Short Communications

A simple method for the quantitative analysis of sugars by ion-exchange chromatography

Several years ago, KHYM AND ZILL[1] showed that borate complexes of sugars could be separated by ion-exchange chromatography. This method has been applied successfully to the quantitative analysis of sugars in plant materials[2,3,4]. Although the chromatographic procedure itself has been well developed, there are only a few investigations concerning the preparation of sugar solutions to be analyzed by this method. Usual procedures for the preparation of sugar solutions are as follows: Plant materials are extracted with hot 80% ethanol, after which the ethanol is removed by distillation *in vacuo*. The resulting aqueous solution is treated with lead acetate to remove proteins and other interfering substances. Excess lead is removed by treating with H_2S (followed by aeration to remove excess H_2S) or with sodium phosphate. The solution is then desalted with a mixture of Amberlite IR-120 (H^+) and Amberlite IR-4B (OH^-) resins and concentrated to 2–5 mg sugar/ml. After adding sodium tetraborate to the solution to a final concentration of 0.001 *M*, the solution is ready for chromatographic separation.

Based on a critical examination of the conditions of these procedures, we proposed a somewhat simplified version which gave the best recovery of sugars with minimum hydrolysis of sucrose[4]. Although sugar solutions obtained by this method are well suited for chromatography, the method itself is still laborious and time-consuming.

In seeking another method to simplify these procedures, we thought of the possibility that sugar-borate complexes might be adsorbed from a 50%-ethanolic solution by a strong-base anion-exchange resin in its borate form, because PONTIS *et al.*[5] had reported that yeast nucleotides in 50%-ethanolic extracts could be successfully chromatographed on a Dowex-1 (Cl^-) column. The idea was tested by using a mixture of authentic sugars. As will be seen in Fig. 1A, the adsorption of sugars on, and the succeeding elution from, the column is nearly quantitative. Another experiment showed that sugars were completely adsorbed on the column even when their concentrations were quite small (as low as 0.3 mg sugar/ml of 50% ethanol).

Results of experiments using extracts from plant materials are shown in Fig. 1, B–D. Extracts to be put on the column were prepared as follows: Plant tissues were extracted three times with hot 80% ethanol in the presence of a small amount of $CaCO_3$. The combined extracts were diluted with water to 50% ethanol. The solution was cooled in an ice bath and treated for 20 min with occasional shaking with a mixture of 5 ml (wet vol.) each of Amberlite IR-120 (H^+) and IR-4B (OH^-) per 100 ml of the diluted ethanolic extract. The resins were filtered off and thoroughly washed with 50% ethanol. A solution of sodium tetraborate was added to the combined filtrates to give a final concentration of 0.001 *M*. The preparation of the Dowex-1 borate column and subsequent chromatographic procedures were carried out according to KHYM AND ZILL.

In the cases of potato and apple extracts, treatment of the extract with ion-exchange resins may be omitted without significant effect on the chromatographic behavior of each sugar. However, in the case of extract from soybean seedlings, elution of sucrose was greatly retarded when the deionization procedure was omitted. It may be, therefore, better to perform the deionization as a routine procedure in order to obtain good separation of component sugars.

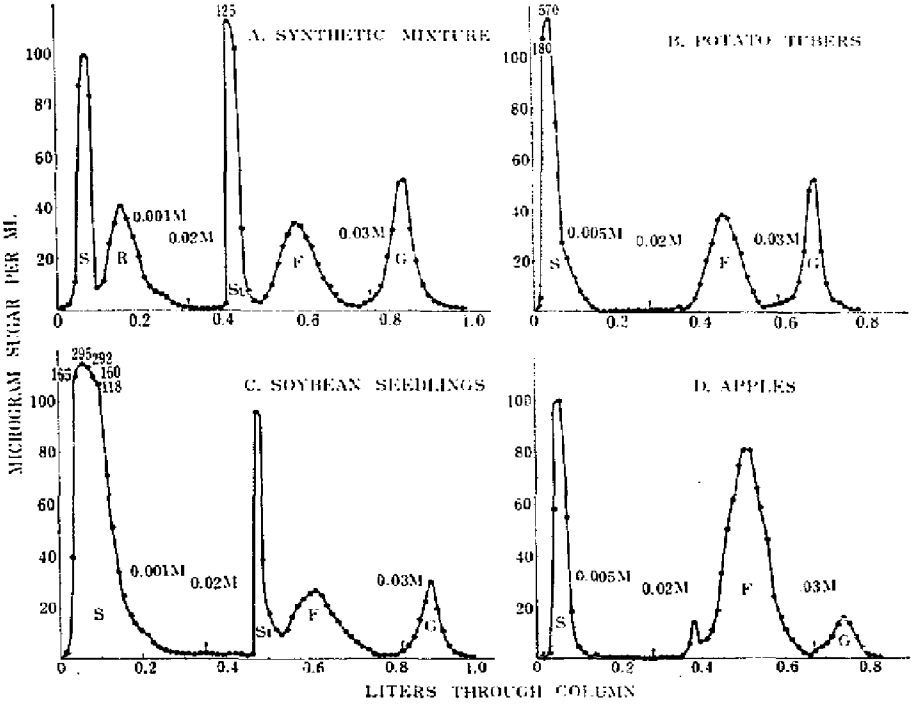

Fig. 1. Separation of Sugars by Ion-exchange Chromatography. The preparation of an analytical column, 0.85 cm^2 × (3.5–4.5 cm), of Dowex-1 X8, borate form, 200–400 mesh, and chromatographic procedures were performed as described by KHYM AND ZILL[1]. Concentrations of sodium tetraborate in the eluting solvents are indicated by arrows in the figure. Flow rate, 1 ml/min. Total sugars were determined by the phenol method of DUBOIS *et al.*[6]. The resorcinol method of ROE was also used for the determination of fructose[7]. Abbreviations used in the figure: S, sucrose; R, raffinose; St, stachyose; F, fructose; and G, glucose. – A. Elution pattern of authentic sugars in a synthetic mixture. The sugar solution added to the column contained 4.00 mg sucrose, 3.56 mg raffinose, 3.53 mg stachyose, 3.95 mg fructose, and 3.70 mg glucose in 20 ml 50% ethanol. Sugars recovered from the column were: 4.06 mg sucrose (101%), 3.50 mg raffinose (98%), 3.68 mg stachyose (104%), 3.81 mg fructose (96%), and 3.60 mg glucose (97%). B. Separation of sugars extracted from potato tubers. Potato tubers (*Solanum tuberosum* L. var. Irish cobbler) which had been stored at 6° for 2 weeks were used. Deionization procedure was omitted. 24 ml of 50%-ethanolic solution, corresponding to 1.6 g fresh potatoes, was put on the column. Sugars found: 16.1 mg sucrose (1.01%), 4.0 mg fructose (0.25%), and 2.6 mg glucose (0.16%). C. Separation of sugars extracted from soybean seedlings. Soybeans (*Glycine max* Merrill var. Nôrin, No. 1) were germinated in the dark for 3 days. Seedlings were homogenized and extracted with hot 80% ethanol. Oils were removed by shaking the extract with petroleum ether. 40 ml of 50%-ethanolic solution, corresponding to 1.7 g fresh weight, was used for chromatography. Sugars found: 19.5 mg sucrose (1.15%), 2.2 mg stachyose (0.13%), 3.3 mg fructose (0.19%), and 1.7 mg glucose (0.10%). D. Separation of sugars extracted from apples. Apples (*Malus pumilla* Miller var. McIntosh, ASAHI) purchased from the market were used. 9.6 ml of 50%-ethanolic solution, corresponding to 0.15 g fresh apple, was used for chromatography. Sugars found: 3.7 mg sucrose (2.47%), 9.3 mg fructose (6.20%), and 1.2 mg glucose (0.80%).

By employing this simplified method, sugar solutions to be analyzed by ion-exchange chromatography can be prepared in a short time, which is a great advantage over the existing methods. Other advantages are the smaller possibilities for the loss of sugars and hydrolysis of sucrose during the preparation of sugar solutions. The chromatographic elution pattern of sugars obtained by this method is, as far as the plant materials we have thus far tested are concerned, essentially the same as those obtained by the standard method.

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[1] J. N. KHYM AND L. P. ZILL, *J. Am. Chem. Soc.*, 74 (1952) 2090.
[2] G. R. NOGGLE AND L. P. ZILL, *Arch. Biochem. Biophys.*, 41 (1952) 21.
[3] T. FUKUI AND Z. NIKUNI, *Nippon Nôgei-Kagaku Kaishi*, in the press.
[4] K. MORI AND M. NAKAMURA, *Bull. Agr. Chem. Soc. Japan*, in the press.
[5] H. G. PONTIS, E. CABIB AND L. F. LELOIR, *Biochim. Biophys. Acta*, 26 (1957) 146.
[6] M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
[7] J. H. ROE, *J. Biol. Chem.*, 107 (1934) 15.

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Changes in the ultraviolet absorption spectrum of trypsin associated with disruption of tertiary structure

Changes in the ultraviolet absorption spectra of several proteins have been demonstrated when these were exposed to a variety of experimental conditions. The changes may arise from the ionization of the tyrosine hydroxyl groups[1,2,3] or from the disruption of hydrogen bonds involving tyrosine hydroxyl groups[4,5]. It has also been suggested that a change in charge of an ionizing group neighbouring a non-ionizable chromophoric group (*e.g.* tryptophan) may result in a shift in the absorption spectrum of the latter to lower wavelengths[6]. Recently, CHERVENKA has shown that the activation of chymotrypsinogen is accompanied by a shift in the absorption of the tyrosine residues to lower wavelengths[7]. He also demonstrated a spectral change accompanying the autolysis of chymotrypsin similar to that which occurred during urea treatment of either the zymogen or the enzyme. Under both conditions of denaturation, the difference spectrum resulted from a wavelength shift in the absorption of tryptophan and probably of all the chromophoric amino acid residues.

We have been unable to demonstrate a significant change in absorption during the activation of trypsinogen, but have observed a characteristic and reproducible difference spectrum when trypsin was autolysed in the absence of calcium or upon treatment with urea. These spectra show a striking similarity to those obtained by CHERVENKA with the chymotrypsin system.

In all experiments at pH 8.00, the reference cuvette of a Beckman DK-1 re-