Gas Chromatographic Determination of Monoterpenes in Spruce Needles (*Picea abies*, *P. omorica*, and *P. pungens*) after Supercritical Fluid Extraction

J. SEDLÁKOVÁ, L. LOJKOVÁ, and V. KUBÁŇ*

Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, CZ-613 00 Brno, Czech Republic
e-mail: kuban@mendelu.cz

Received 12 July 2002

Six monoterpenes (α-pinene, β-pinene, 3-carene, phellandrene, camphene, and limonene) were determined in the needles of *Picea abies, P. omorica*, and *P. pungens* spruces by gas chromatography after supercritical fluid extraction (SFE) with carbon dioxide at the pressure 20 MPa and at the temperature 80 °C. Significant differences among the monoterpene content of individual spruce cultivars were found. Limonene (34.3 %), α-pinene (30.4 %), and camphene (30.1 %); camphene (44.5 %), limonene (24.5 %), and α-pinene (24.7 %), and finally limonene (51.5 %), camphene (29.1 %), and α-pinene (18.2 %) are the main monoterpenes in *P. omorica, P. abies,* and *P. pungens* cultivars, respectively. The relative standard deviations (RSDs) of *ca.* 1.0 %, 7.8 %, and 22.5 % were found in the whole needles, ground samples, and cut samples of the cultivars, respectively. The contents of minor monoterpenes decrease further from β-pinene through phellandrene to 3-carene in all cultivars. The SFE from the whole needles has been found as the very suitable method for isolation of monoterpenes from complex matrices.

The analysis of volatile compounds of plant origin has been already investigated in many studies. Most often, the studies of terpenes in coniferous trees explain differences based on geographic origin and/or to confirm chemotaxonomy [1, 2]. Also the studies of the effect of atmospheric pollution in urban locations on the relative distribution of monoterpenes in essential oil [3, 4] and the role of monoterpenes in atmospheric disturbances [5] were performed. Further, many articles dealt with individual extraction techniques for the isolation and subsequent determination of terpenes in essential oils from various plant materials [6—11].

The terpenes are volatile compounds with strong flavours, which are most often extracted from plant materials [12, 13]. The hydrocarbon terpene skeleton is a base of the terpenes structure, so they belong to the group of isoprenoids. Even though isoprene itself was not found in nature, its polymers, terpenic hydrocarbons and their oxygen derivatives are present in high quantities very often in different species. The terpenes are divided to several groups according to the number of their carbon atoms in the molecule: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), and polyterpenes [14, 15].

The terpenes are distributed in various parts of plants: in florets, fruits, leafs, bark, and roots. They are situated in glandular trichomes, papillas, glandular cells, receptacles, channels, and intercellular spaces. The cross-section of *Picea abies* needle is shown for illustration in Fig. 1. The terpenes have been found in various matrices. For example, (+)-limonene was found in orange, lemon, and caraway essential oil. (-)-Limonene is present in spruce and fir

Fig. 1. Cross-section of *Picea abies* needle.

*The author to whom the correspondence should be addressed.

needles essential oil. α-Pinene is the most important hydrocarbon in the turpentine essential oil and together with β-pinene forms the base of turpentine oil. Citronellol is the main constituent of rose and geranium essential oils. Geraniol is the main component of rose oil. Menthol is a dominant terpenoid in peppermint [16].

Currently, supercritical fluid extraction (SFE) represents a new, dynamically evolving separation technique that is very suitable for isolation of volatile compounds from complex natural matrices [17]. Physicochemical properties of supercritical fluids represent the transition between the properties of gases and liquids, what is nowadays the main reason for the increasing interest in SFE. In comparison to a liquid, supercritical fluid has a higher diffusivity and lower viscosity with high solvent power maintained. From the mass transfer point of view, the properties of gases are combined with the solvation properties of liquids in supercritical fluids [18].

In the SFE, predominantly the carbon dioxide is used [19—21], because of its low critical temperature (31 °C) and pressure (7.38 MPa), nontoxic character, incombustibility, and low reactivity. Its polarity and extraction power is close to hexane and the extraction efficiency is decreasing with a growing analyte polarity. In the determination of volatile, reactive, and thermosensitive terpenes, analyzed in this work, low critical temperature and nonpolar character of supercritical fluid is preferred. In addition to the SFE, the steam distillation [22] according to the standard CSN 58 0110, rapid steam distillation [10] and hexane extraction [23], Soxtec, accelerated solvent extraction (ASE) [9], and microwave oven extraction [1] can be used for the isolation and the gas chromatographic (GC) determination of the volatile components in spruce needles. Volatile compounds can be isolated from the gaseous samples also by a solid phase microextraction (SPME) followed by a thermal desorption with direct injection into a GC column [24].

The GC with flame ionization detector (FID) or mass spectrometry (MS) detection is mostly being used for the determination of volatile compounds. The benefits of high-performance liquid chromatography (HPLC) with UV [25, 26] or polarimetric detection, derivative spectrophotometry in the UV region, and proton magnetic resonance have been recognized [27].

EXPERIMENTAL

Spruce needles of the *Pinaceae* family, in particular of Serbian spruce (*Picea omorica*) and Blue spruce (*Picea pungens*) were collected in the campus of the Mendel University in Brno in 1999, 2000. The needles of Norway spruce (*Picea abies*) were gathered in Soběšice, the northern part of the city Brno, in 1999 and 2000. The spruce needles were cut as a whole branch *ca.* in 2 m height, hermetically sealed in PE bags, immediately transferred into the cooled box (0 °C), transported to the laboratory, where they were separated from the branch and stored in the lap vials at the temperature −18 °C until the extraction.

To determine the content of α-pinene, β-pinene, camphene, 3-carene, phellandrene, and limonene in SFE extracts, a gas chromatograph HP4890D equipped with FID was used. The separation was carried out on an HP-INNOWax column (polyethylene glycol, length × i.d. × film thickness: 30 m × 0.25 mm × 0.25 μm, all Hewlett—Packard) at the helium flow rate 1 cm^3 min^{-1}, injector temperature 240 °C, and detector temperature 250 °C. A column temperature program: 60 °C, 5 °C min^{-1} to 150 °C, 0.01 min, 40 °C min^{-1} to 220 °C, 0.3 min was used. Total time of the analysis was about 20 min. 1 mm^3 of the extract was injected into the column. Final chromatograms were processed by CSW data acquisition program (version 1.7, Data Apex, Prague).

Standards of α-pinene, β-pinene, camphene, 3-carene, phellandrene, and limonene (purity > 99.5 %, Fluka, Switzerland) were used to test the extraction efficiency. An HPLC purity hexane (Merck, Germany) was used for the trapping of extracted substances. Liquid carbon dioxide (for food industry), nitrogen (99.99 % and/or 99.996 %), hydrogen (99.999 %), and medicinal oxygen were used for SFE and GC (all AGA, Brno).

Supercritical Fluid Extraction

Monoterpenes were extracted from the spruce needles by means of supercritical CO_2 in the supercritical fluid extractor SE-1 (SEKO-K, Brno), trapped into hexane in a trapping vial and analyzed *via* GC. Approximately 2 g (± 0.01 mg) of a sample were weighted into an extraction cartridge. The cartridge was inserted into a stainless steel extraction cell of inner volume 7.0 cm^3, according to the volume of a sample, and closed by frits on both sides. The extraction cell, supplied with a depressurization screw, was fastened with an extraction cap. The restrictor heater was adjusted to 120 °C to prevent the restrictor plugging. The carbon dioxide, the extraction medium, came out from the extraction cell through the restrictor, leading to a trapping vial with hexane. A fused silica capillary of i.d. 30 μm was applied as a restrictor. Trapping was carried out at room temperature. Heating and cooling regulation of a trapping vial were off during these procedures.

The instrument was controlled from the front panel and all values were displayed on the screen. Extraction programs created by the users could be stored in the extractor memory. The pressure was adjusted by the pneumatically controlled piston micropump to the values of 7 to 40 MPa. The whole system worked with three gas cylinders (extraction and cooling CO_2 and N_2 as a pressure gas). A minimal N_2 pressure of

1.5 MPa was necessary to achieve the CO_2 working pressure of 40 MPa. The volume of a piston micropump was 10 cm^3. It was not large enough for a long extraction time. Extraction time is influenced by the restrictor length and internal diameter (i.d.). Before the pump filling started, the pump head was cooled by a stream of liquid CO_2 to 3 °C, and then the filling time started to be counted. If the extraction medium stored in the pump was depleted during the extraction, the Valco valve switched automatically and the pump filling started again. The time of pump filling was preset to 2 min. When the pump was filled, the Valco valve was switched again and the pressure was increased up to the required value. The extraction was terminated automatically after the preset extraction time expired.

RESULTS AND DISCUSSION

The needle samples collection and storage is the most crucial operation of the analyses. The method for collection of such materials has not been standardized yet, as can be seen from various studies (see literature). The sampling strategy depends mostly on the extraction method used, since different amounts of plant materials are needed for various methods. For example, 0.01—0.1 g is necessary for the SPME, 0.1—1 g for SFE, and 1—10 g for the sonication while the steam distillation requires 10—100 g. Thus, the sample homogeneity is affected by the amount of a sample, and various methods are used for the monitoring of variations in relative distribution of monoterpenes in the needles, *i.e.* the lesser is the sample mass, the higher is the heterogeneity.

Also the storage of needle samples has a great impact on the content of individual monoterpenes. The needles, although treated immediately after the collection, must be stored at such temperature, under which distinct changes do not occur. The effect of temperature on the samples storage was investigated in the sample of *P. omorica* needles. The influence of temperature rate from 4 °C to −18 °C (refrigerator) and also the storage in liquid nitrogen on quality of results was investigated. The monoterpenes content decreases according to the temperature, at which the sample was stored, *i.e.* the lower storage temperature, the less the monoterpenes content changes. Thus, the refrigerator was used in further experiments to store the samples.

The optimization study of temperature and pressure extraction conditions of the SFE method was carried out. For the determination of monoterpenes in spruce needles, the pressure 20 MPa and the extraction cell temperature 80 °C were found to be the most suitable. At higher temperatures, high-molecular compounds were coextracted, which caused problems both during the extraction (restrictor plugging) and during the GC analysis.

Several modifiers (methanol, ethanol, acetonitrile, acetone, dichloromethane, chloroform, hexane, toluene) were also tested, but the nonmodified CO_2 provides more selective extraction with sufficient extraction efficiency. With modifiers, waxes were coextracted, which caused problems with restrictor plugging and during GC analyses.

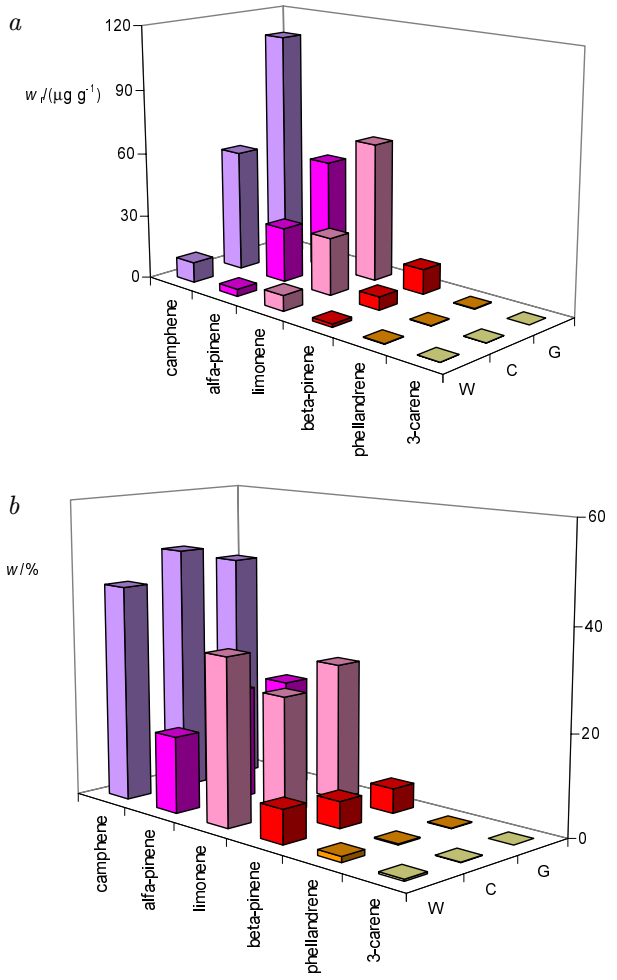

Fig. 2. The effect of sample preparation method on the amount (*a*) and representation (*b*) of monoterpenes in *P. abies*.

The amount of individual monoterpenes in essential oils depends on a particle size and on the sample preparation method. The needle samples of *P. abies* and *P. omorica* were extracted in the form of whole needles, needles cut into small pieces (≈ 1.5 mm), and needles cryogenically ground under liquid nitrogen (≈ 10 μm). The results are presented in Figs. 2 and 3.

The highest amount of monoterpenes was obtained in the case of the ground samples, lesser for the cut samples and the lowest extraction efficiency was gained in the case of whole needles. But at the same time the best reproducibility was ascertained for the whole samples, with the RSD of *ca.* 1.01 %, and in the case of ground sample (RSD was 7.75 %), while the worst RSD was obtained for cut samples (RSD 22.47 %). Thus, the whole needle samples were used

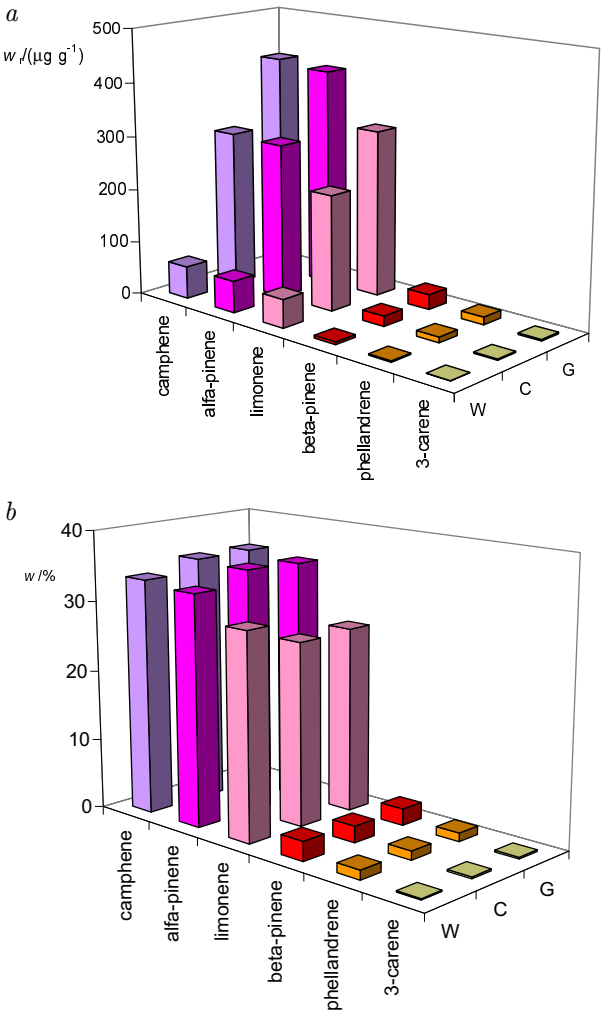

Fig. 3. The effect of sample preparation method on the amount (*a*) and representation (*b*) of monoterpenes in *P. omorica*.

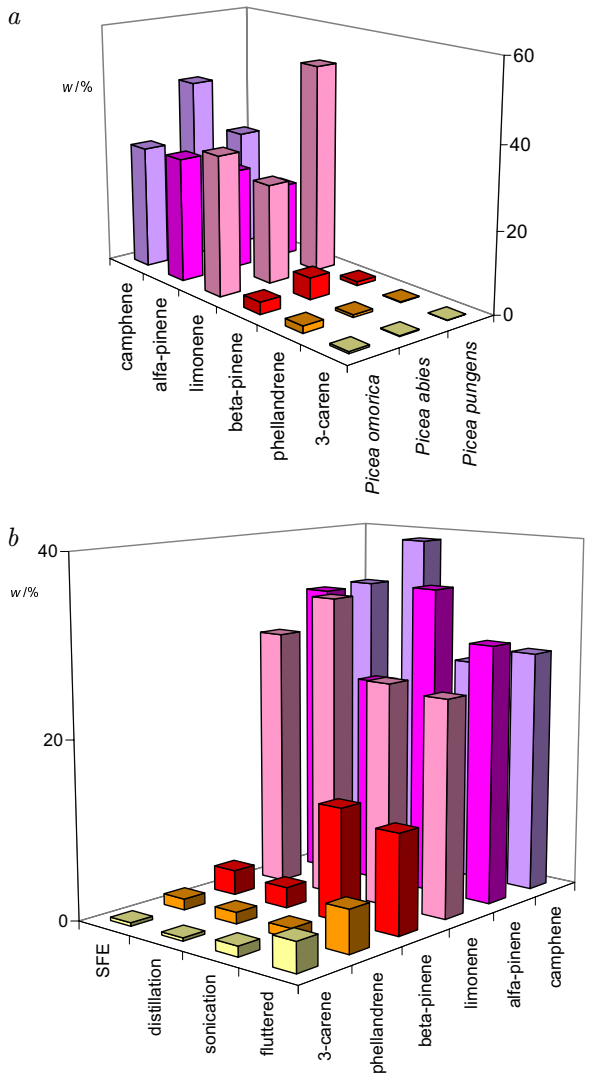

Fig. 4. The amount of monoterpenes in the whole needles of individual cultivars (*a*) and the dependence of the amount of obtained monoterpenes in the whole needles of *P. omorica* on the extraction procedure (*b*).

for further measurement and observation of various dependences, which showed the best RSDs values at the slightly lower extraction efficiency.

The representation of individual monoterpenes in *P. abies* varies according to the sample preparation method (see Fig. 2*b*). The content of α-pinene and camphene increases gradually from the *W* values (whole needles) across the *G* values (ground needles) to the *C* values (cut needles). The content of β-pinene, 3-carene, and phellandrene increases in the order *G*, *C*, *W* and the portion of limonene changes from *C* to *G* and *W*.

As for the monoterpenes from *P. omorica*, the representation of α-pinene, limonene, and phellandrene is consistent with the results obtained for *P. abies* (*cf.* data in Figs. 2 and 3). The representation of 3-carene varies in the rank of *W*, *G*, *C*, the one of camphene from *C*, *G*, *W*, and β-pinene from *G* to *C* and *W*.

In various spruce species from the *Pinaceae* group (*P. abies*, *P. omorica,* and *P. pungens*), contents of six prevailing monoterpenes (α-pinene, β-pinene, camphene, 3-carene, phellandrene, and limonene) were examined. The results are summarized in Fig. 4*a*. As seen from the figure, there are significant differences among the monoterpene contents of individual spruce cultivars. In the *P. omorica*, the mostly representative monoterpenes are limonene (34.3 %), α-pinene (30.4 %), and camphene (30.1 %). Camphene (44.5 %), limonene (24.5 %), and α-pinene (24.7 %) are the most representative monoterpenes in *P. abies*. And finally, limonene (51.5 %), camphene (29.1 %), and α-pinene (18.2 %) are the most representative monoterpenes in *P. pungens* spruce. In all the spruce cultivars, the content of monoterpenes decreases further from β-pinene through phellandrene to 3-carene, which is the least representative one.

The dependences of the monoterpenes amounts on the sample extraction method were investigated us-

ing four extraction methods. The highest amounts of monoterpenes (447.2 μg g^{-1}) were obtained by means of supercritical fluid extraction (pressure 20 MPa, temperature 80 °C, extraction time 60 min) and *via* the steam distillation (274.61 μg g^{-1}) according to CSN 58 0110 (extraction time 4 h). One order of magnitude lower results (24.93 μg g^{-1}) were obtained by sonication (extraction time 4 h) and the lowest recovery was gained by means of the extraction into the fluttered solvent (6.72 μg g^{-1}). The sonication and SFE methods had the best reproducibility with RSDs 1.87 % and 3.27 %, respectively. The other two methods, steam distillation and extraction to the fluttered solvent, had very poor reproducibility, RSDs 18.03 % and 17.65 %, respectively. The used extraction method was found to have a great influence on the representation of individual monoterpenes. The results for the whole needles of *P. omorica* spruce are in Fig. 4*b*.

CONCLUSION

The collection and storage of needle samples has been shown as the most important part of the analyses. The samples were cooled immediately after picking up and stored at the temperature, under which considerable changes do not occur (in the freezer), because of a significant effect of needles storage. For the determination of monoterpenes in spruce needles, SFE from 0.1 g to 1 g amounts of the whole needles was performed under the pressure 20 MPa and at the temperature 80 °C. The best reproducibility (RSD 1.01 %) but lower extraction efficiencies were ascertained for the whole needles.

Significant differences were found among the contents of individual monoterpenes in different spruce cultivars. Limonene (34.3 %), α-pinene (30.4 %), and camphene (30.1 %); camphene (44.5 %), limonene (24.5 %), and α-pinene (24.7 %), and finally, limonene (51.5 %), camphene (29.1 %), and α-pinene (18.2 %) are the main monoterpenes in *P. omorica, P. abies*, and *P. pungens* cultivars, respectively.

The efficiencies of four separation methods for the extraction of monoterpenes from needles (the supercritical fluid extraction, steam distillation, sonication, and extraction into the fluttered solvent) were compared. It was found that the highest amount of monoterpenes was extracted by means of SFE (447.72 μg g^{-1}) and also the RSD of this method is favourable (3.72 %). The used extraction method has also a great impact on the representation of monoterpenes.

Thus, SFE has been shown as a dynamically evolving separation technique, which is very suitable for the extraction of monoterpenes from coniferous needles.

Acknowledgements. Financial support from the Grant Agency of the Czech Republic (Grant No. 203/98/0943) and from the Ministry of Education, Youth, and Sports of the Czech Republic (Grant No. MSM 432100001) is gratefully acknowledged.

REFERENCES

1. Schaefer, P. R. and Hanover, J. W., *For. Sci. 32*, 725 (1986).
2. Glowacki, S., *Sylwan 1*, 27 (1994).
3. Supuka, J., Berta, F., and Chladná, A., *Trees 11*, 176 (1997).
4. Supuka, J. and Berta, F., *Ekologia 17*, 102 (1998).
5. Schürmann, W. and Ziegler, H., *Naturwissenschaften 80*, 276 (1993).
6. Orav, A., Kailas, T., and Koel, M., *J. Essential Oil Res. 10*, 387 (1998).
7. Starmans, D. A. J. and Nijhuis, H. H., *Trends Food Sci. Technol. 7*, 191 (1996).
8. Scheffer, J. J. C., Capasso, F., and Evans, F. J., *Phytother. Res. 10* (Supplement 1), S6 (1996).
9. McDonald, A. G., Stuthridge, T. R., Clare, A. B., and Robinson, M. J., Isolation and Analysis of Extractives from Radiata Pine HTMP Fibre, *ISWPC* 71/1—71/6 (1997).
10. Muzika, R. M., Campbel, C. L., Hanower, J. W., and Smith, A. L., *J. Chem. Ecol. 16*, 2713 (1990).
11. Schönwitz, R., Merk, L., and Ziegler, H., *Trees 1*, 88 (1987).
12. Tomko, J., *Pharmacology 1999,* 69.
13. Vacek, L., in *Organic Chemistry,* pp. 148—154. MZLU, Brno, 1995.
14. Teuscher, E., *Biogene Arzneimittel 1997,* 110.
15. Pavel, J., in *Plant Biochemistry,* pp. 133—135. MZLU, Brno, 1989.
16. Škárka, B. and Ferenčík, M., *Biochemistry 1987*, 238.
17. Sedláková, J., Kocourková, B., and Kubáň, V., *Czech J. Food Sci. 19*, 31 (2001).
18. Churáček, J., in *Textbook of Licensing Studies II*, p. 15. University of Pardubice, 1992.
19. Kallio, H., Kerrola, K., and Alhonmaki, P., *J. Agric. Food Chem. 42,* 2478 (1994).
20. Engelhardt, H. and Gross, A., *J. High Resolut. Chromatogr. Chromatogr. Commun. 11*, 38 (1988).
21. Bounoshita, M., Hibi, K., and Nakamura, H., *Anal. Sci. 9,* 425 (1993).
22. Sedláková, J., Kubáň, V., Holubová, V., and Kocourková, B., in *Book of Abstracts*, pp. 120—126. 2 THETA, Komorní Lhotka, 1998.
23. Bouwmeester, H. J., Davies, J. A. R., and Toxopeus, H., *J. Agric. Food Chem. 43,* 3057 (1995).
24. Mani, V. and Wiooley, C., *LC-GC 13*, 734, 738, 740 (1995).
25. Kovar, K. A. and Bock, E., *J. Chromatogr. 262,* 285 (1983).
26. Seif El Din, A. A., Korany, M. A., and Abdel Salam, N. A., *Anal. Lett. 16*, 891 (1983).
27. Mossa, J. B., El-Obeid, H. A., and Hassan, M. M. A., *Spectrosc. Lett. 13*, 49 (1980).