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Short communication

# Separation of a fructo-oligosaccharide mixture by hydrophilic interaction chromatography using silica-based micropellicular sorbents

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**Abstract**

An amino-bonded, micropellicular stationary phase was prepared and applied to the separation of a fructo-oligosaccharide mixture. Glucose, sucrose, 1-kestose, nystose and fructose-nystose were eluted in sequence on a 10-cm long column. A linear response was obtained by plotting the peak area vs. the sample loading of each carbohydrate. The logarithm difference of capacity factors ($\Delta \log k'$) were estimated from the slopes of the plots of the capacity factor vs. the number of fructosyl residues in the carbohydrates to be 0.58 and 0.47, respectively, for the columns packed with micropellicular and totally porous sorbents. The use of fluid-impervious, micropellicular sorbents led to a higher value of $\Delta \log k'$, compared with totally porous sorbents. This finding could explain why the pellicular stationary phase is effective for the separation of macromolecules like proteins. To reduce the effect of the extra-column dead volume on the chromatographic efficiency, the extra-column tube should be as short as possible, especially for the column packed with micropellicular sorbents. © 1998 Elsevier Science B.V.

*Keywords:* Stationary phases, LC; Fructo-oligosaccharide mixture; Sucrose; 1-Kestose; Nystose; Fructose-nystose

## 1. Introduction

The mode of chromatography in which a polar stationary phase is used with a less polar mobile phase has long been known as 'normal-phase chromatography.' The nomenclature symbol 'hydrophilic interaction chromatography' with the acronym HILIC, proposed in 1990 by Alpert [1], has recently been used in order to emphasize the role of polarity in the mechanism. HILIC is a liquid chromatography carried out by combining a hydrophilic stationary phase and a hydrophobic, mostly organic, mobile phase. Applications of HILIC are mainly in the separation of carbohydrates and resolution of complex carbohydrates [2]. Recent developments in the application of high-performance liquid chromatography based on this mode for carbohydrate separation have been reviewed by Churms in two review papers [3,4]. The sorbents used in HILIC are usually macroporous silica gel preparations, with diameters of 5 or 3 μm and bonded amino, amide, cyano, diol and polyol phases. Among the bonded-phase silica-based sorbents, aminopropyl silica has been widely used in HILIC of sugars and other carbohydrates. Silica-based sorbents with application in the chromatographic analysis of carbohydrates also include

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many packings in which cyclodextrins are bonded to silica beads [5] and a polymer-coated stationary phase that is prepared by incorporation of ethanolamine into a coating of polysuccinimide, covalently bonded to silica [1]. The present paper describes the use of silica-based micropellicular sorbents as the stationary phase for the chromatography of a fructo-oligosaccharide mixture. Pellicular supports have been attracting attention during the last decade particularly in the separation of biopolymers. The fluid-impervious, silica-based supports of small particle diameter have been found effective in the chromatography of proteins based on the modes of reversed-phase, ion-exchange, hydrophobic interaction and affinity interaction [6–13]. We here demonstrate the potential of the use of micropellicular amino-bonded silica in the separation and chromatographic analysis of fructo-oligosaccharide products from sucrose solution under the catalytic action of β-fructofuranosidase.

## 2. Materials and methods

Micropellicular silica was made by reacting 8 ml of tetraamyl orthosilicate with 50 ml of an ammonia-saturated alcohol solution. The alcohol solution was prepared by passing ammonia through water–methanol–2-propanol (1:2:6) under cooling in ice. The synthesis reaction was carried out with stirring at 250 rpm for 24 h. The resulting silica was washed to neutrality with distilled water and methanol, then dried in a vacuum oven at 150°C for 24 h. Tetraamyl orthosilicate was prepared by slowly adding 120 ml of amyl alcohol at a flow-rate of 1 ml/min to 25 ml of silicon tetrachloride in a 500-ml flask with stirring and cooling in ice, followed by a 2-day reflux and fractionation. The fraction of liquid with boiling points greater than 200°C was collected. The nonporous silica was washed with 5% nitric acid at reflux for 1 h prior to modification with silane. Modification of silica with 6% γ-aminopropyltriethoxysilane was carried out under anhydrous conditions [14]. The resulting aminopropyl silica was slurry-packed into a 10 cm×4.6 mm I.D. stainless-steel column using a column packer (Model CPP-085; Chemco). Distilled water and acetonitrile–water (4:1), respectively, were employed as the slurry solvent and the pressure solvent for the column packing. The HPLC equipment consists of a Jasco PU-980 pump and a Shodex RI-71 detector. For the chromatography of the fructo-oligosaccharide mixture, samples dissolved in distilled water are injected through a 5-μl sample loop. The mobile phase was a mixture of acetonitrile and water (4:1). The peak data were collected and integrated with a Chem-Lab system, including an interface and software for computing integrator (Scientific Information Service Corp., Taiwan).

## 3. Results and discussion

### *3.1. Preparation of silica-based micropellicular sorbent*

The prepared silica was estimated to have an average diameter of 3 μm from scanning electron microscopy. The particle size was controlled by the concentration of ammonia, the composition of alcohol solution and the reaction time. The particle size distribution of the resulting silica was monodisperse and the average diameter was greater than previously prepared by following a similar recipe [13]. This was a result of using a higher ammonia concentration in the present work, since the ammonia-saturated alcohol solution was prepared with an ice-water cooling. The higher the concentration of ammonia employed, the larger was the average particle size of the silica. However, a higher ammonia concentration could lead to the erosion of the silica and some concave structures on the silica surface (not assessed here). Introducing aminopropyl groups to the silica surface was achieved by γ-aminopropyltriethoxysilane. In comparison with the unmodified silica, the FTIR spectra of the resultant aminopropyl silica contained an additional peak at 760.3 $cm^{-1}$. The density of amino groups on the silica surface was determined to be 19 μmol/g by titration with 0.1 M HCl. During the titration, the aminopropyl silica was suspended in 4 *M* NaCl, which reportedly gives the HCl free access to the ionizable groups [15].

### *3.2. Chromatography of fructo-oligosaccharides mixture*

A 10-cm long column packed with the amino-propyl phase was examined for the chromatographic analysis of the fructo-oligosaccharides produced from sucrose catalyzed by immobilized β-fructofuranosidase. The fructo-oligosaccharide mixture is mainly composed of 1-kestose ($GF_2$), nystose ($GF_3$), glucose (G) and sucrose (GF). In some samples a very small amount of fructose-nystose ($GF_4$) is also presented. Fig. 1 shows the separation of fructo-oligosaccharide by isocratic elution. This column was evaluated for its chromatographic efficiency using toluene as the non-retained component. The minimum plate height appeared at the flow-rate ranging from 0.2 to 0.4 ml/min. The plate height increased sharply with flow-rate when the latter exceeded 0.4 ml/min. The plate number per unit meter of column length was determined to be 9400 from the results of applying 5 μl of toluene to the column at a flow-rate of 0.3 ml/min.

The retention behavior using this column demonstrates that the kinetics of the interaction between sugar and aminopropyl phase is linear. Calibration graphs could be obtained by plotting the peak area vs. the load of sample for each carbohydrate (Fig. 2). The retention time remained nearly unchanged until the loading of glucose exceed 5 μg. To further examine the mechanism of the interaction between carbohydrates and amino groups bonded on the silica, we plotted the capacity factor vs. the number of fructosyl residues in the fructo-oligosaccharides, denoted $GF_n$ (Fig. 3). The retention data obtained from a commercial column, Supelcosil LC-$NH_2$

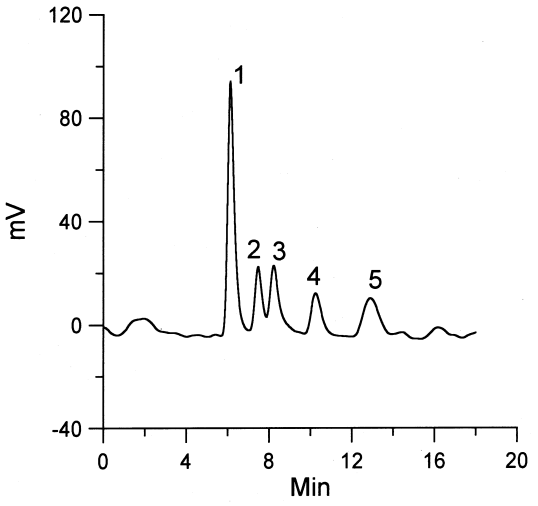


Fig. 1. Separation of fructo-oligosaccharide mixture: solvent (1), glucose (2), sucrose (3), 1-kestose (4) and nystose (5). Flow-rates, 0.5 ml/min.

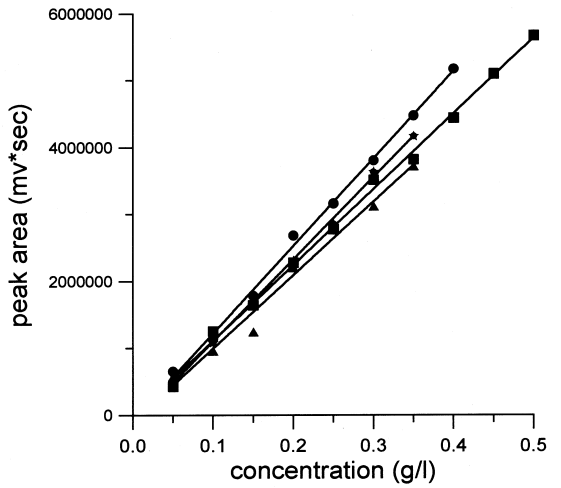


Fig. 2. Calibration curves of glucose (☆), sucrose (●), 1-kestose (■) and nystose (▲).

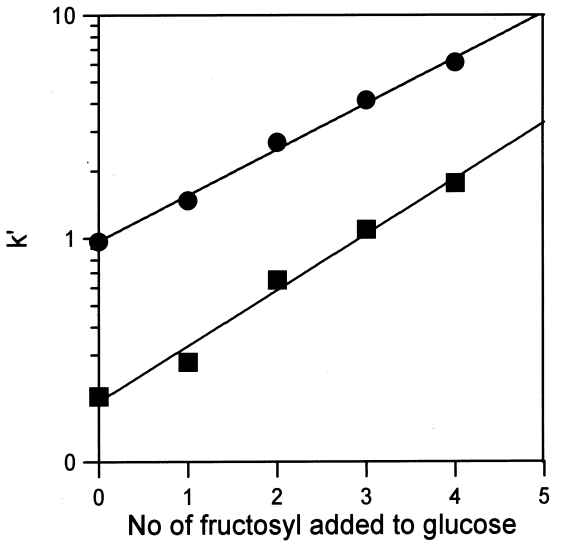


Fig. 3. Variation of the capacity factor as a function of the number of fructosyl residues in fructo-oligosaccharides. The column of Supelcosil LC-$NH_2$ (●) at a flow-rate of 1 ml/min, vs. 0.2 ml/min for the 10-cm micropellicular column (■).

(350×4.5 mm I.D., 5 μm, 10 nm), for the fructo-oligosaccharides were also reported. The composition of mobile phase for both the prepared and commercial columns was acetonitrile–water (4:1). Fig. 3 shows that the linearity was of the following form:

$$\log k' = \log k'_{\text{glucose}} + n(\Delta \log k') \quad (1)$$

The logarithm differences of capacity factors $\Delta \log k'$) were estimated from the slopes of the plots in Fig. 3 to be 0.58 and 0.47, respectively, for the columns packed with micropellicular and totally porous sorbents. The capacity factors for carbohydrates on the column packed with micropellicular sorbents were relatively small due to a low surface area of the stationary phase. However, since the effect of steric hindrance was negligible in the absence of microporous structure, and most of the surfaces were available for solute binding, the use of micropellicular sorbents led to a higher value of $\Delta \log k'$, compared with totally porous sorbents. This finding could explain why the pellicular sorbents are effective for the separation of macromolecules like proteins. A protein molecule contains plenty of functional groups that can interact to the stationary phase. According to the literature [16], the capacity of columns packed with micropellicular stationary phase is relatively low with small molecules, yet with macromolecules it is not much smaller than the capacity of totally porous sorbents. A value of 0.58 for $\Delta \log k'$ corresponds to a decrease in Gibbs free energy of about 800 cal $\text{mol}^{-1}$. This means that an additional fructosyl residue which contains three free hydroxyl groups contributed a significant degree of retention. In the case of $n=1$, Eq. (1) suggests that $\Delta \log k'$ is the logarithm difference of the capacity factors of sucrose and glucose. Data from the work of Nikolov and Reilly [17] suggest that $\Delta \log k'$ increases with the concentration of acetonitrile in the eluent.

### 3.3. Extra-column effect

The total void volume of the column is smaller than that of conventional column packed with totally porous sorbents, reflecting the lack of pore volume. The extra-column volume correspondingly represents a greater percentage of the total dead volume. Thus, its effect on column efficiency was examined in this work. The peak spreading due to extra-column effects can be described by the equation for chromatography in an open-tube column with a zero capacity. According to Taylor [18] and Golay [19], the effective diffusion coefficient that is related to the width of the peak can be expressed as

$$D_{\text{eff}} = D + \frac{u_0^2 R^2}{48D} \quad (2)$$

where $u_0$ is the superficial velocity, $R$ is the radius of the extra-column tube, and $D$ is the molecular diffusivity of the sample component. The variance (second central moment) of the peak, therefore, is obtained as

$$\sigma^2_{\text{t,ext}} = \frac{2Dl}{u_0^3} + \frac{V_{\text{ext-col}}}{24\pi D u_0} \quad (3)$$

In Eq. (3), $l$ is the total length and $V_{\text{ext-col}}$ is the total volume of the extra-column (0.181 ml). A plot of the experimental $\sigma^2_{\text{t,ext}}$ vs. the reciprocal of $u_0$, obtained from the injection of maltose as the sample component, suggests that a straight line with a slope of 0.36 can be obtained at higher values of $u_0$ (Fig. 4). Using this value of slope and Eq. (3), the diffusivity

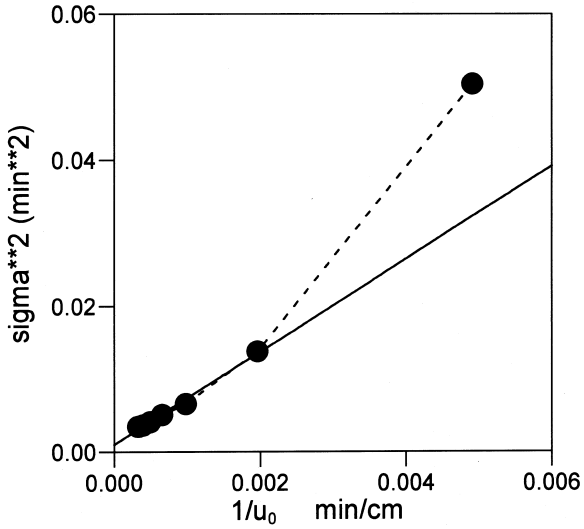


Fig. 4. Effect of flow velocity on the peak variance due to extra-column volume. The solid line was the linear regression of the variance at higher flow velocities.

$D$ was determined to be $0.63\times0^{-5}$ $cm^2/s$. The diffusion coefficient of this level is typical for a low-molecular-mass sugar in the aqueous solution. Experimental data indicate that the variance due to dead volume in the extra-column estimated by using Eq. (3) could be significant in comparison with the total variance of the elution peak. For example, the variance due to extra-column volume was estimated to be 0.05 $min^2$ at a flow-rate of 0.1 min/ml. Based on a comparison with the total variance of an elution peak from the column (0.44 $min^2$), the extra-column volume that is about 20% of the total void volume of the column contributed 11% of the peak spreading. Therefore, the extra-column tube should be as short as possible in order to reduce its effect on peak broadening.

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