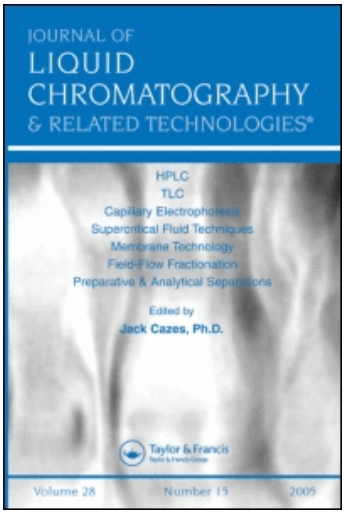

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AN EVALUATION OF ELUENT RECYCLING AND COLUMN LIFE FOR HPLC ANALYSIS OF CARBOHYDRATES

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ABSTRACT

The length of service of amine modified silica columns for the automated analysis of carbohydrates in food and beverages typically exceeds 500 sample injections. Excessive accumulation of carbohydrate in the recirculated eluent results in a gradual decrease in peak height. Detector response is independent of injection volume over the range of 1-50 µl. These results suggest critical parameters for use in automated carbohydrate analysis systems.

Key words: Eluent recycling, HPLC, carbohydrate, automated analysis.

INTRODUCTION

The development of methods for the analysis of carbohydrates using high performance liquid chromatography (HPLC) in chemical

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and biological research and the food industry has received considerable attention in the last five years (1,4,8,9). HPLC techniques are particularly attractive for carbohydrate analysis because they allow rapid, simultaneous separation and quantitation of sugars and polyhydric alcohols without sample derivatization. Further, HPLC methods lend themselves to automated analysis systems which are highly desirable in multiple sample and quality control applications.

Ion exchange columns offer a useful and sensitive means of carbohydrate analysis, however, their use requires extensive sample clean-up and they are relatively short-lived. In a recent report a technique was described (6) for the analysis of soluble carbohydrates using amine modified radially compressed silica columns and eluent recirculation. This report provides an extension of that study by evaluating column life and assessing the import of eluent recirculation. Specific suggestions for the use of amine modified silica columns in automated systems are provided.

MATERIALS AND METHODS

Equipment

The HPLC system consisted of a Waters Model R401 differential refractometer, a Model 6000A pump, Model 710B WISP automated injection system and Model 730 Data Module. A Radial-Pak B silica cartridge (10cm X 8mm ID) (10μ particle size) was employed in a Radial Compression Module in conjunction with an inline filter (2μ). The column was first conditioned with 50ml of acetonitrile: water (70:30) containing 0.1% (v/v) tetraethylenepentamine (TEPA), pH 9.0 at a flow rate of 2ml/min. Then the final recirculating eluent of acetonitrile: water (81:19) containing 0.02% TEPA (v/v) (pH 9.0) was introduced and the system allowed to equilibrate (2-6 hrs) as detailed elsewhere (6).

Standards containing 5 μg/μl of carbohydrates and polyhydric alcohols dissolved in water were run following every 15-20

injections of sample. Eluent flow rate was 2 or 4 ml/min. All sample preparations ended with filtering through a 0.45µm pore filter (Millipore filter type: HAWP). Reproducibility of peak height was greater than 98%.

The modified silica column was considered expended when back pressure reached 2000 psi and/or resolution of peaks remained poor after reversing and backflushing the column.

Sample Preparation

Cereal (sugar coated) - Kellogg's Frosted Flakes (0.5 g) were homogenized in 4ml of distilled water using a teflon-glass pedestal homogenizer, centrifuged for 10 minutes (ca. 900 rpms), the supernatant decanted and the pellet discarded. Three ml of 70% ethanol were added and the mixture centrifuged at 4,000 rpms for 10 minutes. The supernatant was passed through a Sep-Pak C_{18} cartridge following pretreatment of the cartridge by passing 2 ml methanol through the Sep-Pak. The sample was then run through the Sep-Pak and the first ml (dead volume) was discarded.
Corn syrup - Karo syrup was diluted (1:9) with water and filtered.
Beverage (Soft drink): Canada Dry Tahitian Treat Fruit Punch was diluted (1:1) and filtered.
Beverage - Fermented wine stock was filtered only.

RESULTS AND DISCUSSION

Column Life

Column longevity was investigated by making up to 500 sample injections for each of 4 beverages or foods (Fig. 1). The extent of column life was not reached for the cereal, soft drink or corn syrup samples because the resolution of the carbohydrates was maintained through the 500th injection when each test was terminated. Using the fermentation stock the column failed after 80 injections

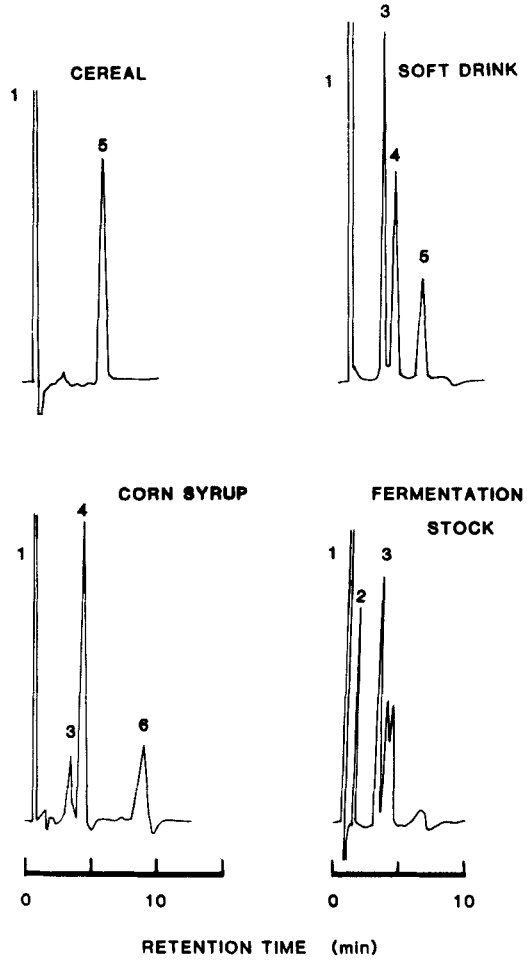

Fig. 1 Sample chromatograms of polyols and sugars separated using a 10 μ silica column (10 cm X 8 mm I.D.). Elution solvent - acetonitrile: H_2O: TEPA (81:19:0.02), pH 9.0, flow rate = 4 ml/min., 700-800 psi.
1. water front, 2. glycerol, 3. fructose, 4. glucose, 5. sucrose and 6. maltose.

and could not be reconditioned to the initial resolution by reversing and backflushing. Additional sample clean-up procedures were not attempted, but will have to be incorporated into this assay to enable a larger number of samples to be run.

A decrease in detector response (peak height) was observed during the course of the longevity studies. This reduction is illustrated in Figs. 2 and 3. These data were obtained by making

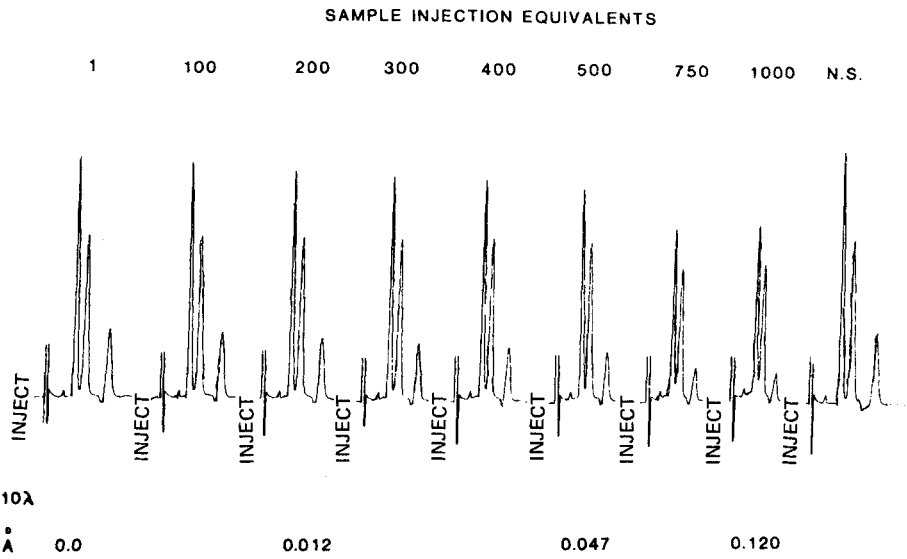

Fig. 2 Chromatograms generated during the addition of 1000 sample injection equivalents to one liter of recycled eluent. Injection volume equals 50 µl of water: soft drink (1:1). The abbreviation "N.S." represents the replacement of the mobile phase with new solvent (1ℓ) while continuing to use the same silica column. Changes in absorbance at 510 λ during progressive eluent adulteration are indicated at the bottom of the figure. Soft drink contains 22 mg/ml fructose, 27 mg/ml glucose and 11 mg/ml sucrose. Flow rate = 4 ml/min at an attentuation of 32X. The static reference on the differential refractometer was purged after each 100 injection equivalents.

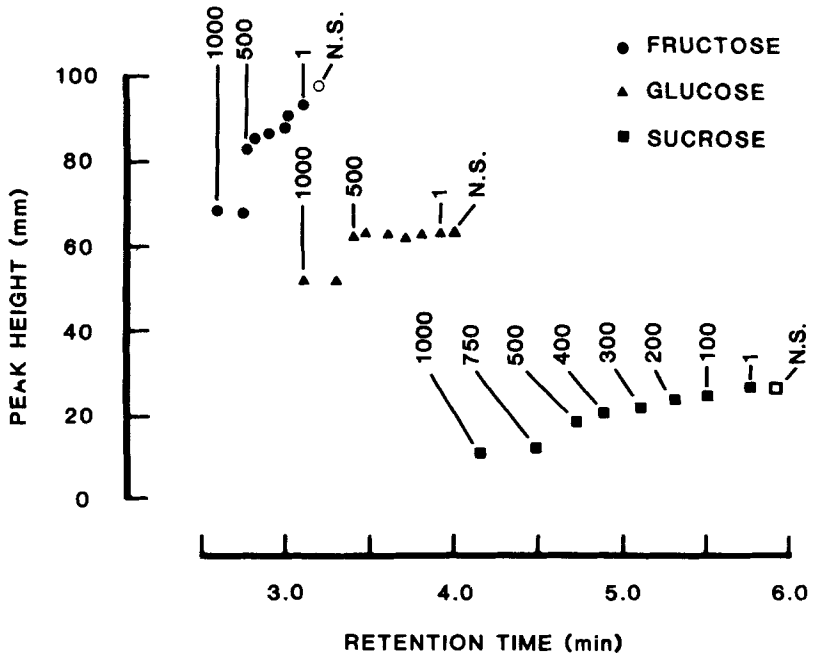

Fig. 3 Summary of changes in retention time and peak height during the addition of 1000 sample injection equivalents as illustrated in Fig. 2.

a 50 µl sample injection of the soft drink every 100 injection equivalents. An injection equivalent refers to the addition of 50 µl of sample directly to the one liter solvent reservoir as a means of simulating a given level of mobile phase adulteration (e.g. 100 injection equivalents = 5.0ml of sample). This procedure produced results directly comparable to those observed in the longevity study. Although the reduction in peak height was minimal during the first 200 injection equivalents, successive additions resulted in a more pronounced decrease. No significant baseline drift was observed.

It should be noted that this soft drink is red in color and during the addition of 1000 injection equivalents the color of the

mobile phase changed from clear to pink. This change corresponded to an overall increase of 0.120 absorbance units at 510 λ (Fig. 2). The replacement of the mobile phase while retaining the same column returned sample peak heights and retention times to their original values (Fig. 3). Therefore the observed reduction in detector response is a function of sample accumulation in the mobile phase and not column degradation or influenced by the red dye.

Eluent Recirculation

In order to determine whether the reduction in peak height was due specifically to carbohydrate accumulation or to other constituents of the sample matrix, the experiment was repeated using a standard solution of glycerol (5 mg/ml, fructose (8.2 mg/ml), glucose (14.2 mg/ml) and sucrose (15.1 mg/ml.). Standard concentrations were selected such that all four peaks would be equal in height regardless of their respective retention times. Previous observations indicated that the extent of peak height reduction was a function of the absolute initial peak height; shorter peaks lost a greater proportion of their initial height during a sequence of injections. In order to investigate this factor 1:1 and 1:6 dilutions of the standard solution were prepared. Duplicate injections of each standard were made during the addition of 200 sample equivalents (Fig. 4).

Although there was no change in the retention time of glycerol, the elution time for sucrose decreased from 5.9 to 5.4 min. (Fig. 4). The initial peak heights of the undiluted standard and the 1:1 dilution represented, respectively 84 and 42% of full-scale deflection at an attenuation of 4X, while the 1:6 dilution was 13%. After the addition of 150-200 sample injection equivalents the compounds in the most dilute standard could no longer be resolved from the baseline (Fig. 4). Peak height of the undiluted glycerol standard decreased by 6% and sucrose by 17% following the same addition.

The introduction of a sample into the recycled mobile phase represents a simultaneous change in two variables - water and

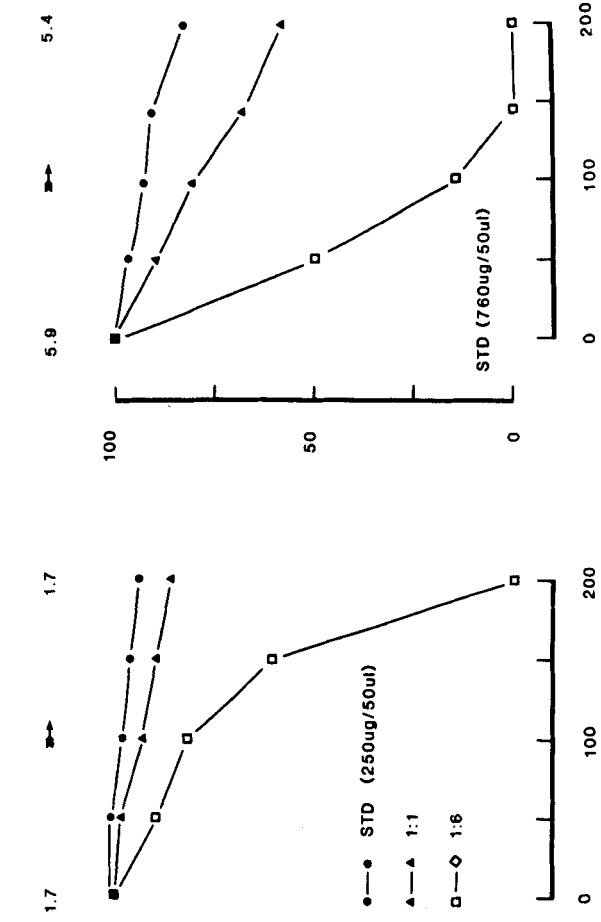

Fig. 4 Changes in retention time and peak height expressed as percentage of initial injection peak height during the addition of 200 sample injection equivalents (50 µl per injection of standard solution).

dissolved carbohydrates. To determine the cumulative effect each factor has on solvent recycling, water and solute equivalent to 200 injections, as in Fig. 4, were added independently. With the addition of 10 ml of water the retention time of sucrose was reduced by 17% and, thereby, predictably increased the relative peak height (Fig. 5). A decrease in the acetonitrile: water ratio reduces retention time, particularly for late eluting compounds (6).

The introduction of solute alone to the eluent produced a reduction in peak height which was directly proportional to the

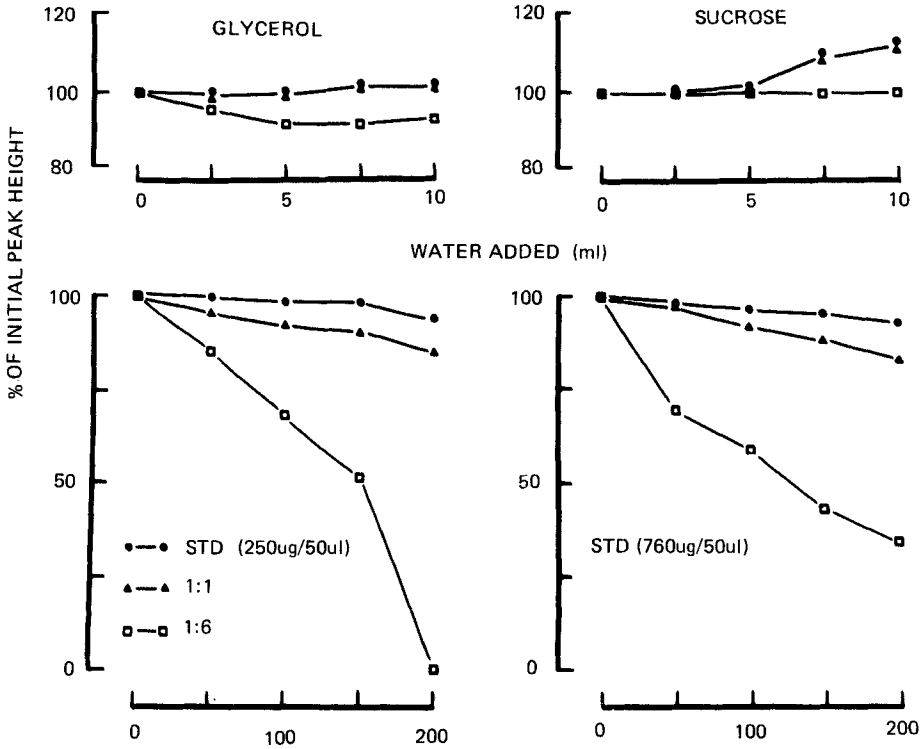

Fig. 5 Relative changes in peak height during the addition of water only (Top) or carbohydrate only (Bottom) equivalent to 200 sample injections as described in Fig. 4.

total amount of accumulated solute. Further, the decrease was similar to that observed in Fig. 4.

Although the idea of solvent recirculation may be disconcerting to persons familiar with traditional methods of HPLC analysis, in this system it offers a considerable savings in solvent expense without significant impairment of analysis. The decrease in detector response is within the limits of most microprocessor based integrator systems to automatically compensate for updating calibration. Not only is solvent use and cost reduced, but time is saved that would otherwise be used for mobile phase preparation.

Injection Volume

A practical problem frequently encountered in HPLC is that of injection volume effects (i.e. as injection volume varies so does retention time, peak symmetry or detector response). Investigators are frequently faced with variable sample matrices that yield different injectable sample volumes. Figure 6 illustrates the independence of injection volume and solute concentration.

Application of Amine Modified Silica Columns to Automated Analysis

Based upon the results of this study and other work in our laboratory involving the measurement of endogenous levels of both animal (2,3,7) and plant (5) carbohydrates, we offer the following suggestions for the general application of this method, particularly with respect to its use in automated analysis systems.

[1] Since some sugars are relatively insoluble in a mixture of acetonitrile and water, standards or samples should be injected in a water carrier. To avoid a precipitation problem between eluent and sample after injection, a small amount of each should be mixed before injection to determine whether a major precipitant forms.

[2] Use small sample injection volumes (50 µl or less). During the course of injecting several hundred samples, small injection volumes will avoid significant changes in the acetonitrile: water ratio and, thereby, avoid changes in retention times. Secondly, at

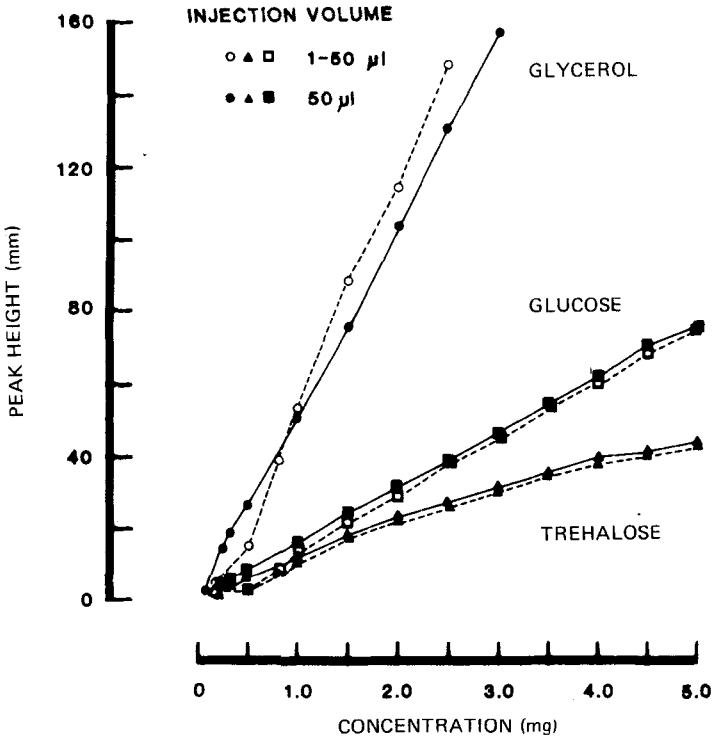

Fig. 6 Effect of variable injection volume on peak height for a range of carbohydrate concentrations. Solid symbols represent a constant 50 µl injection volume using varying solute concentrations. Open symbols represent variable injection volumes (e.g. 20 µl corresponds to 2.0 mg, 45 µl to 4.5 mg).

injection volumes greater than 75 µl severe peak "fronting" may occur.

[3] Minimize the absolute amount of carbohydrate added to the recirculating eluent. This will reduce the rate of decrease in peak height during a series of injections and allow a microprocessor based integrator to automatically update calibration.

[4] An increase in the volume of the eluent reservoir to 2-5 liters serves to minimize the cumulative effects of adding water and solute from samples to the recirculating mobile phase.

[5] Since the reduction in detector response due to the accumulation of solute in the eluent is dependent on the initial absolute peak height, external standards and sample peak heights should be similar (±10%). Peak height reduction may be further minimized by adjusting the attenuation of the refractometer so that peaks are in the 60-90% range of full scale deflection.

[6] Refractometers are sensitive to small temperature changes (2-3°C). Variations in room temperature should be avoided and/or the detector temperature stabilized with a water jacket.

[7] Although only an inline filter was used in the present study, we routinely use a guard column packed with Ax/Corasil (Waters Associates) for the analysis of carbohydrates extracted from plant and animal tissue. This serves to remove material from the sample that could inactivate the column (e.g. ions, lipids and precipitated proteins).

[8] With the build-up of high pressure or a loss of resolution the silica column may be reversed and back-flushed with new solvent in an attempt to return the column to initial operating conditions.

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