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SACCHARIDE SEPARATIONS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING *n*-ALKYL AMINE MOBILE PHASE ADDITIVES

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SUMMARY

A high-performance liquid chromatography method is described for the separation of mono-, di- and trisaccharides on reversed-phase columns using *n*-alkyl amine mobile-phase additives. Saccharide capacity is dependent on solvent conditions and on the amount of adsorbed modifier. When compared with results from an amino column, the additive system requires weaker solvent conditions for the effective resolution of saccharide mixtures. The reproducibility and stability of this modified reversed-phase system is also demonstrated.

INTRODUCTION

Because of their relative abundance and their widespread importance in biology, medicine, agriculture and the food industry, the analysis of carbohydrate mixtures is an area of high interest. Traditionally, paper, ion-exchange, thin-layer and gel filtration chromatography have been used to separate these mixtures[1,2]. For analysis by gas chromatography and mass spectroscopy, trimethylsilane[3,4] butylboronic acid[5,6] and a variety of substituted benzeneboronic acid[7] derivatives of saccharides have been prepared. Sugar separations using high-performance liquid chromatography (HPLC) combining refractive index detection and microparticulate amine phases have been developed[8–11]. Amine mobile-phase additives have also been effective in separating saccharides on silica gel columns[12–15] and on cation-exchange resins[16]. In order to enhance the detection sensitivity of these separation techniques, several ultraviolet (UV) post-column reagents have been introduced[17–20]. Saccharides have also been derivatized with both UV and fluorescent labels prior to separation on silica gel to enhance the detection sensitivity[21–23]. Recently, reversed-phase HPLC has been used to separate classes of oligosaccharides according to their degree of polymerization[24,25].

Due to the stability of reversed-phase columns, several mobile-phase additives have been developed to achieve other difficult separations. Enantiomeric separations of amino acids in modified reversed-phase systems have been achieved using chiral, metal complexes[26–28]. Using its charge-transfer properties, Lochmüller and Jensen

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used a chiral, non-ionic mobile modifier [N-(2,4-dinitrophenyl)-L-alanine-*n*-dodecyl ester] to separate enantiomers of 1-aza-hexahelicene on reversed-phase columns[29]. Enhanced separations of aromatic amines and pyridine derivatives hve also been achieved using a coordinatively unsaturated nickel complex [bis(2,2,6,6-tetramethyl-heptane-3,5-dionate)nickel(II)] [Ni(DPM)] with reversed-phase systems[30]. The present study reports on the utility of *n*-alkyl amine mobile-phase additives in reversed-phase systems in effectively retaining and separating mono-, di- and trisaccharides. These modifiers provide both capacity and selectivity for these saccharides not normally provided by reversed-phase systems. The approach also minimizes the effect of rapid deterioration and spoiling observed when separating saccharides using amine or carbohydrate columns since the amine modifier may be reproducibility removed and replaced with minimal time and expense and, for some, eliminates the need for an amine column for an occasional saccharide separation. Furthermore, the performance differences between the amine column and the amine additives contribute to a more complete understanding of both systems.

EXPERIMENTAL

Apparatus

The chromatographic system incoporated a Varian (Walnut Creek, CA, U.S.A.) Model 8500 solvent delivery system, a Valco (Houston, TX, U.S.A.) Model CV-6-UHPa-N60 7000 p.s.i.g. injection valve fitted with a 10-μl injection loop, a laboratory Data Control (Riviera Beach, FL, U.S.A.) RefractoMonitor Model 1107 and 0.177 mm I.D. stainless-steel tubing. A DuPont (Wilmington, DE, U.S.A.) 25 cm × 4.6 mm I.D. Zorbax-NH_2 column was used to analyze selected saccharides. A 25 cm × 4.6 mm I.D. reversed-phase column was prepared with Partisil 10 (Whatman, Clifton, NJ, U.S.A.) and dichloromethyloctadecylsilane (Petrarch Systems, Bristol, PA, U.S.A.) according to the procedure of Evans *et al.*[31] and upward slurry packed with methanol at 8000 p.s.i.g. The average efficiency for the reversed-phase column was calculated from the mean values of multiple injections of mixtures of benzene, naphthalene and biphenyl with an acetonitrile–water (70:30) mobile phase at 1.0 ml/min using a Varian Vari-Chrom UV–VIS detector Model VUV-10 with an 8-μl flow cell at 254 nm. The column efficiency initially was 3300 and fell to 2517 after eight months of continual use. The average efficiency for the additive system was 650 as calculated from the mean values of multiple injections of mixtures of D-(+)-ribose, fructose and sorbitol with an acetonitrile–water (89:11) mobile phase with 0.150 g of *n*-tetradecylamine adsorbed at 1.0 ml/min using the refractive index detector.

Materials

The *n*-alkyl amines were obtained from Aldrich (Milwaukee, WI, U.S.A.) and were recrystallized twice from water prior to use. The saccharides were also purchased from Aldrich and used without further purification. Acetonitrile and water were Omnisolv-HPLC grade from MCB (Cincinnati, OH, U.S.A.).

Chromatographic measurements

Mobile phases with the *n*-alkyl amine additives were prepared by dissolving the appropriate amount of additive in acetonitrile and diluting with water. All mobile

phases were filtered and degassed before use. The columns were equilibrated by pumping the mobile phase until constant $\Delta k'$ values were obtained for glucose and fructose. All chromatographic measurements were taken at 25°C and at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Table I summarizes the capacity factors (k') of selected mono- and disaccharides on a Zorbax-NH_2 column. The elution order for these saccharides is identical to that found in previous studies with amine bonded phases[8–11]. The observed normal-phase behavior agrees with the proposed retention mechanism of a competitive interaction between water and the saccharides[32–34]. The limitation of primary amine columns is their reactivity with reducing saccharides to form Schiff bases[35] which limits the use of amine columns for common mono- and disccharides such as ribose, arabinose, xylose, mannose, galactose, glucose, fructose and sorbitol[36]. Since secondary amines are less reactive toward reducing saccharides[37], columns incorporating secondary amines (such as Partisil-10-PAC) are recommended when separating reducing saccharides. In this study, *n*-dodecylamine and *n*-tetradecylamine were used as mobile-phase additives since *n*-alkyl amines with less than ten carbons did not retain saccharides even at high concentrations and the low solubility of *n*-hexadecylamine in acetonitrile–water mixtures prevented its use. All these amines could be removed from the column after use, allowing reproducible capacity factors to be maintained.

Table II shows the changes in saccharide capacity ($\Delta k'$) using the dodecylamine additive under various mobile-phase conditions. For mobile-phase compositions greater than or equal to 90% acetonitrile, the retention order is identical to that on the Zorbax-NH_2 column at lower acetonitrile concentrations. As the percentage of acetonitrile increases, the solubility of the saccharides decreases and the reactivity of the reducing sugars with the amines increases.

Table III shows the changes in saccharide capacity ($\Delta k'$) when using the tetradecylamine additive under various mobile-phase conditions. The elution order for

TABLE I

SACCHARIDE CAPACITY FACTORS (k') ON AN AMINE COLUMN*

Saccharide	*Acetonitrile (%)*			
	65	*70*	*75*	*80*
D-(+)-Xylose	0.90	1.24	1.74	2.38
Fructose	1.14	1.67	2.41	3.44
D-Glucose	1.20	1.86	2.63	4.16
Sorbitol	1.29	2.01	2.94	4.60
D-(+)-Galactose	1.33	2.03	2.99	4.74
Sucrose	1.50	2.46	3.94	7.09
D-(+)-Maltose	1.68	2.87	4.66	8.69
Inositol	1.89	3.14	4.87	9.28
Lactose	1.95	3.22	5.59	11.70

* Capacity factors obtained on a Zorbax-NH_2 column at 1.0 ml/min.

TABLE II

n-DODECYLAMINE ADDITIVE DATA

Acetonitrile (%)	*Concn. (10^3 M)*	q_{ads} *(g)*	$\Delta k'$				
			Fructose	D-*Glucose*	D-*Galactose*	*Sucrose*	*Maltose*
80	11.8	0.243	0.20	0.19	0.20	0.00	0.05
85	6.78	0.165	0.38	0.44	0.42	0.30	0.32
90	14.8	0.211	0.97	1.04	1.10	1.15	1.58
90	17.3	0.229	1.02	1.26	1.42	1.54	1.93
95	6.28	0.118	1.21	1.47	1.80	1.80	n.s.*
95	15.1	0.185	1.81	2.43	9.97	2.78	n.s.*

* n.s. = Not seen.

the reversed-phase column modified with tetradecylamine is identical to the Zorbax-NH_2 results except for sorbitol and D-galactose. A plot of $\Delta k'$ *vs.* the amount of adsorbed modifier (q_{ads}) for *n*-tetradecylamine additive at 89:11 acetonitrile–water displays a linear relationship between $\Delta k'$ and q_{ads}. When the acetonitrile–water concentrations are varied, $\Delta k'$ is not proportional to q_{ads} nor to the amine concentration in solution. A linear relationship is found with q_{ads} only when identical acetonitrile–water conditions are used. Once the system reached equilibrium, the capacity factors for these saccharides remained unchanged over several days of continual use. This stability allows changes in the acetonitrile–water percentages to enhance a particular saccharide separation with only minimal equilibration times. Fig. 1 shows a typical saccharide separation with $2.10 \cdot 10^{-3}$ *M* *n*-tetradecylamine in acetonitrile–water (89:11) (q_{ads} = 0.150 g) in this reversed-phase system.

The reproducibility of the *n*-alkyl amine additive system was investigated using a $2.24 \cdot 10^{-3}$ *M* solution of *n*-tetradecylamine in 90:10 acetonitrile–water. After the system reached equilibrium (q_{ads} = 0.165 g), three injections of each saccharide were made. The column was then washed with 1 l of methanol to remove the amine modifier. The system was re-equilibrated with the same *n*-tetradecylamine solution (q_{ads} = 0.165 g), and three injections of each saccharide were made. Table IV lists the results which demonstrate that this approach to saccharide separations is reproducible within the margin of experimental error even though the capacity factors increased slightly between the two trials.

The primary distinction between the results obtained with the amino column and the amine additive is the acetonitrile–water concentration required for adequate retention and resolution of saccharides. The results in Fig. 1 show that an acetonitrile–water (90:10) (with q_{ads} = 0.165 g) mobile phase was required to obtain capacities similar to those obtained on the Zorbax-NH_2 column an acetonitrile–water (70:30) mobile phase. Although some differences in capacity (comparing $\Delta k'$ and k') could be the result of amine concentration, the major difference in mobile-phase conditions resulted from the increased hydrophobicity in the additive system. The hydrocarboneous character of the reversed-phase material as well as the added reversed-phase character of the *n*-alkyl amine chains decreased saccharide capacity requiring the use of weaker solvent conditions. This difference could not be the result

TABLE III

n-TETRADECYLAMINE ADDITIVE DATA

Acetonitrile (%)	*Concn. (10^3 M)*	*q_{ads} (g)*	*Δk'*																	
			D-(+)-*Xylose*	D-(−)-*Ribose*	L-(−)-*Fucose*	L-(+)-*Arabinose*	*Fructose*	D-(+)-*Mannose*	D-*Glucose*	D-(+)-*Galactose*	*Sorbitol*	*Dulcitol*	*Sucrose*	D-(+)-*Maltose*	D-*Cellobiose*	*Inositol*	*Lactose*	*Maltotriose*	α-D-*Melibiose*	D-*Raffinose*
80	2.24	0.167	0.406	0.472	0.506	0.541	0.497	0.453	0.444	0.493	0.565	0.565	0.328	0.387	0.384	0.722	0.384	0.412	0.522	0.437
83	2.25	0.166	0.412	0.467	0.589	0.641	0.613	0.585	0.610	0.689	0.763	0.799	0.570	0.677	0.668	1.17	0.872	0.802	0.982	0.891
87	2.24	0.165	0.538	0.557	0.798	0.889	0.881	0.900	0.987	1.09	1.20	1.27	1.07	1.33	1.34	2.13	1.78	1.87	2.19	2.14
89	2.21	0.165	0.886	0.873	1.28	1.41	1.61	1.76	1.83	2.19	2.48	2.57	2.70	3.39	3.53	4.56	4.69	6.52	6.45	8.05
89	0.480	0.0152	–	–	–	–	1.08	–	1.31	1.51	–	–	1.90	2.61	–	–	–	–	–	–
89	1.40	0.136	–	–	–	–	1.47	–	1.81	2.04	–	–	2.25	2.99	–	–	–	–	–	–
89	15.6	0.351	–	–	–	–	1.80	–	2.49	2.85	–	–	3.21	3.47	–	–	–	–	–	–
90	2.23	0.165	0.965	–	–	–	1.78	–	2.10	2.37	2.82	–	3.48	4.11	–	5.59	5.86	–	–	–
93	2.25	0.162	1.55	1.38	2.13	2.37	3.03	–	3.81	–	–	–	–	–	–	–	–	–	–	–

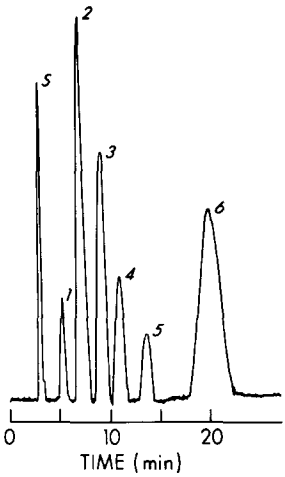

Fig. 1. Separation of saccharides on an octadecyl column with the *n*-tetradecylamine additive (q_{ads} = 0.150 g); mobile phase, acetonitrile–water (89:11); flow-rate, 1.0 ml/min. Peaks: S = water; 1 = D-(+)-xylose; 2 = fructose; 3 = sorbitol; 4 = D-(+)-maltose; 5 = lactose; 6 = D-raffinose.

of a difference in basicity between the *n*-propylamine on the bonded phase and the *n*-tetradecylamine modifier since their pK_a values are nearly equivalent (see Table V).

Another factor influencing this capacity loss in the additive system could be the result of an increase in the effective pore size. Due to the length of both the bound and adsorbed aliphatic chains, the additive system may result in the amine functionality being further from the pore surface than for the amino bonded phase which effectively increased the average pore size. This increase may limit the effectiveness of the amine to buffer the pore environment (because the amine chains are more accessible to solvent) and thus reduce the strength of interaction between the solute and the adsorbed molecule.

TABLE IV

SYSTEM REPRODUCIBILITY TEST*

Solute	$\Delta k'$			
	Trial 1	*Trial 2*	*Average*	*Increase (%)***
D-(+)-Xylose	0.929 (±0.006)	1.00 (±0.3)	0.97 (±0.5)	+7.9
Fructose	1.76 (±0.4)	1.80 (±0.04)	1.78 (±0.2)	+2.2
D-Glucose	2.03 (±0.1)	2.16 (±0.3)	2.10 (±0.8)	+6.4
D-(+)-Galactose	2.26 (±0.4)	2.47 (±0.1)	2.37 (±1.0)	+9.0
Sorbitol	2.70 (±0.4)	2.93 (±0.1)	2.82 (±1.5)	+8.5
Sucrose	3.32 (±1.9)	3.63 (±0.1)	3.48 (±1.9)	+9.1
D-Maltose	4.04 (±0.6)	4.18 (±1.1)	4.11 (±0.9)	+3.5
Inositol	5.54 (±1.6)	5.63 (±1.1)	5.59 (±0.5)	+1.5
Lactose	5.76 (±0.9)	5.95 (±0.9)	5.86 (±0.7)	+3.2

* For each solute, three injections were made. The variance in the parenthesis was computed at 95% confidence limits.

** The percentage increase is computed as $\frac{(\text{trial 2} - \text{trial 1})}{\text{trial 1}} \times 100$.

TABLE V

pK_a VALUES OF *n*-ALKYL AMINES

Carbon No.	*Name*	pK_a	*Ref.*
1	Methylamine	10.62	38
2	Ethylamine	10.63	38
3	Propylamine	10.53	38
4	Butylamine	10.59	38
5	Pentylamine	10.63	39
6	Hexylamine	10.56	39
7	Heptylamine	10.66	39
8	Octylamine	10.65	39
8	Octylamine	10.65	40
9	Nonylamine	10.64	39
14	Tetradecylamine	10.62	39
16	Hexadecylamine	10.61	40

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