Phytochemistry, 1968, Vol. 7, pp. 1915 to 1922. Pergamon Press. Printed in England

ASPECTS OF LIPID METABOLISM IN HIGHER PLANTS—II.

THE IDENTIFICATION AND QUANTITATIVE ANALYSIS OF LIPIDS FROM THE PULP OF PRE- AND POST-CLIMACTERIC APPLES

T. GALLIARD

Agricultural Research Council, Food Research Institute, Colney Lane, Norwich

(*Received* 11 *May* 1968)

Abstract—The identity, composition and concentration of each individual lipid in the pulp of apples has been determined for fruit in both the pre- and post-climacteric stages of ripening. The fatty acid composition of each acyl lipid is similar for apples at both stages of ripening. Although linoleic acid was the main fatty acid component of most of the major acyl lipids, linolenic acid was the predominant acid in mono- and di-galactosyl diglycerides. Glucocerebrosides contained only hydroxylated fatty acids. The decrease in the level of linolenic acid in the *total* lipid at the post-climacteric stage was mainly due to a decreased concentration at this stage of galactosyl diglycerides. The concentration of phosphatidyl glycerol was also lower in the post-climacteric fruit. Most other lipids showed no marked differences in concentration between the two stages. The results, which suggests a selective degradation of plastid membranes during ripening, are discussed in relation to current theories of fruit ripening.

INTRODUCTION

THE INTRACELLULAR changes during the ripening of fruits are not well understood. Two apparently opposed views are currently held. One theory[1] holds that the breakdown of membrane structures at the onset of ripening leads to increased permeability of all membranes resulting in a delocalization of cellular components and increased catabolic processes causing eventual senescence. The other theory, for which evidence has accumulated recently,[2–4] requires an initial synthetic phase at the onset of ripening, during which specific enzymes are synthesized, cellular processes are under strict control and at least some cell organelles, e.g. mitochondria, retain their structure and activity well into the ripening phase.

Changes in the properties of membraneous components of cells could be expected to be reflected in alterations in the nature of those lipids associated with lipo-protein membrane structures. Recent work[2, 5] has shown that the onset of ripening in apples is associated with an increased rate of lipid synthesis. Lipoxidase activity increases markedly during the climacteric rise in respiration of apples[6] and there is evidence[7] that lipoxidase activity and fatty acid metabolism may be involved in the production by apples of ethylene, the ripening "hormone" of many fruits.

The purpose of the present work was to identify the lipid components of apple pulp and to measure quantitatively any significant differences between pre- and post-climacteric fruit in the composition and concentration of each lipid.

[1] J. A. SACHER, *Symp. Soc. Exp. Biol.* **21**, 269 (1967).
[2] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *New Phytologist* **64**, 158 (1965).
[3] A. RICHMOND and J. BIALE, *Plant Physiol.* **41**, 1247 (1966).
[4] A. C. HULME, M. J. C. RHODES, T. GALLIARD and L. S. C. WOOLTORTON, *Plant Physiol.*, in press (1968).
[5] T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 1453 (1968).
[6] L. S. C. WOOLTORTON, J. D. JONES and A. C. HULME, *Nature* **207**, 999 (1965).
[7] T. GALLIARD, M. J. C. RHODES, A. C. HULME and L. S. C. WOOLTORTON, *Phytochem.* **7**, 1465 (1968).

RESULTS

The pulp of apples was selected for study because the main purpose of the work was to examine particularly those lipids associated with lipo-protein membranes and not the waxes and other lipid components of the cutin of the peel tissue. The methods used for the identification and analysis of lipids were basically those described previously[8] for the analysis of lipids from potato tubers.

Identification of Lipids

The two-dimensional TLC* separation of the polar lipids from the pulp of pre- and post-climacteric apples were qualitatively identical and were similar to that obtained earlier for lipids of potato.[8] The nature of each lipid was determined by the combined DEAE-cellulose column and TLC methods previously described.[8]

Phospholipids. PC, PE, PI, PG, DPG, PS and PA were present. A small amount of an unidentified phosphate-containing lipid (not detected on the two-dimensional TLC separation) was eluted from the DEAE-cellulose column with the acidic lipids. This lipid had an R_f greater than that of DPG on one-dimensional TLC analysis in both chloroform–methanol–7 N NH_4OH (65:30:4, v/v/v) and in chloroform–methanol–acetic acid–water (170:25:25:4).

Galactolipids. Mono- and di-galactosyl diglycerides were found. The polygalactosyl diglyceride previously found in potato[8] was present in only trace amounts. An unidentified galactolipid with TLC properties identical to that in potato[8] and liberating fatty acids and galactose on hydrolysis was also found in apple.

Steryl lipids. Steryl glucoside and esterified steryl glucoside were identified by analysis of their products on acid hydrolysis. The neutral lipid fraction contained both free and esterified sterol. The identity of the sterol in each of the above lipids was not determined separately but GLC analysis of the sterols obtained from the non-saponifiable fraction of the total lipids from pre-climacteric apples showed that 98 per cent of the total sterol was contained in a single peak with the same retention time, on a silicone gum column, to that of authentic β-sitosterol (3·1 relative to cholestane). The remaining 2 per cent of the sterol fraction gave a single peak with a retention time of 2·5 relative to cholestane but was not identified.

Glucocerebroside. This lipid had identical chromatographic properties to that of potato,[8] and liberated glucose, a long-chain nitrogenous base and fatty acids (exclusively hydroxy acids) on acid hydrolysis. The identity of the base was not determined but it behaved similarly to sphingosine on TLC and is presumably phytosphingosine or a derivative.

Sulpholipid. Sulphoquinovosyl diglyceride was found in trace amounts.

Neutral lipids. The components of this fraction, which ran as a single spot in the TLC separation and which were eluted from the DEAE-cellulose column as a mixture, were separated by TLC and found to contain, in addition to free and esterified sterol described above, triglyceride together with small amounts of chlorophyll and other pigments. No significant amounts of triterpenes or mono- and diglycerides were observed.

* *Abbreviations used.* TLC = thin layer chromatography; GLC = gas–liquid chromatography; PC, PE, PI, PG and PS = phosphatidyl-choline, -ethanolamine, -inositol, -glycerol, and -serine respectively; PA = phosphatidic acid; DPG = diphosphatidyl glycerol; MGDG, DGDG, PGDG = mono-, di- and polygalactosyl diglyceride respectively; SG = steryl glucoside; ESG = esterified steryl glucoside; SL = sulpholipid; TG = triglyceride; CER = glucocerebroside; NL = neutral lipids; UGL = unidentified galactolipid.

[8] T. GALLIARD, *Phytochem.* **7**, 1907 (1968).

Fatty Acid Composition of Lipids

Table 1 gives data for the distribution of the major fatty acids in the total lipid extract and in isolated lipids from both pre- and post-climacteric apples. Other fatty acids, not shown in Table 1, were present in only trace amounts (< 1 per cent of the fatty acids in each lipid).

TABLE 1. FATTY ACID COMPOSITION OF INDIVIDUAL LIPIDS FROM THE PULP OF PRE- AND POST-CLIMACTERIC APPLES

	Fatty acids (% peak area)									
	Pre-climacteric					Post-climacteric				
Lipid	16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
Total lipid	24·4	2·2	1·6	48·4	23·2	24·5	3·8	6·9	54·0	10·8
Phosphatidyl choline	25·2	4·2	3·8	54·0	12·8	22·5	2·9	7·7	56·9	10·0
Phosphatidyl ethanolamine	23·1	3·1	3·3	60·3	10·2	22·8	2·1	1·5	64·8	8·8
Phosphatidyl inositol	33·6	2·6	1·3	42·9	19·6	39·0	3·4	5·0	38·5	14·1
Phosphatidyl glycerol	45·5	1·9	6·4	29·0	17·2	42·2	2·2	16·8	30·4	8·4
Diphosphatidyl glycerol	14·5	3·1	6·4	58·0	18·0	25·0	3·5	5·3	49·4	16·8
Monogalactosyl diglyceride	2·0	0 6	0·6	5·4	91·4	6·1	0·8	7·1	12·7	73·3
Digalactosyl diglyceride	16·9	4·2	2·2	16·9	59·8	19·5	6·7	7·0	23·8	43·0
Unidentified galactolipid	—	—	—	—	—	30·6	4·4	3·9	43·9	17·2
Esterified steryl glucoside	13·8	6·0	7·9	63·3	10·0	10·0	3·3	6·3	69·2	11·2
Sulpholipid	31·7	6·7	7·9	30·8	22·9	35·2	7·9	5·0	29·8	22·1
Triglyceride	12·5	3·9	5·8	52·9	24·9	18·3	7·7	10·2	51·7	12·1
Glucocerebroside	2-hydroxy acids only (see text)									

In general, the distribution of fatty acids in the same lipid from both pre- and post-climacteric apples was similar except that the proportion of linolenic acid in lipids from post-climacteric fruit was lower than in pre-climacteric fruit and this was particularly marked in the total lipid fatty acid composition. It will be shown later that the main cause of the lower linolenic acid concentration in post-climacteric fruit is a decrease in the concentration of those lipids which are rich in this fatty acid and is not due solely to changes in the fatty acid distribution in individual lipids.

For fruit at both stages of development some general characteristics of fatty acid composition are evident. Linoleic acid is the major component of the total lipid and of many of the individual lipids. The galactosyl diglycerides (particularly MGDG) contain a high proportion of linolenic acid and much less linoleic acid. Palmitic acid is the largest component of phosphatidyl glycerol. In contrast to all other acyl lipids, glucocerebroside contains only hydroxylated fatty acids. Acid hydrolysis of glucocerebroside from pre-climacteric apples liberated fatty acids which, as both free acids and as methyl esters, had TLC characteristics identical to authentic 2-hydroxypalmitic acid and its methyl ester. GLC analysis of the methyl esters gave the following percentage composition of acids in glucocerebroside: 2-hydroxy $C_{16:0}$ (42·2); 2-hydroxy $C_{21:0}$ (1·9); 2-hydroxy $C_{22:0}$ (20·5); 2-hydroxy $C_{23:0}$ (6·4) and 2-hydroxy $C_{24:0}$ (29·0).

Quantitative Measurement of Lipid Concentrations

Using methods previously described,[8] it was possible to determine the amounts of each lipid separated by DEAE-cellulose column and thin-layer chromatography. This was performed for the lipids of both pre- and post-climacteric apples. The results shown in Table 2, have been expressed in terms of both μmoles of lipid and weight of lipid from 1000 g fresh weight of apple pulp.

Phospholipids as a group form almost half of the total weight of lipids. PC and PE are the major components of this group. The concentration of each lipid is similar in both pre- and post-climacteric fruits except for PG which is lower in the post-climacteric phase.

Galactosyl diglycerides show a marked fall in both MGDG and DGDG concentrations in the post-climacteric apples. Polygalactosyl diglyceride is present only in trace amounts.

Steryl lipids. On a molar basis, these lipids have a total concentration of the same order as the phospholipids. Free sterol is the major component (mainly β-sitosterol) and this was present at a higher concentration in the post-climacteric fruit. Other steryl lipids are present at similar concentrations in both stages of development.

Neutral lipids. Free and esterified sterol together with triglyceride comprise almost the total weight of this fraction. The higher chlorophyll content of the neutral lipid from the pre-climacteric apples resulted in a green solution whereas the corresponding fraction from post-climacteric apples was yellow, presumably due to proportionately higher carotenoid content, although this was not determined quantitatively. The higher concentration of neutral lipids in post-climacteric apples is due mainly to a higher free sterol content.

Other polar lipids. Sulpholipid was present in only trace amounts and the unidentified galactolipid was also present at too low concentration for satisfactory determination. Glucocerebroside was a relatively important component of the lipid extract being present at a somewhat higher concentration in post-climacteric fruit.

Related to fresh weight, the total weight of *polar* lipids was very similar in both stages of development, the slightly higher amount of the *total* lipids in post-climacteric apples was due mainly to increased sterol concentration in this tissue.

DISCUSSION

The lipids identified in the pulp of apple are typical of many plant tissues. Qualitatively, the lipid components of apple pulp resemble, for example, those of leaves[9] and storage tissues.[10–12] In addition to the well-characterized lipids, others, whose existence in plants has only recently been recognized, are also present in apple; viz. esterified sterol glucoside,[13,14] glucocerebroside[15] and polygalactosyl diglyceride (possibly trigalactosyl diglyceride).[8,16] Small amounts of an unidentified phospholipid and an unknown galactolipid previously found in potato tubers[8] were also present in apple. The phospholipids and neutral lipids of apple pulp have recently been described[17] and the present work confirms the identities of these lipids.

[9] J. B. MUDD, *Ann. Rev. Plant Physiol.* **18**, 229 (1967).
[10] M. LEPAGE, *J. Chromatogr.* **13**, 99 (1964).
[11] M. LEPAGE, *Lipids* **2**, 244 (1967).
[12] B. W. NICHOLS and A. T. JAMES, *Fette, Seifen, Anstrichmittel* **66**, 1003 (1964).
[13] M. LEPAGE, *J. Lipid Res.* **5**, 587 (1964).
[14] T. KIRIBUCHI, T. MIZUNAGA and S. FUNAHASHI, *Agr. Biol. Chem.* **30**, 770 (1966).
[15] P. S. SASTRY and M. KATES, *Biochemistry* **3**, 1271 (1964).
[16] C. F. ALLEN, O. HIRAYAMA and P. GOOD, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. 1, p. 195, Academic Press, New York (1966).
[17] P. MAZLIAK, *Phytochem.* **6**, 687 (1967).

TABLE 2. QUANTITATIVE COMPOSITION OF LIPIDS IN PULP OF PRE- AND POST-CLIMACTERIC APPLES

Lipid	μmoles lipid/1000 g fresh wt.		Weight lipid mg/1000 g fresh wt.		% by weight of total lipid	
	Pre-climacteric	Post-climacteric	Pre-climacteric	Post-climacteric	Pre-climacteric	Post-climacteric
Phosphatidyl choline	240	272	189	214	21·5	22·7
Phosphatidyl ethanolamine	168	136	124	101	14·4	10·7
Phosphatidyl inositol	62	69	53	59	6·0	6·2
Phosphatidyl glycerol	35	10	27	7·7	3·1	0·8
Diphosphatidyl glycerol	4	3	5·8	4·4	0·7	0·5
Phosphatidyl serine	5	5	4·0	4·0	0·4	0·4
Phosphatidic acid	4	9	2·8	6·0	0·3	0·7
Unknown phospholipid	2	2	—	—	0·2†	0·2†
Total phospholipid	520	506	405·6	396·1	46·6	42·2
Monogalactosyl diglyceride	54	15	42	12	4·8	1·3
Digalactosyl diglyceride	114	52	107	49	12·2	5·3
Polygalactosyl diglyceride	trace	trace	—	—	—	—
Unidentified galactolipid	trace	trace	—	—	—	—
Total galactolipid	168	67	169	61	17·0	6·6
Esterified steryl glucoside	14	18	12	15	1·4	1·6
Steryl glucoside	89	89	51	51	5·8	5·4
Sterol	314	460	129	190	14·7	20·1
Sterol esters	27	32	18	22	2·0	2·1
Total steryl lipids	444	599	210	278	23·9	29·2
Sulpholipid	trace	trace	—	—	1·0	1·0
Glucocerebroside	47	69	34	49	3·9	5·2
Triglyceride	50	55	44	50	5·0	5·3
Chlorophyll	1	0·3	0·9	0·3	0·1	0·03
Combined neutral lipids	—	—	208*	272*	23·1	28·7
Combined polar lipids	—	—	672	675	76·9	71·3
Total lipids	—	—	880*	947*	—	—

* Results from direct weighing.
† Estimated value.

The fatty acid composition of the lipids in general is also typical of plant lipids. The high linoleic acid content of the total lipid is reflected in the composition of the major acyl lipids, PC, PE and TG. ESG in apples has a similar fatty acid composition to the above lipids whereas the ESG of potato tubers[8, 11] has a very high content of saturated fatty acids. The predominant occurrence of α-hydroxy acids in cerebroside is characteristic of this lipid from plant sources.[15, 18] Galactolipids have fatty acid compositions similar to those in leaf tissues.[9] The high content of linolenic acid in the galactolipids of apple pulp is interesting in comparison with the results of Mazliak and Justin[19] with *peel* tissue of post-climacteric apple in which no significant amounts of this acid were found in the galactolipids.

Because of the wide range of molecular weights of component lipids, it is useful to express the concentrations of lipids in both molar and weight terms. Also, although the immediate purpose of the present work was to compare the concentrations of lipids in apples at two distinct phases of development, it is possible to observe some interesting differences in the lipid composition of different plant tissues. For example, green photosynthesizing tissue has a high proportion of galactolipids,[9] etiolated tissue has a relatively lower content of these lipids[20] which is of the same order (on a fresh weight basis) as that found in storage tubers of potato[8] but higher than that found in apple pulp in the present work. In potato, ESG was the major sterol-containing lipid,[8, 11] whereas in apple, free sterol and steryl glucoside are present in much higher amounts.

Comparing the concentrations of individual lipids in pre- and post-climacteric apples, most lipids are present at similar concentrations in both tissues. However, it is significant that the lipids which are present at markedly lower concentrations in the post-climacteric apples, i.e. MGDG, DGDG, and PG, are those lipids associated with plastid structures.[9] A corresponding decrease in chlorophyll content was observed although, relative to the galactolipids, the concentration of this pigment was much lower even in the pulp of pre-climacteric apples than its concentration in leaves.[20]

These results would suggest that during ripening of the apples there is a loss of lipids associated with plastid membranes. Support for this view is obtained from electron microscope studies[21, 22] showing breakdown of chloroplasts during ripening of pears and apples. The lower concentration of galactolipids in post-climacteric apples is the major cause of the lower linolenic acid content of this tissue. This particular result illustrates the importance of a knowledge of lipid concentrations rather than fatty acid compositions alone in comparing differences in lipids between tissues, or between different developmental phases of the same tissue.

Ethylene is known to initiate the ripening of fruits[23-25] and is itself produced in appreciable amounts by many fruits. It has been shown that the onset of ripening in apples is associated with a greatly increased ethylene production and a parallel increase in lipoxidase activity.[6] The possibility that linolenic acid and lipoxidase might be involved in ethylene production in fruits has been suggested[6, 7, 26] but Abeles[27] considered this unlikely on the

[18] H. E. CARTER, R. A. HENDRY, S. NOJIMA, N. Z. STANACEV and K. OHNO, *J. Biol. Chem.* **236**, 1912 (1961).
[19] P. MAZLIAK and A-M. JUSTIN, *Fruits* **22**, 413 (1967).
[20] I. K. GRAY, M. G. RUMSBY and J. C. HAWKE, *Phytochem* **6**, 107 (1967)
[21] J. M. BAIN and F. V. MERCER, *Australian J. Biol. Sci.* **17**, 78 (1964).
[22] M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1 (1967).
[23] R. GANE, *J. Pomol.* **13**, 351 (1935)
[24] S. P. BURG, *Ann. Rev. Plant Physiol.* **13**, 263 (1962).
[25] L. W. MAPSON, *Biol. Rev.*, in press.
[26] M. LIEBERMAN and L. W. MAPSON, *Nature* **204**, 343 (1964).
[27] F. B. ABELES, *Nature* **210**, 23 (1965).

basis of analyses for linolenic acid in apples during ripening. However, the present work indicates that there is a fall in linolenic acid concentration in the pulp and that this is correlated with breakdown of plastid membranes which could liberate linolenic acid for ethylene production or oxidation. Other evidence for the possible involvement of linolenic acid in the production of ethylene by apples is presented elsewhere.[7]

Conclusions drawn from comparisons of fatty acid concentrations at different stages of development must be examined carefully since it is known that even post-climacteric apples are able to synthesize fatty acids very efficiently.[4,28] However, slices of apple peel or pulp when incubated with acetate-1-^{14}C readily incorporated label into saturated and monoenic fatty acids but polyunsaturated fatty acids were labelled only slowly.[5,29] In the present work the decrease in linolenic acid would seem to be due mainly to loss of structures rich in this acid rather than to a decreased rate of synthesis.

It has been demonstrated that mitochondria of apples retain structural integrity and activity through the climacteric phase.[2,21] Gross changes in membrane structure might be expected to show differences in the lipid components of lipo-protein membranes. The phospholipids, common to most membranes of cells and organelles, do not differ appreciably in pre- and post-climacteric applies. It seems probable therefore that the onset of ripening of apples is accompanied not by gross changes in membrane structures in general, but by selective changes in which, for example, plastid membranes disappear while mitochondria and the plasmalemma remain intact.

EXPERIMENTAL

Materials

Apple fruits were picked from Cox's Orange Pippin trees on Malling IX root stocks grown at the Burlingham Horticultural Station, Norfolk. The fruits were picked in the pre-climacteric state and maintained at 12° until used. The respiration of the fruit at 12° was monitored.[30] Pre-climacteric fruits (respiration rate; 63·1 ml CO_2/10 kg/hr) were taken from a batch of apples which commenced the climacteric rise of respiration 13 days after the experimental material was removed and which reached the climacteric peak (112 ml CO_2/10 kg/hr) after a further 7 days. Post-climacteric fruits were taken from a batch of apples (110 ml CO_2/10 kg/hr) 14 days after the climacteric peak. Standard lipids and derivatives, together with other reagents, were obtained as described previously.[8]

Extraction of Lipids

All operations were performed at 0°. Twenty apples were carefully peeled to remove all green tissue and the cores removed. The remaining pulp tissue was weighed in 250-g portions, diced and homogenized and the total lipids extracted and washed by the methods previously described.[8] A total of 1000 g fresh weight of material was used in both pre- and post-climacteric apples.

Chromatographic Separations and Analyses

The methods used for TLC, GLC and DEAE-cellulose column chromatography and for the identification and quantitative analysis of lipids were the same as those used for analysis of lipids from potato tubers[8] except for the modifications and additional methods described below.

Sterol-containing lipids were saponified by refluxing in 15% ethanolic KOH for 2 hr. The liberated sterols were isolated by preparative TLC of the non-saponifiable fraction on silica gel G in hexane–diethyl ether (70:30 v/v). The sterols, eluted from the TLC plate with methanol–ether (10:1) were injected into a 5 ft glass column of silicone gum (SE 30)-3% on 80–100 mesh Gas Chrom. Q. The column was pre-treated with hexamethyl disilazane and run isothermally at 220° with argon carrier gas and flame-ionization detection. The retention volumes of sterols were measured relative to that of authentic cholestane.

28 P. Mazliak, *Phytochem.* **6**, 941 (1967).
29 P. Mazliak, *Compt. Rend.* **261**, 2716 (1965).
30 A. C. Hulme, J. D. Jones and L. S. C. Wooltorton, *Proc. Roy. Soc. Ser.* B, **158**, 519 (1963).

The hexose content of lipids was routinely determined by the orcinol method;[31] the phenol–H_2SO_4 method[32] was found to be more convenient and gave lower blank values than the orcinol method but could not be used for sterol-containing lipids. However, the phenol method was used to check the results obtained for the galactolipids with good agreement. The use of the phenol–H_2SO_4 method for sulpholipid analysis has recently been described[33] but insufficient material was available for analysis in the present work.

The chlorophyll content of the lipid extract was estimated spectrophotometrically.[34] Cerebroside was determined by total nitrogen content.[35] All analyses, except for direct weighing, gave molar concentrations of lipids. To compare all the lipids on a weight basis (Table 1), a weight value was determined from the molar concentration and an estimated molecular weight calculated for each lipid.

Acknowledgements—I am grateful to Drs. A. C. Hulme and M. J. C. Rhodes for help and advice on the physiological aspects of this work, to Mr. A. S. A. Pryke for valuable technical assistance.

[31] L. SVENNERHOLM, *J. Neurochem.* **1**, 42 (1956).
[32] D. S. GALANOS and V. M. KAPOULAS, *Biochem. Biophys. Acta* **98**, 278 (1965).
[33] P. G. ROUGHAN and R. D. BATT, *Analyt. Biochem.* **22**, 74 (1968).
[34] J. BRUINSMA, *Photochem. Photobiol.* **2**, 241 (1963).
[35] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).