QUANTITATIVE CHROMATOGRAPHIC ANALYSIS OF ORGANIC ACIDS IN PLANT TISSUE EXTRACTS

by

F. BRYANT

Research Laboratory, Prestige Ltd., Melbourne (Australia)

AND

B. T. OVERELL

Division of Food Preservation and Transport, Commonwealth Scientific and Industrial Research Organization, Sydney (Australia)

INTRODUCTION

The fundamental importance of organic acids in plant and animal metabolism renders it desirable that methods should exist for their simultaneous identification and determination. The more common methods of analysis for individual acids of the types likely to be found in plant tissues have recently been reviewed[11]. LUGG AND OVERELL[9] have reported the separation and identification of a number of nonvolatile carboxylic acids by paper partition chromatography and the present authors[2] have since briefly indicated how the technique can be utilised for the quantitative analysis of such acids in extracts of apple and carrot tissues. This report is a more complete account of that investigation.

FISHER, PARSONS AND MORRISON[6] showed that the areas of separated spots of amino acids and sugars on a paper chromatogram bear a linear relationship to the logarithm of the concentration of the substance therein and REID AND LEDERER[10] have successfully applied this relationship to the determination of certain fatty acids. We have similarly developed this technique for certain nonvolatile carboxylic acids, but have found it simpler to relate acid concentration to the mass of the filter paper spots which the separated acids occupy on the chromatogram.

Mixtures of organic acids when applied to the chromatogram should be free from contaminating inorganic salts, sugars, and other interfering substances. This state of purity may be realised by adsorbing the acids from tissue extracts on a column of anion exchange resin (Amberlite IRA 400) and subsequently eluting with ammonium carbonate. Such treatment of the extracts gives a solution containing the organic acids which is free from pigments, sugars and cations, but which contains excess ammonium carbonate that can be readily removed by evaporation. Recovery from the resin columns has been found to be 100% for citric, malic and fumaric acids and approximately 90% for succinic.

Ion exchange resins have previously been used for the deacidification of both fruit

References p. 476.

juices and fruit extracts, *e.g.* tartaric acid from pomace and brandy still slops[12] and malic acid from apple juice[3]. CHALLINER AND KIESER[4] have found that resin treatment of apple juice renders it more stable towards fermentation.

EXPERIMENTAL

a. *Standard acids*

Standard solutions of acids were made with A.R. chemicals except in the case of *iso*citric acid which was synthesised following the method described by KREBS AND EGGLESTON[7].

b. *Preparation of tissue extracts*

(i) Carrot. Carrots were obtained fresh from market and stored in moist sand at 1° C until required for use. Discs (80 mm in diameter and 1.0 mm thick) were cut from the xylem of a turgid carrot and rinsed in distilled water before plunging into boiling 80% ethanol. They were refluxed for 90 minutes then macerated in hot ethanol for three minutes in a Waring blender. The suspensions were filtered through sintered glass and the residue washed thoroughly with 80% ethanol. The filtrate, plus washings, were combined and evaporated to dryness on a steam bath. The residue after evaporation was dissolved in approximately 250 ml distilled water and this solution then passed through a resin column as described below. After elution with ammonium carbonate the solution containing the acids and excess elutant was evaporated to dryness and the residue brought to the required volume with distilled water. Dry weight of the discs used was determined by comparison with discs cut from the same carrot and dried at 80°–90° C for 48 hours.

(ii) Apple. Apples ("Statesman" variety) were obtained from an experimental plot at commercial picking time and the tissue analysed without preliminary storage. Three layers of tissue were taken from the fruit 1 cm, 2 cm, and 3 cm, from the epidermis respectively. The dried flesh was ground and the powder suspended in water to dissolve the acids and finally filtered, the residue being thoroughly washed and the filtrate and washings combined. This extract was diluted to 250 ml, passed through a resin column and thereafter treated similarly to the carrot.

c. *Preparation of resin columns*

The resin used was the strong base anion exchanger Amberlite IRA 400. Colouring matter was removed from the resin by successively washing with 1.0 *N* sodium carbonate, water, then 1.0 *N* hydrochloric acid in that order for several cycles over the resin packed in the form of a large column. All fine particles were removed by suction.

A column containing 5.0 g of Amberlite IRA 400 was required to adsorb 50 mg each of at least three acids, *e.g.* citric, malic and succinic. Larger size columns (20 g) were also tried with the same acid concentration but offered no advantage and, indeed, necessitated the use of very large volumes of eluant.

The approximate amount of resin (5 g air-dry weight) was exchanged with 500 ml 1.0 *N* sodium carbonate overnight. As is customary, the columns (0.80 cm internal diameter) were packed wet. After the passage through the column of another 500 ml of 1.0 *N* sodium carbonate, water was passed to wash out all excess alkali. Ammonium carbonate was used in a similar manner although it has been found[8] that the exchange capacity of Amberlite IRA 400 regenerated with ammonium carbonate is less than if regenerated with sodium carbonate.

Flow rates through the 5 g columns were usually maintained at 1.0–1.5 ml/min although where the same quantities of acids were used in larger volumes (1 litre), complete adsorption was obtained with a flow rate as high as 2.5 ml/min.

Elution was performed with sodium or ammonium carbonate, the latter being found preferable because of the absence of any contaminating cation spots on the developed paper chromatograms. Both alkalis at 1.0 *N* concentration will quantitatively elute all the acids investigated except succinic, of which the recovery obtained has only been approximately 90%. Ammonium carbonate is removed when concentrating the effluent fractions and this obviates the necessity for electrolytic desalting[5] or deionising with a cation exchange resin such as Amberlite IR120. Adsorption studies were performed on acid mixtures similar to the above to which sugar (glucose) in an amount fifty times that of the total acid concentration had been added, but the adsorption and elution characteristics of the resin columns were unaffected.

d. *Paper chromatography*

The conventional descending technique in a stainless steel tank was employed with Whatman No. 1 filter-paper (24″ × 24″). Extracts and standard mixtures were applied to the sheet 10 cm from the upper edge and the spots were allowed to dry completely before the sheets were placed in the tank to equilibrate with the aqueous phase of the solvent system. Complete delivery of the

References p. 476.

solution from a micropipette ensured exact replication of the volume applied (approximately 0.005 ml). The solvent system used mostly was made up in the following proportions — 75 ml redistilled mesityl oxide, 75 ml water and 36 ml 85% formic acid. R_F values of a number of acids in this system have already been reported[2]. Water-saturated butanol swamped with formic acid was sometimes employed. Eight hours run was allowed for qualitative analysis of the extracts by which time the mesityl oxide solvent front had descended approximately 35 cm. For quantitative determinations a much shorter run was allowed, usually 2.5–3 hours as the spots obtained were then very regular and quite discrete after development by spraying with an 0.04% alcoholic solution of brom-cresol-green.

Usually twenty-four spots were applied on a filter-paper sheet 24″ × 24″, each extract and control mixture being replicated four times. It is considered that four, and no less than three concentrations of the control mixtures should be applied to ensure a satisfactory reference curve and thus two or three different unknowns can be applied in quadruplicate on the same sheet. The optimum range of concentrations for individual acids should be from 1.0 to 10.0 mg/ml.

A few drops of strong ammonia solution added to the indicator before spraying assisted in producing a vivid contrast between the acid spots and the background. If the concentration of acid is low the spots will be temporarily obliterated but will reappear on standing. Good transmitted illumination is necessary for the spot delineation and an X-ray illuminator has been used for this purpose. The spots were then carefully cut out and weighed to the nearest mg, and in the case of standards acids, the mean weights plotted against the logarithm of the corresponding concentrations.

Even a small amount of inorganic salt present in the extracts causes sufficient tailing of the bands to interfere with quantitative determinations although giving good qualitative separations.

RESULTS

a. *Quantitative paper chromatography*

Each acid has its own characteristic line on a graph of the logarithm of concentration plotted against the mass of filter paper. This line does not necessarily have the same slope nor occupy the same position on the graph from chromatogram to chromatogram, consequently for each acid on every chromatogram a separate curve must be drawn. Such a curve was linear over a range of 1.0–10.0 mg/ml, although there was some evidence of a deviation from linearity at lower concentrations. The average standard error determined from the means derived from twelve chromatograms was $\pm$ 0.3 mg/ml. At lower concentrations this is quite a large percentage error, but if desired, the concentration of any acid in a mixture can be brought to a convenient value by concentration or dilution.

The accuracy of the method depends to a large extent upon the consistency with which the edges of the spots are marked, and it is, therefore, desirable that the operator obtain as much experience as possible in this aspect of the technique. Once skilled, it is possible that three points or, perhaps, even two, may suffice to give quite an accurate graph.

In Table I the quantitative estimations of various applied amounts of acid (between 0.63 mg/ml and 10.00 mg/ml) are given for five different chromatograms.

b. *Acid recovery from resin columns*

In Table II are set out the quantitative results on various acids obtained in the ammonium carbonate eluate of five separate experiments with the 5 g resin columns. Citric, malic and fumaric acids were quantitatively eluted, but succinic to an extent of only approximately 90%. The percentage recoveries of the first three acids have been generally in excess of 100% and it is considered that this may be attributed in part to the presence of inorganic ions causing slightly enlarged spots on the chromatogram. This aspect is at present under investigation and a technique being examined should eliminate the possibility of any such effect.

References p. 476.

TABLE I

ACCURACY OF PAPER CHROMATOGRAM DETERMINATIONS

Acid	*Concentration applied (mg/ml)*	*Concentration determined (mg/ml) Chromatogram No.*				
		1	*2*	*3*	*4*	*5*
Citric	5.00	4.60	4.80	5.50	5.00	5.10
	1.60	1.60	1.55	1.32	1.43	1.51
Malic	10.00	10.00	10.10	10.40	10.00	9.90
	5.00	4.90	5.40	4.85	5.05	4.90
	1.25	1.25	1.25	1.65	1.25	1.47
Succinic	10.00	9.95	10.50	9.70	9.90	9.80
	5.00	4.65	4.80	4.45	5.05	5.00
	1.25	1.17	1.30	1.42	1.35	1.48
Fumaric	2.50	2.50	2.35	2.50	2.50	2.50
	1.25	1.25	1.22	1.31	1.22	1.25
	0.63	0.63	0.59	0.76	0.64	0.66

TABLE II

ACID RECOVERY (%) FROM RESIN COLUMNS

Acid	*Experiment No.*					*Mean*
	1	*2*	*3*	*4*	*5*	
Citric				113	113	113
Malic	108	110	110	105	95	106
Fumaric	111	110	106			109
Succinic	91	87	98	91	83	90

c. *Organic acid analysis of carrot tissue*

In all carrot roots analysed malic acid was by far the greatest contributor to total acidity, but succinic and fumaric were also usually present in appreciable quantities. Three other acids were detected, but these have not as yet been positively identified. In different carrots the concentrations of malic, succinic, *iso*citric and fumaric have been found to vary considerably, but values for a typical root are given in Table III. During these investigations citric and *iso*citric acids were separable, but subsequently with different batches of paper this separation could not be repeated.

d. *Organic acid analysis of apple tissue*

Malic acid constituted virtually the total acidity of the "Statesman" apple. There is an interesting gradient of this acid (on a dry weight basis) in the three different shells of apple tissue. The first and second shells contained practically the same concentration, but the innermost shells had an acid content nearly twice as large as the others. The values given are the mean of two fruits.

References p. 476.

TABLE III

ACIDS DETECTED IN PLANT TISSUE EXTRACTS

(Mg acid per g dry weight of tissue)

R_F (Mesityl oxide system)	R_F (n-butanol system)	Acid	Apple extracts			Carrot extracts
			Outer	Middle	Inner	
0.31	0.30	*Iso*citric	trace	trace	trace	3.9
0.31	0.37	Citric	—	—	—	trace
0.39	0.45	Malic	21.8	21.8	42.5	32.0
0.48	0.50	Unknown	—	—	—	trace
0.54	0.55	Unknown	—	—	—	trace
0.60	0.65	Unknown	trace	trace	trace	1.8
0.70	0.74	Succinic	trace	trace	trace	6.5
0.82	0.84	Fumaric	—	—	—	0.6

DISCUSSION

The general acceptance by plant physiologists that KREB's "Carboxylic Acid Cycle" operates as such, or in a modified form, in plant tissues and the roles ascribed by BENSON *et al.*[1] to organic acids in photosynthesis have directed considerable attention to their identification and determination.

The main obstacle to the use of partition chromatography in this field has been the difficulty of separating the acids in tissue extracts from such interfering materials as sugars, colouring matter and inorganic cations. This difficulty has largely been overcome by the use of ion-exchange resins.

With a simple method for quantitative determinations as has been described, the use of partition chromatography should extend to the investigation of many problems confronting plant physiologists. For instance, in this laboratory the changes occurring in type and concentration of acids in carrot discs treated with malonic acid are being investigated and some significant features in the action of the inhibitor have been discovered. Whilst the inhibition of oxygen uptake of carrot slices from different sources was similar, subsequent analysis indicated qualitative differences in their organic acid compositions.

SUMMARY

A method of estimation of certain organic carboxylic acids has been developed by utilising a paper chromatography technique relating concentration to the weight of paper occupied by the spot. The method has been used in the analysis of apple and carrot tissues, the extracts of which have been freed from sugars and pigments by passage through exchange resin columns.

RÉSUMÉ

Nous avons dévéloppé une méthode d'évaluation de certains acides organiques carboxyliques. Cette méthode est basée sur une technique de chromatographie sur papier, où la concentration est mise en rapport avec le poids du papier occupé par la tache. Nous avons appliqué cette méthode à l'analyse de tissus de pomme et de carotte dont les extraits avaient été libérés de sucres et de pigments par le passage à travers des colonnes de résines échangeuses d'ions.

References p. 476.

ZUSAMMENFASSUNG

Es wurde eine Bestimmungsmethode für gewisse organische Carbonsäuren ausgearbeitet, die die papierchromatographische Technik benützt und die die Konzentration in Beziehung zu dem Gewicht des von dem Tüpfel beanspruchten Papiers bringt. Die Methode wurde bei der Analyse von Apfel- und Karottengeweben angewandt, deren Extrakte von Zuckern und Pigmenten durch das Durchlaufen durch Harzaustauschersäulen befreit wurden.

REFERENCES

1 A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS AND W. STEPKA, *J. Am. Chem. Soc.*, 72 (1950), 1710.
2 F. BRYANT AND B. T. OVERELL, *Nature*, 167 (1951) 361.
3 R. E. BUCK AND H. H. MOTTERN, *Ind. Eng. Chem.*, 37 (1945) 635.
4 W. CHALLINER AND M. E. KIESER, *Nature*, 161 (1948) 1023.
5 R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 41 (1947) 590.
6 R. B. FISHER, D. S. PARSONS AND G. A. MORRISON, *Nature*, 161 (1948) 764.
7 H. A. KREBS AND L. V. EGGLESTON, *Biochem. J.*, 38 (1944) 426.
8 R. KUNIN AND F. X. MCGARVEY, *Ind. Eng. Chem.*, 41 (1949) 1265.
9 J. W. H. LUGG AND B. T. OVERELL, *Aust. J. Sci., Res.* A. 1 (1948) 98.
10 R. L. REID AND M. LEDERER, *Biochem. J.*, 50 (1951) 60.
11 K. V. THIMANN AND W. D. BONNER, *Ann. Rev. Plant Physiol.*, 1 (1950) 75.
12 U.S. Department Agriculture, Information Sheet A.I.C. 14 (1943).

Received August 15th, 1952