A Comparative Study of the Ability of Different Techniques to Extract Menthol from *Mentha piperita*

María Luisa Ruiz del Castillo, Guillermo Santa-María, Marta Herraiz*, and **Gracia P. Blanch**

Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Científicas (CSIC), c/Juan de la Cierva 3, 28006 Madrid, Spain

Abstract

Supercritical fluid extraction, direct thermal desorption, hydroalcoholic extraction, and atomization are used to extract menthol from leaf plants of *Mentha piperita*. The investigated methods are comparatively evaluated on the basis of their reliability to determine the enantiomeric distribution of menthol. The enantioselectivity required for the gas chromatographic analysis is achieved using Octakis (2,6-di-*O*-*n*-pentyl-3-*O*-butyryl)-γ-cyclodextrin as the stationary phase. From the obtained results, it is established that there is a significant effect of the combination of pressure and temperature to achieve the effective isolation and fractionation of the less and most volatile compounds using supercritical fluids.

Introduction

The reliable determination of enantiomeric ratios of specific chiral compounds may be of special interest to establish microbial transformations and genetically controlled biosynthetic mechanisms. Therefore, the knowledge of the true natural enantiomeric composition of chiral flavor compounds not only may help in suggesting biosynthetic pathways, but also can make it possible to distinguish between natural and nature-identical flavors. For that reason, the determination of the enantiomeric purity of relevant chiral compounds decides its medicinal value or its commercial price as raw material for a broad range of applications (e.g., for flavoring food, beverages, cosmetics, or fragrance compositions) (1–6).

In this respect, recent advances in the development of a high number of new chiral stationary phases in gas chromatography (GC) have enabled the stereodifferentiation of a variety of chiral compounds to be performed (7–12). The usefulness of other techniques, namely supercritical fluid chromatography and electrochromatography, to achieve the enantioselectivity required to analyze chiral compounds has also been reported (13–15). However, the achievement of the effective fractionation of difficult matrices (e.g., aromatic and medicinal plants) and the isolation of relevant compounds may demand the use of enrichment techniques that are inadequate in enantiomeric studies. In fact, in some cases, the determination of the true natural composition of specific chiral compounds may become extremely difficult, or even impossible, and experimental conditions that can eventually bring about the racemization of the chiral compound must be applied. Consequently, the sample preparation step usually required prior to the enantiomeric chromatographic analysis may precisely be the most critical point to assure the reliability of the determination.

On the other hand, the potential of supercritical fluid extraction (SFE) as an alternative to conventional extraction procedures has been demonstrated with a wide variety of samples (16–19) including aromatic plants (20–22). Actually, the possibility, which offer supercritical fluids (SFs) concerning the variability of the solvent strength achievable by modifying the pressure and temperature, is of special interest when the low thermal stabilities or high reactivities of the compounds to be analyzed demand the use of mild experimental conditions for sample extraction.

In previous work we have also demonstrated the possibility of analyzing the volatile composition of plant materials by direct introduction of dried and crushed leaves into the programmed-temperature vaporizer injector (PTV) of a gas chromatograph and subsequent thermal desorption (23). Although the procedure was advantageous for the rapid analysis of volatile compounds, the suitability of the procedure to determine the enantiomeric composition of chiral compounds in complex matrices has not yet been investigated.

In any case, it is clear that the influence of the matrix and isolation and concentration conditions must not be neglected when performing enantiomeric analysis (24), and, consequently, the development of methods adequate to perform the reliable determination of the enantiomeric composition of volatile compounds occurring in plant materials is still needed.

The aim of this work was to comparatively evaluate different methods for extraction of enantiomers from leaf plants to establish their reliability and performance. The study was accomplished from a plant (*Mentha piperita*), which is used as raw material for flavoring food and was focused on because of its main aroma compound (i.e., menthol).

* Author to whom correspondence should be addressed.

Experimental

Materials

For identification purposes, a test solution containing D and L-menthol (Sigma-Aldrich Química, Madrid, Spain) was used. To establish the enantiomeric composition of menthol in *Mentha piperita*, different extraction procedures [i.e., SFE, direct thermal desorption (DTD), hydroalcoholic extraction (HAE), and atomization] were comparatively evaluated. In all cases the analyses were performed by GC.

Instrumentation

SFE

Extractions were performed using an SFE module (Iberfluid, Madrid, Spain). The SFE unit was equipped with a pump (450 bar), a flow meter, and a 300-mL extraction vessel connected to two 100-mL separation vessels. The pressure and temperature of the extraction and separation vessels were controlled by metering valves and a thermocouple connected by electronic relays. Carbon dioxide (purity > 99%) was obtained from Carburos Metálicos (Madrid, Spain) and was filtered through active charcoal (activated carbon microcolumn). A cooler placed before the pump was used to condense the carbon dioxide.

Leaves from plants of *Mentha piperita* were air dried and milled before SFE. A 30-g sample weight was loaded into the extraction vessel. A CO_2 stream (40°C, 350 bar, and 50 mL/min measured as liquid flow) was passed through the extractor. The most insoluble and less volatile compounds were collected as a yellow oil in the first separation vessel, which was maintained at 50ºC and 150 bar. A 3-mL volume of ethanol was used to recover the fraction, which was stored at –18ºC until analyzed by GC. The CO_2 leaving the first separation vessel was subsequently passed through the second separation vessel, which was maintained under different conditions to optimize the retention of medium-volatile compounds. Specifically, the pressure in all extractions was maintained at 60 bar while different temperatures (i.e., 2°C, 16°C, and 27°C ± 1°C) were tested. The fraction collected from this separation vessel was recovered with a 3-mL volume of ethanol and was then stored at –18ºC until analyzed by GC. The fractions obtained in both separation vessels were collected through the corresponding sampling valves placed at the bottom of the separators. The CO_2 stream leaving the second separation vessel was passed through a cooling unit made in form of a Dewar flask with dry ice–acetone (–80°C) to retain the most volatile compounds, which were collected in a 20-mL volume of ethanol. The overall extraction time was 3 h.

GC analysis of the SFE extracts

Analysis of the three mentioned SF fractions (i.e., those collected in the two separation vessels and Dewar flask) were performed by sampling a 0.2-µL volume into a Model 8500 PerkinElmer gas chromatograph (Norwalk, CT) fitted with a PTV and a flame ionization detector (FID). A fused-silica column (25-m × 0.25-mm i.d.) of (Octakis (2,6-di-*O*-*n*-pentyl-3-*O*-butyryl)-γ-cyclodextrin (Macherey Nagel, Düren, Germany) was used, and helium served as the carrier gas. Sampling introduction was performed in the PTV injector, which was operated in the splitless mode with 320°C as the injection temperature. The column temperature was maintained at 45°C for 5 min and then programmed (3°C/min) until 85°C, and subsequently at 5°C/min up to 160ºC. The final temperature was held for 30 min. If required, the enantiomeric composition was confirmed running the analysis under isothermal conditions (65°C). Data acquisition from the FID was performed from an HP ChemStation (Hewlett-Packard, Palo Alto, CA). All throughout the experimentation, the FID was operated at 250°C.

DTD

A 3-mg sample weight of dried and crushed leaves from *Mentha piperita* was introduced, without any pretreatment, into the glass liner (80- × 1-mm i.d. × 2-mm o.d.) of the PTV injector between two small plugs of deactivated glass wool. The glass liner was placed in the injector after having interrupted the carrier gas circulation, while maintaining the injector temperature at 35°C. The flow was then established again, and the chromatographic analysis was simply performed by thermal desorption and subsequent transfer of the retained material to the capillary column by raising the injector temperature (at ~ 14°C/s) to 320°C. The end temperature was held for 5 min. The injection was performed with a split ratio of 50:1 while maintaining a septum purge of 2.8 mL/min. The column was temperature-programmed as detailed previously for the SF extracts.

HAE

Mentha piperita was dissolved in a hydroalcoholic medium and extracts were obtained by distillation at 50°C and subsequent vacuum concentration performed at 60°C. A 0.2-µL volume of the hydroalcoholic extract obtained was sampled into the PTV injector, which was operated in the splitless mode at 320°C. The column was temperature-programmed as previously detailed for the SF extracts.

Atomization

Mentha piperita was converted to an aerosol using a liquid transported by a hot air stream at 160°C for 1 s. The sample was then cooled to 70°C and collected for its chromatographic analysis. The atomized material was then placed into the glass liner of the PTV injector and analyzed by thermal desorption under the mentioned conditions for the DTD procedure. The column was temperature-programmed as for the SF extracts.

Results and Discussion

Because our study was aimed at the reliable determination of the enantiomeric composition of menthol from *Mentha piperita*, the experimental conditions were established to minimize the risk of racemization. The obtained results were comparatively evaluated in terms of the enantiomeric excesses (EE, or excess of predominant enantiomer expressed as percent) estimated from the peak areas corresponding to both enantiomers [i.e., EE = ([predominant enantiomer – minor enantiomer]/[predominant enantiomer + minor enantiomer]) × 100].

Figures 1–3 show the chromatograms resulting from a 0.2-µL

injection of the SF extracts obtained from *Mentha piperita* when the experimentation is performed maintaining the temperature in the second separation vessel at 27°C, 16°C, and 2ºC, respectively. Aside from the chromatograms corresponding to the second separation vessel, each figure also includes those chromatograms resulting from the first separation vessel, as well as those obtained from the fractions collected in the Dewar flask.

As can be seen, experimental conditions in the two separation vessels enabled the fractionation of the less and most volatile compounds to be achieved. Actually, pressure and temperature at which the extract is obtained in the first separation vessel (i.e., 150 bar and 50°C) allowed the necessary decrease of the solvating power of the SF leaving the extraction vessel (held at 350 bar and 40°C) to precipitate the most insoluble and less volatile compounds of the extract. In this case, gas chromatograms resulting from SF extracts obtained in the first extraction vessel (Figures 1A, 2A, and 3A) did not show other compounds than the solvent used to recover the extract.

Likewise, the pressure variation from the first to the second separation vessel, namely from 150 to 60 bar, resulted in a lower density of the SF. Actually, pressure and temperature were decreased until the CO_2 became a gas and, thus, the solvent strength was adequate to precipitate the medium volatile compounds.

Considering the experimental conditions tested in the second separation vessel, it is clear that the solute vapor pressure will increase when raising the extraction temperature from 2–27°C at constant pressure (i.e., 60 bar). On the other hand, it is also known that the amount extracted of a compound may lower if the extraction temperature becomes greater while maintaining constant the pressure because of the decrease of the solvating power (13,16). Therefore, the effect on the solubility of menthol of an increase of temperature will depend on the balance between CO_2 density and solute vapor pressure changes. The peak area sum obtained for both D- and L-menthol in chromatograms resulting from the second separation vessel (Figures 1B, 2B, and 3B) showed that experimentation at the medium temperature (i.e., 16°C, Figure 2B) resulted in menthol peak areas more than 30 times higher than those obtained when performing the experimentation at 27°C (Figure 1B) caused by the consequent increase of the supercritical CO_2 density. However, temperatures as low as 2°C in the second separation vessel (Figure 3B) did not permit the obtaining of better recoveries. Concerning the extract collected in the Dewar flask, generally speaking, it is clear that it is useful to avoid losses of volatile compounds when relatively high temperatures must be applied in the separation vessels. In this case, the comparison of Figures 1C and 2C shows that those compounds reaching their vapor pressure can be swept by increasing the temperature.

All in all, from the obtained results, it seems clear that the enhancement of the fractionation of menthol requires a careful selection of the combination of pressure and temperature at which the SFE and fractionation are performed.

Figure 4 gives the chromatograms obtained from (A) the hydroalcoholic extract, (B) the atomized material and (C) the DTD resulting from *Mentha piperita*, with 97%, 42%, and 94%

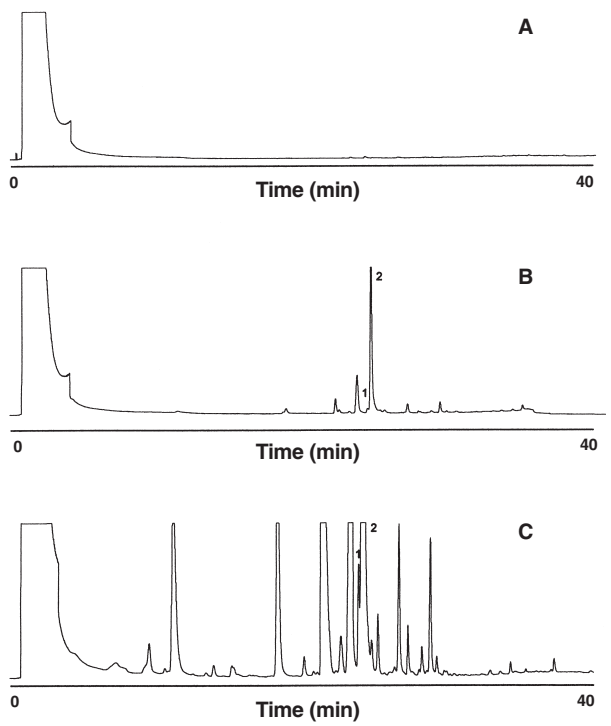

Figure 1. Chromatograms of a 0.2-µL injection of the SF extracts obtained from *Mentha piperita* in the first separation vessel (A), in the second separation vessel maintained at 27°C (B), and in the Dewar flask (C). The extraction time was 3 h. Chromatograms A, B, and C were recorded at the same full range. Identification peak numbers: 1, D-menthol; and 2, L-menthol.

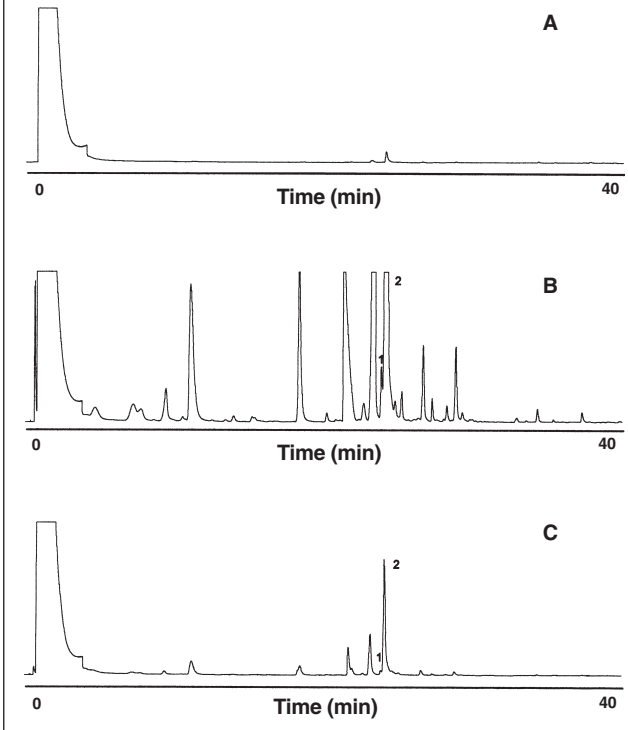

Figure 2. Chromatograms of a 0.2-µL injection of the SF extracts obtained from *Mentha piperita* in the first separation vessel (A), in the second separation vessel maintained at 16°C (B), and in the Dewar flask (C). The extraction time was 3 h. Chromatograms A, B, and C were recorded at the same full range. The identification peak numbers are the same as in Figure 1.

being the enantiomeric excesses calculated for menthol, respectively. As mentioned in the Experimental section, the EE values found for menthol were occasionally confirmed carrying out the analysis under isothermal conditions (65°C). This confirmation was especially necessary when performing the analysis by DTD from the plant (Figure 4C) because the overlapping of D- and L-menthol made the reliable determination of the EE difficult. On the other hand, the GC analysis of the SF extracts obtained in the second separation vessel and in the Dewar flask (Figures 1B–3B and 1C–3C) yielded average EE values of 97% and 95%, respectively. Therefore, it is clear that with the exception of atomization, all of the procedures tested seemed to allow the reliable determination of the enantiomeric distribution of menthol because racemization was not observed. In any case, it should be underlined that the low EE found for menthol in the atomized material (42%) may be attributable either to the coelution of an unknown compound at the retention time of the first enantiomer, or to the fact that occasionally some amounts of the plant that can remain in the atomizer between consecutive processes are subjected up to 30 min at temperatures between 70°C and 160°C. Consequently, racemization can be eventually produced, thereby lowering the EE values finally obtained.

Conclusion

In summation, it is clear that the DTD procedure is advantageous concerning speed of analysis and reduction of risk of analyte loss and degradation. In fact, under the used conditions given in the Experimental section, menthol recovery as high as 99% was obtained, with 0.012 mg of leaf plants being the lowest amount required to detect the compound of interest. Concerning the repeatability of the analysis, a relative standard deviation (RSD) (calculated from three replicates) as low as 1.3% was estimated for the enantiomeric excesses of menthol. In short, the DTD procedure can be proposed as a screening method to establish the enantiomeric composition of chiral compounds occurring in plants.

However, despite the advantages of the DTD procedure, it is also evident that a wide number of analytes other than the compound of interest are transferred to the chromatographic system, thus increasing the risk of peak overlapping when analyzing complex matrices. In this context, the use of SFE appears to be a valuable alternative for the extraction and reliable enantiomeric analysis of chiral compounds for three main reasons: (*a*) SFs make possible the use of mild conditions preventing racemization; (*b*) because no organic solvents are required, SFE offers a versatile procedure that meets both legal regulations concerning the use of contaminant solvents and the increasing demand in the use of natural products; and (*c*) the variable solvent strengths that can be achieved by modifying the pressure and temperature of the SF over the extraction and separation vessels may enable the effective fractionation and isolation of chiral compounds of high added value to be achieved.

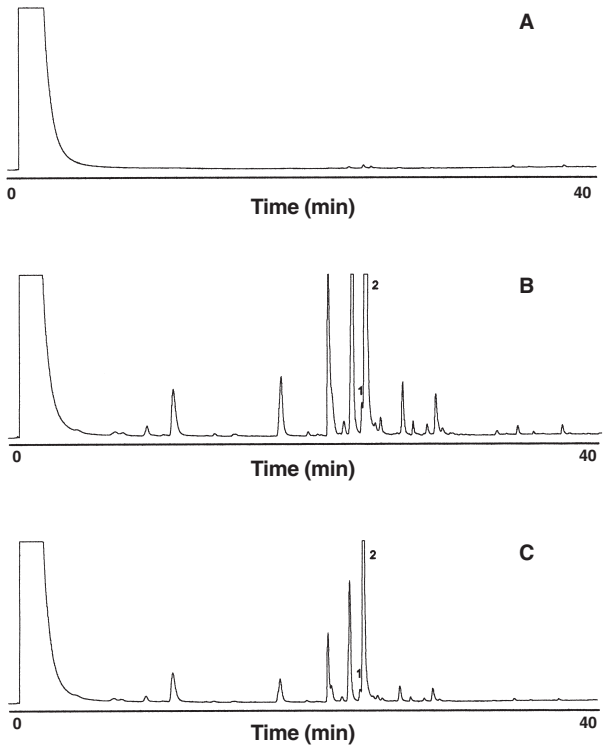

Figure 3. Chromatograms of a 0.2-µL injection of the SF extracts obtained from *Mentha piperita* in the first separation vessel (A), in the second separation vessel maintained at 2°C (B), and in the Dewar flask (C). The extraction time was 3 h. Chromatograms A, B, and C were recorded at the same full range. The identification peak numbers are the same as in Figure 1.

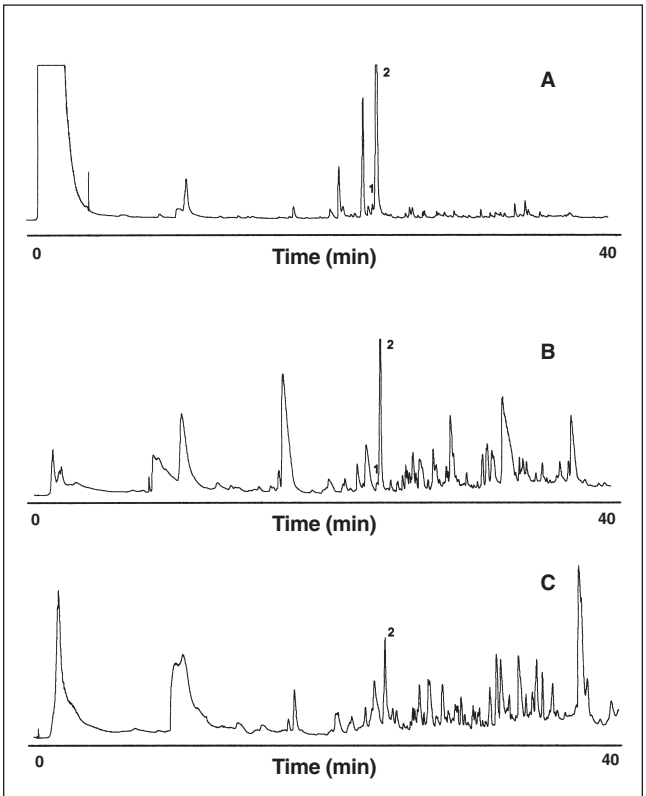

Figure 4. Chromatograms obtained from *Mentha piperita* by hydroalcoholic extraction (A), atomization (B), and DTD (C). The identification peak numbers are the same as in Figure 1.

Acknowledgments

Financial assistance from the Ministerio de Ciencia y Tecnología, Project PPQ2002-03641, is gratefully acknowledged.

References

1. P. Werkhoff, S. Brennecke, W. Bretschneider, M. Güntert, R. Hopp, and H. Surburg. Chirospecific analysis in essential oil, fragrance and flavor research. *Z. Lebensm. Unters. Forsch.* **196:** 307–28 (1993).
2. W.A. König, C. Fricke, Y. Saritas, B. Momeni, and G. Hohenfeld. Adulteration or natural variability? Enantioselective gas chromatography in purity control of essential oils. *J. High Resol. Chromatogr.* **20:** 55–61 (1997).
3. L. Mondello, M. Catalfamo, P. Dugo, A.R. Proteggente, and G. Dugo. Multidimensional capillary gas chromatography for the analysis of complex samples. Determination of the enantiomeric distribution of some components of citrus essential oils. *Essenze Deriv. Agrum.* **67:** 62–85 (1997).
4. A. Mosande and D. Juchelka. Advances in the authenticity assessment of citrus oils. *J. Essent. Oil Res.* **9:** 5–12 (1997).
5. M. Wüst and A. Mosandl. Important chiral monoterpenoid ethers in flavors and essential oils—enantioselective analysis and biogenesis. *Eur. Food Res. Technol.* **209:** 3–11 (1999).
6. W.M. Coleman III and B.M. Lawrence Examination of the enantiomeric distribution of certain monoterpene hydrocarbons in selected essential oils by automated solid-phase microextraction–chiral gas chromatography–mass selective detection. *J. Chromatogr. Sci.* **38:** 95–99 (2000).
7. W.A. König. *Gas Chromatographic Enantiomer Separation with Modified Cyclodextrins.* Hüthig, Heidelberg, Germany, 1992.
8. V. Schurig. Enantiomer separation by gas chromatography on chiral stationary phases. *J. Chromatogr. A* **666:** 111–29 (1994).
9. V. Schurig. "Gas Chromatography—Techniques, Chiral Separations". In *Encyclopedia of Analytical Science*. Academic Press, London, U.K., 1995, Vol. 3.
10. T.E. Beesley and R.P.W. Scott. *Chiral Chromatography*. John Wiley, Chichester, U.K., 1998.
11. C. Bicchi, A.D´Amato, and P. Rubiolo. Cyclodextrin derivatives as chiral selectors for direct gas chromatographic separation of enantiomers in the essential oil, aroma and flavor fields. *J. Chromatogr.* A **843:** 99–121 (1999).
12. V. Schurig. Separation of enantiomers by gas chromatography—review. *J. Chromatogr. A* **906:** 275–99 (2001).
13. M.L. Lee and K.E. Markides, Eds. *Analytical Supercritical Fluid Chromatography and Extraction*. Chromatography Conferences, Provo, UT, 1990.
14. D. Wistuba and V. Schurig. Recent progress in enantiomer separation by capillary electrochromatography. *Electrophoresis* **21:** 4136–58 (2000).
15. G. Gubitz and M.G. Schmid. Chiral separation by chromatographic and electromigration techniques. A review. *Biopharmaceutics & Drug Disposition* **22:** 291–336 (2001).
16. J.W. King. Fundamentals and applications of supercritical fluid extraction in chromatographic science. *J. Chromatogr. Sci.* **27:** 355–64 (1989).
17. M.B. King and T.R. Bott, Eds. *Extraction of Natural Products Using Near-critical Solvents*. Chapman & Hall, Glasgow, U.K.,1993.
18. S.S.H. Rizvi, Ed. *Supercritical Fluid Processing of Food and Biomaterials*. Blackie, Academic & Professional, London, U.K., 1994.
19. T.L. Chester, J.D. Pinkston, and D.E. Raynie. Supercritical fluid chromatography and extraction. *Anal. Chem.* **68:** 487–514 (1996).
20. G.P. Blanch, E. Ibáñez, M. Herraiz, and G. Reglero. Use of a programmed temperature vaporizer for off-line SFE/GC analysis in food composition studies. *Anal. Chem.* **66:** 888–92 (1994).
21. G.P. Blanch, M.M. Caja, M.L. Ruiz del Castillo, G. Santa-María, and M. Herraiz. Fractionation of plant extracts by supercritical fluid extraction and direct introduction in capillary gas chromatography using a programmable temperature vaporizer. *J. Chromatogr. Sci.* **37:** 407–10 (1999).
22. M.C. Díaz-Maroto, M.S. Pérez-Coello, and M.D. Cabezudo. Supercritical carbon dioxide extraction of volatiles from spices. Comparison with simultaneous distillation-extraction. *J. Chromatogr. A* **947:** 23–29 (2002).
23. G. Reglero, M. Herraiz, T. Herraiz, and J. Sanz. Capillary gas chromatographic determination of volatiles in solid matrices by direct introduction using a programmable-temperature vaporizer. *J. Chromatogr.* **483:** 43–50 (1989).
24. G.P. Blanch and J. Jauch. Enantiomeric composition of filbertone in hazelnuts in relation to extraction conditions. Multidimensional gas chromatography and gas chromatography/mass spectrometry in the single ion monitoring mode of a natural sample. *J. Agric. Food Chem.* **46:** 4283–86 (1998).

Manuscript accepted June 3, 2003.