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FATTY ACID PROFILES OF ORANGE AND TANGOR JUICE SAC LIPIDS

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Abstract—Fatty acids from the juice sacs of four orange varieties from early season harvest, one variety from mid- and one variety from late season, and two tangors were quantitatively determined and it was found that the five major fatty acids, viz. palmitic, palmitoleic, oleic, linoleic and linolenic, comprised more than 92 per cent of all acids. There appeared to be no correlation between the major fatty acids and the harvest season of the various orange varieties. The two tangors were distinctly different from the oranges in both their major and minor fatty acid profiles. A theory is proposed for the occurrence of specific iso- and anteiso-branched acids in citrus.

INTRODUCTION

CITRUS taxonomy has been subject to considerable controversy over the years. The genus *Citrus* has been divided into from 16[1] to 157[2] species which, in turn, have been further divided into enumerable varieties. To disinguish the various species, chemical markers or 'fingerprints' have been employed. The most comprehensive studies on citrus chemotaxonomy have been conducted by Scora *et al.*[3] on essential oils and Albach and Redman[4] on flavanones.

The authors previously reported[5] fatty acid profiles from juice and seeds of five citrus species. Distinct fatty acid differences were observed for these species and the question arose as to the possibility of employing fatty acid profiles to distinguish varieties within a species.

Information on fatty acid profiles is important, not only in citrus chemotaxonomy but also may have practical utility in the citrus processing industry. The possibility of employing fatty acid profiles for detection of adulteration and distinguishing juice from early, mid or late season varieties appears feasible.

In this study six orange varieties, viz. Pineapple, Hamlin, Walker Early, Washington Navel, Parson Brown and Valencia, and two tangors were investigated for their fatty acid content. The two tangors, viz. Temple and Temple × Kinnow, were included because Temple is commercially the most important tangor in Florida while the Temple × Kinnow hybrid has potential commercial possibilities.

RESULTS AND DISCUSSION

Purification of fatty acids was determined essentially as reported in our previous paper[5] with slight improvements. An extra purification step was added because of the possibility

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[1] W. T. SWINGLE, *The Botany of Citrus and its Wild Relatives of the Orange Subfamily*, University of California Press, Berkeley (1943).

[2] T. TANAKA, *Citrologia Semi-centennial Commemoration Papers on Citrus Studies*, Citrologia Supporting Foundation, Osaka University, Osaka (1961).

[3] R. W. SCORA, A. B. ENGLAND and W. P. BITTERS, *Phytochem.* **5**, 1139 (1966).

[4] R. F. ALBACH and G. H. REDMAN, *Phytochem.* **8**, 127 (1969).

[5] H. E. NORDBY and S. NAGY, *Phytochem.* **8**, 2027 (1969).

that unsaturated hydrocarbons and dimethyl acetals[6,7] (liberated from aldohydogenic lipids) may not have disassociated from the fatty acid methyl esters even after two $AgNO_3$ thin-layer purifications. To insure maximum purity of the methyl ester preparation, the ester classes, viz. saturated, monoene, diene and triene, after two $AgNO_3$ platings and hydrogenation, were subjected further to multidevelopment on slicia gel G plates in hexane–ethyl ether (95:5). This step completely separates dimethyl acetals, hydroxy fatty acid methyl esters and hydrocarbons from nonhydroxy methyl esters. Examination of the four (hydrogenated) ester classes after TLC multidevelopment showed the saturated and monoene fractions for all citrus to be pure; however, the diene and triene fractions both showed a hydrocarbon band. This hydrocarbon band presumably arose from hydrogenation of unsaturated hydrocarbons which were carried along with the unsaturated fatty acid methyl esters. This band, however, amounted to less than 3% for both fractions. Surprisingly, only the two tangor species showed a weak band in the aldehyde dimethyl acetal region. This band was completely absent from all orange varieties.

In our previous investigation[5] we reported fatty acid profiles for citrus juices obtained from hand-reamed fruit extracted in a household juice extractor. The juice of hand-reamed fruit contained not only juice sacs but some carpellary membrane, core and peel. Lipid extraction of hand-reamed juice produced a fatty acid profile which was a reflection of a heterogeneous mixture rather than a pure source. To impart greater relevance to citrus fatty acid patterns, juice sacs were carefully removed from the fruit and precautions taken not to include membrane, core nor peel in this fraction. A preliminary comparative examination of fatty acids from the carpellary membrane and juice sacs of Pineapple orange revealed that the membrane contained 16·7% linolenic acid as contrasted to 9·1% for its juice sacs. Also, the fatty acid profile from juice of hand-reamed Valencia orange[5] was found slightly different than the profile obtained for Valencia juice sacs reported in Tables 1–4. In this paper, fatty acid profiles were determined only for juice sacs.

Examination of the saturated fatty acids in Table 1 shows that every linear even- and odd-numbered acid from C_{10} to C_{28} is present in all eight varieties. With one exception, palmitic acid is the only linear even-numbered acid found at concentrations greater than 1%. The one exception is lignoceric ($C_{24:0}$) found in Valencia orange at a concentration of 1·2%. Surprisingly, stearic acid was found in all varieties at concentrations less than 1%, i.e. in the range 0·38–0·81%. All linear odd-numbered acids are found at concentrations less than 1%. For all varieties, the major odd acid is $C_{25\ 0}$ with $C_{17:0}$ being the next most abundant. With few exceptions, even- and odd-numbered linear acids are accompanied by iso-branched acids. These iso acids range from a trace (0·001%) to 0·23% and the major acid in all varieties is iso $C_{18:0}$. Anteiso acids are found accompanying only odd-numbered linear acids. Their concentrations range from a trace to 0·055% and the major acid is anteiso $C_{25:0}$. One interesting relationship observed for the iso and anteiso acids is that for similar carbon numbers, the anteiso–iso ratio increases with increase in chain length.

Table 2 reveals few noticeable differences in the monoene composition of the eight citrus. Valencia orange possesses the lowest concentration of oleic acid while the Washington Navel shows the highest. The concentration of $C_{19:1}$ in seven of the eight citrus is between 0·01 and 0·02%; however, in Parson Brown this acid is found at a noticeably higher

[6] Z. L. BANDE, *Chem. Phys. Lipids* **3**, 409 (1969).
[7] H. K. MANGOLD, in *Thin-layer Chromatography* (edited by E. STAHL), p. 373, Springer-Verlag, New York (1969).

value, i.e. 0·09%. An interesting pattern exists in the C_{12}–C_{15} region. In all eight citrus, the concentration of C_{13} is greater than C_{12} and C_{15} greater than C_{14}. This is in contrast to the pattern observed for the saturated acids (Table 1). A number of iso-branched acids are found in the monoene fraction; however, iso $C_{18:1}$ is the only acid found in any appreciable amounts. This iso monoene varies from 0·02 to 0·11% with Parson Brown manifesting the highest concentration. For iso-branched monoenes a general pattern emerges, i.e. iso acids are found only for even-numbered acids above $C_{16:1}$ but below $C_{16:1}$ they may be associated with either even- or odd-numbered acids. The only anteiso monoene found in all eight citrus is $C_{19:1}$. The concentrations of this acid are approximately equal, in most cases, to its linear chain homolog.

Table 3 shows the diene composition. All eight citrus possess both even- and odd-numbered acids from $C_{14:2}$ to $C_{20:2}$ and the even numbered $C_{22:2}$ and $C_{24:2}$. The two tangors (Temple and Temple × Kinnow) show concentrations of $C_{19:2}$ near 0·1%. This concentration is far greater than the trace amounts shown for the six orange varieties and is higher than any value reported for several other citrus species.[5] Only two iso-branched dienes occur with any regularity in the eight citrus, viz. $C_{16:2}$ and $C_{18:2}$. In addition to iso $C_{16:2}$ and iso $C_{18:2}$, Valencia orange is the only citrus to possess a noticeable iso $C_{14:2}$ content, i.e. 0·023%. Only one anteiso diene is detected in all eight citrus, viz. $C_{19:2}$. This acid is found in concentrations two to three times the concentration of anteiso $C_{19.1}$ (Table 2). For the six orange varieties the concentration of anteiso $C_{19:2}$ is ten or more times greater than its linear chain homolog; however, for the two tangors the concentration of this acid is one-half that of its linear chain homolog.

The triene acids are shown in Table 4. In addition to the major triene, $C_{18:3}$, only four other linear chain trienes are present in significant amounts, viz. $C_{14:3}$, $C_{16:3}$, $C_{17:3}$ and $C_{20:3}$. Only one iso triene, $C_{16:3}$, occurs in all eight citrus with any regularity.

Table 5 summarizes the % composition of the five major fatty acids in the six orange and two tangor varieties. Collectively, these acids, viz. palmitic, palmitoleic, oleic, linoleic and linolenic, comprise more than 92% of all fatty acids found in the juice sacs. Of the six orange varieties, Washington Navel is the most distinctive; it has the highest level of oleic acid and the lowest level of palmitic and linoleic acids. The two tangors, viz. Temple and Temple × Kinnow, are also different from each other. Temple shows the highest content of linolenic acid and lowest content of linoleic acid when compared to the Temple × Kinnow and the six orange varieties. High concentrations of linolenic acid have been previously shown to occur only in lime and lemon species.[5]

TABLE 5. PER CENT OF MAJOR FATTY ACIDS IN ORANGE AND TANGOR JUICE SACS

Fatty acid	Variety							
	Walker Early	Parson Brown	Hamlin	Washington Navel	Pineapple	Valencia	Temple	Temple × Kinnow
Palmitic	21·1	20·1	21·8	19·0	20·6	21·9	18·4	18·8
Palmitoleic	5·1	4·9	3·2	6·5	5·0	3·6	4·3	6·2
Oleic	31·9	30·9	27·7	34·1	29·5	25·4	33·0	32·4
Linoleic	28·3	28·3	30·7	25·0	29·6	34·8	21·4	26·5
Linolenic	7·2	8·3	10·4	9·3	8·7	6·6	15·1	9·4

Examination of Tables 1–4 reveals that there are acids present in some orange and tangor varieties which are absent in others. The reported absence of these acids implies only that they were not detected under our experimental conditions and may be present at concentrations below 0·001%. Approximately 0·009–0·09% of the total acids in the juice sacs could not be definitively characterized. These acids possessed effective chain length numbers ending in units of 0·30 and were shown not to be hydrocarbons, dimethyl acetals or hydroxy fatty acids.

In citrus, the most intriguing fatty acids are those containing the iso- and anteiso-branched structures. Plants form these branched acids by employing isobutyrate, isovalerate and 2-methylbutyrate as the initial primer in the two-carbon elongation process.[8–10] Examination of the fatty acid profiles from Tables 1–4 and profiles previously reported for several citrus species[5] reveals the following rules: (a) citrus species do not synthesize even-numbered anteiso fatty acids in any fraction, i.e. saturated, monoene, diene or triene; (b) only even-numbered iso fatty acids are formed; (c) odd-numbered fatty acids are found with either or both iso and anteiso structures.

In our previous paper[5] we reported the presence of 165 anteiso acids in seven different citrus juices and seeds. Examination of the tables in that paper[5] revealed that only four anteiso acids were characterized as even-numbered anteiso structures and these four were found in trace concentrations. To further substantiate our claim for the absence of even-numbered anteiso acids, the fractions containing these four acids were re-examined. After subjecting these fractions to the multidevelopment purification procedure, no even-numbered anteiso acids were detected. Rules (a), (b) and (c) were, therefore, verified.

Hunter and Brogden,[11] from this Laboratory, in characterizing the nonvolatile paraffins in Valencia orange oil found the major linear chain hydrocarbons to be C_{23} and C_{25}. These hydrocarbons were accompanied by small concentrations of their respective iso-branched homologs. Also, a number of even-numbered hydrocarbons were accompanied by either their respective anteiso or iso homologs. Since the biosynthesis of long chain hydrocarbons proceeds through decarboxylation of fatty acids,[9] it was not surprising that the major long chain hydrocarbons found were odd-numbered. While anteiso even-numbered fatty acids do not appear in citrus, anteiso even-numbered hydrocarbons occur. This relationship exists because, e.g. decarboxylation of anteiso C_{25} fatty acid yields anteiso C_{24} hydrocarbon. As a second example, decarboxylation of iso C_{24} fatty acid yields iso C_{23} hydrocarbon. If our theory is correct that citrus does not form anteiso even-numbered fatty acids, then anteiso odd-numbered hydrocarbons should also not be found. The work of Brogden and Hunter[11] shows the complete absence of anteiso odd-numbered hydrocarbons and thus, confirms our theory.

There appears no distinct differences between fatty acid patterns and the harvest season of the orange varieties. Parson Brown, Walker Early, Washington Navel and Hamlin belong to early season; Pineapple to mid- and Valencia to late season. The two tangors, which are generally harvested between mid- and late season, manifest distinct differences in both major and minor fatty acids when compared to the orange varieties. The possibility of employing fatty acid patterns to determine parentage of hybrids appears feasible.

[8] P. E. KOLATTUKUDY, *Biochem.* **5**, 2265 (1966).
[9] P. E. KOLATTUKUDY, *Science* **159**, 498 (1968).
[10] F. D. GUNSTONE, *An Introduction to the Chemistry and Biochemistry of Fatty Acids and Their Glycerides*, p. 183, Chapman & Hall, London (1967).
[11] G. L. K. HUNTER and W. B. BRODGEN, *Phytochem.* **5**, 807 (1966).

EXPERIMENTAL

Isolation and Purification of Juice Lipids

Valencia, Hamlin, Parson Brown and Walker Early oranges were obtained from local groves. Pineapple and Washington Navel oranges and the Temple × Kinnow tangor were obtained from U. S. D. A.'s Whitmore Experimental Farm (Crops Research Division, U. S. D. A., Orlando, Florida). Care was taken that all fruit was collected from all parts of the tree and harvested at or near peak maturity. The Temple tangor was obtained from a local market. The eight citrus were cut in half and the intact juice sacs (vesicles) were carefully separated from core, peel, seeds and carpellary membrane with the aid of a citrus spoon. The juice sacs were ruptured by homogenizing the samples (400 ml) 5 min at slow speed in a Waring Blendor. From this point, the isolation and purification of lipids was carried out as previously described.[5]

Methyl Ester Preparation, Purification and Separation

The esters were prepared, purified and separated as previously described[5] with the following modifications. Di-tert-butyl-cresol (0·1%) was added to all developing solvents to prevent oxidation during development of the thin-layer chromatograms. Improved resolution of the ester classes, viz. saturated, monoene, diene and triene, was obtained through use of improved $AgNO_3$ plates. These plates were prepared in the following manner. Fifty ml of distilled water was heated to 50° and $AgNO_3$ added until a saturated solution ensued. 0·5 ml conc. NH_4OH was added to stabilize the solution. To 150 ml methanol, the $AgNO_3$ solution was added dropwise under constant stirring until saturation occurred. Five ml water and 1 ml conc. NH_4OH were then added to this methanolic solution to bring the excess $AgNO_3$ back into solution. TLC plates coated with 400 μ silica gel H were placed in this $AgNO_3$ methanolic solution and allowed to impregnate for 4 hr. The plates were removed after this period, air-dried for 30 min and activated at 110° for 1 hr. The ester classes were separated on these argentation H plates in solvent systems previously reported.[5]

Hydrogenation, Purification and Gas–Liquid Chromatographic Analyses

A major portion of each unsaturated fraction obtained after $AgNO_3$ partitionment was dissolved in 1 ml of hexane and hydrogenated under 50 lb/in^2 at room temperature for 1 hr with 10 mg of 10% Pd-C catalyst in a Parr apparatus. Following separation of the hydrogenated methyl ester from the catalyst, the ester was re-chromatographed on silica gel G precoated plates (20 × 20 cm, 0·25 mm, Analtech, Inc., Wilmington, Del.). Plates were subjected to multidevelopment by allowing the plates to develop three times to a height of 17 cm in hexane–ether (95:5).[7] This multidevelopment procedure was an additional purification step to our previously reported purification method.[5] The plate was dried, sprayed with Rhodamine 6G and the hydrogenated ester band detected under short wavelength u.v. light. The ester was eluted from the gel with ether, filtered through a very fine sintered glass funnel and reduced to dryness under helium. All esters were stored in 0·1 ml heptane in vials under helium at 6°.

Gas–liquid chromatographic analyses of the fatty acid methyl esters were determined with an F & M Model 5750 gas chromatograph equipped with flame ionization detectors. Dual aluminium 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 employed in quantitative analyses. The total methyl ester sample (nonhydrogenated) was run isothermally at an oven temperature of 200°, detector temperature 250°, injection temperature of 270° and a helium flow rate of 80 ml/min. Methyl ester fractions which had been separated by argentation TLC, hydrogenated and finally subjected to TLC multidevelopment were run on DEGS columns under temperature programmed conditions of 150–230° at 2°/min and held isothermally at 230° until the C_{26}–C_{30} esters had emerged. The helium flow rate was 80 ml/min. Quantitative results were obtained by measurement of peak areas with the aid of a disc integrator and the percentage of each component acid in each fraction was determined as previously described.[5]

Reproducibility of analyses was tested with methyl ester samples prepared from Walker Early oranges and Temple tangors. A total methyl ester sample from Walker Early was divided equally into four parts while the Temple sample was divided into three parts. Each fraction was subjected individually to TLC fractionation and GLC quantitation. The coefficient of variation (% relative standard deviation) determined for the esters in these samples were found to be no higher than 8·2%. The vast majority of esters possessed coefficients of variation between 1 and 5%.