The Determination and Isolation of the Organic Acids in Fruit

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A new method has been evolved which will separate quantitatively many of the acids commonly found in fruit and if necessary can be readily modified so as to allow the constituent acids to be isolated in a relatively pure state and identified. The essential steps in the procedure are the extraction of the acids from an acidified extract of fruit by 50% (v/v) *n*-butanol-chloroform, the preparation of a concentrated solution of the acids in 50% (v/v) *tert*-amyl alcohol-chloroform and their final separation and analysis using a modified partition chromatogram. In each of these steps a column procedure has been used on account of its convenience, mild treatment of the organic acids concerned, and the ease with which the acids can be handled quantitatively. It is necessary to concentrate the dilute solution of the acids to at least 5–10 mg./ml. so that the separation of the acids on the chromatogram will be sharp. The addition of an equivalent amount of the acids in dilute solution gives unsatisfactory separations.

In the present study the partition chromatogram used is a very much modified version of that devised by Martin & Synge (1941), the most noteworthy change being the replacement of the aqueous solution of indicator by 0·5N-H_2SO_4. The bands are then revealed by continuously feeding a small amount of an aqueous solution of an indicator into the solvent emerging from the bottom of the column. 0·5N-H_2SO_4 is used instead of water in order to depress the ionization of acids such as oxalic and fumaric which would otherwise give very wide bands owing to the rapid increase in the partition coefficient with dilution.

EXPERIMENTAL

Reagents

Preparation of silica gel. Silica gel prepared from water glass by the method of Gordon, Martin & Synge (1943) has proved to be unsuitable as a supporting medium for the aqueous phase of the chromatogram because of its adsorptive properties, e.g. in the case of oxalic acid the adsorbed acid is held so firmly that it cannot be extracted from the column even if a large volume of solvent is used. Very pure silica gel (Kargin & Rabinovitch, 1935) shows similar properties which suggests that the adsorption is connected with the structure of the gel and not with the presence of impurities.

The method described in the present paper for the preparation of a non-adsorbent gel is based upon the fact that treatment with 10N-HCl at room temperature for about a day eliminates the ability to adsorb organic acids. The actual procedure consists of three operations.

(1) Silica gel substantially free from Al^{III} and Fe^{III} is prepared from water glass by precipitation with 10N-HCl. A large excess of HCl is used and the gel thoroughly washed with 10N-HCl in order to remove all but traces of Al^{III} and Fe^{III}. Analysis of a dry gel indicated that 0·004 and 0·0002% of Al and Fe respectively remained.

(2) The dry gel is allowed to stand for at least 2 weeks. This is necessary because many freshly prepared gels, while showing no adsorptive properties at first, rapidly change in the course of a few days to give gels which show pronounced adsorptive properties. After 2–3 weeks the gels, seem to be quite stable.

(3) The 'aged' gel is then treated with 10N-HCl at room temperature.

This method has been applied to water glass from three different manufacturers (three samples from Messrs Jos. Crosfield, four from Messrs Boots Chemists, and two from Messrs Timothy Whites; the samples were purchased over a period of 3 years) with successful results in every case.

A sample of the HCl-treated, 'aged' gel has been supplied to Dr G. R. Tristram, Biochemical Laboratory, Cambridge, who has used it for the separation of acetyl-amino acids (Martin & Synge, 1941). He has obtained satisfactory separations and quantitative recoveries and will refer to it in a forthcoming paper.

6 lb. of commercial water glass is diluted with twice its volume of warm distilled water, and is then filtered twice through a hardened filter paper (Whatman No. 50) to remove a small quantity of dark coloured suspended matter. The filtered solution should be absolutely clear and colourless. A little methyl orange (M.O.) is added. 10N-HCl is then added in a thin stream with vigorous stirring, addition being interrupted at intervals and stirring continued to obtain efficient mixing. The solution changes at first slowly, and then rapidly to a thick porridge and all but the smallest lumps are broken up by stirring. When the mixture is acid, a further 200–300 ml. of 10N-HCl are added and the mixture very vigorously stirred. It is allowed to stand for several hours and then filtered on a Buchner funnel using a hardened paper (Whatman No. 541). The filtrate contains a considerable amount of Fe^{III} and Al^{III}. When the residue has been sucked as dry as possible, it is suspended in 5 l. of 10N-HCl and allowed to stand overnight at room temperature. It is again filtered. The gel is then washed with 10 l. of 5N-HCl without allowing the precipitate to crack. The filtrate should then be free from Fe^{III}. The gel is washed with 20 l. of distilled water or until the filtrate is free from chlorides (the M.O. at this point almost ceases to colour the

filtrate), then with 10 l. of absolute ethanol and finally with 5 l. of dry ether. The powder is dried in a current of warm air. After the gel has stood for at least two weeks, it is suspended in 7 l. of 10 N-HCl and allowed to stand overnight. It is then filtered and washed with 10 l. of 5 N-HCl, distilled water, absolute ethanol and finally with dry ether. The absolute ethanol contains 1% (w/w) of 10 N-H_2SO_4. The last traces of ether are removed by drying *in vacuo* over P_2O_5. Yield about 800 g.

The dry gel can be stored in a closed vessel for long periods without deterioration. It is quite suitable without further treatment for the extraction of organic acids from fruit, and for use in the chromatogram. After drying over P_2O_5 the gel should be able to adsorb its own weight of 0·5 N-H_2SO_4 and still appear superficially dry. If this is not the case, the gel must be dried for a further period *in vacuo* over P_2O_5.

Preparation of indicator solution. The indicator chosen must have a pK which is at least one unit above the pH of a dilute solution of the sodium salt of the weakest acid. This is essential in order that the colour changes shall be sharp when the concentration of acid is still low. Assuming that the acid has a dissociation constant of 10^{-6} and that the sodium salt of the indicator has been just decomposed, then the pH of a dilute (indicator used is 0·0004 M) solution of its sodium salt is about 8·5. The pK of the indicator should therefore be 9·5. Three indicators were tried which had a pK about this value—thymol blue (8·9), phenolphthalein (9·7) and thymolphthalein (9·2). Of these thymol blue was the best because the colour change was very sharp and easy to observe. In practice the solution of indicator is rendered more alkaline than is necessary to convert it to its sodium salt, the excess alkali being used to neutralize the acidity of the organic phase emerging from the column.

0·02 g. of thymol blue is ground in an agate mortar with 2 ml. of 0·1 N-NaOH until it has dissolved. The solution is then diluted to 100 ml. with CO_2-free water and filtered (Whatman No. 41) to remove any small insoluble particles which might clog the capillary outlet of the indicator reservoir *B* (see Fig. 1). The indicator in this solution is approximately 0·0004 M.

Solvent. The 50% (v/v) *n*-butanol-chloroform used for the extraction of the acids was equilibrated before use against a solution similar in composition to that of the acidified fruit juice, e.g. in the case of apples the solvent was shaken with a solution containing 35 g. glucose, 200 ml. 0·2 N-H_2SO_4, and sufficient N-NaOH so that when the volume was made up to 500 ml., the pH was 2·0. The solvent was then run through a small column (2 g. of silica gel + 2 ml. of the solution) to free it from suspended drops of solution.

The 50% (v/v) *tert*-amyl alcohol-chloroform was equilibrated against M-Na_2SO_4. *Tert*-amyl alcohol-chloroform was used for the final extract in order to avoid the esterification which occurs when acids such as oxalic are dissolved in mixtures of *n*-butanol-chloroform and left for more than a few hours. The ester appears as a fast moving band ahead of the main band.

The mixtures of *n*-butanol-chloroform (5–35% v/v) used for the development of the partition chromatogram were equilibrated against 0·5 N-H_2SO_4. The maximum concentration has been fixed at 35% because with this solvent it is possible quantitatively to separate malic and citric acids within a reasonable time (2 hr.). The volume of solvent required is less than 200 ml. and the acid blank of the solvent very small. The other concentrations 20, 10 and 5% have been chosen arbitrarily.

Description of partition chromatogram

This is best appreciated by reference to Fig. 1. *A* is a glass tube (int. diam, 1·3 cm., length 25 cm.) furnished with a perforated porcelain disc near the bottom resting on three indentations in the wall of the tube. On this disc is placed a single layer of filter paper (Whatman No. 541) cut very slightly larger than the internal cross section of the tube.

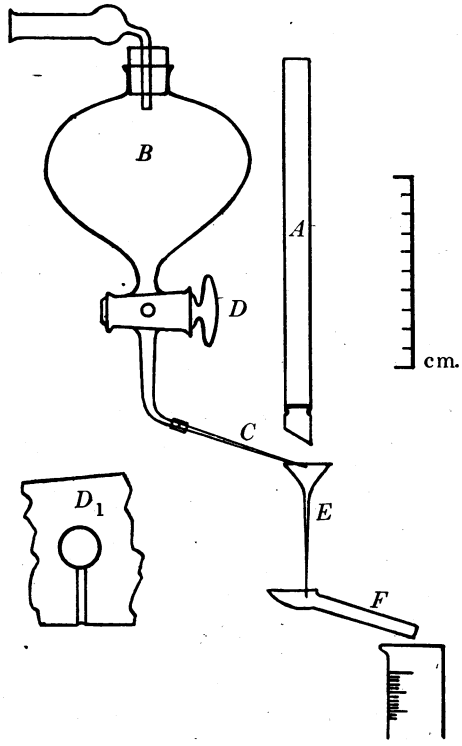

Fig. 1. Diagram of the arrangement of the chromatographic apparatus. *A*, the tube containing mixture of silica, 0·5 N-H_2SO_4 and solvent; *B*, the reservoir of indicator solution; *C*, the capillary outlet from *B*; *D*, the tap which controls flow of indicator (also shown in more detail D_1); *E* and *F*, a device for mixing indicator and solvent.

The lower end of the tube is ground obliquely so that the solvent passing down the tube will drip in a uniform manner from one place. *B* is a separating funnel (500 ml.) containing the solution of indicator. The top is closed with a bung and a soda-lime tube to prevent the solution inside absorbing CO_2. The lower part of the stem of *B* is bent so as to make an angle of about 105° with the upper part, and is then drawn out into a capillary (2–3 mm. diam.). Attached to this capillary by means of a piece of rubber tubing (as supplied for bicycle tyre valves) is a finer capillary *C*. This is about 5 cm. long. The exact length and diameter is adjusted to give a rate of flow from *B* when the tap *D* is fully open of about 0·2 ml./min. The final adjustment of the rate of flow is obtained by manipulating the tap *D*. To facilitate this adjustment, the plug of the tap has two very fine file cuts leading from opposite ends of the hole to about half way

round the plug. One of these file cuts is shown at D_1, the other, of course, is hidden but runs upwards from the other end of the hole. When the tap D is closed so as just to block the main hole, these file cuts create a capillary through which the solution of indicator must pass. The length of this capillary can be varied by turning the tap (through about 90°) and it is an easy matter to adjust the flow to any required amount (within the range 0·2–0·02 ml./min.). The central portion of the tap is not lubricated in order to avoid blocking the file cut with grease. The apparatus is so arranged that the tip of the capillary C is about 3 cm. immediately below the point on the glass tube A from which the solvent drips. The mixture of solvent and indicator is collected by a small filter funnel E, the stem of which is drawn out into a coarse capillary (5–6 cm. long). The main function of E is to ensure the intimate contact of the two liquids. This is achieved partly by the impact of the drop of solvent as it falls on the indicator at the tip of C and partly by the turbulent passage of the two liquids down the capillary as a series of very small drops which mix again at the lower end before falling into the upper end of F. F is an inclined glass tube (int. diam. 6 mm., length 7 cm.) the upper end of which has been enlarged into a small basin. The colour of the mixture flowing down the tube is observed against a white background, particular attention being paid to the drop which forms at the end of the tube. It is essential to have good lighting, and a 80 W. fluorescent lamp has proved very satisfactory when bright daylight is not available.

Extraction of acids from fruit

All pH measurements were made at 20°±1° with a glass electrode and calomel half cell, using a Cambridge Instrument Co. valve potentiometer.

The solvents and chemicals used were the purest which could be purchased commercially.

Procedure. This method is described in terms of apples but has been applied successfully to gooseberries, strawberries, and rhubarb without modification.

Before analysis the apples are frozen at −20° and then ground to a flour which is stored at −20° in a closed vessel until required.

50 g. of the well mixed apple flour are mixed thoroughly with 20 ml. of 0·2 N-H_2SO_4 and the whole allowed to thaw. Stirring is continued until the mixture has completely thawed out; the pH should be 2·0±0·2. A preliminary trial with a small sample is useful to determine the right amount of acid to add. The mixture is then centrifuged, and the clear supernatant fluid decanted and filtered if necessary through a dry filter paper (Whatman No. 541) from any floating particles of cell wall.

10 g. of this extract which should contain organic acids equivalent to 10–20 ml. 0·1 N-NaOH, are added to 12 g. of silica gel and the mixture thoroughly stirred and well worked by pressing small portions against the side of the beaker with the spatula. The preparation of the mixture of silica gel and aqueous phase in order to obtain a powder which is superficially dry and barely adheres to the sides of the beaker will be referred to subsequently as 'thorough mixing'. To this powder is added about 50 ml. of 50% (v/v) *n*-butanol-chloroform, and the mixture of silica gel and solvent quantitatively transferred to a glass tube (diameter 5·2 cm., length 20 cm., similar to A, Fig. 1) and the filtrate collected. The filter paper resting on the porcelain plate at the bottom is usually wetted beforehand with a little of the solvent and gently pressed down with a glass rod. The solvent emerging at the bottom of the tube should be clear and free from any traces of silica gel. The column is allowed to run dry and then the tube filled up with solvent. 350–400 ml. of filtrate are collected. A further 50 ml. are collected separately and titrated with 0·1 N-NaOH to pH 9·4, about 0·2 ml. being required if the extraction is complete. The main bulk of the filtrate is then titrated with 0·1 N-NaOH to pH 9·4 and allowed to stand for 0·5 hr. The lower solvent layer is separated and washed twice with 10–15 ml. of water. The combined aqueous layers are concentrated *in vacuo* to about 2 ml. at a bath temperature not exceeding 35°, and then quantitatively transferred to a small beaker, and dried *in vacuo* over P_2O_5. To the residue in the beaker is added the calculated amount of 2 N-H_2SO_4 necessary to liberate the organic acids present as the sodium salts. The volume of liquid should be between 0·7 and 1·0 ml. which just ensures that all the residue can be thoroughly wetted and dissolved. When the residue has dissolved save for a small amount of flocculent material, 1·0 g. of silica gel is added and the whole 'thoroughly mixed'. To this powder is added about 10 ml. of 50% (v/v) *tert*-amyl alcohol-chloroform and the mixture quantitatively transferred to a glass tube (diameter 1·3 cm., length 25 cm., similar to A, Fig. 1) and the filtrate collected. The procedure thereafter is the same as that described for the extraction with 50% (v/v) *n*-butanol-chloroform except that special care is taken to transfer all the particles of silica gel to the column by using some of the filtrate. Finally the beaker is rinsed with 5 ml. of solvent which is transferred to the tube when the top of the column of silica gel is dry. As soon as this solvent has been absorbed, more is added until the tube is full. 30 ml. of the filtrate are collected (the extraction takes about 2 hr.) and the completeness of the extraction checked by collecting a further 5 ml. and titrating with 0·1 N-NaOH to pH 8·4. Less than 0·1 ml. is required when no organic acids are present.

To calculate the amount of acid present in the fresh fruit, the figure obtained for the acid in the *tert*-amyl alcohol-chloroform extract is multiplied by

$$\frac{50 + 20\text{-dry weight of the cell wall insoluble in water in g.}}{10}$$

The acid is assumed to be wholly present in solution in the fruit juice, the weight of which in the present instance has been taken as the fresh weight of the fruit, minus the weight of the cell wall insoluble in water.

Control experiments on the extraction procedure. Determinations of the *l*-malic acid present in the same batch of frozen apple flour were in excellent agreement with each other, e.g. the amounts found in the case of a sample of Bramley's Seedling apples which had been stored at +7° for 5–6 months were 1·20, 1·21, 1·21% of the fresh weight. Estimations of acetic and citric acids added to mixtures of frozen apple flour and 0·2 N-H_2SO_4 were in satisfactory agreement with the amounts known to be present. In one experiment when 30 mg. of each acid was added (0·06% on fresh weight of fruit) the analysis indicated that 28·8 mg. of acetic and 29·4 mg. of citric acid were present. In a second experiment when 600 mg. of each acid was added (1·2%) the analysis indicated that 588 mg. of acetic and 594 mg. of citric acid were present. These acids were chosen as typical of a volatile and a non-volatile acid respectively, and in

addition were known to be absent from this particular apple flour. In a later paper it is hoped to give the results of experiments in which acids such as *cis*-aconitic, *iso*citric and oxalo-acetic are used.

Separation and analysis of the mixture of acids using the partition chromatogram

Procedure. To 3·0 ml. of 0·5 N-H_2SO_4 in a small beaker is added 3·0 g. of silica gel and the whole 'thoroughly mixed'. The powder is then gently brushed through a brass gauze sieve (90 mesh). The sieved powder is returned to the beaker and about 20 ml. of the appropriate strength of *n*-butanol-chloroform (10–35% v/v) is then added. The slurry of silica gel and solvent is poured into the glass tube *A* (Fig. 1) and the column allowed to pack down until the top surface is dry and until the solvent has ceased to drip from the bottom. During the last stage when the top surface is dry but solvent is still dripping from the bottom the column shrinks 3–4 mm. on a length of 8 cm., and leaves a ring of silica gel, 2–4 mm. above the top of the column. This ring is carefully removed without disturbing the top surface of the column by wiping the glass with a piece of cotton wool twisted round the end of a glass rod. The indicator solution from *B* is adjusted to about 0·1 ml./min. (in the case of 35% v/v *n*-butanol-chloroform) and a measuring cylinder placed under the lower end of *F*. 2 ml. of the solution of acids from the apple tissue in 50% (v/v) *tert*-amyl alcohol-chloroform is added from a pipette dropwise to the top of the column so as not to disturb the silica gel. When this solution has drained into the gel 1 ml. of the appropriate strength of the developing solvent (*n*-butanol-chloroform) is added very carefully so as to wash any traces of the solution on the walls of the tube into the gel. This is allowed to drain into the gel and the process repeated twice. The glass tube is then filled up to the top with the solvent, and more solvent added as required to keep the tube full. The rate at which solvent emerges from the column should be about 1 ml./min. The colour of the mixture of indicator and solvent flowing down the tube *F* is closely observed. The volume of indicator should be $\frac{1}{8}$–$\frac{1}{10}$ of the corresponding volume of solvent in the case of 35% (v/v) *n*-butanol-chloroform and if very different should be adjusted to this ratio. The mixture of solvent and indicator is blue when no organic acid is present, the alkali in the indicator just neutralizing the slight acidity of the solvent. When an organic acid commences to wash out of the column, the colour sharply changes to green and then to yellow, the solvent becoming a distinct pink. If only a trace of organic acid is present the drop of solvent becomes pink even though the colour of the aqueous phase remains substantially blue. At the point when the colour is still green-blue the receiver is changed. When all the organic acid has been washed out the reverse colour change occurs, and the receiver is changed as soon as the colour is blue. The volumes of solvent and indicator in the receiver containing the organic acid are recorded. The whole procedure is repeated each time an organic acid is washed out of the column, and the mixture of acids originally added to the top of the column is resolved into a series of sharply defined fractions. These are titrated with 0·01 N-$Ba(OH)_2$ to pH 8·4 after 20 ml. of CO_2-free water have been added. The mixture of solvent, indicator and alkali is thoroughly mixed by blowing CO_2-free air through the titration vessel.

At pH 8·4 most organic acids, e.g. citric, acetic, and succinic, are completely neutralized in the concentrations (0·005–0·0001 N) likely to result if 10 and 0·1 mg. quantities are passed through the partition chromatogram as described previously and titrated with 0·01 N-$Ba(OH)_2$.

A small correction (approximately 2%) must be added to the observed titre because the mixture of solvent and indicator is slightly more alkaline than pH 8·4. This is evaluated by titrating under exactly the same conditions a known amount of the pure acid.

The analysis of an unknown mixture of acids is best effected by using 35% (v/v) *n*-butanol-chloroform as the developing solvent. If the presence of acids coming through earlier than oxalic and malic is established then a second analysis is made, but in this case the column is developed first with 5% (v/v) *n*-butanol-chloroform if acetic and formic acids have to be separated and then after about 100 ml. of solvent have passed through the column, changed over to 10% (v/v) *n*-butanol-chloroform, then to 20%, and eventually to 35%.

Strength of acid in the aqueous phase of the column. To depress the ionization of such acids as oxalic and fumaric, it is essential to lower the pH of the aqueous phase as much as possible, though the amount of acid in the developing solvent must not be large enough to interfere with the accuracy of the final titration. The amount of acid present will depend upon the concentration and nature of the mineral acid and the composition of the solvent, the alkali titre being highest for 35% (v/v) *n*-butanol-chloroform. In Fig. 2, the amount of 0·01 N-$Ba(OH)_2$ necessary to neutralize

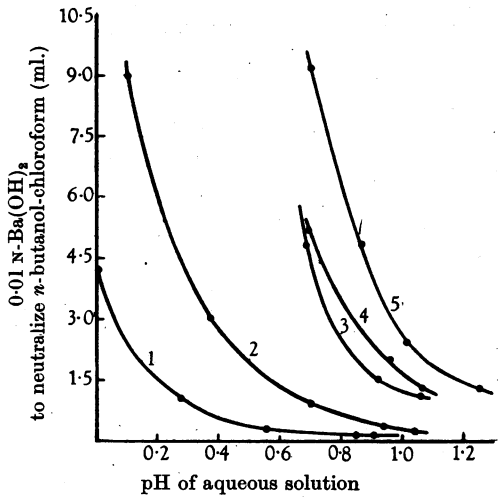

Fig. 2. Variation in the amount of acid present in 35% (v/v) *n*-butanol-chloroform in contact with dilute aqueous solutions of: 1, H_2SO_4; 2, HCl; 3, HNO_3; 4, $HClO_4$; 5, H_3PO_4.

(pH 8·4) 20 ml. of 35% (v/v) *n*-butanol-chloroform which had been equilibrated against a given concentration of acid has been plotted against the pH of the aqueous phase for a series of strong acids. The results indicate that H_2SO_4 is the best acid to use. The concentration has been standardized as 0·5 N which is equivalent to a pH of about 0·55. In

practice, the free mineral acid in the solvent is more than neutralized by the slight excess of alkali present in the indicator solution.

RESULTS

Separation and analysis of organic acids using the chromatogram

Separation. The experimental data obtained with the standard chromatogram for many of the acids which occur in plants have been recorded in Fig. 3.

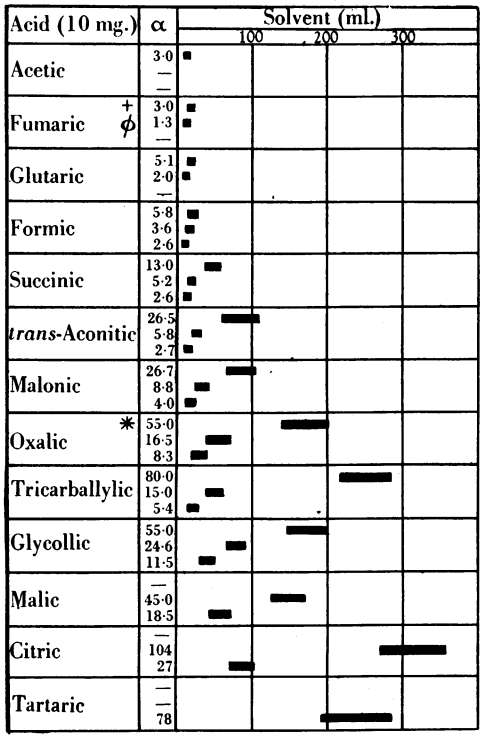

Fig. 3. Effect of varying the composition of the solvent used for development on the volume of solvent required to wash a number of common plant acids through a standard column. Upper line for each acid represents development with 10%, middle line 20%, and lower line 35% (v/v) *n*-butanol-chloroform respectively. The volume of solvent necessary when the acid has reached the bottom of the column is indicated by the length of the line. α, the partition coefficient,

$$= \frac{\text{g. solute/ml. non-mobile phase}}{\text{g. solute/ml. mobile phase}}.$$

\+ 1·5 mg. φ 5·0 mg. * 3·0 mg.

The amount of acid added to the top of the column was usually 10 mg. Each acid is represented by a horizontal line, the beginning of the line indicating the point at which the indicator changed over to the acid colour, and the end, the change back to the alkaline colour. The length gives the amount of solvent required to wash the acid out of the column, the abscissa representing the volume of solvent used to develop the column from the moment the acid was added at the top. The partition coefficient,

$$\alpha = \frac{\text{g. solute/ml. of non-mobile phase}}{\text{g. solute/ml. of mobile phase}}$$

at equilibrium, has been calculated using the formula derived by Martin & Synge (1941). In the present case, the movement of the position of the maximum concentration of solute has been from the top of the column to the bottom. The simultaneous movement of the surface of the developing fluid has been calculated from the volume necessary to wash out half the band, i.e. volume measured to centre of horizontal line (Fig. 3), divided by the cross sectional area of the tube. The assumption that the centre of the horizontal line represents the position of maximum concentration is correct for the majority of the acids because the curve relating the concentration at any one point with the volume of developing solvent is almost symmetrical. This is illustrated by Fig. 4 (1) which depicts the results

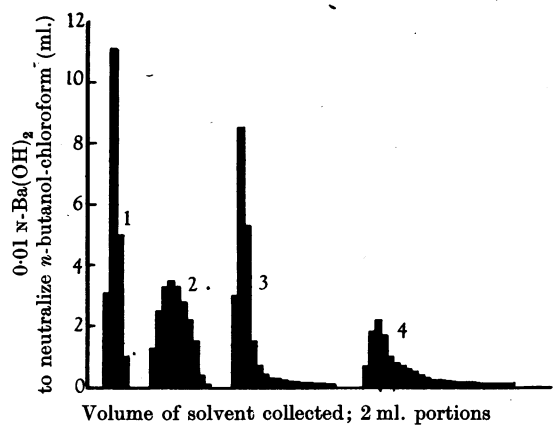

Fig. 4. Concentration of oxalic acid in the solvent emerging from the column. 10 mg. of oxalic acid was added to columns of (1) 3 g. of HCl-treated silica gel + 3·0 ml. 0·5 N-H_2SO_4. (2) 7 g. of potato starch + 2·5 ml. 0·5 N-H_2SO_4. (3) 3 g. of silica gel prepared according to directions of Martin & Synge (1941) + 3·0 ml. 0·5 N-H_2SO_4. (4) 3 g. of HCl-treated silica gel + 3·0 ml. of water. (1) is the standard column recommended.

obtained by titrating equal volumes of the solvent as it issued from the column when an oxalic acid band had just reached the bottom and was being washed out. The results are plotted as a histogram. Oxalic acid was chosen because it is the strongest of the fruit acids and very readily adsorbed by untreated silica gel, circumstances which would be very likely to produce an unsymmetrical curve.

The effect of using untreated silica gel (3) or starch (2) in place of the HCl treated gel (1) and the effect of substituting water for the 0·5N-H_2SO_4 normally used is also recorded. It is noticeable that both starch (Elsdon & Synge, 1944) and the HCl treated silica gel which do not adsorb oxalic acid, give almost symmetrical histograms. Untreated silica gel gives a noticeable tail as does the substitution of water for the 0·5N-H_2SO_4.

From Fig. 3 one can determine at a glance whether a separation of two acids is practicable.

Analysis of mixtures of acids. In Table 1 is recorded the analysis of various mixtures of acids. Special attention has been paid to the separation of malic acid and citric acid which follow each other closely (see Fig. 3).

Table 1. *Separation of mixtures of acids*

(First line gives acid added to top of column and second line amount recovered calculated from titration figure.)

Exp.	Fumaric (mg.)	Succinic (mg.)	Oxalic (mg.)	Malic (mg.)	Citric (mg.)	Tartaric (mg.)
1	3·0	10·0	10·0	10·0	10·0	10·0
	2·95	10·0	9·9	10·0	10·0	10·0
2	—	—	—	10·0	1·0	—
	—	—	—	10·0	0·975	—
3	—	—	—	1·0	10·0	—
	—	—	—	0·97	10·0	—
4	—	—	—	10·0	0·10	—
	—	—	—	10·0	0·09	—
5	—	—	—	0·10	10·0	—
	—	—	—	0·11	10·1	—

10% (v/v) *n*-butanol-chloroform used to separate fumaric and succinic acids. 35% (v/v) *n*-butanol-chloroform for remainder.

Isolation of acids

When the band containing the acid is at the bottom of the column the indicator solution is stopped and then an appropriate volume of solvent collected. The volume necessary is usually determined by a preliminary experiment. The acid, or if it is volatile its sodium salt, is obtained by evaporation of the solvent.

In a small-scale experiment, 10 g. of frozen apple flour gave 95 mg. of *l*-malic acid. The silver salt contained Ag 61·8. Calc. for $Ag_2C_4H_4O_5$:Ag, 62·0%. 9·84 mg. of the free acid + 22·7 mg. $(NH_4)MoO_4$ in 7·2 ml. H_2O gave $[\alpha]_D^{20°} + 560°$. McKenzie & Plenderleith (1923) record $[\alpha]_D^{14·5} = +568°$ under similar conditions. The m.p. of the *p*-nitro-benzyl ester was 124°.

In a second experiment 100 mg. of acetic acid and 100 mg. of citric acid were added to 10 g. of frozen apple flour, and the acids extracted and separated. 92 mg. of sodium acetate and 78 mg. of citric acid were obtained. Each acid was identified as the *p*-bromo-phenylacyl ester, m.p. 85° and 148° respectively.

In both experiments some acid, approximately 30% of the total, remained behind with the insoluble residue in the centrifuge tube.

DISCUSSION

The usual procedure for the extraction of the acids from fruit (Pucher, Wakeman & Vickery, 1941) is very drastic for it transforms the *cis* isomer of aconitic acid into the *trans* (Krebs & Eggleston, 1944). An alternative method, based on the extraction of the acids by solvents such as ethyl acetate (Pucher & Vickery, 1939) in an apparatus first designed by Widmark (1926), has not been found satisfactory as at least 24 and often 48 hr. are required for the complete removal of the organic acids, and in addition some of the H_2SO_4 used to acidify the fruit juice is also extracted.

The method described in the present paper in which the fruit extract is acidified with H_2SO_4 to a pH of 2·0 and then extracted with 50% (v/v) *n*-butanol-chloroform, is very rapid and does not subject the solutions to a temperature above 25°. The time required, from the addition of the 0·2N-H_2SO_4 to the finely ground frozen fruit to the end of the extraction, is not more than 4 hr. Volatile acids which would be lost if the fruit were dried, can be extracted quantitatively. In addition the *n*-butanol-chloroform extract can be readily manipulated so as to yield a concentrated solution suitable for analysis by the partition chromatographic method without any loss of volatile acids.

The partition chromatogram used for the analysis of the fruit acids is based upon the same principles as that of Martin & Synge (1941) but differs from it in several important details.

(1) The most fundamental change is that the indicator has been placed outside the column. This has been done in order to overcome the difficulties which were experienced in attempting to use the unmodified partition chromatogram—it was found that the concentration of *n*-butanol in the mobile phase had to be at least 30–40% for malic and citric acids and at this concentration, the acid forms of any of the indicators recommended previously (Gordon, Martin & Synge, 1944; Lidell & Rydon, 1944) were immediately leached from the silica gel. In addition, it was found impossible to obtain an indicator which would function reversibly on the column for all fruit acids because their relative strengths varied so widely, e.g. pK of first H· of oxalic acid is 1·19 while that of malic acid is 3·40 (for the function of the indicator and the causes underlying the non-ideal behaviour of unmodified partition chromatograms see Lester Smith, 1942). In the modified partition chromatogram, a constant

stream of an indicator sensitive to the weakest acid likely to be present is injected into the solvent flowing from the column. The indicator has no influence on the working of the column.

(2) Another important change is the replacement of the aqueous non-mobile phase by 0·5N-H_4SO_4 in order to depress the ionization of organic acids which otherwise would give very wide bands on the column. With water the separation of a relatively strong acid such as oxalic from those that might follow it is impossible.

(3) In general, higher concentrations of *n*-butanol-chloroform (10–35 % v/v) have been used. This is obligatory but has the advantage that the association of the organic acids in the mobile phase appears to be slight. Association in the mobile phase has the same effect on the apparent partition coefficient as dissociation in the aqueous phase. Some acids associate markedly in chloroform (e.g. formic acid, Shilov & Lepin, 1922).

(4) The adsorptive properties of silica gel towards organic acids have been eliminated by treatment with 10N-HCl, 'ageing' in the dry state for 2–3 weeks, and then retreatment with 10N-HCl.

The total effect of these changes is to produce a partition chromatogram which agrees very closely with the mathematical picture of Martin & Synge (1941); the shape of the curve relating the concentration of the solute at any one point on the column with the volume of the solvent used for development is very nearly symmetrical (see Fig. 4 (1)). This is assumed to indicate the absence of any significant interference either by adsorption or by changes in the apparent partition coefficient with dilution (Martin & Synge, 1941; Lester Smith, 1942).

The ratio of the volume of indicator to solvent is very important if one of the acids is only present in small amount. Consider the curve for oxalic acid (1) in Fig. 4. Assuming that the characteristics of the curve can be expressed in terms of the normal curve of error, as suggested by the mathematical analysis of Martin & Synge (1941), then it can be shown that 99·8 % of the acid will have been collected when the concentration in the organic phase has fallen to 1 % of the maximum value. This applies whether the total quantity of acid is 10 mg. or 0·1 mg. though the limiting absolute concentrations in the two cases for oxalic acid are about $0{\cdot}25 \times 10^{-3}$M and $0{\cdot}25 \times 10^{-5}$M respectively. These figures mean that if the excess alkali present in the indicator solution, above that necessary to neutralize the mineral acid in the solvent, is equal to a concentration of oxalic acid of $0{\cdot}25\text{M} \times 10^{-3}$, then a quantity of 10 mg. added to the top of the column will be recovered to the extent of 99·8 % whereas a quantity of 0·1 mg. will not show up at all. It follows therefore that in order to determine small quantities of an acid the excess alkali in the mixture of solvent and indicator must be reduced to the minimum. The limit to the sensitivity of the indicator device is set by the concentration of acid necessary to effect a demonstrable change in the colour of the indicator. This concentration can be calculated assuming that the indicator salt must be decomposed to the extent of about 25 % (Britton, 1942) before the change in the colour is detectable and that all the organic acid in the solvent is transferred to the indicator solution. The minimum concentration of acid in the organic phase then works out at $0{\cdot}0004 \times 0{\cdot}25 \times 0{\cdot}1 = 1 \times 10^{-5}$M (using a solvent/indicator ratio of 10) which is less than the limiting absolute concentration required in order to obtain a 99·0 % recovery of oxalic acid when only 0·1 mg. is taken for analysis. On theoretical grounds therefore the indicator device should be sufficiently sensitive to enable even quantities as small as 0·1 mg. to be analyzed satisfactorily. In practice an overall accuracy of about 1 % on a sample of 10 mg. is regarded as satisfactory. On 0·1 mg. the error, mainly due to the titration, is about 10 %.

SUMMARY

1. A simple method has been devised to extract both the volatile and the non-volatile acids from fruit without subjecting any of the solutions to a temperature above 35°. The acidified fruit juice is absorbed into silica gel and extracted in the form of a column with 50 % (v/v) *n*-butanol-chloroform, the fruit acids in this solution then being concentrated and dissolved in 50 % (v/v) *tert*-amyl alcohol-chloroform for analysis.

2. The mixture of acids is separated using a modified partition chromatogram, the non-mobile phase consisting of 0·5N-H_2SO_4 and the mobile phase of mixtures of *n*-butanol-chloroform. The acid present in each fraction can be isolated by evaporation of the solvent, the solution of indicator which feeds into the solvent leaving the column being stopped so as not to contaminate the acid. Volatile acids are isolated as their sodium salts.

3. The modified partition chromatogram avoids several of the defects associated with the original design of Martin & Synge (1941). The most important improvements are the use of an external indicator instead of an internal one, the replacement of water by 0·5N-H_2SO_4, and the use of a non-adsorbent silica gel. The working of the column then closely approaches that required for an ideal chromatogram, on the basis of the mathematical treatment of Martin & Synge (1941).

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The Fixation and Retention of Ascorbic Acid by the Guinea-pig

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In this communication experiments are described which are an extension and an elaboration of an investigation on guinea-pigs and man which has been in progress for some time (Johnson & Zilva, 1934; Zilva, 1936; Kellie & Zilva, 1938, 1939; Zilva, 1941), the general object being to coordinate experimental data obtained on guinea-pigs with observations made on human beings. Since the scope of experimentation on man is for obvious reasons limited, many gaps in our knowledge can only be filled in by systematic and detailed experimental work on animals. Such information can not only throw light on the general metabolic problem but can also help in assigning proper value to observations made on man. The present experiments deal with the absorption and retention of ascorbic acid by the tissues of the guinea-pig and the bearing of this phenomenon on the development of scurvy.

TECHNIQUE

Experimental animals and diet. All animals employed in this investigation were young growing guinea-pigs weighing approximately 300 g. The basal scorbutic diet had the following composition (% by weight):

Bran	24
Barley meal	16
Weatings	21
Fishmeal	8
Oats (crushed)	26
Salt mixture	5

In addition each animal received reconstituted full cream milk which had been boiled for 1 hr. after the addition of 1 ml. 0·5% $CuSO_4$/100 ml. of milk. This preliminary treatment of the milk was carried out since it was considered that the nature of the investigation required a basal diet as free as possible from vitamin C. On this diet, guinea-pigs increased in weight for about 14 days and then decreased rapidly. The first macroscopic lesions of scurvy appeared after *c.* 15 days and the animals succumbed to the disease in about 25 days.

Preparation and analysis of the tissues. The guinea-pigs were killed by stunning and bleeding and the blood was collected in oxalate. The tissues were then prepared for analysis as follows. Whole blood (5 ml.) was precipitated with 15 ml. 6% trichloroacetic acid. The liver, kidney and muscle samples and also the stomach and intestinal tissues, after removal of the contents and subsequent washing and drying the tissues with filter paper, were extracted twice with 2 parts by weight of 10% trichloroacetic acid and by grinding with sand and centrifuging. Measured portions of the resulting extracts (not more than 4 ml.) were diluted to 20 ml., the final trichloroacetic acid concentration being adjusted to 4%. The adrenal, spleen and marrow were extracted with 20 ml. 4% trichloroacetic acid. Since the amount of marrow obtained from one animal was small, the sample analyzed was taken from the femurs and tibias of three guinea-pigs killed at the same time. Leucocytes were obtained by a procedure similar to that employed by Kellie & Zilva (1938), the pooled white cells from six animals being used for each experiment. Eighteen hours before the guinea-pigs were killed, they were injected intraperitoneally with 30 ml. of meat infusion broth containing 1% peptone and 0·5% NaCl. The leucocytes thus obtained (yield, 0·5–0·8 g.) were extracted with 20 ml. 4% trichloroacetic acid. Ascorbic acid was then determined by the 2:4-dinitrophenylhydrazine method of Roe & Kuether (1943). Using this technique we have found that added ascorbic acid is recovered with an error of less than 5%. No interference