Improvement in the Reproducibility of the Enzymatic Analysis of Sucrose in Stored Golden Delicious Apples

The precision of the enzymatic analysis of sucrose in stored Golden Delicious apples is improved by removing the natural glucose.

The coefficients of variability for sucrose content (Golden Delicious apples, harvest 1971) after 195 and 216 days of storage were respectively ±18.0 and ±16.3% (Gorin, 1973). These high coefficients were explained by the high relative content of natural glucose to sucrose. Original glucose was not destroyed because the ratio of glucose to sucrose was lower than 5:1 (Boehringer Mannheim GmbH, 1971).

When estimating sucrose enzymatically as previously described (Gorin, 1973) but with stored apples of harvest 1972, natural glucose was removed with glucose oxidase and catalase (Boehringer Mannheim GmbH, 1971) from the moment that the coefficient of variability became higher than ±10%. This point was chosen because glucose was no longer analyzed and therefore the ratio of glucose to sucrose was unknown.

Table I. Estimation of Sucrose in Golden Delicious Apples,[a] Harvest 1972, after Removing Natural Glucose

	$\bar{x}$	$s \pm$	CV, %
	62 Days of Storage[b]		
Sample I	1.21	0.02	1.3
Sample II	1.45	0.03	2.2
Sample III	1.29	0.03	2.7
	90 Days of Storage[b]		
Sample I	1.06	0.03	2.8
Sample II	1.12	0.01	1.1
Sample III	1.41	0.01	0.8

[a] Apples were purchased from the same fruit grower (Puiflijk, Netherlands) as those of 1971. Values are expressed as grams/100 g fresh weight. They are not corrected for initial fresh weight, *i.e.*, in respect to zero time storage. $\bar{x}$ = average of three estimations; $s = (\Sigma d^2/n - 1)^{1/2}$; CV (%) = $s100/\bar{x}$. [b] Storage at 4° in a controlled atmosphere (1973) (Gorin, 1973).

Table I records the data of sucrose enzymatically estimated after removing natural glucose. Without this step the coefficient of variability was higher than ±10% as was confirmed with apples stored for 62 days.

From Table I we may conclude that the precision (Fischer and Peters, 1968; Crockford and Nowell, 1956) of the enzymatic analysis of sucrose is improved by removing natural glucose.

In addition we would recommend applying this procedure to Golden Delicious apples before the ratio of glucose to sucrose becomes 5:1.

ACKNOWLEDGMENTS

We wish to thank William Klop for valuable discussions.

LITERATURE CITED

Boehringer Mannheim GmbH, Food Analysis, Sucrose, Western Germany, 1971.
Crockford, H. D., Nowell, J. W., "Laboratory Manual of Physical Chemistry," Wiley, New York, N. Y., 1956, p 7.
Fischer, R. B., Peters, D. G., "Basic Theory and Practice of Quantitative Chemical Analysis," 3rd ed, W. B. Saunders, Philadelphia, London, Toronto, 1968, pp 75-93.
Gorin, N., *J. Agr. Food Chem.* **21**, 670 (1973).

Natalio Gorin*
Hendrik Zonneveld

Sprenger Instituut
Wageningen, The Netherlands

Received for review November 19, 1973. Accepted March 15, 1974.

Determination of Sucrose, Glucose, and Fructose by Liquid Chromatography

A simple and rapid method for the determination of sucrose, glucose, and fructose in plant foods by elution with water from a cation exchange resin and detection with a differential refractometer is described. The separation of the three sugars in deionized juices or food extracts can be completed in as little as 10 min and microgram quantities of the individual sugars can be measured. Examples of application to food products are shown. The method can also be utilized for determination of a limited number of other sugars.

Plant foods are primary sources of free sugars in the diet. Sucrose, glucose, and fructose predominate in plants, other sugars seldom occurring in more than trace quantities. Thus, the food scientist and nutritionist, as well as the plant biochemist, often wish to determine these three sugars in plant tissues. Recent evidence that the ingestion of particular sugars encourages development of dental caries and possibly cardiovascular disease provides additional impetus for measuring the dietary intake of individual sugars.

There is a scarcity of data on the concentration of individual sugars in plant foods. The most extensive compilation available is that of Hardinge *et al.* (1965). These authors present the data as a "temporary expedient," recognizing the lack of uniformity in sampling and analytical techniques. Nor is there any information on the effect of variety, origin, maturity, or storage, except to state that the samples were fresh foods in a stage of maturity as commonly eaten. Additional data on sugars in fruits and vegetables have become available recently. Dako *et al.* (1970) used enzymatic methods to determine sucrose, glucose, and fructose in 14 different fruits, and to show the

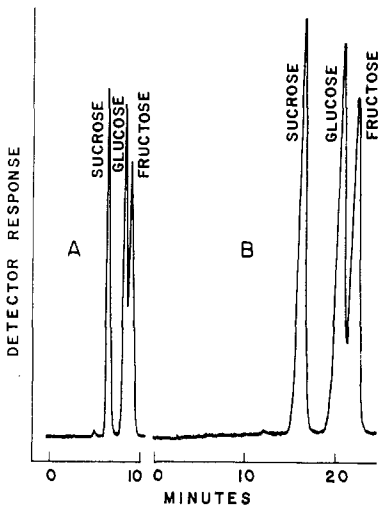

Figure 1. Separation of sucrose, glucose, and fructose at (A) 1.85 ml/min and (B) 0.85 ml/min; sample, 20 μl of standard solution containing 7.5 mg/ml of each sugar; sensitivity, 100 mV full scale.

marked influence of variety, ripeness, and sample origin on the sugar contents. Lee *et al.* (1970) utilized paper chromatography to obtain data on 14 fruits and some 50 vegetables. In most cases several varieties were analyzed, with some limited data on the effect of maturity and ripeness. Although a variety of gas chromatographic (Clamp *et al.*, 1971) and ion exchange methods (Martinsson and Samuelson, 1970; Hough *et al.*, 1972; Hobbs and Lawrence, 1972) for separating sugar mixtures have been described, these are relatively complex systems which have rarely been applied to the routine analysis of sugars in foods (*e.g.*, see Mason and Slover, 1971; Reineccius *et al.*, 1972).

The present communication describes a method (adapted from a preparative procedure of Saunders, 1968) primarily designed for the determination of sucrose, glucose, and fructose in plant foods.

EXPERIMENTAL SECTION

Liquid Chromatography. The following equipment and procedures were used: 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 variable (usually 0.15–0.25 in./min); columns, length 60 cm, No. 316 stainless steel, ⅜ in. o.d. × 0.028 in. wall thickness (about 6 mm i.d.); column packing, Aminex Q-150-S (Bio-Rad Laboratories, Richmond, Calif.), a cation exchange resin of 8% cross-linkage, particle size 20–35 μ, converted to potassium form and packed wet; column temperature, 60°; eluent, distilled water; flow rate, 0.75–2.75 ml/min at pressures up to about 400 psi. Samples were injected with a syringe (Precision Sampling Corporation, Baton Rouge, La.).

Sugar Standards. Sugars used were of the highest purity available from Sigma (St. Louis, Mo.) and were dried overnight *in vacuo* at about 60°. No impurities were ever detected during our analyses. Standard solutions were prepared in water.

Food Samples. Juice samples were normally diluted so injections of 20 μl would yield readily measurable peak heights at 100–200 mV full scale (*e.g.*, attenuation 4 on refractometer, 50 mV full-scale recorder). These diluted samples were deionized by passing them through a mixed bed (0.8 × 3.4 cm) of 200–400 mesh AG-50 (H^+) and AG-3 (OH^-) (Bio-Rad Laboratories, Richmond, Calif.). Lee *et al.* (1970) used a similar procedure. One column could be used to deionize at least ten samples. Five milliliters of a sample was more than sufficient to flush the column free of the previous sample and the subsequent 1 ml was then collected for analysis. Preliminary trials with standard solutions and with juice samples showed no loss of sugars during deionization. Some samples were analyzed without deionization. Where necessary (*e.g.*, orange juice) particulate matter was removed prior to deionization by passage through a coarse or medium sintered filter.

Cereal or grain samples were finely ground and 5-g samples were extracted with occasional stirring at 75–80° for 1 hr with 75 ml of 80% ethanol, including a small quantity of $CaCO_3$ to neutralize any acidity. The mixture was cooled, filtered, and evaporated to dryness in a rotary evaporator *in vacuo* (water aspirator) and a bath temperature of about 50°. The residue was washed repeatedly with hot water to dissolve the sugars and the combined washings were made up to 100 ml.

The fresh banana tissue was thinly sliced, frozen, and ground to a fine powder in liquid nitrogen and stored at −40°. Extraction of this fine powder was carried out essentially as described above for cereals, except that the tissue:solvent ratios were varied and the tissue was refluxed for 2 hr in 80% ethanol. The extracts were deionized before analysis.

Table I. Chromatographic Data on Separation of Sucrose, Glucose, and Fructose

Flow rate, ml/min	Anal. time, min[a]	Resolution[b] of glucose and fructose	Peak heights,[c] mm		
			Sucrose	Glucose	Fructose
0.85	23	0.98	454	427	368
1.25	17	0.85	416	392	330
1.85	11	0.79	379	361	299
2.75	8	0.62	342	336	268

[a] This time can be reduced approximately in half after the first sample by injecting subsequent samples when the "front" peak appears or just after the sucrose peak. [b] Resolution $= V_2 - V_1/0.5(W_2 + W_1)$, where V and W refer to the retention times and peak widths, respectively. When $R = 1$, there is a peak overlap of about 2%. [c] Resulting from injection of 20 μl of a standard mixture containing 7.5 mg/ml of each sugar. Calculated to attenuation 1 at 50-mV full scale.

RESULTS AND DISCUSSION

Figure 1 shows a typical separation of sucrose, glucose, and fructose at two flow rates, one about twice the other. These chromatograms illustrate several things. First, sucrose was completely resolved from glucose and fructose at all flow rates tested (up to 2.75 ml/min). Second, the resolution of glucose and fructose was not complete, but it was improved by decreasing the flow rate. Third, there was a significant increase in sensitivity at the lower flow rate. Table I summarizes data on the separation of glucose and fructose which provide guidelines for matching the separation to the problem at hand.

Separation was carried out at 60° because the resolution of glucose and fructose was markedly improved as the temperature was raised from 25°. Temperatures higher than 60° yielded only slight additional improvement, at the risk of accelerating resin breakdown (Marinsky, 1966).

Standard curves were obtained by analysis of a solution containing equal weights of the three sugars, plotting the peak heights. The standard curves were linear over at least a 30-fold range (20–675 μg injected), although the slopes of the curves varied significantly with flow rate and for the individual sugars (Table I). The coefficient of variation for ten repeated 20-μl injections of the same sample was 1% or less for all three sugars. Variations in injection volume over the range 20–80 μl had no effect on peak

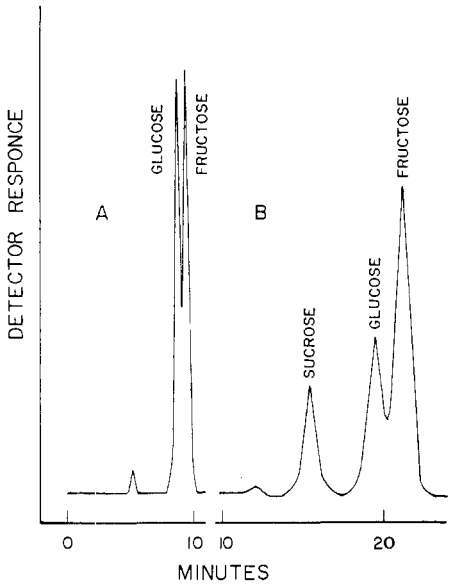

Figure 2. Separation of sugars in (A) grape juice at 1.85 ml/min and (b) apple juice at 0.8 ml/min; sample, 20 μl of deionized juice, diluted fivefold with water; sensitivity, (A) 200 mV full scale; (B) 160 mV. The small peak at about 5 and 12 min, respectively, is a "front" peak, presumably resulting from elution of juice pigments or other unretained substances in the juice.

heights. Resolution was noticeably reduced with injection volumes of 250–500 μl.

Since glucose and fructose were incompletely resolved (Table I) significant errors could occur when analyzing samples containing a high proportion of either sugar (Snyder and Kirkland, 1971). A series of samples was prepared containing known concentrations of these two sugars in varying proportions. The sugar concentrations were determined on the liquid chromatograph, using the standard curves prepared as above (1:1 ratio of fructose:glucose) to calculate the sugar concentrations. At flow rates in the range 0.8–1.85 ml/min, accurate data (coefficient of variation 2%) for fructose and glucose were obtained if the fructose:glucose ratio did not exceed 1.5:1. As the ratio increased, from 2:1 to about 5:1, the determined fructose concentrations were 3–8% low and the glucose concentrations correspondingly higher. Glucose could not be determined at higher flow rates (1.30 and 1.85 ml/min) if the ratio exceeded about 8:1. At 0.85 ml/min, the glucose peak height could be measured at the 8:1 fructose:glucose ratio, but the calculated concentration was about 133% of the actual. Both sugars could be accurately (±2%) determined when the glucose concentration exceeded the fructose concentration, even when the glucose concentration was ten times that of fructose.

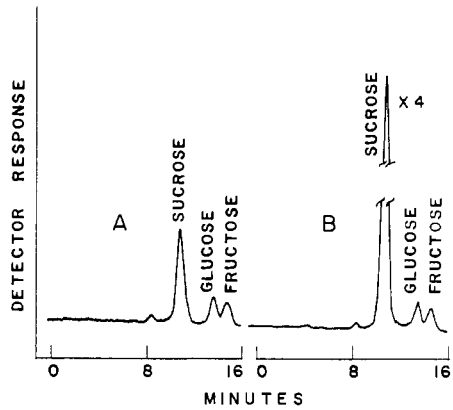

Figure 3. Separation of sugars in (A) corn flakes and (B) "sugar frosted" corn flakes; sample, 20 μl of deionized extracts; sensitivity, 100 mV full scale; flow rate, 1.25 ml/min.

These results indicated that the incomplete resolution of fructose and glucose will not be a problem when analyzing fruits and vegetables, since almost without exception the fructose:glucose ratio is 1:1 (Hardinge *et al.*, 1965; Dako *et al.*, 1970; Lee *et al.*, 1970). In the rare exceptions *(e.g.*, apples, pears), fructose tends to exceed glucose by ratios in the range 2:1 to 5:1, and these can be handled by operating at the lower flow rates and applying small correction factors. For all other samples with the usual 1:1 ratio, flow rates of 1.5–2.0 ml/min appear to be optimal, yielding separations in 10–15 min with no sacrifice in precision or accuracy.

Figure 2 shows typical separations of sugars from deionized fruit juices. The separations were comparable to those obtained with the test mixtures and the sugar concentrations, calculated from such runs, were always within the range reported in the literature (Hardinge *et al.*, 1965; Dako *et al.*, 1970; Lee *et al.*, 1970). The coefficient of variation for repeated determinations with the juice samples was normally under 2% for all three sugars. Table II shows typical data for the recovery of sugars added to fruit juices. These results indicate that the described method has adequate precision and accuracy for analysis of these sugars in plant tissues, especially in light of the results of Dako *et al.* (1970) which indicate that the *within variety* variation of sugar content in fruits is typically ±25% and quite often approaches ±50%.

Figure 3 shows the separation of sugars extracted from corn flakes and from "sugar frosted" corn flakes. It is apparent that the "frosting" was achieved by addition of a large quantity of sucrose (3.4% *vs.* 37.1% sucrose). The peaks for glucose and fructose in Figure 3 each represent about 11 μg of these sugars. This is approaching the lower limits of the assay. The precision was about 1% for duplicate assays at this level.

Table II. Recovery of Sugars Added to Fruit Juices

	Sucrose			Glucose			Fructose		
	Initial concn, mg/ml	Added, mg/ml	Recovery, %	Initial concn, mg/ml	Added, mg/ml	Recovery, %	Initial concn, mg/ml	Added, mg/ml	Recovery, %
Apple juice	31.7	25.0	97.5	27.1	25.0	100.0	64.2	25.0	98.2
	19.0	40.0	96.7	28.6	40.0	96.5	64.1	20.0	91.8
	18.6	40.0	99.0	29.3	40.0	97.9	64.9	40.0	95.5
				28.9	40.0	99.4	64.9	40.0	101.8
							64.9	80.0	102.1
Grape juice				79.8	20.0	103.4	86.4	20.0	93.9
	~1.5	50.0	102.0	79.8	50.0	98.8	85.7	50.0	99.0
Orange juice							28.9	30.0	96.3
							28.9	30.0	94.7
Recovery all samples, % ± sd	97.6 ± 1.0			99.3 ± 2.3			97.0 ± 3.5		
Coeff. of variation, %	1.0			2.3			3.6		

Table III. Relative Retention Times of Sugars on the Aminex Q-150-S Column

Sugar	Rel retention time,[a] glucose = 1.00
Stachyose	0.67
Raffinose	0.71
Sucrose	0.78
Maltose	0.80
Lactose	0.80
Galactose	1.00
Xylose	1.02
Mannose	1.06
Rhamnose	1.06
Fructose	1.06
Fucose	1.10

[a] Approximate values calculated from runs at various flow rates and temperatures. Partial separation was achieved at the lower flow rates when relative retention times differed by at least 0.03; base-line separation requires differences of about 0.1.

The method has also been utilized to determine the changes in sugar content of fresh banana pulp during ripening. The data calculated from these assays showed agreement with earlier studies (Poland *et al.*, 1938) both with regard to total sugar content and in the relative proportions of sucrose, glucose, and fructose at each stage of ripening.

Since the deionization procedure is so simple, we have routinely deionized samples (such as in Figures 2 and 3) before injection. However, our recent studies have indicated two possibilities for avoiding this preliminary deionization. First, a deionizing pre-column (6 × 0.3 cm i.d.; mixed AG-50 (H^+) and AG-3 (OH^-)) was installed between the injection port and the separation column. A large number of 20-μl injections of juice samples were passed through this column with no evidence of ion breakthrough. However, there was some peak broadening, probably arising from dead volume in the connectors. The second possibility is to simply inject the samples without deionization. In this case, with juices, a "front" peak of about the same height as the subsequent sugar peaks appears, representing both anions and potassium ions displaced from the column. An approximate calculation from the detector response for citric acid (Palmer and List, 1973) and the capacity of the resin (about 1.8 mg/ml of resin bed) indicated that it would require at least 100 injections (20 μl) of apple or grape juice to displace 1% of the potassium ions from the separation column. Presumably an occasional overnight flushing with a dilute KNO_3 solution would serve to regenerate the column if any deterioration in resolution was observed. In limited trials we have obtained exactly the same resolution and precision with and without deionization.

These results illustrate the usefulness of the described method for determining sucrose, glucose, and fructose. The relative retention data for other sugars (Table III) indicate additional possibilities. For example, stachyose and raffinose are sufficiently well separated from sucrose to allow determination of all three of these sugars in legumes such as beans or peas, which often contain significant proportions of stachyose and raffinose (Lee *et al.*, 1970). Also, lactose could be rapidly determined in dairy products. We have recently used the method to estimate lactose in whey and in baked goods prepared from whey. However, the data of Table III also indicate potential interferences. For example, maltose cannot be separated from sucrose and this would be a problem in a few cases where these sugars occur together (*e.g.*, certain varieties of grapes; Lee *et al.*, 1970).

Ethanol is another potential interference. It has a retention time about the same as fructose. This means that the usual 80% ethanol extracts of plant tissues must be freed of ethanol before analysis. We have acomplished this by evaporation to dryness *in vacuo* and redissolving the sugars in hot water (see Experimental Section). Our data from bananas suggest little or no loss of sugars during this procedure, but this will have to be confirmed with additional samples.

If the presence of interfering sugars is suspected, or for confirmation of the identity of a sugar responsible for a peak, it is a simple matter to collect the effluent during emergence of a peak, concentrate by evaporation, and check purity by thin-layer chromatography or other means. Similarly, the separated sugars are readily collected for estimation of radioactivity during labeling experiments.

This simple and rapid procedure appears to have broad applicability for the estimation of sucrose, glucose, and fructose in plant foods and plant tissues in general. Within rather wide limits, the separation and estimation of these sugars are not particularly sensitive to operating variables such as flow rate, injection size, age of column, etc. However, we have not been able to significantly improve the separations of Figure 1 by manipulation of these variables and the method appears to have little or no potential as a general method for separation of more complex sugar mixtures.

LITERATURE CITED

Clamp, J. R., Bhatti, T., Chambers, R. E., *Methods Biochem. Anal.* **19**, 229 (1971).
Dako, D. Y., Trautner, K., Somogyi, J. C., *Schweiz Med. Wochenschr.* **100**, 897 (1970).
Hardinge, M. G., Swarner, J. B., Crooks, H., *J. Amer. Diet. Ass.* **46**, 197 (1965).
Hobbs, J. S., Lawrence, J. G., *J. Chromatogr.* **72**, 311 (1972).
Hough, L., Jones, J. V. S., Wusteman, P., *Carbohyd. Res.* **21**, 9 (1972).
Lee, C. Y., Shallenberger, R. S., Vittum, M. T., *N. Y. Food Life Sci. Bull.* **No. 1** (1970).
Marinsky, J., "Ion Exchange," Vol. I, Marcel Dekker, New York, N. Y., 1966.
Martinsson, E., Samuelson, O., *J. Chromatogr.* **50**, 429 (1970).
Mason, B. S., Slover, H. T., *J. Agr. Food Chem.* **19**, 551 (1971).
Palmer, J. K., List, D. M., *J. Agr. Food Chem.* **21**, 903 (1973).
Poland, G. L., Manion, J. T., Brenner, M. W., Harris, P. L., *Ind. Eng. Chem.* **30**, 340 (1938).
Reineccius, G. A., Andersen, D. A., Kavanaugh, T. E., Keeney, P. G., *J. Agr. Food Chem.* **20**, 199 (1972).
Saunders, R. M., *Carbohyd. Res.* **7**, 76 (1968).
Snyder, L. R., Kirkland, J. J., "Modern Liquid Chromatography," The American Chemical Society, Washington, D. C., 1971.

James K. Palmer*[1]
Walter B. Brandes[2]

Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
[1] Microbial Chemistry Group
Pioneering Research Laboratory
U. S. Army Natick Laboratories
Natick, Massachusetts 01760
[2] U. S. Army Medical Laboratory
Fort Sam Houston
San Antonio, Texas 78234

Received for review September 14, 1973. Accepted April 5, 1974. The authors thank Waters Associates for partial financial support.