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Resolution and signal-to-noise in analysis of carbohydrate isomers by graphitised carbon chromatography with charged aerosol detection

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ABSTRACT

The effects of co-eluents and additives on separation and signal-to–noise ratio in analyses of monosaccharides by graphitised carbon chromatography (GCC) in combination with charged aerosol detection were studied. Design of experiments was used to model and predict the elution of two monosaccharide isomers, galactose and glucose and the corresponding amine at varying isocratic conditions, including concentration of water-soluble co-eluent, flow and temperature. The study confirmed the well-known order of eluent strength of the co-eluents investigated but showed that the eluent strength of MeOH was significantly lower than that of ACN, and at co-eluent concentrations ≥5% (v/v) IPA approached that of THF. Addition of NH_3 increased retention and improved both peak shape and separation but the detector response decreased with increasing NH_3 concentration lowering the signal-to-noise ratio. The best combination of response, retention and separation was obtained at 0.1% NH_3. Increasing column temperature in the range of 15–50 °C resulted in decreased retention times and resolution. The corresponding Van't Hoff correlations showed negative adsorption enthalpies indicating an exothermic adsorption process driven by a decrease in entropy minimising the surface energy of the system. Isocratic elution with MeOH as co-eluent offered limited possibilities for optimisation of resolution due to the opposite effects of changes in co-eluent concentration and changes in flow rate. Elution with acetonitrile as co-eluent showed possibilities for optimisation of the resolution within the range of flow rates of 0.6-0.95 mL/min and co-eluent concentrations of 0.1–0.3%, with the highest resolution predicted at 0.1% acetonitrile and a flow rate of 0.81 mL/min. Saccharides in the size range DP1–4, including amino, acetamido, and deoxy sugars, were separated using a binary gradient method. Higher retention was observed for increasing degree of polymerisation (DP) and N-acetylated saccharides were retained longer than non-substituted saccharides of corresponding DP. Partial resolution of two lacto-N-tetrasaccharide positional isomers was obtained.

1. Introduction

Separation of carbohydrates remains a challenge due to the variety of isomers with high polarity and similar chemical properties. Graphitised carbon chromatography (GCC) has been used for analyses and preparative separations of a wide range of saccharides and their derivatives in combination with different detection methods, including refractive index (RI), UV, and pulsed amperometric detection (PAD), respectively [1–3]. The surface of porous graphitic carbon (PGC) is planar and retains analytes through dispersive and charged-induced interactions, offering unique selectivity and the possibility to resolve isomeric and closely related compounds. Elution patterns are based on the molecular size and planarity, with larger and more planar molecules generally retained more strongly. Molecular conformation and type and positioning of functional groups in relation to the PGC surface are important properties affecting the area of contact and charge-induced dipole interactions with the stationary phase, and thereby contributing to the retention [4]. The adsorption mechanism of fluorescence-labelled malto-oligosaccharides was found to depend on the type of organic modifier used in the mobile phase. Positive adsorption enthalpies and entropies were observed using 35% (v/v) acetonitrile as mobile phase in the temperature range of 53–67 °C, which are explained by the displacement of a dense and highly ordered acetonitrile solvation layer on the PGC surface. On the other hand, negative enthalpies were observed with either 10% (v/v) tetrahydrofuran or 17.5% (v/v) 2-propanol as mobile phase [5].

The exceptional chemical and physical stability of the PGC allows repeated use without loss of performance or reproducibility. As PGC is completely unaffected by strongly acidic or alkaline

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conditions and can be used throughout the entire pH range, separations with a much wider range of solvents become feasible [1,6]. PGC has successfully been used to separate aldonic acids of cello-oligosaccharides and their corresponding lactones by acetonitrile gradients in water with acidic and alkaline additives respectively [7], and CAD was used for detection of C1 and C4 oxidised cello-oligosaccharides with ammonium acetate as additive in optimal concentrations above 10 mM [8]. The response of the CAD is known to depend on the composition of the mobile phase, with higher response at increasing organic solvent concentrations [9,10]. Nevertheless, the effect of organic additives to eluents on chromatographic separation and the sensitivity of detection has received relatively limited interest.

Design of experiments has previously been applied successfully for statistical analysis, modelling and prediction of chromatographic methods [11]. Such systematic approaches minimise the number of experiments needed to collect the data necessary for statistical analyses and modelling the relationships between experimental variables and the corresponding responses. The resulting modelling and prediction can be utilised for directed development of elution strategies [11,12].

The present study focussed on analysis of unmodified carbohydrates by graphitised carbon chromatography (GCC) in combination with charged aerosol detection (CAD). The aim was to study the effects of co-eluents and additives on separation and signal-to–noise ratio in analyses of monosaccharides by GCC in combination with CAD and evaluate the compromise between resolution and the signal-to-noise ratio. Retention and adsorption mechanisms of epimers in the temperature range of 15–50 °C are reported. Design of experiments was used to model and predict the elution of three monosaccharides at isocratic conditions, the variables investigated were flow rate, type and concentration of co-eluent. The model was used to predict chromatographic conditions resulting in optimal peak resolution. In addition, results on the separation and detection of saccharides in the size range DP1–4, including amino, acetamido, and deoxy sugars are presented and briefly discussed.

2. Materials and methods

2.1. Materials

Acetonitrile (ACN, LC–MS grade) and 2-propanol (IPA, HPLC grade) were from VWR Chemicals, methanol (MeOH, HPLC grade), tetrahydrofuran (THF, ≥ 99.9%) and trifluoroacetic acid (TFA, 99%) were from Sigma-Aldrich, and ammonium (NH_3, 25% w/w solution) was from J.T. Baker. Water (H_2O) of Type I purity (ELGA PURELAB flex) and organic co-eluents were sparged with nitrogen (N_2, quality 3.0) for 10 min, and sonicated (Elma Transsonic Digital water bath) for 20 min.

D-Glucose (Glc),D- galactose (Gal), L-fucose (Fuc, S-1266, approx. 95%), D-melibiose (Mel, ≥ 98%) D-maltotriose (Glc3, ≥ 95%), D-maltotetraose (Glc4, 96%), and *N,N′,N″*triacetylchitotriose (GlcNAc3, approx. 95%) were from Sigma-Aldrich, glucosamine (GlcN) was from AppliChem, D-lactose (Lac) was from Merck, D-maltose (Mal) was from MP Biomedicals, and *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), *N*-acetyllactosamine (LacNAc), *N,N′*-diacetylchitobiose (GlcNAc2), lacto-*N*-triose II (LNT2), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT) were from Carbosynth.

2.2. Software

Dionex Chromeleon 7 (7.2.1, Thermo Fisher Scientific) was used to acquire the chromatographic data, MATLAB (R2015b, The MathWorks, Inc.) was used for data processing, and R (3.1.2, The R Foundation for Statistical Computing) was used for statistical analyses.

2.3. Chromatographic conditions

Saccharides in water (0.2 g/L, 10 μL inj) were analysed using an Ultimate 3000 system (Dionex) with a Corona Veo charged aerosol detector (N_2 pressure 35 psi, nebulizer temp. 50 °C, range 100 pA, filter constant 5 s). The column used was a HyperCarb porous graphitic carbon column (150 x 4.6 mm, with 10 x 4 mm guard, Thermo Scientific) maintained at 25 °C. Eluents consisted of water and MeOH, with 0.1% NH_3 in both eluents. Flow rate was 0.5 mL/min. Deviations from this reference elution method are specified below.

Isocratic elutions of Glc, Gal, and GlcN were used to study the effects of additives in amounts of 0.1% and 0.25% TFA, and 0.05%, 0.1%, and 0.25% NH_3, respectively. Column temperatures were investigated at 5 °C intervals in the range 15–50 °C. Eluent strength of organic solvents MeOH, ACN, IPA and THF were studied at isocratic co-eluent concentrations of 1%, 3% and 5% respectively. Varying flow and co-eluent concentration of MeOH or ACN were performed according to the experimental designs described.

Gradient elutions of Glc, Gal, GlcN, GlcNAc, GalNAc, GlcNAc2, Lac, LacNAc, Mel, Mal, Glc3, Glc4, GlcNAc3, LNT2, LNT, and LNnT were performed at 15 °C, with 20 μL of samples injected, using water, MeOH and ACN according to the following elution profile: 100% water to 5% MeOH at 5 min, to 0% MeOH and 4% ACN at 10 min, to 44% ACN at 60 min (24% ACN at 35 min).

2.4. Design of experiments

In the low concentration range of organic co-eluent the experimental set-up for varying flow and concentration of co-eluent was minimized using a design-of-experiments approach. Elution was defined by the two variables flow (*F*, 0.5–1.0 mL/min) and concentration of organic co-eluent (*O*, 0.1–1.9%). The variable ranges were determined from preliminary experiments and HPLC-system specifications using the epimers Glc and Gal, and GlcN as amine substituent of Glc.

Circumscribed central composite (CCC) optimization design was used, giving a circular design space with each variable investigated at five levels. The design was performed in two replicates and with five samplings of the centre point, giving 9 unique HPLC analyses of 26 in total. For model validation, experimental test sets were performed. These partially randomized combinations of variable values were biased towards even distributions around the centre point, and performed in one replicate, with five samplings of the centre point, giving 9 unique analyses of 13 in total.

The responses analysed were observed retention time (t_R, min.) and peak width (w_b, min.) as prerequisites for calculation of resolution (R_s) of neighbouring peaks [13] based on equation (1):

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2} = \frac{2\Delta t_R}{4\sigma + 4\sigma} = \frac{\Delta t_R}{4\sigma} = \frac{\Delta t_R}{4\bar{\omega} t'_R} \quad (1)$$

σ : standard deviation; t_R' : retention time adjusted for system void time; $\bar{\omega}$: slope of the peak width where $w_b = 4\sigma$ and $\sigma = \bar{\omega} t_R'$.

Before statistical analysis, retention time was adjusted for system void time by subtracting $\frac{2}{F}$, resulting in adjusted retention times (t_R'). Distributions of responses were investigated using normal probability plots and the Shapiro-Wilk normality test [14]. Transformation parameters were estimated using the Box-Cox method [15].

Experimental data were assessed using percent relative standard deviation (%RSD), while the transformed responses (y^*) were analysed by analysis of variance (ANOVA) and regression modelling

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