

THE ORIGIN OF CITRUS FLAVOR COMPONENTS—I

THE ANALYSIS OF CITRUS LEAF OILS USING GAS-LIQUID CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY, AND MASS SPECTROMETRY*

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Abstract—Steam-distilled leaf oils from 11 varieties of citrus were analysed and 28 compounds identified as constituents of one or more oils. Gas-liquid chromatographic (GLC) analyses were carried out on both analytical and semi-preparatory scales. Thin-layer chromatographic (TLC) data was obtained using both silica gel and aluminum oxide coated plates in conjunction with solvent systems of varying polarity. Mass spectral (MS) studies were conducted on a time-of-flight mass spectrometer.

INTRODUCTION

RECENT investigations¹⁻⁵ at this laboratory have been concerned with the identification of volatile organic compounds contributing to the flavor and aroma of orange juice. As a consequence of this research, 50 components were isolated and identified. These included 6 terpene hydrocarbons, 6 esters, 12 aldehydes and ketones, 20 alcohols, and 6 volatile organic acids.

Interest has now been shifted from the volatile constituents of the juice of the mature fruit to a study of the origin of these compounds in the citrus trees. Accordingly, present investigations are concerned with volatile oils extracted from active, growing tissues of the tree other than the mature fruit; namely, leaves, blossoms, and various stages of immature fruit. This paper describes a qualitative analysis of the oils from mature leaves of 11 *Citrus* varieties (from five species and one hybrid). Preliminary gas chromatographic identifications were confirmed by thin-layer chromatography and mass spectral analysis. These identifications supersede earlier results,⁶ based on gas chromatography alone, obtained during efforts to use leaf oil analysis to differentiate citrus species.

RESULTS

A total of 28 compounds have been identified as constituents of the steam distilled oils from one or more of the 11 varieties of citrus leaves studied. Six compounds have been tentatively identified and partial structures have been assigned to six other compounds not

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¹ J. A. ATTAWAY, R. W. WOLFORD and G. J. EDWARDS, *J. Agric. Food Chem.* **10**, 102 (1962).

² R. W. WOLFORD, G. E. ALBERDING and J. A. ATTAWAY, *J. Agric. Food Chem.* **10**, 297 (1962).

³ R. W. WOLFORD, J. A. ATTAWAY, G. E. ALBERDING and C. D. ATKINS, *J. Food Sci.* **28**, 320 (1963).

⁴ J. A. ATTAWAY, R. W. WOLFORD, G. E. ALBERDING and G. J. EDWARDS, *J. Agric. Food Chem.* **12**, 118 (1964).

⁵ J. A. ATTAWAY and R. W. WOLFORD, *Proc. 5th Int. Symp. Gas Chromatogr., Brighton, England*, Sept. 8-10, (1964).

⁶ J. W. KESTERSON, A. P. PIERINGER, G. J. EDWARDS and R. HENDRICKSON, *Proc. Am. Soc. Hort. Sci.* **84**, 199 (1964).

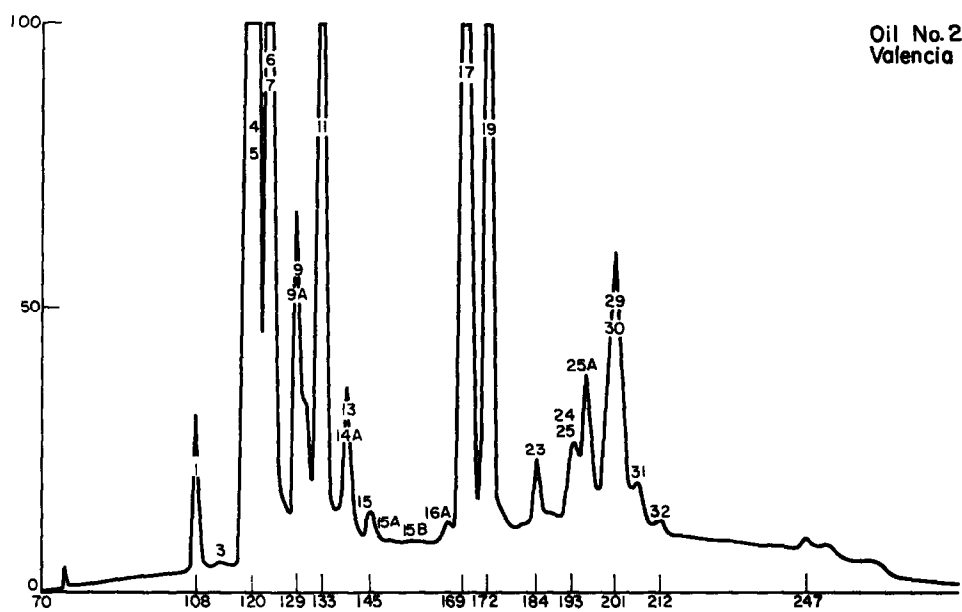
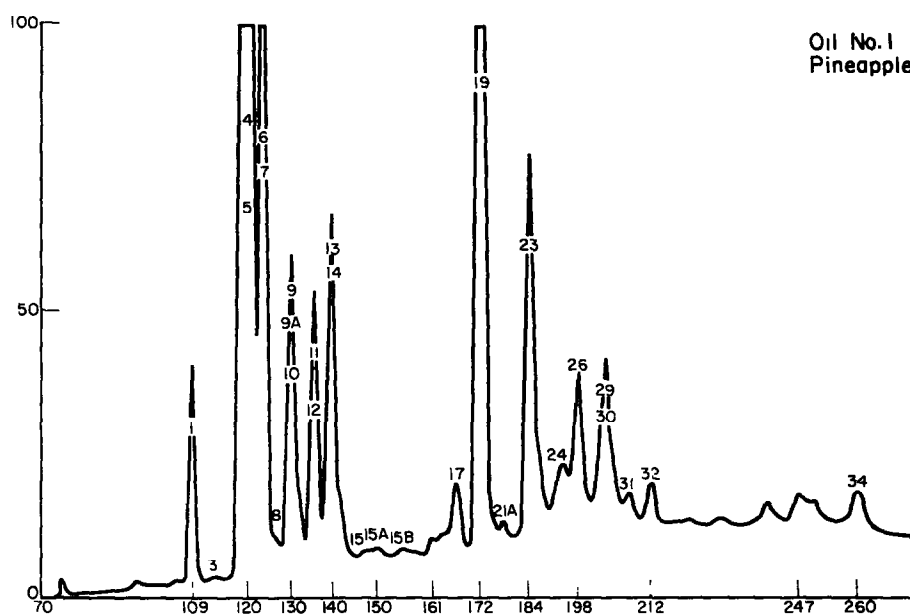
yet identified. These compounds are listed in Table 1 with the criteria for their identification. The oil numbers shown in Table 1 and on the chromatograms in Fig. 1-4 indicate in which oils the compounds have been found. The numbers above and within the peaks in Figs. 1-4 correspond to the compound numbers in Table 1 thus giving a quick reference to peak identity. (The first peak on each chromatogram is the air peak.)

TABLE 1. COMPOUNDS IDENTIFIED IN CITRUS LEAF OILS

Number	Compound [*]	Retention temperature C	Criteria for identification			Oils where identified [†]
			GLC	TLC	MS	
1	α -Pinene	109	+	+	-	all
2	α -Thujene?	109	+	?	-	9, 10
3	Camphene?	113	+	-	-	all but 4
4	β -Pinene	120	+	+	+	all
5	Sabinene	120	+	+	-	all
6	Myrcene	123	+	+	-	all
7	δ -3-Carene	123	+	+	-	1-3, 5, 6, 11
8	α -Terpinene	124	+	?	+	1, 5, 6, 9, 10
9	(+)-Limonene	131	+	-	-	all
9A	C ₁₀ H ₁₆	132	-	?	C ₁₀ H ₁₆	1-3, 5-8, 10
10	Cineole?	132	+	?	-	1
11	β -Ocimene	134	+	+	+	all
12	γ -Terpinene	134	+	+	+	1, 8
13	<i>p</i> -Cymene	140	+	+	-	all
14	Terpinolene	140	+	+	+	1, 3-5, 7-11
14A	2,4(8)-Menthadiene?	141	+	+	C ₁₀ H ₁₆	2, 6, 10 and others
15	Methyl heptenone	144	+	+	+	1-3, 5, 6, 11
15A	C ₁₀ H ₁₆	149	-	?	C ₁₀ H ₁₆	1-3, 5-9, 11
15B	C ₁₀ H ₁₄ O	153	-	?	C ₁₀ H ₁₄ O	all but 4
16	<i>p</i> -Isopropenyltoluene	161	+	+	-	8, 10
16A	C ₁₀ H ₁₈ O	167	-	?	C ₁₀ H ₁₈ O	2, 5, 7, 9
17	Citronellal	169	+	+	+	1-3, 5, 6, 11
18	Decanal	172	+	-	+	7, 11
19	Linalool	173	+	+	+	all
20	Menthone	174	+	?	-	11
21	Linalyl acetate	177	+	+	+	4
21A	C ₁₀ H ₁₈ O	178	-	?	C ₁₀ H ₁₈ O	1, 3, 5, 7
22	Thymyl methyl ether	181	+	+	+	8, 10
23	Terpinen-4-ol	184	+	+	+	all but 10
24	β -Caryophyllene	191	+	+	C ₁₅ H ₂₄	1, 2, 5, 6, 9, 11
25	Neral	191	+	+	-	2, 5, 6, 11
25A	C ₁₀ H ₁₆ O	198	-	?	C ₁₀ H ₁₆ O	2, 3, 5, 9
26	α -Terpineol	198	+	+	-	1, 4, 6-8, 10, 11
27	Cadinene?	198	+	?	C ₁₅ H ₂₄	5
28	Humulene?	201	+	?	C ₁₅ H ₂₄	6
29	Geranial	201	+	-	+	1, 2, 4-6, 11
30	Neryl acetate	201	+	+	-	1, 2, 4, 6
31	Nerol	208	+	+	-	1-5, 7, 11
32	Geraniol	211	+	+	-	1-5, 7, 11
33	Thymol	248	+	+	+	8, 10
34	Unknown	260	-	?	+	5-8

* Tentative identification shown by ?.

† The sources of the oils are: 1-3, *Citrus sinensis* varieties "Pineapple", "Valencia", and "Hamlin" respectively; 4, *C. aurantium*; 5 and 6, *C. paradisi* varieties "Duncan" and "Marsh"; 7 and 8, *C. paradisi* > *C. reticulata*, varieties "Orlando" and "Seminole"; 9 and 10, *C. reticulata*, varieties "Murcott" and "Dancy"; and 11, *C. limon*, "Rough Lemon".



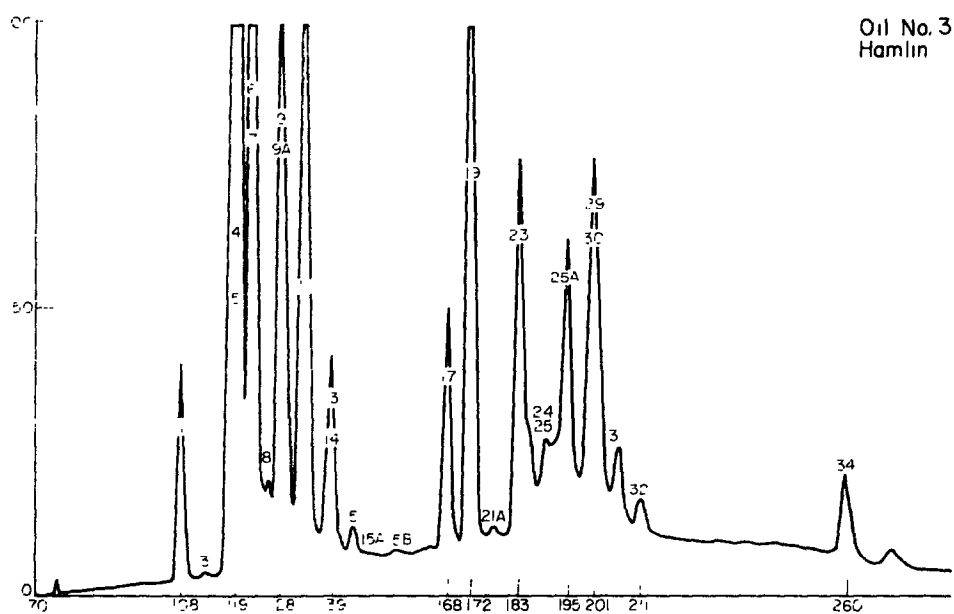


FIG. 1c.

FIG. 1. GAS CHROMATOGRAMS OF "PINEAPPLE", "VALENCIA", AND "HAMLIN" ORANGE LEAF OILS, ALL OF THE SPECIES *Citrus sinensis* (Linn.) Osbeck.

Ordinates-- Response, %; Abscissa--Retention temperature, °C.

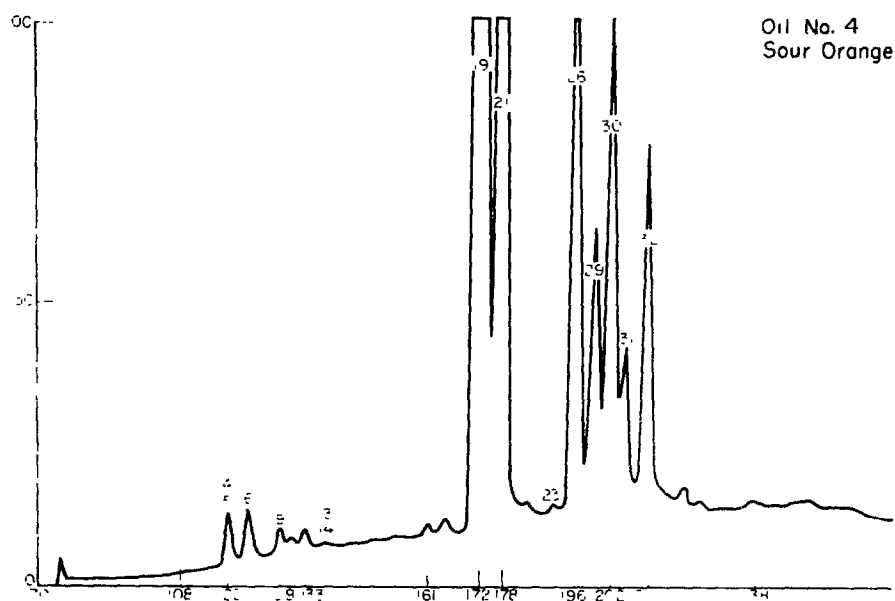


FIG. 2A.

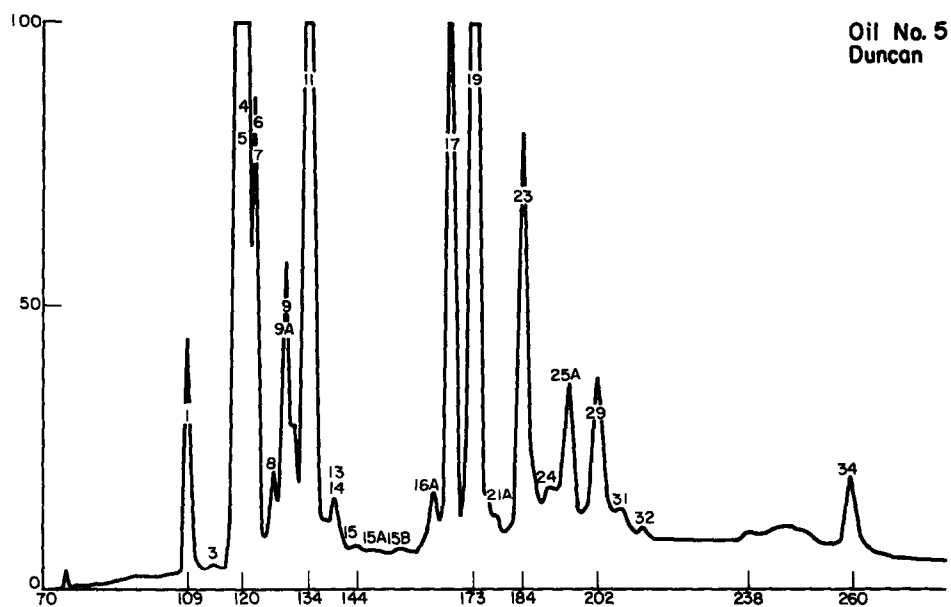


FIG. 2B.

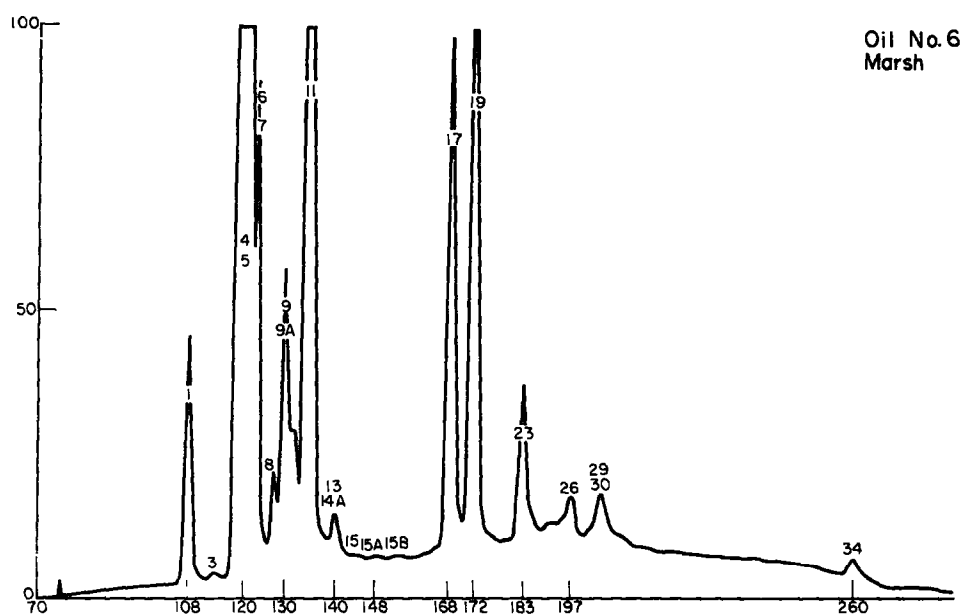


FIG. 2C.

FIG. 2. GAS CHROMATOGRAMS OF SOUR ORANGE LEAF OIL, *Citrus aurantium* Linn., AND "DUNCAN" AND "MARSH" GRAPEFRUIT LEAF OILS, BOTH *Citrus paradisi* MacF.

Ordinates—Response, %; abscissa—Retention temperature, °C.

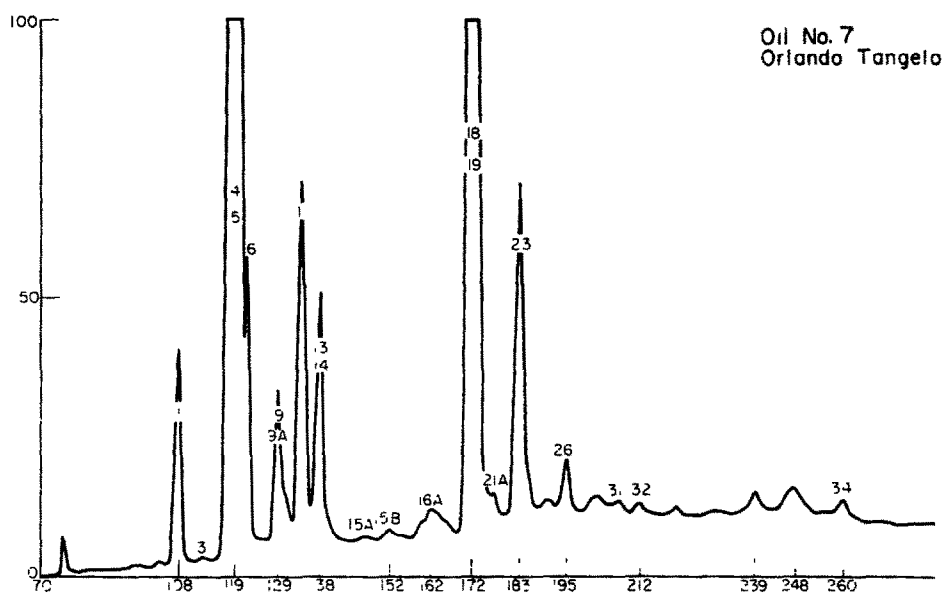


FIG. 3A

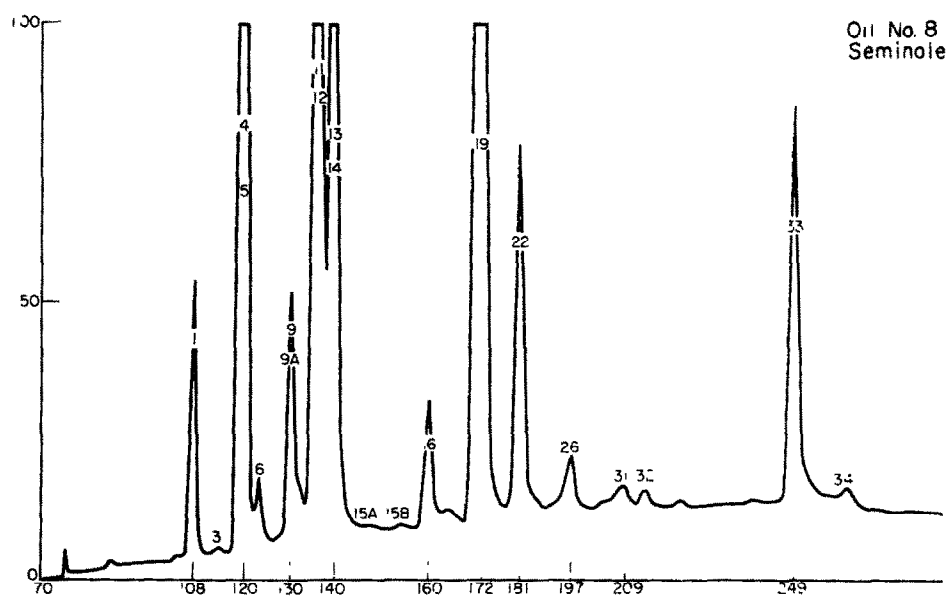


FIG. 3B.

FIG. 3. GAS CHROMATOGRAMS OF "ORLANDO" AND "SEMINOLE" TANGELO LEAF OILS, BOTH *Citrus paradisi* MacF. \times *Citrus reticulata* Blanco.

Ordinates—Response, %; abscissa—Retention temperature, °C.

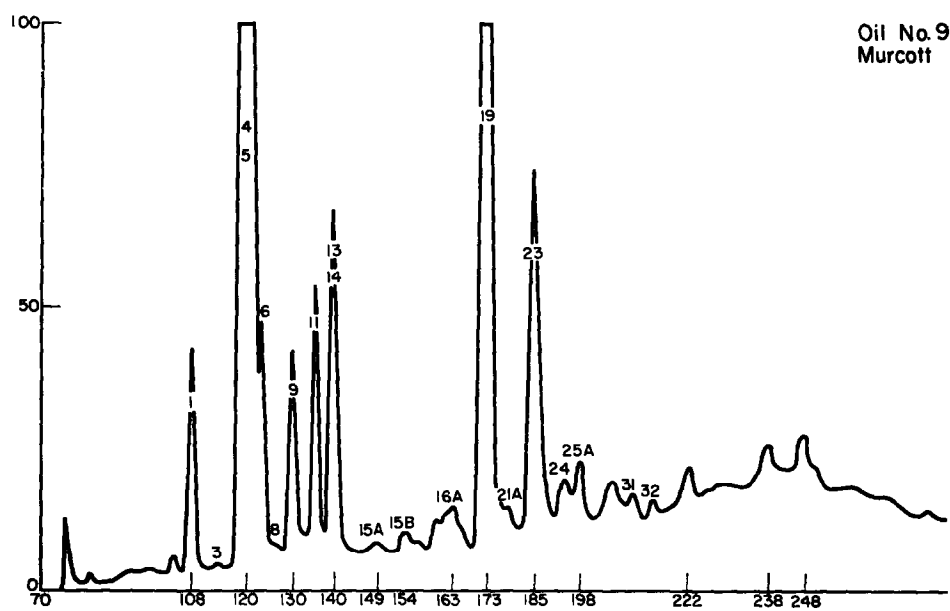


FIG. 4A.

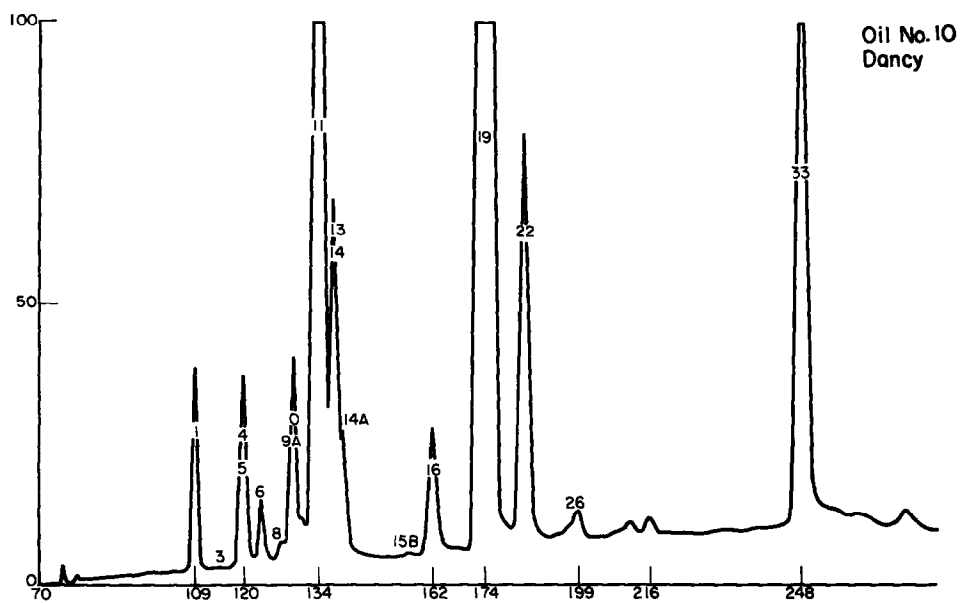


FIG. 4B.

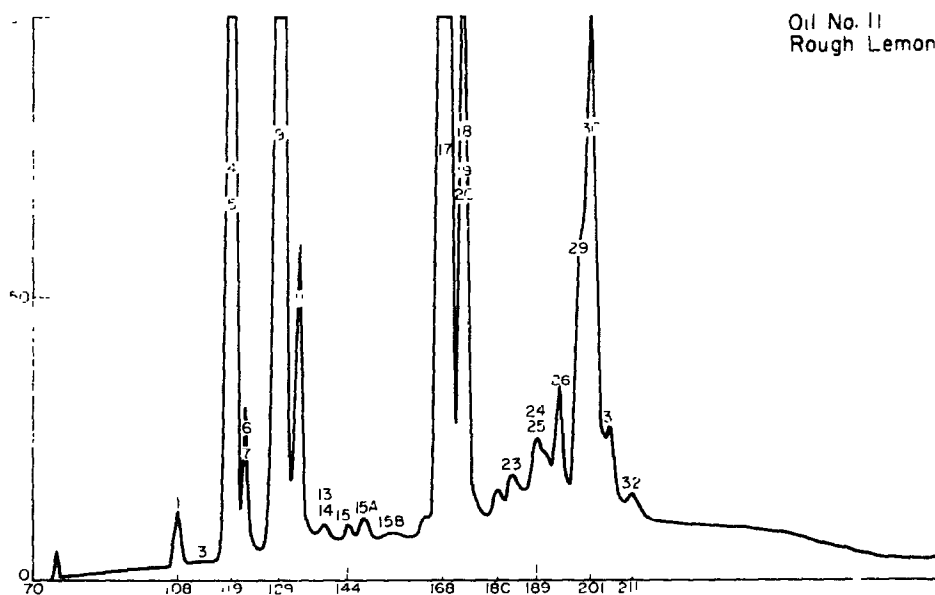


FIG. 4c

FIG. 4. GAS CHROMATOGRAMS OF "MURCOTT" AND "DANCY" TANGERINE LEAF OILS, *Citrus reticulata* Blanco AND ROUGH LEMON LEAF OIL, *Citrus limon* (Linn.) Burm.
Ordinates -Response, "%; abscissa -Retention temperature, °C

DISCUSSION

A large number of compounds were found to be common to all or many of the citrus leaf oils analysed, while a few were found only in oils of certain varieties. Table 1 and Figs. 1-4. The most striking examples of the latter are thymyl methyl ether and thymol, compounds 22 and 33 respectively (Table 1), which are major constituents of "Seminole" tangelo, *Citrus paradisi* MacF. \times *Citrus reticulata* Blanco, and "Dancy" tangerine, *Citrus reticulata* Blanco, but which were not found in the leaf oils of the other varieties studied. Thymyl methyl ether has not been previously identified in any type of citrus oil. The presence of these compounds in even very minute quantities is easily detected using thin-layer chromatography because of their bright pink color reactions with the vanillin/ H_2SO_4 spray reagent. The hydrocarbon *p*-isopropenyltoluene (compound 16, frequently referred to in the literature as *p*-alpha-dimethylstyrene) was also found only in the oils of "Seminole" tangelo and "Dancy" tangerine. It was revealed on the chromatoplates by its lavender color reaction with the vanillin spray reagent. The presence of this compound is interesting as it was only recently reported in citrus⁷⁻¹⁰ and was considered by some investigators to be an artifact resulting from commercial processing rather than a naturally occurring material of biogenetic origin.

The vanillin spray reagent was found to be particularly useful in distinguishing the acyclic terpene hydrocarbons myrcene and ocimene from the alicyclic terpenes, as described in detail in another publication.¹¹

⁷ J. J. LOORI and A. R. COVER, *J. Food Sci.* **29**, 576 (1964).

⁸ R. TERANISHI, T. H. SCHULTZ, W. H. MCFADDEN, R. E. LUNDIN and D. R. BLACK, *J. Food Sci.* **28**, 541 (1963).

⁹ E. SZ. KOVATS, *Helv. Chim. Acta* **46**, 2705 (1963).

¹⁰ E. KUGLER and E. SZ. KOVATS, *Helv. Chim. Acta* **46**, 1482 (1963).

¹¹ J. A. ATTAWAY, J. L. BARABAS and R. W. WOLFORD, *Anal. Chem.* **37**, 1289 (1965).

Compounds present in all of the leaf oils were the hydrocarbons α -pinene, β -pinene, sabinene, myrcene, limonene, *p*-cymene, and β -ocimene, and the alcohol linalool. Terpinen-4-ol, α -terpineol, terpinolene, and β -caryophyllene, were identified in most of the oils, with terpinen-4-ol missing only in "Dancy" tangerine oil. Ocimene was found to be a major terpene hydrocarbon in all leaf oils. This was unexpected as this compound had not been identified in orange juice at this laboratory⁵ or in orange peel oil by Hunter and Brogden.¹² Its absence from peel oil indicates that leaf and peel terpenes may be formed by different biogenetic mechanisms.

An examination of the chromatograms in Figs. 1–4 further reveals that the alcohol linalool was present in a greater quantity than any other single constituent in most if not all oils. Previously it was found to be a major volatile organic compound, and the major alcohol, in orange juice essence both at this laboratory,⁴ and by Schultz *et al.*¹³ Hunter and Moshonas¹⁴ found it to be the major alcohol in the peel oil of "Valencia" oranges.

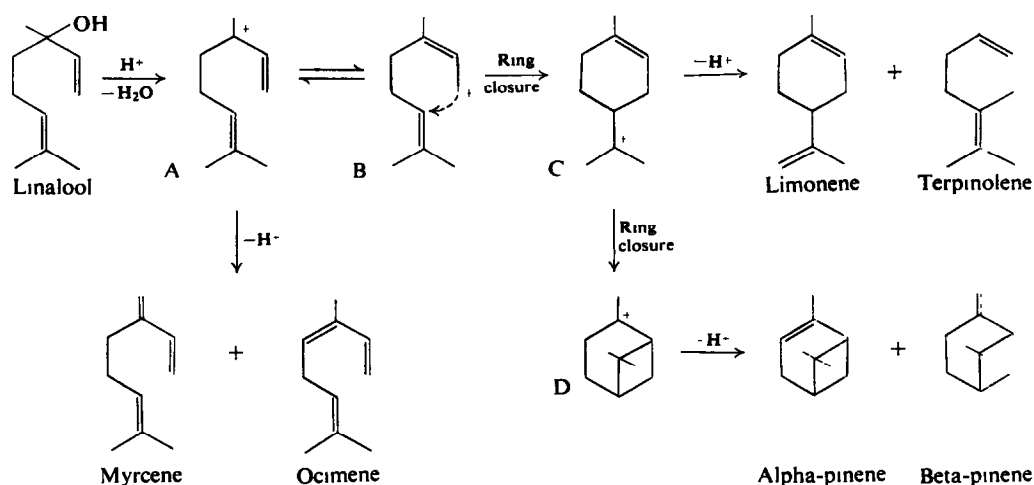


FIG. 5. PROPOSED MECHANISM OF CITRUS TERPENE FORMATION THROUGH LINALOOL INTERMEDIATE.

As a consequence of these findings it seems reasonable to believe that linalool may be a precursor in the biogenesis of many of the citrus flavor components. The formation of the major terpene hydrocarbons identified in this study can be accounted for by the mechanism shown in Fig. 5. Linalool is first attacked by a proton which causes the elimination of H_2O to form carbonium ion A. This ion can stabilize itself by losing a proton to form a mixture of myrcene and ocimene, or it can rearrange to form ion B which cyclizes to give C. Ion C may lose a proton to give a mixture of limonene and terpinolene, or it may cyclize to ion D which upon loss of a proton will give a mixture of α - and β -pinenes. Of these six terpenes, all but terpinolene were definitely identified in all eleven citrus leaf oils, and terpinolene was found in nine of them. The possibility that small quantities of terpinolene are present in the other two oils cannot be excluded. Radiocarbon techniques will be used in future studies directed toward verifying this mechanism.

¹² G. L. K. HUNTER and W. B. BROGDEN, Jr., *J. Food Sci.* **30**, 1 (1965).

¹³ T. H. SCHULTZ, R. TERANISHI, W. H. MCFADDEN, P. W. KILPATRICK and J. COURSE, *J. Food Sci.* **29**, 790 (1964).

¹⁴ G. L. K. HUNTER and M. G. MOSHONAS, *Anal. Chem.* **37**, 379 (1965).

Very small quantities of linalool oxide were detected in some leaf oils which were stored at 40° F for several weeks prior to analysis. This material was thought to be an artifact.

In summary, small quantities of citrus leaf oils may be studied in considerable detail by these techniques. The complete analysis using GLC, TLC, and MS was usually carried out on no more than 100 μ l of sample. Consequently, these oils should provide a useful base for studies on the biogenesis of citrus terpenes using radioactive isotopes and other related techniques. This study further indicates that thin-layer chromatography may be a rapid, inexpensive technique to use in distinguishing species or varieties through their leaf oils.

EXPERIMENTAL

Preparation of Samples

Leaf oil samples were prepared in the same manner as reported earlier.¹⁵

Gas-Liquid Chromatography

The gas chromatograms, Figs. 1-4, were obtained using an F & M Model 720 dual column, temperature programmed instrument equipped with a thermal conductivity detector. Packed columns of 20% Carbowax 20 M on 60-80 mesh Gas-Chrom Z, 12 ft \times $\frac{1}{8}$ in. were used. Helium, at a flow rate of 45 ml/min and inlet pressure of 60 lb/in² was the carrier gas. The column temperature was programmed from 70 to 260° at a rate of 4°/min. Retention temperatures were recorded from the pyrometer which was found to give more reproducible values than the readout dial. In addition, the F & M Model 1609 single column, programmed temperature gas chromatograph and the Perkin Elmer Model 800 dual column, temperature programmed gas chromatograph, both equipped with flame ionization detection, were used when increased sensitivity was thought desirable.

Fractions used for mass spectral identification were collected by slipping capillary tubes, $\frac{1}{2} \times \frac{1}{16}$ in. i.d., onto a syringe needle attached to the exit port of the Model 720. The pure compound, or simplified mixture, issuing from the gas chromatograph was then condensed by passing the opposite end of the capillary through a small hole bored into the side of a plastic jar containing Dry Ice.

Fractions for thin-layer chromatographic analysis were trapped either in the manner just described or in $\frac{1}{8}$ in. i.d. glass U-tubes cooled in Dry Ice-acetone mixture.

Thin-layer Chromatographic Analysis

Thin-layer analyses were conducted on Silica Gel G or aluminum oxide chromatoplates prepared as described earlier.¹⁶ Fractions from the gas chromatograph were transferred to the thin-layer plates as Skellysolve B (hexane) solutions. From 5 to 10 μ l of Skellysolve B, depending on the quantity of fraction collected, were used to accumulate the condensed material into a droplet, after which 1 or 2 μ l from the droplet were transferred to the plate using a 10 μ l Hamilton syringe. Terpene hydrocarbons were identified using either Skellysolve B or a fluorocarbon as the developing solvent as described earlier.¹¹ Thymyl methyl ether was also chromatographed with Skellysolve B. Esters, aldehydes, ketones, and alcohols were analysed using methylene chloride as the developing solvent. Most compounds could be detected using the vanillin/H₂SO₄ spray reagent.¹⁶ The materials not sensitive to this

¹⁵ A. P. PIERINGER, G. J. EDWARDS and R. W. WOLFORD, *Proc. Am. Soc. Hort. Sci.* **84**, 204 (1964).

¹⁶ J. A. ATTAWAY, R. W. WOLFORD and G. J. EDWARDS, *Anal. Chem.* **37**, 74 (1965).

reagent were located on the plates with $\text{KMnO}_4/\text{H}_2\text{SO}_4$, except that aldehydes and ketones were more conveniently located with dinitrophenylhydrazine spray.¹⁶

Mass Spectrometry

The Bendix Model 12-100 Time of Flight Mass Spectrometer was used. The ion source was operated at 50° and 70 eV. The spectrum was scanned from m/e 20 to m/e 200 in 4 min and recorded on an X-Y recorder (Electronic Associates, Inc., Variplotter 1110). Pure compounds and simplified mixtures were introduced into the mass spectrometer from the capillary tubes described above and the fragmentation patterns compared with known patterns.

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