Phytochemistry, 1969, Vol. 8, pp. 2027 to 2038. Pergamon Press. Printed in England.

FATTY ACID PROFILES OF CITRUS JUICE AND SEED LIPIDS

HAROLD E. NORDBY and STEVEN NAGY

U.S. Fruit and Vegetable Products Laboratory, Winter Haven, Florida*

(*Received* 12 *April* 1969)

Abstract—Fatty acids from the juice and seeds of six citrus varieties were quantitatively determined for the region C_{12}–C_{26}. The total number of juice acids observed ranged from eighty-one (Key limes) to one hundred and two (Eureka lemons) and the total number of seed acids ranged from sixty-two (Valencia oranges) to sixty-eight (Key limes and Eureka lemons). Thirty-eight different iso and twenty-six anteiso acids were observed for the first time in edible plant material in concentrations ranging from less than 0·001 per cent to greater than 1 per cent. These single branched acids were verified by comparison of retention times with standards and by their characteristic mass spectrographic fragmentation patterns.

INTRODUCTION

THERE is considerable evidence that the deterioration in flavor of canned citrus juice on storage is due in part to the oxidation of lipids contained in the suspended matter or "cloud" of the juice.[1,2] Degradation products derived from the oxidation of unsaturated fatty acids have been implicated as one of the causative factors in citrus off-flavor development. During processing of citrus fruits, seeds are often inadvertently cut and crushed. The extruded seed lipid has been postulated to contribute to the juice "cloud" lipid.[3] A comparative study of both juice and seed fatty acids should prove informative as to the contribution of each to the "cloud" of processed citrus juice.

Preliminary gas–liquid chromatographic (GLC) analyses of citrus juice indicated the presence of branched and/or multibranched fatty acids. Branched acids have been reported from several animal sources, viz. wool wax,[4] butter fat,[5] ewe milk fat,[6] human sebum,[7] human milk[8] and human blood.[9] In plants, Mold[10] reported the presence of branched acids in tobacco, and Iverson[11] showed these acids to be present in several seed nut oils; however, their structural composition of the seed nut oil fatty acids, i.e. iso, anteiso, etc., was not mentioned. Information on the structural and quantitative composition of these branched acids in citrus juice and seed should be of considerable academic and practical interest.

* One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

[1] A. J. NOLTE and H. W. VON LOESECKE, *Food Res.* **5**, 457 (1940).
[2] A. L. CURL, *Fruit Products J.* **26**, 329 (1947).
[3] L. J. SWIFT, *J. Am. Oil Chem. Soc.* **26** (8), 438 (1949).
[4] A. W. WEITKAMP, *J. Am. Chem. Soc.* **67**, 447 (1945).
[5] R. P. HANSEN and F. B. SHORLAND, *Biochem. J.* **50**, 207 (1951).
[6] T. GERSON, F. B. SHORLAND and C. R. BARNICOAT, *Biochem. J.* **68**, 644 (1958).
[7] A. T. JAMES and V. R. WHEATLEY, *Biochem. J.* **63**, 269 (1956).
[8] W. INSULL, JR., and E. H. AHRENS, JR., *Biochem. J.* **72**, 27 (1959).
[9] A. T. JAMES, J. F. LOVELOCK and J. P. W. WEBB, *Biochem. J.* **73**, 106 (1959).
[10] J. D. MOLD, R. E. MEANS and J. M. RUTH, *Phytochem.* **5**, 59 (1966).
[11] J. L. IVERSON and P. G. HARRILL, *J. Assoc. Official Anal. Chemists* **50**, 1335 (1967).

There were two purposes in the study. The first part entailed a study of juice and seed fatty acid compositions of Duncan and March seedless grapefruit, Key and Persian limes, Eureka lemons, Valencia oranges and Dancy tangerines. For the second part, a relationship was sought between fatty acid composition and storage stability of a citrus juice. The implication of this relationship is that those juices with a high percentage of unsaturated fatty acids should be more susceptible to oxidative deterioration than those juices which contain mainly saturated fatty acids.

RESULTS AND DISCUSSION

Extraction of lipids from several citrus juices and purification through a Sephadex column gave quantities of material which varied from 0·067 per cent for Duncan grapefruit to 0·10 per cent for Marsh seedless grapefruit. This range is similar to that reported by Swift.[12] The use of chloroform–methanol ensured a more complete extraction of phospholipids and other polar lipids. Coupled with Sephadex column purification this extraction procedure ensured a lipid product free of all pectin and other contaminants. The yields of purified seed lipid per dry weight of seed varied from 24 per cent for early season Duncan to 36 per cent for late season Marsh grapefruit. The oil content of dry seeds usually coincides with the optimum maturity of the fruit;[13] thus, these yields of seed lipids were within the natural range.

Esterification of the total lipid left, in the case of lemon, lime and tangerine juices, a residue insoluble in hexane or heptane. This residue may contain hydroxy fatty acid esters, since it has been reported[4] that crystallization from petroleum ether is a means of separating hydroxy acids from nonhydroxy fatty acids. Although this insoluble material was not investigated further, an isothermal GLC analysis of crude esters containing this insoluble material did not differ from the normal purified ester chromatogram. Transmethylation in the case of citrus juices left a product that was contaminated with pigments, carotenoids and terpene products. An ester product free of all colored materials was not obtainable with the different developing solvents that were investigated. The possibility that oxidized terpenoids were in the purified ester product upon TLC purification is obtained from the fact that a major peak (*ca.* 8 per cent), corresponding to methyl myristate in a chromatogram of purified lemon juice, was not present in the chromatogram of an unpurified ester sample. This peak had the same retention time as carvone, a rearrangement product obtained when limonene is passed through a silica gel column. This impurity is removed, however, by the two silver nitrate preparative TLC steps. The completeness of the BF_3–methanol esterification procedure on polar lipids present in citrus was tested by comparing this method with transesterification with sodium methylate in absolute methanol. The analyses of the products by TLC and GLC showed no differences in the two methods.

The yields of purified methyl esters from Sephadex-purified juice and seed lipids varied from 20·3 per cent for Persian lime juice to 84·5 per cent for Marsh grapefruit and Key lime seeds. TLC analyses showed the lipids of the two fruit sections to be quite different, the seed lipid being composed almost completely of triglycerides while the juice lipid contained a multitude of subfractions. This accounts in part for the high yield of esters from seed lipid.

Silver nitrate-impregnated TLC plates offered very clean separation of the saturated and the three unsaturated fractions. Bands containing more highly unsaturated fatty acids, e.g. tetraenes, were not observed. Rechromatography of each band on silver nitrate plates

[12] L. J. SWIFT, *J. Assoc. Official Agric. Chemists* **29** (4), 389 (1946).
[13] R. HENDRICKSON and J. W. KESTERSON, Bull. 698, Agr. Expt. Station, University of Florida, p. 47 (1965).

produced an extremely clean fraction as determined by GLC on DEGS columns before hydrogenation.

Attempts were made to spike the various fractions with internal standards such as methyl hexa-, hepta-, nonadecanoate or tricosonate for qualitative, as well as quantitative analyses. However, the attempts were not successful since all four of these acids are already present in the saturated fractions and they could not be resolved from a number of the unsaturated acids. The fractions were thus hydrogenated [11] and all peaks were qualitatively compared to the retention time of heptadecanoate, an acid present in every sample in fairly uniform concentrations. Hydrogenation of all unsaturated fractions decreased the time and temperature needed to give symmetrical peaks for the C_{20} to C_{26} acids. This step also assured us that oxidation of the dienoic and trienoic acids could not take place before the sample was subjected to GLC analyses. Of even greater importance was the fact that this additional step assured that all samples could be assayed under conditions where nearly all reference standards were available.

The per cent composition of each fatty acid in the juices are given in Tables 1A to 1D and for the seeds in Tables 2A to 2D. The normal chain fatty acids comprise 97–99·4 per cent for grapefruit, orange and tangerine; thus, it is understandable that branched acids were not detected before in citrus. Normal chain acids comprise 95·7 per cent for Key lime, 93·9 per cent for Persian lime and only 91·7 per cent for lemon juice. Table 1A shows that the saturated acids in the juice comprise 25–31 per cent of the total fatty acids; every even and odd carbon number acid from C_{12} to C_{26} is present. This observation has been shown before [11] in nut oils where overloading and increased GLC sensitivity were employed. In addition, most of these citrus species contained normal, iso and anteiso acids in the C_8 to C_{12} and C_{27} to C_{29} ranges. These acids were only tentatively identified and, therefore, were not included in the fatty acid tabulations. Of the minor straight-chain saturated acids, the concentration of even-numbered acids is generally three times the concentration of odd-numbered acids. Only three of these acids (C_{14}, C_{18}, C_{24}) are found in concentrations over 1 per cent and never are more than two of them found over 1 per cent in any one species.

All species contain approximately 20 per cent palmitic acid, except Duncan grapefruit with 24·6 per cent and Key lime with 17·3 per cent. Stearic acid is present at $1 \pm 0{\cdot}5$ per cent in all species and thus is characteristic of citrus juices. Behenic ($C_{22:0}$) to cerotic ($C_{26:0}$) collectively comprise from 1·8 (tangerine) to 3·1 per cent (lemon) of the total acids in the juice.

With only two exceptions there are iso saturated fatty acids for every carbon number from C_{14} to C_{26}, the concentrations ranging from a trace ($<0{\cdot}001$ per cent) to 1·55 per cent for lemon. Anteiso saturated acids are found with one exception for every odd carbon numbered acid from C_{13} to C_{25}. Isostearic acid in Persian lime (1·30 per cent) and lemon (1·55 per cent) is found in a greater concentration than stearic acid.

Table 1B reveals that the normal monoenes, palmitoleic and oleic acid, vary a great deal from species to species. Although the number of monoene branched acids detectable are less than half the number of saturated branched acids, the general pattern consists of an anteiso acid accompanying an odd number straight-chain and an iso acid with the even acids. Almost all concentrations are in the trace to 0·05 per cent range except $C_{18:1}$ iso and $C_{19:1}$ anteiso. Comparing these two acids with their linear chain homologs, it is manifest that these two branched acids are found in greater concentrations than the normal acids for lemon and both lime species.

Table 1C shows that normal dienes are present to nearly the same extent as monoenes although their concentrations are somewhat less. Fewer branched acids are present although

TABLE 1A. PER CENT OF SATURATED FATTY ACIDS IN CITRUS JUICES

Carbon no.	M. grapefruit			D. grapefruit			K. lime			P. lime			E. lemon			V. orange			D. tangerine		
	N*	I†	A‡	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12	0·30			0·15			0·076			0·062			0·12			0·16			0·46		
13	0·072			0·017	T	0·014	0·089		0·047	0·016	0·003	0·011	0·009	T	0·004	0·001		T	0·005		0·001
14	0·93	0·48		0·35	0·009		0·89	0·15		0·23	0·023		0·34	0·023		0·43	0·001		1·04	0·013	
15	0·37	T	0·004	0·25	0·015	0·016	0·30	T	0·072	0·25	0·022	0·077	0·20	0·066	0·055	0·32	0·001	0·001	0·47	0·002	0·012
16	21·26	0·60		24·61	0·15		17·26	0·87		20·12	0·60		21·95	0·24		20·33	0·054		20·30	0·057	
17	0·21	0·004	0·014	0·32	0·030	0·047	0·32	T	0·34	0·39	0·068	0·77	0·33	0·025	0·054	0·35	0·004	0·007	0·42	0·002	0·009
18	1·54	0·21		1·01	0·25		0·87	0·63		1·00	1·30		1·44	1·55		1·21	0·16		0·90	0·19	
19	0·065	0·009	T	0·020	0·003	0·003	0·029	0·003	0·012	0·014	0·002	0·079	0·019	0·002	0·072	0·016	0·011	0·010	0·021	T	0·006
20	0·27	0·007		0·35	0·008		0·25	0·015		0·22	0·012		0·32	0·014		0·25	0·003		0·36	0·002	
21	0·08	0·008	T	0·069	0·006	T	0·047	0·016	0·004	0·045	0·006	0·004	0·045	0·012	0·005	0·041	0·012	T	0·09	0·007	0·001
22	0·38	0·010		0·51	0·034		0·42	0·045		0·43	0·13		0·46	0·076		0·32	0·027		0·63	0·027	
23	0·24	0·005	T	0·21	0·019	0·003	0·16	0·076	0·013	0·19	0·031	0·071	0·28	0·046	0·050	0·15	0·030	0·003	0·19	0·016	0·007
24	0·79	0·027		0·95	0·059		1·12	0·093		1·18	0·17		1·22	0·12		0·90	0·017		0·72	0·005	
25	0·32	0·021	T	0·31	0·025	0·013	0·33	0·13	0·005	0·44	0·057	0·053	0·55	0·055	0·045	0·27	0·018	T	0·14	0·008	0·007
26	0·31	0·050		0·40	0·072		0·52	0·12		0·12	0·18		0·65	0·14		0·39	0·024		0·17	0·012	

* Normal linear chain fatty acid.

† Iso branched acid possessing the general structure, $CH_3-\underset{\displaystyle CH_3}{\overset{\displaystyle H}{\underset{|}{\overset{|}{C}}}}-(CH_2)_x-CO_2H$.

‡ Anteiso branched acid possessing the general structure, $CH_3-CH_2-\underset{\displaystyle CH_3}{\overset{\displaystyle H}{\underset{|}{\overset{|}{C}}}}-(CH_2)_x-CO_2H$.

T = trace, less than 0·001 per cent.

TABLE 1B. PER CENT MONOUNSATURATED FATTY ACIDS IN CITRUS JUICES

Carbon no.	M. grapefruit			D. grapefruit			K. lime			P. lime			E. lemon			V. orange			D. tangerine		
	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12	T			T			T			T			T			T			T		
13	T		T							T		T									
14	0·030	0·001		0·015			0·017	0·004		0·022	0·007		0·012	T	T	0·001			0·027		
15	0·28		0·019	0·15			0·20	T	T	0·25	0·008		0·22	0·003	T	0·15	T		0·16	T	
16	1·90			3·41	0·007		3·79	0·030		2·65	0·028		0·64	0·017	T	2·84	T		3·53	0·010	
17	0·42		0·003	0·35	T	0·001	0·37	T	0·023	0·32	0·003	0·040	0·24	T	0·036	0·68	T	T	0·53	0·001	T
18	20·76	0·28		22·83	0·33		14·03	1·04		15·08	1·38		6·29	1·96		26·91	0·27		37·00	0·14	
19	0·085		0·028	0·011		0·032	0·053		0·23	0·027		0·46	0·003		0·90	0·092		0·046	0·035		0·031
20	0·43	0·007		0·35	0·010		0·41	0·046		0·35	0·063		0·18	0·080		0·90	T		0·065	0·003	
21	0·046		0·006	0·003			0·043			0·006		0·004	0·001		0·002	T			0·004		
22	0·16			0·20			0·21			0·30	0·006		0·039	0·003		0·53			0·34	0·002	
23	0·043			0·006			0·004			0·006			0·001		T	0·001			0·007		
24	0·062			0·053			0·10			0·22	0·008		0·012	0·002		0·11	T		0·064		
25	0·007			0·005			T			0·008		0·002	T		T						
26	0·009			0·014						0·059	0·003		0·002								

TABLE 1C. PER CENT DIUNSATURATED FATTY ACIDS IN CITRUS JUICES

Carbon no.	M. grapefruit			D. grapefruit			K. lime			P. lime			E. lemon			V. orange			D. tangerine		
	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12																					
13																					
14	0·009	T		0·004			T			T			0·007	T		0·003	T		0·008		
15	0·040	T	0·027	0·008			0·009			0·034			0·009	0·003		0·003			0·009		
16	0·27	0·005		0·15	0·005		0·084	0·007		0·082	0·001		0·11	T		0·16	T		0·080		0·003
17	0·27		0·011	0·15		T	0·12	T	T	0·12	0·002	T	0·38		T	0·16		T	0·065	T	0·001
18	35·77	0·16		33·49	0·049		34·80	0·12		31·81	0·12		38·83	0·91		33·08	0·043		23·26	0·030	
19	0·029		0·085	0·015		0·13	0·056		0·14	0·015		0·20	0·004		1·21	0·032		0·041	0·006	T	0·065
20	0·083	0·002		0·037	0·004		0·18	0·004		0·077	0·003		0·29	0·034		0·081	0·002		0·053	T	
21	0·027		0·004							T			0·001		T	T			T		
22	0·022			0·005			0·071			0·008			0·003	0·002		0·003			0·028		
23	0·007			0·002						T						T					
24	0·031	T		0·003			0·031			0·005			0·004	0·002		T			0·018		
25																					
26										T											

TABLE 1D. PER CENT TRIUNSATURATED FATTY ACIDS IN CITRUS JUICES

Carbon no.	M. grapefruit			D. grapefruit			K. lime			P. lime			E. lemon			V. orange			D. tangerine		
	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12																					
13																					
14	T			T			T			0·002			T			T			T		
15	0·002	T		T			T			0·006		T	0·004		T	0·002		0·003	T		
16	0·020			0·004	T		0·058			0·012	T		0·006	T		0·005	0·001		0·007	0·001	
17	0·036	T		0·015		0·002	0·068			0·033		0·002	0·025	0·002		0·019	0·001	T	0·005		T
18	10·58	0·002		7·82	0·005		18·25	0·006		16·72	0·003		16·37	0·005		8·19	0·026		8·08	0·003	
19	T			T			T			0·004			0·003			0·025		0·003	T		
20	0·026			0·014			0·062			1·03			0·061	T		0·025	T	0·005	0·025		
21	T									T						0·019		0·001			
22	T			T			T			0·012			0·005			0·015			0·002		
23										0·002						0·007			0·001		
24										0·011			0·003	T		0·013	0·001		T		
25																					
26										0·004			T								

TABLE 2A. PER CENT OF SATURATED FATTY ACIDS IN CITRUS SEEDS

Carbon no.	M. grapefruit			D. grapefruit			K. lime			E. lemon			V. orange			D. tangerine		
	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12	T			T			T			T			T			T		
13	T			T			T		T	T			T			T		
14	0·089	0·003		0·061	0·006		0·049	0·002		0·046	T		0·015	T		0·052	T	
15	0·073		0·011	0·020		0·004	0·018		0·004	0·020		0·004	0·019		0·003	0·022		T
16	28·98	0·009		29·90	0·022		24·10	0·033		20·42	0·026		27·12	0·010		22·16	0·022	
17	0·10	0·006	0·064	0·085		0·076	0·054		0·12	0·073		0·18	0·064		0·060	0·094		0·10
18	3·49	0·096		3·60	0·080		4·56	0·16		2·49	0·17		4·47	0·074		4·59	0·11	
19	0·001		0·003	0·002		0·010	0·001		0·021	0·003		0·030	0·002		0·008	T	T	0·016
20	0·25	0·017		0·21	0·008		0·37	0·009		0·26	0·011		0·42	0·006		0·21	0·006	
21	0·029		0·001	0·002		0·004	0·004		0·003	0·008		0·005	0·002		0·002	0·002		0·001
22	0·14	0·003		0·048	0·004		0·099	0·014		0·12	0·009		0·043	0·004		0·16	0·009	
23	0·07	T	0·002	0·035		0·005	0·043	T	0·004	0·037	T	0·006	0·036		0·001	0·036	T	0·007
24	0·20	0·002		0·14	0·005		0·14	0·006		0·067	0·005		0·097	0·003		0·23	0·002	
25	0·020	T	0·003	0·024	T	0·005	0·012		0·002	0·015		0·004	0·026	T		0·025		0·001
26	0·011	T		0·019	0·004		0·011	0·003		0·013	0·004		0.024	0·001		0·022	T	

TABLE 2B. PER CENT OF MONOUNSATURATED FATTY ACIDS IN CITRUS SEEDS

Carbon no.	M. grapefruit			D. grapefruit			K. lime			E. lemon			V. orange			D. tangerine		
	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12	T			T			T			T			T			T		
13																		
14	0·010			0·002			0·004			0·004			0·004			0·001		
15	0·016			0·007			0·006			0·008			0·006			0·019		
16	0·29	0·002		0·17	0·003		0·40	0·005		0·17	T		0·064	T		0·75	0·001	
17	0·049		0·004	0·035		0·001	0·049		0·005	0·059		0·003	0·021	T	T	0·062		T
18	19·76	0·032		21·40	0·022		21·87	0·002		26·34	0·064		28·12	0·006		22·38	0·065	
19	0·009		0·030	0·002		0·006	T		0·017	0·007		0·037	0·003		0·008	0·004		0·050
20	0·12	0·004		0·014	T		0·065	0·001		0·028	0·003		0·029	T		0·13	T	
21				0·001			T			T		T	T					
22	0·006			0·003			0·002			0·001			0·002			T		
23				0·002			T			T			0·001					
24				0·005			T			T			0·002	T				
25																		
26																		

TABLE 2C. PER CENT OF DIUNSATURATED FATTY ACIDS IN CITRUS SEEDS

Carbon no.	M. grapefruit			D. grapefruit			K. lime			E. lemon			V. orange			D. tangerine		
	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12																		
13																		
14	0·003			0·002			T			T			T			T		
15	T			T			T			T			T			T		
16	0·022			0·017			0·027	T		0·014			0·020			0·043		
17	0·014		T	0·014	0·003	T	0·016			0·009			0·005			0·026	T	0·003
18	41·01	0·005		38·66	T		37·12	0·003		38·08	T		36·07	0·001		44·56	T	
19	0·001		0·006	T		T	T		T	*0·001*		*0·006*	T		0·001	0·004		T
20	0·011			0·007			0·006			0·012			0·002			0·011		
21																		
22				T			0·001			T						T		
23							T			T								
24							0·001			T								
25																		
26																		

TABLE 2D. PER CENT OF TRIUNSATURATED FATTY ACIDS IN CITRUS SEEDS.

Carbon no.	M. grapefruit			D. grapefruit			K. lime			E. lemon			V. orange			D. tangerine		
	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A	N	I	A
12																		
13																		
14										T								
15	T			T						T			T					
16	T			0·002			0·006			0·008			0·005			0·007		
17	0·001			0·001	T		0·002	0·001		0·003	0·001		T			0·001	T	0·002
18	4·81	T		5·46	0·001		10·54	T		11·11	0·003		3·11	0·003		3·95	0·002	
19	T		T	T			0·002		T	T			T			T		
20	0·006	T		0·001			0·004			0·003			0·006			0·011		
21																		
22				T												T		
23																		
24																		
25																		
26																		

lemon contains an anteiso acid ($C_{19:2}$ anteiso) greater than 1 per cent. Trienes in juice (Table 1D), with the exception of linolenic acid and eicosatrienoic acids are less than 1 per cent; the major portion of the acids being in the C_{16}–C_{20} area. The number of branched acids in each species varies from one for Key lime to ten for orange. Concentrations are normally in the trace to 0·01 per cent range. The concentration of linolenate varies from around 8 per cent for Duncan grapefruit, orange and tangerine to 18 per cent for Key lime.

Table 2A shows the saturated acid composition of citrus seeds. Stearic acid is found in larger concentrations in seeds (2·5–4·6 per cent) than in juices. Palmitic acid in seeds varies from 20 to 30 per cent in the different species and has no correlation with the percentage found in the juice of the respective specie. The number of straight-chain acids found in the seed is about the same as in the respective juice; however, their respective concentrations are quite different. Unlike the branched-chain saturates in juice, the iso acids are nearly all of even carbon numbers and the anteiso acids nearly all of odd carbon numbers.

Palmitoleic acid (Table 2B) is found in less than 1 per cent concentrations in all six seed species. Oleic acid in seeds, unlike that found in juice, is quite constant at 19·8–22·4 per cent for the grapefruit, limes and tangerines. However, the concentration increases to 26·3 per cent for lemon and 28·1 per cent for orange. The number and concentrations of branched monoenes are greatly reduced from that of their respective juice components.

Linoleic and linolenic acids are the only major dienoic and trienoic acids, respectively, found in citrus seeds (Tables 2C and 2D). The concentration of linoleic acid is quite constant varying from 36·1 per cent for orange to 44·6 per cent for tangerine. Key lime seed has the largest number and concentration of dienes which consists mainly of normal chain length acids. Linolenic acid is present from 3·1 per cent in orange to 10·5 and 11·1 per cent in Key lime and lemon, respectively. All other trienes are observed to be in the trace to 0·01 per cent region. As expected, C_{16} to C_{20} straight-chain fatty acids are the main components of these trienes.

Examination of the various tables reveals that there are acids present in some species which are absent in others. The reported absence of these acids implies only that they were not detected under our conditions and may be present in very minute quantities ($<0·001$ per cent).

Although similar comparisons can be made between the composition of fatty acids in seeds and their respective juices, their overall compositions appear to be quite different. We have recently observed (unpublished data) that the total fatty acid patterns from fresh commercially processed juice compared closely with the fatty acid patterns reported in this paper. From these observations and the basic dissimilar fatty acid patterns reported for juices and their respective seeds, we concluded that seeds do not contribute appreciably to the juice lipids of processed juice.

Although urea clathration has been used as a means of separating branched acids from straight-chain fatty acids, complete separation of the two isomers cannot be obtained in a single clathration. When the method was applied to saturated lemon juice methyl esters a 26-fold increase in branched acids was obtained. The ratios of the branched acids to each other, however, were quite different from the ratios observed before clathration. Thus, this method, while serving a useful purpose, could be used only for qualitative analysis and enrichment.

As expected in natural products, all fatty acids could not be definitely characterized. From 0·03 to 0·23 per cent of fatty acids in the juice of the various species did not have carbon numbers characteristic of the three different structural acids recorded in the tables. These

uncharacterized acids generally possessed carbon numbers ending in units of 0·30. Rehydrogenation and TLC replating of these fractions showed these uncharacterized acids to be truly hydrogenated compounds; their effective chain lengths and concentrations were unchanged as evidenced by GLC.

An acid derived from phytol, a natural plant constituent, is phytanic acid. Its biosynthetic relationship to phytol made it a natural contender for being one of the minor unknown peaks in citrus juices. Under temperature programmed conditions standard phytanic acid had a retention time nearly the same as heptadecanoic acid on DEGS columns. Under normal conditions of urea clathration a tetra branched fatty acid would remain in the non-clathrated filtrate or washings. In the chromatogram of the non-clathrated filtrate from a clathrated saturated lemon juice methyl ester sample, the peak corresponding to a carbon number of 17·0 was completely absent. Therefore, there is good evidence that phytanic acid is not a constituent of citrus juice.

Two recent articles clearly demonstrated the use of mass spectrometry in characterizing iso and anteiso structures of long-chain hydrocarbons.[10,14] The mass spectrograms of similar fatty acid methyl esters, however, were more complex due to the presence of the ester moiety.[15–17] Examining the spectrograms of the saturated methyl ester fraction (C_{14}–C_{28}) of Persian lime juice revealed that the parent ion in all cases corresponded to the molecular weight of the methyl ester characterized by GLC analyses. It was evident, therefore, that if multibranched acids were present in these areas their concentrations would be insignificant.

Further evidence for the presence of monomethyl branched acids in citrus was obtained from studies of their fragmentation patterns. Straight- and branched-chain methyl esters yielded very similar fragmentation patterns. With chain lengths greater than seven, common major fragments, $m/e = 74$, 87 and 143, were observed for both structures. However, minor but very characteristic differences were observed between straight and branched esters in the parent ion (M) to M-80 m/e region of the spectrogram.

All esters yield an acylium ion, m/e = M-31, an ethyl ion, m/e = M-29 and a propyl ion, m/e = M-43. For straight-chain esters the intensity ratios of the M-31 to the M-29 ion varied from 3:1 for C_{14} to 4:3 for C_{26}. In all cases the acylium ion was in greater amount than the ethyl ion.[17] For iso branched acids the intensities of these two ions have a 1:1 ratio.[16] This ratio prevailed for each iso acid from C_{16} to C_{26}.

In addition the iso branched acids had a very small M-65 ion which has been reported[16] as being characteristic of iso branched fatty esters with chain lengths greater than seven. Iso branched esters gave M-15 ions of greater intensity than their isomeric esters. This ion has been shown to form by the loss of the easily fragmented terminal branched methyl moiety. Anteiso fatty esters were observed in all cases to have an M-29 ion of greater intensity than the acylium ion (M-31) for that specific ester. This has been reported[16] to be due to the increased probability of ethyl ions being eliminated from the isobutyl moiety of anteiso compounds. The M-57 ion, characteristic of anteiso methyl esters fragmented on the ester side of the tertiary carbon, was observed for the three major anteiso esters, C_{15}, C_{17} and C_{19}, found in lime juice. These findings lend further support to the many newly discovered acids reported in this paper.

Comparative examination of the degree of fatty acid unsaturation of a citrus juice and its

[14] G. L. K. HUNTER and W. B. BROGDEN, JR., *Phytochem.* **5**, 807 (1966).

[15] S. ABRAHAMSSON, S. STALLBERG-STENHAGEN and E. STENHAGEN, in *Progress in the Chemistry of Fats and other Lipids* (edited by R. T. HOLMAN), Vol. 7, p. 41, Pergamon Press, New York (1963).

[16] R. RYHAGE and E. STENHAGEN, *Ark. Kem.* **15**, 291 (1960).

[17] R. RYHAGE and E. STENHAGEN, *Ark. Kem.* **13**, 523 (1959).

susceptibility to oxidation showed no correlation. This comparative study was mainly prompted by the observation that tangerine juice is the most susceptible citrus juice to develop off-flavor on storage.[3] Comparison of the fatty acid profile of tangerine juice and seed to other citrus species manifested no marked difference, thus negating the hypothesis that the degree of unsaturation is mainly responsible for off-flavor development. Proneness to oxidation may be due to a number of factors, e.g. lack of a sufficient natural antioxidant concentration or to the possibility that unsaturated acids may be concentrated in a specific type of lipid (e.g. phospholipid) which is more susceptible to enzymatic breakdown.

EXPERIMENTAL

Isolation and Purification of Juice and Seed Lipids

Dancy tangerines, Eureka lemons, Marsh seedless grapefruit, Duncan grapefruit and Persian limes were obtained from local markets. Key limes and Valencia oranges were obtained from local groves. The fruits were hand-reamed and extracted in a household juice extractor with the seeds being carefully removed, washed and dried in a vacuum desiccator for 24 hr or more. Organic solvents used in these experiments were analytical grade and were distilled before use.

Utilizing a method modification of Swift,[18] 400 ml of freshly extracted juice were mixed with 20 g of Celite 545 and allowed to settle 30 min in a refrigerator. After cooling, the mixture was passed over 20 g of a Celite pad on a coarse porosity 11 cm sintered-glass funnel under slightly reduced pressure. The vacuum was gradually increased over a 20-min period leaving a clear juice filtrate and a Celite pad containing the lipid material and some residual water. This Celite pad was homogenized in a Waring blendor with 200 ml of Folch's reagent ($CHCl_3$–methanol, 2:1, v/v) for 5 min. After this treatment, the mixture was returned to the glass funnel, the extract removed by suction and the Celite washed with the Folch reagent (3×100 ml). The washings and extract were combined, the lower organic phase was removed and the upper residual aqueous phase re-extracted with $CHCl_3$ (2×50 ml). The combined $CHCl_3$–methanol extracts were reduced to near dryness *in vacuo* in N_2 at 30°. The crude lipid was taken up with successive small volumes (2–5 ml) of the lower phase of a $CHCl_3$–methanol–water (200:100:75, v/v/v) system and transferred to a Sephadex column for purification by the method of Wuthier.[19] Purified lipid extracts were reduced to dryness (*in vacuo*, in N_2 at 30°), taken up in $CHCl_3$ and dried, and made up to 10 ml with $CHCl_3$.

Dry seeds, approximately 3 g, were homogenized for 15 min with 50 ml Folch reagent in a Waring blendor microcup. The lipid extract was filtered through a coarse porosity sintered-glass funnel and the residue re-extracted with 50 ml Folch reagent. The combined filtrates were washed with water (2×100 ml), concentrated and purified by Sephadex chromatography. The purified seed lipids were dried in a similar manner to that conducted for juice lipids. Weight determinations were obtained for both juice and seed lipids by drying a 1-ml aliquot in a vacuum desiccator.

Methyl Ester Preparation and Purification

Esterification was carried out by the BF_3-methanol method of Metcalfe *et al.*[20] with slight modifications. Aliquots of the dry $CHCl_3$ extracts of seed and juice lipids (3 and 5 ml, respectively) were reduced to dryness under vacuum and subjected to methyl esterification. The esters were extracted with heptane,[21] the heptane layer washed with water saturated with NaCl and, finally, the heptane layer was dried. With lemon, lime and tangerine juice considerable residue accompanied the methyl esters in the heptane phase. This residue was removed by centrifugation and the resulting supernatant reduced in volume under helium.

The crude esters were purified by preparative chromatography on silica gel G precoated plates (20×20 cm, 0·25 mm, Analtech, Inc., Wilmington, Del.) with benzene–hexane (7:4). The plates were dried in a vacuum desiccator, sprayed with Rhodamine 6G, and the ester band detected under short wavelength u.v. light. Esters were eluted with freshly distilled ether (30 ml), filtered through a very fine sintered-glass funnel and reduced to dryness under helium. All esters were stored in 0·1 ml of heptane in vials under helium at 6°.

Separation of Methyl Esters by Argentation TLC According to the Degree of Unsaturation

Five plates (20×20 cm) were coated to a thickness of 0·5 mm with silica gel H (40 g/95 ml water). The plates were air-dried for 30 min and stored in a drying oven at 110° until ready for impregnation with $AgNO_3$. The

[18] L. J. SWIFT, *Food Res.* **16**, 142 (1951).
[19] R. E. WUTHIER, *J. Lipid Res.* **7**, 558 (1966).
[20] L. D. METCALFE, A. A. SCHMITZ and J. R. PELKA, *Anal. Chem.* **38**, 514 (1966).
[21] D. VAN WIJNGAARDEN, *Anal. Chem.* **39**, 848 (1967).

H plates were removed from the oven, cooled to room temperature and placed in a developing tank containing a solution of 5 ml of water-saturated $AgNO_3$ and 95 ml of methanol. The plate was allowed to develop in this impregnating system for 4 hr. The impregnated plates were removed, air-dried and activated at 110° for 1 hr.

After cooling to room temperature the $AgNO_3$ plates were streaked with the purified ester sample and developed in hexane–ethyl ether (90:10). The plates were dried, sprayed with Rhodamine 6G and the four bands (saturates, monoenes, dienes and trienes) eluted with ether as described previously. The saturate and monoene fractions were separately re-chromatographed on 0·25 mm silver nitrate-impregnated plates prepared as outlined above and developed in hexane–ether (95:5). The diene and triene fractions were also re-chromatographed on silver nitrate plates in hexane–ether (90:10) and (60:40), respectively. Each fraction was recovered by ether elution of the gel and completeness of separation verified by isothermal GLC analyses.

Urea Enrichment of Branched Acids

A sample of the saturated fraction (5 mg) from lemon juice was mixed with *ca.* 15 mg urea, 5 drops of hexane, 5 drops of methanol and allowed to stand covered overnight at room temperature. The mixture was filtered, the filtrate washed once with water and reduced to near dryness for GLC analysis.

Hydrogenation and Gas–Liquid Chromatographic Analyses

A major portion of each of the unsaturated fractions obtained from silver nitrate partitionment was dissolved in 1 ml of hexane and hydrogenated under 50 lb/in^2 pressure at room temperature for 1 hr with 10 mg of 10% Pd—C (Parr apparatus). Samples were filtered through a fine porosity sintered-glass funnel, transferred to vials with hexane, reduced to dryness under helium and kept under refrigeration.

Gas chromatographic analyses were run on an F & M Model 5750 gas chromatograph equipped with dual flame ionization detectors. Dual aluminum columns (3·05 m in length and 3 mm i.d.) coated with 10% stabilized DEGS (Analabs, Inc., Hamden, Conn.) on 100/120 mesh, DMCS treated, acid-washed Chromosorb W were used for all quantitative analyses. Analyses on the total purified methyl esters were run isothermally at an oven temperature of 200°, detector temperature 285°, injection temperature of 290° and a flow rate of 40 ml/min. Samples of 3 μl containing 200–400μg of methyl esters were injected. Average time for the C_{26} acid to emerge was 40 min. Three or more injections were made on each sample to obtain ratios between the four major peaks, i.e. palmitate, oleate, linoleate and linolenate until these ratios showed a deviation less than 1 per cent. Areas under the peaks were measured with the aid of a disc integrator.

The four fractions of each lipid sample, separated on basis of saturation by argentation TLC and catalytically hydrogenated, were run on the DEGS columns under programmed temperature conditions of 150–230° at 2°/min then held at limit until the C_{26} to C_{29} esters had emerged. The flow rate was 40 ml/min. Ester samples in heptane were injected in 6-μl aliquots. The amount of material injected was varied from 0·1 to 1 mg depending on the type of sample analyzed. The sample was first run to determine every minor peak,[22] its relative intensity and retention time. Quantitative data was obtained by attenuating the recorder for each emerging peak so that each peak was kept on scale and in its proper perspective to each other peak.

The percentage of each component fatty acid in the purified methyl ester fraction from the various seed and juice species was determined as follows:

(*a*) The total methyl ester sample was subjected to GLC and the percentage of palmitate (16:0) in the mixture determined. Area ratios of 18:1, 18:2 and 18:3 to 16:0 were next recorded.

(*b*) The total ester sample was separated into four fractions (saturate, monoene, diene and triene) by argentation TLC and each unsaturated fraction hydrogenated. For each fraction, area ratios were tabulated for each acid relative to a reference acid of that fraction. For example, in the monoene fraction (hydrogenated), the ratio of hydrogenated palmitoleate to hydrogenated oleate (reference) for orange juice was 0·105. As a second example, in the diene fraction (hydrogenated), the ratio of hydrogenated eicosadienoate to hydrogenated linoleate (reference) for orange juice was 0·024.

(*c*) To determine the percentage of each acid in the total mixture, the percentage of palmitate in the total mixture was multiplied by the area ratios obtained in parts (*a*) and (*b*), e.g.

$$\% \text{ palmitoleate} = (\% \text{ palmitate})\left(\frac{\text{oleate}}{\text{palmitate}}\right)\left(\frac{\text{palmitoleate}}{\text{oleate}}\right).$$

For verification of the effective chain lengths of the various acids, the samples were run on two non-polar columns. Dual aluminum columns (1·83 m in length and 3 mm i.d.) coated with 3% OVIOl (Western Analytical Service, Orinda, California) on 100/120 mesh, DMCS treated, acid-washed Chromosorb W and on dual stainless-steel columns (1·83 m in length and 1·5 mm i.d.) coated with UC W98. Both columns were programmed under various rates and flows from 40 to 80 ml/min.

[22] J. L. IVERSON, *J. Assoc. Official Anal. Chemists* **50**, 1118 (1967).

Retention times were recorded for each peak on the three columns and ratios of retention times to methyl heptadecanoate recorded. A series of standard curves was prepared from standards of normal straight-chain fatty acids methyl esters obtained from Hormel Institute, Austin, Minn., and branched-chain esters obtained from Applied Science Labs., State College, Pa. These curves related the relative retention time ratios to their carbon number. For every iso branched acid, the acid had an effective carbon number 0·5 less than its parent acid on the DEGS column. An anteiso acid had an effective carbon number 0·25 less than its parent acid under the same conditions. Thus, every characterized peak was classified as being a normal, iso or anteiso acid based on its retention time relative to the retention time of methyl heptadecanoate. For the two non-polar columns the separation factor between the two branched acids was not as great; thus, when both isomeric acids were present it was not always possible to distinguish an iso from an anteiso acid.

In order to test for possible spurious peaks appearing on the chromatogram, complete procedural blank runs were conducted with all reagents, solvents and adsorbents used in these experiments. The reagent blanks were subjected to programmed GLC analyses analogous to the parameters and sensitivity employed for methyl ester identification and quantitation.

Mass Spectrometry

A Persian lime sample of a saturated methyl ester fraction was injected into a Model 9000 LKB GLC-mass spectrometer. A glass column (2·44 m in length and 1·5 mm i.d.) coated with 10% stabilized DEGS was operated under the same programmed conditions as previously described. The mass spectrometer was operated at 70 eV with the separator temperature maintained at 270° and the ion source at 290°.

Acknowledgements—The authors are indebted to Dr. Henry M. Fales, National Institutes of Health, Bethesda, Maryland, for mass spectrometry of the methyl esters.