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Determination of Organic Acids in Foods by Liquid Chromatography

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A simple and rapid method for the determination of organic acids in foods by high efficiency anion exchange chromatography is described. Separation of microequivalent amounts of up to 15 of the commonly occurring food acids can be completed in about 75 min. These acids include Krebs cycle acids (citric, isocitric, α-ketoglutaric, succinic, fumaric, malic, and oxalacetic), alicyclic acids (quinic, shikimic), and various other acids (acetic, galacturonic, lactic, malonic, oxalic, and tartaric). Separation of the predominant acids in particular food samples often requires only 20 to 40 min. A special precolumn injection system sharpens the acid peaks and makes possible the analysis of liquid samples (fruit juices, acidulated beverages, etc.) or aqueous extracts with little or no pretreatment. Use of a differential refractometer detector simplifies the quantitative measurements and facilitates collection of effluent samples for confirmation of identity.

A considerable number of organic acids occur in foods and they are often major constituents of plant foods. More than 36 acids are reported to occur in fruits, for example (Hulme, 1970). These acids often influence flavor, stability, and keeping quality and have been proposed as an index of maturity, ripeness, or spoilage in some foods. Certain organic acids (citric, fumaric, lactic, malic, and tartaric) are commonly added to foods as acidulants or flavor modifiers. Organic acids may also be produced during fermentations or other processing operations.

Data on the organic acids in foods are increasingly required by the food industry for quality control to meet legal requirements and as labeling information. Similar data are also required by nutritionists, biochemists, and food scientists during research on the metabolism and function of the organic acids. The "official" chemical methods for organic acids in foods (AOAC, 1970) are simply too time-consuming for most purposes and, in any case, "official" methods are available for only a few acids.

A number of chromatographic methods have been developed for determining organic acids in biological samples. Partition chromatography on silica gel [first utilized for foods by Isherwood (1946)] and anion exchange chromatography [as originally proposed by Busch *et al.* (1952)] have emerged as the methods of choice, since other methods such as gas chromatography have significant disadvantages (Stahl *et al.*, 1972). Numerous applications or adaptations of these two techniques have been reported (Bengtsson and Samuelson, 1969, 1971; Kesner and Muntwyler, 1969; Palmer, 1955; Palmer and Wyman, 1965; Von Korff, 1969). However, these are slow and cumbersome procedures, at least in relation to recent developments in high efficiency liquid chromatography (Kirkland, 1971). Separation of nanoequivalent quantities of organic acids has been accomplished in high efficiency systems by both partition (Stahl *et al.*, 1972) and anion exchange chromatography (Bengtsson and Samuelson, 1971). These procedures apparently have adequate resolution only at the nanoequivalent level, thus limiting the sample size and requiring the use of rather complicated photometric detection systems. The ion exchange system also requires up to 16 hr to separate the commonly occurring organic acids. For most food analyses (and indeed for many other biological samples) simplicity, speed, reliability, and ease of sample preparation are more important criteria than ultimate sensitivity.

The general approach in this study has been to adapt the anion exchange procedure of Palmer (1955) to a high efficiency system, including the use of fine, spherical resins and a differential refractometer for detection. The resulting method combines simplicity and minimum sample preparation with adequate speed, sensitivity, and precision for food analysis. The method appears to have application to the determination of organic acids in other biological systems.

EXPERIMENTAL SECTION

Liquid Chromatography. Following is a list of instruments, materials and conditions used in the liquid chromatographic operation. Waters Associates ALC-100, differential refractometer detector, sensitivity 1×10^{-7} refractive index units; attenuation 2–16. Texas Instruments Servo/riter II recorder, 50 mV full scale, chart speed 0.15 in./min. Columns: precolumn, length 3.6 cm; separation columns, length 75–90 cm; all columns of 316 stainless steel, ¼ or ⅜ in. o.d. by 0.028-in. wall thickness. Column

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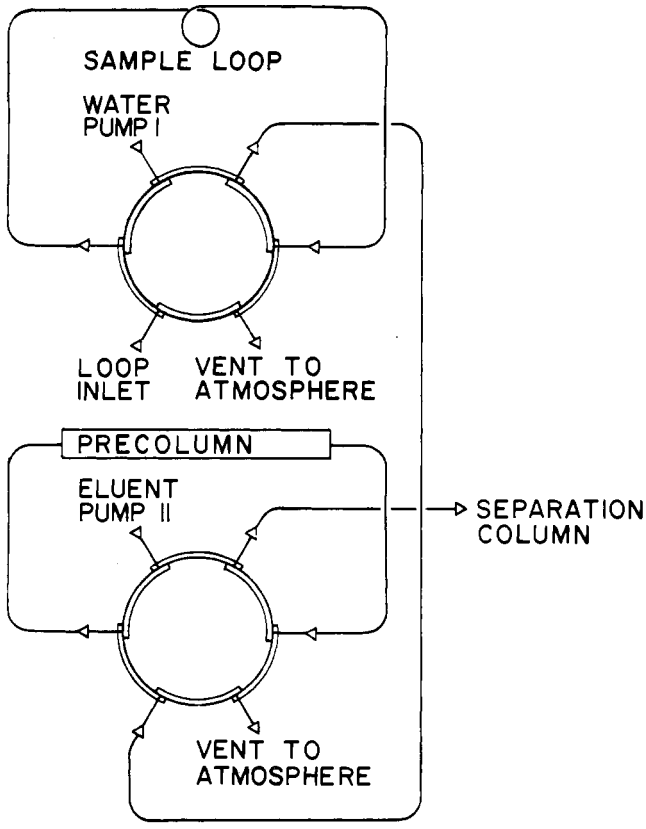

Figure 1. Flow diagram for the loading and preliminary purification of organic acids.

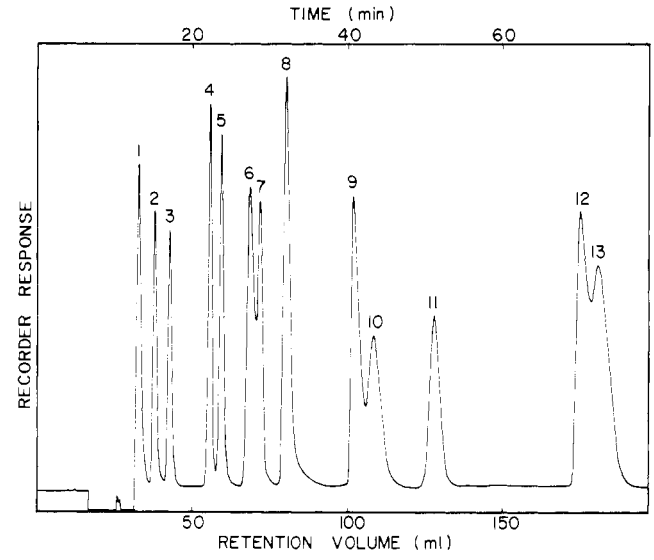

Figure 2. Typical chromatogram of a multicomponent mixture of organic acids. Eluent, 1.0 *N* sodium formate; separation column, 75 cm, 3/8 in. o.d.; flow rate, 150 ml/hr. See text and Table I for other data.

packing: Aminex A25 (Bio-Rad Laboratories, Richmond, Calif.), a strongly basic anion exchange resin, particle size 17.5 ± 2 μ, converted to formate form and packed wet. Column temperatures: precolumn, 25°; separation column, 70°. Eluent: sodium formate, usually 1.0 *N*. Flow rate: 60 to 150 ml/hr at pressures up to about 500 psi.

Acid Standards. Organic acids of the highest purity available from Calbiochem were used (San Diego, Calif.). No acidic impurities were ever detected during our analyses. Standard solutions were prepared in water.

Food Samples. Juice, beverages, and aqueous extracts were filtered (if necessary) to remove any insoluble material.

Procedure. The aqueous samples were injected *via* a precolumn system (Figure 1). A sample loop of known volume (0.5–2.0 ml) was filled with sample (utilizing a detachable 10-ml syringe) and the loop contents were pumped to the precolumn with Pump I. The acids were retained at the top of the column. Neutral and basic components were washed out by pumping water through the precolumn for a minimum of 12 min at about 1.2 ml/min. The precolumn was then connected to the top of the separation column and Pump II was utilized to pump sodium formate eluent through the precolumn and then through the separation column.

After appearance of the negative water peak on the recorder, the precolumn was disconnected from the separation column, and elution of the separation column continued to complete the desired separation. During the course of the separation, the precolumn was again washed with water for at least 12 min at about 1.2 ml/min and the next sample was loaded onto the precolumn, as described above. All connections and disconnections of the pumps and columns were accomplished rapidly with six-way valves, as indicated in Figure 1. The precolumn pump (Pump =I) is not standard equipment on the liquid chromatograph. It could be replaced with a pressurized reservoir of water if a second pump were not available.

RESULTS AND DISCUSSION

Figure 2 shows a typical separation of a mixture of 13 organic acids injected as an aqueous solution *via* the sample loop–precolumn system and eluted with sodium formate. Table I identifies the acids of Figure 2 and provides data on column efficiency and resolution. The peaks were generally quite sharp and baseline separation was achieved in most cases, 75 min being required for the complete run. Among the acids studied, *cis*-aconitic and phosphoric were the only acids which consistently failed to yield measurable peaks; they both yielded flat, barely discernible peaks with maximum height at the retention volumes indicated in Table I. The pairs malic–malonic and *cis*-aconitic–isocitric were the only acids not resolved in this system.

The most significant peak overlap in Figure 2 is that between malic and citric acids, since those acids commonly occur together as the predominant organic acids in plant foods. However, the resolution (Kirkland, 1971) of malic and citric acids can be increased from 0.44 to an adequate 1.04 by increasing the eluent concentration from 1.0 to 1.05 *N*. This decreases the resolution of the acetic–lactic pair from 1.45 to 1.05, which is still a satisfactory separation. Malic and citric acids can be completely separated with an eluent concentration of 1.1 *N* or somewhat higher. However, citric and lactic acids form a single peak under these conditions and the higher eluent concentrations can only be used where lactic acid occurs in negligibly small amounts. This is the case for most plant tissues.

Such adjustments of eluent concentration make it possible to adapt the method to virtually any sample. In practice, some prior knowledge of the approximate organic acid composition of samples is generally available, which enables selection of the appropriate eluent concentration. Many food samples contain only one or at most five predominant acids and the separation time can be shortened to 20 to 40 min. If information on the organic acids is lacking, the unknown sample can be eluted with 1.0 *N* sodium formate, the identity and purity of the acids responsible for the peaks can be checked by paper, thin-layer, or gas chromatography, and the eluent concentration can be adjusted accordingly. The nondestructive refractometer detector makes collection of peak fractions a simple matter. If it will interfere in the subsequent chromatography, the sodium formate in the collected samples can be converted to formic acid by treatment with a cation exchang-

Table I. Chromatographic Data from Organic Acid Separation of Figure 2

Peak number, Figure 2	Acid	Amount injected, micro-equivalents	Net retention volume, V_N, ml	Peak width, W, ml	Theoretical plates, N	Effective theoretical plates, N_{eff}
1	α-D-Galacturonic	2	15.8	3.00	1710	580
2	D-Quinic	1	21.1	2.45	3590	1190
3	Shikimic	1	25.8	2.70	3730	1460
3A	(Phosphoric)[a]		28.7	[b]		
4	Acetic	20	39.1	2.55	7210	3700
5	D(—)-Lactic	5	42.5	2.90	6390	3430
6	Citric	10	51.4	5.15	2700	1600
7	(—)-Malic	5	54.8	3.55	6220	3780
7A	(Malonic)[a]		55.5	3.76	5700	3480
7B	(Succinic)[a]		61.3	4.03	5810	3700
8	Tartaric	10	63.4	4.40	5100	3290
9	Oxalic	20	84.5	5.40	5470	3910
10	D,L-Isocitric	20	90.9	7.35	3370	2460
10A	(*cis*-Aconitic)[a]		91.0	[b]		
11	Oxalacetic	30	110.5	6.55	5950	4580
12	Fumaric	10	158.6	8.51	6690	5550
13	α-Ketoglutaric	15	159.3	9.55	5340	4430

[a] The acids in parentheses and labeled 3A, 7A, etc., were not included in this particular run. The data given were calculated from comparable analyses of other mixtures containing these acids. [b] Broad, flat peaks, often with considerable tailing.

Table II. Precision of Organic Acid Analyses[a]

Sample	Acid	Mean concentration ± standard deviation, mequiv/l.	Coefficient of variation, %
Standard mixture	Quinic	1.99 ± 0.024	1.2
	Malic	9.78 ± 0.14	1.4
Grape juice	Malic	48.8 ± 0.55	1.1
	Tartaric	41.5 ± 1.20	2.9

[a] Calculated from peak height measurements of ten repeated injections of 0.84 ml of each sample. Chromatographic conditions as in Figure 3.

er in the hydrogen form and the solution evaporated to dryness to remove most of the formic acid.

The results with different eluent concentrations suggest that the best approach for complete resolution of all peaks would be an increase in column length, thus increasing the number of theoretical plates. Contrary to the data of Bengtsson and Samuelson (1969), substitution of magnesium ion for sodium ion yielded inadequate separation of the acids.

Table I shows the amount of each acid (in microequivalents) responsible for the peaks in Figure 2 and indicates the markedly different response of the detector to the different acids. This differential response can be as high as 50-fold, and standard curves must be prepared for each acid to be analyzed. For individual acids injected in a 2-ml volume, the standard curves are linear over at least a 50-fold range in concentration, ranging from about 1/10 of the amount indicated in Table I up to about 120 microequivalents. Peak tailing becomes a problem above 120 microequivalents, especially with the acids eluted after tartaric acid. Peak height measurements are adequate for the acids eluted before oxalic; the remaining acids exhibit somewhat nonlinear isotherms and peak area is a more reliable correlation with concentration. Any variations in the day to day response are easily determined by injection of an appropriate standard mixture.

A significant feature of the present method is the use of a precolumn. It serves two essential purposes. First, the total acidity of samples (up to about 250 microequivalents) can be accumulated and subsequently transferred to the separation column as a narrow band. This results in sharper peaks, thus increasing both resolution and sensitivity. Since there is no limitation to sample volume, solutions containing trace amounts of acids can be analyzed. Second, the precolumn provides the necessary cleanup of the sample by allowing removal of all potentially interfering neutral and basic substances, including most pigments.

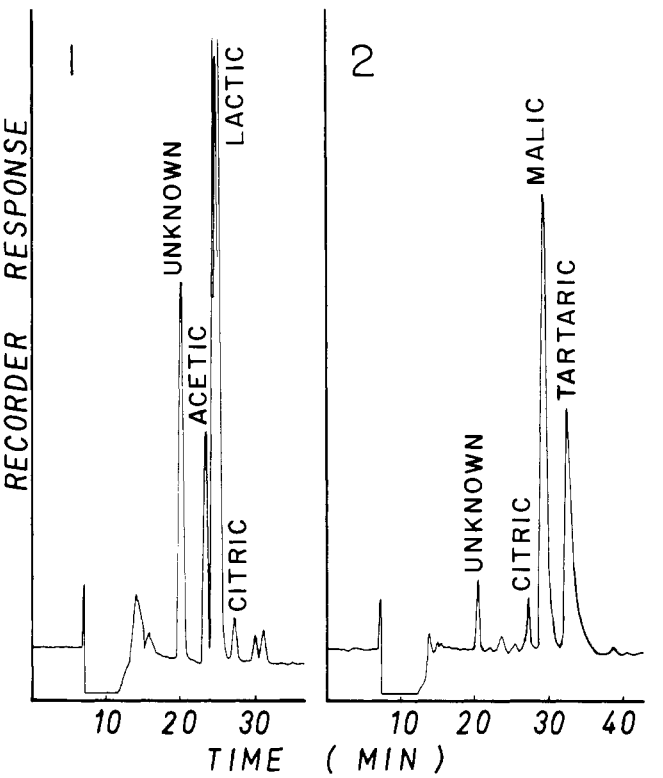

Figure 3. Separations of organic acids in juices. Samples: (1) sauerkraut juice; (2) grape juice. Eluent, 1.0 *N* sodium formate; separation column, 90 cm, 1/4 in. o.d.; flow rate, 62 ml/hr.

Separation was carried out at 70° because the organic acid peaks were markedly sharpened as the temperature was raised from 25°. Higher temperatures were not employed because of rapid deterioration of the column packing, presumably due to breakage of cross-linkages and conversion of ammonium to amino groups (Bengtsson and Samuelson, 1971; Marinsky, 1966). Even at 70° there was

some loss of theoretical plates within 6 weeks. However, the useful life of a column was at least 4 months, if the upper 1 cm of deteriorated and irreversibly loaded resin in both precolumn and separation column was replaced whenever a decrease in resolution of the standard mixture was apparent.

In preliminary studies, excellent separation of the predominant organic acids in a series of beverages, fruit juices, cider and wine vinegar, and sauerkraut juice was obtained. The acid peaks were sharp, the baseline was stable, sensitivity was more than adequate (often requiring 5- to 20-fold dilution to bring samples into the normal working range), and the identity and quantity of the predominant acids found in the samples were consistent with data in the literature or on the labels. Figure 3 shows typical separations of two juice samples.

Preliminary studies of the chromatographic method (Table II) indicate excellent precision. For complex mixtures of standard acids (as in Figure 2) the standard deviation for repeated injections did not exceed ±3%, except for citric and malic acids. For this incompletely resolved pair (1.0 N sodium formate eluant), the standard deviation approached ±15%, when the concentration of one of the acids was four times the other. Further studies are underway to establish the precision and accuracy of the method for determining the predominant acids in a wide variety of liquid and solid food samples, especially in comparison with "official" methods. The anion exchange method will also be tested as a means for determining the complete profile of acids, including trace components, in various plant and animal tissues, since the method appears to have potential for application in metabolic studies.

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The authors are indebted to Walter Brandes, who carried out preliminary studies on the feasibility of the described method.

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Method of Extrapolation for Yield-Decay-Type Data

Edward J. Robel

A method employing the use of polynomials has been devised for the extrapolation of yield-decay data to obtain original or true yield values (A_0). The method was applied to amino acid data and performs effectively with hydrolysis and loss rates that cause inaccuracy with conventional methods of extrapolation because of significant yield losses, which occur before the entire amount (A_0) is hydrolyzed. Once the method is programmed, data can be handled simply on a small desk computer.

The limited accuracy of amino acid analysis was established prior to Robel and Crane (1972) for correcting amino acid losses during hydrolysis (Hirs *et al.*, 1954; Mahowald *et al.*, 1962; Noltmann *et al.*, 1962; Smith and Stockell, 1954; Smith *et al.*, 1954). A recent report (Robel and Crane, 1972) presents a method of extrapolation for determining true or original amino acid amounts at zero hydrolysis time (A_0) and compares its usefulness over established methods of calculation—the logarithmic method with least squares linear regression analysis or semilogarithmic plotting to zero time. However, the new method (Robel and Crane, 1972) requires a large computer to correct to zero hydrolysis time. In some locations, processing data in this manner may not be possible. This report presents a method of extrapolation requiring only a desk computer and illustrates the applicability and accuracy of this method using practical hydrolysis rates and loss rates encountered with protein hydrolysates.

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METHODS AND MATERIALS

Method Application. The method for estimating A_0 values was applied to artificial error-free "data" and run on an Olivetti Underwood Programma 101 electronic desk computer. The "data" were generated using eq 4 (Robel and Crane, 1972):

$$B(t) = \frac{A_0 h}{h - l}(e^{-lt} - e^{-ht})$$

After the program equations were formed, time and corresponding yield data were entered in the computer and true yield values (A_0) were obtained.

Derivation of Equations. The polynomial method for estimating the original amino acid content of protein utilizes data to include a hydrolysis range spanned by two curvilineal fits. The residue yield plus destruction is the resultant curve from zero to maximal yield time (first curvilineal fit) and the resultant curve from maximal yield time to infinite time (second curvilineal fit). The calculations, therefore, involve a hydrolysis range spanned by two curvilineal fits. The maximal yield values determined from the first curvilineal fit, added to the amounts of de-