SEPARATION OF SUGAR ANOMERS BY CAPILLARY ELECTROCHROMATOGRAPHY

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Abstract

Capillary electrochromatography (CEC) as one of the most efficient separation techniques has been successfully applied to carbohydrate analysis. CEC columns are pressure-packed with an octadecyl silica stationary phase and exhibit a linear velocity of electroosmotic flow up to 1.25 mms^{-1} and separation efficiencies up to 220 000 plates/metre for thiourea. PMP-derivatives of sugars are successfully separated with large separation efficiencies (up to 600 000 plates/metre for PMP-mannose). This study shows that it is possible to separate the anomers of some sugars, PMP-glucose, galactose and lactose, based on their complexation with borate. The optimal eluent consists of 15 mM boric acid in 75% MeCN, 25% water (v/v). The anomeric separation of PMP-mannose is not possible even at elevated concentrations of boric acid.

Introduction

Capillary electrochromatography (CEC) is an electromigration technique that has received increasing attention in the past few years [1-3]. In CEC, the separation of solutes is based on their partitioning between the stationary phase and the eluent which is pumped by means of electroosmotic forces. Since the flow is electrically driven, the flow profile is plug-like contrasting to the pressure driven flow in HPLC, which is one of the reasons why CEC gives higher resolution and better separation efficiencies than micro-HPLC on the same column. These and other advantageous features have made CEC an established analytical technique in some fields of research.

A limiting factor in the application of CEC is that the preparation of packed columns is still a cumbersome procedure. Also, CEC is susceptible to excessive Joule heating and/or bubble formation [4], especially when concentrated buffers are employed. These obstacles can limit the interest and success of CEC. There are commercial

suppliers of CEC columns, which means that packing a column is not necessarily prerequisite and there are also commercial instruments that allow pressure to be applied on both sides of the column to prevent (or diminish) bubble formation. Despite of this, the packing of columns and the operation of CEC under ambient pressure still represents quite a challenge [5].

There are several ways to pack capillaries with a stationary phase, including pressure packing [6], electrokinetic packing [7] and packing with a supercritical fluid carrier [8]. The most delicate part of the whole packing procedure is frit formation since the quality of the frits directly affects the performance of the column and can be a major source of bubbles when running CEC.

CEC has been successfully applied for many compounds of interest, including pharmaceuticals [9, 10], peptides and proteins [11], nucleosides [12] and mono- and oligosaccharides [13, 14]. All these investigations clearly demonstrate the usefulness of CEC for the quantitative analysis of different classes of compounds, and broaden the range of possibilities of CEC.

Carbohydrate analysis especially is destined to profit from the high separation power of CEC. Namely, the separation and quantification of carbohydrates is complicated by the diversity of carbohydrate species. This heterogeneity arises from configurational and conformational differences. Not only is it important to determine the monosaccharide composition of a given carbohydrate, it is also needed to know in which isomeric form a constituent part is present, since the biological role depends on the anomeric form of a given monosaccharide or the type of linkage between various units [15].

Separation of anomeric forms (α-, β-) of sugars and other closely related carbohydrates is achieved on graphitised carbon columns where the separation process is based on adsorption onto the flat surface of the packing [16, 17]. Also, when using special columns, the anomers can be separated by reversed phase HPLC [18]. Nevertheless, it is usually undesirable in HPLC to observe anomers due to the peak splitting.

Carbohydrate analysis is additionally complicated because most sugars do not possess chromophoric groups to enable UV detection. To achieve this, a derivatisation must be performed prior to analysis or post-column. Derivatisation with 1-phenyl-3-methyl-5-pyrazolone (PMP) is one of the most attractive possibilities, owing to its sensitivity of detection and the formation of a single derivative [19]. Especially important, from the point of view of anomer separation, is that PMP-derivative analysis exhibits no anomer separation neither by HPLC [20, 21] nor by CZE [22, 23].

In this study, the possibility of anomer separation by CEC is investigated. First, the packing procedure for CEC columns used in our previous study [13] is modified to produce columns with improved characteristics. Since the apparatus employed allows only UV detection, sugars are derivatised with PMP. Second, the separation of anomers is studied using different buffers. In previous accounts on PMP-derivatives separation [20-23], the separation of anomers is not shown. However, there is a report on the separation of p-nitrophenyl derivatives [14], showing the possibility of anomer separation. This study shows that the advantageous characteristics of CEC permit an efficient separation of sugar anomers.

Experimental

Chemicals

All chemicals were of analytical grade and used as supplied. Sodium dihydrogen phosphate (NaH_2PO_4) and boric acid (H_3BO_3) – both used for buffer preparation – were purchased from Kemika (Zagreb, Croatia). HPLC grade acetonitrile was purchased from Rathburn (Walkerburn, Scotland) and deionised water was additionally purified using the Milli Q water purification system (Millipore, USA). Sugar standards (D-glucose, D-galactose, D-mannose, α-lactose and β-lactose) and the derivatising agent PMP were supplied by Sigma-Aldrich (Steinheim, Germany). Fused silica capillaries (100 μm I.D. and 360 μm O.D.) were purchased from Supelco (Bellefonte, USA). The octadecyl silica (ODS, 5 μm) stationary phase and bare silica (5 μm) were supplied by Phase Separations

(Deeside, UK). The HPLC pump was from Milton Roy (now ThermoSeparationProducts, San Jose, CA, USA).

Instrumentation and CEC procedure

The instrumentation comprised a 270A-HT Capillary Electrophoresis System (Applied Biosystems, Perkin Elmer) equipped with Turbochrom software. The shortest capillary that the instrument can accommodate is 50 cm and the packed capillaries were of such a length or exceeding it. The length to the detector is 30 cm. The injections were performed electrokinetically and the PMP derivatives of saccharides were detected at 245 nm. Different buffers were used either containing sodium dihydrogen phosphate (from 1 mmol/L to 5 mmol/L) or boric acid (from 5 mmol/L to 30 mmol/L). The content of acetonitrile varied from 40% to 80% (v/v).

Preparation of PMP-derivatives

Derivatisation was performed according to Honda *et al*. [19]. Briefly, a sample of a saccharide (100 nmol) or a mixture of saccharides is dissolved in 0.3 mol/L NaOH (50 μL). Afterwards, a 50 μL portion of 0.5 mol/L methanolic solution of PMP is added and the mixture left at 70 ^{o}C for 30 min. The resulting solution is then neutralised with 50 μL of 0.3 mol/L HCl and additionally diluted with 50 μL of water. The solution is directly analysed by CEC without removing the derivatising reagent.

Column packing

The procedure for column packing is given in our earlier paper [13]. In our case, the frit formation is substantially simplified. The capillary is cut to its correct length (usually 60 cm, to allow some extra length if the capillary has to be cut) and dipped repeatably into dry bare silica until the silica is trapped into the capillary for approximately 2 mm. The silica is then heated using a burner for one minute (the

temperature in the middle of the flame is estimated to be around 800 °C). In this way a retaining frit is made. The open end is then attached by a fitting to an empty HPLC column used as the slurry reservoir. A schematic of the assembly is given in Figure 1.

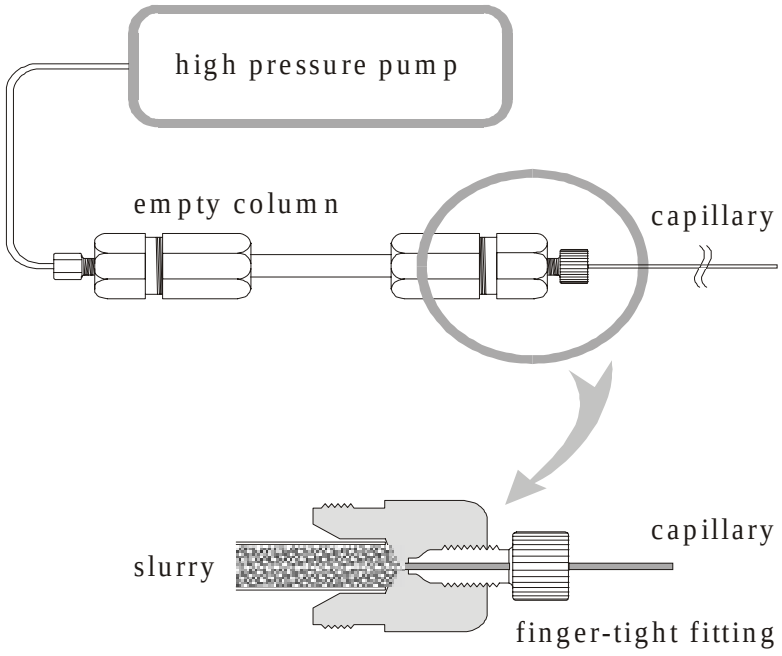

Figure 1: Schematic representation of the packing assembly and a detailed arrangement of capillary and the slurry reservoir.

A slurry of the packing material is prepared in methanol, sonicated for 15 min and transferred into the slurry reservoir. The capillary is attached to the slurry reservoir by means of a finger-tight capillary fitting (Upchurch Scientific, Bremehaven, Germany). It is very important that the capillary does not protrude into the reservoir - the details are given in the exploded view in Figure 1. Once the capillary is properly attached, the pressure is slowly raised to 400 bar and the capillary left to pack for an hour. Sometimes, the first packing does not produce a fully packed column. In this case the packing is repeated. Once the column is full, it is sonicated for 5 minutes with the frit out. Some

reports use sonication during the packing procedure, however, we decided against it because the frit can be damaged allowing the packing to escape. After sonication, acetonitrile is pumped through the column. The frit on the detector end is made in the same way as the inlet frit. The column is then checked and conditioned in the instrument (20 min at each voltage up to 30 kV, with increments of 5 kV, buffer is usually 1 mmol/L NaH_2PO_4, 80% acetonitrile and 20% water (v/v)). After conditioning, the column is taken out and a pressure of 400 bar is applied again. During conditioning, the particles will rearrange and an empty space will appear at the outlet end. Pressure is then applied again and the empty section of the column is removed and a new frit made. This re-fritting and re-conditioning procedure is usually repeated 2 to 3 times. Only then is the column ready for use.

Since the capillaries we use have a transparent UV coating, it is not necessary to make a detection window which makes the columns more durable, and care only has to be taken for the inlet and outlet frit. The sensitivity of detection is reduced [14] but this does not represent a problem because we do not use the technique for trace analysis.

Results and discussion

The characteristics of the packed columns

The packing procedure produces fully packed columns which are durable and easy to use and flush if the bubbles form inside the column. When using 1 mmol/L NaH_2PO_4, 80% acetonitrile and 20% water (v/v) as a buffer, thiourea elutes at 4 min at an applied voltage of 30 kV (600 V/cm) at 25 °C. The calculated linear velocity is 1.25 mm/s and the column efficiencies for thiourea are in the order of 220 000 plates per metre.

We also studied the temperature dependence of the electroosmotic flow (EOF). The results are depicted in Figure 2.

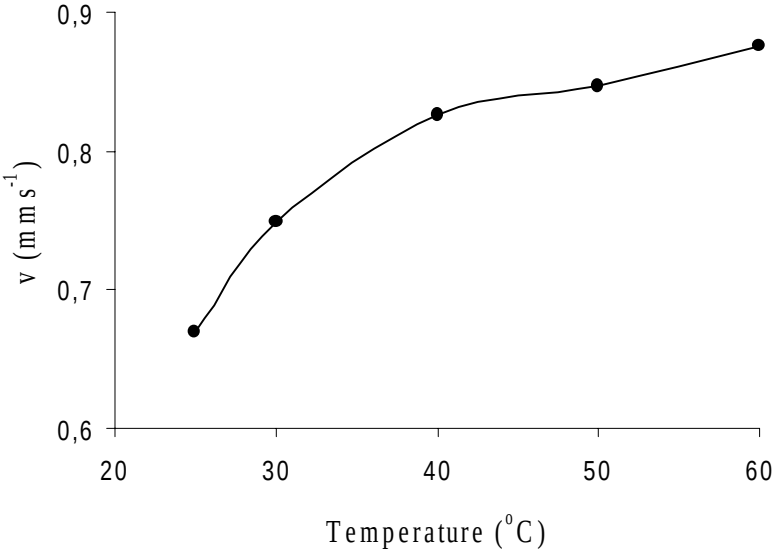

Figure 2: The temperature dependence of EOF (i.e., its linear velocity). Conditions: thiourea as a marker; 30/50 cm, 100 μm I.D. capillary, packed with 5μm ODS; UV detection at 254 nm through the packed section at 30 cm from the column inlet; mobile phase: 1 mM NaH_2PO_4 in 80% acetonitrile, 20% water (v/v); injection: 5 s at 10 kV; analysis: 20 kV.

The data agree with Cahours *et al.* [10]; i.e. the flow rate increases with temperature, but not regarding column efficiencies, which our experiments show do not change with increasing temperature. One reason could be the different stationary phase, which was phenyl in the cited reference, whereas we use ODS.

Anomer separation

In the analysis of sugars by reverse phase HPLC, peak splitting can occur due to the partial separation of anomeric forms. This phenomenon is usually seen as undesirable. We wanted to examine the possibilities for anomer separation using CEC which can overcome this problems by its high separation efficiency.

First, we derivatise the sugars with PMP to facilitate UV detection. The separation of PMP derivatives of some monosaccharides is described in our earlier paper [13]. When using 5 mM NaH_2PO_4 in 80% acetonitrile, 20% water (v/v) buffer and a column which exhibited efficiencies up to 150 000 plates/metre for thiourea, we do not observe any peak splitting. The column efficiencies (N) for five important sugars are given in Table 1. Note a high N for PMP-mannose. The reason for such a sharp peak is currently not known and can be attributed to a focusing effect which was also observed in the case of some other substances, like pharmaceuticals [9].

Table 1: Column efficiencies (plates/m) for some PMP-derivatives separated on an ODS capillary column. Conditions: 30/50 cm, 100 μm I.D. capillary, packed with 5μm ODS; UV detection at 245 nm through the packed section at 30 cm from the column inlet; mobile phase: 5 mM NaH_2PO_4 in 80% acetonitrile, 20% water (v/v); injection: 5 s at 10 kV; analysis: 10 kV

Substance	Column efficiency (plates/m)
PMP-mannose	600 000
PMP-glucose	400 000
PMP-galactose	170 000
PMP-α-lactose	90 000
PMP-β-lactose	110 000

When the buffer is changed to 1 mM NaH_2PO_4 in 80% acetonitrile, 20% water (v/v) and the column is replaced with a superior one (with N up to 220 000 for thiourea) and the separation voltage is only 7 kV, we observe peak splitting which is especially pronounced for lactose and galactose (Figure 3). This gave us an idea to indulge further in examining this interesting possibility with the use of different running buffers, namely, boric acid buffers.

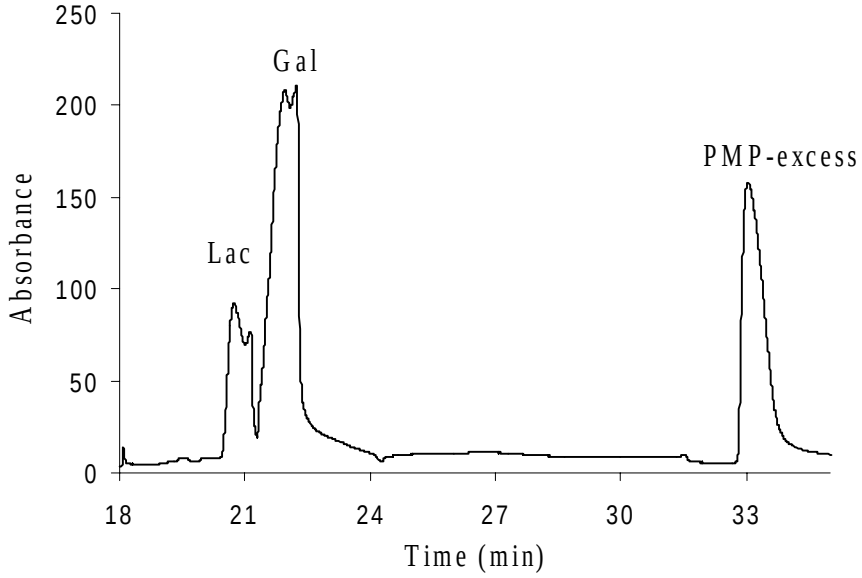

Figure 3: Peak splitting in the case of lactose and galactose with 1 mM phosphate buffer. Conditions: the same as in Figure 2, except: UV detection at 245 nm; run at 7 kV.

It is shown that using a borate buffer [14], anomers of p-nitrophenyl-monosaccharides are successfully separated. Thus the buffer was changed to borate buffer with different concentrations of boric acid and acetonitrile content. It is observed that at elevated concentrations of boric acid (up to 30 mM) the baseline becomes unstable.

It is observed that the separation of anomers of PMP-mannose is not possible even at elevated boric acid concentrations (up to 30 mM). In Figure 4, an electrochromatogram of PMP mannose is depicted, using 15 mM buffer, showing a slight tailing of the peak which presumably arises from anomers but its shape could be also attributed to other reasons.

Boric acid buffer is very efficient for separating the anomers of glucose, galactose and lactose. The optimal boric acid concentration is 15 mM and the acetonitrile content 75% (v/v). If the boric acid concentration is lower than 15 mM, no anomer separation is achieved, whereas at higher concentrations a very noisy baseline is observed and the

peaks are distorted. β- anomers elute earlier than their α-counterparts because the former interact stronger than the latter with borate as described by Yang and El Rassi [14]. The running voltages are usually 8 kV, giving separation of anomers in less than 35 min. Figure 5a) shows separation of anomers of PMP-glucose and 5b) PMP-galactose. The anomer separation of PMP-mannose is not possible even at elevated boric acid contents.

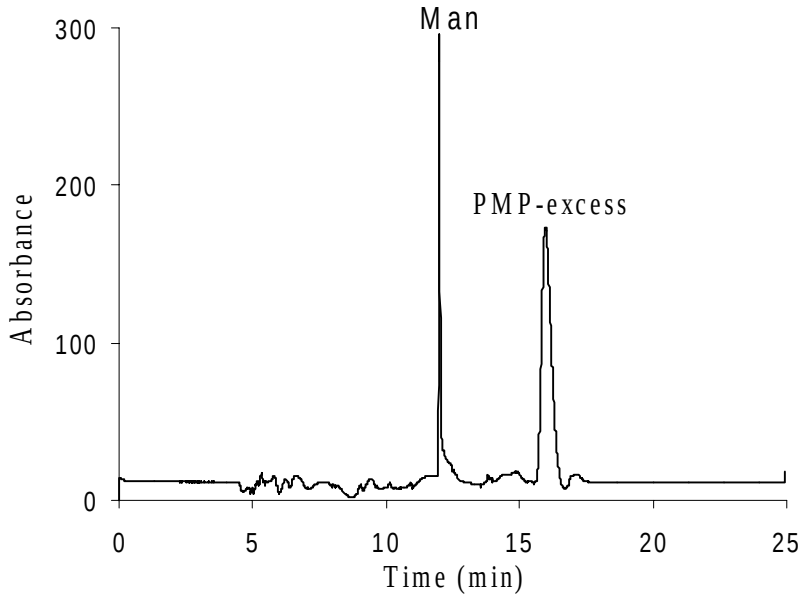

Figure 4: Electrochromatogram of PMP-mannose, showing no anomer separation. Conditions: the same as in Figure 2, except: detection at 245nm; 30 mM boric acid in 75% MeCN, 25% water (v/v) as buffer; injection: 5 s at 5 kV; run at 15 kV.

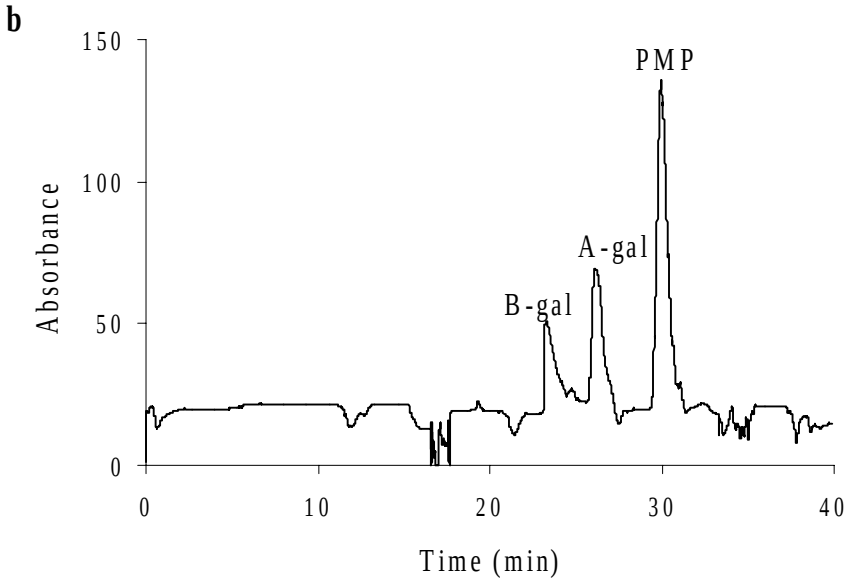

Figure 5: Separation of anomers of PMP-derivatives: a) PMP-glucose and b) PMP-galactose. Conditions: the same as in Figure 2, except: detection at 245 nm; 15 mM boric acid in 75% MeCN, 25% water (v/v) as buffer; injection: 5 s at 5 kV; run at 8 kV (for glucose) and 10 kV (for galactose).

Conclusions

In this paper, we present a detailed description for packing capillary columns for CEC. We succeeded to produce fully packed columns with very high efficiencies of separation. We noticed that the EOF increases at elevated temperatures, without the separation efficiency being compromised.

We also developed a method for anomer separation of sugars, using boric acid buffers. Sugars are derivatised with PMP to facilitate UV detection. The choice of buffer concentration is crucial for good separation since at lower contents no separation is achieved whereas at higher contents the anomers elute as very diffuse peaks on a noisy baseline. By means of using boric acid buffer, a successful separation of PMP-glucose, galactose and lactose is made possible, but PMP-mannose resists the separation even at elevated boric acid contents in the buffer.

In our research of saccharides using CEC we plan to use other possible derivatisation methods for analysis of glycoproteins, thus broadening the scope CEC.

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Povzetek

Kapilarna elektrokromatografija (CEC) se kot ena najbolj učinkovitih separacijskih tehnik uspešno uporablja tudi pri analizi ogljikovih hidratov. Kolone za CEC so napolnjene z modificiranim silikagelom (ODS) in omogočajo linearno hitrost elektroosmotskega toka do 1.25 mms^{-1} ter separacijske učinkovitosti do 220.000 teoretskih podov/meter.
PMP-derivati nekaterih sladkorjev so bili uspešno ločeni z visokimi separacijskimi učinkovitostmi (do 600 000 podov na meter za PMP-manozo). Pričujoča študija kaže, da je mogoče ločiti anomere nekaterih sladkorjev na osnovi njihove kompleksacije z boratom. Optimalni eluent vsebuje 15 mM borne kisline v 75% MeCN, 25% vode (v/v). Separacija anomer PMP-manoze ni možna niti pri povišanih koncentracijah borne kisline.