STUDIES IN THE NATURAL COATING OF APPLES

I. PREPARATION AND PROPERTIES OF FRACTIONS

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[*Manuscript received May* 10, 1951]

Summary

The preparation and properties of the major fractions of the natural coating of apples are described. These include the oil, wax, ursolic acid, and "cutin" fractions. Particular attention has been given to the oil fraction, which contains unsaturated esters, and the "cutin" fraction, which gives complex hydroxy acids on saponification. The use of ammonium oxalate for separating apple skin is described.

Methods are given for quantitative determination of the coating fractions. The distribution of these fractions in the cuticle and epidermis is discussed.

I. INTRODUCTION

The natural coating of apples is of considerable importance in the physiological behaviour of the fruit as it forms the major barrier to diffusion of water vapour and other gases. Respiration and resistance to diffusion cause the internal atmosphere of the fruit to have a lower concentration of oxygen and a higher concentration of carbon dioxide than the outside air. Trout *et al.* (1942) have shown that most of the resistance to gaseous diffusion is in the skin.

Sando (1923) separated the apple skin mechanically from the flesh after soaking in dilute hydrochloric acid. He extracted the skin with light petroleum and obtained a wax, which was purified by removing coloured impurities with 80 per cent. acetone. He found a saturated hydrocarbon and a saturated secondary alcohol as major constituents of the wax. Subsequently Chibnall *et al.* (1931) identified nonacosane, heptacosane, *d*-10-nonacosanol, *n*-hexacosanol, *n*-octacosanol, and *n*-triacontanol. Most of the alcohol fraction was present in the free form. The presence of nonacosane and 10-nonacosanol was confirmed by Markley, Hendricks, and Sando (1932).

Sando (1923) extracted apple skin with ether (after previous extraction with light petroleum) and obtained a further fraction. The crude material was crystallized from alcohol and gave a melting point of 280°-282°. Subsequently Sando (1931) obtained a more highly purified product, melting at 285°, which he named ursolic acid and showed to be identical with preparations from other sources. Later workers have obtained melting points varying from 284° to 291°.

Gane (1931) extracted coating material from whole apples by immersion in ether. He separated the fractions by crystallization and distillation and

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obtained an oil in addition to the wax and ursolic acid. The oil was shown by its saponification and iodine numbers to be mainly unsaturated esters.

After extraction with solvents the residual skin is still water-repellent and retains lipoid stains. The material responsible for these properties is known as "cutin." Markley and Sando (1933) determined "cutin" by an empirical procedure. After preliminary extraction with dilute alkali and acid, the residual skin was boiled for three hours with 3 per cent. alcoholic potassium hydroxide. The loss of weight in the last treatment was taken as "cutin" originally present.

Markley and Sando (1931) followed the changes in the natural coating during maturation on the tree and subsequent storage at 0°C. The "total ether extract" (containing oil, wax, and ursolic acid) was first weighed and the ursolic acid was then determined by titration. The "oily fraction" was calculated by subtracting ursolic acid from "total ether extract." They obtained increases in the surface concentration of both fractions during maturation and storage. Subsequently Markley and Sando (1933) found that the concentration of "cutin" increased with maturity.

The present authors have made further studies of the fractions of apple skin. Particular attention was given to the oil fraction, which had been largely neglected, probably on account of its low concentration in freshly picked apples. Changes during storage were investigated, and the various fractions were usually determined by direct separation and weighing. The work was mainly concerned with the Granny Smith variety, and all results are for this variety unless the contrary is stated.

II. Extraction and Preparation of Fractions

The method of soaking tissue in dilute hydrochloric acid, as used by Sando (1923), has not been found very effective for separating the skin. Ammonium oxalate solution (1-2 per cent.) has been found more effective, and is less likely to alter the constituents. The ammonium oxalate solution probably dissolves a pectic layer between the epidermal and underlying cells. A solution (pH 4) containing 1.6 per cent. ammonium oxalate and 0.4 per cent. oxalic acid separated the skin of apple peelings in 48 hr. at 37°C. and 16 hr. at 50°C. The solution containing free acid gave generally better results than ammonium oxalate alone unless the apples themselves were fairly high in acidity. The separated skin was composed of cuticle and epidermis. It was washed with water and then dried in air at room temperature.

Extraction of the skin with light petroleum (50°-70°C.) in a Soxhlet apparatus removed both oil and wax fractions. The extract was evaporated and the residue dissolved in boiling acetone (about 30 ml. per g. of material) to give a somewhat turbid solution. The solution was cooled to 0°C. with stirring, when the wax separated out. After standing overnight at 0°C., the wax was filtered off and washed with a small volume of cold acetone. The wax was re-dissolved and separated from acetone twice. The combined acetone filtrates

were concentrated to a small volume and cooled to 0°C., when a small quantity of additional wax separated. The oil fraction was obtained on evaporating the final filtrate.

Subsequent extraction of the skin with ether gave the ursolic acid fraction as a light powder. Further extraction with ethanol gave only a small quantity of soluble material. The ethanol-insoluble residue was found to yield a high proportion (about 50 per cent.) of ether-soluble acids after boiling with 0.5N ethanolic potassium hydroxide for 2 hr. After saponification the ethanol solution was filtered through a sintered glass filter and the residue washed with boiling ethanol. The filtrate was evaporated to a small volume and diluted with water. A small quantity of neutral material was extracted with ether. The aqueous solution was then acidified, and the precipitated acids extracted with ether. The yield of acids obtained by this procedure can be regarded as a measure of "cutin" content, and is probably preferable to the weight loss method of Markley and Sando (1933).

The oil and wax were obtained more readily, and with less loss of oil, by extracting whole apples with boiling light petroleum for 15 min. The solvent was boiled in the bottom of a cylindrical vessel, and the apples were placed on a platform above the boiling solvent. The condensed solvent flowed back over the apples and removed the oil and wax. Successive batches of apples could be extracted with the same charge of solvent, and 1 l. of light petroleum was found adequate for 18 kg. of apples. The extract was dried with anhydrous sodium sulphate or calcium chloride and filtered before evaporation of solvent.

The combined oil, wax, and ursolic acid fractions can be obtained by extracting whole apples with boiling carbon tetrachloride for 30 min. The wax and oil can then be separated from the ursolic acid fraction with light petroleum. This method, however, has not proved very satisfactory. The ursolic acid fraction is only sparingly soluble in carbon tetrachloride. The crude extract is difficult to dry and contains a little extraneous material from the flesh, which can be removed only by re-extraction.

It is estimated that about half the total lipoid or light petroleum-soluble material of the apple is in the skin. The lipoid material in the flesh was extracted with ethanol, and the ethanol extract was evaporated and re-extracted with light petroleum. A typical figure for the concentration of lipoid in the flesh is 0.06 per cent., hence an apple weighing 120 g. would contain about 72 mg. of flesh lipoid. The concentration of lipoid in the skin is about 0.5 mg. per sq. cm., which corresponds to 70 mg. per apple (of weight 120 g. and surface area 139 sq. cm.).

The insoluble "cutin" material, which gives ether-soluble acids on saponification, is almost entirely confined to the skin. The disintegrated flesh was extracted with ethanol until all the soluble material was removed. The insoluble residue gave a negligible yield of ether-soluble acids on saponification.

III. Properties of Fractions

(*a*) *Oil*

The oil is readily soluble in ethanol, acetone, ether, chloroform, carbon tetrachloride, and light petroleum. The characteristics of oil from Granny Smith apples are approximately as follows: saponification no. 110-150, acid no. 10-30, hydroxyl no. 50-70, iodine no. (Wijs) 80-140. The characteristics of oil from Jonathan and Sturmer apples come within the same range.

Saponification of the oil gave 70-85 per cent. of fatty acids, 15-30 per cent. of unsaponifiable matter, and a negligible yield of glycerol. It was found that undesirable changes could be minimized by carrying out the saponification and subsequent treatment entirely at room temperature. The oil was dissolved in 2N ethanolic potassium hydroxide and allowed to stand for 16 hr. at 20°C. in an atmosphere of nitrogen. After diluting with four volumes of water, the unsaponifiable portion was extracted with ether. The aqueous layer was then acidified and the fatty acids extracted with ether.

A sample of fatty acids was found to have the following characteristics: acid no. 173, hydroxyl no. 122, iodine no. (Wijs) 109. The hydroxyl number indicated the presence of hydroxy acids, but these did not amount to more than one-quarter of the total acids. About 75 per cent. of the acid mixture was dissolved by boiling light petroleum (30 ml. per g.), and the soluble portion had a very low hydroxy content. The insoluble somewhat tarry residue may contain products of oxidation.

The corresponding sample of unsaponifiable matter had a hydroxyl no. of 148 and an iodine no. (Wijs) of 86. Both the acid and unsaponifiable portions were highly unsaturated.

(*b*) *Wax*

The chemical composition of the wax has already been studied thoroughly by Chibnall *et al.* (1931). Samples of wax from Granny Smith apples gave the following characteristics: saponification no. 20-40, acid no. 5-7, hydroxyl no. 33, iodine no. (Wijs) 10-15. Wax from Jonathan and Sturmer apples did not differ significantly. A sample of wax from Granny Smith apples gave 10 per cent. of acids on saponification.

(*c*) *Ursolic Acid Fraction*

The ursolic acid was recrystallized from 99 per cent. ethanol and dried at 120°C. *in vacuo* for 20 hr. The mono- and di-acetyl derivatives were prepared.

Ursolic acid.—m.p. 286-8°C. (284-291°C. in literature).
Found: C, 78.9; H, 10.6.
Calculated for $C_{30}H_{48}O_3$: C, 78.9; H, 10.5.
Monoacetyl derivative.—m.p. 287-8°C. (285-296°C. in literature).
Found: C, 77.3; H, 9.8.
Calculated for $C_{32}H_{50}O_4$: C, 77.1; H, 10.0.
Diacetyl derivative.—m.p. 197-9°C. (198-200°C. in literature).
Found: C, 75.9; H, 9.5.
Calculated for $C_{34}H_{52}O_5$: C, 75.6; H, 9.6.

The presence of ursolic acid as major constituent was confirmed for Granny Smith apples.

Other constituents are present in the crude ursolic acid fraction. Acid numbers from 85 to 105 were found for the crude fraction compared with 120 for the recrystallized ursolic acid. Saponification numbers for the crude fraction varied from 120 to 150, indicating some esterification. A small amount of waxy material, soluble in light petroleum, was obtained on saponification.

(*d*) *"Cutin"*

The acids obtained by saponifying the "cutin" fraction gave the following characteristics: acid no. 150-180, hydroxyl no. 208, iodine no. (Wijs) 50. Boiling light petroleum (30 ml. per g.) dissolved only 7 per cent. of the acid mixture. Complex hydroxy acids appear to be the major constituents.

IV. Determination of Fractions

Each fraction of the apple coating was determined quantitatively by separation and weighing. The methods of separation already described are applicable to quantitative determination with certain exceptions. Separated skin is not suitable for quantitative determination of oil, as appreciable losses may occur during soaking and separation. In one test 24 per cent. less oil was obtained from separated skin than from extraction of whole apples. The wax and ursolic acid fractions can be determined in separated skin, if the apples have not been previously extracted. But if the whole apples are first extracted with light petroleum, about 13 per cent. less of the ursolic acid fraction is obtained from the separated skin. Apparently removal of the oil and wax facilitates mechanical loss of the ursolic acid fraction.

In studying changes during storage, the following methods were used for determination of coating fractions. For determination of oil, whole apples were extracted with boiling light petroleum (50°-70°C.) for 30 min. The oil and wax were separated with acetone as previously described. Reasonably accurate results were obtained by washing the wax from the first separation with a little cold acetone and avoiding the tedious re-precipitation. For determination of wax, extracts of both whole apples and separated skin were used.

The ursolic acid fraction was determined most readily by extraction of separated skin with ether after previous extraction with light petroleum. It was also extracted from whole apples with boiling carbon tetrachloride for 30 min., separated from oil and wax, and purified by re-extraction.

The "cutin" fraction was determined in the insoluble skin that had been previously used for determination of other fractions and finally extracted with ethanol to remove all soluble material. The insoluble skin was boiled with 0.5N ethanolic potassium hydroxide for 2 hr., and the acids were separated as previously described.

The results were expressed as mg. of material per sq. cm. of apple surface. To determine the surface area of a sample, the mean weight per apple

was determined separately for each size. The specific gravity of a representative sample was determined and used for calculating the volume. The surface area of a sphere of equal volume was calculated, and this area was multiplied by 1.028 to obtain the surface area of the apple. The correction factor was obtained from measurements by Mr. E. W. Hicks.

"Total fatty acids," which give a significant correlation with oil content, were determined in some cases. They were titrated after saponification of the total light petroleum extract (mixed oil and wax). A sample of about 20 apples was extracted with boiling light petroleum (50°-70°C.) for 30 min. The extract was dried with anhydrous sodium sulphate or calcium chloride, filtered, and evaporated. It was saponified by boiling under reflux with 10 ml. of 0.2N ethanolic potassium hydroxide for 45 min. Ethanol (40 ml.) was then added and the solution titrated with 0.1N hydrochloric acid. It was necessary to keep the solution warm during titration to avoid separation of wax. The titre was subtracted from the blank and the concentration of "total fatty acids" was calculated as μE per sq. cm.

V. Structure of Cuticle

A transverse section of the separated skin, stained with Sudan IV, is shown in Plate 1, Figure 1. The skin consists of cuticle and one layer of cells (epidermis). The uptake of the lipoid stain by the cuticle is ascribed to the presence of insoluble "cutin," as all the soluble lipoids are removed before staining.

It is still uncertain whether "cutin" is composed entirely of condensed lipoid material or contains cellulose in association On soaking skin sections in 0.5N ethanolic potassium hydroxide for about two weeks at room temperature the cuticle disappeared while the cell walls of the epidermis remained intact (Plate 1, Fig. 2). It was necessary to cover the section with a collodion film before treatment to prevent loss from the slide. There was no evidence of a cellulose residue in the cuticle, though this might have been lost from the section through complete breakdown of structure.

Chemical fractionation of the residual skin gave some evidence of an association between cellulose and lipoid material. The residual (ethanol insoluble) portion of the skin was first boiled with 1 per cent. ammonium oxalate solution for 1 hr. to remove pectin. The residue was soaked in cuprammonium solution (25 g. CuO in 1000 ml. 15N NH_4OH) at 37°C. for 24 hr. to remove cellulose. The remaining material was then saponified by boiling with 0.5N ethanolic potassium hydroxide for 4 hr. The residue from saponification was treated again with cuprammonium solution. The percentage loss in weight resulting from each treatment was:

Ammonium oxalate	12.0
Cuprammonium (first treatment)	2.5
Saponification with ethanolic KOH	51.3
Cuprammonium (second treatment)	24.9
Final residue	9.3

These results suggest that a large proportion of the cellulose is combined in some way with lipoid material and only becomes available to the cuprammonium reagent after saponification. The cell walls of the epidermis may contain some lipoid material although they do not stain strongly.

The distribution of oil, wax, and ursolic acid in the cuticle is still largely unknown. It is probable that each fraction forms a separate phase, as neither of the solid fractions is appreciably soluble in the oil at room temperature. The wax dissolves in the oil on heating but separates out on cooling.

VI. Acknowledgments

The authors wish to thank Miss M. Hindmarsh, Linnean Macleay Fellow, Botany School, University of Sydney, who prepared the sections of apple skin and treated them to remove the cuticle; and Mr. R. B. Bradbury, Division of Industrial Chemistry, C.S.I.R.O., for determinations of carbon and hydrogen.

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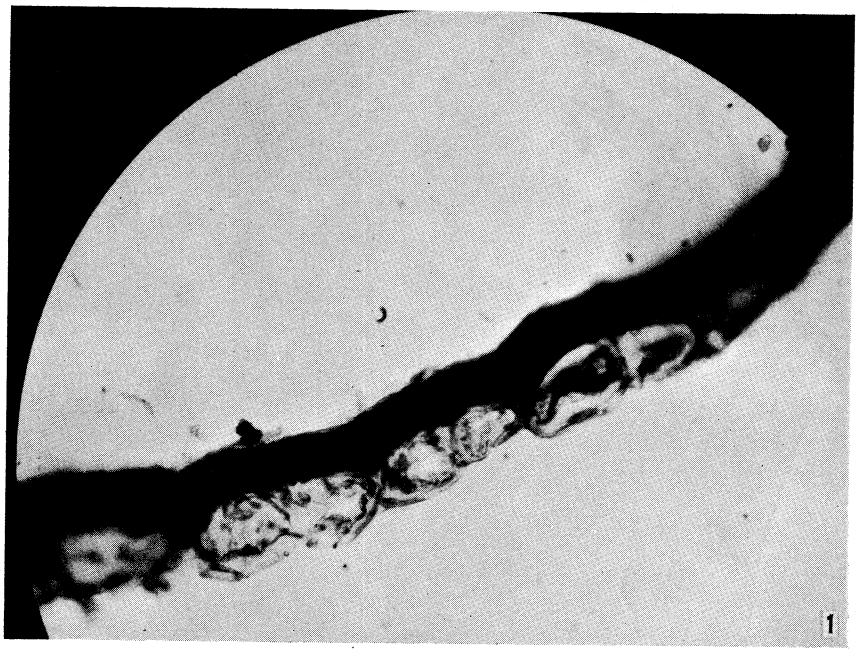

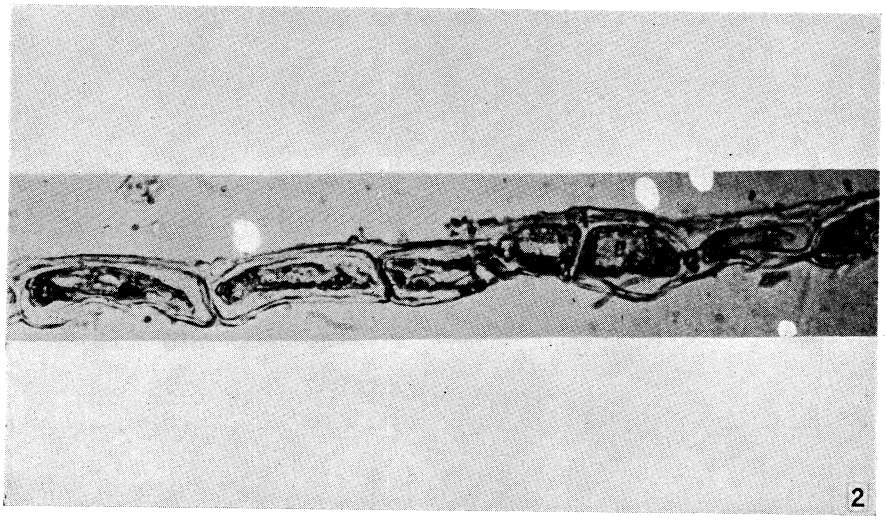

Fig. 1.—Section of separated skin (x480). The cuticle is stained with Sudan IV.
Fig. 2.—Epidermis from separated skin (x520). The cuticle was removed by ethanolic KOH.