HPLC Isolation of the Sesquiterpene Hydrocarbon Germacrene B from Lime Peel Oil and Its Characterization as an Important Flavor Impact Constituent

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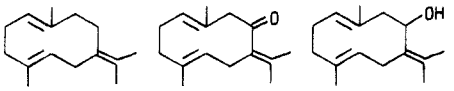
A general, normal-phase high-performance liquid chromatography (HPLC) method has been developed for the semipreparative fractionation of mono- and sesquiterpene hydrocarbon mixtures using three silica columns in tandem and elution with hexane. The method allows for efficient determination of gas–liquid chromatography and mass spectrometry (GC–MS) data on the collected fractions. This method has made possible the isolation of a labile sesquiterpene from the higher hydrocarbon fraction of Key lime peel oil, which has been identified by a variety of spectroscopic techniques and confirmed by synthesis to be germacrene B (1). Quantitative capillary GC analysis indicates this hydrocarbon to represent ≈0.35% of the whole oil. Germacrene B has a potent, warm, sweet, woody-spicy, geranium-like odor and is very important to the fresh lime peel character of the oil.

The use of HPLC in the analysis and isolation of flavor constituents has recently been reviewed (Bitteur, 1984), and we (Chamblee et al., 1985a) have just reported a general HPLC method for the prefractionation of the oxygenated constituents of essential oils, especially terpenes. This method is not useful for terpene hydrocarbons, however, as they tend to coelute early without proper resolution.

Several methods for separating terpene hydrocarbons by reversed-phase HPLC have been reported (Strack et al., 1980; Morin et al., 1986). The use of aqueous solvents in these methods requires time-consuming extraction before the samples can be concentrated and analyzed by GC–MS and other spectroscopic techniques. A reported (Kubeczka, 1981) normal-phase separation of mono- and sesquiterpene hydrocarbons at −15 °C with pentane appears cumbersome to practice.



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We report here a semipreparative, normal-phase HPLC method using three silica columns in tandem and eluting with hexane at room temperature. The method is quick, easy, reproducible, and particularly useful for the final separation prior to identification of heat-labile compounds. It has allowed both a very good separation of the higher hydrocarbon fraction of lime peel oil and the isolation of a new lime constituent, germacrene B (1), which has been found to be an important lime flavor impact compound.

Two types of commercial lime oils, expressed and distilled, are produced in Mexico from the Key lime (*Citrus aurantifolia* Swingle). The most widely used type is distilled lime, produced by crushing the whole fruit and steam distilling the oil (Arctander, 1960; Haro-Guzman and Huet, 1970). The other type is isolated directly from the peel of the fruit and is referred to as expressed, centrifuged, or cold pressed. Both types differ widely from each other in chemical composition and aroma character. Most of the major constituents of lime oils have been identified (Haro and Faas, 1985); however, new trace constituents have only recently started to be identified (Chamblee et al., 1985a,b), largely due to the adoption of HPLC techniques. A review of the literature yields no information on the constituents responsible for the warm-spicy, dry-out note of lime peel oil, which is important in distinguishing it from lemon.

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EXPERIMENTAL SECTION

Materials. Germacrone (2) isolated from Zdravets oil and recrystallized once (80% ethanol) was obtained as a gift from Dr. A. F. Thomas, Firmenich SA, Genève, and used as received. Woelm silica gel and alumina were used. The 1 M LAH in THF was from Aldrich.

Chromatography and Spectroscopy. The HPLC system was a Waters Model ALC/GPC 201 that included a M6000 pumping system, a M U6K universal injector, and a MR 401 differential refractometer. These columns were employed in tandem: two Whatman Partisil-PXS columns, consisting of 25 cm × 4.6 mm (i.d.) stainless-steel tubing packed with 10- or 5-μm microparticulate silica, and a Waters Radial-PAK 51 column, 10 cm × 8 mm (i.d.), 10-μm triple pack. The Partisil 10 column was placed before the Partisil 5 column followed by the Waters radial compression column. Slightly better separation could possibly be achieved by putting the 5-μm column first as is theoretically preferable. A guard column, consisting of 7 cm × 2.1 mm (i.d.) stainless-steel tubing and packed with Whatman HC-Pellosil, was used. A flow rate of 2 mL/min was employed for all separations. The hexane (HPLC grade) mobile phase was first filtered through SiO_2 (40 g, activity 1) and Al_2O_3 (40 g, activity 1) as recommended by Woelm. This eliminates the problem of polymeric solvent residue adhering to the pump check valves, causing them to malfunction.

Fractions were collected from the HPLC, concentrated as necessary, and monitored by GC or GC–MS. Capillary GC analysis of the lime hydrocarbon fraction before and after collection of the total effluent from HPLC showed no alteration by HPLC. It is, however, prudent to periodically "deactivate" and clean the column by pumping some polar solvent for a short time.

GC analyses were performed on a Varian 3700 equipped with a 1075 capillary injector and using helium as a carrier. Split ratios of 40:1 or 100:1 with injections of 0.4–0.8 μL were employed, depending on sample concentration. Either a J&W, DB-5 fused silica 30 m × 0.25 mm (i.d.), 1-μm film thickness, column or a Hewlett-Packard (HP), Carbowax 20m fused silica 50 m × 0.32 mm (i.d.), 0.5-μm film thickness, column was employed. The oven temperature

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was programmed from 70 to 180 °C at 2 or 5 °C/min with 10-min initial hold. The injector and detector were maintained at 160 and 250 °C, respectively. For quantitation, standard solutions in CH_2Cl_2 were prepared with weighed amounts of sample and tetradecane and appropriate response factors used.

Preparative GC fractionation was performed by collection with $^1/_8$-in. glass U-tubes cooled in an ice bath using a F&M 810 GC equipped with a TC detector (fid), a 12 ft × $^1/_4$ in. (i.d.) glass column configured for on-column injection, packed with HP-5% Triton X-305 on Chromosorb W (80–100 mesh) and programmed at 2 °C/min. Germacrene B (1) is very heat labile, and a faster program rate destroyed much of the material.

GC–MS analyses were carried out on a HP 5985 GC–MS equipped with a HP 5840 GC. IR spectra were determined on a PE-281 or 221 as solutions in CCl_4. ^1H NMR (300 MHz) were determined on a Bruker CXP-300 as solutions in $DCCl_3$–CCl_4 using the $HCCl_3$ proton as internal standard.

Capillary Odor Evaluation. Odor evaluations were performed on a Varian 3700 GC fitted with an empty TC detector tower that functions as a thermostated oven exit. HP 50 ft × 0.32 mm (i.d.) fused silica capillaries coated with either Carbowax 20m or 5% phenyl methyl silicone were used with a 1085 direct capillary injector. The end of the capillary column is fitted with a SGE VSOS 123630 outlet splitter consisting of a union connection to two empty fused silica capillaries of appropriate diameter to yield a 10:1 split. The larger capillary is diverted through the heated oven outlet (sniff port) to the atmosphere, while the smaller capillary goes to the fid for simultaneous detection. The component of interest remains as a gas up to the point of sniffing with no condensation or band broadening.

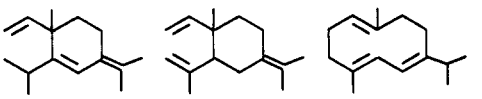
Isolation of the Higher Hydrocarbon Fraction of Lime. Commercial Mexican expressed lime oil (185 g) was distilled through a glass ASCO rotary film molecular still (2-in. i.d.). A similar model is now available from Pope Scientific. A lower boiling fraction (141 g, 76%), which consisted of monoterpene hydrocarbons and lower boiling oxygenates, was collected at 70–74 °C (12.5–14 mmHg). The pot residue (40 g) was redistilled and 17 g (9% of original charge) of volatiles removed at 81–83 °C (4–5 mmHg). The second pot residue (21 g, 11%) contained generally the higher boiling volatiles, including a considerable fraction of the sesquiterpenes and all the non-GC-volatile residue, but some monoterpenes remained. The distillation recovery was quantitative.

An open LC column (27-mm i.d.) was packed with silica gel (65 g, activity III). The pot residue of the lime oil distillation (≈4 g) was eluted with hexane (≈50 mL) until the point when no further compounds eluted as determined by GC. This hydrocarbon fraction consisted of a complex mixture of mono- and sesquiterpenes in addition to waxes.

Germacrene B (1). Germacrone (2) was reduced as described (Ognjanov et al., 1958) with LAH to yield germacrol (3). Germacrol was in turn reduced to germacrene B (1) by a procedure (Corey and Achiwa, 1969) proven useful for allylic terpene alcohols. Germacrol (1 g, 4.5 mmol) in THF was allowed to react at 0 °C with pyridine–sulfur trioxide (1.08 g, 6.8 mmol) for 5 h to form the sulfate monoester. The ester was reduced in situ with an excess of LAH (1 M in THF, 27.2 mL), added with stirring over a period of 1.5 h (0 °C) followed by reaction for 1 h (room temperature). The reaction was cooled, saturated Rochelle salts were added dropwise until all the salts precipitated, the liquid was decanted, and the salts were washed with ether (5 × 15 mL). The combined organic layer was washed with water (3 × 5 mL) and saturated NaCl (1 × 10 mL). The extract was dried over Na_2SO_4, concentrated in vacuo to ≈2 mL, and separated by on-column LC with activity III silica as previously described by eluting with hexane to yield a hydrocarbon fraction with one major peak (70% of total peak area by HPLC): germacrene B.

RESULTS AND DISCUSSION

The expressed Key lime oil has a warm, woody, sweet dry-out note and, in some samples, a green or herbaceous top note. These characteristic notes account for much of the difference between lime and lemon peel oils. The first ≈90% of a Mexican-expressed lime oil was distilled over in a thin-film dropping molecular still to yield a pot residue (10%) that contained 50–60% non-GC-volatile material and appreciable amounts of the sesquiterpene hydrocarbons and sesquiterpene alcohols. This pot residue is very important to the distinctive expressed lime flavor since the distilled fraction representing 90% of the whole oil has an odor somewhat similar to lemon. In order to learn more about the distinctive lime flavor, the pot residue was separated by open-column LC on silica gel into a hydrocarbon and an oxygenated fraction. Evaluation of the hydrocarbon fraction by smelling the GC effluent showed that only one peak out of the 25–30 sesquiterpenes resolved had any odor at the low concentration used for capillary GC. This peak is 61 (Figure 1; Table I) and represents ≈0.35% of the whole lime oil. The MS of peak 61, from a GC–MS run of the whole oil, was very similar to α- or γ-elemene (4, 5). α-Elemene (4) was synthesized by dehydration of elemol and found not to be the peak of interest since its GC R_t was very different from that for 61 and it did not have the characteristic odor.



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In order to prefractionate the lime hydrocarbon fraction for easier GC–MS analysis and in particular to isolate peak 61, a general HPLC method for terpene hydrocarbons was needed. Using three silica columns, a 10-μm, a 5-μm, and a radial compression column arranged in tandem, and a mobile phase of 100% hexane at room temperature, a very good separation was achieved for the lime hydrocarbons. If one has been using polar solvents, it is necessary to precondition the columns by pumping hexane for several hours. Even then there is a slight increase in R_t as the column becomes more active during continued use of the nonpolar mobile phase.

A typical HPLC chromatogram (Figure 2) yielded 13 peaks, each of which was collected and concentrated in vacuo and its GC/MS determined. A GC of the starting lime hydrocarbons and two representative subfractions is shown (Figure 3). As can be seen, the HPLC prefractionation facilitates collection of MS data by providing less complex samples. Tentative identification of a few major components by GC–MS and relative retention time demonstrates that the separation is particularly good for the sesquiterpenes, with some eluting in almost every fraction, while the monoterpenes elute mainly in peaks 4–8 (Figure 2). According to GC analysis, peak 12 (Figure 2) contains the desired lime impact compound and large amounts of another major sesquiterpene. Collection of the target compound from the lime hydrocarbon fraction by prepa-

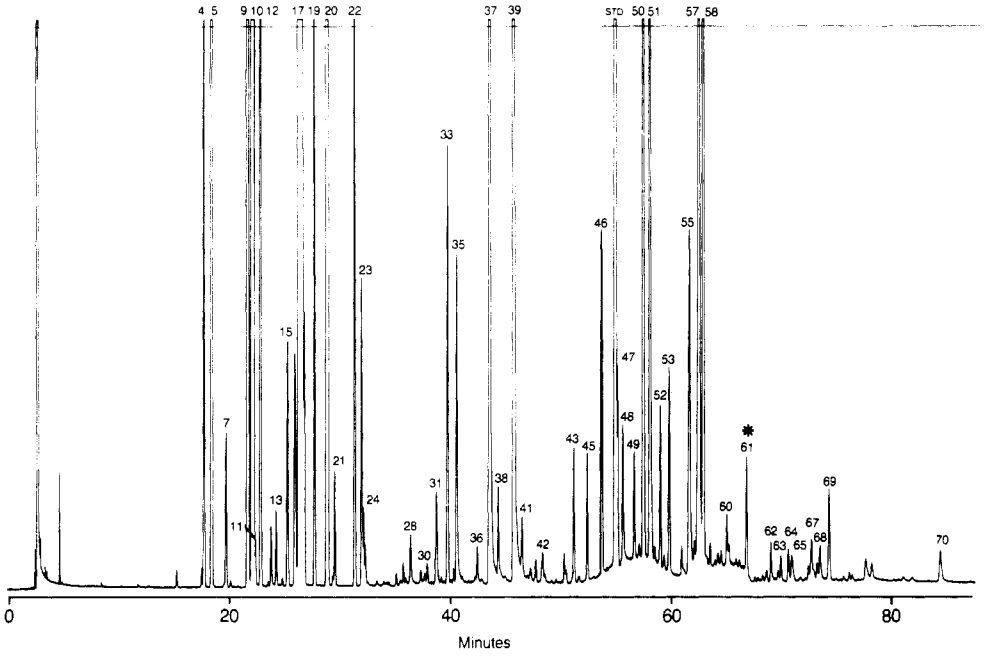


Figure 1. Capillary GC on DB-5 of expressed lime oil. Peak 61 is germacrene B.

Table I. Identified Peaks[a] in a Mexican-Expressed Lime Oil (See Figure 1)

peak no.	compound	peak no.	compound
4	α-thujene	41	bornyl acetate (t)
5	α-pinene	42	undecanal
7	camphene	43	δ-elemene
9	sabinene	46	gernayl acetate
10	β-pinene	47	β-elemene
11	6-methyl-5-hepten-2-one	48	dodecanal
12	myrcene	49	*cis*-α-bergamotene
13	α-phellandrene	50	caryophyllene
15	α-terpinene	51	*trans*-α-bergamontene
16	*p*-cymene	52	β-farnesene
17	limonene	53	α-humulene
19	monoterpene hydrocarbon	55	β-copaene (t)
20	γ-terpinene	57	α-farnesene
21	monoterpene ethers	58	β-bisabolene
22	terpinolene	60	α-bisabolene
23	linalool	61	germacrene B
24	nonanal	62	tetradecanal
28	citronellal	63	sesquiterpene alcohol
30	borneol	64	sesquiterpene alcohol
31	terpinen-4-ol	65	sesquiterpene alcohol
33	α-terpineol	67	2,3-dimethyl-3-(4-methyl-3-pentenyl-2-norbornanol) (t)
35	decanal		
36	nerol		
37	neral	68	campherenol
38	geraniol	69	α-bisabolol
39	geranial	70	hexadecanal

[a] Peak identifications are based on comparisons with file spectra of known standards or published spectra and relative retention time. If a question remained, a known standard was used for MS and peak enrichment. If a standard was not available the peak is marked tentative (t).

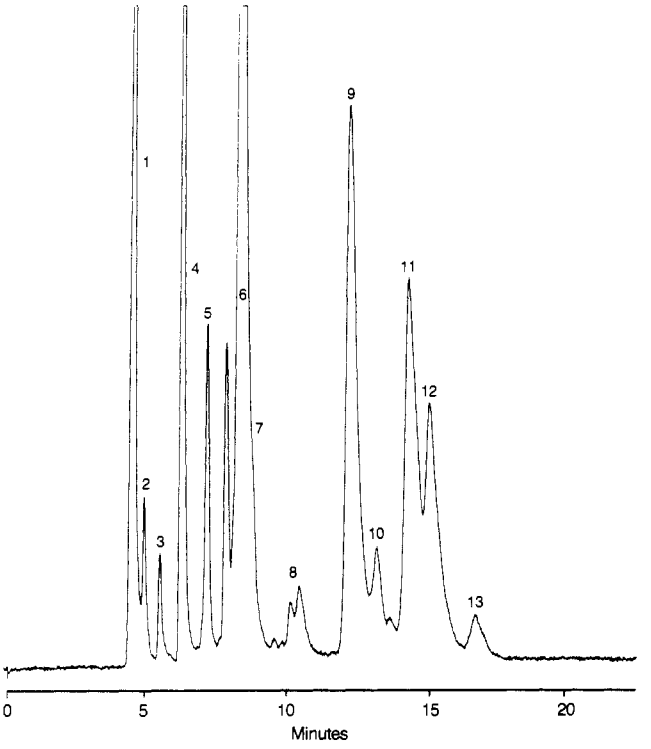


Figure 2. HPLC of higher hydrocarbon fraction of expressed lime oil, 100% hexane mobile phase, with only a *few* representative compounds listed: peak 1, solvent; peaks 2 and 3, trace amounts of monoterpenes; peak 4, α-thujene, β-pinene; peak 5, α-elemene, *cis*- and *trans*-α-bergamotene; peak 6, sabinene, limonene, β-santalene; peak 7 (sh), γ-terpinene, caryophyllene, *trans*-β-farnesene; peak 8, *p*-cymene, β-elemene; peak 9, β-bisabolene; peak 10, α-bisabolene; peak 11, α-humulene, α-farnesene; peak 12, germacrene B; peak 13, trace hydrocarbons.

rative GC also results in impure lime impact compound (60% purity). However, the use of preparative GC followed by HPLC of the fraction containing the target compound led to pure material suitable for IR, ^{1}H NMR, and UV analyses (≈98% purity by GC on Triton X-305). A summary of the isolation of germacrene B is provided in Scheme I.

The IR spectra (Figure 4) exhibited typical hydrocarbon absorption with strong bands at 2970 (s), 2920, and 2870 cm^{-1}, medium bands at 1445 cm^{-1} and a triplet of 1382, 1370, and 1360 cm^{-1}, and weak adsorption at 1338, 1250, 1220, 1180, 1140, 1110 and 1090 (d), 1050, and 900 cm^{-1}. The IR spectra ruled out γ-elemene (**5**) since the required strong terminal vinyl absorption at 900 cm^{-1} was absent. Germacrenes [either B (**1**) or C (**6**)] were suspected since they readily rearrange to elemenes on gentle heating by a Cope rearrangement in accord with the MS results. The germacrenes A and D were eliminated due to terminal

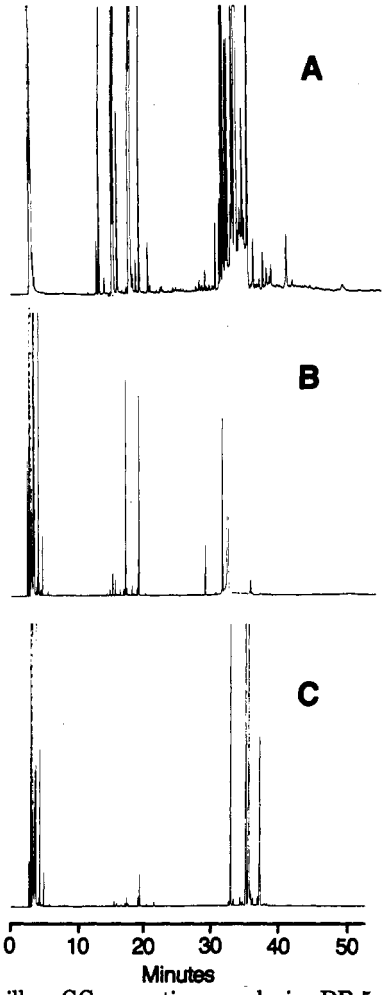
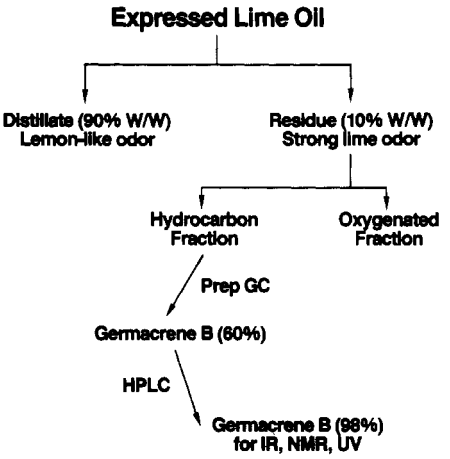


Figure 3. Capillary GC separations employing DB-5: (A) starting material, expressed lime oil—higher hydrocarbon fraction; (B) peak 5 (see Figure 2) collected from the HPLC; (C) peak 10 (see Figure 2) collected from the HPLC.

Scheme I. Isolation of Germacrene B



vinyls. The 300-MHz ^{1}H NMR (Figure 5) had two vinyl protons very far upfield at 4.67 and 4.48 ppm for endocyclic vinyl(s), which were required by the IR spectra. Also it exhibited two methyls on a double bond "too far" upfield at 1.47 and 1.50 ppm. Examination of the literature showed these anomalies were characteristic of germacrenes and were due to cross-ring shielding by each respective vinyl group (Sathe et al., 1968.) A low-resolution 60-MHz ^{1}H NMR spectra of germacrene B (1) provided by Professor Sutherland, University of Manchester, appeared quite similar to our spectra. The UV maxima of 220 nm was compatible with germacrene B and due to an interesting transannular conjugation reported for germacrenes (Sorm, 1971).

Germacrene B (1) was synthesized from germacrone (2) in a manner similar to that reported (Brown et al., 1975). The synthetic material, purified by open-column LC followed by HPLC, was identical with the compound isolated from lime as shown by 300-MHz ^{1}H NMR and IR. The odor of the synthetic material was identical with that from lime also.

Germacrene B is partially decomposed–adsorbed on the nonpolar liquid phase DB-5, and the best GC results are obtained on Carbowax 20m or Triton X-305. In our hands the best GC methods resulted in a 10–15% decomposi-

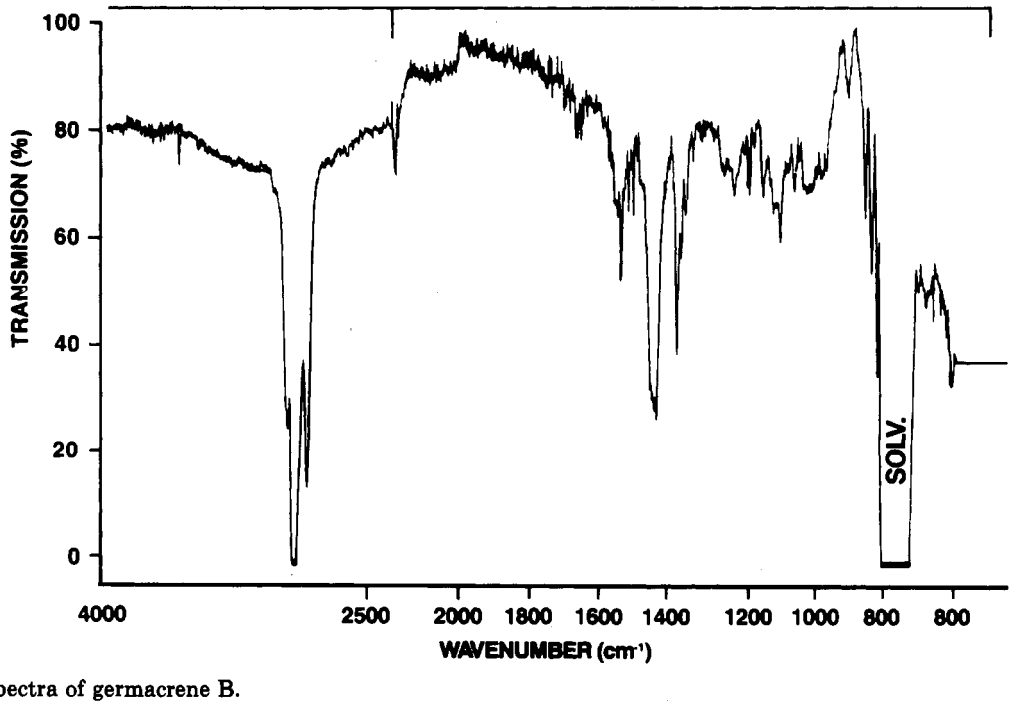


Figure 4. IR spectra of germacrene B.

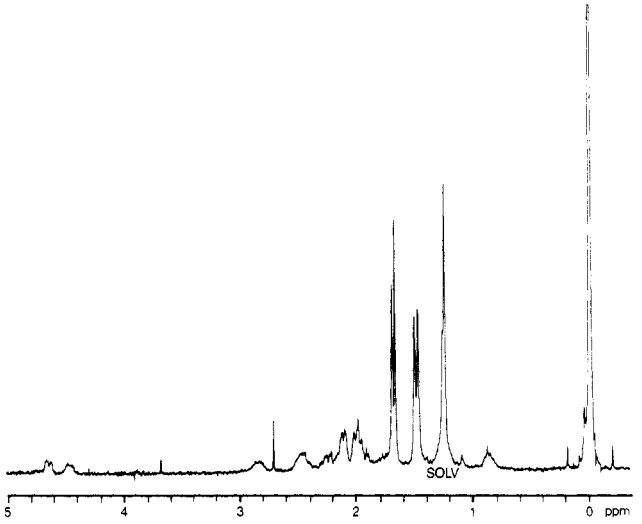


Figure 5. 300-MHz ^{1}H NMR spectra of germacrene B.

tion–adsorption relative to HPLC.

Germacrene B has been identified in 17 species belonging to 12 different terpene-producing families of plants; however, several of these reports offer little chemical evidence for its occurrence. It has only been reported once in *Rutaceae* (*Citrus junos*, a Japanese species), but no substantiating data were provided (Nishimura et al., 1969). Even though the compound has been reported as a major component of hop oil (Hartley and Fawcett, 1969), there is apparently no description in the literature of its odor. One should qualify this statement by noting that flavor articles, particularly reviews, are not always abstracted.

Germacrene B has been judged by a few experts in our laboratories to have a warm, sweet, woody-spicy, geranium-like odor and to be very important to the fresh lime peel character. It is definitely an impact compound of expressed lime that is very important in distinguishing it from lemon. Hydrocarbon impact compounds are rare (Ohloff et al., 1985) and extremely rare in citrus. The organoleptic properties of germacrene B are being evaluated further.

ACKNOWLEDGMENT

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