Determination of the Enantiomeric Composition of Limonene and Limonene-1,2-epoxide in Lemon Peel by Multidimensional Gas Chromatography with Flame-Ionization Detection and Selected Ion Monitoring Mass Spectrometry

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Abstract

The enantiomeric excess of limonene and limonene-1,2-epoxide in lemon peel samples is determined. The enantiomers were separated by multidimensional gas chromatography with flame ionization detection and gas chromatography–mass spectrometry in the selected ion monitoring mode. With this system, it is possible to measure limonene-1,2-epoxide, which appears at low concentrations in lemon peel. The enantiomeric excess of R-(+)-limonene in lemon peel is between 97.1 and 97.4% and that of (1S,2R,4R)-(+)-limonene-1,2-epoxide is between 88.0 and 91.9%. The quantitative determination of limonene in lemon peel is carried out using the optical antipode of the limonene to be quantitated as an internal standard.

Introduction

The analysis of fruits is becoming increasingly important, particularly as a method of quality control and in view of possible toxicants present. The monoterpene limonene (4-isopropenyl-1-methyl-1-cyclohexene) (see Figure 1) is found in all citrus fruits (1–5) and is widely used as flavoring material in food products. It is the main component of lemon peel oil, and the concentration varies with the maturity of the fruits (4).

Although humans routinely consume limonene without adverse effects, it has been found that when dosed orally to adult male rats, R-(+)-limonene (Figure 1A) causes the male rat-specific protein α2u-globulin to accumulate in phagolysosomes of renal proximal tube cells (6). This toxicity is described as hyalne droplet nephropathy (7). Investigations have shown that limonene or its metabolite(s) bind reversibly to α2u-globulin, and this binding appears to be a prerequisite to the accumulation of the protein in the kidney (8).

Because the 1,2-epoxides of limonene (4-isopropenyl-1-methyl-1-cyclohexen-1,2-epoxide) (Figures 1C–1F) can occur in nature, it is plausible that the human system can epoxidize the two double bonds of limonene when this compound is ingested in a food source (e.g., citrus fruits). Epoxides are known to be involved in the metabolism of a variety of aliphatic and aromatic compounds in plants (9). They are by nature biologically toxic compounds, and in view of their possible antimitotic activity, we quantitated the amount of limonene, precursor of the epoxides (10), in lemon peel.

The volatiles contributing to the aroma of a foodstuff are generally in the form of a complex mixture. Accurate determination of the enantiomeric purity by gas chromatography (GC) of the single components is often difficult because of the high probability of peak overlapping in the chromatographic system. This problem can be greatly reduced by the use of multidimensional GC (MDGC) (11) with an achiral precolumn and a chiral main column (12–15). Only cut fractions are introduced to the chiral column, thus protecting it from contamination, and the difference in polarity between the two columns greatly reduces the probability of peak overlap in the chiral separation. Progress in the development of derivated cyclodextrins as chiral selector phases (16–18) enables the enantiomeric separation. Since the introduction of MDGC, numerous modifications have been proposed (16,19–22), and the technique has been demonstrated to be a powerful method for the stereo-

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analysis of chiral volatiles (16,23,24).

The steam distillation solvent extraction (SDE) described in 1964 by Likens and Nickerson (25) has received increasing attention. Several modifications of the original design have subsequently been proposed (26–29). Recently an improved SDE version using a more efficient cooling surface and allowing operation with solvents of both higher and lower density than water with only a single configuration was presented (28).

The object of the present work is the quantitation of R-(+)-limonene, precursor of the epoxides, and the separation and enantioselective determination of limonene and limonene-1,2-epoxides extracted from lemon peel samples by SDE extraction using an off-line MDGC with flame-ionization detection (FID) and mass spectrometric (MS) analysis in the selected ion monitoring (SIM) mode.

Experimental

Sample preparation

Four lemon (*Citrus limon*) peel samples were purchased from a local market. The peel was ground and homogenized using a mechanical blender. Ground lemon peel samples were kept refrigerated (5°C) until extraction.

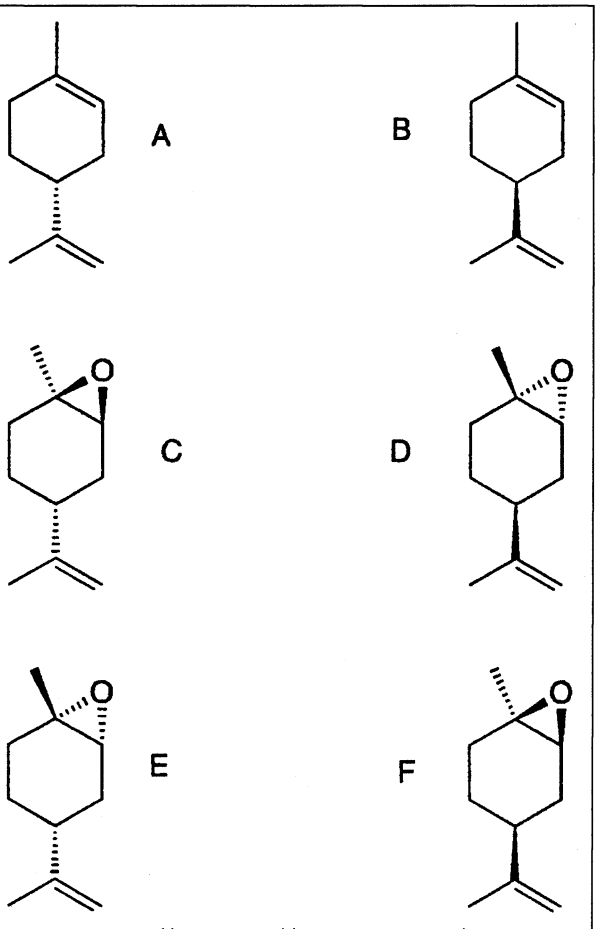

Figure 1. Structures of limonene and limonene-1,2-epoxides. (A) R-(+)-limonene, (B) S-(-)-limonene, (C) (1R,2S,4R)-(+)-limonene-1,2-epoxide or *cis*-(+)-limonene-1,2-epoxide, (D) (1S,2R,4S)-(-)-limonene-1,2-epoxide or *cis*-(-)-limonene-1,2-epoxide, (E) (1S,2R,4R)-(+)-limonene-1,2-epoxide or *trans*-(+)-limonene-1,2-epoxide, and (F) (1R,2S,4S)-(-)-limonene-1,2-epoxide or *trans*-(-)-limonene-1,2-epoxide.

The SDE device used in this study was a modification of the micro version designed by Godefroot et al. (27) and was constructed by Blanch et al. (28). Ground lemon peel and 100 mL of distilled water were placed in the 250-mL flask of the SDE device and brought to pH 7 with NaOH. Dichloromethane (2 mL, Sigma-Aldrich, Steinheim, Germany) was used as an extraction solvent. On the basis of our previous experience, extractions were carried out under the following conditions: the sample heating bath temperature was 149°C, the solvent heating bath temperature was 67°C, the coolant temperature was –15°C, and the extraction time was 2 h. The SDE was carried out under a nitrogen atmosphere.

Enantiomeric determination of R-(+)-limonene by MDGC

GC was performed on a Siemens Sichromat-2 MDGC (Siemens, Mannheim, Germany) with two separate ovens, an on-column injector, and two FIDs. Column switching was achieved with a pneumatically controlled six-port valve (Valco, Schenko, Switzerland). Peak broadening was minimized by cooling the first 10 cm of the chiral column with air that had been precooled with liquid nitrogen, thus focusing the cut fraction.

The achiral column was a 20-m × 0.3-mm-i.d., glass capillary column coated with crosslinked PS255 (stationary phase film thickness [d_f], 2 µm). Hydrogen was used as the carrier gas; the head pressure (p_i) in the column was 40 kPa. The column was held at 70°C for the first 3 min, programmed to increase 4°C/min to 220°C, and held at this upper temperature for 20 min. Samples (0.6 µL) were injected on-column.

The chiral column was a 25-m × 0.25-mm-i.d., fused-silica capillary column coated with Chirasil-γ-Dex (permethyl-γ-cyclodextrin) (30) (d_f = 0.25 µm) (Chrompack, Middelburg, The Netherlands). Hydrogen (p_i = 90 kPa) was used as the carrier gas. The column was held at 50°C isothermal.

Quantitative analysis of R-(+)-limonene

A 2-g amount of ground lemon peel and 100 mL of distilled water were placed in a 250 mL flask, and 10 µL of S-(-)-limonene (Fluka, Buchs, Switzerland) was added as an internal standard. The SDE extraction was carried out as described above, and the time of extraction was optimized. A second extraction was carried out similarly but without the addition of the optical antipode, S-(-)-limonene. An aliquot of each extract was injected on-column. The chromatographic conditions were as described in the previous section.

Enantiomeric determination of *cis*-(+)- and *trans*-(+)-limonene-1,2-epoxide

MDGC–GC–FID

The low concentration of the limonene-1,2-epoxides precluded the determination of its enantiomeric purity by conventional MDGC using FID. The modified MDGC instrument described above was coupled off-line with a second GC (Chrompack CP-9000). To improve the sensitivity of the determination, the cut fractions from two to six injections were trapped in a short, uncoated, fused-silica capillary (80 cm ×

0.25-mm i.d.) cooled with liquid nitrogen. This capillary was then installed in the second GC instrument, connected to the Chirasi-γ-Dex analytical column, and heated at 40°C. GC conditions for the achiral column were as described in the previous section.

The order of elution was assigned by injection of enantiomerically pure standards obtained from Fluka (*cis*-[+]- and *trans*-[+]-limonene-1,2-epoxide) and Aldrich (*cis*-[-]- and *trans*-[-]- limonene-1,2-epoxide).

MDGC–GC–MS–SIM

To confirm the observed enantiomeric ratios, GC–MS analysis in the SIM mode was carried out. Because our GC–MS instrument is not equipped with MDGC facilities, the off-line approach described above was taken. The cut fractions from several injections were combined and analyzed.

The GC–MS analyses were performed with a Carlo Erba Fractovap 2900 (Carlo Erba, Milano, Italy) coupled to a Varian MAT 112S (Varian, Bremen, Germany) in the SIM mode; the recorded ion was m/z = 153. The electron energy was 80 eV. Columns and chromatographic conditions were as described in the previous section.

The identification of the volatiles obtained from SDE of lemon peel was carried out with the GC–MS described above using a glass capillary column (20 m × 0.3-mm i.d.) coated with PS225 (d_f = 2 µm). Chromatographic conditions were as described previously.

Results and Discussion

The chromatogram of the volatiles isolated from fresh lemon peel of intermediate maturity by simultaneous SDE is shown in Figure 2. All compounds listed in Table I have been tentatively identified by GC–MS. The advantage of the SDE

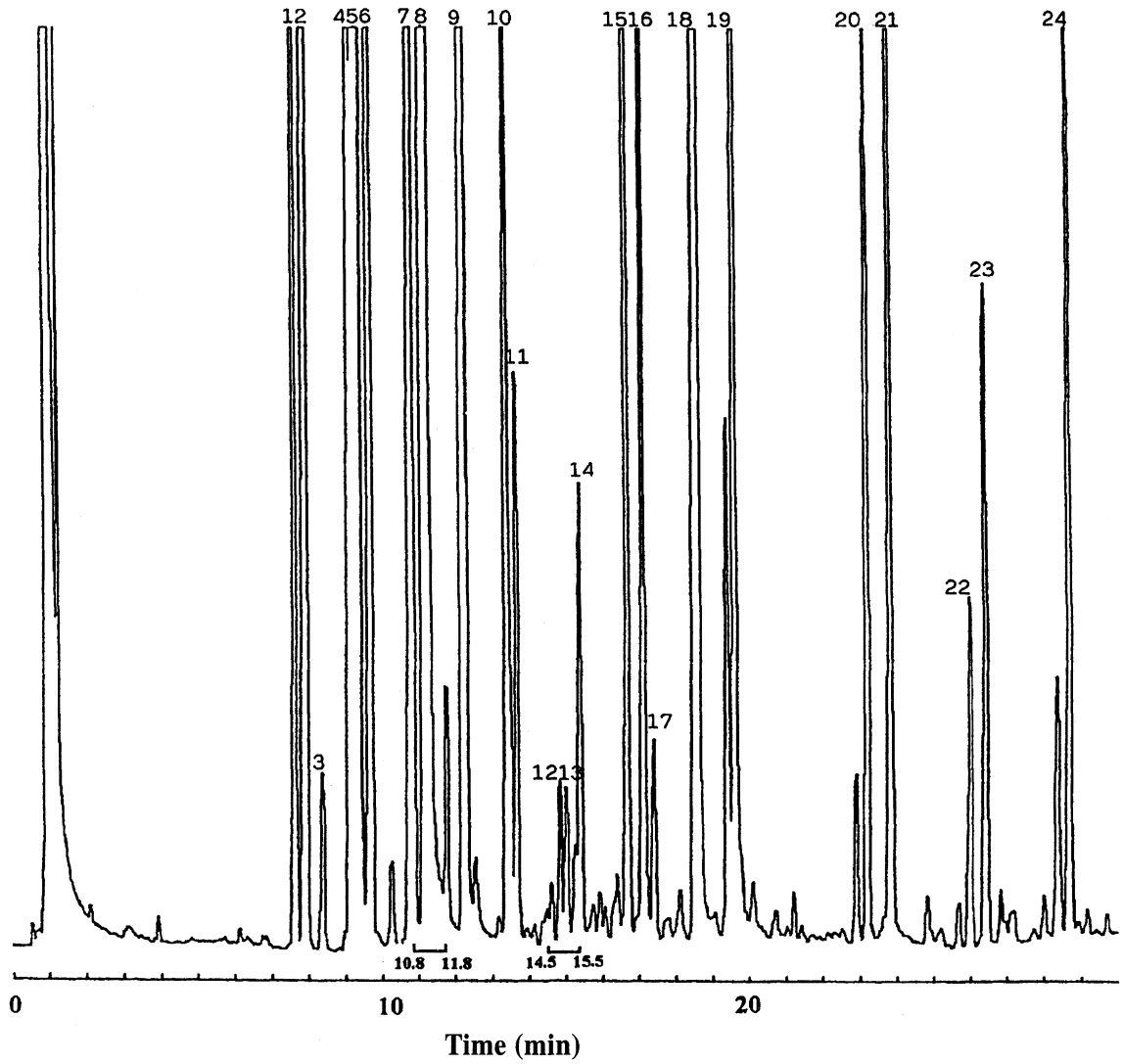

Figure 2. GC chromatogram of the volatiles obtained from SDE of lemon peel samples on a glass capillary column (20 m × 0.3-mm i.d.) coated with PS255 (d_f = 2 µm). The column temperature was programmed from 70°C (3 min) to 220°C (20 min) at 4°C/min. H_2 carrier gas, p_i = 40 kPa. The fractions indicated were transferred to the chiral column.Tentative peak identification is given in Table I.

method was the high quality of the extract obtained (absence of fatty acids), which requires no further purification. Preliminary studies of lemon oil indicated that the extraction method did not cause any changes in the composition of the flavor compounds (31).

As previously mentioned, limonene is the main component of lemon peel oil, constituting 54–76% (32), and is the precursor of limonene epoxide (10). The concentration of limonene in lemon peel was determined by the internal standard method using synthetic S-(-)-limonene as the added standard, thus better compensating for possible errors arising from incomplete recovery, decomposition, and losses during extractions that can occur when alkanes or diastereomers are used (33). Figure 3 shows the chromatograms of the separation of limonene isomers.

Because the calculation of the amount of R-(+)-limonene present in a sample is based on the known amount of synthetic S-(-)-limonene added as an internal standard, the proportions of the respective antipodes in the standard and in the sample must be first determined. The S-(-)-limonene used as an internal standard had an enantiomeric excess (EE) of 94.8%. The EE of R-(+)-limonene in the measured lemon peel samples was between 97.1 and 97.4% (Table II), which was in agreement with previous results (34,35).

Table I. Volatile Flavor Compounds Tentatively Identified by GC–MS in Lemon Peel Extract

Peak*	Compound	Relative peak area (%)
1	α-thujene	0.43
2	α-pinene	1.87
3	camphene	0.070
4	sabinene	1.75
5	β-pinene	14.4
6	myrcene	1.39
7	*p*-cymene	2.26
8	limonene (A and B in Figure 1)	61.0
9	γ-terpinene	10.3
10	linalool	0.56
11	nonanal	0.209
12	*trans*-limonene-1,2-epoxide (E and F in Figure 1)	0.055
13	*cis*-limonene-1,2-epoxide (C and D in Figure 1)	0.065
14	citronellal	0.031
15	terpinen-4-ol	0.49
16	α-terpineol	0.50
17	decanal	0.074
18	neral	1.12
19	geranial	0.87
20	neryl acetate	0.39
21	geranyl acetate	0.76
22	caryophyllene	0.148
23	*trans*-α-bergamotene	0.260
24	β-bisabolene	0.367
Total		99.4
Unidentified		0.6

* Numbering of peaks as shown in Figure 2.

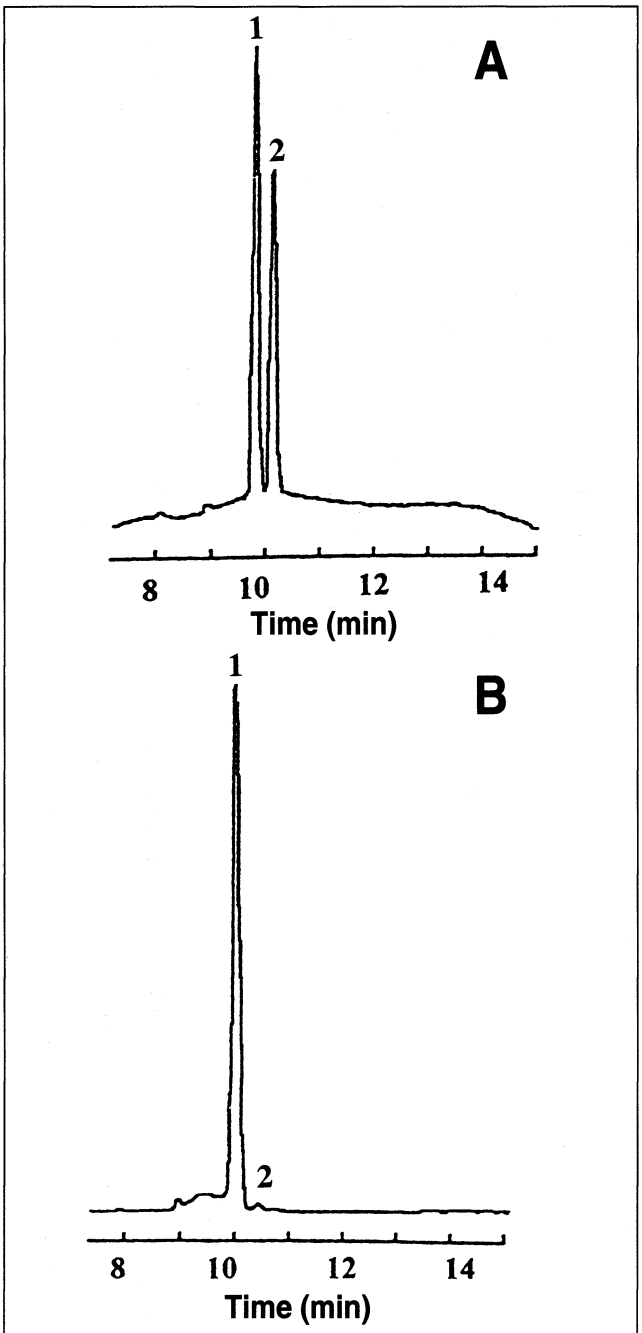

Figure 3. Enantiomeric separations of cut fractions obtained from MDGC of (A) racemic limonene and (B) lemon peel extract. Peaks: 1, R-(+)-limonene; 2, S-(-)-limonene. For chromatographic conditions, see the Experimental section.

Table II. Enantiomeric Excess (%) of R-(+)-Limonene in Lemon Peel Extract by MDGC and *trans*-(+)-Limonene-1,2-epoxide in Lemon Peel Extract by MDGC Coupled Off-Line with GC–FID and GC–MS–SIM

Sample	R-(+)-Limonene MDGC–FID	*trans*-(+)-Limonene-1,2-epoxide GC–FID	GC–MS–SIM
1	97.1	91.9	91.6
2	97.4	91.2	91.9
3	97.1	90.3	90.6
4	97.2	88.0	89.8

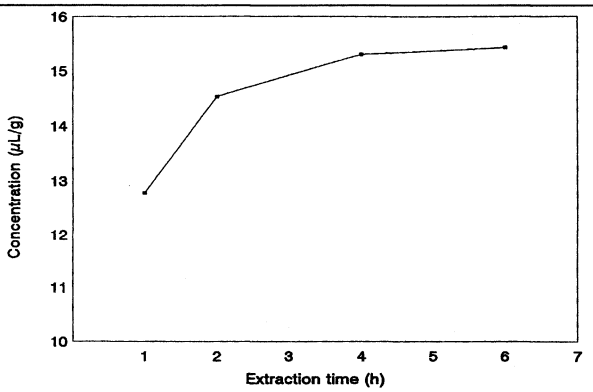

Figure 4. Concentration (µL/g) of R-(+)-limonene in lemon peel samples as a function of the extraction time.

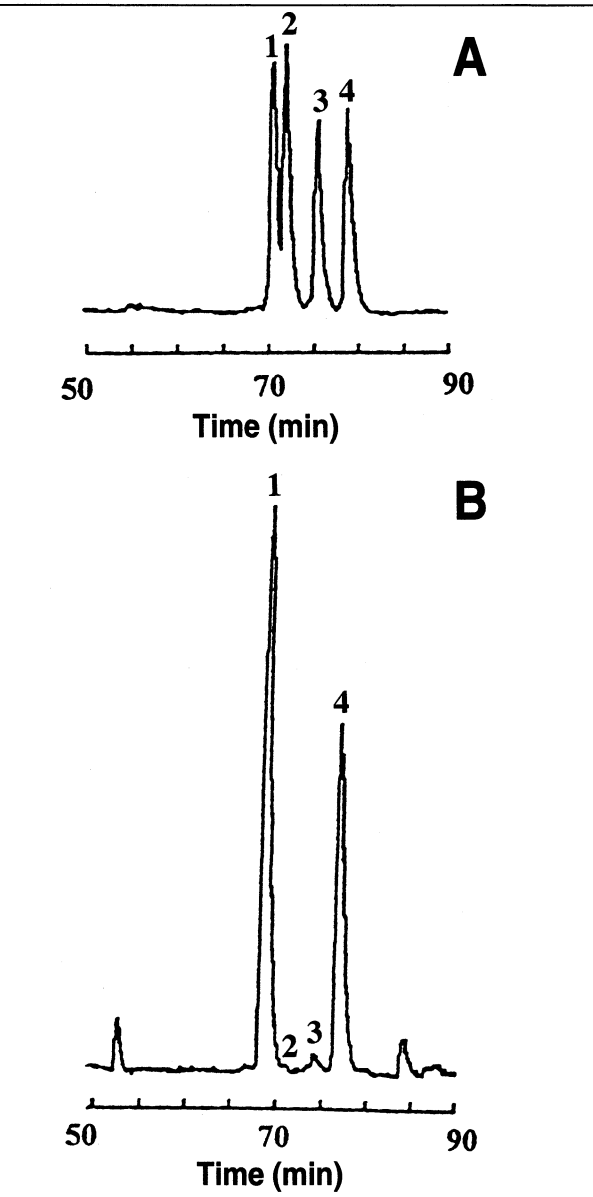

Figure 5. Enantiomeric separations of cut fractions obtained from MDGC–GC–FID of (A) racemic limonene-1,2-epoxide and (B) lemon peel extract. Peaks: 1, (1R,2S,4R)-(+)-; 2, (1S,2R,4S)-(-)-; 3, (1R,2S,4S)-(-)-; and 4, (1S,2R,4R)-(+)-limonene-1,2-epoxide. For chromatographic conditions, see the Experimental section.

As with the conventional internal standard technique, the standard should be added before any manipulations are executed in order to ensure that both standard and samples are subjected to identical treatment. A further prerequisite is the homogeneous distribution of the internal standard and the substance to be determined (i.e., the identical extractability of these compounds). This was checked by studying the influence of the extraction time on the quantitative result. Figure 4 depicts the effect of the extraction time on the recoveries of R-(+)-limonene. It can be seen that limonene was extracted from the lemon peel samples at a slightly slower rate than the S-(-)-limonene added as an internal standard. After 4 h of extraction, the R-(+)-limonene was seen to be completely extracted from the lemon peel because no further rise in its concentration was observed. The average concentration of R-(+)-limonene in the lemon peel samples studied was determined as 15 µL/g. This represents 61% of the total volatiles from the relative peak areas (Table I).

Previous studies on the formation of limonene epoxides under mild conditions with peroxo complexes have shown that the primary oxidation products of R-(+)-limonene are limonene-1,2-epoxides (Figures 1C and 1E). The regioselectivity was very high, and formation of limonene-8,9-epoxides was not observed (36). The monoepoxides also generate diepoxides that could not be detected in samples of fresh lemon peel oil. They were found in orange oil stored for over 25 years (37) and were probably artifacts from oxidation of limonene.

To study the extent of recovery for the extraction of limonene-1,2-epoxides by the SDE device under the applied conditions, extractions of water spiked with these compounds were carried out. Recoveries were calculated by adding *n*-undecane as an internal standard to the final extract and comparing the relative areas (epoxide area/alkane area) with the relative areas of a calibration mixture prepared by dissolving the same aliquot of the epoxides and the standard in 2 mL of dichloromethane. Recovery values for *cis*-(+)- and *trans*-(+)-limonene-1,2-epoxides were higher than 85%. The possibility of racemization during the extraction was excluded by carrying out the same extraction procedure with a single enantiomer each of *cis*-(+)- and *trans*-(+)-limonene-1,2-epoxide. To obtain the optimum separation of the enantiomers, MDGC was used. Enantiomeric separation of the compounds in Figures 1C–1F was obtained for the first time to the best of the authors' knowledge (Figure 5A). The compounds in Figures 1C and 1D were found in higher concentration (0.065%) than those in Figures 1E and 1F (0.055%). This showed that the endocyclic double bond was preferentially epoxidized at the *cis*- configuration with respect to the methyl and isopropenyl groups.

Quantitative determination of the epoxides proved difficult because of the low concentration involved. The amount of limonene epoxides transferred by a single cut from the achiral precolumn to the chiral capillary was insufficient for FID detection and quantitation of all enantiomers. The EEs of the two diastereomeric epoxide pairs were measured on cuts from several injections on an achiral thick-film (d_f = 2 µm) column (selected to increase the sample load) that were transferred to a trap capillary and analyzed on the chiral column that was installed in a second GC equipped with an FID (Figure 5B).

Using this strategy, the substances of interest could be readily concentrated by repeated injection. The results were confirmed using the same off-line MDGC technique but with MS–SIM detection (Figure 6).

The enantiomeric excess of R-(+)-limonene and limonene-1,2-epoxide from lemon peel extracts is given in Table II. Incomplete resolution of the enantiomers combined with widely differing peak heights prevented the exact determination of the enantiomeric excess of the compound in Figure 1C.

Conclusion

Although present at low concentration levels, the EE of limonene-1,2-epoxides was conveniently determined from lemon peel samples by MDGC, measured on cuts from several injections on an achiral thick-film column, transferred to a trap capillary, and analyzed on a capillary column coated with Chirasil-γ-Dex that was installed in a second GC. The results showed that the formation of the epoxides was not completely enantioselective, and further studies with the object of elucidating the biosynthetic pathways and mechanisms of detoxifications of limonene-1,2-epoxide in vivo appear to be worthwhile.

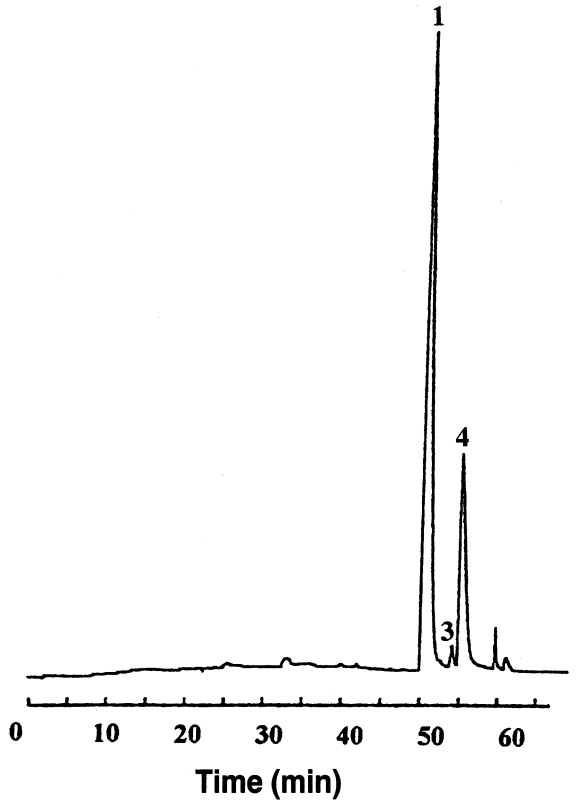

Figure 6. Enantiomeric separation of cut fractions obtained from MDGC–GC–MS–SIM (*m/z* =153) of lemon peel extract. Peak assignments are listed in Figure 5. For chromatographic conditions, see the Experimental section.

Acknowledgment

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