HPLC of Structural Isomers Using Cyclodextrin–Poly(vinylamine)-Coated Silica Columns, Part II: Retention Behavior and Separation

G. Crini and **M. Morcellet***

Laboratoire de Chimie Macromoléculaire, U.R.A. 351 CNRS, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

Abstract

The liquid chromatographic retention behavior and separation by isocratic elution of aromatic compounds by using several β-cyclodextrin bonded stationary phases is investigated. The high-performance liquid chromatographic supports are based on silica beads coated with a poly(alkylamine) (poly[vinylamine] or PVA). The ability of these supports to separate *ortho, meta,* and *para* isomers of some disubstituted benzene derivatives is studied. The contribution of the amino groups of the poly(vinylamine) and of the β-cyclodextrin cavity to the separation process is discussed. Several interactions (inclusion complex formation, acid–base and hydrophobic interactions) are discussed. The effect of the composition of the mobile phase on the separation is also examined.

Introduction

Disubstituted benzene derivatives, in particular substituted phenols, are the subject of increasing interest in recent years (1–5). These toxic compounds are products of many industrial processes, and they may be found in trace quantities in water. Therefore, it is necessary to have adsorbant resins that are able to totally eliminate them. There are several methods of qualitative and quantitative analysis, including chromatographic techniques (gas and liquid).

The ability of β-cyclodextrin (β-CD) to form inclusion complexes with various molecules, especially aromatics, was used in this study. β-CD is a torus-shaped cyclic oligosaccharide that is made up of seven α-1,4-linked D-glucopyranose units. The apolar cavity can selectively include various phenolic derivatives. The inclusion process is influenced mainly by shape, size, and polarity of guest molecules (6,7). Hence β-CD complexation is a procedure of choice for separation of compounds, and it has been used as an advantage in high-performance liquid chromatography (HPLC) (8–16).

β-CD can be used in HPLC in two main ways: it can be chemically bonded to silica stationary phases (10–12) and it can be used as a selective component of the mobile phase in a

Table I. Characterization of β-CD Bonded Phases (Type I Supports)

Name*	Polymer deposit† (mg/g)	β-CD‡ (μmol/g)	Cross-linking agent
SiPVAβCD7ret.	99	7	Epichlorohydrin
SiPVAβCD24ret.	121	24	BUDGE
SiPVAβCD15ret.	92	15	Epichlorohydrin
SiPVAβCD36ret.	72	36	Epichlorohydrin
SiPVAβCD53ret.	64	53	Epichlorohydrin

* SiPVAβCDxret refers to a support coated with poly(vinylamine) (PVA) functionalized with β-CD. The resulting system has *x* μmole of β-CD per gram of support. Lastly it is cross-linked (reticulated or *ret*).
† Amount of polymer (milligrams) per gram of support.
‡ Amount of β-CB (μmicromoles) per gram of support.

Table II. Characterization of β-CD Bonded Phases (Type II Supports)

Name*	Polymer deposit† (mg/g)	β-CD‡ (μmol/g)	Cross-linking agent
SiPVA	99	–	
SiPVA		–	
SiPVAret.		–	Epichlorohydrin
SiPVAret.	132	–	BUDGE
SiPVAret.βCD12	158	12	Epichlorohydrin
SiPVAret.βCD23	204	23	Epichlorohydrin
SiPVAret.βCD36		36	BUDGE
SiPVAret.βCD46	175	46	Epichlorohydrin
SiPVAret.βCD67	186	67	Epichlorohydrin
SiPVAret.βCD95		95	Epichlorohydrin

* SiPVAβCDxret refers to a support coated with poly(vinylamine) (PVA) functionalized with β-CD. The resulting system has *x* μmole of β-CD per gram of support. Lastly it is cross-linked (reticulated or *ret*).
† Amount of polymer (milligrams) per gram of support.
‡ Amount of β-CB (μmicromoles) per gram of support.

* Author to whom correspondence should be addressed.

reversed-phase system (13,14). Attempts were also made to prepare stationary phases by grafting β-CD on organic gels like polyacrylamide gel beads (15) or agarose (16). More recently, a method that consists of coating and cross-linking a polymer onto the surface of silica beads has been investigated. Supports for ion exchange (17), affinity chromatography (18) or size exclusion chromatography (19) were also prepared in this way.

In a previous publication (20), we discussed the synthesis and characterization by solid state nuclear magnetic resonance of new stationary phases for HPLC. These stationary phases were based on silica beads coated with a β-CD-containing poly(vinylamine) (PVA). This paper reports the HPLC separation of substituted phenols by isocratic elution and detection at the ultraviolet wavelength by using these supports. In this study, we investigated phenomena of the retention of solutes and attempted to establish a correlation between the chromatographic behavior of supports and their physicochemical characteristics (which are dependent on the method of preparation, as shown in the first part of this work [20]).

Experimental

Apparatus

The chromatographic analyses were carried out using a Merck L.6200A Intelligent Pump from Merck-Clevenot (Nogent sur Marne, France) with a Rheodyne 7125 injection valve (sample loop, 20 µL) connected to an L.4250 UV–Vis variable wavelength monitor and a D.2500 Chromato-Integrator.

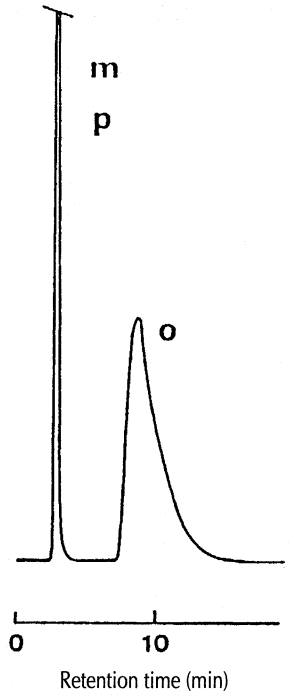
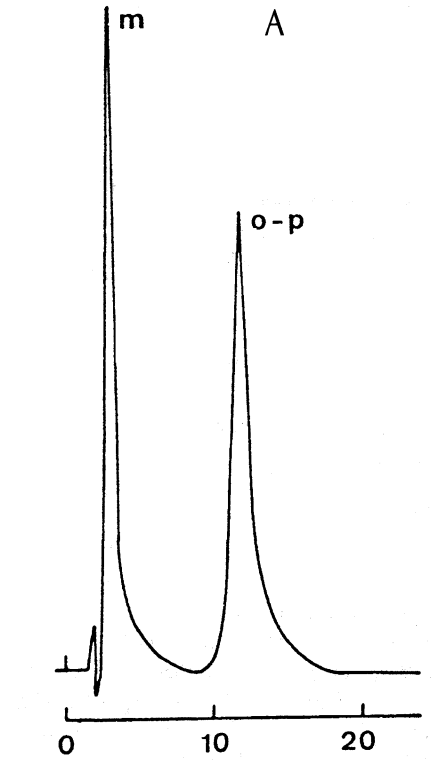


Figure 1. Chromatogram of nitrobenzoic acids on a SiPVA column. The eluent was 0.01M phosphate buffer (pH 6). *o*, *m*, and *p* denote *ortho*, *meta*, and *para* isomers, respectively.



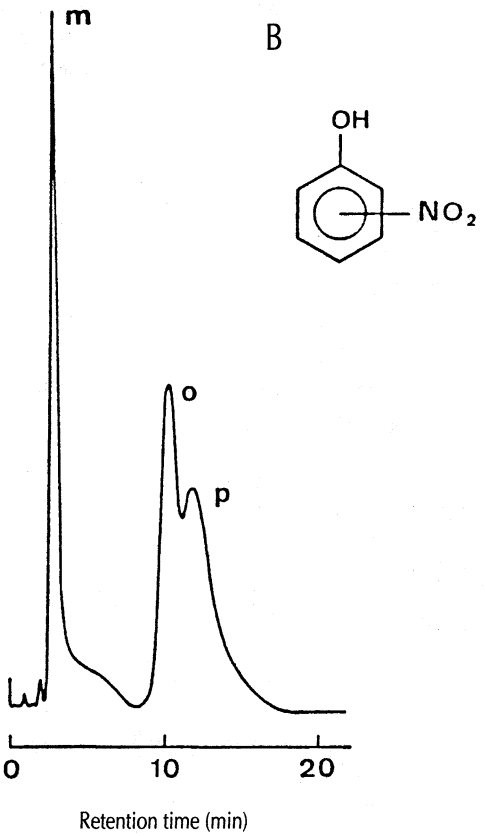


Figure 2. Separation of nitrophenol isomers on two stationary phases: A, SiPVA; B, SiPVAret. The mobile phase was H_2O–MeOH (75:25).

Columns

The supports were prepared as reported previously (20). The columns (100 × 4.6 mm) were filled by a slurry packing technique. A silica support (1.25 g) was suspended in 10 mL of carbon tetrachloride, sonicated for 1 min, and packed into the column at 200 bar with dichloromethane. The columns were then washed with methanol and methanol–water mixtures before use.

HPLC procedure

Experiments were carried out at room temperature. The flow rate was 1 mL/min. The detector was operated at 280 nm. The void volume of the column was determined by injecting potassium nitrite. Methanol, acetonitrile, and water were HPLC grade from Merck. The mobile phases used were mixtures of methanol–water and acetonitrile–water. The solutions and the buffers were filtered through a 0.2-µm Millipore HA membrane and degassed by ultrasonic vibration before use. Samples were dissolved in methanol (or water) at a concentration of 50 µg/mL. Twenty-microliter volumes were injected. The elution order was established from consecutive injections of the individual isomers.

Table III. Influence of the Cross-linking Agent on the Resolution Factor of the Nitrophenol Isomers

Support*	Coating solvent	Cross-linking agent	Resolution (%)
SiPVAret1	MeOH	Epichlorohydrin	52
SiPVAret2	H_2O	Epichlorohydrin	33
SiPVAret3	MeOH	BUDGE	69

* Silica was coated with poly(vinylamine) (PVA) in different solvents then cross-linked (reticulated) with different cross-linking agents.

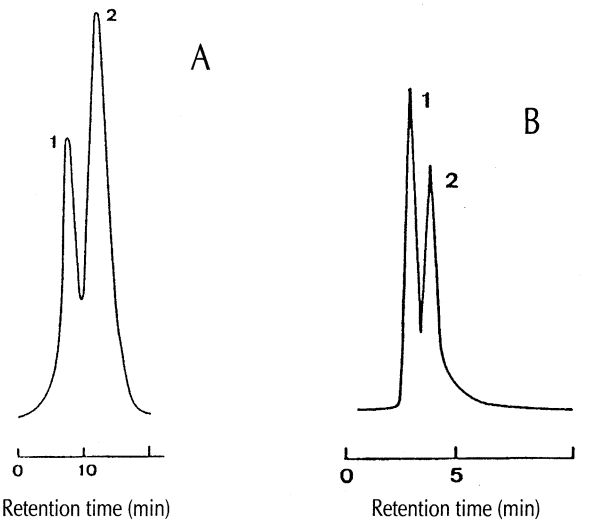


Figure 3. Chromatograms: A, *p*-chlorophenol (peak 1) and *p*-hydroxybenzoic (peak 2); B, *p*-aminobenzoic acid (peak 1) and *p*-nitrobenzoic acid (peak 2). An SiPVAret column (BUDGE cross-linking agent) was used, and the eluent was 0.01M phosphate buffer (pH 6).

Results and Discussion

The main features of supports prepared by methods I and II are given in Tables I and II (for more details, see reference 20).

Role of the polymer coating

Figure 1 shows the resolution of nitrobenzoic acid isomers on a stationary phase containing Si100 coated with PVA

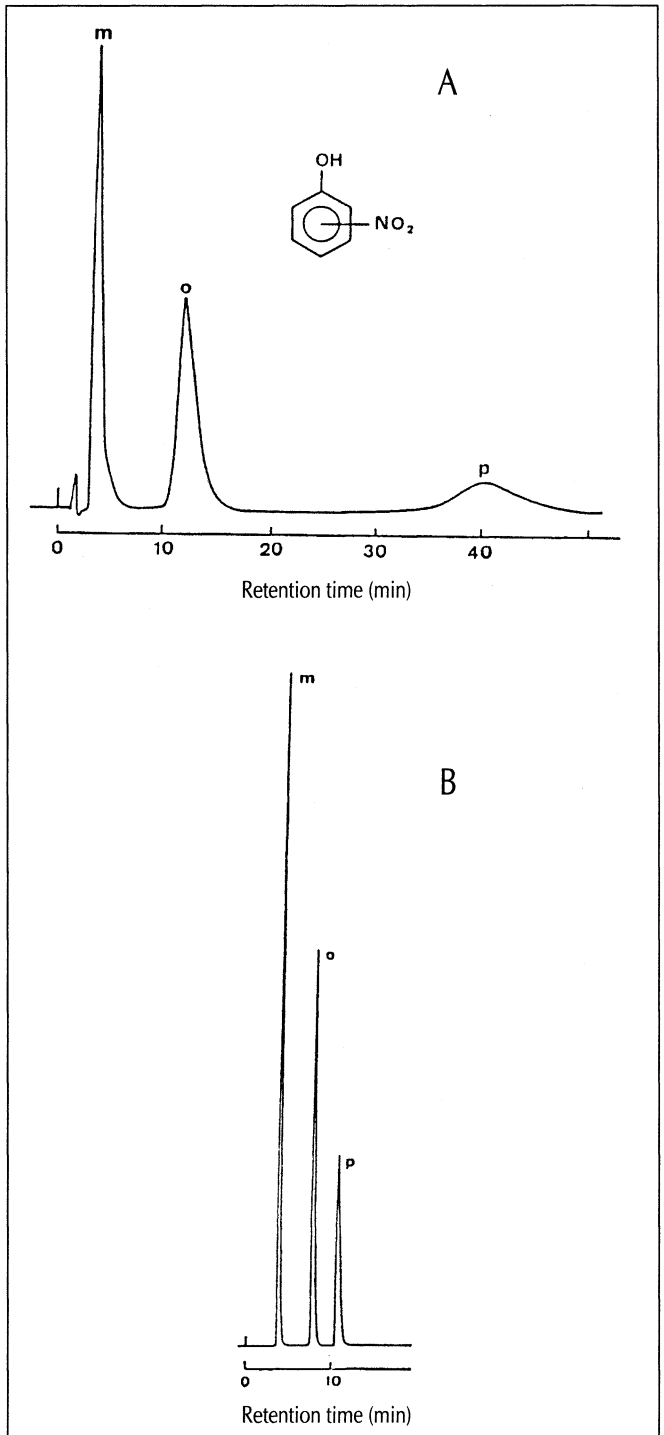


Figure 4. Liquid chromatogram of nitrophenol isomers on an SiPVA β-CD12ret column. The mobile phase was H_2O–MeOH (75:25) (A) or H_2O–CH_3CN (65:35) (B).

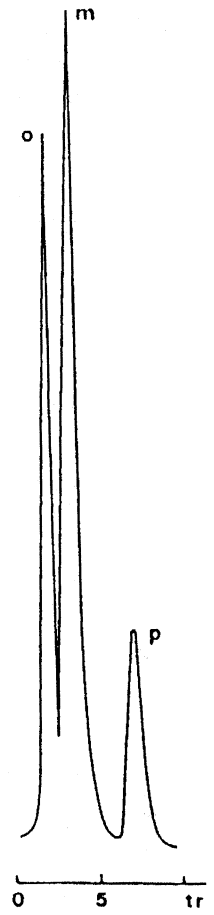


Figure 5. Liquid chromatogram of nitrobenzoic acid isomers on an SiPVA-β-CD36ret column (250 x 4.6 mm). The mobile phase was H_2O–CH_3CN (75:25) adjusted with 0.01M phosphate buffer (pH 6).

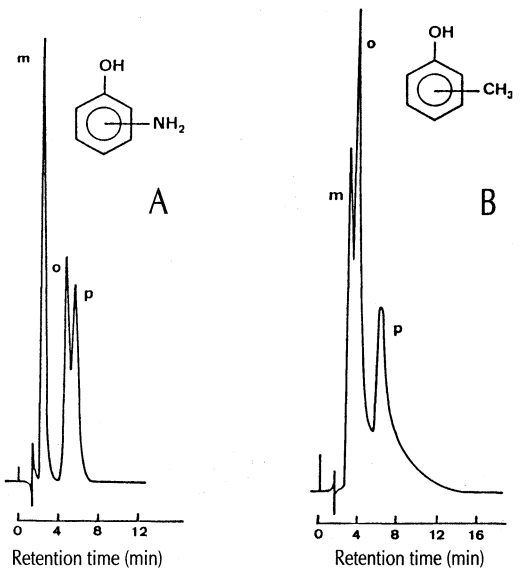


Figure 6. Chromatograms of aminophenol (A) and cresol (B) isomers on an SiPVAret-β-CD67 column. The mobile phase was H_2O–MeOH (85:15), and the flow rate was 0.7 mL/min.

(SiPVA). Nitrobenzoic acid isomers were not retained at all on native silica and were eluted at the void volume of the column.

The *meta* and *para* isomers were eluted (but not resolved) first on the SiPVA column; this is in relation to the *p*K values of the different isomers (*ortho*, 2.16; *meta*, 3.47; and *para*, 3.41). In this case, it can be seen that the separation is based mainly on the acid–base interactions (ion-exchange, hydrogen bonding) between the amino groups of the stationary phase and the solute. The *meta* and *para* derivatives, which are more basic, eluted first.

The chromatogram in Figure 2A confirms this hypothesis. For the nitrophenol isomers, the *meta* isomer (*p*K = 8.28), which is more basic, eluted first, whereas the *ortho* (*p*K = 7.17) and *para* (*p*K = 7.15) isomers are not resolved. These three compounds were not at all retained on native silica and were eluted at the void volume of the column.

When the amount of polymer coated on silica increased, the retention times increased and were accompanied by a broadening of the peaks. This is explained by an increase of the acid–base interactions.

Role of the cross-linking agent

When the polymer was cross-linked with epichlorohydrin, the *ortho* and *para* nitrophenol isomers were partially resolved despite the fact that their *p*Ks are equal (Figure 2B). This partial resolution was obtained only for a given composition of the mobile phase (75:25) and disappeared for a 30:70 water–methanol mixture. This suggests that hydrophobic interactions also play a role. This assumption is supported by the fact that the resolution between the *ortho* and *para* isomers also depended on the nature of both the polyamine and the cross-linking agent (21). The resolution factor increased when butanediol 1,4 diglycidyl ether (BUDGE) (which is also more hydrophobic) was used instead of epichlorohydrin (Table III).

In Figure 3A, *p*-chlorophenol and *p*-hydroxybenzoic acid are resolved on a stationary phase that contains Si100 coated with PVA and cross-linked with BUDGE. These two compounds were retained on SiPVAret column (epichlorohydrin was used as cross-linking agent) but not resolved. This chromatogram confirms that the cross-linking agent introduces hydrophobic interactions.

The *para* aminobenzoic acid (*p*K = 4.92) and *para* nitrobenzoic acid (*p*K = 3.41) were also partially resolved (Figure 3B) by using an SiPVAret column but eluted at the void volume of the SiPVA column despite the fact that their *p*K values are different. The acid–base interactions are equal and the separation in this case is based only on hydrophobic interactions between the polymer stationary phase and the solute. Addition of 5% methanol to the aqueous buffer suppressed any resolution.

Apart from the effect on the resolution of the isomers, the cross-linking of the polyamine layer ensured the long time stability of the support by avoiding the desorption of the polymer.

Separation of isomeric compounds in the presence of β-CD

Figure 4 shows the resolution of nitrophenol isomers on a SiPVA-β-CD12ret column. They eluted in the sequence *meta*, *ortho*, and *para*, following the order of the stability for binding to β-CD (22). Nevertheless, this order was the same as in Figure

2B for a separation without CD. Thus, in that case, CD only increased the resolution factors.

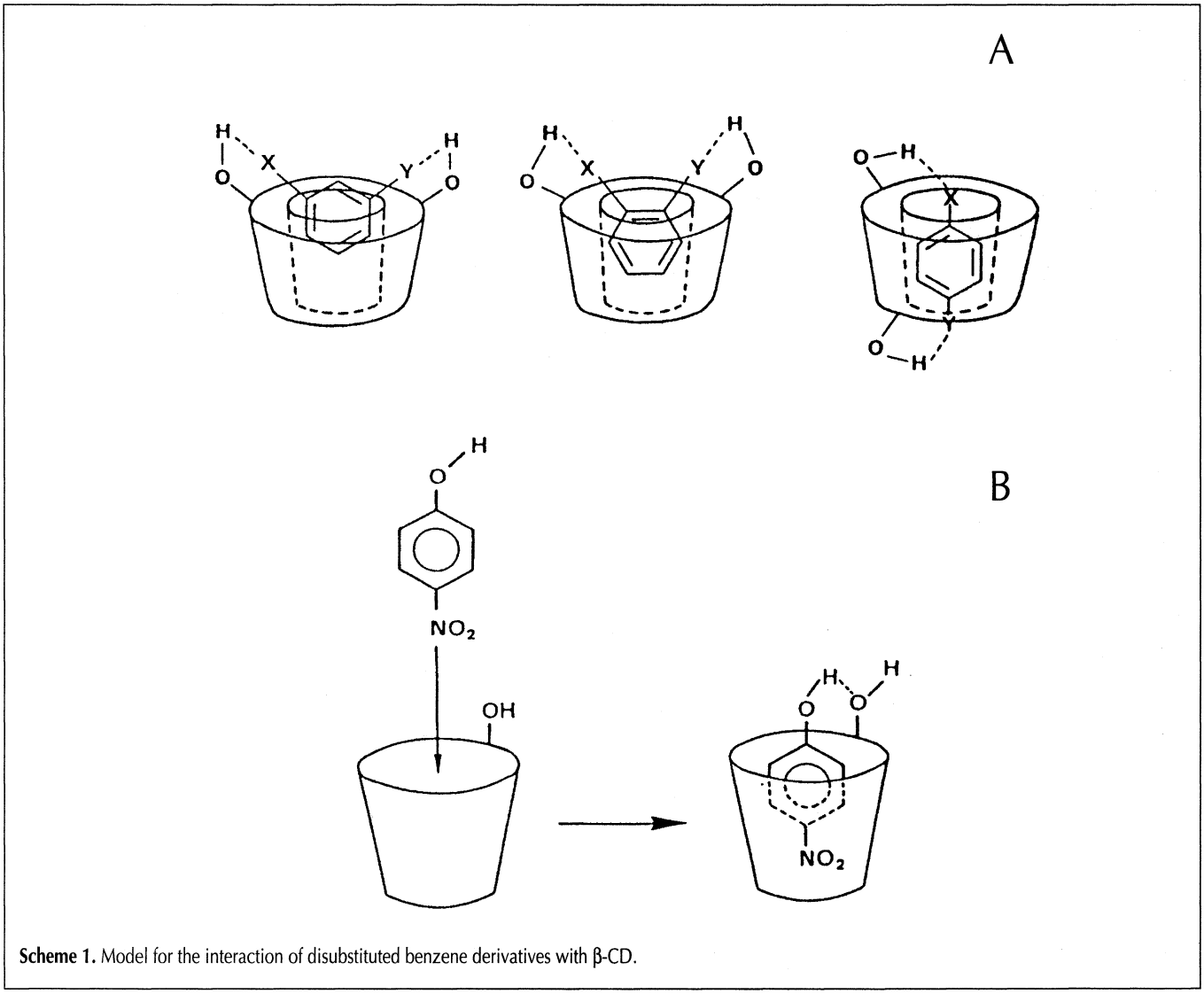
The separation of isomers of nitrobenzoic acid is shown in Figure 5. The elution order in that case is different from that obtained without CD. Thus the formation of inclusion complexes was responsible for the retention of the three isomers. The elution order is *ortho*, *meta*, and *para*, and that of nitrophenol isomers was *meta*, *ortho*, and *para*. The elution order of these two sets of compounds was affected by the properties and the position of the substituted groups. It should be noted that the observed orders agreed with the order for nitrophenol isomers (*meta* < *ortho* < *para*) and for nitrobenzoic acid isomers (*ortho* < *meta* < *para*) that is reported in the literature using different β-CD-bonded stationary phases (10–14).

Figure 6 shows the resolution of cresol and aminophenol isomers on a SiPVAret-β-CD67 column. These compounds were not retained at all on the SiPVAret column, which excludes the role of hydrophobic interactions. In addition, the cresol isomers have very close *p*K values (range, 10.01–10.2) and thus the separation cannot result from acid–base interactions. In these two examples the mechanism is based mostly on the inclusion complex formation.

According to Hinze (23), in the case of the nitrophenol complexes, the nitro group locates into the β-CD cavity and the hydroxyl groups interact with the secondary hydroxyl of the β-CD (Scheme 1). Accordingly the poor resolution of the *ortho* and *para* isomers of aminophenol should be due to the replacement of the nitro group by an amino group. In the case of cresol, the more hydrophobic methyl group was included in the cavity and the hydroxyl group interacted with β-CD, leading to better resolution.

Effect of the organic solvent content in the mobile phase

The effect of mobile phase composition on the retention time of nitrophenol isomers was investigated by changing the organic modifier-to-water ratio in the mobile phase as shown in Figure 7. The retention time decreased linearly with the amount of methanol in the mobile phase, especially for the *para* isomer which gave the more stable complex with β-CD (Figure 7A). This was related to the decreasing stability of the inclusion complexes when the amount of organic solvent increased. When the amount of MeOH was low, the high value of the retention time reflected a broadening of the peaks. When acetonitrile was used instead of methanol as a component of the



Scheme 1. Model for the interaction of disubstituted benzene derivatives with β-CD.

eluant, the retention times were much lower, which indicated that the inclusion complexes of the three isomers with β-CD were more destabilized by acetonitrile than methanol. The retention time also decreased with the amount of acetonitrile, but the variation was not linear (Figure 7B).

The elution order for each solute was independent of the organic modifier content in the mobile phase. The selectivity of separation of nitrophenols was maintained for all water–organic solvent ratios. Table IV shows that when the amount of methanol increased, the resolution of the three isomers of cresol decreased.

Table IV. Influence of the Amount of Methanol on the Resolution Factor of the Cresol Isomers

H_2O–MeOH	Resolution factor (*m/p*)*	Resolution factor (*o/m*)*
65:35	47.6	0
75:25	75	24
80:20	74.5	25.4
85:15	71.4	31.2

* *m/p* and *o/m* are the resolution factors for *meta* vs *para* and *ortho* vs *meta*, respectively.

Effect of pH on retention

The effect of pH on the capacity factor (k') was investigated by changing the pH of the mobile phase from 5 to 7 with phosphate buffers (0.01M). The relationship between the capacity factor for nitrophenols and nitrobenzoic acids isomers and pH is plotted in Figure 8. For these two compounds, there was no change in elution order with a change pH in the range examined. In the case of nitrophenol isomers (Figure 8A), an increase in retention time was observed when the pH was increased. However, for nitrobenzoic acids isomers, the k' values and the resolution decreased with increasing pH, as shown in Figure 8B. Differences in the course of this relationship were reported (14) to reflect the varying degrees of dissociation of the individual isomers and the varying ability of the ions produced to interact with β-CD. It was assumed, for example, that the neutral form of *p*-nitrobenzoic acid interacted with the β-CD cavities more strongly than the anionic form and the reverse was true for *p*-nitrophenol (14). In our case, we also considered the interactions with the amino groups of the stationary phase. As for all polyelectrolytes, the pK_a value of PVA changed with its

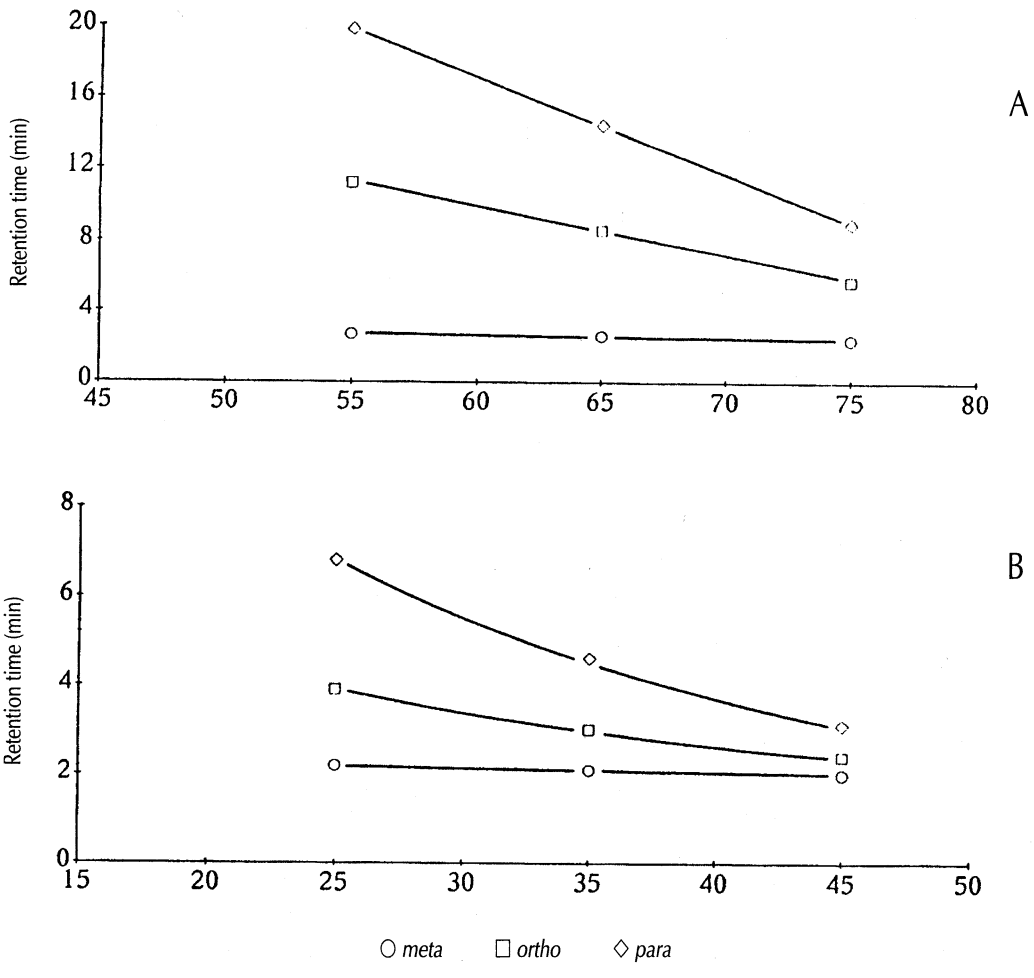


Figure 7. Variation of the retention time vs the concentration of methanol (A) and acetonitrile (B) in the mobile phase. An SiPVAret—CD67 column was used.

ionization state. A potentiometric study gave a $pK_{1/2}$ value of approximately 8, which corresponds to half ionization (average value). Thus, PVA was fully charged at pH 5 and remained charged at pH 7. Nitrophenols were uncharged at pH 5 and did not interact strongly with the charged amino group. When the pH was increased, they became negatively charged and their interaction with the stationary phase increased strongly. Nitrobenzoic acid isomers are always fully ionized in the pH range 5–7 and the decrease of the k' values reflected only the decrease of the charge of the polymer. In our opinion, these interactions are more conclusive than differences in the affinities of β-CD for the neutral and anionic forms of the solutes.

Effect of the amount of β-CD

When the β-CD concentration increased, the retention time of each isomer increased, which corresponds to increasing interactions between the cavity of β-CD and the different substrates. This clearly indicates that the inclusion phenomenon plays the major role in the separation. This effect was more pronounced for the *para* isomer, which gave the more stable complex. The increase of the retention times was accompanied by broadening of the peaks. From these results, it appeared that a very low β-CD concentration (as low as 47 µmol/g) is enough to allow a good separation. Nevertheless, the use of columns with high β-CD concentrations allowed an increase in the proportion of organic solvent, which led to an increased lifetime of the columns.

Figure 9 shows the variation of the theoretical plate height versus the concentration of β-CD. The theoretical plate height passes through a minimum value around 15–20 µmol β-CD per gram (type I supports) and 30–35 µmol β-CD per gram (type II supports). This minimum value in the variation of theoretical plate height was also observed for PEI-based supports (21) but we have no explanation for this.

Comparison of the curves in Figures 9A and 9B for the two types of supports shows that the value of the theoretical plate height for type II is about 2 times smaller than that for type I. Thus, a better efficiency in the separation was obtained when the functionalization step by CD is the last step of synthesis. The NMR study in Part I of this work has shown that the mobility of both the polymer chain and the CD cavity differ strongly between the two types of supports. It seems that a lower mobility leads to a better chromatographic efficiency.

In addition, for substrates such as nitrophenols, the peaks of

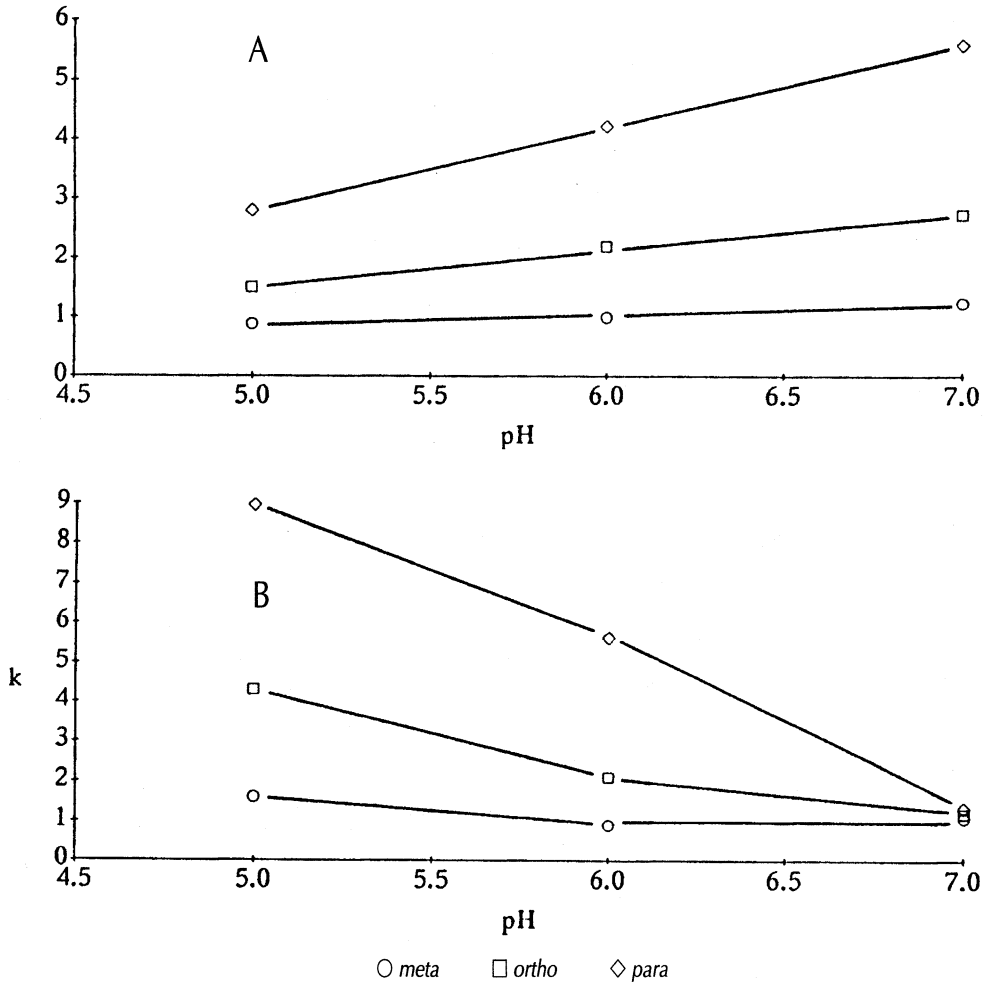


Figure 8. Effect of the pH of the mobile phase on the capacity factor: A, nitrophenol isomers (acetonitrile–phosphate buffer [15:85]); B, nitrobenzoic acid isomers (100% phosphate buffer) (SiPVA-β-CDret34 column).

the three isomers are more symetrical when type II supports were used. Nevertheless, type I supports allowed the resolution of compounds that were not separated with type II supports.

Separation of other compounds

Figure 10 shows another example of separation with the Si PVAret-β-CD95 column. A mixture of caffeine and theophyllin was well-resolved by using pure water as the mobile phase. When a reversed-phase C_{18} column was used, a much more complex mobile phase had to be used (91:4:4:1 water–2-propanol–acetonitrile–acetic acid).

Figure 11 shows the chromatograms obtained for several drugs on an SiPVA-β-CD15ret column. When a type II support was used, all these drugs eluted at the void volume of the column.

Figure 12 shows that the relationship between the column length and the number of theoretical plates is linear.

Conclusion

The stationary phases described may be used for the resolution of geometric isomers of disubstituted benzenes (e.g., nitrophenol, nitrobenzoic acid, cresol) and other molecules such

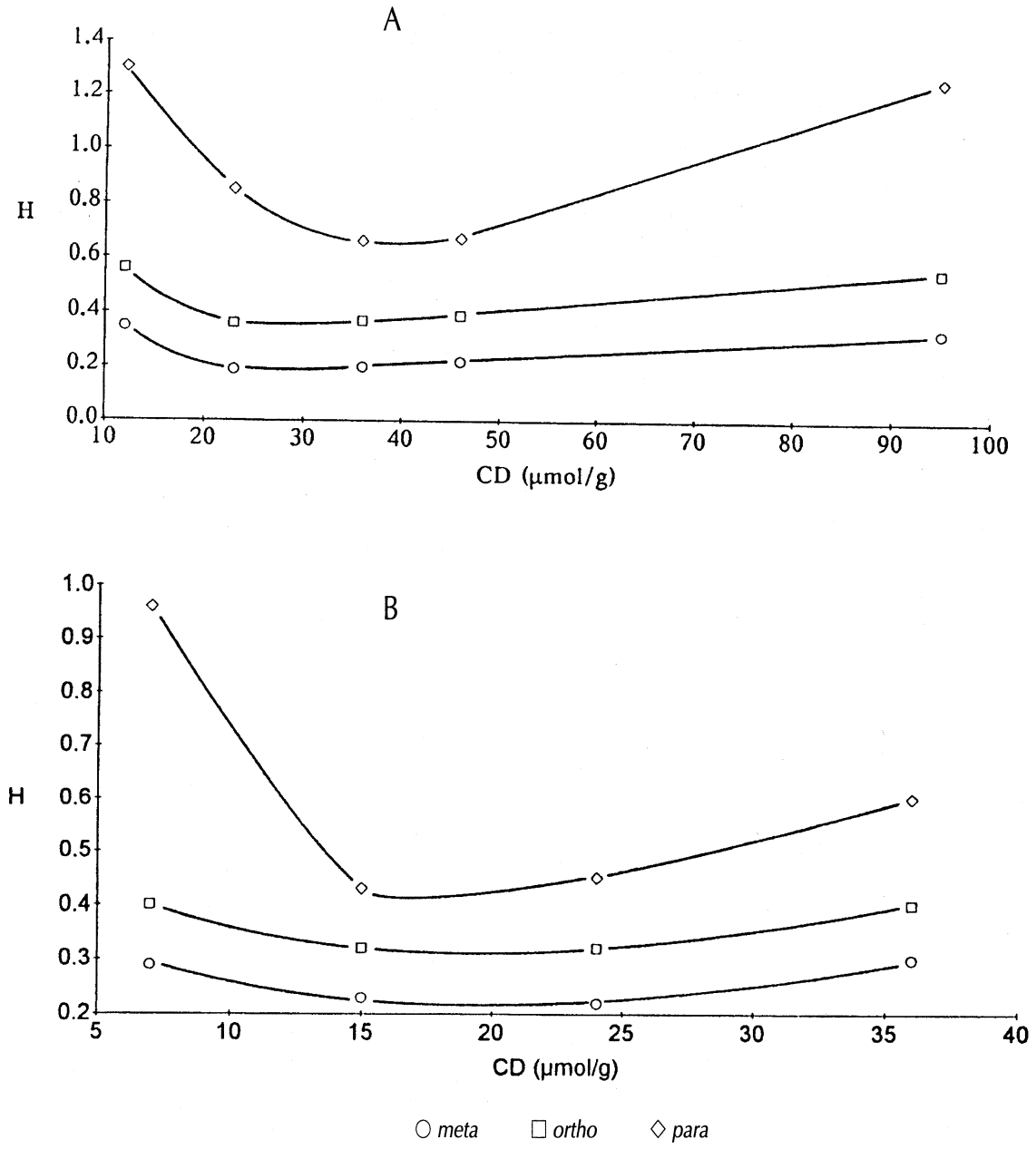


Figure 9. Effect of the concentration of β-CD on the efficiency of the stationary phase (separation of nitrophenol isomers). H is the theoretical plate height. Type II supports (A) and type I supports (B) were used. The mobile phase was H_2O–MeOH (75:25).

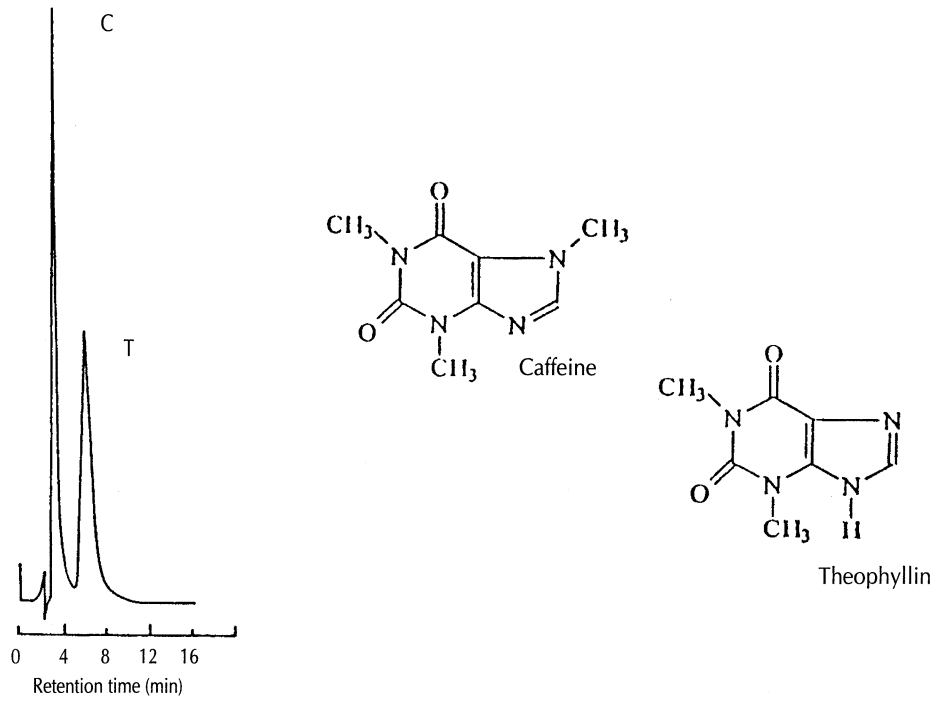


Figure 10. Liquid chromatogram of a mixture of caffeine (C) and theophyllin (T) on an SiPVA-βCD12ret column. The mobile phase was 100% H_2O.

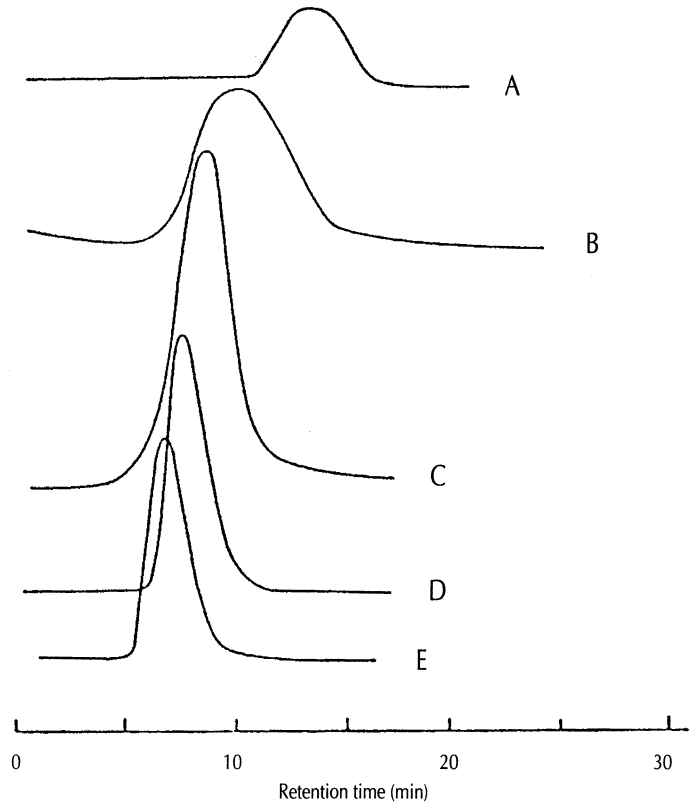


Figure 11. Liquid chromatogram of propanolol (A), warfarin (B), indomethacin (C), furosemide (D), and phenylbutazone (E) on an SiPVA—CD24ret column. The mobile phase was 0.01M phosphate buffer (pH 7).

as caffeine, theophyllin, and indomethacin. The retention mechanism is based mainly on the formation of inclusion complexes, and additional effects (such as acid–base interactions between the substrates and the polymer layer and hydrophobic interactions) contribute to retention. Some columns have been used for months under different pH and eluent conditions without changes in the retention times if the polymer layer was cross-linked. The long time stability of these systems was excellent with the eluents used. This was due to the strength of the interaction between the amino polymer and the silica surface and also to the reticulation of the coated layer, which prevented the loss of polymer even for long time use.

The separation properties of the chromatographic phases depended on the way in which they were functionalized with CD. It seems clear that the separation ability depends on the more or less high mobility of the coating layer. Studies are in progress in this direction.

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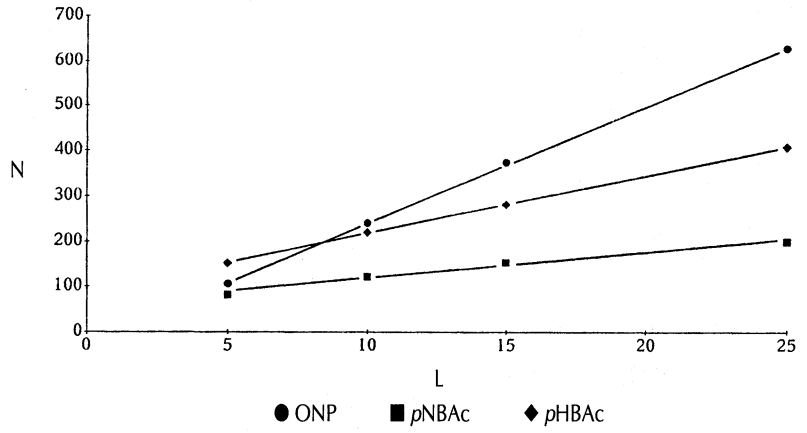

Figure 12. Effect of the column length (L) on the number of theoretical plates (N). An SiPVA-β-CD15ret column was used. The mobile phase was H_2O–CH_3CN (75:25). Substrates: *o*-nitrophenol (*o*NP), *p*-nitrobenzoïc acid (*p*NBAc), and *p*-hydroxybenzoïc acid (*p*HBAc).

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