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# RETENTION MECHANISM OF NUCLEOTIDES, NUCLEOSIDES AND THEIR BASES ON POLYVINYL ALCOHOL

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## SUMMARY

The basic chromatographic properties of a polyvinyl alcohol (PVA) column material were investigated. By pH titration, it was found that the PVA gel surface was negatively charged. The chromatographic behaviour of nucleotides on the gel was compared with that on an ODS column and found to be similar. However, the behaviour of nucleosides and bases were quite different from that on the ODS column. Since the retention characteristics of both types of solutes on the PVA column could be explained reasonably by the solvophobic theory, this result may suggest that the behaviour of these solutes on the ODS column is unusual. The difference in retention between AMP isomers and cAMP can be explained by the differences in their conformations.

## INTRODUCTION

In order to detect and identify molecules of biological importance in living bodies, various types of chromatography have been utilized. The columns used most frequently in size exclusion and reversed-phase chromatographies are silica-based. Since the effect of the remaining free silanol groups is known to be undesirable for some compounds[1-3], the development of other matrix-based columns has been extensive. However, except for a few columns, these columns had undesirable properties such as strong interactions with the samples. Therefore, it is extremely important to know the basic chromatographic characteristics of newly developed columns, particularly when the columns are based on polymer gels because their characteristics are highly dependent on the matrix. For instance, a number of carboxyl groups are known to remain on polymethyl methacrylate gel. Therefore, ionic interactions can occur between functional groups on the gel matrix and those of the solutes.

Polyvinyl alcohol (PVA) gel is a new type of column packing material introduced independently by Showa Denko and then Asahi Kasei, and was previously employed for size exclusion chromatography. However, the basic properties of the column have not yet been fully investigated. It has been demonstrated[4-6] that the retention mode of PVA columns for large molecules such as carbohydrates, polyeth-

ylene glycols[4] and proteins[6] is size exclusion, while for small molecules such as fatty acids, peptides with molecular weights less than 500, nucleotides, etc., the retention mode is similar to that of reversed-phase chromatography[5]. However, a determination of the detailed fundamental retention mechanism could not be made, although there was a possibility that the gel surface was slightly charged. Therefore, in order to reveal the overall retention mechanism of PVA columns, each chromatographic factor should be identified and studied in more detail. Major factors to be investigated first are the effects of the hydrophobic properties and charge of the surface of the PVA gel.

In reversed-phase chromatography the retention mechanism can be explained largely by the solvophobic theory[7,8] which describes how the hydropohobic interaction between the hydrophobic surface of the gel and the solutes, in the aqueous phase, can be produced. However, the retention behaviour of nucleotides, nucleosides and their bases is exceptional[9-11]. For instance, a nucleoside is retained more strongly than its base on an ODS column, although the former has higher hydrophilicity due to the hydroxyl groups in the ribose ring compared to the latter. The unusual behaviour of these two series of compounds has not been explained unambiguously.

In the present study, therefore, in order to demonstrate that the solvophobic theory cannot also be used to explain the behaviour of solutes on ODS columns, the chromatography of nucleotides, nucleosides and their bases on the PVA packing material was investigated in detail and the results obtained compared to those on ODS columns.

EXPERIMENTAL

The nucleotides, nucleosides and their bases employed as samples are listed in Table I. They were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Kohjin Co. (Tokyo, Japan) and Yamasa (Chiba, Japan). Other chemicals were obtained from Nakarai Chemicals (Kyoto, Japan). Water was purified by a MILI R/Q water purifier (Millipore, Bedford, MA, U.S.A.).

An HLC-803 high-speed liquid chromatograph (Toyo Soda, Japan) equipped with an UVIDEC 100 UV spectrometer (Jasco, Japan) was employed. The column effluents were monitored at 260 nm. The Asahipak GS-320 column (50 cm × 7.6 mm I.D.) containing 9-$\mu$m microparticulate polyvinyl alcohol gel was obtained from Asahi Chemical Ind. Co. (Tokyo, Japan). A pH titration was performed by use of a Horiba digital pH meter F-7$_{LC}$ (Kyoto, Japan).

RESULTS AND DISCUSSION

Previous papers described the highly complicated retention mechanism of peptides on the PVA column[5]. For example, the increase in the retention of the peptides caused by the addition of a salt indicated the occurrence of hydrophobic interactions between the solutes and the gel matrix. However, the retention volumes of the same substrates were found to be small for hydrophobic interaction and also were small even when they had been separated by size exclusion chromatography. This suggested the coexistence of a repulsion effect due to the charges on the solutes and the gel matrix.

TABLE I

COMPOUNDS USED AND THEIR ABBREVIATIONS

A = Sigma Chemical Company; B = Kohjin; C = Yamasa.

| Compound | Abbreviation | Source |
|---|---|---|
| Adenine | Ade | B |
| Adenosine | Ado | B |
| Adenosine 5'-phosphate | (5'-)AMP | B |
| Adenosine 2'-phosphate | 2'-AMP | A |
| Adenosine 3'-phosphate | 3'-AMP | C |
| Adenosine 5'-diphosphate | ADP | C |
| Adenosine 5'-triphosphate | ATP | C |
| Adenosine 3',5'-cyclic phosphate | cAMP | B |
| Guanine | Gua | B |
| Guanosine | Guo | B |
| Guanosine 5'-phosphate | GMP | B |
| Guanosine 5'-diphospohate | GDP | A |
| Guanosine 5'-triphosphate | GTP | C |
| Cytosine | Cyt | B |
| Cytidine | Cyd | B |
| Cytidine 5'-phosphate | CMP | A |
| Cytidine 5'-diphosphate | CDP | C |
| Cytidine 5'-triphosphate | CTP | A |
| Uracil | Ura | B |
| Uridine | Urd | B |
| Uridine 5'-phosphate | UMP | A |
| Uridine 5'-diphosphate | UDP | A |
| Uridine 5'-triphosphate | UTP | A |
| Thymine | Thy | B |
| Thymidine | Thd | C |
| Thymidine 5'-phosphate | TMP | B |
| Thymidine 5'-diphosphate | TDP | A |
| Thymidine 5'-triphosphate | TTP | A |

First, therefore, in order to explore the dissociative characteristic of the PVA gel surface, a pH titration was carried out on a suspension of the gel. A 1.0-g amount of dried PVA gel was suspended in 50 ml of water and the suspension was titrated with 0.005 $M$ hydrochloric acid. Pure water was used as a reference. Prior to the experiment both the samples were adjusted to pH 11.30 by addition of 0.1 $M$ sodium hydroxide. During the titration the samples were stirred continuously with a magnetic stirrer. The results are presented in Fig. 1. Below pH 7.0, the titration curve obtained for the suspension of PVA gel was found to deviate from that for pure water, implying that there are dissociable groups on the PVA gel surface, although the amount of such groups is quite small. The $pK_a$ value of the charged group lies between 4 and 5.

The effect of the surface charge was then investigated, using pyrimidine and purine compounds as samples. In reversed-phase chromatography, the $pK_a$ values of these substrates are known to be an important factor for the retention. Therefore, experiments were carried out by changing the pH of the eluents.

First, 5'-monophosphate nucleotides (AMP, GMP, UMP, CMP and TMP)
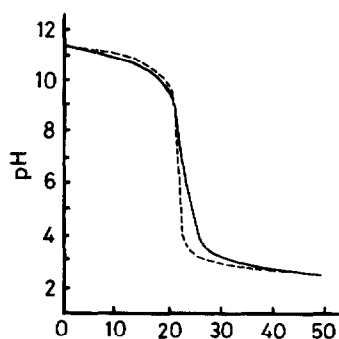
Titration Volume of 0.005 N HCl (ml)

Fig. 1. Titration curves for a suspension (1 g per 50 ml) of PVA gel (—) and for pure water (50 ml) (—
–). Before the pH titration the suspension and pure water were adjusted to pH 11.30 by addition of 0.1
$M$ hydrochloric acid.

were studied. The change in the elution volumes with pH is shown in Fig. 2. It was
found that purine nucleotides were eluted more slowly than pyrimidine nucleotides,
except that TMP was eluted later than AMP at pH 3.0. The largest elution volumes
for 5′-monophosphate nucleotides other than 5′-AMP were obtained at pH 3.0 and
the retention decreased with increasing pH of the eluent; the largest elution volume
of 5′-AMP was obtained at pH 5.0 and the highest values for CMP occurred at both
pH 3.0 and 5.0. These results can be explained in terms of the structural changes in
the ionogenic groups of the substrates upon changing the pH. In the case of 5′-
monophosphate nucleotides other than AMP and CMP, the extent of the dissociation
of phosphate groups led to a decrease in retention because the purine ring of GMP
(p$K_a$ for N-7 is 2.4) is uncharged at pH 3.0 and the two pyrimidine nucleotides (UMP
and TMP) do not have dissociable groups other than the phosphate group at this
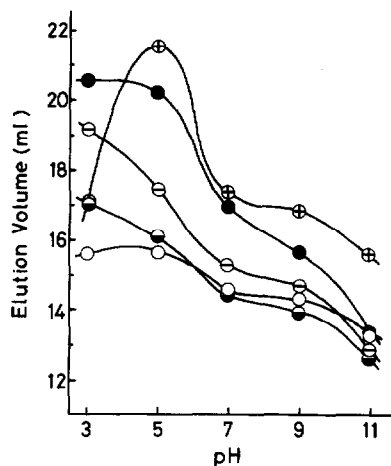pH. The nitrogen (N-1) of the adenyl ring of AMP (p$K_a$ 3.8) is positively charged at



Fig. 2. Effect of pH on the retention volume of monophosphate nucleotides: ⊕—⊕, AMP; ●—●, GMP;
⊖—⊖, TMP; ◓---◓ UMP; ○—○, CMP. Conditions: flow-rate, 1 ml/min; sample volume, 20 μl; detec-
tion, UV at 260 nm; temperature, ambient.

pH 3.0. Hence the solute surface may be covered largely with an aqueous phase because of the formation of its cationic form resulting from the protonation at N-1. At pH 5.0, the adenyl ring of AMP is uncharged. Since the decrease in the hydrophilicity of the adenyl ring caused by its neutralization can result in an increase in the hydrophobic interaction of the solute with the matrix, the retention volume of AMP at pH 5.0 is larger than that at pH 3.0. A similar trend in retention was observed for 5′-CMP. The pyrimidine ring of CMP ($pK_a$ for N-3 is 4.5) is positively charged at pH 3.0. Therefore, CMP cannot be retained strongly on the gel surface at this pH. The small retention of AMP and CMP at pH 3.0 implies that the ionic interaction between negative charge of the gel and positive charge of the solutes is not sufficiently large to compete with the rapid elution caused by the decrease in hydrophobicity due to the positive charge. This is evident because at pH 3.0 the gel surface is mostly neutralized ($pK_a$ 4–5).

Di- and triphosphate nucleotides were also chromatographed with eluents at different pH values. The retention volumes of these substrates are summarized in

TABLE II

EFFECT OF pH ON THE ELUTION VOLUMES (ml) OF NUCLEOTIDES, NUCLEOSIDES AND THEIR BASES

Conditions: flow-rate, 1 ml/min; detection, UV at 260 nm; sample volume, 20 μl; temperature, ambient.

| Compound | pH | | | | |
|---|---|---|---|---|---|
| | 3 | 5 | 7 | 9 | 11 |
| Adenine | 20.72 | 63.06 | 78.16 | 71.46 | 48.88 |
| Adenosine | 23.32 | 57.28 | 65.50 | 59.02 | 61.20 |
| AMP | 16.92 | 21.56 | 17.36 | 16.90 | 15.60 |
| ADP | 14.88 | 16.06 | 15.12 | 14.88 | 13.92 |
| ATP | 13.96 | 14.32 | 14.00 | 13.80 | 13.10 |
| Guanine | 26.82 | 48.48 | 47.28 | 41.44 | 25.02 |
| Guanosine | 34.10 | 37.16 | 39.84 | 31.84 | 24.08 |
| GMP | 20.56 | 20.26 | 16.96 | 15.70 | 13.44 |
| GDP | 16.40 | 15.96 | 15.10 | 14.24 | 12.78 |
| GTP | 14.96 | 14.40 | 14.14 | 13.48 | 12.36 |
| Cytosine | 16.28 | 20.00 | 22.22 | 21.96 | 23.20 |
| Cytidine | 16.56 | 21.26 | 22.36 | 23.58 | 21.64 |
| CMP | 15.58 | 15.66 | 14.52 | 14.36 | 13.40 |
| CDP | 14.34 | 13.80 | 13.60 | 13.22 | 12.12 |
| CTP | 13.58 | 12.98 | 13.04 | 12.88 | 12.20 |
| Uracil | 25.64 | 25.48 | 25.20 | 23.38 | 17.76 |
| Uridine | 23.38 | 23.72 | 24.58 | 21.00 | 15.86 |
| UMP | 17.08 | 16.08 | 14.40 | 13.98 | 12.60 |
| UDP | 14.80 | 13.92 | 13.60 | 13.22 | 12.12 |
| UTP | 13.86 | 13.08 | 13.12 | 12.70 | 11.84 |
| Thymine | 32.52 | 32.42 | 32.52 | 31.30 | 20.56 |
| Thymidine | 32.22 | 32.36 | 34.20 | 30.00 | 18.96 |
| TMP | 19.22 | 17.48 | 15.26 | 14.72 | 12.84 |
| TDP | 15.54 | 14.26 | 13.86 | 13.58 | 12.20 |
| TTP | 14.28 | 13.22 | 13.20 | 12.96 | 11.90 |

Table II. Triphosphate nucleotides were eluted more rapidly than diphosphate nucleotides and both series of solutes were eluted faster than monophosphate nucleotides. This is because of the larger number of phosphate groups in the samples which can influence the hydrophilicity and solubility of the solutes. The retention orders of the substrates in each series of nucleotides were analogous to those of the monophosphate nucleotides. These results are similar to these obtained for the nucleotides on an ODS column[9,12,13].

Analogous experiments were carried out for nucleosides and their bases. The results are also listed in Table II. All the elution volumes obtained at each pH were large compared to those of the nucleotides. This is because of the lack of the highly hydrophilic phosphate moiety in these substrates and because increased hydrophobicity leads to a decrease in the solubility of the solute and to longer retention. The overall retention order of the substrates at pH 3.0 can be explained as follows. The guanyl ring of Guo ($pK_a$ for N-7 is 1.6) is uncharged at this pH. Therefore, the solutes have substantial hydrophobic interaction between the purine ring and the gel. Since the hydrophobicity of Thy is considered to be larger than that of Ura because of the methyl group on the ring, Thy and Thd were retained more strongly than Ura and Urd, respectively. Thd and Urd have a ribose ring which can result in an increased solubility of these solutes. Therefore, the nucleosides were eluted more rapidly than the bases. Half of the nitrogen (N-7) of Gua ($pK_a$ for N-7 is 3.2) is positively charged at pH 3.0. Therefore, the solubility of this solute is still relatively high at this pH. For this reason, Gua was eluted before Guo, Thd and Thy. Probably, because of the weak hydrophobicity of the pyrimidine ring, Ura and Urd were eluted before Gua. The purine rings of Ade and Ado have $pK_a$ values of 4.15 and 3.5 respectively for N-1 and are mainly in the ionic form at pH 3.0. Therefore, these two compounds were eluted more rapidly than the above six compounds and their elution order, Ade and Ado, was a function of the extent of the adenyl ring being positively charged at N-1. This means that the effect of the charge on the decrease in retention is larger than that of the hydrophilic ribose ring. Cyt and Cyd ($pK_a$ values for N-3 are 4.5 and 4.15, respectively) were eluted most rapidly because they were chiefly in the positively charged form and because of the weak hydrophobicity of the ring.

Also, it was found that the elution order of the nucleosides at various pH values is dependent on the order of the bases at the same pH, which is analogous to that for the nucleotides. Also, a characteristic retention order was observed between each nucleoside and its base. At pH 3.0, the orders for each pair were Ade < Ado, Gua < Guo, Cyt < Cyd, Ura > Urd and Thy > Tyd, and at pH 7.0, Ade > Ado, Gua > Guo, Cyt < Cyd, Ura > Urd and Thy < Thd. On the other hand, in a previous study of the same pairs on ODS columns, different elution orders were obtained[9-14]. Especially, at pH 7.0, the orders were completely opposite to those on PVA columns. This extremely characteristic elution behaviour of solutes on the PVA column at pH 7.0 is caused by reversed-phase retention. Since the retention order of the solutes on the PVA gel can be explained reasonably as mentioned above, the order on ODS columns is rather unusual. At pH 7.0, part of the surface of the PVA column is covered with negative charges, which may explain the difference in behaviour from that of the free silanol groups of the ODS column.

The effect of the charged groups on the retention of ionic compounds was also explored for samples such as AMP isomers (5'-, 3'- and 2'-AMP) and 3',5'-cyclic

TABLE III

EFFECT OF pH ON THE ELUTION VOLUMES (ml) OF cAMP AND AMP ISOMERS

Conditions as in Table II.

| Compound | pH | | |
|---|---|---|---|
| | 3.0 | 5.0 | 7.0 |
| 5'-AMP | 16.80 | 23.86 | 19.42 |
| 2'-AMP | 17.36 | 24.64 | 20.44 |
| 3'-AMP | 18.34 | 29.42 | 22.24 |
| cAMP | 21.06 | 43.38 | 49.56 |

AMP (cAMP). These substrates were chromatographed at pH 3.0, 5.0 and 7.0. There were two notable differences in the retention between cAMP and the isomers and between the isomers as shown in Table III. cAMP always had a retention larger than those of the three isomers in the pH range applied. Also, the difference in the retention between cAMP and the isomers was much larger at pH 5.0 and 7.0 compared to that at pH 3.0. The conformation of cAMP and the three isomers are illustrated in Fig. 3. In cAMP, the phosphate group is fixed on the ribose ring by the two ester bonds and therefore has little contact with the adenyl ring which has a positive charge at N-1. On the other hand, the phosphate groups of the three isomers are attached to ribose rings through only one ester bond and can rotate relatively freely. For all the four samples, the adenyl rings are considered to participate in hydrophobic interactions with the gel matrix, while the phosphate groups interrupt these interaction through their hydrophilicity, which increases the solubility of the solutes. Also repulsive force arising from the negative charges on the solutes and the gel matrix may decrease the retention. Thus, cAMP, in which hydrophilicity of the phosphate group can hardly affect the hydrophobic influence of the adenyl ring because of the long distance between these groups, has larger retention than the isomers.

The combination of the charges on the phosphate group and on N-1 of the adenyl ring can also contribute to the retention mechanism. Since the phospohate moiety of cAMP is attached to the ribose ring by two ester bonds, it can only have charge of $-1$ at pH 3.0, 5.0 and 7.0 (p$K_a$ of cAMP phosphate is ca. 2). On the other hand, the phosphate groups of the three isomers have a charge of $-1$ at pH 3.0 and 5.0 (p$K_a$ of primary dissociation is less than 1) and $-2$ at pH 7.0 (p$K_a$ for the secondary dissociation is ca. 6) and can rotate so freely that they can interact with the
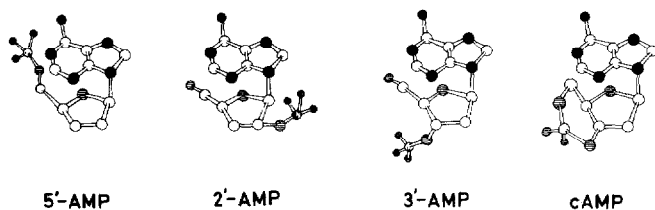


Fig. 3. Conformations of cAMP and the AMP isomers. $\bigcirc$ = Carbon, $\bullet$ = nitrogen, $\ominus$ = oxygen and $\oslash$ = phosphorus.

positively charged adenyl rings. The extent of the interaction of the AMP isomers with the gel matrix is strongly dependent on the position of these freely rotating phosphate groups. At pH 3.0, the adenyl rings of cAMP and the isomers have a charge of $+1$ at N-1 (the $pK_a$ value for N-1 is 3.7–3.8). Therefore, the hydrophobic interaction of the solutes with the gel matrix caused by the adenyl ring is weakened by the hydrophilic interference of the positive charge at N-1 of the adenyl rings. The solubility increase caused by the charge at N-1 decreases the retention of all the solutes on the PVA column and results in a decrease in the difference in retention between cAMP and the three isomers. In this case, the interaction between the positive charge of the adenyl ring and the negative charge of the gel matrix cannot compete with the hydrophilic effect because the extent of the negative charge on the gel is small at pH 3.0. Therefore, at pH 3.0, all the four solutes had smaller retentions than at pH 5.0 and 7.0 and the difference in retention between cAMP and the isomers was not large. At higher pH (5.0 and 7.0), the retention of all the solutes was larger than that at pH 3.0. At these pH values, the nitrogen (N-1) of the adenyl rings of cAMP and the isomers is now uncharged. The hydrophobic interaction between the adenyl ring and the gel matrix is strengthened by the increase in hydrophobicity of the ring in its neutral form. Also, particularly at pH 7.0, a large difference in retention between cAMP and the AMP isomers was observed compared to the difference at pH 3.0. This can be explained by considering the charges and the conformational characteristics of the solutes. At pH 7.0 the secondary dissociation of the phosphate groups of all the AMP isomers has taken place. Therefore, the charge on the phosphate moiety of the AMP isomers is $-2$, while cAMP still has a charge of $-1$ on the phosphate moiety. The presence of the additional negative charge on the phosphate groups increases the hydrophilicity and solubility of the samples, weakening the interaction of the adenyl ring with the gel matrix and resulting in lower retention. On the other hand, for cAMP there the negative charge on the phosphate moiety is $-1$ at the three pH values. Therefore, the hydrophilicity of cAMP arising from the phosphate group is unchanged at these pH values while the hydrophobicity is much increased by neutralizing the adenyl ring.

Next, the dissociation of the gel matrix was considered. Because the $pK_a$ value for this dissociation was determined to be 4–5, the surface of the gel is considered to be negatively charged at pH 5.0 and 7.0, while at pH 3.0 it is neutral. Therefore, at pH 5.0 and 7.0, there is a repulsive force between the negatively charged solutes and the gel surface. This force may be higher for the AMP isomers than cAMP because of the larger number of negative charges on the isomers. For the above reasons, the retention of cAMP increased much more than that of the AMP isomers upon raising the pH of the eluent. Consequently, at pH 5.0, the difference in the extent of the repulsive force between the negative charges on the gel surface and on the phosphate group and in the extent of the hydrophilic interference of the phosphate group with the hydrophobic interaction of adenyl ring results in the difference in the retention volume between cAMP and the AMP isomers. In addition to the above two differences which governed retention of the solute, at pH 7.0, secondary dissociation of the phosphate groups of the isomers was involved.

The difference in retention between the three isomers can also be explained as follows. Since the structure of the adenyl ring and the phosphate group is not substantially different between the isomers at the three pH values, almost equal retention

is expected for these solutes at each pH. As seen in Table III, however, the retention order of these substrates as always 5'-, 2'- and 3'-AMP. Therefore, a slight difference in the overall structure of the solutes may be used to account for the difference in the retention. As shown in Fig. 3, of the isomers, the phosphate group of 5'-AMP is nearest to the adenyl ring. In the case of 3'-AMP, the phosphate moiety is kept away from the aenyl ring because its rotation is restricted by the hydroxymethyl group of the ribose ring. The accessibility of the phosphate group of 2'-AMP to the adenyl ring can be considered to represent intermediate between these two cases. Thus, the differences in the extent of the hydrophilicity of the adenyl ring, arising out of the accessibility of the phosphate group, can explain the elution order of 5'-, 2'- and 3'-AMP.

The largest elution volumes obtained for all the AMP isomers at pH 5.0 can be explained by the increase in hydrophobicity of the adenyl ring due to the formation of the neutral form of the adenyl ring (at N-1), as described in the explanation of the retention of 5'-AMP. In the case of cAMP, since N-1 of the adenyl ring is uncharged and secondary dissociation of phosphate group does not take place at pH > 5.0, the elution volume increased with increasing pH. The trend in the elution order obtained on the PVA column is similar to that on ODS.

In summary, the negative charge formed on the PVA gel surface had little effect on the overall retention of the solutes studied. The effect of the charge on the solute retention was also quite different from that observed on ODS columns. For substrates having no phosphate groups, nucleosides and their bases, this negative charge gave rise to an elution order which can be explained in terms of the solvo-phobic theory, while the order obtained on ODS columns is not readily explained by this theory. The trend observed on the PVA column for substrates having phosphate groups was found to be similar to that on ODS columns. Considering the above results, it may be concluded that the negative charge on the PVA matrix does not produce undesirable and incomprehensible interactions between the solutes and the gel, which have often been observed with ODS columns and cannot easily be explained.

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