100	ov	100	ov	100	ov	nd
(-)-limonene	<5	nd	<5	nd	27.4	100	52.0	100	31.8	nd	6.1	100
(+)-pulegone	100	100	100	100	99.3	100	100	100	98.9	100	99.8	100
(+)-neomenthol	94.9	100	98.5	100	98.1	100	97.2	100	96.7	100	91.2	100
(-)-menthol	100	100	100	100	100	100	100	100	100	100	nd	nd

<sup>a</sup> Sample as dried plant material. <sup>b</sup> Sample as infusion. <sup>c</sup> Predominant enantiomer in brackets. <sup>d</sup> Not detected. <sup>e</sup> Overlapped with other matrix components.

which was labeled as 100% pure *M. pulegium* L. In fact, the use of *M. piperita* L. instead of *M. pulegium* L. is relatively frequent in the industry owing to its pleasant aroma and lower economic cost. Nevertheless, we considered it interesting to include a pure *M. piperita* L. sample (sample 7) in our study to confirm the presence of this herb in samples 1–5. This specific analysis was performed in the same experimental conditions as those used for *M. pulegium* L. samples. As a result, a qualitative profile comparable to that provided by samples 1–5 was obtained, menthol (70.8%) and menthone (24.5%) being the major components. The similarity between these plants confirms in short the occurrence of *M. piperita* L. in all *M. pulegium* L. samples studied, except in sample 6. In this respect, it is important to keep in mind that both herbs are used for different purposes. Whereas *M. piperita* L. is generally used as a flavoring agent, the main application of *M. pulegium* L. is found in its effectiveness as a digestive tonic. This reflects the need for the control of the composition of commercial herbs to guarantee their adequate application.

As far as the infusions are concerned, qualitative and quantitative differences in the aromatic composition of the brew and the plant used in its preparation were observed. First, in contrast to the dry herb samples,  $\beta$ -pinene could be certainly determined as no overlapping was observed. Second,  $\alpha$ -pinene and limonene either did not occur or were present at extremely low levels. In addition, some extra compounds were identified in the infusion. Specifically, sabinene,  $\alpha$ -terpinene, 1,8-cineole, 3-octanol, and linalool were detected in most samples, although these compounds were not further considered. In any case, the most remarkable variation in samples 1–5 between plants and infusions was found in pulegone, whose content decreased so drastically during the preparation of the infusion that it became a minor compound. With regard to menthol, its content not only remained constant but also increased slightly in most cases. From these results and taking into account the hepatotoxicity of pulegone, samples 1–5 might be, in principle, more recommendable as an infusion than as a powder.

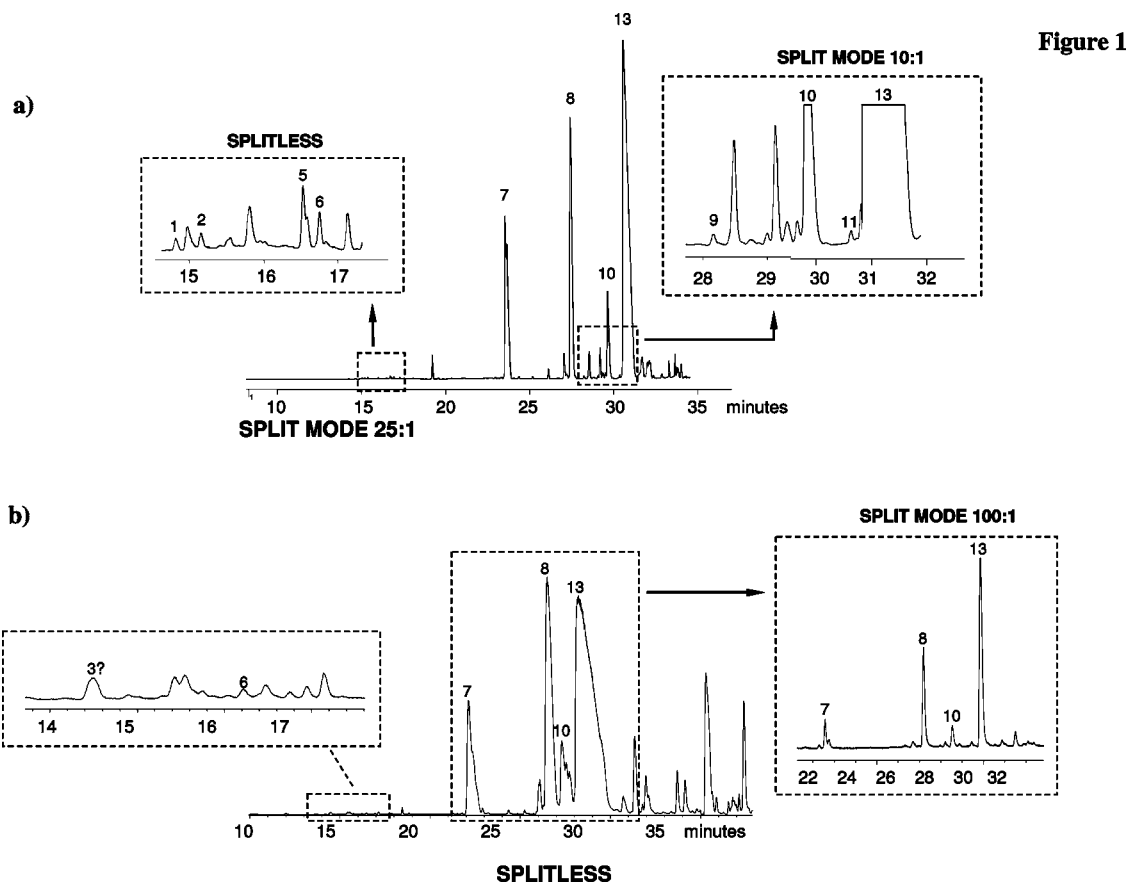
In contrast to samples 1–5, sample 6 did not show such a notable modification in its composition by adding hot water to the dry material. In fact, the most relevant aspect of this sample was the substantial decrease of neomenthol. A slight increase in the pulegone level was also observed. Consequently, except a small change in the sensory perception, the properties of sample 6 did not appear to be affected by its use either as an infusion or as a dry herb.

All changes observed in the volatile constituents during the preparation of the infusion are reasonable as the isolation of the compounds from the dry plant implies exclusively their release and subsequent retention according to their volatility and affinity for the SPME fiber, whereas the determination of the compounds in the infusion involves, prior to the release from the matrix, the extraction of the compounds with boiling water.

All in all, although no control of the dehydration treatment used (temperature/duration) or the subsequent storage conditions of the samples included in this study was carried out, the results found in the present work allow us to gain insight into the way the boiling water can affect the aromatic fraction of herbs and, as a consequence, modify their properties.

Table 2 depicts the enantiomeric excess of the compounds identified by SPME–GC–MS and separated into their enantiomeric pairs on the Chirasil- $\beta$ -Dex column in *M. pulegium* L. dry plants and infusions. In all cases, the enantiomeric excesses were calculated from peak areas obtained from the FID signals, and the excess of the predominant enantiomer was expressed as a percent, i.e., [(predominant enantiomer – minor enantiomer)/(predominant enantiomer + minor enantiomer)]  $\times$  100.

It is worth pointing out that this stationary phase did not allow menthone to be enantiomerically resolved. This fact, together with, as previously mentioned, the overlapping of  $\beta$ -pinene observed in all plants, made it necessary to additionally accomplish the analyses by using an alternative chiral stationary phase. The results of this latter study are briefly commented on later. As listed in Table 2, some of the studied compounds occurred at very high enantiomeric purity. Specifically, (-)- $\beta$ -



**Figure 1.** SPME–GC–MS analyses of volatile compounds in the headspace of dried plant material of (a) sample 5 (labeled as 100% *M. pulegium* L.) and (b) sample 7 (labeled as 100% pure *M. piperita* L.) by using permethylated  $\beta$ -cyclodextrin. See the text for further details. Peak identification is as follows: (1) (–)- $\alpha$ -pinene, (2) (+)- $\alpha$ -pinene, (3) (+)- $\beta$ -pinene, (4) (–)- $\beta$ -pinene, (5) (–)-limonene, (6) (+)-limonene, (7) ( $\pm$ )-menthone, (8) (+)-pulegone, (9) (–)-pulegone, (10) (+)-neomenthol, (11) (–)-neomenthol, (12) (+)-menthol, (13) (–)-menthol.

**Table 3.** Enantiomeric Excess (%) and Predominant Enantiomer of Chiral Volatile Compounds Identified by SPME–GC–MS in *M. pulegium* L. Dried Plant Material and in the Infusions Obtained from Them by Using 2,3-Diacetoxy-6-*O*-(*tert*-butyldimethylsilyl)- $\gamma$ -cyclodextrin as the Stationary Phase

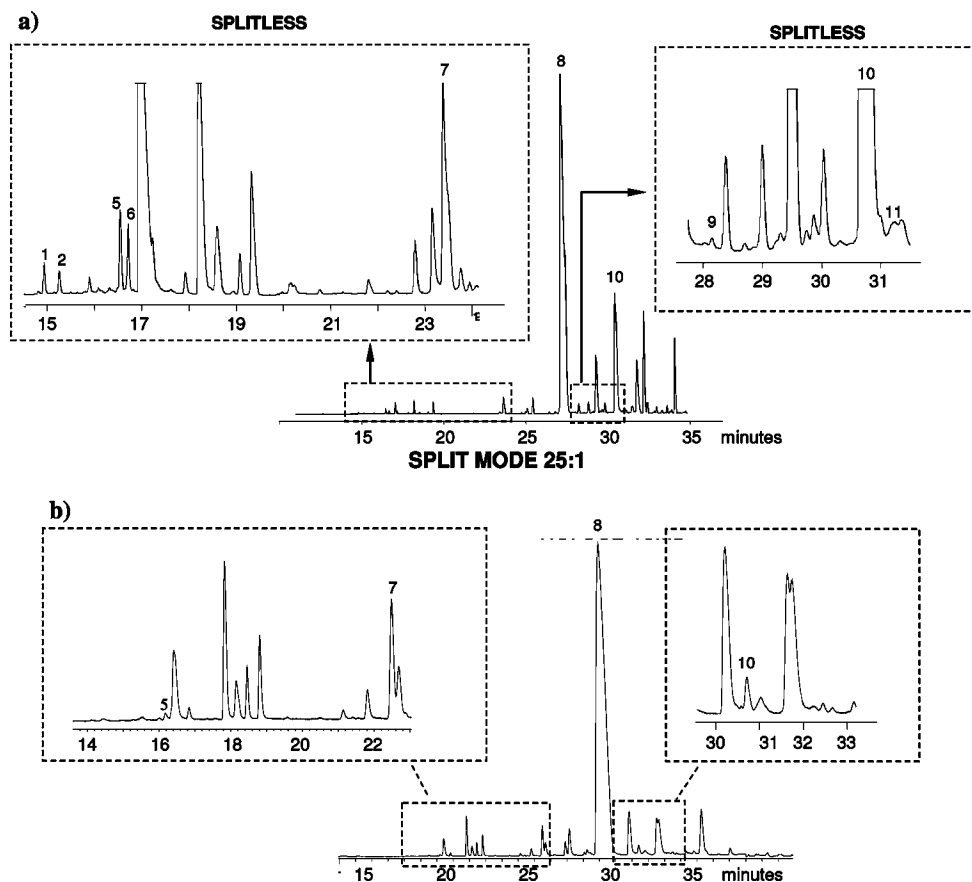
compound	sample 1		sample 2		sample 3		sample 4		sample 5		sample 6	
	P <sup>a</sup>	I <sup>b</sup>	P	I	P	I	P	I	P	I	P	I
(+)- $\alpha$ -pinene <sup>c</sup>	6.7	nd <sup>d</sup>	nd	2.1	3.0	nd	5.9	nd	0.5	nd	28.9	28.9
(–)- $\beta$ -pinene	4.1	100	nd	100	10.6	100	18.8	100	6.0	100	2.9	2.9
(–)-menthone	100	100	100	100	100	100	100	100	100	100	100	100
(+)-pulegone	100	100	100	100	100	100	100	100	100	100	100	100
(+)-neomenthol	94.0	nd	95.5	100	94.3	100	94.4	97.3	98.2	100	94.3	100
(–)-menthol	100	100	100	100	100	100	100	100	100	100	nd	nd

<sup>a</sup> Sample as dried plant material. <sup>b</sup> Sample as infusion. <sup>c</sup> Predominant enantiomer in brackets. <sup>d</sup> Not detected.

pinene, (+)-pulegone, (+)-neomenthol, and (–)-menthol were present practically as pure enantiomers. On the contrary, for  $\alpha$ -pinene and limonene, both enantiomers could be detected with different enantiomeric excesses according to the specific sample considered. The occurrence of both enantiomers of  $\alpha$ -pinene is in accordance with results previously found in our laboratory (15). In fact, the presence of both enantiomers in the same plant is relatively common for monoterpenes, and it is indicative of the existence of two different sets of stereospecific enzymes in the plant (16). It is also interesting to emphasize that the enantiomeric composition of  $\alpha$ -pinene varied within a reasonably narrow range with the plant studied. This small variation is probably due to the different proportion of *M. piperita* L. in each *M. pulegium* L. sample as a consequence of the occurrence of (+)- and (–)-enantiomers of  $\alpha$ -pinene in both *M. pulegium* L. and *M. piperita* L. herbs as minor components (15). On the

other hand, it is worth mentioning the tendency to form the pure (–)-limonene as a consequence of adding hot water to the dry herb for samples 3, 4, and 6. This can be explained by the effect of the high temperature used during the procedure. In any case, in spite of the difference in the aroma described for (+)- and (–)-limonene (12), the minor proportion of this compound in the samples studied along with the strong flavor shown by the major components (i.e., menthol, menthone, and pulegone) makes it quite unlikely to observe any alteration in the sensorial perception of *M. pulegium* L. plants and infusions owing to the presence of one or another enantiomer.

As an example, Figure 1 shows the chromatograms obtained from the SPME–GC–MS analyses of (a) sample 5 and (b) sample 7 on the permethylated  $\beta$ -cyclodextrin column. Both chromatograms were recorded at the same full range. As can be seen, the qualitative and quantitative compositions of the



**Figure 2.** SPME–GC–MS analyses of volatile compounds in the headspace of sample 6 (labeled as 100% pure *M. pulegium* L.) as (a) the plant material and (b) the infusion by using permethylated  $\beta$ -cyclodextrin. See the text for further details. Peak identification is as in **Figure 1**.

two samples were similar, even when sample 5 was labeled as pure *M. pulegium* L. As previously mentioned, (–)-menthol, (+)-pulegone, ( $\pm$ )-menthone, and (+)-neomenthol were the chief components. It is important to mention that menthone could not be separated into its enantiomers by using permethylated  $\beta$ -cyclodextrin. Likewise, the quantitative differences between the minor and major compounds made it necessary to use distinct split ratios to reliably establish the identity and absolute areas of all compounds detected. This aspect has been reflected in **Figure 1**.

Similarly, **Figure 2** illustrates the chromatograms resulting from the SPME–GC–MS analyses of sample 6 (a) as the dried plant material and (b) as the infusion. Both chromatograms were recorded at the same full range. Once more, different split ratios were required to determine the minor and major compounds.

As mentioned above, to confirm the results obtained by using Chirasil- $\beta$ -Dex as well as evaluate the enantiomeric composition of  $\beta$ -pinene and menthone, a chiral phase other than permethylated  $\beta$ -cyclodextrin was in addition employed. In particular, the phase used was 2,3-diacetoxy-6-*O*-(*tert*-butyldimethylsilyl)- $\gamma$ -cyclodextrin, as detailed in the Materials and Methods. With respect to the composition of the samples, the results obtained on Chirasil- $\beta$ -Dex were confirmed since similar qualitative and quantitative profiles were obtained in all samples. Concerning the enantiomeric excesses, **Table 3** displays the enantiomeric composition of the studied compounds separated into their enantiomeric pairs on the  $\gamma$ -cyclodextrin column in *M. pulegium* L. plants and infusions. The main aspects to be highlighted are that, on one hand, menthone could be on this column successfully separated into its enantiomers in such a way that the occurrence of just (–)-menthone was established in all cases and, on the other hand, the enantiomeric composition of

$\beta$ -pinene could be certainly determined in plants and infusions as no overlapping was found by running the samples on the  $\gamma$ -cyclodextrin column. Finally, data on the enantiomeric distribution of limonene provided by  $\beta$ -cyclodextrin could not be confirmed on the  $\gamma$ -cyclodextrin column as this latter column did not enable limonene enantiomers to be resolved.

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