

VOLATILE PRODUCTS OF APPLES

I. IDENTIFICATION OF ACIDS AND ALCOHOLS

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Summary

The volatile substances given off to the air by Granny Smith apples at 20°C. were condensed at low temperature. The acids obtained on saponification were found to be virtually free from carbonyl, hydroxy, or unsaturated groups. By conversion to hydroxamic acids followed by chromatographic separation on paper they were identified as formic, acetic, propionic, butyric (probably normal), valeric, and caproic acids. All these acids were shown to be present in the esterified form. Formic and acetic acids were shown to occur in both the free and esterified forms. All these acids do not necessarily occur in every sample of volatiles. For instance, formic acid has been found in some samples but not in others.

The alcohols obtained on saponification were found to be predominantly primary and saturated and the major constituents were found to be methanol, ethanol, and *n*-propanol. Ethanol and *n*-propanol were identified by paper chromatography after conversion to hydroxamic acids and methanol by a specific colour test.

I. INTRODUCTION

There is evidence that the volatile substances given off to the atmosphere by apples are responsible for superficial scald, a functional disorder of cold-stored fruit (Brooks, Cooley, and Fisher 1919). A systematic study of these volatile substances is being made and this paper describes methods for the identification of the acids and alcohols present in free and esterified forms.

Power and Chesnut (1920) identified methyl, ethyl, and amyl esters of formic, acetic, caproic, and caprylic acids in the steam distillate of the parings of certain apple varieties.

White (1950) found esters of formic, acetic, propionic, butyric, and caproic acids in a concentrate of volatiles distilled from apple juice. Methanol, ethanol, 2-propanol, and butanol were identified as the alcoholic components of the esters. A high proportion of the volatile concentrate was composed of free alcohols which were identified as methanol, ethanol, *n*-propanol, 2-propanol, butanol, *isobutanol*, *d*-2-methyl-1-butanol, and hexyl alcohol. The carbonyl compounds comprised acetaldehyde, acetone, caproaldehyde, and 2-hexenal.

Of the substances given off to the atmosphere by fresh apples at ordinary temperatures, carbon dioxide, ethylene (Gane 1935), and acetaldehyde (Power and Chesnut 1920) have been identified. Walls (1942) has given evidence for the presence of esters of amyl alcohol and of formic and acetic acids among

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the volatile substances from whole Lane's Prince Albert apples after absorption on granular calcium chloride.

In the present work the volatile substances given off to the air by Granny Smith apples at 20°C. were collected by condensation at low temperatures and the constituent acids, free and esterified, and alcohols, free and esterified, were identified.

II. FREE AND ESTERIFIED ACIDS

(a) Collection and Preparation of Sample

Air, purified by combustion of organic matter and absorption of carbon dioxide, was passed over 10 kg. of Granny Smith apples at 20°C. The volatiles were condensed for two to five days in a spiral absorber cooled in liquid oxygen. The esters were hydrolysed by addition of 0.2N sodium hydroxide. The alcohols were removed by distillation from the alkaline solution, which was then acidified and the acids distilled. A preliminary test indicated that acids up to decanoic would be recovered in this procedure. The acid distillate was brought to pH 8 with sodium hydroxide and evaporated to dryness. The sodium salts of all the acids, both free and esterified, were thus obtained.

Tests were first made to determine whether these acids were predominantly simple saturated fatty acids or whether they contained carbonyl, hydroxy, or unsaturated groupings.

(b) Test for Carbonyl Group

The sensitive method of Friedman and Haugen (1943) for the estimation of carbonyl compounds was used. This method measures the colour produced by reaction with 2,4-dinitrophenylhydrazine in acid solution followed by the addition of sodium hydroxide. The colorimeter readings were converted to equivalents of carbonyl compounds, using pyruvic acid as standard. The mean molecular weight of the mixed sodium salts was assumed to be equal to that of sodium butyrate. This assumption was justified by the identification of the individual acids. The tests indicated not more than one carbonyl group in 1400 moles of mixed acids.

(c) Test for Hydroxyl Group

The hydroxy content of the mixed sodium salts was estimated by acetylation. The mixed sodium salts (20 mg.) and acetyl chloride (15 drops) were allowed to stand for 15 minutes. Water (approximately 2 ml.) was added and allowed to stand for 30 minutes to decompose the excess acetyl chloride. The acetyl derivatives were separated by extraction with ether. The ether extract was treated with hydroxylamine in alkaline solution to give acethydroxamic acid, which was estimated colorimetrically (Thompson 1950). Lactic acid was used to standardize the method. The test indicated not more than one hydroxy group in 165 moles of mixed acids. The hydroxy content may be appreciably less, as small amounts of anhydrides may resist hydrolysis and be included in the determination.

(d) Test for Unsaturation

With the small amount of material available, the only satisfactory method of determining unsaturation was found to be hydrogenation with a palladium catalyst in a Warburg flask with manometric estimation of the hydrogen absorbed (Milton and Waters 1949). The figure obtained indicated a ratio of one mole of hydrogen (corresponding to one double bond) to 385 moles of mixed acids.

(e) Identification as Hydroxamic Acids

The sample of sodium salts was converted to a mixture of hydroxamic acids in the following manner:

Thionyl chloride (0.2 ml.) was added to the dry sodium salts (10 mg.) and allowed to stand for 15 minutes. Absolute ethyl alcohol (5 ml.) was added and allowed to stand for a further 30 minutes. Water was added and, after allowing several minutes for the decomposition of the thionyl chloride, the esters were extracted from the solution with ether (30 ml.). The ether solution was dried over calcium chloride and neutralized with alcoholic sodium hydroxide. The hydroxamic acids were formed by reaction with hydroxylamine as described in an earlier paper (Thompson 1950). The efficiency of conversion of carboxylic to hydroxamic acid was found to be approximately 70 per cent. with *n*-butyric acid.

The chromatographic separation of the hydroxamic acids was carried out as described previously (Thompson 1951). By comparing the R_F values with those of known hydroxamic acids run on the same paper the acids derived from apples were identified (Plate 1 and Plate 2, Fig. 1). The acids are generally described by the older names which only indicate number of carbon atoms, as in most cases it was impossible to distinguish between isomers.

The relative proportions of the acids varied somewhat with different samples. A sample examined in 1949 was shown to contain acetic, propionic, butyric, valeric, and caproic acids (Plate 1*d*). The chromatogram was obtained with benzene-acetic acid. This test did not prove the absence of formic acid, as in 1949 a solution of ferric perchlorate containing excess perchloric acid was used to develop the chromatograms. Under these conditions formhydroxamic acid is unstable. However, by means of a separate colorimetric test based on reduction to formaldehyde (Grant 1948) and reaction with chromotropic acid (MacFadyen 1945), it was shown that the proportion of formic acid was negligible.

A sample from less mature apples examined in 1950 was found to contain formic and acetic as predominant acids (Plate 2, Fig. 1*b*). A small amount of propionic acid and only traces of higher acids were present. Butanol-acetic acid was used for this test. The spots were developed with ferric chloride to which formhydroxamic acid is quite stable. The chromotropic acid test for formic acid was strongly positive.

III. ESTERIFIED ACIDS

For identification of esterified acids the volatile substances from apples were more conveniently condensed in ether (30 ml.) in a spiral absorber cooled in a mixture of solid carbon dioxide and ethanol. The efficiency of collection of esters in this absorber was about 95 per cent. The ether layer was separated from the water layer and dried. The esters were converted directly to hydroxamic acids by reaction with hydroxylamine (Thompson 1950), and identified by paper chromatography as already described.

For each sample of apples examined all the acids obtained on hydrolysis were represented in the esters. The esterified acids from the 1949 sample of apples were found to include acetic (trace), propionic, butyric (major constituent), valeric (trace), and caproic acids. This is shown in Plate 3, Figure 1 (caprylic alcohol-oxalic acid) and Plate 1*b* (benzene-acetic acid). In Plate 3, Figure 2 (benzene-acetic acid), the absence of higher acids is demonstrated. Storage of these apples at 0°C. for various periods up to 30 weeks made no significant difference to the composition of the esterified acids.

TABLE 1
R_F VALUES OF BUTYRO-HYDROXAMIC ACIDS FROM ESTERS

Paper	Test Solutions		
	Normal Butyrate	"Apple" Butyrate	isoButyrate
1	0.127	0.125	0.133
	0.148	0.143	0.148
	0.143	0.142	0.160
	0.134	0.140	0.168
2	0.136	0.152	0.162
	0.150	0.151	0.148
	0.137	0.134	0.145
	0.104	0.117	0.136
3	0.112	0.120	0.135
	0.122	0.132	0.132
	0.127	0.125	0.158
	0.125	0.125	0.147

From the less mature apples examined in 1950 only formic acid and acetic acid were identified amongst the esterified acids. Butanol-acetic acid was used as solvent mixture (Plate 2, Fig. 1*a*).

An attempt was made to determine whether the butyrate present was normal or *isobutyrate*. It was possible, by paper chromatography with benzene and acetic acid, to effect a small separation of normal and *isobutyro*-hydroxamic acids. By statistical treatment of the R_F values (Table 1) of the two isomers and of the butyrohoxamic acid derived from apples, it was

shown that the probability of the "apple" butyrate being *isobutyrate* is less than 0.001, whereas the probability of it being normal butyrate is greater than 0.2 (Table 2).

TABLE 2
ANALYSIS OF VARIANCE OF DATA IN TABLE 1

Source of Variation		D.F.	Mean Squares	Ratio
Between solutions (<i>n</i> -butyrate, "apple" butyrate and <i>iso</i> -butyrate)	(S)	2	0.000950	16.10***
Between papers	(P)	2	0.000550	9.32**
S × P		4	0.000024	N.S.
Between replicates within papers	(R)	9	0.000337	5.71***
R × S		18	0.000059	
Total		35		
"Apple" butyrate v. <i>isobutyrate</i>		1	0.001080	18.31***
<i>n</i> -Butyrate v. (mean of "apple" and <i>isobutyrate</i>)		1	0.000820	13.90**
"Apple" butyrate v. <i>n</i> -butyrate		1	0.000070	1.19 (N.S.)
<i>iso</i> Butyrate v. (mean of "apple" butyrate and <i>n</i> -butyrate)		1	0.001830	31.02***

N.S. = not significant; * = significant at 5 per cent. level; ** = significant at 1 per cent. level; *** = significant at 0.1 per cent. level.

IV. FREE ACIDS

The volatile substances from apples were collected in ether cooled in a solid carbon dioxide-alcohol bath. The ether solution, with a similar volume of water, was neutralized with 0.1N sodium hydroxide using phenolphthalein as indicator and shaking vigorously throughout. The aqueous layer was separated and evaporated to dryness. The sodium salts of the free apple acids were converted to hydroxamic acids as described in Section II and separated by paper chromatography. Only the free acids from the less mature apples in 1950 were examined by this procedure. Using phenol or butanol-acetic acid (Plate 2, Fig. 2), formic and acetic acids were identified but no higher acids. Formic acid was also identified by the chromotropic acid test.

It had previously been shown that the esterified acids from these apples were mainly formic and acetic. It appears, therefore, that formic and acetic acids, when present, occur free as well as esterified. The higher acids predominated in the esterified acids from the more mature apples in 1949, but unfortunately no attempt was made to identify the free acids from these apples. Hence there is no evidence that the higher acids occur free but the possibility is not excluded.

V. FREE AND ESTERIFIED ALCOHOLS

(a) Preparation of Solution

The distillate from the alkaline solution of saponified volatiles collected in 1949 (see Section II (a)) was used for the identification of alcohols. A microhydrogenation test indicated negligible unsaturation, amounting to approximately one double bond in 600 moles. This calculation was based on the weight of sodium salts obtained on oxidation, assuming an average molecular weight equal to sodium acetate.

A colorimetric test (Friedman and Haugen 1943) showed the presence of trace amounts of carbonyl compounds. The distillate was therefore treated with the 2,4-dinitrophenylhydrazine solution and distilled under reduced pressure, with ice water in the condenser, to obtain a distillate free from carbonyl compounds.

(b) Test for Secondary Alcohols

An aliquot of the carbonyl-free distillate was oxidized with dilute chromic acid and again distilled. The distillate of oxidized products was free from carbonyl compounds, indicating the absence of secondary alcohols before oxidation.

(c) Identification as Hydroxamic Acids

The carbonyl-free solution of primary alcohols was oxidized with chromic acid and the acids distilled off. The distillate was brought to pH 8 with sodium hydroxide and evaporated to dryness. The sodium salts of the fatty acids were converted by hydroxamic acids by the method previously described (Section II (e)).

Hydroxamic acids containing two, three, and six carbon atoms (the last only in traces) were identified on the chromatogram (Plate 1c). The presence of ethanol and *n*-propanol and a trace of six-carbon alcohol in the original solution was thus established. The use of ferric perchlorate-perchloric acid solution as developer prevented the detection of formhydroxamic acid and hence methanol in the solution of alcohols.

However, the presence of methanol was shown by a specific test on a solution of apple alcohols. After oxidation with permanganate (Feigl 1943) the solution gave a positive test for formaldehyde with chromotropic acid. No formaldehyde was detected before oxidation.

VI. DISCUSSION

The author has found saturated fatty acids containing one, two, three, four, five, and six carbon atoms and saturated alcohols containing one, two, three, and six carbon atoms in the volatiles from fresh apples. It is probable that each acid and alcohol can occur both in the free and esterified form. All the acids have been found as esters, and formic and acetic acids have also been found free. The higher acids—free and esterified—were virtually absent from the only sample tested for free acids.

No other acids were found by White (1950) and Walls (1942), but an acid with eight carbon atoms was found by Power and Chesnut (1920). Walls (1942) and Power and Chesnut (1920) found amyl alcohol, and White (1950) found alcohols containing one, two, three, four, five, and six carbon atoms. Although White's work was based on the volatile fraction of apple juice, it is probable that every member of this series was present in the original fresh apples from which the juice was derived, as all except the four-carbon alcohols have been found either by Walls (1942) or the author in the volatiles from whole fresh apples.

The volatile fraction of apple juice analysed by White (1950) was found to contain 92 per cent. of free alcohols, 6 per cent. of carbonyl compounds, and only 2 per cent. of esters. The ratio of esters to free alcohols in the volatiles evolved by fresh apples would probably be higher since the esters may more readily pass through the lipoid phase of the cuticle and be preferentially evolved. Moreover, in preparation of the juice, part of the esters may be retained in the pressed residue, and there may also be subsequent loss of esters by hydrolysis.

The author's results, in conjunction with those of other workers, provide definite evidence that saturated acids and alcohols containing one, two, three, four, five, and six carbon atoms can occur in the volatiles from fresh apples and enter into a large variety of ester combinations. The relative proportions of the different components may vary from sample to sample, thus reflecting the differences in aroma and flavour associated with variety, maturity, and other factors.

The presence of homologous series of acids and alcohols with both odd and even numbers of carbon atoms is unusual among natural products. Products containing only even-numbered acids are more common, but recently Weitkamp, Smiljanic, and Rothman (1947) have demonstrated the presence of a complete series of odd-numbered fatty acids with seven to 17 carbon atoms, as well as even-numbered acids, in human hair fat.

Some comments may be made on the mechanism by which these substances are formed in the apple. Presumably each alcohol is interconvertible with the corresponding acid by oxidation and reduction. The current biochemical theories for the synthesis of fatty acids *in vivo* account only for the production of even-numbered straight chains. An important contribution to the mechanism of fatty acid synthesis has been made by the recent discovery of Stadtman and Barker (1949) that cell-free extracts of *Clostridium kluyveri* catalyse the linkage of ethanol and acetate to fatty acids of four and six carbon atoms. Both ethanol and acetate are closely related to the respiratory cycle. The linkage of odd-numbered acids or alcohols with two-carbon fragments has not been investigated but there remains the possibility of obtaining the higher odd-numbered acids by this means. A one-carbon fragment may be necessary for the initial condensation and this could be readily provided as methanol, which is always present in apple tissue as the methyl ester of pectic acid. The reduction of three-carbon intermediates of respiration to propionic acid is also possible. To obtain branched chains, linkage of fragments at other than end

groups would be necessary. This mechanism could not account for the formation of the secondary alcohol 2-propanol, which was found by White (1950) in the volatile concentrate from apple juice.

VII. ACKNOWLEDGMENTS

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APPENDIX I

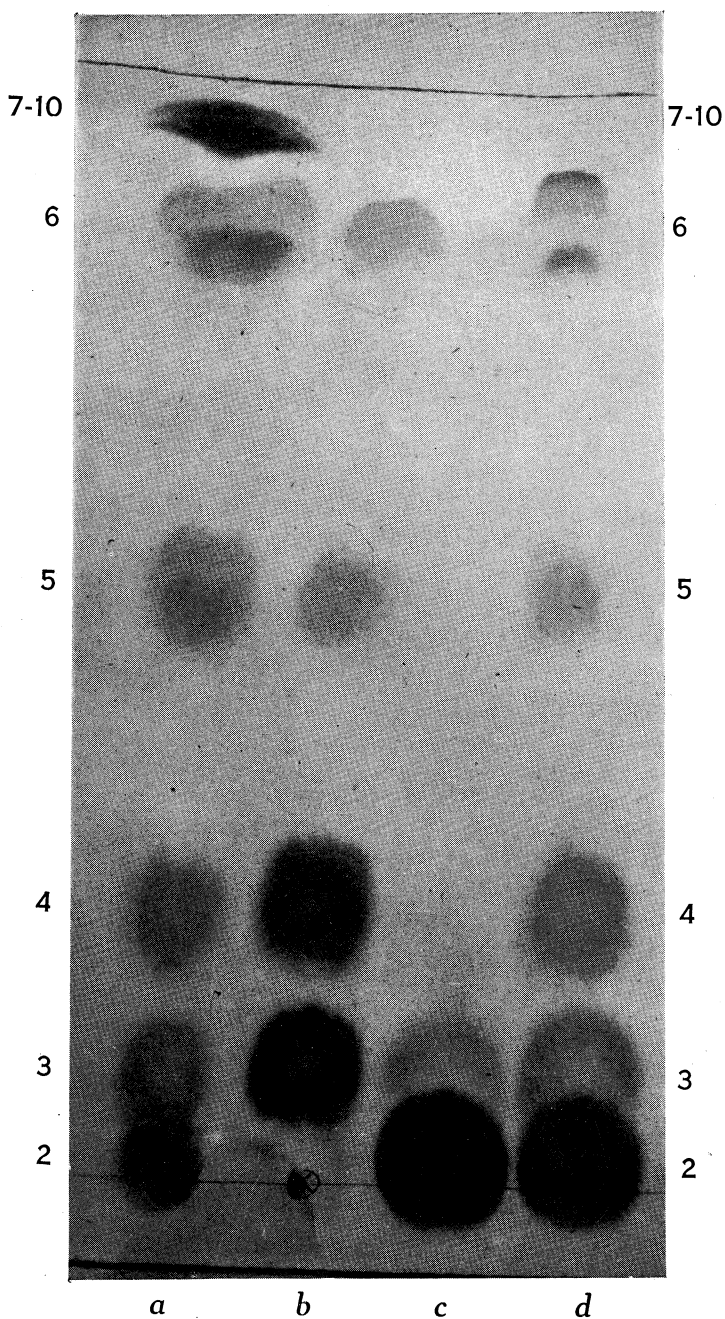
MASS SPECTROMETRIC ANALYSES

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In conjunction with the work reported in the above paper, a mass spectrometric examination of the apple volatile products was made, giving results in substantial agreement with those obtained above.

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VOLATILE PRODUCTS OF APPLES. I



Paper chromatogram run with benzene-acetic acid showing ferric hydroxamate spots derived from:

- (a) An artificial mixture of esters containing acids with one to ten carbon atoms;
- (b) Esterified acids from apples (1949);
- (c) Free and esterified alcohols from apples (1949);
- (d) Free and esterified acids from apples (1949). The appearance of two spots in the C6 position is due to "shadowing" (Thompson 1951).

VOLATILE PRODUCTS OF APPLES. I

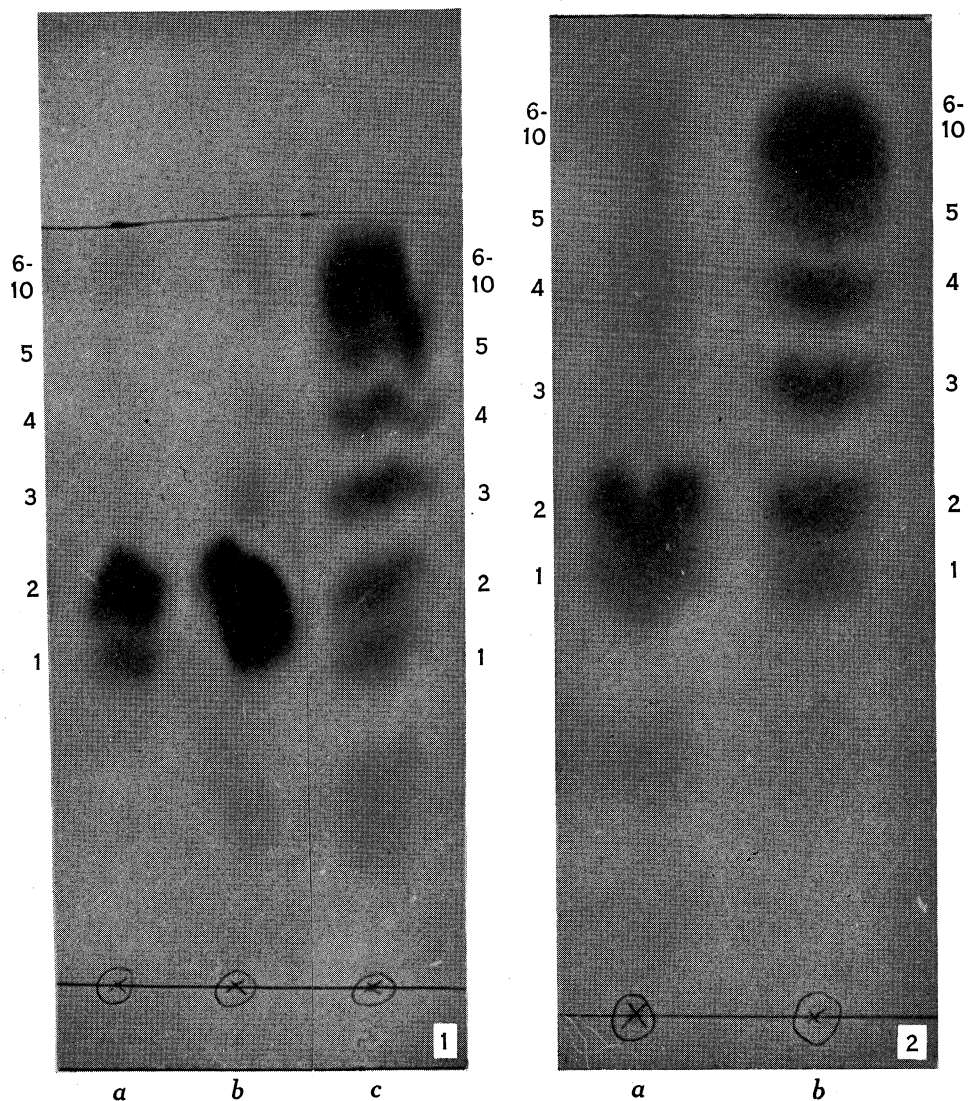
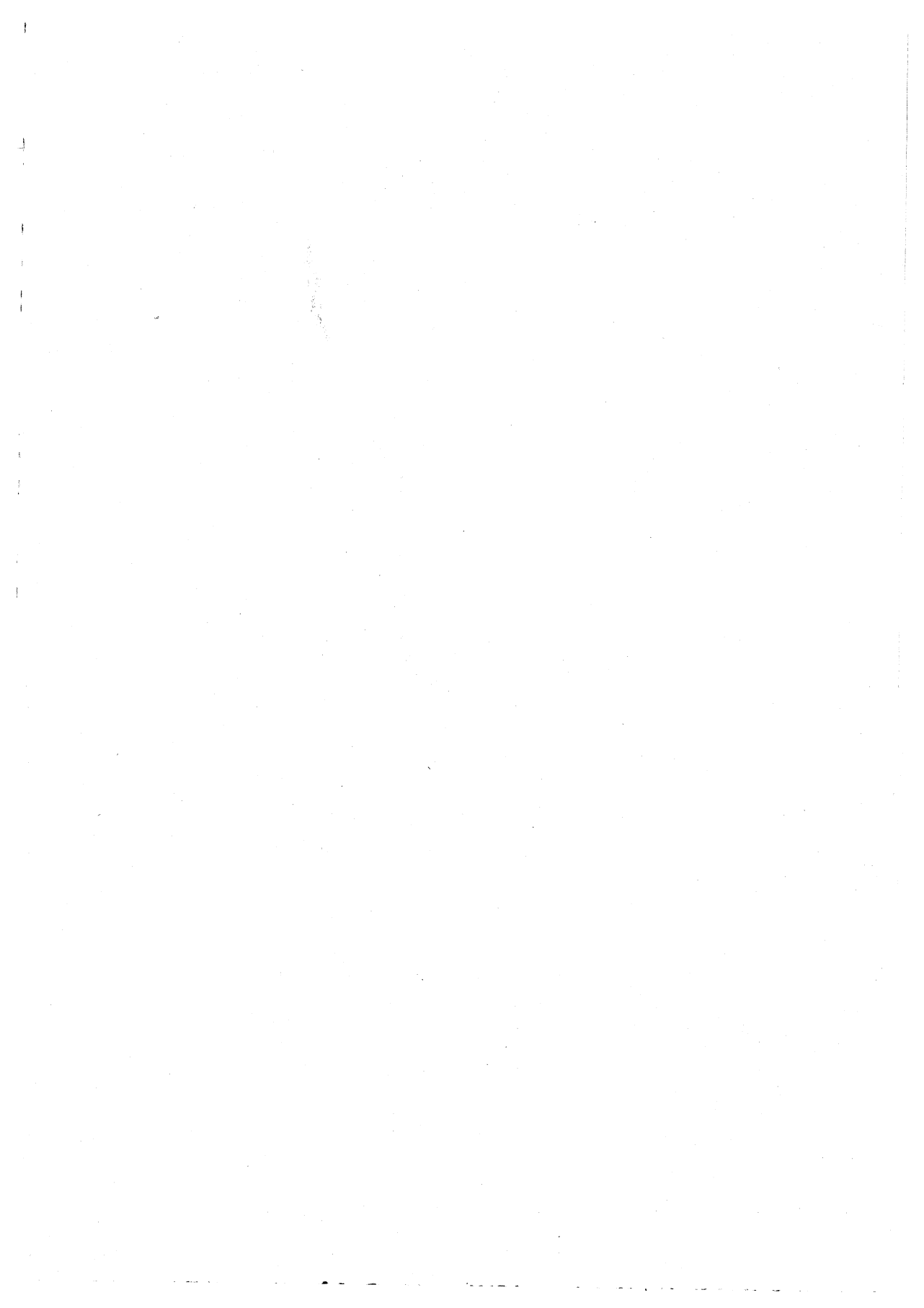


Fig. 1.—Paper chromatogram run with butanol-acetic acid showing ferric hydroxamate spots derived from:

- (a) Esterified acids from apples (1950);
- (b) Free and esterified acids from apples (1950);
- (c) An artificial mixture of esters containing acids with one to ten carbon atoms.

Fig. 2.—Paper chromatogram run with butanol-acetic acid showing ferric hydroxamate spots derived from:

- (a) Free acids from apples (1950);
- (b) An artificial mixture of esters containing acids with one to ten carbon atoms.



VOLATILE PRODUCTS OF APPLES. I.

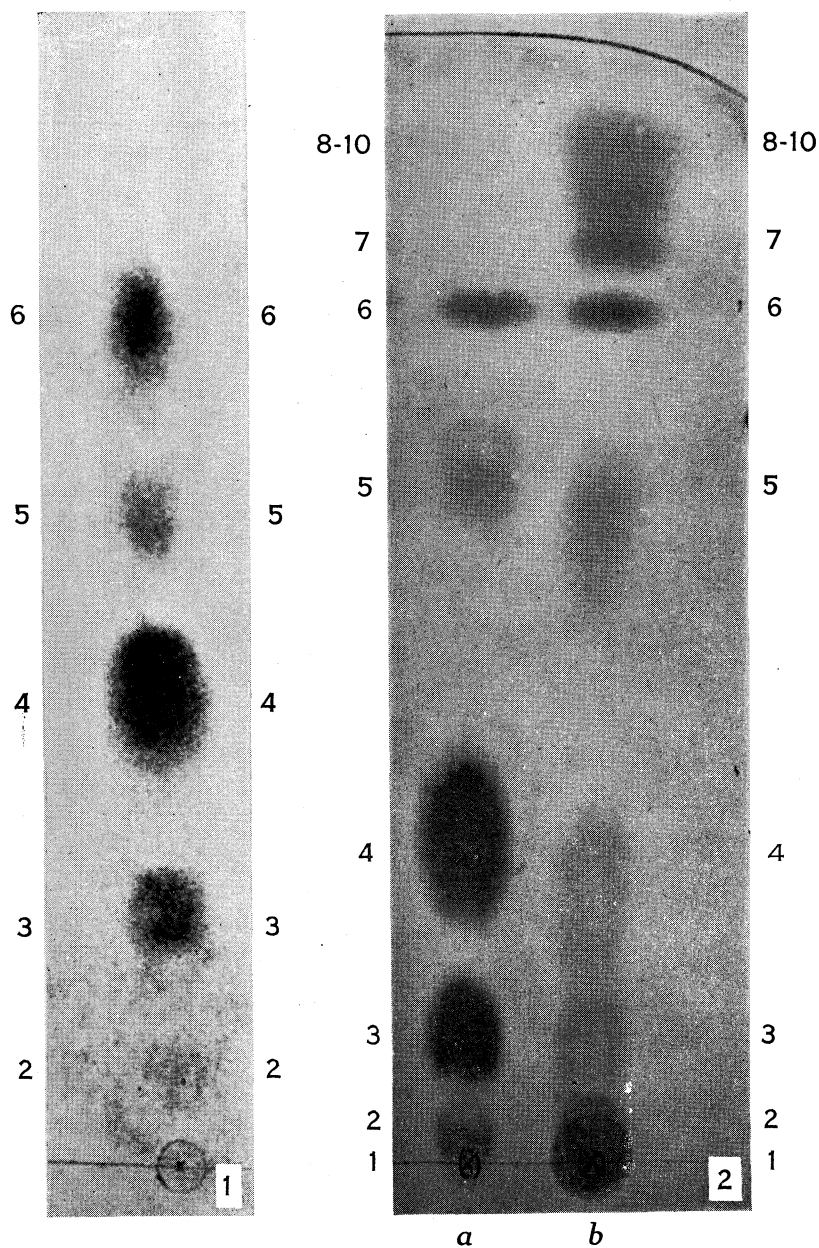


Fig. 1.—Paper chromatogram run with caprylic alcohol-oxalic acid, showing ferric hydroxamate spots derived from esterified acids from apples (1949).

Fig. 2.—Paper chromatogram run with benzene-acetic acid showing ferric hydroxamate spots derived from:

(a) Esterified acids from apples (1949);

(b) An artificial mixture of esters containing acids with one to ten carbon atoms.



The apple volatile products were collected as two samples, the less volatile (1) in an ice-calcium chloride trap, and the more volatile (2) in a liquid-air trap.

The mass spectra of these samples were measured, when it was found that the first was heavily contaminated with water, and the second with CO₂. It did not prove possible to separate the water by fractionation, but the CO₂ could be removed by this method.

The mass spectrum of the more volatile sample (2) after fractionation was complex, prominent peaks being observed at masses 74, 102, 116, 130, 136, 206. It was thought that the samples contained aliphatic esters, and examination of the spectra of a number of such esters with parent peaks at these values indicated that ethyl *n*- and *isobutyrate*, and ethyl *n*- and *isovalerate* were more probable than esters such as *n*-butyl acetate. This evidence was, however, not conclusive.

TABLE 3
COMPARISON OF MASS SPECTRA OF SAMPLES A AND B WITH THOSE OF
ALIPHATIC MONOCARBOXYLIC ACIDS

Mass	Ion Peak Heights					Sum of Mass Spectra of Pure Acids
	Sample	Acetic	Propionic	<i>n</i> -Butyric	<i>n</i> -Valeric	
Sample A						
43	249	49	110	265	27	451
45	357	45	180	133	14	372
60	466	29		375	47	451
73	205		120	106	16	242
74	205		198	6	4	208
87	20			6	1	7
88	17			16		16
Sample B						
43	674	692	37			729
45	588	625	61			686
60	422	422				422
73	43		40			40
74	66		66			66

At this stage the volatiles of type RCOOR' were hydrolysed to the corresponding alcohols R'OH and acids RCOOH, which were separated by distillation. The acids were converted to the sodium salts, sample A, and the alcohols oxidized with chromic acid to the corresponding acids, these also being converted to the sodium salts, sample B.

The free acids were obtained when desired for analysis by treatment of the Na salts in vacuo with vacuum-dried H₃PO₄, and the mass spectra of the acids in samples A and B measured.

The mass spectra of purified samples of the aliphatic acids acetic, propionic, *n*- and *isobutyric* and *n*- and *isovaleric* were obtained and compared with the spectra of samples A and B. (Table 3.) The *iso*- acids gave large peaks at mass numbers where there were none observed in the spectra of the acids from samples A and B, so it was assumed that only normal acids were present in these.

The agreement obtained between the sum of the patterns of the aliphatic acids and the patterns produced by the samples is not very good; this is probably due to the fact that the acids employed for obtaining the calibration spectra were not of high purity. Nevertheless, the results indicate that sample A probably contains acetic, propionic, *n*-butyric, and *n*-valeric acids, and that sample B contains acetic and propionic acids as major constituents.

The esters present in the original sample would therefore be ethyl and propyl acetate, propionate, *n*-butyrate, and *n*-valerate.