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Natural variability of the enantiomeric composition of bioactive chiral terpenes in *Mentha piperita*

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Abstract

The variability of the enantiomeric distribution of biologically active chiral terpenes in *Mentha piperita* plants from different geographical origins was evaluated by solid phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS). The optimisation of some parameters (i.e. exposure temperature, extraction time and type of fibre) affecting SPME-extraction enabled relative standard deviations ranging from 4 to 13% to be achieved. The use of two different chiral stationary phases allowed to separate the identified chiral terpenes into their corresponding enantiomers as well as to verify the enantiomeric excesses of those compounds which were enantiomerically resolved on both phases. For all chiral terpenes, the enantiomeric composition varied within a very narrow range all over the samples. Consequently, it may be stated that the enantiomeric composition of chiral terpenes in *Mentha piperita* appears to be independent of the geographical origin of the plant and, thus, any alteration in the characteristic value may be related to an adulteration or inadequate sample handling. These results support the usefulness of the enantiomeric composition of bioactive chiral terpenes in *Mentha piperita* in authenticity studies.

Keywords: *Mentha piperita*; Terpenes; Chiral; Enantiomer separation

1. Introduction

Aromatic plants are at present widely studied for their large therapeutical interest and benefits. These benefits depend largely on some “active principles” which, in general terms, occur in all herbs [1]. In this regard, it is well known that plants contain a complex mixture of bioactive compounds covering a number of demands for the human health. Among these compounds, terpenoids have shown special interest over the last few years due to the great variety of biological properties displayed by most of them.

Specifically, menthol exhibits numerous pharmacological activities including anti-inflammatory, analgesic, antifungal and central nervous system excitation effects [2]. This monoterpene is also the responsible for the typical minty smell and flavour shown by some commercial products. Menthol is mainly obtained from *Mentha piperita* and *Mentha arvensis*, which contain up to 50 and 70% menthol, respectively [3], along with minor quantities of other terpenes that also possess biological properties. These plants belong to *Labiatae* family and their pleasant aroma makes them useful as flavouring agents in the food industry. In fact, the extracts obtained from *Mentha* species are nowadays extensively used in the manufacture of a wide range of products such as confectionery, candy, alcoholic and non-alcoholic beverages and chewing gum.

On the other hand, the interest of the consideration of stereochemical aspects in analytical chemistry, in general, and in food analysis, in particular, has been widely demonstrated [4,5]. Differences in sensory properties and biological activities between pairs of enantiomers have already been published in the literature [6]. Specifically for menthol, some researchers have reported that only (−)-menthol, of the eight stereoisomers of menthol (i.e., (+/−)-menthol, (+/−)-neoisomenthol, (+/−)-neomenthol and (+/−)-isomenthol), exhibits the greatest cooling activity as well as the typical peppermint odour [7,8]. It is also interesting to point out that many of the chiral components that occur in nature are enantiomeric mixtures formed by specific proportions because of the enzymatic pathways involved in their biogenesis. The knowledge of the characteristic ratio in which both

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enantiomers are naturally present may be used as a tool for quality control purposes as any alteration in this ratio can be associated with an adulteration or with a specific technological process. Actually, the addition of racemic mixtures of chiral compounds, such as menthol, to plant extracts is relatively frequent in the industry to enhance the natural flavour of some foodstuffs and, thus, the consumer acceptance.

Considering the different biological activity shown by some enantiomeric pairs of certain chiral compounds, the addition of both enantiomers in the same proportion implies not only the adulteration of the sample and, therefore, the consequent economic fraud but also a risk for consumer health as the undesirable enantiomer may possess unknown biological activity.

In this context, it is evident the necessity for establishing whether the enantiomeric ratio for a chiral compound may vary depending on different factors, such as geographical origin and climatic conditions, as the variability of natural products might make the enantiomeric ratio insufficient for authenticity control.

Regarding sample preparation, solid-phase microextraction (SPME) technology has already demonstrated to be a valuable alternative to conventional techniques for different matrices and applications including the determination of the volatile terpenoid composition from plant material [9]. As far as the stereochemical analysis is concerned, it has also been occasionally reported that SPME offers the possibility of isolating selectively the chiral constituents from complex matrices under non-racemization conditions [10–12].

Although a number of articles on the enantiomeric composition of some menthol isomers have previously been published by other authors [13–15], in most cases the reported studies refer to essential oils from *Mentha* species instead of to the plant material. Moreover, multidimensional chromatographic techniques have been so far mainly applied [15], thus demanding a careful and sometimes complex optimisation to be done before the analysis can be performed.

Taking into account, on the one hand, that the manufacture of essential oils can, on certain occasions, lead to the racemization of chiral compounds and that, on the other, SPME is a far more accessible and simpler methodology than coupled analytical techniques, the goal of this study was to prove the usefulness of the enantiomeric composition of bioactive chiral terpenes, particularly menthol, in *Mentha piperita* plants as an authenticity parameter by using SPME–GC–MS. For that purpose, the variability of the enantiomeric ratio of these compounds in samples from different geographical origins was evaluated.

2. Experimental

2.1. Samples and materials

All chemicals used in the confirmation of some identities were supplied by Sigma–Aldrich (Dorset, UK) except sabinene and thujene, which were provided by Extrasynthese (Genay, France). Sabinene, thujene, α-phellandrene and α-terpineol standards were obtained as racemic mixtures whereas for menthol, menthone, neomenthol, limonene, α-pinene and β-pinene, both pure enantiomers were separately purchased. In the case of isomenthol and β-*trans*-caryophyllene, only the (+)- and (−)-enantiomer, respectively, could be obtained (99% enantiomeric purity for both of them). However, even in "highly pure" standards small amounts of the other enantiomer could be clearly detected.

Mentha piperita from different geographical origins was used as dried plant material. Also, direct analysis of leaves taken from the living plant was occasionally performed. All samples were obtained from the supermarket and from the industry.

2.2. Solid-phase microextraction

An SPME holder (Supelco, Madrid, Spain) was used to accomplish the experiments. A fused silica fibre coated with a 100 μm layer of polydimethylsiloxane (PDMS) was used to trap the terpenes released from the plants. A 0.05 g weight, approximately, of each herb was placed into a 5.0 ml vial, which was sealed with a plastic film. The vial was subsequently heated at 40 °C for 10 min prior to the extraction to enrich and stabilize the sample headspace in the compounds of interest. Finally, the extraction of the volatile terpenes was performed by exposing the fibre to the headspace of the sample for 15 min at 40 °C.

2.3. Gas chromatography–mass spectrometry analysis

A Hewlett-Packard Model 6890 gas chromatograph fitted with a split/splitless injector and coupled to an Agilent 5989A quadrupole instrument (Palo Alto, CA) was used. The GC separation was first performed on a 25 m × 0.25 mm i.d. fused silica column coated with a 0.25 μm layer of permethylated β-cyclodextrin (Chirasil-β-Dex, Chrompack) and, subsequently, on a 30 m × 0.25 mm i.d. fused silica column coated with a 0.25 μm layer of 2,3-di-acetoxy-6-*O*-*tert*-butyl dimethylsilyl γ-cyclodextrin (Restek). Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The GC-column was programmed at 4 °C/min from 45 °C (5 min) to 100 °C (3 min), subsequently at 2 °C/min up to 125 °C and finally at 6 °C/min to 180 °C (5 min) when permethylated β-cyclodextrin was used and at 5 °C/min from 40 °C (5 min) to 120 °C, subsequently at 3 °C/min to 135 °C/min and finally at 5 °C/min up to 180 °C when 2,3-di-acetoxy-6-*O*-*tert*-butyl dimethylsilyl γ-cyclodextrin was employed. The fibre desorption was carried out at 250 °C for 5 min while the injector was operated in either the splitless mode or the split mode according to the sensitivity required. The source and the quadrupole temperatures were set at 230 °C and 100 °C, respectively. In all cases identifications of terpenes in samples were based on comparisons of mass spectra

with those provided by the Wiley library. When necessary, further confirmation of some peak identities was performed using both the mass spectra and retention time data provided by the standards run under the same experimental conditions. Data acquisition from the MS was performed with the HP-ChemStation system.

Additionally, the chromatograms resulting when using a flame ionization detector (FID) operated at 250 °C to monitorize the enantiomeric separation of the chiral terpenes were also recorded.

3. Results and discussion

The experimental conditions used during the SPME extraction procedure (40 °C, 15 min, 100 μm layer of PDMS) were selected as a result of the optimisation of the method in which several exposure temperatures (30, 40, 50 °C), extraction times (1, 5, 15, 25 min), and SPME-fibres (100 μm layer of polydimethylsiloxane, 65 μm layer of PDMS/DVB and 85 μm layer of polyacrylate) were tested. Initially, the PDMS fibre was used on the basis of reported data in the literature on its usefulness to extract terpenes from different matrices [16,17]. Subsequently, each exposure temperature was combined with all studied extraction times, to select those values that provided the highest peak areas for the target compounds. As a result, 40 °C and 15 min were chosen as extraction temperature and time, respectively. Although the obtained results allowed to determine reliably the terpenic composition of *Mentha piperita*, two different SPME-fibres (65 μm layer of PDMS/DVB and 85 μm layer of polyacrylate) were additionally tested under the selected temperature and time conditions in order to evaluate their performance. The choice of the last two mentioned fibres was based on our previous experience in the extraction of terpenes from different matrices, other than plants [18,19] and on the theoretical adequacy of polyacrylate fibre for terpenic compounds, respectively. However, neither of these fibres provided higher peak areas than those obtained when PDMS was utilised.

The repeatability of the headspace sampling technique was estimated by measuring the relative standard deviation for all studied compounds from three replicates of all samples using the optimum experimental conditions. The value ranged from 4.2 to 13.1%.

As previously mentioned in the Experimental section, the SPME–GC–MS analysis of the samples considered in this study was performed on two different chiral stationary phases to increase the reliability of the obtained results. Table 1 outlines the terpenes identified by SPME–GC–MS in *Mentha piperita* plants from different locations using permethylated β-cyclodextrin as a chiral phase. The obtained qualitative terpene profile was practically identical for all *Mentha piperita* plants considered regardless of their geographical origin. On the contrary, quantitative differences in the terpene level were apparent depending on the origin of the plant. The identification of β-myrcene, γ-terpinene, 1,8-cineole, isomenthone and menthyl acetate was tentative as it was solely based on MS data. However, for the rest of the compounds (namely, (+)-α-thujene, (−)-α-pinene, (+)-sabinene, (+)-α-pinene, (+)-α-phellandrene, (+)-limonene, (+)-β-pinene, (−)-β-pinene, menthone, (+)-α-tepineol, neomenthol, isomenthol, menthol, and (−)-β-*trans*-caryophyllene) retention times of standards were also used to confirm the identities. The elution order for racemic mixtures was obtained from previous studies and from the literature. To illustrate the differences existing in terpene levels among the samples considered, data in Table 1 are expressed as the relative amount (%) of each terpene with respect to the total peak area sum in each chromatogram of the analysed chiral terpenes obtained from the signal recorded by the FID. This information was sufficient for the purpose of comparison of enantiomeric excesses within plants from different locations. As expected [20], menthone, menthol and menthyl acetate, followed by 1,8-cineole, were the major constituents in most cases. A clear exception was found in the sample from Gerona in which the terpene quantitative profile was substantially different to that showed by the rest of the samples. In this regard, the presence of (+)-limonene (33.37%) and 1,8-cineole (30.75%) as major components was particularly remarkable. This considerable difference is most likely due to the fact that this was the only sample that was directly taken from the living plant instead of using the dried plant material. As a consequence, it was more difficult the reliable extraction of the volatile compounds by SPME due to the greater moisture in the sample headspace.

As an example, Fig. 1 shows the chromatogram resulting from the SPME–GC–MS analysis using permethylated β-cyclodextrin as the chiral phase of a *Mentha piperita* plant from Soria under the optimised experimental conditions. In spite of the complexity of the sample, the method proposed allowed us to identify reliably the main constituents in *Mentha piperita* as well as to separate the minor chiral terpenes into their corresponding enantiomers. As can be seen, some extra components were also extracted and subsequently analysed by SPME. Table 2 depicts the enantiomeric excesses of the minor chiral terpenes identified by SPME–GC–MS in *Mentha piperita* plants from different geographical origins and separated into their enantiomeric pairs on the Chirasil-β-Dex column. In all cases, the enantiomeric excesses were calculated from peak areas obtained from the FID signals and excess of predominant enantiomer was expressed as a percent, i.e.: [(predominant enantiomer − minor enantiomer)/(predominant enantiomer + minor enantiomer)] × 100. As listed in Table 2, all minor chiral terpenes in *Mentha piperita* enantiomerically resolved on permethylated β-cyclodextrin occurred as the pure (+)-enantiomer, except β-*trans*-caryophyllene, whose (+)-enantiomer could not be detected, and α- and β-pinene, which exhibited characteristic ratios of both (+)- and (−)-enantiomers. The occurrence of both enantiomers of a single compound has been described as common for monoterpene hydrocarbons and, on the contrary, as unusual for sesquiterpenes [21]. As β-*trans*-caryophyllene, the only sesquiterpene in Table 2, occurred as

Table 1
Relative amounts (%) of each terpene with respect to the total sum of the volatile terpenes identified by SPME–GC–MS in *Mentha piperita* plants from different geographical origins by using permethylated β-cyclodextrin as the stationary phase

Terpenes	Geographical origins							
	Unknown1	Barcelona (Spain)	Gerona (Spain)	Italia	Madrid (Spain)	Unknown2	León (Spain)	Soria (Spain)
(+)-α-Thujene	n.d.[a]	0.10	n.d.	n.d.	0.07	n.d.	0.08	n.d.
β-Myrcene	n.d.	0.63	9.83	n.d.	0.13	0.34	0.06	0.12
(−)-α-Pinene	0.51	0.15	0.75	0.12	0.20	0.20	0.27	0.20
(+)-Sabinene	0.40	0.34	7.21	0.11	0.19	0.29	0.32	0.26
(+)-α-Pinene	0.54	0.16	1.05	0.11	0.18	0.18	0.25	0.22
(+)-α-Phellandrene	n.d.	0.39	2.10	0.25	0.11	0.16	0.11	0.21
(+)-Limonene	0.47	1.24	33.37	0.37	0.73	0.89	1.00	2.77
(+)-β-Pinene	0.79	0.17	1.87	0.21	0.29	0.27	0.10	0.23
(−)-β-Pinene	0.78	0.14	1.83	0.24	0.31	0.32	0.12	0.22
γ-Terpinene	0.26	0.09	n.d.	0.18	0.13	0.01	0.20	0.07
1,8-Cineole	16.75	6.36	30.75	4.47	4.22	4.59	6.95	6.07
Menthone	20.35	58.83	1.03	42.37	56.46	56.06	9.39	32.71
Isomenthone	1.15	1.80	n.d.	0.22	0.91	0.52	3.47	0.23
Menthyl acetate	32.39	7.87	2.51	7.02	7.77	10.21	46.65	14.19
(+)-α-Terpineol	0.66	0.11	1.72	0.20	0.22	0.48	0.43	0.21
Neomenthol	6.97	0.26	n.d.	5.01	2.37	2.68	4.09	3.55
Isomenthol	6.65	0.72	0.93	5.04	0.69	0.37	7.10	0.21
Menthol	8.56	19.10	1.19	31.51	24.91	22.41	17.67	36.6
(−)-β-*trans*-Caryophyllene	2.72	1.47	3.81	2.55	0.04	0.17	1.55	1.93

[a] n.d.: not detected.

the pure (−)-enantiomer whereas α- and β-pinene showed mixtures of both enantiomers, it can be stated that the results of this study are in accordance with those previously reported. Furthermore, the presence of both enantiomers in the same plant is indicative of the existence of two different sets of stereospecific enzymes. On the other hand, it is also convenient to underline that the enantiomeric ratios found for α- and β-pinene were considerably low (<10%) in most cases. Although this fact might, in principle, seem surprising, similar enantiomeric purities for α-pinene have been equally reported in earlier studies in essential oils [21,22]. It is also worth mentioning that the predominant enantiomer for α- and β-pinene could not be reliably established due to the similar proportion in which both enantiomers occurred. In any case, it is important to emphasize that the enantiomeric excess for each terpene was similar in all samples (except, as already mentioned, in *Mentha piperita* from Gerona) and, hence, independent of the geographical origin. From the results obtained from the analysis on permethylated β-cyclodextrin, it may be initially concluded that the enantiomeric composition of the minor chiral terpenes in *Mentha piperita* appeared to be naturally invariable.

In contrast to the minor terpenes, the enantiomeric pairs of the major components (menthol and related compounds) could not be properly resolved on the permethylated β-cyclodextrin column used due to its lack of enantioselectivity

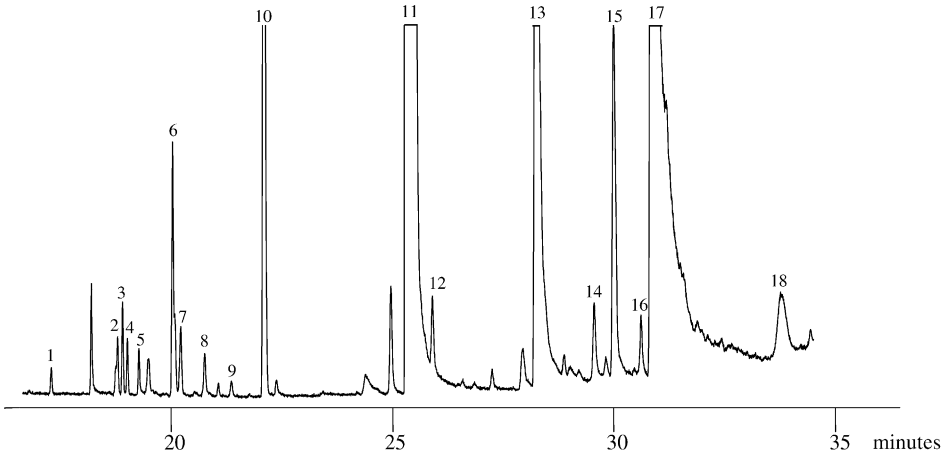

Fig. 1. SPME–GC–MS analysis by using permethylated β-cyclodextrin of volatile terpenes in a *Mentha piperita* plant from Soria (Spain). Peak identification is as follows: 1. β-myrcene, 2. (−)-α-pinene, 3. (+)-sabinene, 4. (+)-α-pinene, 5. (+)-α-phellandrene, 6. (+)-limonene, 7. (+)-β-pinene, 8. (−)-β-pinene, 9. γ-terpinene, 10. 1,8-cineole, 11. (+/−)-menthone, 12. (+/−)-isomenthone, 13. (+/−)-menthyl acetate, 14. (+)-α-terpineol, 15. (+/−)-neomenthol, 16. (+/−)-isomenthol, 17. (+/−)-menthol, 18. (−)-β-*trans*-caryophyllene.

Table 2
Enantiomeric excess (%) and predominant enantiomer of some chiral terpenes in *Mentha piperita* plants from different geographical origins by SPME–GC–MS using permethylated β-cyclodextrin as the stationary phase

Terpenes	Geographical origins							
	Unknown1	Barcelona (Spain)	Gerona (Spain)	Italia	Madrid (Spain)	Unknown2	León (Spain)	Soria (Spain)
(+)[a]-α-Thujene	n.d.[b]	100	n.d.	n.d.	100	n.d.	100	n.d.
α-Pinene	<10%	<10%	(+)-16.6	<10%	<10%	<10%	<10%	<10%
(+)-Sabinene	100	100	100	100	100	100	100	100
(+)-α-Phellandrene	n.d.	100	100	100	100	100	100	100
(+)-Limonene	100	100	100	100	100	100	100	100
β-Pinene	<10%	<10%	<10%	<10%	<10%	<10%	<10%	<10%
(+)-α-Terpineol	100	100	100	100	100	100	100	100
(−)-β-*trans*-Caryophyllene	100	100	100	100	100	100	100	100

[a] Predominant enantiomer into brackets.
[b] n.d.: not detected.

to these compounds. For this reason, the employment of a chiral stationary phase based on the use of γ-cyclodextrin was tested. Specifically, the second chiral phase employed was 2,3-di-acetoxy-6-*O*-*tert*-butyl dimethylsilyl γ-cyclodextrin, while the previously optimised extraction conditions were maintained. Fig. 2 displays the pattern of terpenes obtained from the SPME–GC–MS analysis of the same *Mentha piperita* plant analysed in Fig. 1. As can be observed, on this occasion, the selectivity of the used chiral column enabled menthyl acetate, menthone, neomenthol, menthol and isomenthol to be successfully separated into their corresponding enantiomers. As expected [21], the elution order obtained for the minor chiral terpenes on the γ-cyclodextrin column was opposite to that provided by Chirasil-β-Dex. This fact increases the security in assigning the enantiomeric composition of chiral terpenes enantiomerically resolved on both chiral phases. With regard to the major constituents, as can be seen in Fig. 2, the large amounts of these terpenes released by the sample and subsequently retained in the SPME fibre resulted in saturated chromatographic signals. Obviously, under these circumstances, the measurement of the enantiomeric ratio of these components could not be reliably

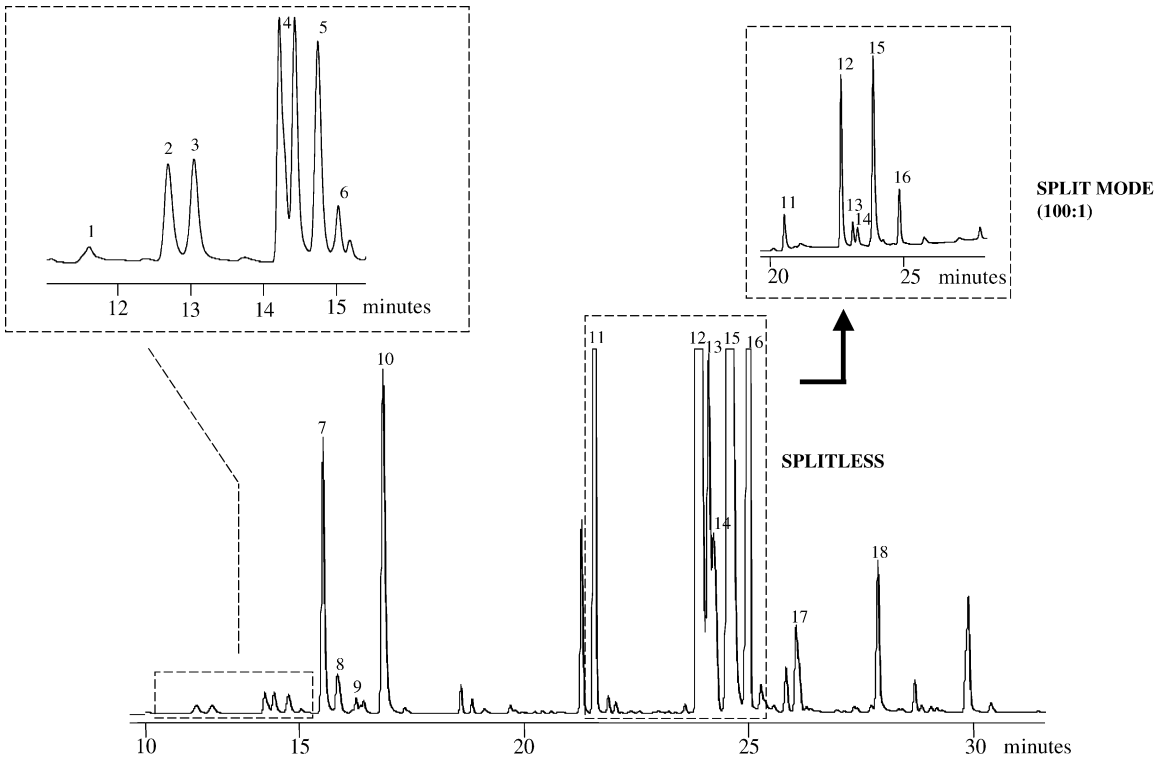

Fig. 2. SPME–GC–MS analysis by using 2,3-di-acetoxy-6-*O*-*tert*-butyl dimethylsilyl γ-cyclodextrin of volatile terpenes in a *Mentha piperita* plant from Soria (Spain). Peak identification is as follows: 1. β-myrcene, 2. (+)-α-pinene, 3. (−)-α-pinene, 4. (+)-sabinene, 5. (−)-β-pinene, 6. (+)-α-phellandrene, 7. (+)-limonene, 8. (+)-β-pinene, 9. γ-terpinene, 10. 1,8-cineole, 11. (−)-menthyl acetate, 12. (−)-menthone, 13. (+)-neomenthol, 14. (−)-neomenthol, 15. (−)-menthol, 16. (+)-isomenthol, 17. (+)-α-terpineol, 18. (−)-β-*trans*-caryophyllene.

Table 3
Enantiomeric excess (%) and predominant enantiomer of some chiral terpenes in *Mentha piperita* plants from different geographical origins by SPME–GC–MS using 2,3-di-acetoxy-6-*O*-*tert*-butyl dimethylsilyl γ-cyclodextrin as the stationary phase

Terpenes	Geographical origins							
	Unknown1	Barcelona (Spain)	Gerona (Spain)	Italia	Madrid (Spain)	Unknown2	León (Spain)	Soria (Spain)
(+)[a]-α-Thujene	n.d.[b]	100	n.d.	n.d.	100	n.d.	100	n.d.
α-Pinene	<10%	<10%	<10%	<10%	<10%	<10%	<10%	<10%
(+)-Sabinene	100	100	100	100	100	100	100	100
(+)-α-Phellandrene	n.d.	100	100	100	100	100	100	100
(+)-Limonene	100	100	100	100	100	100	100	100
β-Pinene	<10%	<10%	<10%	<10%	<10%	<10%	<10%	<10%
(−)-Menthyl acetate	100	100	100	100	100	100	100	100
(−)-Menthone	100	100	100	100	100	100	100	100
Neomenthol	<10%	<10%	<10%	<10%	<10%	<10%	<10%	<10%
(−)-Menthol	100	100	100	100	100	100	96.8	100
(+)-Isomenthol	100	100	100	100	100	100	100	100
(+)-α-Terpineol	100	100	100	100	100	100	100	100
(−)-β-*trans*-Caryophyllene	100	100	100	100	100	100	100	100

[a] Predominant enantiomer in brackets.
[b] n.d.: not detected.

performed. For this reason, an additional experiment using a lower sample amount (i.e., 0.02 g) as well as the split mode (split ratio, 100:1) during the chromatographic analysis was accomplished for all samples. In this way, the enantiomeric composition of the major constituents in *Mentha piperita* plants could be eventually estimated. The obtained values are indicated in Table 3. As depicted, menthyl acetate, menthone, menthol and isomenthol occurred, in general, as pure enantiomers, prevailing the (−)-enantiomer in all cases except for isomenthol, which only exhibited the (+)-enantiomer. In contrast, in the case of neomenthol, the presence of both enantiomers with similar proportions was obvious in all plants. Equally to α- and β-pinene, the predominant enantiomer could not be reliably established due to the low enantiomeric purity found for neomenthol in all samples (<10%). The results shown in the present work agree with those published by other authors, who have described the presence of (+)-menthyl acetate and (+)-menthol in peppermint oil as indicators of an addition and, therefore, adulteration of the sample [13]. As far as the minor terpenes are concerned, data obtained from the chromatographic analysis on the 2,3-di-acetoxy-6-*O*-*tert*-butyl dimethylsilyl γ-cyclodextrin (Table 3) were quite similar to those previously found when permethylated β-cyclodextrin was used (Table 2).

4. Conclusion

We can conclude that the enantiomeric distribution of bioactive chiral terpenes in *Mentha piperita* seems to be independent of the geographical origin of the plant. As a consequence, the enantiomeric composition of these compounds may be used as a powerful tool in authenticity studies. In this regard, the employment of SPME as the sample preparation technique and the consideration of the enantiomeric purity of a relatively high number of chiral terpenes is recommended for quality purposes.

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