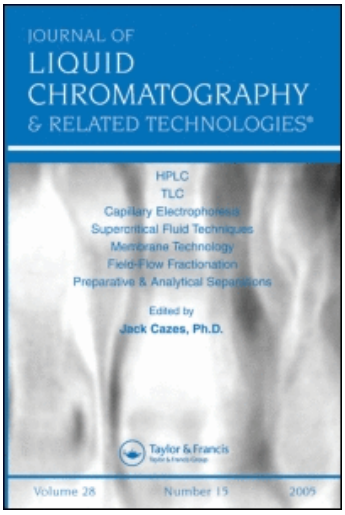

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Hua-tao Feng[a]; Xue-ying Huang[bc]; Chu-ping Luo[b]; May May Lee[a]
[a] Bioprocessing Technology Institute, Agency for Science, Technology and Research (A*STAR), Centros, Singapore [b] Sepax Technologies, Inc., Delaware Technology Park, Newark, Delaware, USA [c] Sepax-Nanjing University Lab, Science City, Changzhou, P. R. China

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Preparation of a Ligand Exchange Column for Carbohydrate Separation and Glucose Determination in Cell Culture Medium

Hua-tao Feng,[1] Xue-ying Huang,[2,3] Chu-ping Luo,[2] and May May Lee[1]

[1]Bioprocessing Technology Institute, Agency for Science, Technology and Research (A*STAR), Centros, Singapore
[2]Sepax Technologies, Inc., Delaware Technology Park, Newark, Delaware, USA
[3]Sepax-Nanjing University Lab, Science City, Changzhou, P. R. China

Abstract: The packing and testing of a ligand exchange column for the separation of carbohydrates was investigated. Resin with a 5 μm particle size and 8% crosslinking provided the best overall column performance. A range of column packing pressures were tested and revealed an optimum of 750 psi to provide good separation and low back pressure. Baseline separation of standard carbohydrates was achieved using an isocratic elution with pure water as the mobile phase and a column temperature of 85°C. The column was applied to the separation of a range of carbohydrates used commonly in the food industry, as well as the specific analysis of glucose in tissue culture medium successfully.

Keywords: Carbohydrates, Cell culture medium, Column packing, Glucose, HPLC, Ligand exchange

Correspondence: Hua-tao Feng, Bioprocessing Technology Institute, Agency for Science, Technology and Research (A*STAR), 20 Biopolis Way, Centros, Singapore 138668. E-mail: feng_huatao@bti.a-star.eu.sg

INTRODUCTION

Carbohydrates are used widely in the food industry for a variety of functions including natural sweeteners and the enhancement of nutritional value. The analysis of carbohydrates is, therefore, an important requirement for both quality control and in regulatory administration.[1,2]

A number of techniques have been proposed for the quantitative analysis of carbohydrates including colorimetric/enzymatic procedures for total carbohydrate analysis and gas chromatography for the analysis of carbohydrates following derivatization. One of the most common methods for qualitative and quantitative analysis of carbohydrates is by high performance liquid chromatography (HPLC).[3–5]

The sample preparation for HPLC is straightforward and rapid since non-derivatized carbohydrates can be injected directly.[5–7] A variety of detection methods can be used such as refractive index (RID),[8] ultraviolet,[9] amperometry,[10–13] and evaporative light scattering detection.[14,15] Carbohydrate separation using reversed phase columns has been reported.[16,17] Based on the weak acidic nature of carbohydrates, ion exchange columns had also been applied to achieve the highly selective separation of carbohydrates since each carbohydrate would carry a different negative charge in a suitable pH environment.[3] High performance anion exchange chromatography (HPAEC) became one of the most common methods for high efficiency separation of carbohydrates.[18–21] However, the above methods need either organic solvents or high concentration sodium hydroxide. A special device might be necessary when LC is coupled with MS.[22] In contrast, the mobile phase for ligand exchange columns could be pure water,[5,23,24] which is harmless and environmentally friendly. The stationary phases in ligand exchange columns were made by loading calcium, sodium, or lead ions onto a cation exchange resin containing sulfonic groups. Subsequently, the resin acquired an additional anion exchange function.

This study investigates the packing conditions and evaluation of a ligand exchange stationary phase with calcium loading and its possible applications in carbohydrate analysis. The separation of different carbohydrates was studied using an isocratic elution method. So far, most studies about ligand exchange columns were applied in the determinations of carbohydrates in foods and drinks,[5] few studies had investigated the determination of carbohydrates in a cell culture medium. Glucose, a soluble hexose sugar, is widely used in bioprocessing as a component of the cell culture medium. Its main function is to provide energy and fuel for the cells. Energy derived from glucose is stored in the form of high energy phosphate bonds in adenosine triphosphate, other nucleotide triphosphates, and as energy rich hydrogen atoms associated with the coenzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide

phosphate. In a batch feeding strategy, the nutrients should be present at levels high enough to satisfy the requirements of the cells for the full duration of the culture. Depletion of nutrients leading to energy starvation would result in poor culture viability and low productivity. Conversely, a glucose level that is too high may not be tolerated by the cells and again result in a poor culture. A compromise would be the fed batch strategy, which introduces feeding at regular intervals to prolong the culture life and productivity.[25] The determination of glucose concentration in culture supernatant is necessary for the understanding and development of the feeding strategy and also, for the design of culture medium. Therefore, the ligand exchange column was applied to the separation of a panel of standard carbohydrates as well as the determination of glucose in Chinese Hamster ovary (CHO) cell culture medium. Both ultraviolet and refractive index detections (RID) were tested.

EXPERIMENTAL

Materials and Apparatus

All reagents used were of analytical grade. Standard carbohydrates (melezitose, maltose, glucose, mannose, fructose, adonitol, maltotetraose, maltotriose, surcose, lactose, galactose) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Two ion exchange resins were supplied by Sepax-tech (Newark, DE, USA) with 5 μm particle size and either 8% or 10% crosslinking.

Chromatographic testing was performed using a Waters (Milford, MA, USA) 600E HPLC system equipped with a Waters 486 tunable absorbance detector and a Waters 626 pump. Stainless steel tubes (300×7.8 mm i.d.; 300×4.6 mm i.d.) and other accessories for column packing were purchased from Iso-tech (Hopedale, MA, USA). Packing pressure was applied using a Haskel air driven fluid pump (Burbank, CA, USA).

For the determination of glucose in cell culture medium, a Shimadzu HPLC system (Kyoto, Japan) was used, consisting of SCL-10A VP controller, CTO-10AC VP oven, LC-10 AD VP pump, SPD-10A VP UV detector, and RID-10A refractive index detector.

Column Packing and HPLC Testing

The columns were downward packed using 50% (v/v) acetonitrile in water as the driving solvent. For a 300×7.8 mm i.d. column, 10 g of dry resin was suspended in 50% (v/v) acetonitrile. For each column dimension and resin type, packing was performed for 3 hours at either

750 psi or 1100 psi. Following packing, the columns were conditioned with mobile phase until a stable baseline was obtained.

Stock solutions of all carbohydrates were made by dissolving carbohydrates in ultrapure water at a concentration of 40 mg/mL. Stock solutions were stored at 4°C. Separations were achieved using ultrapure water as mobile phase and the temperature of the HPLC oven was set at 85°C. Isocratic elution was carried out at a flow rate of 0.40 mL/min for 30 min. Sample injection volume was 10 mL and UV detection was set at 192 nm.

CHO Cells were grown in-house for four days. Cells and culture medium were centrifuged for 5 min at 1000 rpm at 4°C. The supernatant was collected and centrifuged at 14000 rpm (18000 rcf) at 4°C for 10 min before the injection into the HPLC system.

RESULTS AND DISCUSSION

A UV detector was used in the initial packing test and for subsequent analysis of carbohydrates, and a low wavelength of 192 nm was applied. Mobile phase was pure water and the mode of elution was isocratic. Due to the high boiling point of water the column temperature could be increased to 85°C. There are two advantages for using elevated temperature, first, a high temperature reduces the viscosity of the mobile phase and increases the permeability of columns. Second, better resolution was achieved at high temperature. Chromatograms showed that carbohydrate peaks were broad even with a temperature of 70°C; the phenomenon might result from the decrease of carbohydrate ionization at lower temperature. To compare the performance of columns packed by different conditions, a flow rate of 0.4 mL/min was used, since a higher flow rate was not suitable for the columns packed under high pressure.

Effect of Packing Pressure

The stationary phase was packed into a 300×7.8 mm i.d. column at 1100 psi. The column showed high resolution for the six carbohydrates used to test the system (Figure 1). However, the column back pressure was too high at a flow rate of 0.4 mL/min due to the compactness of stationary phase. The back pressure increased from 1200 to 2000 psi when the flow rate was increased to 0.5 mL/min. This implied that such a column would be limited by the number of analyses preformed pre unit time.[26] Although it was noted that the column could tolerate a back pressure of 2000 psi, the flow rate was reduced to 0.4 mL/min and it was found that similar retention times and column efficiencies were obtained.

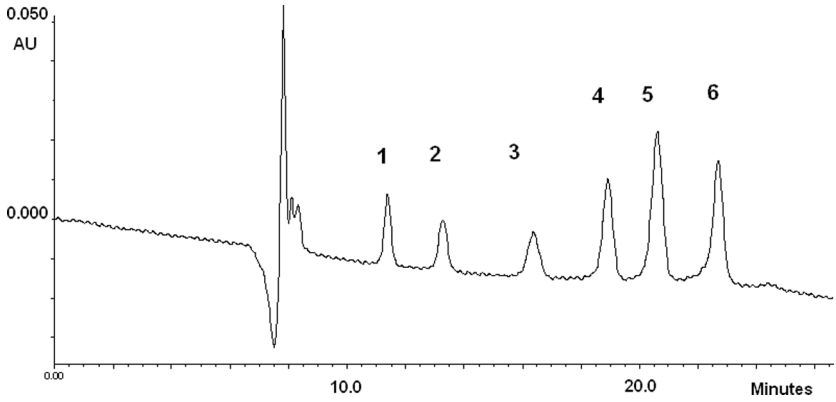

Figure 1. Separation of six carbohydrates using a column packed at 1100 psi. Column dimension: 300 × 7.8 mm i.d.; Resin: 8% cross linking; Mobile phase: water; Flow rate: 0.4 mL/min; Column Temperature: 85°C. Sample injection volume: 10 μL. Detection: UV at 192 nm. Peaks: 1, melezitose; 2, maltose; 3, glucose; 4, mannose; 5, fructose; 6, adonitol. Concentration of each carbohydrate: 6 mg/mL.

Next, a higher pressure (4000 psi) was applied for half an hour before returning the flow rate to 0.4 mL/min. Analyses following this showed that the retention times were now significantly increased. It is likely that the packing of stationary phase was affected by high pressure. However, the column recovered the previous retention times after one day. The solid phase resin is therefore able to withstand a pressure of up to 4000 psi.

In order to allow the column to operate at lower pressures and also allow for a greater range of flow rates, a packing pressure of 750 psi was used for the next test. As a result of the looser packing, a stable back pressure of 680 psi was obtained with a flow rate of 0.8 mL/min at 85°C. Using the same 0.4 mL/min flow rate, the separation efficiencies of maltose, glucose, mannose, and fructose were reduced to around 70% from the 1100 psi packed column, while the plate counts of peaks of melezitose and adonitol remained unchanged. Baseline separation was still achieved and the resolution for six carbohydrates were higher than 1.7. Therefore, 750 psi was adopted as optimal packing pressure.

Effect of Stationary Phase Crosslinking

Another alternative to reduce column back pressure is by a higher degree of crosslinking. This can improve the strength of the resin as well as avoiding an overly compact packing. A 10% crosslinked resin was packed

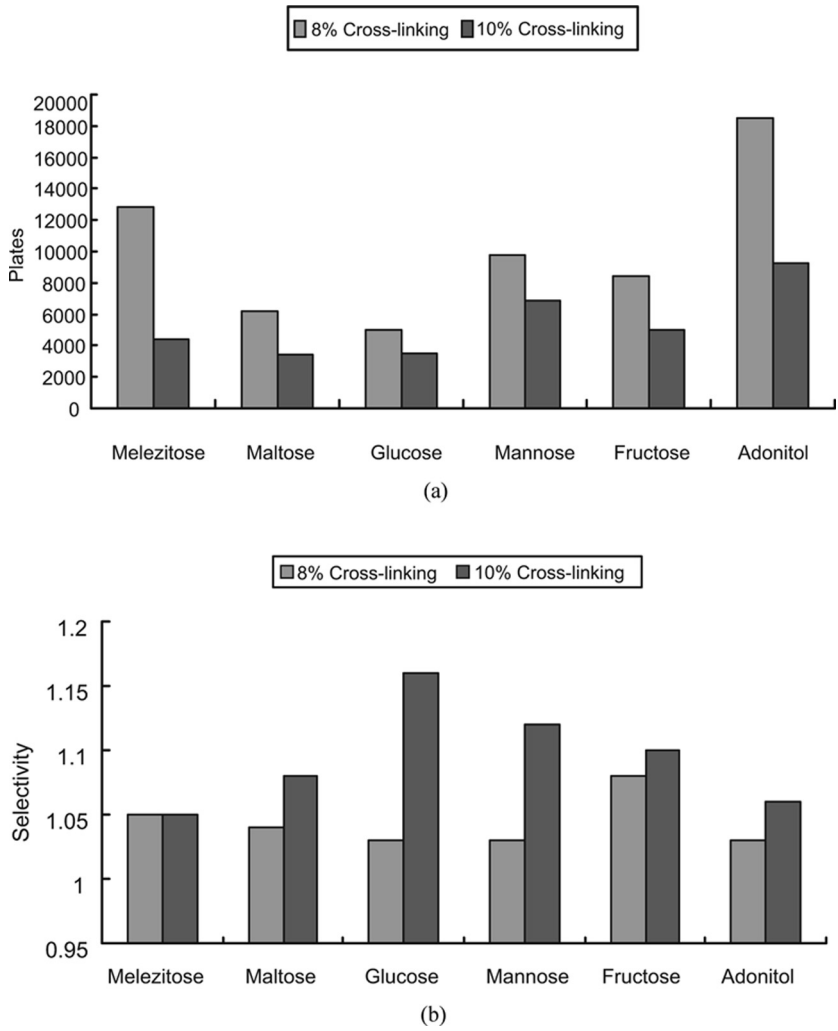

Figure 2. Comparisons of the column performances obtained using resins exhibiting different degrees of cross linking. Packing pressure: 750 psi. Other conditions as in Figure 1.

into a 300×7.8 mm i.d. column using a pressure of 750 psi. The column had a back pressure of 190 psi at a flow rate of 1.0 mL/min. However, the separation efficiencies and selectivities decreased significantly (Figure 2) with the increase of cross linkage. In addition, the first carbohydrate peak (melezitose) comigrated with the system peak.

A 10% crosslinked resin was also packed using a pressure of 1500 psi. However, the separation performance didn't improve significantly.

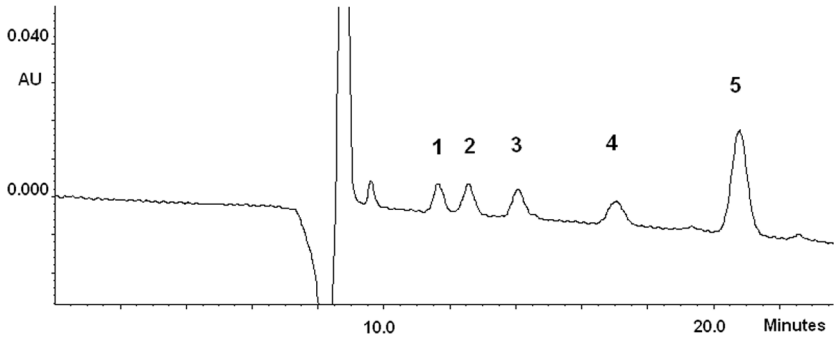

Figure 3. Separation of five common carbohydrates existed in beer. Packing pressure: 750 psi. Other conditions as in Figure 1. Peaks: 1, maltotetraose; 2, maltotriose; 3, maltose; 4, glucose; 5, fructose.

Instead, there was an immediate relaxing of the resin when the high packing pressure was stopped. Based on these observations, an 8% crosslinked resin was adopted for the stationary phase.

The optimal separation that had now been obtained can be applied to the separation of carbohydrates commonly used in the food industry. For example, the separation of five common carbohydrates present in beer[14] could be achieved using this column (Figure 3).

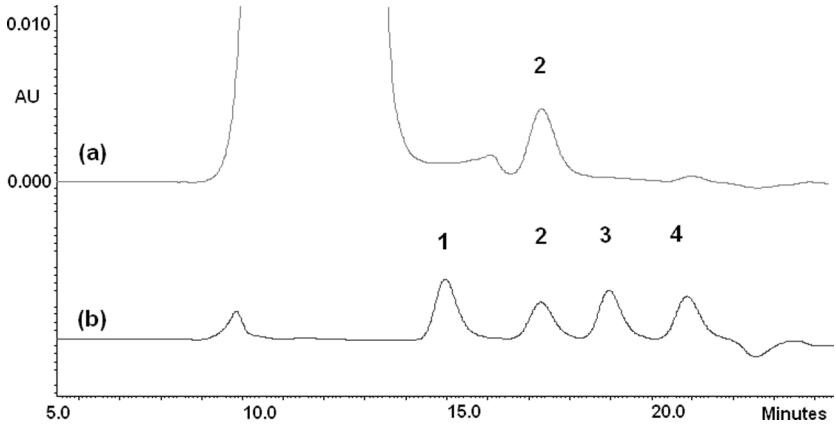

Figure 4. Determination of glucose in CHO cell culture medium using a refractive index detector. Packing pressure: 750 psi. Column Temperature: 80°C. (a), Cell culture medium after centrifugation; (b), Standards of four sugars: 1, lactose; 2, glucose; 3, galactose; 4, fucose; 1.0 g/L each.

Table 1. Quantification data of four carbohydrates using RID

Analytes	RSD (n = 6, %, Peak areas)	RSD (n = 6, %, Retention times)	LOD (mg/L)	LOD with UV detector (mg/L)	Linearity (R^2)
Lactose	1.89	0.12	0.41	38	0.9998
Glucose	3.45	0.18	0.62	86	0.9997
Galactose	2.94	0.26	0.46	27	0.9995
Fucose	2.79	0.14	0.38	19	0.9997

Determination of Glucose in Cell Culture Medium

A column packed with 8% crosslinked resin at a pressure of 750 psi was used for the determination of glucose in a cell culture medium. The maximum column temperature (80°C) allowed by the Shimadzu HPLC system was applied during separation. The cell culture solution was injected and analyzed following a minimal sample preparation method (Figure 4). The glucose concentration after four days of culture was 1.86 g/L, which was reasonable since the glucose concentration was 3.00 g/L at the beginning of this culture. The recovery (95.1%) was tested by spiking 500 mg/L of glucose into the culture medium with 10 times dilution.

The reproducibility of the separation using this column was evaluated by making six replicate injections of a standard solution containing four different carbohydrates, each at 20 mg/L, and calculating the RSD of retention times and peak area (Table 1). To test the quantification of the method, six mixtures of carbohydrate with concentration levels from 5 to 1000 mg/L were measured. The calibration curves exhibited a linear dynamic range with good correlation coefficients (R^2). The detection limits (S/N = 3) of carbohydrates with RID were lower than 1 mg/L, i.e., better than that by UV detection.

CONCLUSIONS

Ligand exchange packing resin loaded by calcium ions can be applied to the separation of carbohydrates using a simple isocratic elution profile. Using pure water as a mobile phase, it was possible to achieve baseline separation of six different carbohydrates. The 8% crosslinked resin and a column packing pressure of 750 psi gave the best column performance. The glucose concentration in CHO cell culture medium was determined and the complex background matrix did influence the separation. This ligand exchange column can be expanded to other potential applications of carbohydrate analysis in subsequent studies.

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