APPLICATION OF THE LIQUID EXTRACTION METHOD FOR THE DETERMINATION OF TOTAL ORGANIC ACIDS IN PLANT SAP[1]

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(WITH ONE FIGURE)

Introduction

The method of PUCHER, VICKERY, and WAKEMAN (**6**) for the estimation of total organic acids by titration in tobacco leaf tissue has proved useful in studies of organic acid metabolism. The method described by PUCHER and co-workers was devised for the extraction of organic acids from dried plant tissues. The present paper describes the application of the liquid extraction method for the titrimetric estimation of total organic acids in expressed plant sap with a discussion of the advantages offered by, and problems arising from, such a procedure. The method is based on the observation that the organic acids can be directly extracted by ether in an efficient liquid extraction apparatus, provided the sap is previously acidified with sulphuric acid (**10**).

Description of apparatus

A sketch of the apparatus is shown in figure 1. The continuous ether liquid extractor is designed from a Soxhlet extractor as described by VICKERY and PUCHER (**10**). The apparatus is fitted with ground glass joints to make the ether extraction more efficient. The condenser (B, fig. 1) is built with a double condenser unit to economize on solvent etc., as described in a recent publication by YIP (**13**). A separate vial (C, fig. 1) having a height[2] suitable for the aliquot desired is placed into the Soxhlet (A, fig. 1). A funnel (D, fig. 1) having small apertures at the bottom is placed in position inside the vial. The extraction flask (E, fig. 1) contains 100 ml. of H_2O_2-free ether. Heat is supplied by a constant temperature water bath maintained at a temperature of 50° C. It is desirable that the temperature be sufficiently high to adequately resupply solvent (after condensation) for rapid, continued extraction. The condensed ether dropping into the funnel causes a pressure head to be developed. The solvent is forced through the small openings of the funnel at the bottom of the vial removing the organic acids as it passes through the liquid to be extracted. The stream of ether globules produces enough agitation of the liquid to insure stirring at all

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[2] It is important that the height of the ether layer be relatively short so that the extracting solvent may be rapidly replaced by the incoming flow of fresh ether. The rate of partition-extraction is thus enhanced.

times. Extraction is continued for a period of 20 to 24 hours to insure completeness. For plant tissues containing organic acids slightly soluble in ether, the time limit may have to be extended (see table I, sample 11).

Method and results

Barley plants were grown according to the method of HOAGLAND and BROYER (**4**). Sap was expressed from frozen excised barley roots by the

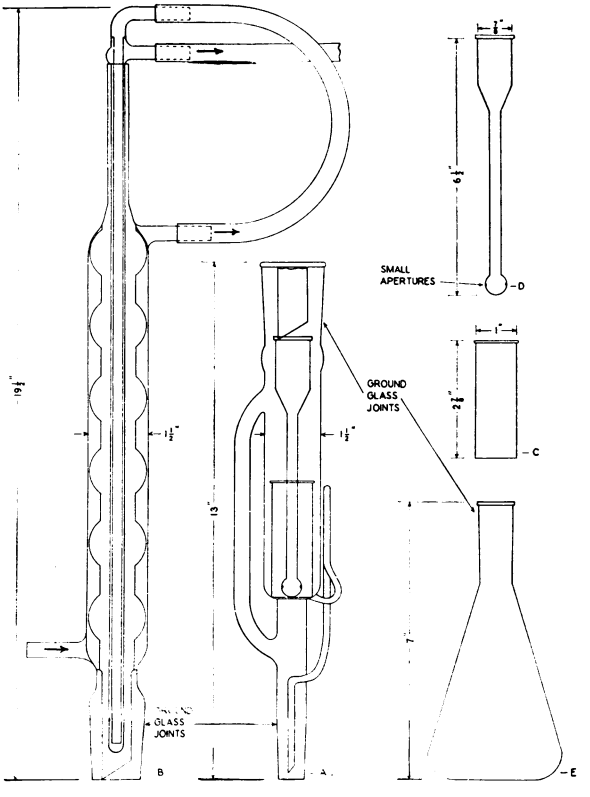

FIG. 1. Sketch of apparatus for liquid extraction of ether soluble organic acids. A. Soxhlet extractor with standard ground glass joints. B. Double consender unit with standard ground glass joint. C. Glass vial. D. Funnel unit consisting of a funnel and attached Folin bulb. E. Receiving flask with standard ground glass joint.

experimental technique of BROYER and HOAGLAND (**1**). As has been pointed out in another paper (**2**), certain methods of pretreatment are considered as unsatisfactory in organic acid estimations. Although in the present study barley root saps were employed, the method may also be applied to plant tissues.

A suitable aliquot of fluid, 10 to 15 ml., is acidified to pH 1 with sulphuric

acid[3] and placed in the liquid extraction vial within the Soxhlet. The funnel is inserted, receiving flask attached, and the extraction started. A blank is conducted with distilled water, parallel with the organic acid sample, being refluxed for the same period of time, and subsequently carried through the same series of operations.

After the extraction is completed, 25 ml. of CO_2-free water is added to the receiving flask. The contents are gently agitated to distribute the acids into the aqueous phase. The ether is carefully distilled off under reduced pressure at approximately 50° C. and condensed for re-utilization. The aqueous solution is transferred to a 100-ml. beaker and diluted to approximately 50 ml. with CO_2-free water. With the aid of a glass electrode, the extract is titrated between the pH limits 7.8 and 2.6, according to the principle of the method of VAN SLYKE and PALMER (**9**). If sulphuric acid is used in the titration the resultant mixture may be subsequently used for the individual determination of the component organic acids.

Calculations

The titration value obtained for the extract necessitates a correction factor owing to the fact that none of the common barley root acids are titrated to the extent of 100 per cent. (**6**, p. 141). A correction factor of 1.05 was calculated from the dissociation constants of the known organic acids in barley root sap between the two given pH limits selected. The total acidity[4] was expressed in terms of milliequivalents per gram fresh weight of original plant tissue.

In the presence of oxalic acid it is necessary to carry out an independent determination of oxalic acid in order to correct the final result (**11**), since oxalic acid behaves as a monobasic acid under the conditions described. A similar correction would be necessary in the presence of large amounts of phosphoric or tartaric acids; and to a lesser extent nitric acid. (The amounts of oxalic and phosphoric acids found in barley root sap did not necessitate a correction factor.)

For certain experimental treatments organic acid factors might have to be estimated for each individual treatment. This would be necessary if and when the individual acids involved are differentially modified (**3**). Under such conditions, because of the laborious procedures involved in separating and estimating individual organic acids, a method not involving acid corrections might prove more suitable. Such a method has been proposed by

[3] A highly ionized mineral acid is employed, since in the final titration traces of these acids will be present as the salts of strong electrolytes and will not alter the titration value for total organic acids between pH 7.8 and pH 2.6 (**9**, p. 570).

Sulphuric acid is selected rather than hydrochloric acid since the latter has been shown experimentally to convert glucose to organic acid on heating (**12**, p. 808).

[4] Any carbonic acid in the original sample is completely expelled during the extraction.

ULRICH (**8**). As pointed out elsewhere, however, such a procedure may be subject to other, more serious limitations. Under the experimental conditions so far studied, the individual acids have been modified in similar manner, so that a correction factor established for the control material may be satisfactorily applied to the experimental treatments. It may be noted that the procedure of direct titration of ether-soluble non-volatile organic acids (**8**) would include the sum of the equivalents of sulphate, phosphate, and nitrate present in the material.

In the material tested, ether-soluble organic bases occurred in extremely small amounts and did not significantly influence the results. If other plant

TABLE I

TOTAL ORGANIC ACIDS IN EXPRESSED SAP OF EXCISED BARLEY ROOTS

SAMPLE NO.	ORGANIC ACIDS IN MILLIGRAM EQUIVALENTS × 10^2 PER GRAM OF FRESH TISSUE	
	LIQUID EXTRACTION METHOD	BUFFER TITRATION CURVES pH 7.8 TO 2.6
	mg. eq. × 10^2	*mg. eq.* × 10^2
1	3.44 3.30 3.32	3.60
2	3.76 4.10 4.13	4.02
3	3.37 3.30	3.29
4	4.75 4.77	4.56
5	3.40 3.40	3.62
6	2.40 2.38	2.09
7	3.71 3.80	3.04
8	4.18 4.19	4.22
9	4.00 4.00	4.34
10	4.43 4.60 4.77	4.50
11	6.11* 6.10 6.09	6.36

* Extraction time, 56 hours. An average value of 4.3 was obtained by the normal 24-hour treatment.

tissues are studied a minor modification of the correction factor may be necessary.

In order to show the applicability of the method, these organic acid estimates were compared with results obtained on comparable samples of sap by calculating total organic acids from buffer titration curves between the pH limits of 7.8 and 2.6. The results of the two independent determinations are presented in table I. Replicate determinations by the liquid extraction method were generally satisfactory. Favorable agreement was obtained between the two methods compared. These facts indicate that this liquid extraction procedure yields a valid quantitative estimate of the total organic acids present in the sample.

Discussion

PUCHER and VICKERY (**7**) have described a mechanically operated continuous liquid extraction apparatus. This equipment affords certain advantages such as reduced fire hazard, direct extraction of large volumes of fluids, and use of solvents (ethyl acetate) more efficient than ether in extracting organic acids. The extract obtained, however, is not applicable for subsequent acid titration (**7**, p. 657). The method herein applied incorporates essentially all of the features (aside from fire hazard, which can be minimized in other ways (see fig. 1)) offered by the Widwark apparatus, as modified by PUCHER and VICKERY. The present technique presents no difficulty with respect to titration errors arising from solvent decomposition.

It should be pointed out that all methods so far proposed for extraction and titration of total organic acids under various metabolic treatments, entail certain assumptions. The direct titration of sap, obtained from tissues after adequate freezing and pressing, equal to the sum of the individual titers between the pH of the original sap and about pH 2.5 and pH 8.0, respectively, yields an acid equivalent value which will serve at least as an approximation of the total organic acids. Under reasonably controlled conditions such values may serve adequately to interpret certain generalized metabolic changes (**5**). In order to estimate more carefully the total organic acids, a separation and estimation of the individual acids, in order to apply a satisfactory correction on the total, is necessary. The technique herein used allows for the application of these various procedures on an individual aliquot of unknown material.

The liquid extraction method for the determination of total organic acids has several advantages not offered by methods involving dehydration (**6, 8, 11**) of plant tissues or fluids. The organic acids are extracted directly and immediately, thus insuring maximum stability of these compounds. This may prove valuable in studies of organic acid metabolism involving unstable intermediates, in that there is no time lag for the activity of microorganisms to become important. The direct extraction minimizes any possible loss of

volatile organic acids. The design of the apparatus permits the use of a wide range of aliquots, within semi-micro quantities. The use of a vial prevents siphoning over of impurities, including fixed acids, encountered with thimble extraction. This method, besides being theoretically sound, is quicker than others which require a lengthy preparation of the sample. As previously indicated, the method may be applied to fluid from other types of tissues with a possible modification of the correction factor. It is suggested that fresh tissues may be directly used, since ether will denature proteins and destroy the permeability of the cells, thereby causing a release of organic acids.

Summary

1. The liquid extraction method for the determination of organic acids is reviewed and a design of the apparatus given.

2. The method has been successfully applied to excised barley root sap.

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