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DISPLACEMENT CHROMATOGRAPHY ON CYCLODEXTRIN–SILICAS

I. SEPARATION OF POSITIONAL AND GEOMETRICAL ISOMERS IN THE REVERSED-PHASE MODE

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SUMMARY

The retention behaviour of several charged and uncharged solutes on β-cyclodextrin–silica was studied as a function of the methanol concentration, ionic strength and pH of the eluent in order to develop efficient displacement chromatographic separations for positional and geometric isomers. These retention curves were used to predict the eluent (carrier solvent) compositions that result in solute retentions in excess of $k' = 10$. The adsorption isotherms of several cationic detergents were determined in these carrier solutions and were found to be convex. The adsorption isotherms of several positional isomers used as test solutes were also determined in these carrier solutions. The adsorption isotherms permitted the development of efficient displacement chromatographic separations for the isomers tested. Column loadings as high as 58 mg were achieved on a regular 4.6 mm I.D. analytical-scale cyclodextrin silica columns.

INTRODUCTION

Cyclodextrins have been increasingly used in liquid chromatography to effect the separation of positional isomers, geometric isomers and enantiomers. The toroidally shaped β-cyclodextrin molecule contains seven glucose units, which are connected through α-(1,4) linkages[1–3]. The inner surface of the cyclodextrin cavity is relatively hydrophobic and has a high electron density, whereas the exterior surface of cyclodextrin is hydrophilic owing to the presence of clockwise projecting 2-hydroxyl groups and counter-clockwise projecting 3-hydroxyl groups at the rim of the larger opening of the cavity, and primary 6-hydroxyl groups at the smaller opening of the cavity. Cyclodextrins readily form 1:1 and 1:2 guest–host complexes with molecules that penetrate into their cavities. The stability of the complex depends on the "snugness" of the fit and the subsequent stabilization of the complex via secondary intermolecular interactions[4].

In liquid chromatography, cyclodextrins are used either as mobile-phase additives[5] or as stationary phases[3,6–10]. With cyclodextrins as mobile-phase additives[5], separation is accomplished in the reversed-phase mode, and is based on the hydro-

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phobicity change of the solutes that is caused by inclusion complex formation. This approach works well in analytical separations, but it is impractical in preparative separations. Cyclodextrin stationary phases are either insoluble cyclodextrin polymers[11] or cyclodextrin units immobilized on a silica support[12–18]. Cyclodextrin polymers have high capacity, but lack chromatographic efficiency[11]. The first high-performance liquid chromatographic (HPLC)-grade cyclodextrin-silicas contained ethylenediamine[12] or diamide bridges[13], had low coverage (50 μmol/g immobilized cyclodextrin) and were hydrolytically unstable. Stable, higher capacity cyclodextrin–silicas, immobilized via alkyl spacers, have been developed[14] and are now commercially available from ASTEC (Whippany, NJ, U.S.A.)[16] with α-, β- or γ-cyclodextrin moieties. Derivatized bonded cyclodextrins, which posses different selectivities, have recently been synthetized by carbamoylation[17] and acetylation[16].

Owing to the special geometry of cyclodextrin and its inclusion complex formation, cyclodextrin–silicas can be used for the separation of positional isomers, geometric isomers and enantiomers. The positional isomers of polysubstituted benzenes[19–23], benzoic[22] and aminobenzoic acids, methylindoles, prostaglandins and steroids and the geometric isomers of polyaromatic hydrocarbons and derivatives have been separated successfully[20]. The separations of *cis–trans* isomers of stilbenes[20], prostaglandins[24], cyclic nitrosamines[25], acyclic nitrosamines[26], cyclohexane derivates[27] and tamoxifen[28] have been reported. Enantiomer separations are actively pursued aspects of cyclodextrin research and have been reviewed[7,8,10,29], but are not dealt with in this paper.

Chromatographic separations can be effected in the elution mode, frontal mode and displacement mode[30,31]. Elution is more suitable for analytical separations (dilute solutions, linear sorption isotherm); frontal and displacement modes (concentrated solutions, non-linear sorption isotherms) offer advantages (high throughput, yield and sample concentration) for preparative separations. Although displacement chromatography has been known for a long time[32], it was revived only when Horvath *et al.*[33] reported efficient separations using HPLC equipment. Recent reviews demonstrate its rapid growth[31–36].

In displacement chromatography, the column is first equilibrated with the carrier solution that has the least affinity for the column. Then the sample, whose components are adsorbed more strongly, is introduced, followed by the displacer, which has the strongest affinity for the stationary phase. As the front of the displacer moves down the column, it displaces the sample components which, in turn, displace each other according to their adsorption strength. If the adsorption strengths of the components are sufficiently different and the column has the necessary efficiency, the components occupy adjacent zones and move at the same velocity in the fully developed displacement train. Component concentrations in the isotachic train depend only on the respective adsorption isotherms and the concentration of the displacer.

Solute concentrations and column loadings that are orders of magnitude higher than in elution chromatography have been achieved in the displacement mode[33–36]. While much has been learned about the role of the operational parameters (column efficiency, capacity, dispersion, mass transfer rate, relative sample loading)[37–39], little is yet known about the rules of displacer selection and selectivity control. The factors that hamper most the wider acceptance of displacement chromatography include the lack of knowledge of solute adsorption isotherms and the lack of well characterized

displacers. Displacer selection is still done by trial-and-error methods. Most modern displacement chromatographic separations were carried out in the reversed-phase mode and dealt with small polar molecules, antibiotics, oligopeptides and small proteins[33–40].

However, displacement chromatographic separations with cyclodextrins, combining the unique selectivity of cyclodextrins and the preparative efficiency of displacement chromatography, as reported here, have not previously been described. This combination permits efficient and unique preparative separations hitherto unavailable, separations which are important in the field of chemical and biomedical sciences and technologies. In this paper, we discuss the displacement chromatographic separations of positional and geometric isomers in the reversed-phase mode. In forthcoming papers we shall describe the displacement chromatographic separations of positional and geometric isomers in the normal-phase mode[41] and the displacement chromatographic separations of enantiomers in the reversed-phase mode[42].

EXPERIMENTAL

A computer-controlled displacement chromatograph, shown in Fig. 1, was constructed from commercial components. It is based on designs described previously[38,40] and consists of two LC 2010 liquid chromatographic pumps (Varian, Walnut Creek, CA, U.S.A.), two computer-controlled, pneumatically activated Type 7001 switching valves (Rheodyne, Cotati, CA, U.S.A.), a computer-controlled, pneumatically activated Type 7125 injection valve (Rheodyne), an LC 2050 variable-wavelength UV detector (Varian), a Series RI-3 refractive index (RI) detector (Varian) and a Powermate II personal computer (NEC, Computer Access, College Station, TX, U.S.A.). Detector signals were recorded by a Maxima Workstation (Waters Assoc.,

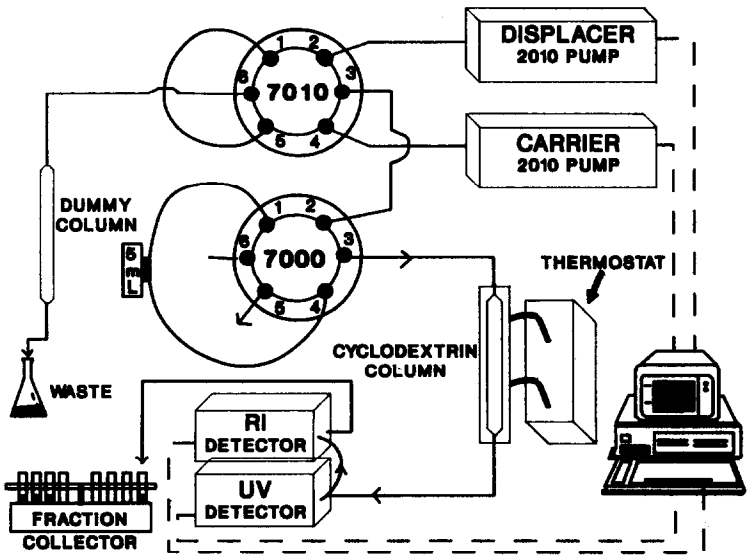

Fig. 1. Schematic diagram of the displacement chromatograph.

Dynamic Solutions, Milford, MA, U.S.A.). Retention volume measurements from elution experiments, individual excess surface adsorption isotherms from frontal chromatographic measurements and preparative-scale displacement chromatographic separations alike can be effected with this instrument. Consumption of chemicals during the isotherm determination steps was minimized by using small-bore columns, packed in our laboratory with commercially available α- and β-cyclodextrin–silicas (ASTEC, Whippany, NJ, U.S.A.). Columns of different length were used in the frontal chromatographic measurements in order to insure comparable adsorption isotherm accuracies for both the strongly and the slightly adsorbed components. The displacement chromatographic separations were completed using the same stationary phases, custom-packed into 250 mm × 46 mm I.D. stainless-steel columns. As retention varies sensitively with temperature, all measurements were carried out with water-jacketed columns thermostated at 30°C.

An integration-algorithm-based interactive graphics program, written in QUICKBASIC for the IBM AT-compatible NEC Powermate II personal computer, was developed to analyze the digitalized frontal chromatograms. Chromatograms measured by the Maxima system were transfered as ASCII files for post-run evaluation by this program. The SAS PC program package (SAS Institute, Cary, NC, U.S.A.) was used to determine the adsorption isotherm parameters.

All solutes were from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. The components used as displacers were from Sigma (St. Louis, MO, U.S.A.). Eluents were prepared from HPLC-grade methanol (Mallinckrodt, St. Louis, MO, U.S.A.) and water produced by a Milli-Q unit (Millipore, Bedford, MA, U.S.A.).

RESULTS

The parameters that influence elution-mode solute retention on cyclodextrin–silicas were investigated first, then the individual excess surface adsorption isotherms of a few displacers, both ionic and non-ionic, were determined, followed by the adsorption isotherms of selected solutes. Finally, displacement chromatographic separations of these solutes were carried out on cyclodextrin-silicas to demonstrate the feasibility of the proposed preparative separation method.

Retention as a function of the methanol concentration in the eluent

The retention curves (log capacity factor, k', *vs.* methanol concentration) of a few polar positional isomers (naphthols, nitroanilines, ethoxyanilines and chloroanilines) are shown in Figs. 2 and 3 for methanol–water eluents. As it was found with other solutes[41], these curves on β-cyclodextrin silica show poorer linearity, and the slopes for the different solute types vary more than on "regular" alkylsilicas. The common feature of these curves is that the solute retention is very low (and almost constant) when the eluents are rich in methanol (*i.e.*, above 60–70% methanol), and increases rapidly as the methanol concentration is decreased.

Retention as a function of the ionic strength and pH of the eluent

It has been stated that on cyclodextrin–silicas solute retention decreases as the ionic strength of the eluent increases, because the eluent cations occupy the cyclo-

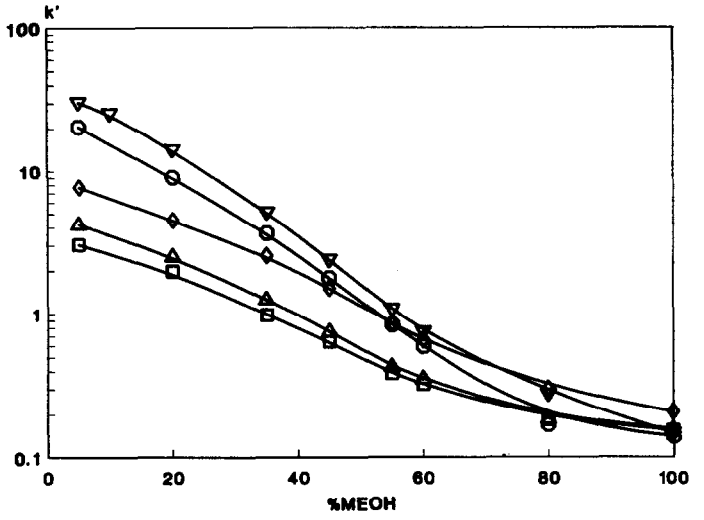

Fig. 2. Retention of the naphthol and nitroaniline isomers on β-cyclodextrin–silica as a function of the methanol (MEOH) concentration of the eluent. ◇ = *p*-Nitroaniline; △ = *o*-nitroaniline; □ = *m*-nitroaniline; ○ = 2-naphthol; ▽ = 1-naphthol.

dextrin cavities and exclude the solute molecules[16]. We found that the role of ionic strength is much more complex and that it depends on both the type of solute and the pH of the eluent. The log *k'* *vs.* ionic strength curves for quinine and quinidine at pH 3.66 (50 m*M* phosphate buffer, ionic strength adjusted with sodium bromide) are

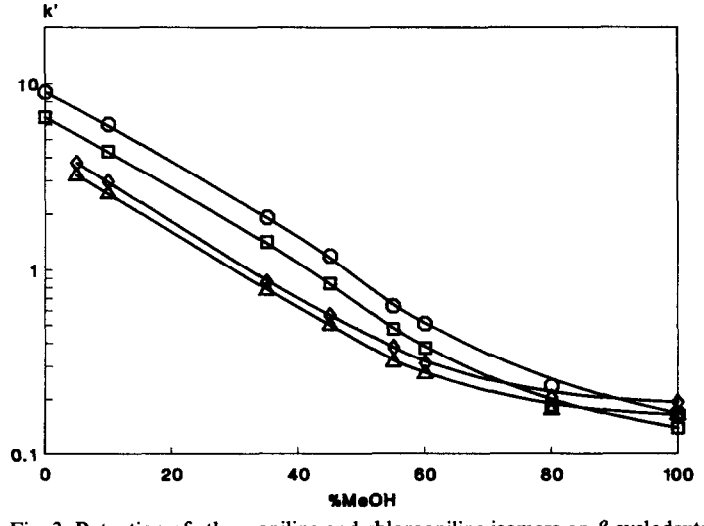

Fig. 3. Retention of ethoxyaniline and chloroaniline isomers on β-cyclodextrin–silica as a function of the methanol (MeOH) concentration of the eluent. ◇ = *p*-Ethoxyaniline; △ = *m*-ethoxyaniline; ○ = *p*-chloroaniline; □ = *m*-chloroaniline.

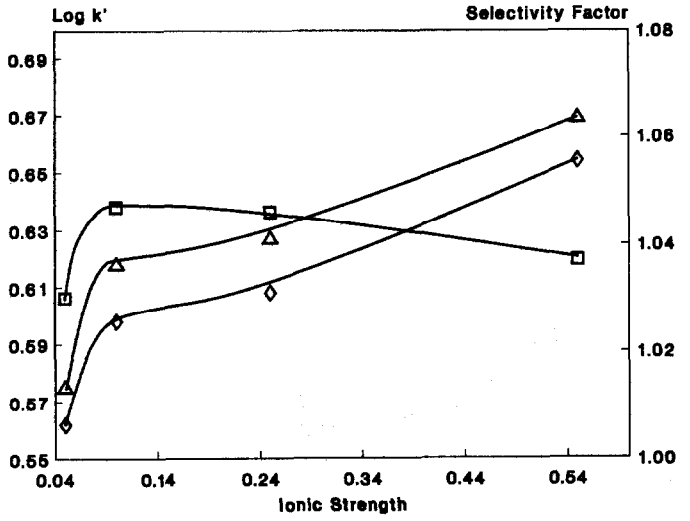

Fig. 4. Retention of (◇) quinine and (△) quinidine and (□) selectivity factor as a function of the ionic strength of the eluent at pH 3.66 (50 m*M* phosphate buffer and NaBr).

shown in Fig. 4. These positively charged components become more retained as the ionic strength increases. However, at higher pH, such as pH 5.55 (Fig. 5) and 6.55 (Fig. 6), their retention decreases with increasing ionic strength. As the pK_a value of the protonated quinine is 6.74, the solutes remain positively charged in the pH range 3.5–6.5. Hence there is no change in the extent of solute ionization to explain the increased (by an order of magnitude) retention. Instead, an ion-exchange mechanism,

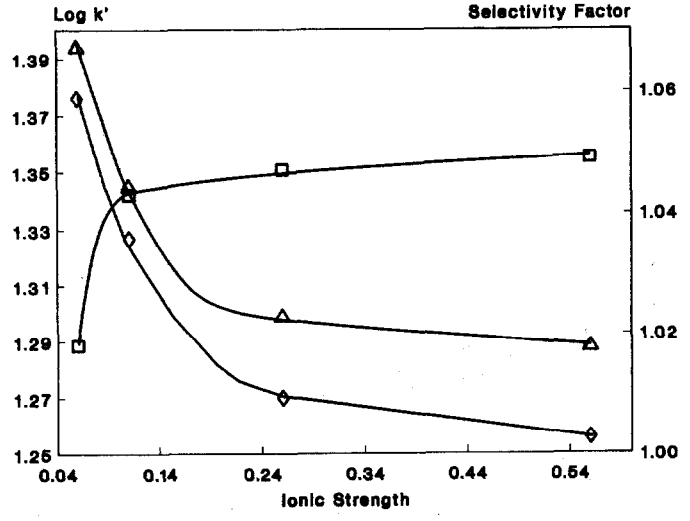

Fig. 5. Retention of (◇) quinine and (△) quinidine and (□) selectivity factor as a function of the ionic strength of the eluent at pH 5.55 (50 m*M* phosphate buffer and NaBr).

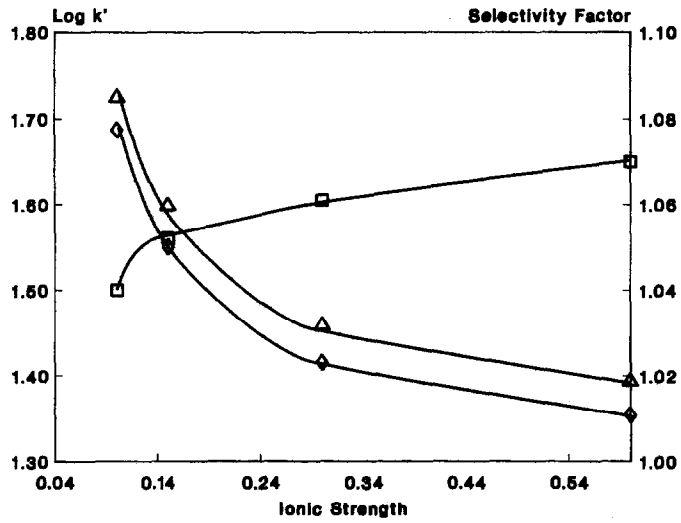

Fig. 6. Retention of (◇) quinine and (△) quinidine and (□) selectivity factor as a function of the ionic strength of the eluent at pH 6.55 (50 m*M* phosphate buffer and NaBr).

similar to that reported by Papp and Vigh[43,44] for alkylsilicas, is operative. At higher pH the silanol groups of the silica support are dissociated more completely, and ion exchange contributes to the retention of positively charged solutes more strongly than inclusion complex formation. Therefore, cation retention decreases in the eluents of higher ionic strength where more competition is present. Simultaneously, and importantly, as ion exchange is repressed by the salt, the chances of selective retention by the cyclodextrin moiety improve, *i.e.* the separation selectivity factor increases, as shown in Figs. 5 and 6.

When there is no such ion exchange, *i.e.*, when the silanol groups are not yet dissociated sufficiently (as in Fig. 4), or when the solute is negatively charged (as the dansylphenylalanine enantiomer pair in Fig. 7) or uncharged (as the naphthol isomers in Fig. 8), retention increases with increasing ionic strength, as in ordinary reversed-phase systems. Unfortunately, as the hydrophobic interaction becomes stronger, the differentiating ability of cyclodextrin–silica decreases and the selectivity factor decreases (Figs. 7 and 8).

Hence it can be seen that the effects of ionic strength and eluent pH are much more complex on cyclodextrin–silicas than is commonly perceived. The retention trends are different for the positively and negatively charged solutes and the uncharged solutes. The retention changes are large enough to necessitate a closer examination of these factors during the development of a preparative separation.

Individual excess adsorption isotherms of selected displacers

The success of any displacement chromatographic separation scheme depends critically on the finding of an appropriate displacer. Therefore, the individual excess surface adsorption isotherms of a few detergents were determined on cyclodextrins using frontal chromatographic measurements. The isotherms of several quaternary

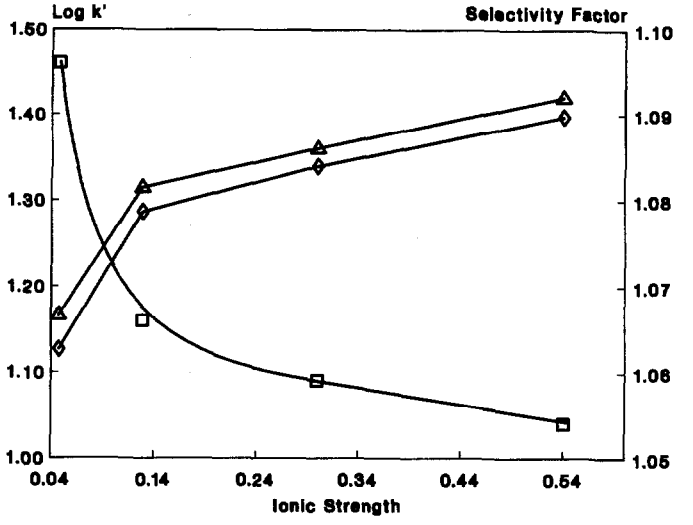

Fig. 7. Retention of the dansylphenylalanine enantiomers (◇ = D-; △ = L-) and (□) selectivity factor as a function of the ionic strength at pH 5.55 (50 m*M* phosphate buffer and NaBr).

ammonium salts are shown in Fig. 9 [β-cyclodextrin–silica, 13% (v/v) methanol–water solution, no pH or ionic strength control[45,46]].

The individual excess surface adsorption isotherms of the symmetrical tetraalkylammonium bromides (tetrabutyl and tetrapentyl) rise much more slowly than the isotherms of the detergents that have a single, long alkyl chain. When the detergents contain a cetyl chain, such as the cetylpyridinium, cetrimide and

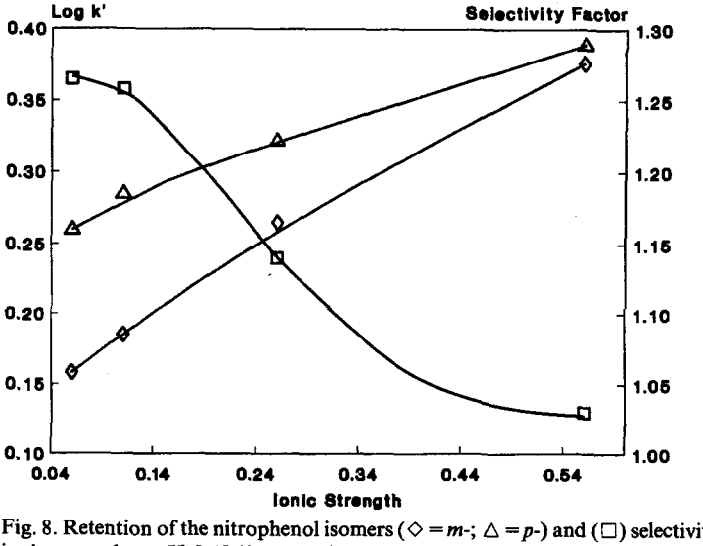

Fig. 8. Retention of the nitrophenol isomers (◇ = *m*-; △ = *p*-) and (□) selectivity factor as a function of the ionic strength at pH 5.55 (50 m*M* phosphate buffer and NaBr).

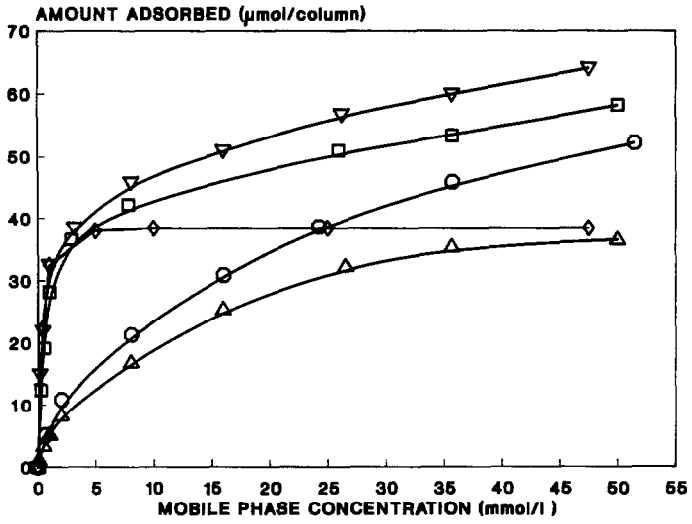

Fig. 9. Adsorption isotherms of the quaternary ammonium salts on β-cyclodextrin–silica from 13% (v/v) methanol–water. ◇ = Cetrimide; △ = tetrabutylammonium bromide; ○ = tetrapentylammonium bromide; ▽ = benzylcetyldimethylammonium chloride; □ = cetylpyridinium bromide.

benzylcetyldimethylammonium cations, there is a very steep initial rise in the isotherm. The isotherm of benzylcetyltrimethylammonium bromide soon levels off. However, the isotherms continue to rise if the detergents contain a bulky group (*e.g.*, a six-membered ring) in addition to the cetyl chain. None of the isotherms follows the simple Langmuir equation. The methanol concentration, pH and ionic strength of the solution also have an important effect on the extent of excess surfactant adsorption and the shape of the isotherm[45,46].

Displacement chromatographic separations on β-cyclodextrin silica

The naphthol and nitroaniline positional isomers were selected to demonstrate the feasibility of displacement chromatographic separations on β-cyclodextrin–silica. As their retention behaviour in methanol–water eluents was known (Fig. 2), a composition of the carrier in which the initial solute retention is sufficiently large ($k' > 10$),

TABLE I

INITIAL BREAKTHROUGH VOLUMES OF CATIONIC DETERGENT-TYPE DISPLACERS IN 13% (V/V) METHANOL–WATER CARRIER SOLUTION

Detergent	*Solution concentration (mM)*	*Breakthrough volume (ml)*
Tetrabutylammonium bromide	0.51	3.86
Tetrapentylammonium bromide	0.65	5.06
Cetyltrimethylammonium bromide	0.54	32.68
Cetylpyridinium bromide	0.50	44.57
Benzyldimethylcetylammonium chloride	0.43	47.84

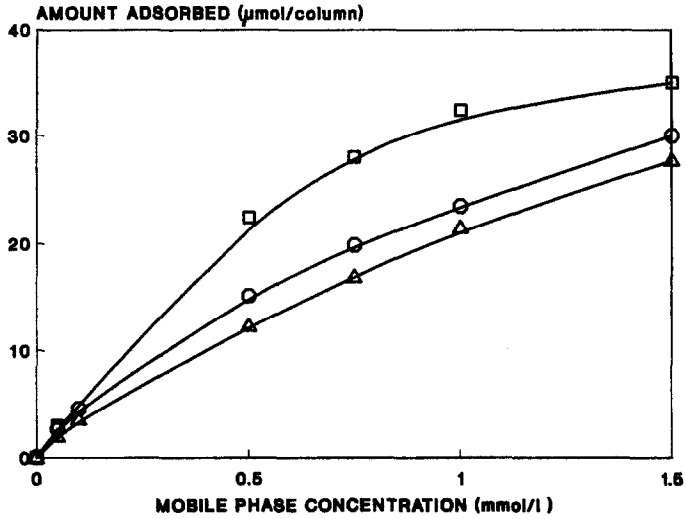

Fig. 10. Adsorption isotherms of (□) cetrimide and the (○) 1- and (△) 2-naphthol on β-cyclodextrin–silica from 13% (v/v) methanol–water.

could be selected *i.e.*, containing 13% (v/v) methanol. The adsorption isotherms of the displacers were also determined in this solution (Fig. 9). The initial breakthrough volumes belonging to the first measured point of the isotherms are shown in Table I. As the extrapolated retention volumes of the naphthol isotherms are larger than the breakthrough volume of tetrapentylammonium bromide, but smaller than that of cetrimide, the latter was considered as a possible displacer. The adsorption isotherms of the naphthol isomers and the cetrimide displacer in 13% (v/v) methanol are shown in Fig. 10. The isotherms of the naphthol isomers run below the isotherm of cetrimide, indicating that the contemplated displacement chromatographic separation may be feasible.

A sample of 8 μmol of 2-naphthol and 10 μmol of 1-naphthol was loaded (from a total volume of 1.8 ml) onto two 250 mm x 4.6 mm I.D. β-cyclodextrin-silica columns in series, pre-equilibrated with the 13% (v/v) methanol–water carrier. The 1.5 m*M* cetrimide displacer solution was introduced at a flow-rate of 0.5 ml/min. The displacement chromatogram, shown in Fig. 11, was recorded using a refractive index (RI) detector. Three individual steps can be observed in the chromatogram. Elution-mode HPLC analysis of the collected fractions showed that the first plateau corresponds to 2-naphthol, the second to 1-naphthol and the third to cetrimide. To our knowledge, this is the first displacement chromatogram ever obtained on β-cyclodextrin–silica columns.

The displacement chromatogram of the nitroaniline isomers was obtained to demonstrate that sometimes the approximate conditions of a first displacement chromatographic separation can be determined solely from the extrapolated retention volumes and the initial breakthrough volumes (adsorption isotherms) of the displacers, without a detailed knowledge of the actual adsorption isotherms of the pure solutes. The extrapolated retention volumes of the nitroaniline isomers with 13% (v/v) methanol–water eluent were calculated from the log k' *vs.* methanol concentra-

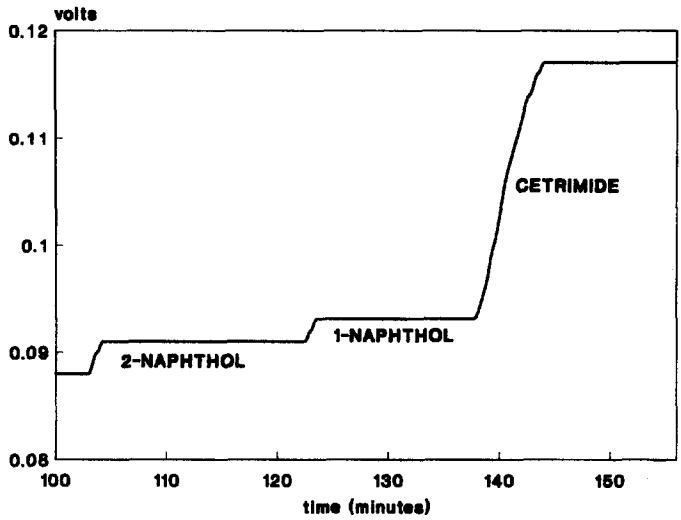

Fig. 11. Displacement chromatogram of the naphthol isomers on β-cyclodextrin–silica with 1.5 m*M* cetrimide [in 13% (v/v) methanol–water] as displacer. Flow-rate, 0.5 ml/min.

tion curves (Fig. 2) to be above $k' = 10$, the minimum retention value generally considered conducive to a successful displacement chromatographic separation. These retention volumes were compared with the initial displacer breakthrough volumes in Table I. Benzylcetyldimethylammonium chloride was found to have a much larger initial breakthrough volume than the retention volumes of the nitroanilines; consequently, its selection as a possible displacer can be considered a reasonably safe proposition.

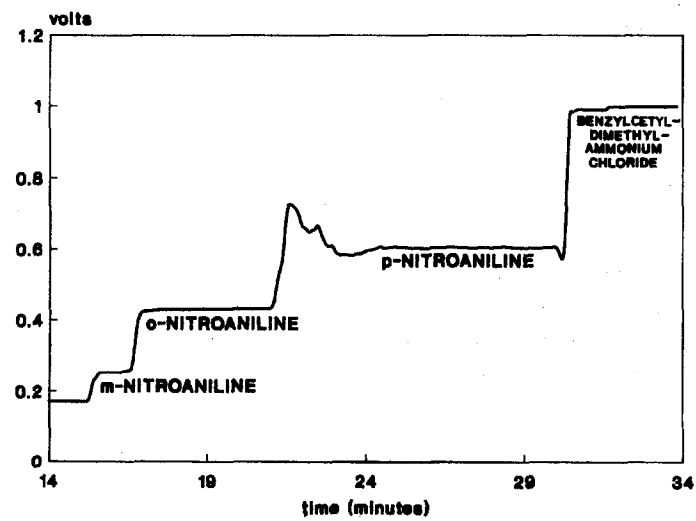

Fig. 12. Displacement chromatogram of the nitroaniline isomers on β-cyclodextrin–silica with 52.6 m*M* benzylcetyldimethylammonium chloride [in 13% (v/v) methanol–water] as displacer. Flow-rate, 0.5 ml/min.

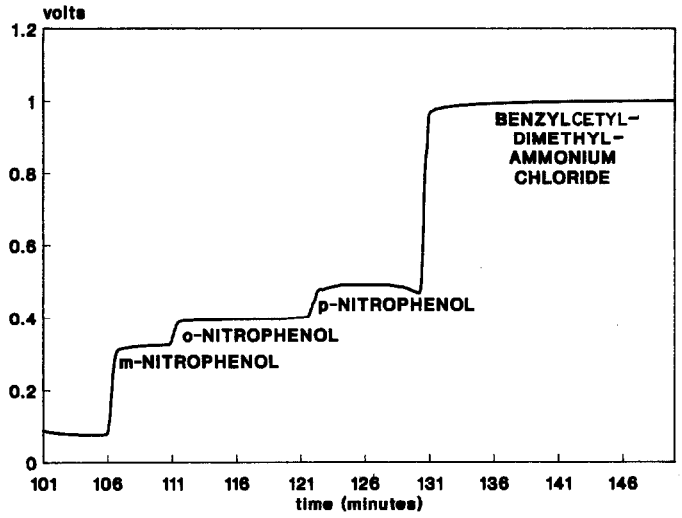

Fig. 13. Displacement chromatogram of the nitrophenol isomers (*m*-, 12 mg; *o*-, 25 mg; *p*-. 21 mg) on β-cyclodextrin–silica with 10% (v/v) methanol–water as carrier and 64 m*M* benzylcetyldimethylammonium chloride as displacer. Flow-rate, 0.5 ml/min.

A sample that contained 0.7 μmol of *m*-nitroaniline, 1.6 μmol of *o*-nitroaniline and 4.8 μmol of *p*-nitroaniline was injected, from 5 ml of 13% (v/v) methanol carrier solution, onto two 250 mm × 4.6 mm I.D. β-cyclodextrin–silica columns in series, then a 52.6 m*M* solution of benzylcetyldimethylammonium chloride was introduced at a flow-rate of 0.5 ml/min as displacer. The displacement chromatogram, shown in Fig. 12, was recorded with an RI detector. Four steps can be observed in the chromatogram. HPLC analysis of the collected fractions showed that the plateaus correspond to *m*-, *o*- and *p*-nitroaniline and benzylcetyldimethylammonium chloride. Some unknown contaminants were also found at the front section of the *p*-nitroaniline fraction. Their origin was traced back to the *o*-nitroaniline standard used, but the identity of the contaminants was not determined.

The displacement chromatogram of 12 mg of *m*-nitrophenol, 25 mg of *o*-nitrophenol and 21 mg of *p*-nitrophenol (a total of 58 mg of sample loaded onto a 4.6 mm I.D. analytical column!) is shown in Fig. 13. The carrier was 10% (v/v) methanol–water and the displacer 64 m*M* benzylcetyldimethylammonium chloride, at a flow-rate of 0.5 ml/min. The yields corresponding to the 99% purity level were calculated from the reconstructed chromatograms and turned out to be 89% for *m*-, 80% for *o*- and 75% for *p*-nitrophenol. Notwithstanding the high load excellent separation of the isomers was achieved.

CONCLUSIONS

It was shown that although the log *k'* *vs.* methanol concentration relationships for polar solutes, especially the positively charged type, are not as simple as on ordinary alkylsilica reversed-phase packings, they are regular enough to permit the

use of a reasonably small number of actual retention measurements to predict (by extrapolation) the carrier solvent compositions that result in sufficiently large initial solute retentions ($k' > 10$ or, preferably, $k' > 25$).

The adsorption isotherms of cationic detergents were found to be convex, although the simple Langmuir isotherm equation could not be used to describe the individual excess adsorption isotherms. The stationary-phase concentrations of the detergent-type displacers varied more sensitively with the methanol concentration of the carrier solution than is observed on regular alkylsilica-type reversed-phase stationary phases.

Using estimated solute retention volumes, predicted carrier compositions and actual displacer adsorption isotherms, succesful displacement chromatographic separations of up to 60-mg samples of positional isomers have been achieved on regular analytical-scale columns. Hence the unique separation selectivity of β-cyclodextrin–silicas, as known from elution chromatographic separations, was successfully combined with the efficient displacement chromatographic mode of operation and resulted in hitherto unavailable preparative separations.

Further work is in progress in our laboratory (and will be reported soon) to study in detail: (1) the retention behaviour of a large number of solutes, representing the three unique application fields of cyclodextrin–silicas (positional, geometric and optical isomers); (2) the adsorption isotherms of selected displacers, both ionic and non-ionic detergents, and "designer displacers" specifically synthesized to meet the requirements of cyclodextrin–silicas; (3) the general rules of displacer selection for cyclodextrin–silicas; and (4) the operating conditions that allow the separation of a large number of positional and geometric isomers and enantiomers of industrial significance.

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