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Separations of Cycloinulooligosaccharides via Hydrophilic Interaction Chromatography (HILIC) and Ligand-Exchange Chromatography

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A homologous series of three cycloinulooligosaccharides were separated on the β-cyclodextrin and on the silica based strong cation exchange columns using high organic content aqueous solvents. The elution order on the cyclodextrin column was from a low degree of polymerization (DP) to high DP of oligosaccharides, whereas the elution order on the cation exchange column was mixed and determined by both the metal cations on the stationary phases and the organic solvent in the mobile phases. In comparison, α-, β- and γ-cyclodextrin were also separated using the same or similar mobile phases on these columns. With no exception, the elution order was from α- to γ-cyclodextrins.

Keywords β-cyclodextrin column; cation-exchange; cyclofructan; HILIC; ligand-exchange

INTRODUCTION

Cycloinulooligosaccharides (cyclofructans) are β-(2 → 1)-linked cyclic fructofuranose oligomers (Fig. 1). They are produced via fermentation of inulin by *Bacillus circulans* or alternatives from the enzyme cycloinulooligosaccharide fructanotransferase which can be isolated from this culture (1,2). The major cyclofructan (CF) produced is cycloinulohexaose (CF6), although both cycloinuloheptaose (CF7) and cycloinulooctaose (CF8) also are produced in smaller amounts (1). CFs have a unique crown ether skeleton as the central core (3,4). The crown ether core for CF6 is 18-crown-6, which makes CF6 attractive for metal complexation. In addition, $CF6 \cdot 3H_2O$ forms crystals in methanol, and can be easily purified (1). Many host-guest studies of CF6 and derivatized CF6 thus have been carried out since its first discovery in 1989 (5–10). On the other hand, CF7 and CF8 are far less studied, largely due to the lack of facile purification methods. The purification of CF7 was briefly reported on the QAE-Toyopearl 550C strong cation exchange (SCX) resin using 70% aqueous ethanol solvents. Unfortunately no information was provided on the counter cation employed (1,6). Shizuma et al. briefly mentioned purifying CF7 with a potassium (K^+)/calcium (Ca^{2+}) charged cation exchange resin (9). In a recent study, Jiang et al. reported different chiral discriminating capabilities between sulfated-CF6 and sulfated-CF7 (11). In their study, 72% pure CF7 was used (with CF6 as the major impurity). To better study the host-guest chemistry of CFs, both appropriate analytical and preparative separation methods for CFs are needed.

Separations of other carbohydrates were achieved on ion-exchange columns mainly in the following three modes (12):

(a) they were either ionized in very basic mobile phases (pH > 13) or complexed with borate anions to adopt an anionic form, and were subsequently separated on anion-exchange columns (13,14);
(b) carbohydrates were separated via a ligand-exchange mechanism on calcium or lanthanum cation charged cation-exchange columns (15);
(c) carbohydrates were separated using high acetonitrile (or ethanol) aqueous solvents on cation- or anion-exchange columns (HILIC mode) (16,17).

Many other polar stationary phases have also been used to separate carbohydrates in the HILIC mode (18–23). In HILIC, the polar stationary phases provide more retention for carbohydrates than reversed-phase stationary phases. In this paper, we present the HILIC and/or ligand-exchange separation of CFs on a β-cyclodextrin column and a silica based SCX column charged with different metal cations. Possible separation mechanisms are discussed.

EXPERIMENTAL

Materials

Lithium chloride, sodium acetate, potassium chloride, rubidium nitrate, silver nitrate, barium acetate, α-, β-, and γ-cyclodextrins were purchased from Sigma-Aldrich (Milwaukee, WI, USA). HPLC grade ACN, methanol

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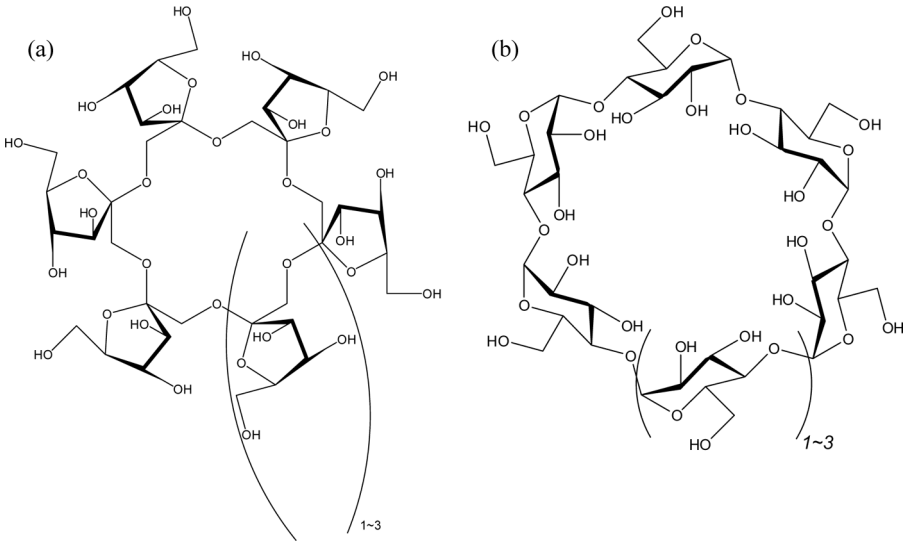

FIG. 1. Chemical structure of (a) cyclofructans and (b) cyclodextrins.

(MeOH) and water were purchased from VWR (Bridgeport, NJ, USA). The β-cyclodextrin column CYCLOBOND I 2000 250×4.6 mm, 5 µm was provided by Astec (Whippany, NJ, USA). The silica based SCX column TSK-Gel SP-2SW 250×4.6 mm, 5 µm was purchased from VWR. The mixture of CF6, CF7, and CF8 was a generous gift from Mitsubishi Kagaku Co. (Tokyo, Japan).

Instrumentation

All LC-ESI-MS experiments were performed on a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system coupled to a Thermo Finnigan LXQ Advantage API ion-trap mass spectrometer with an ESI ion source. A Shimadzu LC-6A pump was used to charge the SCX column with different cations by pumping corresponding 0.5 M metal salts for 1 h at 0.5 ml/min.

Methods

The mixture of CF6, CF7, and CF8 were used directly for analytical separations as it was received from Mitsubishi Kagaku Co. Samples were prepared at 0.5 mg/ml using water or ACN/water 70/30 (v/v, all solvents are indicated in volume percentage unless otherwise noted) as solvents. All separations were carried out at room temperature with 0.4 ml/min flow rate. To enhance the ionization of oligosaccharides, 0.1% w/v sodium acetate dissolved in water/ACN 10/90 was added into the mobile phase post column via a Y-type mixing tee at 4 µl/min using a syringe pump. The ESI-MS conditions were as follows: spray voltage 3.2 kV; sheath gas flow rate, 37 arbitrary units (AU); auxiliary gas flow rate, 6 AU; capillary voltage 11 V; capillary temperature, 350 C; tube lens voltage 105 V; and all oligosaccharides were detected in the SIM mode for $[M + Na]^+$ cations. The Xcalibur software was used for data analysis.

RESULTS AND DISCUSSION

Separation of CFs in the HILIC Mode

The separation of α-cyclodextrin (CD6), β-cyclodextrin (CD7), and γ-cyclodextrin (CD8) on cyclodextrin columns has been reported previously (24). Cyclofructans are isomers of cyclodextrins, and are readily separated on a β-cyclodextrin column using hydro-organic mobile phases with high percentages of ACN (Fig. 2a). The retention and separation factors decrease as the amount of water increases in the mobile phase (Fig. 3).

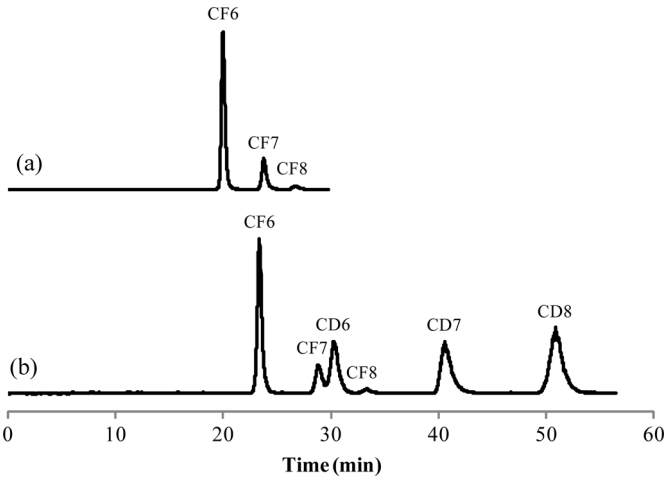

FIG. 2. Separation of (a) CFs and (b) CFs and CDs on the β-cyclodextrin column. Mobile phases: (a), ACN/water 70/30; (b) ACN/water 72/28.

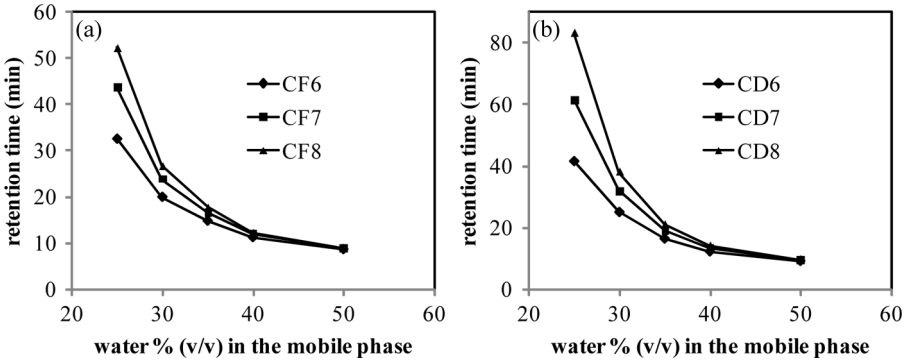

FIG. 3. Plots of retention time of (a) CFs and (b) CDs vs. water percentage in the aqueous ACN mobile phases on the β-cyclodextrin column.

In addition, the mixture of CD6–8 and CF6–8 can be separated simultaneously on the β-cyclodextrin column as shown in Fig. 2b. CFs and CDs of the same degree of polymerization (DP) have the same number of hydroxyl groups, but cyclofructan isomers are less retained than their cyclodextrin counterparts (Fig. 2b). Despite the same total number of hydroxyl groups for CF and CD isomers, all hydroxyl groups are not equally available for solute-stationary phase interactions (e.g., hydrogen-bonding). The separation of CF and CD isomers can be attributed to their different numbers of simultaneously available hydroxyl groups for solute-stationary phase interactions. As a matter of fact, the separation of isomers of oligosaccharides has been reported on the β-cyclodextrin columns before (18,20,21,23).

HPLC separation of CFs was also demonstrated on a silica based strong cation exchange column, TSK-Gel SP-2SW charged with Li^+. The retention of CFs follows the typical HILIC mechanism—the higher the DP, the greater the retention (Fig. 4a); retention increases as acetonitrile percentage increases in the aqueous mobile phase (Fig. 4b).

Separation of CFs via Ligand-Exchange Mechanism

The possession of a crown ether skeleton makes CFs different from other oligosaccharides. Uchiyama et al. studied the complexation of CFs with different metals via thin-layer chromatography (5). They also obtained complexation coefficients in 50% aqueous MeOH solvents based on the equation (Eq. (3)) derived by Briggs et al. (25).

$$K = 1/RF' - 1 \tag{3}$$

where K is the complexation coefficient, and RF' is the real migration rate relative to the solvent front. Table 1 summarizes the complexation coefficients between CFs and selected metal cations in 50% aqueous MeOH. The differences of the complexation coefficients between CFs of different DPs and metal cations are also listed in Table 1.

Figure 5a shows the separation of CFs using a neat water mobile phase on a Ba^{2+} charged SCX column. The retention of all CFs and the separation between CF7/8 and CF6 increases with increasing amounts of ACN in the mobile phase. When using a 40% aqueous ACN mobile phase, the CF7 and CF8 coelute at 8.6 min, whereas the CF6 is retained for 107.5 min (chromatogram not shown). In all aqueous ACN mobile phases tested, the CF6 is always much more strongly retained. The strong retention of CF6 is attributed to its strong binding to the Ba^{2+} on the stationary phase surface.

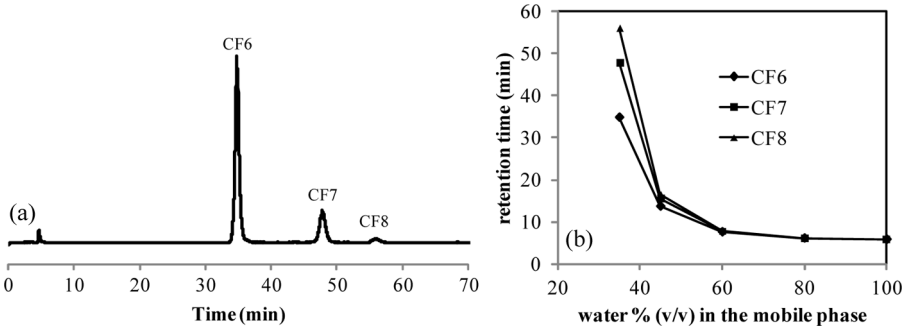

FIG. 4. (a) Separation of CFs on the Li-SCX column using an ACN/water 65/35 mobile phase. (b) Plot of retention time of CFs vs. water percentage in the aqueous ACN mobile phases on the Li-SCX column.

TABLE 1
Complexation coefficients between CFs and different metal cations (summarized from reference 5)

	K_{CF6}	K_{CF7}	K_{CF8}	$\Delta K_{CF6,7}$	$\Delta K_{CF7,8}$
Li^+	0.00	0.00	0.00	0.00	0.00
Ba^{2+}	4.65	0.06	0.01	4.59	0.05
Ag^+	1.66	0.84	0.33	0.82	0.51
Rb^+	1.40	0.09	0.09	1.31	0.00
K^+	1.18	0.11	0.04	1.07	0.07

Our HPLC separations are in good accordance with the complexation difference between CFs obtained by TLC. Ba^{2+} binds CF6 much stronger than CF7 and CF8, and the Ba-SCX provides greatest retention for CF6. In this case, the direct interaction between the CFs and Ba^{2+} on the solid support, instead of hydrophilic partitioning, plays the dominant role in the separation. Furthermore, compared with other metal cations, the Ba^{2+} provides the largest binding difference between CF6 and CF7/CF8, and correspondingly, Ba-SCX produces the best separation between CF6 and CF7/CF8.

Silver cation (Ag^+) provides the largest binding difference between CF8 and CF7 (refer to Table 1), and Ag^+ charged SCX column provides best separation for CF7 and CF8 (Fig. 5b). Using aqueous MeOH mobile phases, CF8 is retained less than CF7 and CF6 due to its weaker affinity for Ag^+.

Mixed Mode Separation of CFs

Rubidium cation (Rb^+) provides a minimal binding difference between CF7 and CF8, whereas it binds strongly to CF6. Figure 6 shows the separation of the cyclofructans on the Rb-SCX column using a 65% aqueous ACN mobile phase. The longer retention of CF6 is the result of the stronger CF6-Rb^+ interaction, whereas relative retention of CF8 and CF7 follow a more conventional HILIC mechanism like that shown in Fig. 4a. In this case, both the hydrophilic interaction and CFs-metal cation complexation are reflected in the chromatogram. In fact, the relative importance of these two interactions can be affected by simply modifying the mobile phase composition as demonstrated on the K-SCX column below (Fig. 7).

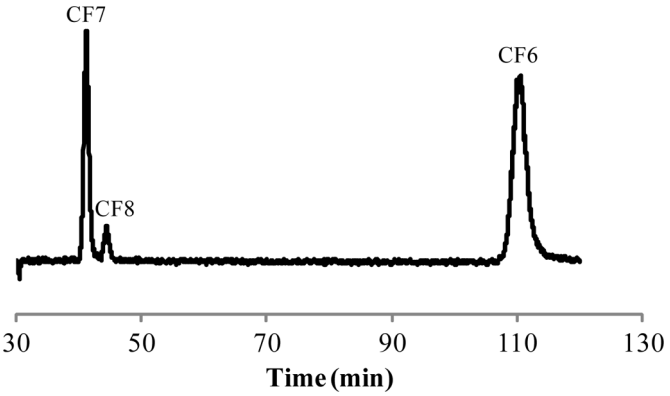

FIG. 6. Separation of CFs on the Rb-SCX column using an ACN/water 65/35 mobile phase.

Potassium cation (K^+) binds strongest to CF6, and it also binds slightly more to CF7 than CF8. In aqueous

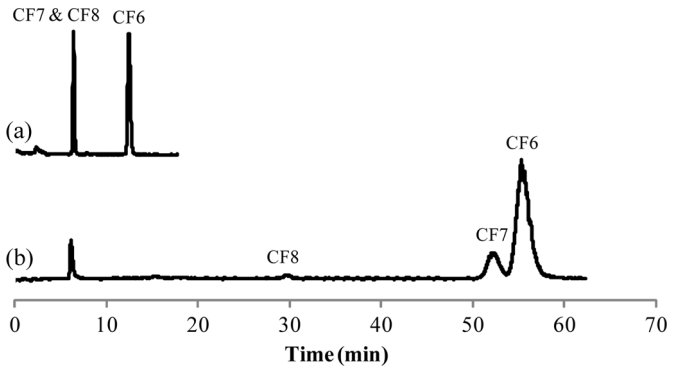

FIG. 5. Separations of CFs on (a) the Ba-SCX column and (b) the Ag-SCX column. Mobile phases: (a) water; (b) MeOH/water 80/20.

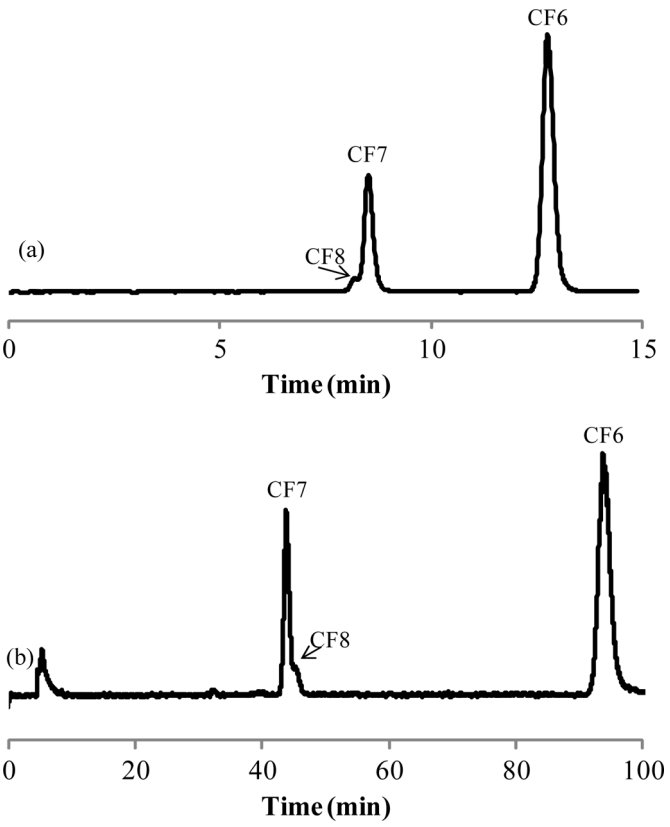

FIG. 7. Separation of CFs on the K-SCX column using different mobile phases: (a) ACN/water 40/60; (b) ACN/water 65/35.

TABLE 2
List of capacity factors for CF6, CF7, and CF8 under different chromatographic conditions

Columns	k'_{CF6}	k'_{CF7}	k'_{CF8}	Mobile Phases[a] (v/v)
β-CD	2.4	3.1	3.5	ACN/WTR 70/30
Li-SCX	5.0	7.2	8.5	ACN/WTR 65/35
Ba-SCX	1.1	0.1	0.1	WTR 100
Ag-SCX	8.6	8.0	4.1	MeOH/WTR 80/20
Rb-SCX	18.0	6.1	6.6	ACN/WTR 65/35
K-SCX	1.2	0.5	0.4	ACN/WTR 65/35

[a]ACN, acetonitrile; WTR, water; MeOH, methanol.

ACN mobile phases, CF6 always elutes last on the K-SCX coulumn. The elution order of CF7 and CF8 can be manipulated by changing mobile phase compositions as shown in Fig. 7. In the 40% aqueous ACN mobile phase, the direct CF-K^+ complexation determinates the elution order, and CF8 is eluted first (Fig. 7a). With a 65% aqueous ACN mobile phase, however, the hydrophilic interaction is a more important contributor to the elution order, and CF7 elutes first (Fig. 7b).

Separation of CDs on the SCX Column

Carbohydrates are in general good ligands for metal cations, and ligand-exchange chromatography has been used for separation of carbohydrates using aqueous mobile phases (26). However, most carbohydrate-metal cation complexation is much weaker than that of CF-metal complexation. In our study, the elution order of cyclofructans were greatly affected by the countercations on the SCX column (Table 2), whereas the elution order of cyclodextrins is, with no exception, from CD6 to CD8 under all tested chromatographic conditions (Fig. 8).

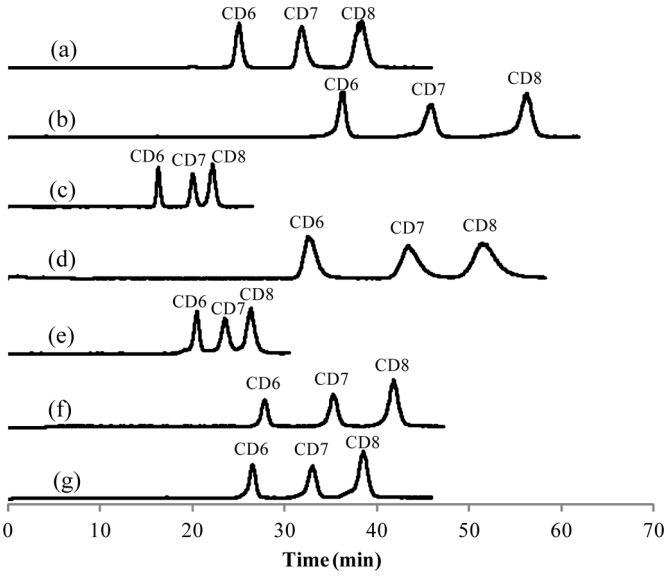

FIG. 8. Separation of CDs under various tested conditions: (a) β-cyclodextrin column, ACN/water 65/35; (b) Li-SCX column, ACN/water 65/35; (c) Ba-SCX column, ACN/water 55/45; (d) Ag-SCX column, MeOH/water 97/3; (e) Ag-SCX column, ACN/water 70/30; (f) Rb-SCX column, ACN/water 65/35; and (g) K-SCX column, ACN/water 65/35.

CONCLUSIONS

The separation of CF6, CF7, and CF8 were achieved on both the β-cyclodextrin column and various metal cation charged silica based SCX columns by HILIC and/or ligand-exchange mechanisms. Typical HILIC elution order, i.e, from CF6 to CF8, was observed on the cyclodextrin column. On the SCX column however, the complexation between CFs and the countercation on the column (ligand-exchange mechanism) played an important role in the retention and separation. The elution order of CFs can be manipulated by changing both countercations on the column and the organic solvent composition in the mobile phase. The ligand-exchange separation of CFs based on their specific complexation differences with metal cations provided higher selectivity than the separations based on simple generic hydrophilic interactions. In comparison, cyclodextrins were eluted in the order of CD6 < CD7 < CD8 in all chromatographic conditions tested.

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