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# GENERAL METHOD FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PREFRACTIONATION OF ESSENTIAL OILS AND FLAVOR MIXTURES FOR GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS

## IDENTIFICATION OF NEW CONSTITUENTS IN COLD PRESSED LIME OIL

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SUMMARY

A general method for the high-performance liquid chromatographic profiling and separation of essential oils, especially those rich in mono- and sesquiterpenes, has been developed. This method was applied to the semi-preparative fractionation of several types of lime oil. The oils were fractionated on three different silica columns in tandem using a mobile phase of 8% ethyl acetate in hexane–methylene chloride (1:1), with refractive index detection. Fractions were collected, concentrated and analyzed directly by capillary gas chromatography–mass spectrometry. The method is simple, reproducible, easily scaled up, and requires no sample work-up.

The results clearly demonstrate that normal-phase high-performance liquid chromatography is valuable both as a profiling technique and as a prefractionation procedure prior to gas chromatographic–mass spectrometric identification. Twenty-three new constituents were tentatively identified in cold pressed lime oil using this technique. At least 40 yet unidentified new constituents, including many sesquiterpene alcohols, were well resolved by gas chromatography–mass spectrometry of the high-performance liquid chromatographic fractions.

## INTRODUCTION

The combination of gas–liquid chromatography and mass spectrometry (GC–MS) is generally accepted as the method of choice for separating and identifying the volatile constituents of flavors and essential oils. A more complete volatile analysis can usually be achieved, however, if a prefractionation step is incorporated prior to GC–MS.

Traditional methods for prefractionation of flavors and essential oils include distillation, preparative GC and column liquid chromatography on silica gel. All

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three techniques are subject to certain disadvantages: the first two methods often introduce thermal transformations, while the third is time consuming, provides very limited resolution and is not completely reproducible.

The application of high-performance liquid chromatography (HPLC) to flavor analysis has been restricted by the limited resolution that can be achieved compared with capillary GC and to a lesser extent by the lack of a sensitive universal detector. Nevertheless, the available published information[1] indicates that: (1) HPLC is preferred for the separation of thermally unstable flavor volatiles; (2) it has potential as a profiling technique unrestricted by differences in volatility; and (3) it is useful as a prefractionation procedure during identification of the components of complex flavors and essential oils. In the latter context three general approaches have been discussed in the literature[1]. The first involves the use of reversed-phase columns with aqueous solvents, isocratic or gradient elution, and UV detection[2–6]. The UV detector is highly sensitive to certain flavor compounds; however, its selectivity for specific chromophores is a disadvantage if a quantitative profile is desired. Short-wavelength UV monitoring can minimize this problem but it severely restricts the choice of solvent[4].

In the second approach, normal-phase HPLC is used with isocratic non-aqueous solvent mixtures and, in most cases, refractive index (RI) detection[7–13].

The third procedure involves the use of gel permeation techniques[2,14,15]. Although restricted by the narrow range of molecular weights usually found in flavor mixtures, the potential of this approach has not yet been fully explored.

The main objective of this work was to develop a simple, reproducible and fast HPLC method that would allow separation and prefractionation of complex essential oil mixtures such as lime oils. This would lead to more efficient GC–MS and other spectroscopic analysis of lime oil constituents as well as providing additional means of profiling essential oils, especially the non-volatile components which are not accessible through GC. Several criteria were chosen for the method: simplicity, ease of reproducibility, avoidance of aqueous solvents for faster work-up, acquisition of quantitative data with minimal use of response factors, easy scaling-up to semi-preparative levels and easy application to most essential oils. In our previous work on the semi-preparative HPLC separation of monoterpene mixtures[9,10] we found that normal phase, two silica columns in tandem, RI detection and isocratic elution with non-aqueous solvents best suited these criteria. The use of RI detection is especially useful for the quantitative analysis of citrus and other essential oils, since their constituents have extreme variability in UV absorptivity thus making UV detection unsuitable unless response factors are employed. A similar approach has been followed by others[11–13] for the separation of mono- and sesquiterpene mixtures.

Lime oils are of major importance to the flavor and fragrance industry. Although various production methods and different varieties of fruit are utilized, three well-defined products have commercial significance: cold pressed (expressed) lime oil, distilled lime oil and terpeneless* distilled lime oil[16]. The distilled oil is the most widely produced and accounts for a large proportion of the total world production of lime oils. Distilled lime is quite different in taste and composition from cold pressed lime, since it is generally prepared by steam distilling crushed fruit by a variety of

* The term "terpeneless" is used within the citrus oil industry to identify an oil that has had the terpene hydrocarbons substantially removed.

processes[17].

Despite the economic importance of lime oils many of their constituents remain unidentified. Generally, capillary GC analysis of a lime oil will yield a chromatogram containing 120 or more resolved peaks. A total of *ca.* 70 GC volatile compounds have been reported in all types of lime oil[18–21]. In the most recent report, 36 volatile components in cold pressed lime are identified[21].

## EXPERIMENTAL

The cold pressed and distilled lime oils were commercial samples of Mexican origin. The terpeneless distilled oil was derived from the distilled oil by column LC on Woelm activity III silica gel[22]. The oils were stored in the dark at 5°C and fresh samples were used if any oxidation products started to appear. Most samples were filtered through a 0.5-$\mu$m Millipore filter prior to chromatography. A summary of the quantitative composition, by compound class, of a cold pressed and distilled lime oil is given in Table I.

The HPLC system was a Waters Model ALC/GPC 201 which included a M6000 pumping system, a M U6K universal injector and a M R 401 differential refractometer. The columns were Whatman Partisil-PXS consisting of 25 cm × 4.6 mm I.D. stainless-steel tubing packed with 10- or 5-$\mu$m microparticulate silica. The Partisil 10 column was placed before the Partisil 5 column followed by a Waters radial compression column: Radial-PAK 51, 10 cm × 8 mm I.D., 10-$\mu$m triple pack. 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.

Fractions were collected from the chromatograph, concentrated as necessary on a rotary evaporator, and monitored by GC. In addition, a standard mixture consisting of caryophyllene, neryl and geranyl acetate and terpinen-4-ol was run

TABLE I

QUANTITATIVE GC ANALYSES OF LIME OILS

| | *Weight percent** | |
|---|---|---|
| | *Cold pressed* | *Distilled* |
| $C_{10}$ Hydrocarbons | 76.99 | 73.36 |
| $C_{15}$ Hydrocarbons | 5.60 | 2.47 |
| Alcohols | 1.23 | 8.58 |
| Aldehydes | 5.08 | 0.31 |
| Esters | 0.42 | 0.21 |
| Unidentified | 0.60 | 12.16 |
| Total GLC volatiles | 90.01 | 97.09 |
| Limonene | 42.10 | 45.19 |
| $\alpha$- and $\beta$-Pinene | 21.50 | 2.79 |
| Citral | 4.51 | 0.16 |
| Non-volatiles | 9.99 | 2.91 |

* Corrected for GLC flame detector response and using tetradecane as internal standard.

TABLE II

RESULTS OF BINARY AND TERNARY SOLVENT SYSTEMS FOR THREE TYPES OF LIME OIL

| | *Binary solvent* | | | | | | | | | *Ternary solvent* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *15% Ethyl acetate–hexane* | | | *5% Ethyl acetate–methylene chloride* | | | *10% Ethyl acetate–toluene* | | | *8% Ethyl acetate–(hexane–methylene chloride)* | | |
| | *Cold pressed* | *Distilled* | *TD** | *Cold pressed* | *Distilled* | *TD* | *Cold pressed* | *Distilled* | *TD* | *Cold pressed* | *Distilled* | *TD* |
| Number of peaks | 18 | 17 | 25 | 17 | 22 | 26 | 18 | 20 | 15 | 22 | 21 | 24 |
| Time (min) | 30.3 | 24.5 | 35 | 18.6 | 30.3 | 30.5 | 26.3 | 20 | 20.5 | 28.5 | 30 | 29 |
| Percent resolution** | 56 | 65 | 60 | 47 | 73 | 54 | 67 | 70 | 80 | 55 | 67 | 58 |

* Terpeneless distilled.

** (Number of baseline resolved fractions/number of peaks) × 100.

periodically. Capillary GC analysis of each oil before and after collection of the total effluent from HPLC showed essentially no alteration by HPLC.

Analyses by GC were performed on a PE-900 or Varian-3700 equipped with an on-column injector, a flame ionization detector, and a 12 ft. × 1/8 in. I.D. glass column packed with 5% Triton X-305 on Chromosorb W H.P. 80–100 mesh. The oven temperature was generally programmed from 70°C to 170°C at 5°/min with 5-min initial hold. The injector and detector were maintained at 150°C and 250°C, respectively. A flow-rate of 35 ml/min of helium was employed. Capillary GC analyses were performed on a Varian-3700 capillary instrument employing a glass 50-m Carbowax 20M column and programmed at 70–180°C at 2°/min with 10-min initial hold. The molecular distillation was carried out on an ASCO still; a very similar model is available from Pope Scientific.

GC–MS analyses were conducted with a DuPont 21-490 mass spectrometer, equipped with a DuPont 21-0948 data system, and interfaced to a Perkin-Elmer 3920 gas chromatograph through a glass jet separator. GC conditions were identical with those described previously for capillary GC analyses. The mass spectrometer was operated at 70 eV and 100 μA with source and separator temperatures at 180°C. Peak assignments were made by comparing spectra of unknowns with those of standards or published spectra.

## RESULTS AND DISCUSSION

### *Optimization of a general prefractionation procedure for lime oils*

Using our previous work[9,10] as a guide for initial conditions, a series of HPLC runs were made with the three types of lime oil using both binary and ternary solvent systems. In addition to the two silica columns used previously[10], it was found advantageous to add a silica radial compression column. The latter resulted in some improvement in resolution and an increase in capacity factors which facilitated collection of fractions. The optimum results for each system are summarized in Table II. The best separation for cold pressed lime using a binary solvent system was achieved with a mobile phase of 10% ethyl acetate in toluene. The distilled lime, which contains more alcohols and is thus more polar, was best separated with 5% ethyl acetate in methylene chloride, as was the very polar, terpeneless distilled oil. The pertinent chromatograms are given in Fig. 1. Since the first objective was good prefractionation, chromatograms were analyzed on the basis of both the number of peaks and the number of discrete fractions with approximate baseline resolution as shown in Fig. 1C and Table II.

To establish a general procedure for the semi-preparative runs, it was desirable to standardize the mobile phase. It was also useful to eliminate toluene owing to its relatively high boiling point (111°C) and poor baseline stability which interfered with quantitative analysis.

A solvent composition intermediate in polarity between ethyl acetate–hexane and ethyl acetate–methylene chloride appeared to offer the best compromise. It was found that for each type of lime oil 8% ethyl acetate in a 50:50 mixture of methylene chloride–hexane gave 21 or more peaks with 55–67 "percent resolution"* within a

* "Percent resolution" is defined here as the number of baseline resolved fractions/the total number of peaks × 100 (see Fig. 1C).

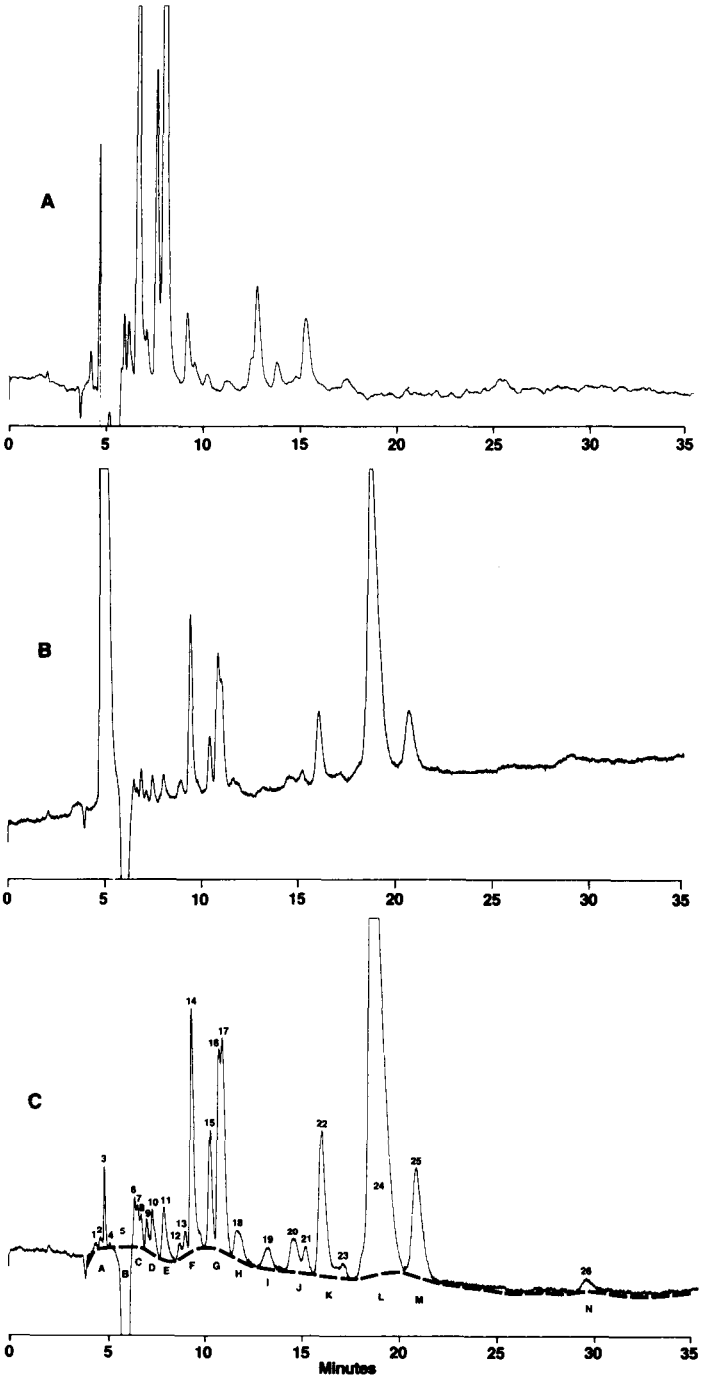



Fig. 1. HPLC separation of lime oils with binary solvent systems. (A) Cold pressed lime oil with ethyl acetate–toluene (10:90, v/v); (B) distilled lime oil with ethyl acetate–methylene chloride (5:95, v/v); (C) terpeneless distilled lime oil with ethyl acetate–methylene chloride (5:95, v/v). Individual peaks are numbered, discrete fractions are lettered.

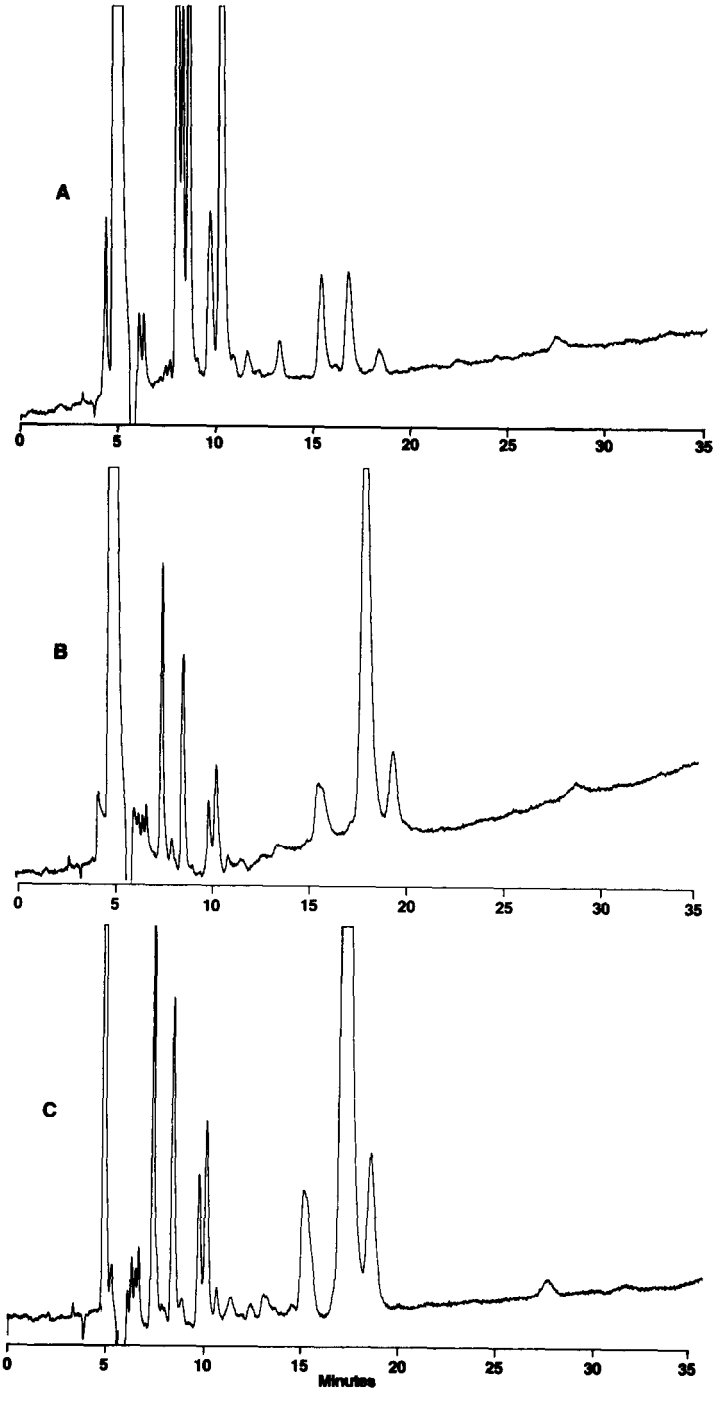



Fig. 2. HPLC separation of lime oils with a ternary solvent system, ethyl acetate–methylene chloride–hexane (8:46:46, v/v). (A) Cold pressed lime oil; (B) distilled lime oil; (C) terpeneless distilled lime oil.

30-min run (Table II). Duplicate analyses gave identical results. Representative chromatograms are given in Fig. 2. Hydrocarbons eluted mainly in the first major peak and to some extent in the second peak while oxygenated components were distributed through all peaks except the first.

*Analysis of cold pressed lime oil. HPLC resolution of an oxygenated fraction*

It is generally agreed that oxygenated constituents contribute much more than hydrocarbons to the characteristic flavor of citrus oils; consequently, it was of interest to examine the potential of HPLC for prefractionating oxygenated components of these oils for GC–MS and other spectroscopic analyses. Cold pressed lime oil was chosen as the initial example for study. The hydrocarbons were removed from the oil by column LC on activity III silica using hexane elution, and the oxygenated constituents were obtained in two fractions of approximately equal volume by further quick elution with methylene chloride.

The second oxygenated fraction from the column LC, which was analyzed by HPLC using the standard conditions already described, gave the chromatogram shown (Fig. 3). Material of each peak was collected as the numbered fractions shown, concentrated *in vacuo* and analyzed by GC–MS. Capillary GC of the starting material and two representative HPLC fractions are given in Fig. 4. As can be seen the HPLC prefractionation greatly facilitates the GC analyses, providing well resolved compounds for MS determinations. In Table III are listed 23 compounds found and identified for the first time in cold pressed lime oil. It should be noted that many peaks were resolved that have not yet been identified and that not all the oxygenated fraction of lime oil was analyzed.

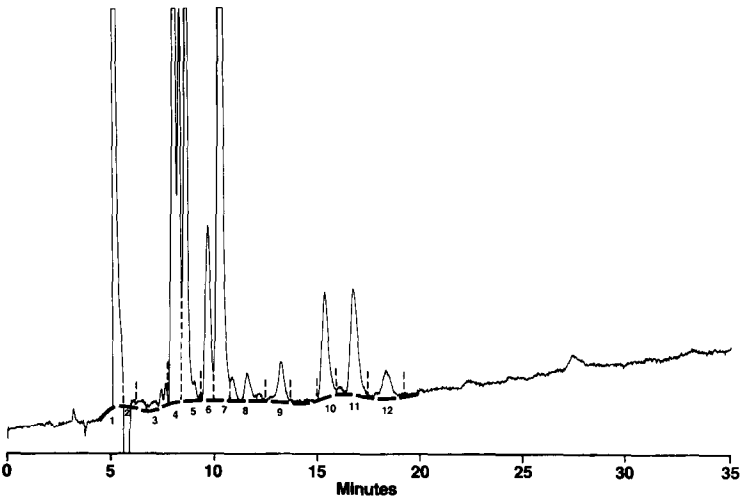



Fig. 3. HPLC separation of an oxygenated fraction from cold pressed lime oil using the solvent system of Fig. 2, with a few representative compounds listed for each peak. Peaks: 1 = $C_{10}$ hydrocarbons, caryophyllene; 2 = decanal, dodecanal, esters; 3 = 6-methyl-5-hepten-2-one, perilla aldehyde, sesquiterpene alcohols (SQA); 4 = neral, SQA; 5 = neral, geranial, 1,8-cineole, SQA; 6 = α-bisabolol; 7 = terpinen-4-ol, SQA; 8 = linalool, β-terpineol; 9 = *trans*-pinocarveol, SQA; 10 = oxides, SQA; 11 = borneol, *p*-cymen-8-ol; 12 = α-terpineol, verbenol, α-cadinol.

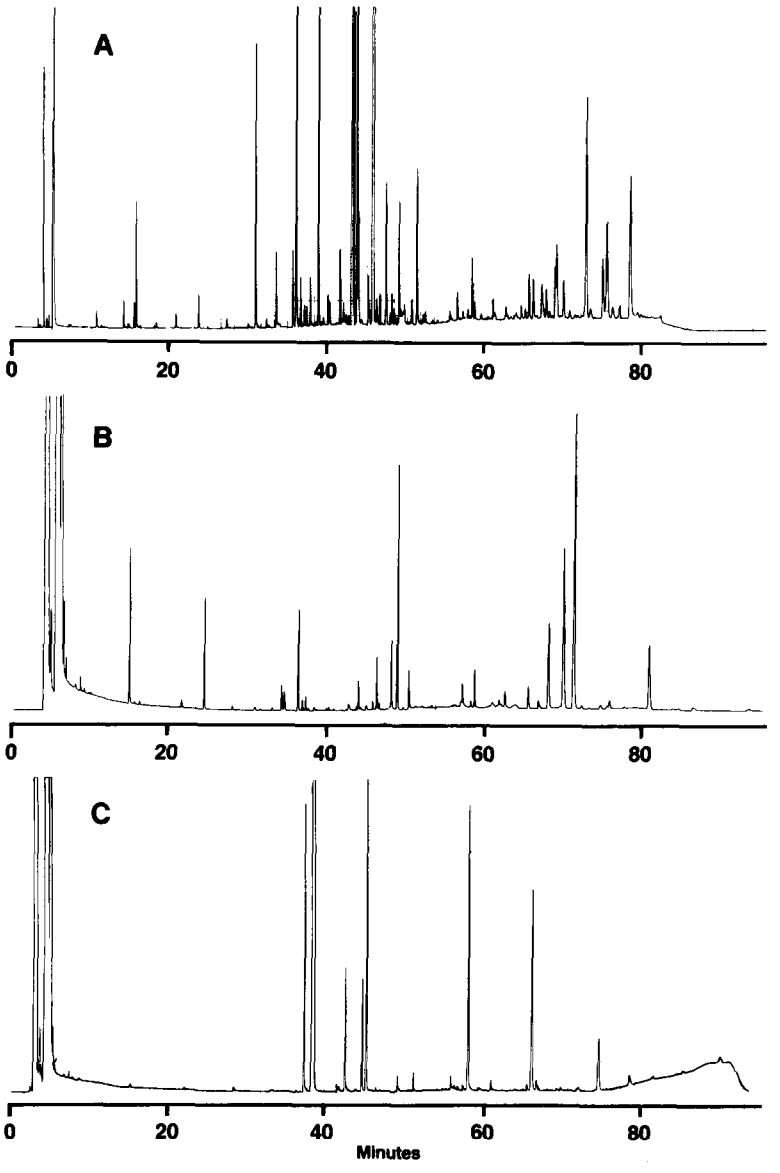



Fig. 4. Capillary GC separations of the oxygenated cold pressed lime oil fraction and two representative HPLC subfractions. (A) Total oxygenated fraction shown in Fig. 3; (B) subfraction 3 from Fig. 3; (C) subfraction 7.

The method is particularly good for separating terpene alcohols. For example, terpinen-4-ol (compound 1) elutes in Fraction 7 and the closely related α-terpineol (compound 2) in Fraction 12. At least eighteen sesquiterpene alcohols and ketones, which represent *ca.* 0.5% of the total volatiles of cold pressed lime, are also very well separated. For example, one or more sesquiterpene alcohols appear in every fraction

TABLE III

NEW COMPOUNDS IDENTIFIED IN COLD PRESSED LIME OIL

| | |
|---|---|
| Dodecyl acetate (2)* | *p*-Menth-3-en-1-ol (12) |
| 1,4-Cineole (2)** | Myrtenol (11) |
| A farnesal (3) | *trans*-Pinocarveol (9) |
| 3-Hexanone (5) | Sabinol (9) |
| 6-Methyl-5-hepten-2-one (3) | β-Terpineol (8) |
| Piperitone (7) | Verbenol (12) |
| *cis*-Carveol (10) | α-Bisabolol (6) |
| *trans*-Carveol (10) | α-Cadinol (12) |
| Citronellol (12) | A farnesol (12) |
| *p*-Cymen-8-ol (11)*** | 1,3-Dimethyl-3-(4-methyl-3-pentenyl)-2-norbornanol (3)§ |
| Isopiperitenol (8) | 2,3-Dimethyl-3-(4-methyl-3-pentenyl)-2-norbornanol (3)§ |
| γ-Isogeraniol (12) | |

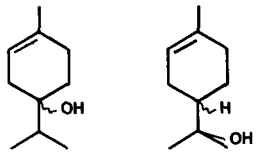
* Occurs for example in Fraction 2 of Fig. 3.

** Inferred to be present in cold pressed lime oil, but not clear[24,25].

*** Previously identified in lime essence[18].

§ Essentially identical mass spectra to those reported[26], however, the compounds were not confirmed[26] by synthesis.

from F-3 to F-10 except F-8. They are reported here for the first time* in lime; however, only a few tentative specific identifications have been made. In the case particularly of the sesquiterpene alcohols and to a lesser extent with monoterpene alcohols this HPLC technique provides very significant resolution and very different order of elution compared with GC.




1 2

The work described here also permits some comments on the potential of this method as a profiling technique for non-volatiles. Cold pressed lime oil usually contains an appreciable non-volatile fraction, especially if it has not been sufficiently winterized (de-waxed). The oil used in the current work contained *ca.* 10% non-volatiles based on quantitative capillary GC analysis. Several peaks in the chromatogram in Fig. 2A were suspected to be due in part to non-volatile material. This was confirmed by passing the oil through a thin film rotary molecular still to remove a substantial portion of the GC volatiles. Our HPLC technique proved to be useful for monitoring the progress of the distillation and also pinpointing peaks which contained significant amounts of non-volatile material. This capacity for detecting both volatiles and non-volatiles, as well as its value in the analysis of terpenes that

* Nerolidol and farnesol have been reported[23] in a rare oil of lime (*Limonette petitgrain* oil). The oil had been harshly saponified.

are too unstable for GC, strongly suggests that HPLC should be investigated further as a profiling technique for essential oils and flavors.

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