total limonoids. However, even in this sample the major limonoid was still limonin.

Extractor Pressure Effects. As previously reported by Attaway (1977) and shown in Figure 3, juice limonin concentrations increase rapidly with increasing extractor pressure. As the fruit was squeezed harder, the amount of juice recovered is increased, but the juice was higher in limonoid content and, thus, more bitter. The increase in bitterness has generally been attributed to extraction of limonoids from other fruit parts such as segment membrane, central core, and seeds in which limonoid concentrations are much greater. The three extractor pressures in Figure 3 correspond to a soft, hard, and very hard squeeze. It is interesting to note that in the soft and hard squeezes the seedy Duncan cultivar had less limonin and nomilin than the seedless Marsh cultivar. Under very heavy extractor pressure there was no cultivar difference in limonin or nomilin contents of the juice. Both nomilin and limonin concentrations doubled as extractor pressure was increased from 14 to 45 psi. As extractor pressure was increased to 64 psi the limonin content of Duncan grapefruit juice more than tripled and Marsh doubled, whereas the nomilin concentration of Marsh grapefruit juice remained unchanged and of Duncan increased only slightly. Therefore, under soft and hard squeeze conditions both limonin and nomilin concentrations increase in roughly the same proportion. Under very harsh squeeze conditions limonin concentrations continue to increase whereas nomilin concentrations are relatively unaffected.

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Quantitative Analysis of Cold-Pressed Lemon Oil by Glass Capillary Gas Chromatography

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Cold-pressed lemon oil, derived from California and Arizona fruit, was analyzed by glass capillary gas chromatography. Thirty-seven components were determined in a single chromatographic run by using the internal standard method and a computing integrator. Six of these components, α-thujene, 3-carene, octanol, nerol, geraniol, and nonyl acetate, had not previously been quantitated in lemon oil. Because of the much higher resolution of capillary columns, compositional data from the present study should be more accurate than those previously obtained by packed column techniques.

The advantages of glass capillary gas chromatography for analysis of complex mixtures of volatiles have been discussed extensively in the recent literature. The unparalleled resolution, inertness, and speed of analysis offered by glass capillary columns have prompted their use in place of packed columns and more than compensate for the somewhat greater care and dexterity required for their proper installation. The commercial availability of excellent capillary columns has made high-resolution gas chromatography available to most laboratories.

Recently, Jennings (1979) has surveyed some applications of glass capillary columns for food and essential oil analysis. The inability of packed columns to adequately separate such samples was clearly illustrated, and the benefits of analysis by capillary techniques were summarized.

Citrus oils are ideal candidates for analysis by such techniques. Their components display a wide range of functionality, polarity, and volatility, yet certain groups of components, such as the mono- and sesquiterpene hydrocarbons, require high column efficiency for complete separation. Although incompletely understood, sensory properties are known to depend critically on the presence of several minor components in the correct proportion (Shaw, 1977).

The economic importance of citrus oils and their widespread use in the flavor and fragrance industries make the acquisition of accurate compositional data highly desirable.

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Quantitative analytical data on cold-pressed citrus oils were most recently reviewed by Shaw (1979). The fact that most components were determined by packed column gas chromatography, usually without the aid of response factors, indicated the need for additional work. Accordingly, Wilson and Shaw (1980) employed glass capillary gas chromatography to quantitate 32 components of Florida, cold-pressed grapefruit oil, using a weighed calibration mixture and an internal standard for highest accuracy.

This study describes a similar application of glass capillary gas chromatography to the analysis of cold-pressed lemon oil and represents the most thorough quantitative study of this essential oil yet reported.

EXPERIMENTAL SECTION

Cold-pressed lemon oil was a commercial blend of California and Arizona oils. Its values for percent citral (2.4% by hydroxylamine method), refractive index (1.474), optical rotation (+65.5°), and specific gravity (0.852) were within the ranges specified in "Food Chemicals Codex" (1981). Compounds used as calibration standards were obtained from commercial sources, with the exceptions noted below, and were purified by suitable combinations of distillation and chromatography. Citronellyl acetate was obtained by acetylation of citronellol; α-thujene and sabinene were obtained by fractionation of cubeb oil; caryophyllene, *trans*-α-bergamotene, and β-bisabolene were obtained by fractionation of cold-pressed lemon oil. The identity of each compound was confirmed by its infrared spectrum.

Peak assignments were made by the technique of coinjection and peak enhancement. All of the compounds had been previously established as components of cold-pressed lemon oil by spectroscopic identification. A calibration mixture, containing weighed amounts of each component to be quantitated and approximating the composition of cold-pressed lemon oil, was used to determine response factors. Methyl myristate was used as the internal standard. Minor components were weighed accurately on a Cahn 25 Automatic Electrobalance by introduction into a septum-stoppered weighing vessel with a microliter syringe.

Analytical gas chromatography was carried out on a Shimadzu GC-Mini 2 equipped with a glass-lined inlet splitter and flame ionization detector. The detector signal was taken to a Spectra-Physics SP-4100 computing integrator which was programmed for the internal standard method. The sample and calibration mixture were injected in triplicate. The column was a 60-m glass capillary coated with SE-54 (Supelco, Inc., Bellefonte, PA). The He flow ($\bar{u}$) was 24 cm/s at 160 °C and 25 psi of pressure. Both capillary inlet and detector were held at 250 °C. Oven temperature was held at 85 °C for 18 min, raised to 230 °C at 3 °C/min, and held at 230 °C for 15 min. Sample size was 0.3 μL of undiluted oil or calibration mixture, split 50/1. Integration parameters were 6, 12, and 100 for peak width, peak threshold, and minimum area, respectively.

RESULTS AND DISCUSSION

Using a weighed calibration mixture and an internal standard, we determined the concentration of 37 components of cold-pressed lemon oil. The components are listed in Table I in order of their retention on SE-54, a nonpolar, silicone phase. Six of these components, α-thujene, 3-carene, octanol, nerol, geraniol, and nonyl acetate, are quantitated here for the first time. For the remaining components, the table also includes the previously reported range of values.

The concentrations of 16 components lay outside the reported range, with all but one (neryl acetate) of these being lower than any previous value. This result is similar to that obtained by Wilson and Shaw (1980) and is undoubtedly due to the much higher efficiency of capillary compared to packed columns. Commercially, the quality of lemon oil is judged primarily on its citral (neral plus geranial) content. Our value for total citral (1.98%) is predictably lower than that (2.4%) found by the traditional, nonspecific, hydroxylamine method. However, the sum (2.3%) of our values for all aldehydes determined chromatographically is in surprisingly good agreement with total aldehyde determined chemically.

Little can be said relating our quantitative results to lemon flavor. While other components besides citral are clearly necessary for a complete flavor, no work using high-resolution chromatography to correlate the concentration of minor components with flavor quality has been reported.

The internal standard method, as applied here in conjunction with glass capillary gas chromatography, provides the most accurate and thorough quantitative analysis of a citrus oil. Its routine use, for screening or quality control, is usually precluded by the difficulty of obtaining pure compounds as reference standards and of formulating the calibration mixture. The limited chemical stability of many citrus oil components and of the calibration mixture itself complicates the logistics of analysis. For research purposes, however, where weight percent data are desirable, the internal standard method is indicated. Naturally, routine application of the method becomes more feasible if it is known that only a few components have a significant flavor impact. The techniques described here are currently

Table I. Volatile Components of Cold-Pressed Lemon Oil

component	concn, wt %	lit. range,[a] wt %
α-thujene	0.38	
α-pinene	1.72	0.4-5.0
camphene	0.05	0.2-0.5
sabinene	1.65	0.5-1.5
β-pinene	10.13	2.2-13.9
6-methyl-5-hepten-2-one	0.002	0.06
myrcene	1.50	0.9-12.7
octanal	0.07	0.1-0.15
α-phellandrene	0.04	0.2
3-carene	0.004	
α-terpinene	0.32	0.7
p-cymene	0.04	0.6-1.1
limonene	68.72	54-80
γ-terpinene	8.55	2.9-14
octanol	0.01	
terpinolene	0.39	0.6-0.9
linalool	0.14	0.08-0.3
nonanal	0.12	0.09-0.3
citronellal	0.07	0.03-0.2
terpinen-4-ol	0.14	0.01-0.4
α-terpineol	0.22	0.2-0.5
decanal	0.04	0.06-0.2
octyl acetate	0.004	0.04
nerol	0.04	
neral	0.76	0.4-1.3
carvone	0.007	0.04
geraniol	0.04	
geranial	1.22	0.6-2.3
nonyl acetate	0.006	
citronellyl acetate	0.02	0.04-0.2
neryl acetate	0.50	0.1-0.4
geranyl acetate	0.43	0.1-1.0
dodecanal	0.02	0.1
caryophyllene	0.24	0.3
trans-α-bergamotene	0.37	0.4
α-humulene	0.02	0.2
β-bisabolene	0.56	0.14-1.4

[a] Shaw (1979).

being used to study geographic and seasonal influences on citrus oil composition.

We found the stationary phase SE-54 to be superior to the widely used Carbowax 20M for analysis of citrus oils. It not only affords good separation of aldehydes, but its greater chemical and thermal stability also provides longer column life and shorter analysis time.

CONCLUSION

Existing data on the quantitative composition of cold-pressed lemon oil probably suffered from the inaccuracies due to the relatively low resolution and high residual activity of packed gas chromatography columns. The application of glass capillary gas chromatography allows quantitation of major, minor, and trace components in a single chromatographic run when used with a calibration mixture for determination of detector response factors.

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Reduction of Limonin Bitterness in Navel Orange Juice Serum with Bacterial Cells Immobilized in Acrylamide Gel

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Limonin debittering of navel orange juice serum was successfully demonstrated with *Arthrobacter globiformis* cells immobilized in acrylamide gel. Treatment of 30 mL of serum (10–27 ppm of limonin), for instance, on a 1.5 cm diameter column packed with 1.6 g of immobilized cells (16-mL bed volume) reduced limonin content by 70% or more. This column was used 17 times without losing its effectiveness. The treatment did not affect juice composition as measured by total acids, total soluble solids, pH, and sugars. 17-Dehydrolimonoate A-ring lactone was identified as the major metabolite, showing the involvement of limonin D-ring lactone hydrolase and limonoate dehydrogenase in the debittering process.

Limonin (I; see Scheme I) is a bitter member of a group of limonoids which are chemically related triterpene derivatives found in Rutaceae and Meliaceae. Bitterness due to I in certain citrus processed products such as navel orange juice is one of the primary determinant factors for product acceptability and has significant economic impact on the industry. The intact fruits do not normally contain I but rather a nonbitter precursor, limonoate A-ring lactone (II), and this nonbitter precursor converts to bitter I under acidic conditions after extraction of juice (Maier and Beverly, 1968; Maier and Margileth, 1969). This conversion is also accelerated by action of limonin D-ring lactone hydrolase which has been shown to be present in citrus (Maier et al., 1969). This phenomenon is referred to as delayed bitterness.

In dealing with this bitterness problem one of the approaches taken at our laboratory is to develop a process which converts I and II in the juice to nonbitter compounds with limonoid-metabolizing enzymes. During the course of this study, we have isolated from soil several species of bacteria which metabolize limonoids (Hasegawa et al., 1972a,b; Hasegawa and Kim, 1975), and have established that limonoids are metabolized in bacteria and citrus through at least two pathways: one via 17-dehydrolimonoids (Hasegawa et al., 1972b, 1974) and the other via deoxylimonoids (Hasegawa et al., 1972a, 1980).

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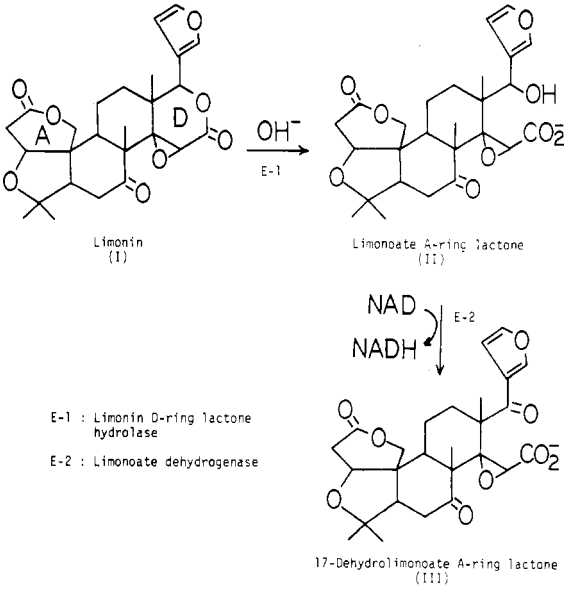
Scheme I. The Major Limonoid-Metabolizing Enzyme System in *A. globiformis* Cells Immobilized in in Acrylamide Gel



Among the species of bacteria isolated, *Arthrobacter globiformis* produces limonoate dehydrogenase, which catalyzes the conversion of II to nonbitter 17-dehydrolimonoate A-ring lactone (III) (Hasegawa et al., 1972b). Possible uses of limonate dehydrogenase enzymes of *A. globiformis* and *Pseudomonas* 321-18 for removal or prevention of limonin