Phytochemistry, 1965, Vol. 4, pp. 863 to 871. Pergamon Press Ltd. Printed in England

FATTY ACID METABOLISM IN THE APPLE FRUIT DURING THE RESPIRATION CLIMACTERIC

D. F. MEIGH and A. C. HULME

Ditton Laboratory, Larkfield, Maidstone, Kent

(*Received* 27 *March* 1965)

Abstract—The respiration rate, lipoxidase activity and free and esterified fatty acid content of peel were determined at intervals in apple fruits passing through the respiration climacteric when either developing on the tree or in storage at 12°. While respiration rate followed the normal course, lipoxidase activity increased rapidly both "on" or "off" the tree. In the fruits developed "on" or "off" the tree there was also an initial build up of fatty acids and esters, both saturated and unsaturated. The quantities of esterified acids always greatly exceeded those of the free acids, and the unsaturated C_{18} acids formed a large proportion of each group. In fruits developing "on" the tree there was a subsequent phase of fatty acid breakdown.

INTRODUCTION

Markley and Sando,[1] in 1931, first showed that the waxy surface coating of apple skin increased in quantity during maturation on the tree and in subsequent storage. By taking advantage of differential solubilities in organic solvents they found that this coating could be divided into three crude fractions. At about the same time these three fractions—an oil, a wax, and ursolic acid—had also been distinguished by Gane,[2] and Chibnall *et al.*[3,4] identified some of the constituents of the wax fraction.

In 1951 Huelin and Gallop[5] reported that when Granny Smith apples were stored at 1° the content of the oil fraction trebled during a period of 27 weeks. If the fruit was kept at 18–20° the increase was of similar magnitude but more rapid, while apples transferred from storage at 1° to a temperature of 18–20° after the accumulation had occurred, suffered a rapid loss of the oil fraction. These changes were closely paralleled by variation in the iodine number of the oil and the total fatty acid content. By comparison, the increase in the wax and ursolic acid fractions during storage was small. Richmond and Martin's findings[6] tend to confirm these results.

With the advent of gas chromatography a detailed comparison of the fatty acid composition of the fractions became possible. Mazliak[7] found that the wax fraction was rich in the saturated C_{20} to C_{30} acids while the oil fraction contained a high proportion of unsaturated C_{18} acids. In his studies of apples developing on the tree,[8] he showed that as the fruit matures the oil fraction increases rapidly and the wax fraction more slowly. After picking, the changes depend on temperature. At 15° the oil fraction accumulates rapidly and then diminishes

[1] K. S. MARKLEY and C. E. SANDO, *J. Agric. Res.* **42**, 705 (1931).
[2] R. GANE, *Dep. Sci. Ind. Research Food Invest. Bd. Rep.*, 1931, p. 242, H.M.S.O., London (1932).
[3] A. C. CHIBNALL, S. H. PIPER, A. POLLARD, J. A. B. SMITH and E. F. WILLIAMS, *Biochem. J.* **25**, 2095 (1931).
[4] A. C. CHIBNALL, S. H. PIPER, A. POLLARD, E. F. WILLIAMS and P. N. SAHAI, *Biochem. J.* **28**, 2189 (1934).
[5] F. E. HUELIN and R. A. GALLOP, *Australian J. Sci. Research* **4B**, 533 (1951).
[6] D. V. RICHMOND and J. T. MARTIN, *Ann. Appl. Biol.* **47**, 583 (1959).
[7] P. MAZLIAK, *Compt. Rend.* **250**, 182, 2250 (1960); **251**, 2393 (1960); **252**, 1507 (1961).
[8] P. MAZLIAK, *La Cire Cuticulaire des Pommes*, Doctorate Thesis, Paris (1963).

again within a period of about 10 days; at 4° the rise is greater but prolonged over 100 days, followed by a comparable fall; at 0° a shallower peak is observed. During storage at 0° or 4°, the individual acids of the oil fraction behave differently; linoleic and linolenic acids continue to accumulate over the whole period while the saturated acids and oleic acid follow the course of the oil fraction as a whole. Mazliak also observed that the total wax content of the apple at 4° and 15° follows a course similar to respiration rate, rising to a peak during the climacteric.

The aim of the work described here was to study the production of fatty acids in greater detail during the climacteric phase in fruit both attached to and detached from the tree, with concurrent determinations of respiration rate and lipoxidase activity.

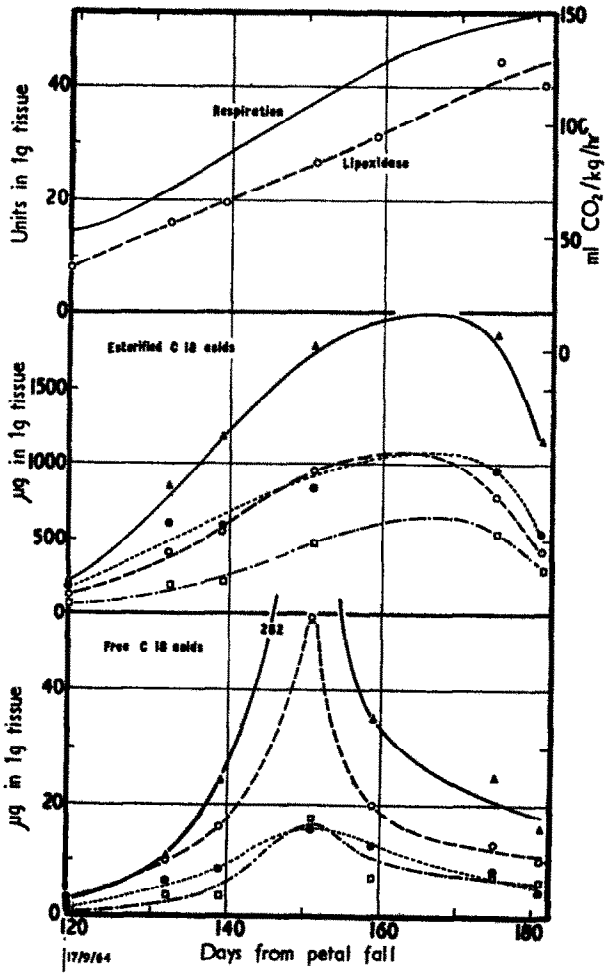

FIG. 1. COX'S ORANGE PIPPIN APPLES DEVELOPING ON THE TREE.

Respiration of whole fruit, lipoxidase activity and C_{18} fatty acids of peel.

–·□–·–·□–· stearic acid (18:0); –○—○– oleic acid (18:1); —△——△— linoleic acid (18:2); ··●···●·· linolenic acid (18:3).

RESULTS

Figures 1 and 2 show changes in respiration of the whole fruit, and lipoxidase activity and C_{18} fatty acid (both free and esterified) content of the peel over the climacteric for "on" and "off" the tree fruit respectively. The respiration rates follow the usual course.[9] "On" the tree the rise was continuous to the end of the period, by which time falling fruit made further

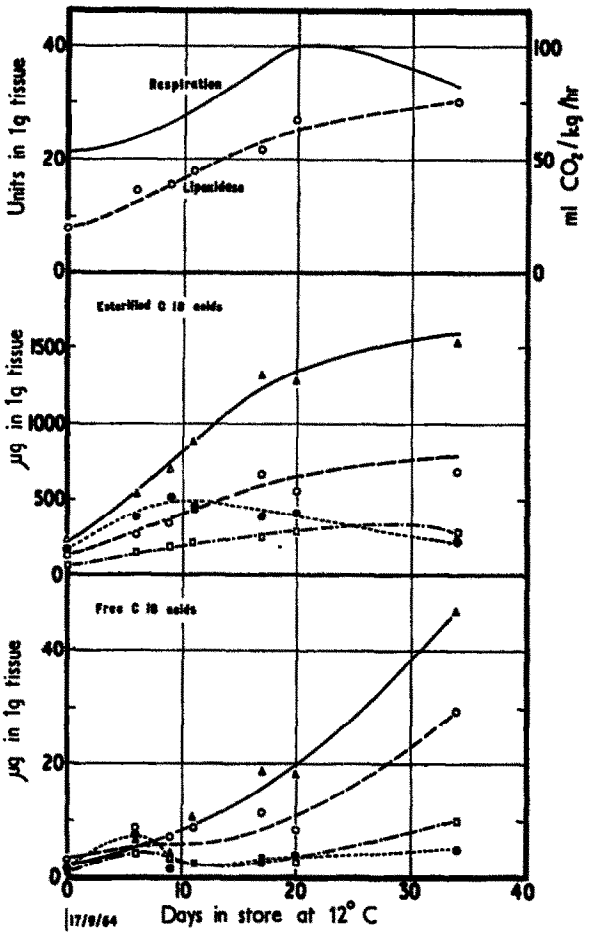

FIG. 2. COX'S ORANGE PIPPIN APPLES STORED AT 12°.
Respiration of whole fruit, lipoxidase activity and C_{18} fatty acids of peel.
–·⊡–·–·⊡–· stearic acid (18:0); –⊙—⊙– oleic acid (18:1); —△——△— linoleic acid (18:2); ··●····●·· linolenic acid (18:3).

experiments unreliable (Fig. 1). "Off" the tree the respiration rose to the peak and then began to fall off (Fig. 2). In both series lipoxidase activity rose rapidly, though the rate of increase tended to fall in the "off" the tree fruit as the climacteric peak was passed.

The analytical figures for the more abundant normal free and esterified acids found in each series of fruit, together with comparable total figures for saturated and unsaturated acids, are given in Tables 1 to 4. The minor constituents which are not individually recorded

[9] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. London* 158B, 514 (1963).

Table 1. Free fatty acid composition of Cox's Orange Pippin apple skin; fruit "on" the tree

Fatty acid	Amount of acid (μg/g fresh weight) at various times (days) after initial picking*						
	0	13	20	31	39	56	62
$C_{12:0}$	0·3	1·9	0·5	6·0	6·7	2·4	2·7
$C_{14:0}$	0·8	2·8	1·8	9·4	9·2	5·2	1·8
$C_{15:0}$	0·4	1·2	0·7	5·5	3·7	1·7	1·1
$C_{16:0}$	4·2	11·0	10·7	33·1	22·7	18·9	8·2
$C_{16:1}$	0·3	2·3	2·0	11·3	9·3	5·5	0·8
$C_{18:0}$	1·4	3·6	3·9	17·6	7·0	7·1	6·1
$C_{18:1}$	3·0	10·2	15·9	52·8	20·2	12·9	9·9
$C_{18:2}$	2·4	10·8	24·2	282·0	33·7	24·5	16·2
$C_{18:3}$	1·6	6·7	8·7	15·6	13·1	8·5	4·8
$C_{20:0}$	1·2	1·1	1·5	5·1	2·1	2·3	1·5
$C_{22:0}$	19·5	20·3	13·0	28·1	16·7	10·2	0·1
$C_{22:1}$	0·1	2·4	3·5	14·0	10·3	5·9	0·6
$C_{24:0}$	42·7	35·8	46·5	86·9	46·2	24·2	8·0
$C_{24:1}$	49·4	18·8	6·1	9·7	5·4	6·5	6·6
Total							
Saturated	92	175	95	276	157	81	86
Unsaturated	58	61	61	390	97	65	40

* 17th September, 120 days after petal fall.

Table 2. Esterified fatty acid composition of Cox's Orange Pippin apple skin; fruit "on" the tree

Acid	Amount of acids (μg/g fresh weight) at various times (days) after initial picking*					
	0	13	20	31	56	62
$C_{12:0}$	11·9	16·8	13·6	29·6	30·9	23·9
$C_{13:0}$	1·6	3·1	2·6	12·2	6·3	5·4
$C_{14:0}$	9·5	14·9	15·7	37·3	25·8	17·7
$C_{14:1}$	1·3	1·3	1·8	7·0	13·0	2·0
$C_{15:0}$	3·7	2·7	3·1	12·3	10·5	6·6
$C_{16:0}$	178·0	318·0	337·0	591·0	573·0	405·0
$C_{16:1}$	8·0	9·7	9·5	13·4	4·5	13·2
$C_{17:0}$	1·9	5·6	9·0	15·2	22·4	5·8
$C_{18:0}$	71·5	198·0	220·0	471·0	545·0	295·0
$C_{18:1}$	138·0	427·0	567·0	969·0	786·0	430·0
$C_{18:2}$	231·0	864·0	1180·0	1770·0	1850·0	1150·0
$C_{19:0}$	6·6	19·0	24·4	42·3	77·0	64·9
$C_{18:3}$	162·0	627·0	626·0	861·0	956·0	543·0
$C_{20:0}$	47·7	15·2	19·5	33·8	31·5	21·7
$C_{22:0}$	43·0	120·0	142·0	365·0	237·0	159·0
$C_{24:0}$	92·5	108·0	105·0	—	—	101·0
Total						
Saturated	568	1810	2150	4760	4150	3130
Unsaturated	556	1950	2390	3640	3610	2160

* 17th September, 120 days after petal fall.

TABLE 3. FREE FATTY ACID COMPOSITION OF COX'S ORANGE PIPPIN APPLE SKIN; FRUIT "OFF" THE TREE

Acid	Amount of acid (μg/g fresh weight) after storage at 12° for various times (days) after picking						
	0	5	9	11	16	19	33
$C_{12:0}$	0·3	3·4	0·9	2·5	5·1	2·3	8·1
$C_{14:0}$	0·8	3·0	5·2	1·5	8·9	3·0	1·2
$C_{15:0}$	0·4	2·8	1·8	1·0	3·7	0·9	0·4
$C_{16:0}$	4·2	9·0	13·6	8·9	17·6	8·9	30·4
$C_{16:1}$	0·3	1·1	4·9	2·3	7·7	2·0	14·7
$C_{18:0}$	1·4	4·1	3·2	2·8	3·3	2·7	9·9
$C_{18:1}$	3·0	9·8	7·8	9·2	11·8	8·2	29·7
$C_{18:2}$	2·4	7·0	3·8	10·9	18·9	18·4	47·0
$C_{18:3}$	1·6	7·6	1·4	3·0	2·3	3·2	5·2
$C_{20:0}$	1·2	—	3·1	0·7	9·5	2·7	2·7
$C_{22:0}$	19·5	22·1	20·1	12·8	7·3	17·6	16·0
$C_{22:1}$	0·1	6·2	8·3	3·2	—	3·7	6·3
$C_{24:0}$	42·7	58·5	52·0	32·8	17·8	62·0	39·8
$C_{24:1}$	49·4	39·1	2·9	3·7	4·6	10·5	6·5
Total							
Saturated	92	122	120	88	98	118	195
Unsaturated	58	71	30	35	51	48	116

TABLE 4. ESTERIFIED FATTY ACID COMPOSITION OF COX'S ORANGE PIPPIN APPLE SKIN; FRUIT "OFF" THE TREE

Acid	Amount of acid (μg/g fresh weight) after storage at 12° for various times (days) after picking						
	0	5	9	11	16	19	33
$C_{12:0}$	11·9	7·6	18·6	10·4	12·9	16·5	25·9
$C_{13:0}$	1·6	0·8	4·8	0·8	1·2	10·3	1·7
$C_{14:0}$	9·5	13·8	20·2	18·6	18·0	23·7	24·5
$C_{14:1}$	1·3	1·0	1·5	0·9	2·7	1·7	8·3
$C_{15:0}$	3·7	4·3	4·6	3·4	14·0	5·3	13·9
$C_{16:0}$	178·0	301·0	321·0	333·0	325·0	392·0	463·0
$C_{16:1}$	8·0	14·0	14·1	12·9	16·4	13·4	19·2
$C_{17:0}$	1·9	3·4	8·0	4·5	65·3	12·3	13·0
$C_{18:0}$	71·5	152·0	192·0	210·0	249·0	292·0	281·0
$C_{18:1}$	138·0	277·0	352·0	468·0	668·0	554·0	679·0
$C_{18:2}$	231·0	537·0	704·0	889·0	1320·0	1290·0	1540·0
$C_{19:0}$	6·6	1·4	2·1	1·9	11·9	39·5	2·8
$C_{18:3}$	162·0	392·0	518·0	459·0	399·0	413·0	232·0
$C_{20:0}$	47·7	72·0	95·1	129·0	299·0	309·0	174·0
$C_{22:0}$	43·0	101·0	80·0	333·0	358·0	235·0	143·0
$C_{24:0}$	92·5	72·7	85·3	95·2	196·0	151·0	132·0
Total							
Saturated	568	1910	2020	2310	4700	2390	3280
Unsaturated	556	1240	1630	1850	2400	2360	2580

here included, in the both the free and esterified acid fractions, the $C_{11:0}$, $C_{12:1}$, $C_{20:1}$ and $C_{21:0}$ acids and a number of peaks tentatively identified as branched chain acids. The esterified fraction also contained the $C_{26:0}$, $C_{28:0}$ and $C_{30:0}$ acids. In the free acid fraction the $C_{12:1}$, $C_{13:0}$, $C_{14:1}$, $C_{15:1}$, $C_{17:0}$, $C_{19:0}$ and $C_{23:0}$ acids were found in minor amounts.

The quantities of esterified acid always exceeded those of the free acids by some ten to fifty times. The only published data with which this can be compared is that of Fernandes *et al.*[10] who recorded ratios of free to esterified acids in cold-stored Bramley Seedling apples from about 1:5 to 1:16. They however were only concerned with surface and occluded waxes. The acid composition given here is comparable with that of Edward VII[11] and Calville Blanc[7] apples. During the period for which results are available there were substantial increases in the free and esterified groups of fatty acids of both the "on" and "off" the tree series. Subsequently, analysis of apples "on" the tree showed a fall in the free and esterified groups, the change in free acids being most marked. The results for the individual acids show that these changes were accentuated in the $C_{18:1}$ (oleic) and $C_{18:2}$ (linoleic) acids.

DISCUSSION

In considering the changes that occurred in the fatty acid composition of the apple during the climacteric phase it must be remembered that the method used for extraction yielded lipid material from two different regions of the fruit. Lipids extracted from the cell contents and, possibly, from the lipid membrane within the cell can be considered to be available for further metabolism, but a large proportion of the extract came from the surface of the apple skin or from isolated fissures within the horny layer of cutin. The mechanism by which comparatively large quantities of fatty material migrate to the external surface of the apple, and the time sequence involved, are imperfectly understood but it can be assumed that when once extruded, the lipid deposit is no longer immediately available to the living cell. Aerial oxidation will, however, cause changes in its chemical composition and when suitably ventilated the more volatile compounds will be lost by evaporation. We might therefore expect that the less stable and more volatile unsaturated acids in the surface layer would be lost at a greater rate than the stable saturated ones, partly by oxidative decomposition and partly by evaporation. Upon these changes is superimposed the varying activities of the enzymes responsible for the synthesis and degradation of the fatty acids of the epidermal cells. In the following discussion the fatty acids present in minor amounts, including the branched chain and C_{26} to C_{30} acids, are not taken into account, except in so far as they contribute to the totals given in the tables.

In fruits that remain attached to the tree two main phases of fatty acid metabolism are apparent. In the first phase (120–150 days from petal fall) there is a substantial build up of fatty esters and free acids. Though considered to follow different chemical routes (and for the apple Mazliak[8] has provided evidence of this in work with radioactive tracers), the synthesis of saturated and unsaturated compounds appear to remain in step with each other. Since the concentration of free acids rises steeply, it appears that esterification does not keep pace with their formation. If this synthetic phase involves acetate derived from carbohydrate it would help to account for the high R.Q. values observed in fruit passing through the climacteric.[12] From the 150th day after petal fall the transition to the second phase begins,

[10] A. M. S. FERNANDES, E. A. BAKER and J. T. MARTIN, *Ann. Appl. Biol.* 53, 43 (1964).
[11] D. F. MEIGH, *J. Sci. Food Agr.* 15, 436 (1964).
[12] A. C. HULME, *Advances in Horticultural Science and their Applications* (Edited by J. C. GARNAUD), Vol. 1, p. 77, Pergamon Press, Oxford (1961).

in which breakdown of the fatty acids proceeds almost as rapidly as their previous synthesis. Initially the fall in free acids may be due to continued esterification but subsequently a loss of esterified acids is also evident. At this stage several problems present themselves. To what extent is lipase available for hydrolysis of the fatty esters? Is hydrolysis a necessary preliminary to their breakdown and which pathways can be considered mainly responsible for it? Lipoxidase activity has reached a high level and can be assumed to account for the rapid decrease in linoleic and linolenic acids, but to what extent is α- or β-oxidation occurring, and are the lost acids completely converted to carbon dioxide or is there a net production of sugars or organic acids? The present results do not answer these questions.

Although sampling from the "off" the tree series was continued to a point at which respiration rate was declining, the free fatty acid content continued to rise in a manner similar to that of the "on" the tree fruits, while the accumulation of esterified acids was only tending to level off. The $C_{18:3}$ (linolenic) acid is an interesting exception in that it appears to reach a peak just as the climacteric rise in respiration begins. The esterified saturated acids reached a maximum in the sample taken 16 days after picking, while the esterified unsaturated acids levelled off at this point. Since these two esterified fractions constitute the major part of the fatty acid group it follows that, as a whole, the group passes through a maximum at about the peak of respiratory activity. This corresponds with Mazliak's finding[8] that the total wax content increased and decreased in step with the changing respiration rate over the climacteric.

Interest lies not only in changes in fatty acids as such but in the fact, shown by Dalgorno and Birt,[13] that free fatty acids may interfere with mitochondrial activity in the cell by uncoupling oxidation and phosphorylation. It has been shown[14,15] that tight coupling of oxidation and phosphorylation appears to be maintained by the mitochondria at least up to the climacteric peak in apples. At first sight, therefore, the overall increase shown here in free fatty acids in the tissue is not having the "expected" effect. Nevertheless, until attempts have been made to differentiate between the lipid reserves within the cell and the lipid material extruded on the surface of the skin, it will not be possible to say with any certainty how the lipid metabolism within the cell is related to other processes taking place over the respiration climacteric.

EXPERIMENTAL

Fruit Used

This was taken from 29 Cox's Orange Pippin trees on Malling IX rootstocks. Petal fall (the date at which approximately 90 per cent of the flowers had shed their petals) was 21 May 1964.

Measurement of the Respiration of Whole Fruits

For the detailed study throughout the climacteric in detached fruits ("off" the tree fruit) in store at 12°, the "individual fruit method" described in detail by Hulme *et al.*[9] was used. By this method a fruit can be selected for analysis on the basis of its actual respiration rate during the climacteric rise. This is important since the whole period from minimum to maximum rate of respiration is, "off" the tree, only 20 days at 12°. The respiration rate for

[13] L. DALGORNO and L. M. BIRT, *Biochem. J.* **87**, 586 (1963).
[14] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *New Phytologist*. In press.
[15] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *New Phytologist*. In press.

fruit developing the climacteric attached to the tree ("on" the tree fruit) was measured on a bulk sample of 20 fruits by the method described by Hulme.[16] The first picking for the "on" the tree fruit was 17 September 1964. On this date also the fruits for the "off" the tree series were picked and placed in their individual containers, at 12°, for following the respiration climacteric and lipid and lipoxidase changes.

Lipoxidase Activity

This was determined by the method of Surrey,[17] with minor modifications. From the mitochondrial preparation as described by Hulme *et al.*,[9] the polyethylene glycol precipitate (PEG 2) from the supernatant fraction was used. This was suspended in 0·25 M sucrose with a Kontes Duall Homogenizer and 0·5 ml of this suspension was used for the determination. The reaction was carried out at pH 6 which was found to be the optimum. The mitochondrial fraction had no lipoxidase activity. Optical densities were measured on a Cary recording spectrophotometer Model 14. A unit of lipoxidase is defined as that activity which will produce a change in optical density of 0·01 in 1 min at a wavelength of 234 mμ, in a total volume of 10 ml of 60% ethanol solution.

Extraction of the Fatty Acids

The fatty acids of the apple peel were extracted by a method based on that of Folch *et al.*[18] Peel was removed from an apple using a stainless steel household peeler. A representative 5 g sample was immersed in chloroform-methanol mixture (100 ml, 2:1 v/v) contained in a 35 × 200 mm glass tube. In this the mixture was homogenized for 30 sec using an Ultra Turrax macerator (120 V setting). The cutters were then examined for entrained portions of unchanged peel, these loosened and the homogenization repeated. The product was filtered through glass paper (Whatman type GF/C), the residue washed with chloroform (10 ml) and the occluded liquid squeezed through. The combined chloroform-methanol extract was then transferred by pipette to a 100 ml beaker immersed in a vessel containing 900 ml of water. After standing overnight the aqueous phase was withdrawn from the beaker by suction, using a fine glass tube, in order to isolate the chloroform extract. All the above operations were conducted at 1°.

To the chloroform extract was added methanol (30 ml) followed by methanolic potassium hydroxide (2 ml, 2 N). After mixing, the solution was extracted three times with 100 ml portions of water, 1 ml of 2 N potassium hydroxide solution being added to the second and third portions. The accumulated aqueous washings were extracted twice with petroleum ether (b.p. 40–60°, free of aromatic hydrocarbons) and acidified with dilute sulphuric acid. The liberated fatty acids were extracted with three portions of the same grade of petroleum ether and dried over anhydrous sodium sulphate. This formed the free acid fraction. The yield of free acids was also determined in a sample of the original extract by titration with standard alkali solution. Comparison with the product of alkaline aqueous extraction showed that no appreciable saponification of esterified acids occurs during the extraction.

The chloroform solution was combined with the petroleum ether washings from the alkaline aqueous solution, evaporated to dryness at low temperature under vacuum and saponified for 3 hours in methanolic potassium hydroxide solution under reflux. After extracting the unsaponifiable material with three portions of the same grade of petroleum

[16] A. C. HULME, *Dep. Sci. Ind. Research Food Invest. Bd. Rep.*, 1937, p. 133, H.M.S.O., London (1938).
[17] K. SURREY, *Plant Physiol.* **39**, 65 (1964).
[18] J. FOLCH, I. ASCOLI, M. LEES, J. A. MEATH and F. N. LE BARON, *J. Biol. Chem.* **191**, 833 (1951).

ether, the solution was acidified with dilute sulphuric acid and the liberated fatty acids extracted with petroleum ether as before. This formed the esterified acid fraction.

Separation and Estimation of the Fatty Acids

An aliquot of each sample was esterified with freshly distilled diazomethane in ethereal solution at 0° to obtain the methyl esters. These were then analysed with a gas chromatograph constructed in the laboratory[11] and fitted with a glass column (184 cm long, 4 mm bore) containing 60–85 mesh Celite coated with diethylene glycol succinate (100:15 w/w). Solutions of the esters in carbon disulphide were applied to the column through the heated injection port with a 10 μl Hamilton microsyringe. A chromatogram similar to that of the fatty acids of Edward VII apple skin[11] was obtained. The identity of the acids was checked with a graph of log retention times against carbon chain length of the acids and comparison with further separation after hydrogenation of the sample with Adams platinum oxide catalyst in ethanolic solution.[19] For quantitative estimation, standard solutions of authentic methyl esters of the C_{12}, C_{14}, C_{16} and C_{18} normal fatty acids were run under the same conditions. In view of the characteristics of the flame ionization detector unsaturated compounds were assumed to give the same response as saturated ones. Recorded peak areas were estimated by triangulation.

Acknowledgements—Lipoxidase activities were determined by Mr. L. S. C. Wooltorton. Mr. A. A. E. Filmer carried out the analysis of fatty acids.

[19] S. Siggia, *Quantitative Organic Analysis via Functional Groups*, (2nd Ed.), p. 74, Chapman & Hall, London (1954).