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LIQUID CHROMATOGRAPHIC FRACTIONATION OF OLIGOMERS OF NONYLPHENYL-OLIGO(ETHYLENE GLYCOL)

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SUMMARY

Underivatized silica and silica lightly derivatized with octadecyl groups produced better separations of oligomers of nonylphenyl-oligo(ethylene glycol) than a more extensively derivatized silica packing. In addition, the resolution was shown to be adversely affected by the presence of 0.01 *M* sodium iodide or by 10% of water in an eluent of either acetonitrile or methanol.

INTRODUCTION

Poly(ethylene glycol) (PEG) is an interesting oligomeric system to study because successful fractionations using silica in normal-phase chromatography[1–3], reversed-phase chromatography (RPC)[4,5] and size exclusion chromatography (SEC) have been reported[6–9]. Furthermore, column packings derivatized with octadecyl groups have been used more extensively than others, such as cyano[10].

Using RPC, Melander *et al.*[4] found that a small change in the solvent composition caused a change in the elution order of the PEG oligomers. On a C_{18} column, large oligomers were retained longer than smaller oligomers in 12% tetrahydrofuran. However, after a small increase to 15%, the smaller oligomers were retained longer. This illustrates a change from an adsorption to a size-exclusion type of behavior, which was attributed to a change in the conformation of the PEG.

In addition to the work by Melander *et al.*, Kraus and Rogers[6] demonstrated that in an SEC mode, the addition of salt to the mobile phase decreased the effective hydrodynamic volume of the PEG oligomers. Therefore, different molecular weights eluted at the same time at high salt concentrations.

The present study originated with an interest in examining the effects of added salts on the behavior of PEG oligomers in different percentages of acetonitrile. However, preliminary attempts to repeat the basic work of Melander *et al.* were unsuccessful. PEG oligomers did not elute from C_{18} in the low percentages of acetonitrile in water that were reported by Melander *et al.* Much larger percentages, about 80%

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of acetonitrile, were needed and then the PEG eluted as a single peak having one or more shoulders. However, good oligomeric resolution was later obtained on underivatized silica (and then on a lightly loaded C_{18} column) using either pure acetonitrile or acetonitrile containing 5% (or less) of water.

As a result, two modified goals were set. One was to study the effect of solvents alone and in the presence of sodium iodide using underivatized silica instead of C_{18} derivatized silica, and the second goal was to examine the effect of the extent of derivatization of the silica surface on the oligomeric separations.

EXPERIMENTAL

Chemicals

PEG samples consisting of nonylphenyl-oligo(ethylene glycol) with various ethoxide chain lengths were obtained (Union Carbide, Southern Charleston, WV, U.S.A.) under the trade name of Tergitol NP. The samples had average ethoxide chain lengths of 4, 8 and 13, which corresponded to molecular weights of 396, 572 and 792, respectively.

Most organic solvents were used as received from J. T. Baker (Phillipsburg, NJ, U.S.A.). Methanol, acetonitrile and methylene chloride were of HPLC grade. Toluene, diethyl ether, acetone, nitrobenzene, pyridine, carbon tetrachloride, propan-2-ol and cyclohexanol were of analytical-reagent grade. Uracil was obtained from Aldrich (Milwaukee, WI, U.S.A.). Nitrobenzene was obtained from Fisher Scientific (Fair Lawn, NY, U.S.A.). House-distilled water was deionized and then distilled again before being collected in glass bottles. All solvents were filtered using 0.5-μm FH filters for organics or 0.45-μm HA filters for aqueous materials (Millipore, Bedford, MA, U.S.A.) and were thoroughly degassed with helium before use. Nitrogen and helium were obtained from Selox (Atlanta, GA, U.S.A.).

Silica packings were used as received or were derivatized in our laboratory. Octadecyldimethylchlorosilane (ODCS) (Petrarch Systems, Bristol, PA, U.S.A.) was used to derivatize some portions of LiChrosphere Si 100 (E. Merck, Darmstadt, F.R.G.) while other portions of LiChrosphere Si 100 were used as received. The commercial C_{18} packing LiChrosorb RP-18 (E. Merck) consisted of 10-μm irregularly shaped particles that had been fully end-capped.

Apparatus

The RPC system consisted of a Model 7000 Liquid Chromatographic System (Micromeritics, Norcross, GA, U.S.A.) a Model 7125 injection valve (Rheodyne, Cotai, CA, U.S.A.) a Model 770 variable-wavelength detector (Kratos, Ramsey, NJ, U.S.A.) set at 275 nm and a Microscribe 4500 chart recorder (Houston Instruments, Austin, TX, U.S.A.).

The normal-phase or liquid–solid chromatographic (LSC) system consisted of a Model 110A pump (Altex, Berkeley, CA, U.S.A.), a Rheodyne 7125 injection valve, a Model 1840 variable-wavelength detector (Isco, Lincoln, NB, U.S.A.) set at 275 nm and a Model 585 chart recorder (Linear, Reno, NV, U.S.A.).

High-performance liquid chromatographic (HPLC) columns were packed in 0.46 cm I.D. stainless-steel tubing (Alltech, Deerfield, IL, U.S.A.) cut to 25-cm length. The columns were fitted with stainless-steel Swagelock zero-dead-volume 6.35 to 1.59

mm reducing unions (Georgia Valve and Fitting, Atlanta, GA, U.S.A.) and Alltech 2.0-μm column frits.

The solvent filtering systems consisted of a 2.0-μm low-pressure inlet filter and a 0.5-μm high-pressure filter, both SSI brand, purchased from Alltech. The filters prevented particulate matter from entering the pump system and sample injector, respectively.

Silica derivatization

Two C_{18} bonded-phase packings were synthesized in a manner similar to a procedure reported by Berendsen and De Galan[11]. A weighed amount of silica was dried at 190°C under vacuum to remove any physically adsorbed water. The bonding reaction took place under nitrogen in a reflux apparatus that had been flame dried. To a mixture of toluene, silica and pyridine (dried with CaH_2) was added an amount of ODCS. The mixture was heated and occasionally stirred. Care had to be taken when stirring the mixture to minimize the breaking of particles. The C_{18} bonded phase was then washed successively in a sintered-glass funnel with toluene, methanol, methanol–water (1:1), water, methanol, diethyl ether and acetone. The C_{18} packing was dried for 24 h at 90°C *in vacuo*.

Ordinarily, reversed-phase packings are end-capped with trimethylchlorosilane (TMCS) after the C_{18} is bonded. Our packings were purposely not reacted with TMCS so that some active silanol sites would remain.

Fines were removed by adding the C_{18} bonded phase to a 500-ml graduated cylinder that contained methanol. The mixture was stirred and the fines were decanted after the larger particles had fallen to the bottom of the cylinder.

Elemental analyses

The results from the elemental analyses of bonded phases are shown in Table I. The surface coverage was calculated according to Berendsen and De Galan[11] for the number of silanols per unit surface area.

C_{18}:15.49% load. This packing was synthesized using 130 ml of toluene, 4.0 g of LiChrosphere Si 100, 3.0 ml of pyridine and 4.0 g of ODCS. The mixture was heated at 65°C for 24 h.

TABLE I

SURFACE COVERAGES AND EFFICIENCIES OF HPLC COLUMNS

Column	*Packing*	*Carbon*			*Theoretical plate number*
		Wt.-%	*μmol/m²*	*mmol/g*	
A	LiChrosphere Si 100	0.29*	0.00	0.00	2366
B	RP-18	22.72	8.45	1.41	3012
C	LiChrosphere Si 100 derivatized using ODCS	15.49	3.32	0.83	3935
D	LiChrosphere Si 100 derivatized using ODCS	3.09	0.54	0.13	709

* There should not be any carbon on this silica.

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C_{18}:3.09% load. This packing was synthesized using 130 ml of toluene, 4.0 g of LiChrosphere Si 100, 1.1 ml of pyridine and 0.8 g of ODCS. The mixture was heated at 40°C for 2.75 h. Instead of washing the packing with methanol–water (1:1) a ratio of 9:1 was used.

Column packing

The viscosity technique was used to pack the columns[12]. To the slurry bomb was added carbon tetrachloride, the slurry of packing (cyclohexanol–propan-2-ol, 3:1) and methanol. The columns were packed at 9000 psi using methanol as the push solvent.

Preparation of samples

PEG samples were dissolved in methanol or acetonitrile. If, for example, the mobile phase was methanol–water, the sample was dissolved in methanol. Acetonitrile phases were handled similarly. The concentration of the PEG sample was 2 mg/ml unless specified otherwise. All samples were filtered prior to use in the same manner as the solvents. This helped to prevent any particulate matter from entering the solumn inlet and eventually causing a blockage.

Test samples, toluene and uracil dissolved in acetonitrile or methanol and nitrobenzene dissolved in *n*-hexane, had a concentration that showed a pen deflection at 0.04 absorbance unit.

Analytical procedures

Solvents were degassed for approximately 20 min with helium before use, followed by purging each solvent line with approximately 25 ml of solvent. A precolumn, 5 cm × 0.46 cm I.D. × 0.635 cm O.D., used with columns A and D, dry packed with the corresponding stationary phase was placed between the pump and the injector in order to saturate the mobile phase with the stationary phase. Before sample injection, the column was flushed with twenty column volumes of the mobile phase. The flow-rate of the mobile phase was held constant at 1.5 ml/min for RPC separations and 0.5 ml/min for LSC separations, unless specified otherwise. The flow-rate was verified by measuring the effluent for a suitable amount of time. Each sample was chromatographed a minimum of four times to make sure that its retention time was reproducible. The retention times agreed to within ± 0.03 min. The first and second injections on each day were repeated at the end of the series to check for any drift. The temperature used for the experiments was kept at 25°C by using a column oven.

Two organic compounds were used to determine the efficiency of the columns used. Toluene was eluted using methanol–water (70:30) as the mobile phase on columns B and C. Column D was tested with methanol–water (1:1) as the mobile phase. Nitrobenzene was eluted using *n*-hexane as the mobile phase on column A. The results are shown in Table I.

Calculations

The theoretical plate number, N, was calculated according to the following equation[3]:

$$N = 5.54\ (t_r/t_{w_{\frac{1}{2}}})^2$$

where t_r is the retention time and $t_{w_{\frac{1}{2}}}$ is the width of the band at half-height. In order to compare results with those of Melander *et al.*[4], the capacity factor (k) was calculated using[3]

$$k = (V_r - V_0)/V_0$$

where V_0 is the void volume, a mesure for an unretained compound, and V_r is the retention volume for a retained compound. The void volume was determined by the change in refractive index caused by a new solvent front.

RESULTS

Normal-phase HPLC

Oligomeric separation. Fig. 1 illustrates the separation of NP-4, -8 and -13 on underivatized silica using acetonitrile as the mobile phase. Each sample shows a separation with satisfactory oligomeric resolution. Retention times increased as the ethoxide chain length increased owing to increased adsorption on the silica surface. Using methanol as the solvent, the oligomeric separations were similar to those for acetonitrile, but the retention times were shorter.

Effect of salt. It has been reported previously that the addition of salt to the

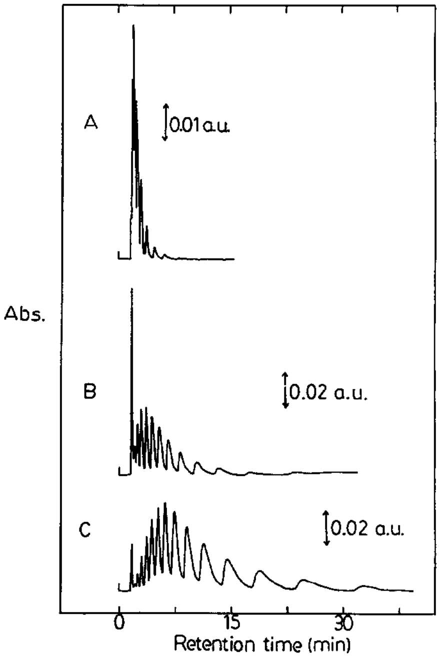


Fig. 1. Separation of PEG oligomers using 100% acetonitrile and column A at a flow-rate of 2.0 ml/min: (A) NP-4, 2 mg/ml; (B) NP-8, 10 mg/ml; (C) NP-13, 10 mg/ml.

mobile phase caused a change in the hydrodynamic volume of PEG; in SEC, this effect was reflected in longer retention times[6]. In an adsorption mode, however, the effect was opposite and led to shorter retention times, as shown in Fig. 2 for NP-8 in the presence of sodium iodide. The addition of $1.00 \cdot 10^{-3}$ *M* sodium iodide dramatically reduced the elution time of the separation. Note that the mobile phase flow-rate was reduced to 0.5 ml/min in order to be able to observe the resolution of oligomers. Also, it resulted in earlier elution of more strongly retained oligomers that had been buried in the baseline of the separation for salt-free 100% acetonitrile. Higher concentrations of sodium iodide gave even shorter retention times. Studies using NP-4 and NP-13 gave similar results.

Effect of water. The effect of the addition of water to the acetonitrile mobile phase is shown in Fig. 3. Interestingly, a small amount of water greatly decreased the retentions of the oligomers and their resolutions. The decrease in resolution may have been caused by water molecules covering up the active silanol sites.

Effect of salt plus water. Fig. 4 shows that the combined effects of salt and water in the mobile phase further decreased the resolution. Alone, $1.00 \cdot 10^{-2}$ *M* sodium iodide had a greater effect than 10% water, but together they caused almost all resolution to be lost.

Test sample. Uracil was used in order to determine if the addition of salt was affecting primarily the PEG solutes in the sample. Within experimental error, the retention times were the same (within ±0.02 min) for separations with and without salt. A k value of 0.43 was obtained when using a mobile phase of 100% acetonitrile without salt; k values of 0.41 and 0.38 were obtained with sodium iodide at concen-

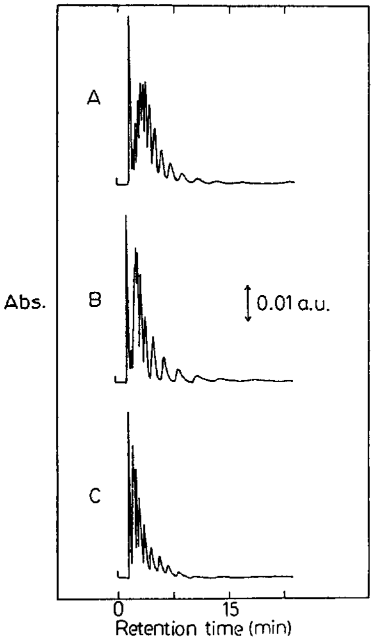
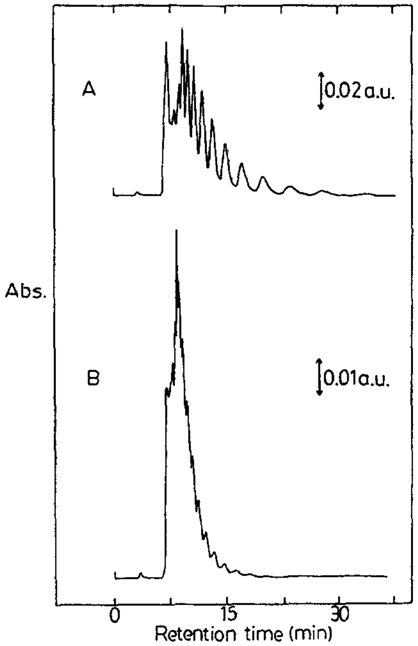


Fig. 2. Effect of NaI in 100% acetonitrile on oligomer resolution for NP-8 (10 mg/ml) using column A at a flow-rate of 2.0 ml/min: (A) $1.00 \cdot 10^{-3}$ *M*; (B) $1.00 \cdot 10^{-2}$ *M*; (C) $5.00 \cdot 10^{-2}$ *M*.



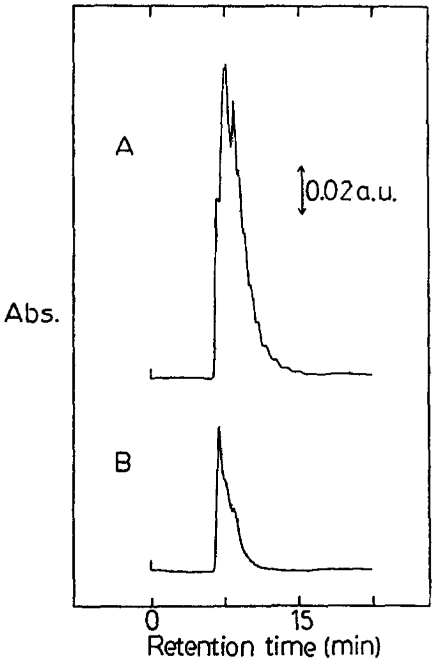


Fig. 3. Effect of water in the mobile phase on the separation of oligomers of NP-8 (10 mg/ml) using column A at a flow-rate of 0.5 ml/min: (A) acetonitrile–water (95:5); (B) acetonitrile–water (90:10).

Fig. 4. Effect of NaI and water on the fractionation of NP-8 with acetonitrile–water (90:10) as the mobile phase using column A at a flow-rate of 0.5 ml/min: (A) $1.00 \cdot 10^{-2}$ M, 10 mg/ml; (B) $5.00 \cdot 10^{-2}$ M, 2 mg/ml.

trations of $1.00 \cdot 10^{-2}$ and $5.00 \cdot 10^{-2}$ M, respectively. Using acetonitrile–water (90:10) without salt as the mobile phase, a k value of 0.12 was obtained; k values of 0.12 and 0.13 were obtained with sodium iodide at concentrations of $1.00 \cdot 10^{-2}$ and $5.00 \cdot 10^{-2}$ M, respectively. As a result, one can conclude that the salt was affecting the PEG sample.

Reversed-phase HPLC

Effect of carbon percentage. Derivatized C_{18} silicas with different percentages of carbon or carbon loads were compared in order to determine if a proper mixture of C_{18} and silanols would provide an adequate separation. The C_{18} columns were studied using acetonitrile–water mobile phases with and without salt. All three molecular weights of PEG were examined but only the data for NP-8 are shown because the results were qualitatively the same for all three samples.

Fig. 5 illustrates the effect of column B, which has a high carbon load of 22.72%, probably with few exposed silanol sites. The coverage slightly resolved oligomers of NP-8 using acetonitrile–water (95:5) as the mobile phase. With the same solvent composition, $1.00 \cdot 10^{-2}$ M sodium iodide was added to examine the effect of salt. A decrease in oligomeric resolution clearly indicated that a salt effect took place.

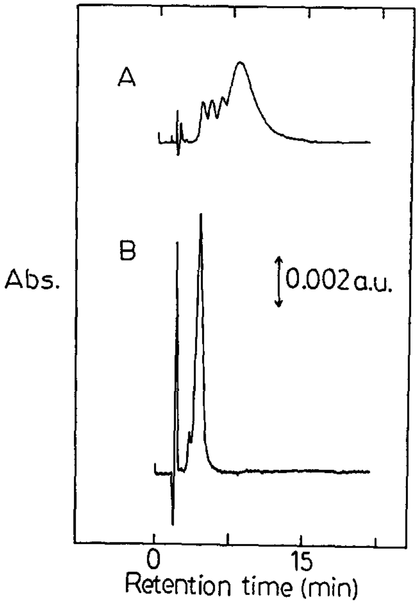
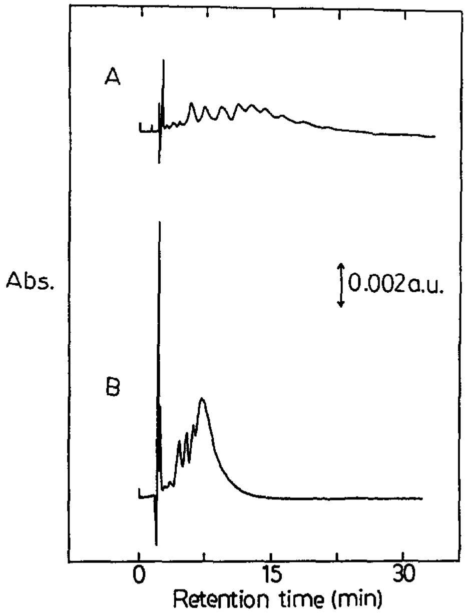


Fig. 5. Separation of NP-8 (2 mg/ml) on column B at a flow-rate of 1.5 ml/min: (A) acetonitrile–water (95:5); (B) acetonitrile–water (95:5) and $1.00 \cdot 10^{-2}$ M NaI.

A lighter loaded C_{18} packing that had more accessible silanols on the surface, as indicated by its 15.49% carbon load, was derivatized. Fig. 6 shows the separation of NP-8 using acetonitrile–water (95:5) as the mobile phase, where there was an increase in the number of oligomers resolved compared with that in 100% acetonitrile. The addition of $1.00 \cdot 10^{-2}$ M sodium iodide again resulted in a decrease in oligomeric resolution. For both carbon loads (22.72 and 15.49%), greater peak heights occurred when salt was added to the mobile phase. This behavior indicated that later eluting oligomers in a mobile phase without salt will elute earlier when salt is added.

A C_{18} packing was derivatized with a very low carbon load of 3.09%. More than 80% of the residual silanols remained underivatized. This medium showed the behavior of both normal- and reversed-phase packings. Fig. 7 shows the separation of NP-8 on column D using an acetonitrile–water mobile phase with and without salt. A mobile phase of 100% acetonitrile effectively separated the oligomers. There was greater resolution for earlier than later eluting oligomers. Adding 5% of water to the mobile phase effectively compressed the oligomeric separation to shorter retention times, more so than it did on underivatized silica. The salt effect also produced a greater decrease in resolution than on underivatized silica.

Effects of water and salt in methanol. In methanol, the resolution of NP-8 oligomers by column C was much worse than in acetonitrile. The presence of 5% of water caused a decrease in resolution and 10% of water a further decrease. However, the presence of $1.00 \cdot 10^{-2}$ M sodium iodide in all three media produced only one large peak that eluted early and had one or two shoulders. Clearly the trends seen in acetonitrile were also found in methanol.



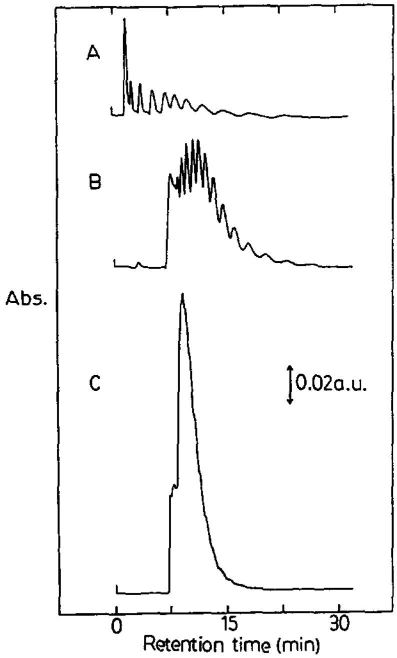


Fig. 6. Separation of NP-8 (2 mg/ml) on column C at a flow-rate of 1.5 ml/min: (A) acetonitrile–water (95:5); (B) acetonitrile–water (95:5) and 1.00 · 10^{-2} *M* NaI.

Fig. 7. Separation of oligomers of NP-8 (10 mg/ml) on column D: (A) 100% acetonitrile, 2 ml/min; (B) acetonitrile–water (95:5), 0.5 ml/min; (C) acetonitrile–water (95:5) and 1.00 · 10^{-2} *M* NaI, 0.5 ml/min.

Test sample. Using toluene as a test sample in column C, *k* values were calculated for acetonitrile–water (95:5) and methanol–water (95:5) mobile phases. Within experimental error, retention times were the same within ±0.01 min for separations with and without salt. *k* values unchanged at 1.03 resulted when using acetonitrile–water (95:5) as the mobile phase without salt and with 1.00 · 10^{-2} *M* sodium iodide. *k* values of 1.09 resulted when using methanol–water (95:5) as the mobile phase without salt and with 1.00 · 10^{-2} *M* sodium iodide. This indicated that the salt was affecting the PEG sample,

DISCUSSION

Underivatized silica provided an oligomeric separation of PEG oligomers. On the other hand, derivatized C_{18} silica having higher carbon loads did not provide significant oligomeric separations. However, a lower load of 3.09%.carbon closely resembled the separation achieved on underivatized silica.

When different derivatized C_{18} silicas were compared, a carbon load of 15.49% gave better oligomeric resolution than a load of 22.72%. The increased resolution resulted from a combination of a greater number of exposed silanol groups that provided adsorption sites and a decrease in the hydrophobic nature of the column. The smallest carbon load, 3.09%, resulted in a separation that was similar to that on underivatized silica in 100% acetonitrile except that early eluting oligomers were better resolved on the 3.09% loaded phase. From the above evidence, it appears that the column used by Melander *et al.*[4] must have had only a small fraction of its surface derivatized, possibly as a result of partial decomposition of the octadecyl-derivatized silica.

The effect of salt interacting with the PEG oligomers was noted by the decrease in resolution in the separations. An increase in the salt concentration sometimes caused the oligomeric separation to collapse into one peak. The addition of lower concentrations appeared to cause later eluting oligomers to become visible as peaks. Hence, this salt effect might be used to advantage by using a gradient of a salt in order to determine the number of oligomers. Otherwise, the later oligomers would be lost in the baseline.

The addition of water to the mobile phase probably covered some of the active silanol sites on the surface. The addition of 10% of water had more effect than 5% of water, as indicated by a greater decrease in the oligomeric resolution. However, there was a greater decrease in the oligomeric resolution with salt than with water.

The addition of salt to the mobile phases used with the different C_{18} carbon-loaded columns provided similar results. It was evident that the sodium iodide interacted with the PEG in the mobile phase. Increased peak heights, for each C_{18} phase, indicated that many oligomers were eluting at the same retention time. Without salt in the mobile phase the oligomers were retained longer on the packing surface. Hence the presence of salt affects the adsorption behavior, probably through a change in conformation just as it did in the SEC study[6].

A mobile phase of acetonitrile or methanol combined with water on column C resulted in an oligomeric separation. The addition of 5% of water to acetonitrile provided more of a baseline-resolved oligomeric separation than the addition of 5% of water to methanol which did not baseline resolve the oligomers. The addition of salt had a greater effect in methanol than in acetonitrile. All oligomeric resolution was lost with $1.00 \cdot 10^{-2}$ *M* sodium iodide in methanol–water (95:5). The same salt concentration in acetonitrile–water (95:5) did not completely collapse the oligomers into one peak.

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