Table I. Effect of Multiple Passage of Solvent on R_f Values of Amino Acids

Amino Acid	R_f Value: Single passage	Triple passage	Clayton and Strong (1)
Leucine	0.32	0.82	0.81
Methionine	0.22	0.66	0.65
Tyrosine	0.17	0.59	0.57
Proline	0.12	...	0.51
Alanine	0.10	0.40	0.47
Glutamic acid	0.08	0.35	0.44
Glycine	0.07	0.29	0.39
Asparagine	0.05	0.19	0.30
Lysine	0.04	0.14	0.25
Cystine	0.02	0.07	0.14

Table II. Identification of Amino Acids in Potato Extracts by Comparing R_f Values with Standard Solutions

Amino Acids	R_f Values: Standards	Potato extract
Leucine	0.82	...
Isoleucine	0.79	...
Phenylalanine	0.76	0.76
Tryptophan	0.70	...
Methionine	0.66	0.67
Valine	0.63	...
Tyrosine	0.59	0.59
β-Alanine	0.49	...
Alanine	0.40	0.39
Glutamic acid	0.35	0.34
Glycine	0.29	...
Aspartic acid	0.26	0.25
Glutamine	0.21	0.22
Asparagine	0.19	0.20
Lysine	0.14	0.15
Cystine	0.07	...

8-inch paper than was reported by Clayton and Strong for a single passage of their solvent on the large sheets.

When 1- to 50-μl. volumes of potato extracts (concentrated to 50 ml. by the ion exchange method) were chromatographed, nine distinct spots were revealed. Two-microliter aliquots gave small, poorly defined spots, whereas 4- and 5-μl. volumes resulted in well defined spots of optimum size and intensity after development with ninhydrin. Volumes of 10 μl. or larger gave large spots with some streaking. Well defined spots were obtained with volumes of 2 to 4 μl. of the 0.01M standard amino acid solutions.

A tabulation of the R_f values for amino acids on chromatograms prepared from standard solutions and from concentrated potato extracts is given in Table II. In all cases, the R_f values of the amino acids in the potato extract agreed within 0.01 R_f unit of the standards. Asparagine, the identification of which might be dubious on the basis of R_f value alone, gave a brown color when treated with ninhydrin. Amino acids identified from potato samples included phenylalanine, methionine, tyrosine, alanine, lysine, glutamic acid, glutamine, aspartic acid, and asparagine. Other investigators (*5*, *6*) reported some amino acids in potatoes which were not found in this investigation. These discrepancies might be due to varietal differences or to cultural and environmental factors, particularly the latter. The Florida potatoes were produced during the winter and early spring months of the year in contrast to summer and early fall production in most other areas.

When ninhydrin in acetone was used for detection of the amino acids, no significant differences were observed between the spray and dip techniques, and no background color was developed. However, spraying the paper with ninhydrin in butanol resulted in a light yellow background.

The methods of isolation and concentration of amino acids in potato extracts and the separation and identification method described in this paper were applied to the analysis of amino acids in a large number of potato-tuber samples having different storage and environmental histories. A visual quantitative estimate of the amount of each amino acid was made by determining the lowest detectable amount of each amino acid and the intensity of the color corresponding to a spot containing 2 μl. of a 0.01M solution. Since all spots had an intensity between the two limiting concentrations, it was possible to estimate the amount of amino acid in each spot once the spot had been identified. Before it would be possible to predict the quantitative effect of amino acids on the browning reaction in the processing of potatoes, many more analyses and correlation studies would have to be made to evaluate the effects of storage and environment.

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COMPOSITION OF CITRUS OILS

Isolation, Identification, and Gas Chromatographic Estimation of Some Esters and Alcohols of Lemon Oil

ROBERT M. IKEDA[1] and ELIZABETH M. SPITLER[2]

Fruit and Vegetable Chemistry Laboratory, Pasadena, Calif.

Saponification and acetylation values of essential oils are commonly used to estimate their ester and alcohol content. These values usually are expressed in terms of a major ester or alcohol constituent. One of the difficulties encountered in the use of saponification values when applied to lemon oil is that compounds such as coumarins and psoralens (*11*) may be hydrolyzed and calculated as esters. Another problem is that acetylation values do not account for the tertiary alcohols, which are the major alcohols present in lemon oil. Therefore, saponification and acetylation values are not always reliable for estimating the ester and alcohol content of essential oils.

Reports on the ester and alcohol content of lemon oil have been reviewed by Guenther (*3*), and more recently by Kefford (*4*). During the past few years, the composition of lemon oil has been investigated by a number of workers using gas chromatography (*1*, *5*, *6*, *8*). In most of these investigations, the oils were separated into hydrocarbon and oxygenated fractions before examination by gas chromatography.

[1] Present address: Phillip Morris Co. Research Center, Richmond, Va.
[2] Present address: Tucson, Ariz.

In the isolation of esters and alcohols from lemon oil, terpene hydrocarbons were first removed by silicic acid chromatography, and then carbonyl components were removed with Girard's "T" reagent. Linaloöl, α-terpineol, terpinene-4-ol, nonyl acetate, citronellyl acetate, neryl acetate, and geranyl acetate were isolated from the remaining fraction and identified by their infrared spectra. A routine procedure for the gas chromatographic estimation of the esters and alcohols in lemon oil is described. Estimations of the ester and alcohol content of 17 samples of commercial lemon oil are given.

This report is part of a comprehensive study of the chemical composition of citrus oils and is concerned with the separation and identification of the individual esters and alcohols in lemon oil and the development of a method for their estimation. Previously developed methods (*9*, *10*) were used to remove the hydrocarbon and carbonyl fractions from the whole oil before analysis of the esters and alcohols in the remaining fraction.

Experimental Procedure and Results

Apparatus. Gas chromatographic analyses were carried out on a commercial unit equipped with a 4-filament detector. Two stainless steel columns were used—a 1/4-inch × 10-foot column packed with 20% w./w. phenyldiethanolamine succinate (PDEAS) on 60- to 80-mesh chromasorb P, and a 1/2-inch × 5-foot preparative scale column packed with the same material. The 5-mv. recorder was equipped with a Disc Integrator to determine the areas under peaks.

Isolation and Identification. For isolation of the esters and alcohols, 1 pound of California lemon oil was chromatographed on a 5- × 30-cm. silicic acid column. The terpene hydrocarbons were eluted with 2 liters of hexane. The oxygenated compounds were removed with 2 liters of isopropyl alcohol, and the volume was reduced to approximately 500 ml. in a rotating vacuum evaporator. To this residue was added 25 grams of Girard's reagent and 5 grams of IRC-50 ion exchange resin (H form), and the mixture was heated for 1 hour as described by Teitelbaum (*12*) to remove carbonyl compounds. The reaction mixture was diluted with 500 ml. of water and extracted with 150 ml. of hexane, then with two 100-ml. portions of hexane. The extracts were combined and dried over anhydrous sodium sulfate. Hexane was removed in a rotating vacuum evaporator to yield approximately 20 grams of a fraction containing the esters and alcohols. A portion of this fraction was gas chromatographed, as shown in the upper chromatogram in Figure 1. Approximately 100 μl. of the fraction were saponified in 0.5*N* KOH in diethylene glycol for 1 hour at 100° C. The reaction mixture was diluted with water and extracted

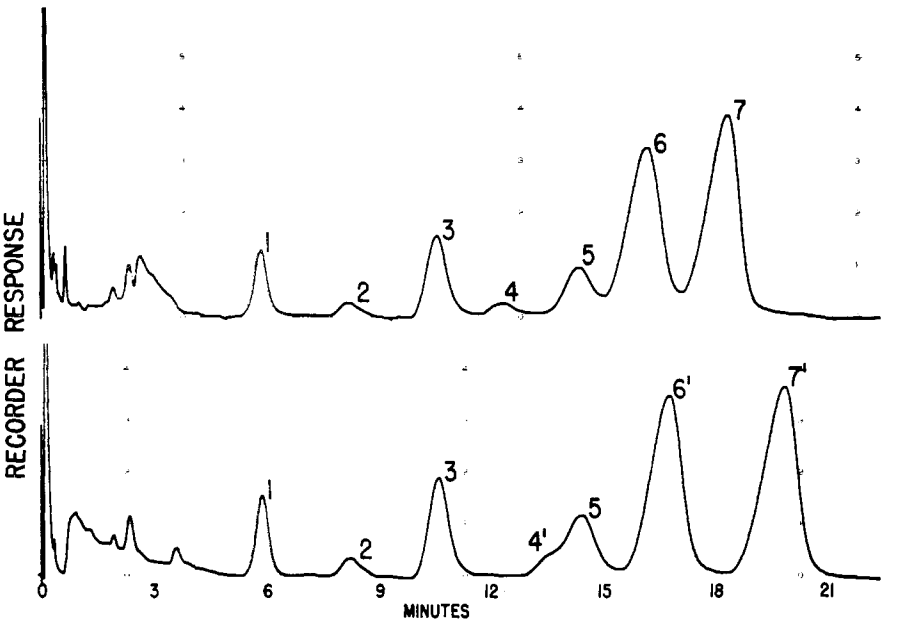

Figure 1. Gas chromatograms of ester-alcohol fraction of lemon oil before (upper) and after (lower) saponification

(1/4-inch × 10-foot PDEAS column; temperature, 139° C.; helium inlet pressure, 25 mm.; flow rate, 180 ml. per minute; peak identities: 1, linaloöl; 2, terpinene-1-ol; 3, terpinene-4-ol; 4, citronellyl acetate; 5, α-terpineol; 6, nery acetate; 7, geranyl acetate; 4', citronellol; 6', nerol; 7', geranio)

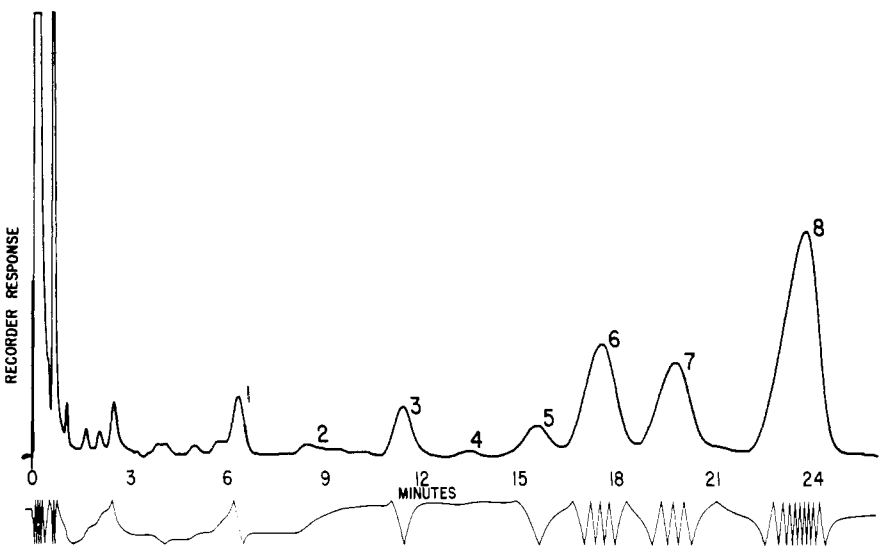

Figure 2. Gas chromatogram used for the quantitative estimation of esters and alcohols in lemon oil

(1/4-inch × 10-foot PDEAS column; temperature, 136° C.; helium inlet pressure, 25 mm.; flow rate, 180 ml. per minute; peak identities same as Figure 1; peak 8 is *n*-propyl benzoate)

with diethyl ether. After the diethyl ether was removed, the saponified material was similarly gas chromatographed, as shown in the lower chromatogram in Figure 1. Since the retention times of peaks 1, 2, 3, and 5 did not change, they were considered to be alcohols. Peaks 4′, 6′, and 7′ should represent the corresponding alcohols.

To determine if free acids were present in the ester-alcohol fraction, a small amount of this fraction was diluted with diethyl ether and extracted with an aqueous solution of sodium carbonate. The diethyl ether was removed, and the residue was gas chromatographed. No changes were observed in the areas of the major peaks on the chromatogram, thus indicating the absence of free acids.

The remaining ester-alcohol fraction was distilled at 2 mm. of pressure in a vacuum distillation apparatus, and four fractions boiling from 60° to 110° C. were collected. These fractions were used in the identification of the following four compounds.

Linaloöl, α-Terpineol, Neryl Acetate, and Geranyl Acetate. Peaks 1, 5, 6, and 7 in Figure 1 corresponded in retention times to linaloöl, α-terpineol, neryl acetate, and geranyl acetate, respectively. Materials having retention times of the above four compounds were collected by gas chromatography from the $^1/_2$-inch column from appropriate distillation fractions. When necessary, the materials were rechromatographed until the infrared spectra were identical with the known compounds similarly purified. Linaloöl, α-terpineol, neryl acetate, and geranyl acetate were identified in the distillation fractions described above.

The ester-alcohol fraction was isolated from a second pound of the same lot of California lemon oil, as described previously, and chromatographed on a 5- × 30-cm. silicic acid column. The column was initially developed with 0.5% v./v. of ethyl acetate in hexane, and the ethyl acetate concentration was gradually increased to 2% over 24 liters. The remaining material was eluted with 2 liters of ethyl alcohol. Altogether 1580 fractions were collected from the column in 18- × 150-mm. test tubes with a fraction collector and used for the identification of the following two compounds.

Terpinene-4-ol (*p*-menth-1-en-4-ol). Gas chromatographic analysis of the combined material from eluate tubes 381 to 450 indicated the presence of a substance corresponding to peak 3 in Figure 1. This material was purified on the $^1/_2$-inch column, and an infrared spectrum was taken on the purified material. From evidence of retention times on various stationary phases, this material was assumed to be terpinene-4-ol. Since no terpinene-4-ol was available for comparison, the compound was prepared by the hydration of α-thujene by a method similar to that described by Wallach (*13*). A sample of 2.5 ml. of α-thujene was shaken with 50 ml. of 5% H_2SO_4 at room temperature for 24 hours. The reaction mixture was extracted with diethyl ether, dried over anhydrous Na_2SO_4, and the diethyl ether removed. Gas chromatographic analysis of the residue indicated the presence of a material with retention time identical to peak 3, Figure 1. This material was isolated by preparative scale gas chromatography. The infrared spectrum of the material prepared by the hydration of α-thujene was identical to that of the material isolated from elution fractions 381 to 450. The latter material was subjected to nuclear magnetic resonance (NMR) analysis. The only structure that would fit all the NMR data was that of terpinene-4-ol. Poore (*7*) reported the presence in lemon oil of an unidentified tertiary alcohol which did not yield tests for linaloöl or α-terpineol. Presumably the alcohol could have been terpinene-4-ol.

Citronellyl Acetate. The retention time of peak 4, Figure 1, corresponds to that of citronellyl acetate. Eluate fractions 291 to 330 contained a material corresponding in retention time to citronellyl acetate. This material was purified on the $^1/_2$-inch column. The infrared spectrum of the purified material was identical to the spectrum of known citronellyl acetate similarly purified by gas chromatography.

Nonyl Acetate. An ester-alcohol fraction was isolated from a 4.4-pound sample of the same lot of California lemon oil, the procedure similar to that described above. This fraction was fractionally vacuum distilled in a spinning brush column. Seven fractions were collected. Fraction 4, boiling from 94° to 101° C. at 10 mm. pressure, contained a small quantity of material having a retention time that corresponds to nonyl acetate. The material was collected from the $^1/_2$-inch column. The infrared spectrum was identical to that of nonyl acetate, also purified by gas chromatography.

Quantitative Estimation of Esters and Alcohols. For the quantitative estimation of esters and alcohols, approximately 1% *n*-propyl benzoate (an internal standard) was accurately weighed into a previously tared sample of 10 ml. of lemon oil. This mixture was chromatographed on a 1.3- × 34-cm. silicic acid column. The terpene hydrocarbons were removed by using approximately 50 ml. of hexane, and the oxygenated compounds were eluted with 40 ml. of methanol. This eluate was diluted to approximately 75 ml. with methanol and heated for 1 hour with 2.5 grams of Girard's reagent and 0.5 gram of IRC-50 ion exchange resin (H form) (*10*). The reaction mixture was diluted with 250 ml. of water and extracted with 75 ml. of hexane. The hexane phase was separated and dried over anhydrous sodium sulfate and evaporated in a rotating vacuum evaporator. A 15- to 20-μl. sample of the residue (ester-alcohol fraction) was chromatographed on a $^1/_4$-inch × 10-foot PDEAS column. The chromatogram is shown in Figure 2.

The area of the *n*-propyl benzoate peak is calculated as a percentage of the areas of all the peaks. This gives the per cent *n*-propyl benzoate in the ester and alcohol mixture since area is related to the weight of material. Then:

$$\text{wt. of esters and alcohols in sample} = \left(\frac{\text{wt. of } n\text{-propyl benzoate used}}{\%\ n\text{-propyl benzoate in mixture}}\right) - (\text{wt. of } n\text{-propyl benzoate})$$

and:

$$\%\ \text{esters and alcohols in the oil} = \left(\frac{\text{wt. of esters and alcohols}}{\text{wt. of oil used for sample}}\right) \times 100$$

To verify the detector response of the esters and alcohols and to determine recovery in the Girard's procedure, a mixture of known composition of some of the compounds found in lemon oil plus *n*-propyl benzoate was prepared. All of the esters and alcohols used to prepare this mixture, except *n*-propyl benzoate, were purified by gas chromatography. The known mixture was gas chromatographed directly to determine the detector response of the various compounds. To check recoveries, two 100-μl. samples of the known mixture were run through the Girard's procedure to analyze for the esters and alcohols. Results are tabulated in Table I.

Results of the analyses of 17 commercial lemon oils obtained from several sources are tabulated in Table II.

Discussion

In the authors' earlier attempts to analyze for the esters and alcohols in citrus oils, including lemon oil, the first hexane extract from the determination of the relative concentration of major aldehydes (*10*) was used. The hexane was removed by evaporation, and the residue was chromatographed on silicic acid. After the terpene hydrocarbons were eluted with hexane, the ester-alcohol fraction was removed with methanol. Gas chromatographic analysis of the ester-alcohol fraction indicated that the alcohols were being formed by the hydration of monoterpene hydrocarbons during the Girard procedure. As mentioned previously, terpinene-4-ol was formed from the hydration of α-thujene. To demonstrate this possibility, 10 ml. of lemon oil were separated into terpene hydrocarbon and oxygenated fractions on silicic acid. The Girard's procedure was carried out on both fractions. The terpene hydrocarbon fraction contained approximately 0.2% alcohols based on the original 10 ml. of

Table I. Detector Response and Recoveries of Some Esters and Alcohols

Composition by:	Compound in Synthetic Mixture, % Linaloöl	α-Terpineol	Geranyl acetate	n-Propyl benzoate
Weight	5.44	15.88	47.44	31.24
Direct gas chromatography	5.1	15.9	48.0	30.9
	5.6	16.2	46.8	31.4
Gas chromatography after Girard procedure, 1st run	3.5	14.0	50.3	32.2
	4.1	14.2	50.1	31.5
2nd run	3.7	13.9	50.3	32.0
	4.1	13.6	50.1	32.2

Table II. Estimation of the Esters and Alcohols of Lemon Oil

Total Ester-Alcohol Content of Oil, %	Relative Percentages of Individual Esters and Alcohols Linalool	Linalyl acetate	Terpinene-4-ol	Citronellyl acetate	α-Terpineol	Neryl acetate	Geranyl acetate	Geraniol
			From California					
1.47	6.5	0.2	2.1	0.3	8.3	38.2	44.2	...
1.46	10.3	...	19.2	0.2	13.5	29.4	27.4	...
1.42	9.4	...	4.7	0.5	10.3	38.4	35.2	...
1.32	8.5	...	2.1	2.0	10.4	36.5	40.3	...
1.39	11.1	...	4.0	0.2	12.0	40.9	31.8	...
1.40	7.1	...	3.1	1.2	9.0	37.4	42.3	...
1.90	11.5	...	10.9	0.4	12.7	32.9	30.4	...
1.18	7.9	...	8.7	0.2	7.6	38.9	36.4	...
0.98	12.1	0.3	4.5	0.3	11.8	34.8	36.3	...
1.42	6.7	0.1	5.5	0.2	7.7	38.4	41.4	...
1.47	9.3	...	5.3	0.5	9.9	36.4	38.6	...
			From Arizona					
0.78	11.6	...	1.8	0.7	10.6	51.6	23.8	...
0.85	11.6	...	3.7	Tr	12.9	54.0	17.7	...
0.75	20.0	...	4.6	...	21.7	45.7	8.9	...
			Foreign					
1.65	9.1	...	5.4	1.0	14.1	25.9	39.5	4.4
1.82	16.2	18.0	4.8	0.3	13.2	19.2	25.5	2.1
1.91	9.9	Tr	7.1	0.9	18.6	26.6	32.3	4.3

lemon oil; the relative composition was 50% terpinene-4-ol, 25% α-terpineol, 20% peak 2, and 5% linaloöl. Thus, for the routine procedure for the estimation of the esters and alcohols, lemon oil was deterpenated prior to reaction with Girard's reagent.

Trace quantities of nerol, geraniol, linalyl acetate, octyl acetate, and ethyl caproate were indicated by their retention times, but they were not present in sufficient quantities for isolation and identification. Thus, these compounds and nonyl acetate do not appear on the chromatograms.

The material represented by peak 2 appeared in small quantity in some lemon oils. The amount of this material was insufficient for infrared analysis, but the retention time corresponded to a minor constituent in a commercial sample of mixed terpineols. α-Terpineol, β-terpineol, and terpinene-1-ol have been reported in commercial terpineol (*2*). The material corresponding to peak 2 in lemon oil was isolated from a commercial sample of mixed terpineols by vacuum fractional distillation and by preparative scale gas chromatography. This material was verified by NMR analysis as terpinene-1-ol.

Data in Table I show that the detector response of the esters and alcohols are sufficiently similar to the weights of the individual compounds that no correction factor is necessary. Other esters and alcohols found in lemon oil may have similar detector responses because of their similarity in structure.

Also shown in Table I is the loss of alcohols when the known mixture was subjected to the Girard's procedure. Approximately a 30% loss of linaloöl was observed. This loss was probably due to the greater solubility of linaloöl in the aqueous alcohol mixture from which the ester alcohol fraction was extracted. However, if one considers linaloöl content on a whole oil basis, the amount would be about 0.15% from values given in Table II. Thus the actual value for linaloöl would be nearer 0.20%. Because of this loss of linaloöl, and the inherent errors involved in a gas chromatographic analysis, this procedure is considered as an estimation. When run under identical conditions, it should give comparable results.

Variations were observed in the esters and alcohols of lemon oil shown in (Table II). Until the advent of gas chromatography, no simple method for the analysis of these individual esters and alcohols was available. Thus the role these compounds play in determining the quality of lemon oil is virtually unknown.

Large variations were observed in terpene alcohols present in lemon oil. A part of these alcohols may have been formed from the hydration of terpenes during the extraction of lemon oil from the fruit. In this process, lemon oil is in intimate contact with water containing traces of citric acid. Hydration is not unreasonable since this reaction was observed during the Girard procedure on the terpene hydrocarbon fraction of lemon oil. These terpene alcohols found in major concentrations in lemon oil were tertiary alcohols while primary alcohols such as citronellol, nerol, and geraniol were found mainly as acetate esters.

Poore (*7*) reported the average ester content by saponification of 72 samples of California lemon oil as 2.34% calculated as linalyl and geranyl acetates. The ester content shown in Table II is much lower than that reported by Poore since this method analyzes only those esters observed on the gas chromatogram. In the authors' analyses, linalyl acetate was present in only small or undetectable quantities in all but one sample of lemon oil.

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