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ORIGINAL

Evaluation of the Chromatographic Performance of Conventional, Polar-Endcapped and Calixarene-Bonded Stationary Phases for the Separation of Water-Soluble Vitamins

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Abstract The performance of four different reversed-phase columns which included a conventional C_{18} phase, a C_{18} polar-endcapped phase, an ether-linked phenyl polar-endcapped phase and a calixarene-bonded phase has been systematically compared for the separation of mixture of some water-soluble vitamins containing basic, neutral and acidic compounds of different polarities, as well as different functional groups at three pH levels and different proportions of buffer/methanol. The characteristics of water-soluble vitamins make this combination of compounds very useful as a test mixture to check column performance with real samples. Due to the physical and chemical differences between these compounds, the type of chosen column has a significant influence on the chromatographic behavior. Results of this comparison show that the C_{18} polar-endcapped phase was the most suitable for the separation of this group of vitamins. The presence of a polar group as an endcapping agent does not seem to influence the overall hydrophobic nature of the polar-endcapped stationary phases. At the same time, these phases displayed enhanced hydrogen bonding and silanol activity.

Keywords Reversed-phase chromatography · Calixarene-bonded phases · Polar-endcapped phases · Water-soluble vitamins

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Introduction

The most widely used mode in high-performance liquid chromatography (HPLC) is by far reversed-phase chromatography (RPC), based on the broad applicability of that mode of separation to a wide range of compounds and sample matrices. One advantage of RPC over other HPLC techniques such as normal-phase chromatography (NPC) or hydrophilic interaction liquid chromatography (HILIC), which is becoming a well-accepted alternative to reversed-phase chromatography for polar analytes, ion-exchange chromatography (IEC) or size exclusion chromatography (SEC), is the vast number of available stationary phases [1]. Due to its simplicity and better column performance, RP-C_{18} as stationary phase is usually the best starting point within the field of reversed-phase chromatography and remain the most widely used of all of the available stationary phases. A recent survey on HPLC columns shows clearly that C_{18} phases (octadecylsilane) were most popular followed by C_8 (octylsilane) [2]. In general, the retention time for C_8 columns is shorter compared to C_{18} columns under the same mobile phase conditions, so that almost all analytes are eluted faster on C_8 columns. C8 may be therefore more useful than C_{18} when shorter retention times are required, and reduced amount of solvents are needed (especially for more hydrophobic analytes). Despite the widespread popularity and acceptance of alkyl-bonded phases, there is a need, or at least an opportunity, for new column chemistries. Novel phases can provide an alternative and complementary separation for many analyses performed on C_{18} columns.

In recent years, significant improvements have been made in the quality of bonded silica particles used in HPLC. Consequently, lately there has been a dramatic increase in the number of improved reversed-phase

columns available to the chromatographer, including different functionalities. Polar-endcapped stationary phases are modifications of the classical C_{18} chemistry with the addition of a polar functional group used as an endcapping agent. These polar or hydrophilic endcapping chemicals allow the silica surface to be wetted with water and enable the full interaction with the longer alkyl chains, making the retention of polar analytes under highly aqueous conditions more reproducible. Perhaps due to the relatively recent introduction of these types of phases, there have been few studies that have attempted to critically evaluate or characterize their performance [1, 3, 4].

Phenyl-bonded phases have been utilized in reversed-phase HPLC for many years [5]. These phenyl phases have been reported to exhibit some π–π as well as steric recognition interactions [6]. An ether-linked phenyl phase could also maximize retention and selectivity for polar and aromatic analytes.

Calix[n]arenes, which are stable macrocyclic compounds composed of phenol units linked by methylene bridges at positions ortho to the hydroxyl groups, have also been found to be useful in HPLC when bonded to silica packing material. Calixarene stationary phases have several applications in LC. Meyer and Jira reviewed and summarized the application possibilities and interactions of calixarenes as a stationary phase in liquid chromatography [7]. These novel LC stationary phases provide different selectivity, which could be new tools to solve classical problems in pharmaceutical analysis [8–10].

These novel LC stationary phases, which could have an enhanced selectivity for some molecules, are providing new tools to solve classical problems in pharmaceutical analysis. One of these problems is the analysis of water-soluble vitamins.

During the last decade, there has been an increasing interest for the simultaneous determination of water-soluble vitamins. Thus, separation techniques such as capillary electrophoresis [11–13], micellar electrokinetic chromatography [14–16] and liquid chromatography [17–21] have been developed. RP-LC without ion-pair reagents [18, 19] has been applied. However, problems with low reproducibility in the retention time of some vitamins occurred. RP-LC with ion-pair reagent was also applied as mobile phase additives to allow successful separation of these polar substances on reversed-phase high-performance liquid chromatography columns, but the complex mobile phases associated with this technique make the column equilibration and the total run time of the analysis longer [17]. In addition, the simplified mobile phase lowers the cost of analysis and avoids interferences with the separation and problems with detection during gradient elution. LC column with amide-based stationary phase was also used but the total time of analysis remains long (45 min) [20].

These compounds present an analytical problem because of their different chromatographic behavior due to the physical and chemical differences between these compounds. On the other hand, though a lot of work has been done for quantitative analysis of the vitamins, the retention mechanism of these compounds still require further to be understood, especially, on different stationary phases.

All these characteristics of water-soluble vitamins make this combination of compounds very useful as a test mixture to check the chromatographic performance of different stationary phases with real samples. Since all test mixtures contain usually different substances such as uracil, benzene derivatives, phenol, benzylamine, naphthaline which are chemical substances and are not used in any pharmaceutical formulation. The selected water-soluble vitamins are, however, not only a test mixture, due to their chemical characteristics, but they are also usually contained in many pharmaceutical formulations as different pharmaceutical preparations (capsules, tablets, syrups and injections) and have been selected as a real-life case study. It can also be tested how the stationary phases fit into the prevision, because the mixture contains not only acidic, neutral and basic compounds with very different polarities, but also different functional groups as can be observed in Table 1. Therefore, a comparison for the chromatographic performance among a group of columns containing a conventional C_{18} phase, a polar-endcapped phase, an ether-linked phenyl polar-endcapped phase and a calixarene-bonded phase for the separation of some water-soluble vitamins has been done. Figure 1 shows the structures of the different stationary phases.

Materials and Methods

Reagents and Chemicals

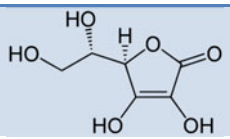
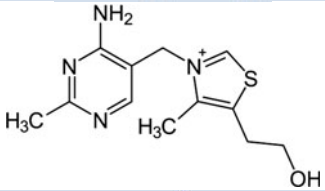
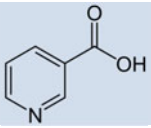
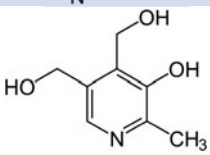
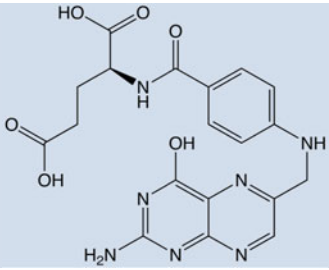
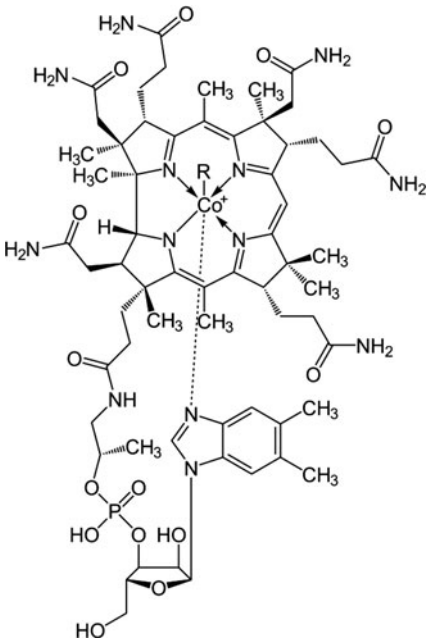
Vitamins B_1, B_6, B_{12}, B_3 (nicotinic acid), C (ascorbic acid) and B_9 (folic acid) of analytical grade were purchased from Sigma-Aldrich (Steinheim, Germany). Phosphoric acid (85 %), potassium hydroxide (85 %) and potassium dihydrogen phosphate (99.5 %) were obtained from Merck (Darmstadt, Germany).

HPLC gradient grade methanol was purchased from Acros Organics (NJ, USA). Water was obtained by bi-distillation.

Standard Solution

Individual standards used for retention time determination were daily prepared by weighing the appropriate amount of standard, in the range of 0.5–10 mg, using a microbalance and then dissolving in 10 mL of water. Folic acid was dissolved in an aqueous solution of 1 M $NaHCO_3$.

Table 1 Names and structures of the used water-soluble vitamins

Generic name	Chemical name	Structure
Vitamin C	Ascorbic acid	
Vitamin B_1	Thiamine	
Vitamin B_3	Nicotinic acid	
Vitamin B_6	Pyridoxine	
Vitamin B_9	Folic acid	
Vitamin B_{12}	Cyanocobalamin	

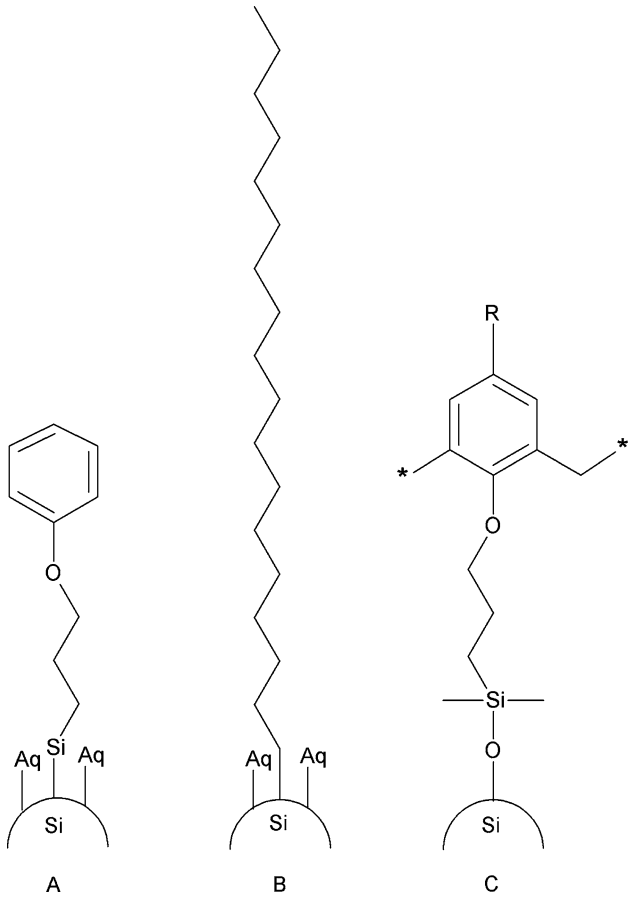


Fig. 1 Representative structures for stationary phases **a** ether-linked phenyl polar-endcapped phase, **b** C18 polar-endcapped phase, **c** calixarene-bonded phase, where R=H or $(CH_3)_3C$ in a ratio of 1:1

Equipment

Chromatography was performed on HP 1090 series II (Hewlett Packard, Waldbronn, Germany) equipped with diode array detectors (Agilent Technologies, Waldbronn, Germany).

The pH value of the solutions was adjusted with a Knick pH meter (Berlin, Germany).

Columns

The columns used were as follows: Thermo Scientific BDS Hypersil® C_{18} (100 × 4.6 mm, 2.4 μm) pore size 120 Å, Phenomenex Synergi polar-RP® (150 × 4.6 mm, 4 μm) pore size 80 Å, YMC-Pack ODS-AQ (150 × 4.6 mm, 3 μm) pore size 120 Å, Caltrex® Science (125 × 4 mm, 5 μm) pore size 100 Å.

For Caltrex® Science column, the ligands were immobilized via hydrophobic spacers on endcapped silica (Kromasil Si 100, 5 μm, specific surface area/BET: 300 $m^2\ g^{-1}$, manufacturer: EKA Chemicals (Bohus, Sweden)). Calixarenes, which are modified with olefin-containing groups with linkers at the oxygen group via ether function, were utilized. In Caltrex® Science materials, a 50:50 w/w mixture of calix[4]arenes and *p-tert*-butylcalix[4]arenes was used for the modification of the silica gel. Thus, both selectivities of unmodified and modified calix[4]arenes are combined in one material. The Caltrex® Science column was kindly supplied by Syntrex GbR (Greifswald, Germany).

Chromatographic Conditions

The experiments were performed with isocratic elution. The binary mobile phase consisted of different proportions of methanol in the aqueous solution (see figures). The two components of mobile phase (aqueous and organic) were mixed 1st time inside the apparatus. All pH values were measured in the aqueous component of the mobile phase. Phosphoric acid and potassium hydroxide were used for pH adjustment. The eluents were degassed with helium gas before running. In all cases, the column temperature was set at 40 °C. The injection volume was 5 μL. Different flow rates are used to retain the back pressures for all columns under 200 bar. For the Synergi polar and the Caltrex Science, there was no problem to work with flow rate of 1 mL min^{-1}. However, for the BDS Hypersil and the YMC-Pack ODS-AQ, the small particle size made it necessary to work with lower flow rates. The flow rates were as follows: 0.7 mL min^{-1} for the conventional C_{18} phase, 0.8 mL min^{-1} for the C_{18} polar-endcapped phase and 1 mL min^{-1} for the ether-linked phenyl polar-endcapped phase and the calixarene-bonded phase. The dead times (t_0) were determined via methanol. Detection was achieved at 254 nm.

Results and Discussion

A mobile phase containing potassium dihydrogen phosphate as buffering compound is suitable to assure a good chromatographic separation of water-soluble vitamins. The pH values of mobile phases were adjusted by addition of potassium hydroxide or phosphoric acid. Methanol is used as the organic modifier in the mobile phase, as the achieved separation of the test mixture on all phases was superior to that with acetonitrile as an organic modifier. This could be interpreted based on two facts. First, using acetonitrile for low retained polar compound (also in low percentage) will decrease the retention and lead also in some cases to non-reproducible retention times. On the other hand, methanol is preferred to be used as an organic modifier in the case of aromatic RP phase, since acetonitrile suppresses π–π interactions between the solute and the aromatic π–π active moiety of the stationary phase, as it is the case for aromatic RP phases (calixarene- and ether-linked phenyl-bonded

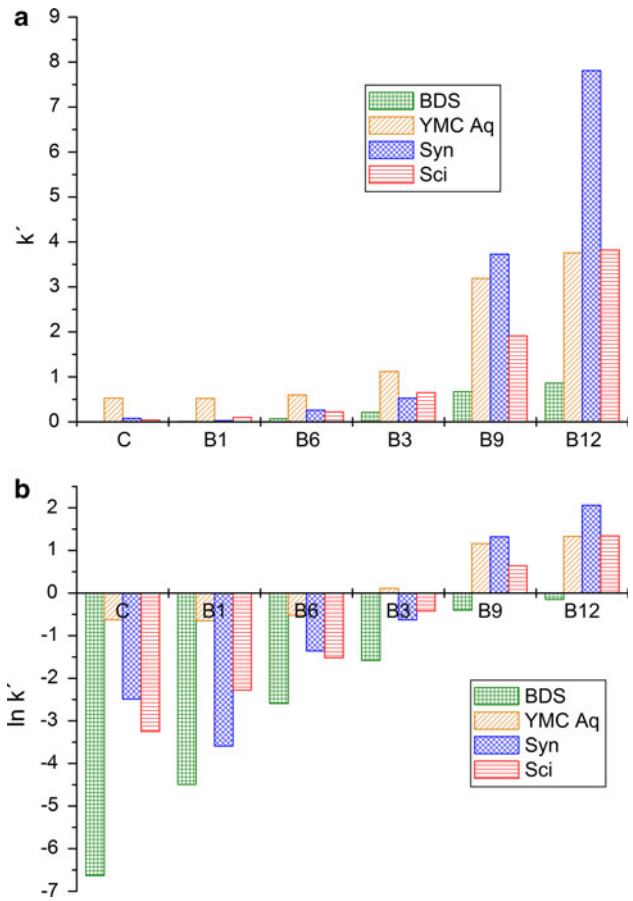


Fig. 2 Comparison of retention between the tested phases (pH 3) Conditions: Mob. phase: 25 mM potassium dihydrogen phosphate/methanol, 75/25 (v/v). Stat. phases: BDS (Thermo Scientific BDS Hypersil® C_{18}), YMC Aq (YMC-Pack ODS-AQ), Syn (Phenomenex Synergi polar-RP®), Sci (Caltrex® Science). Vitamins: C, B_1, B_6, B_{12}, B_3, B_9

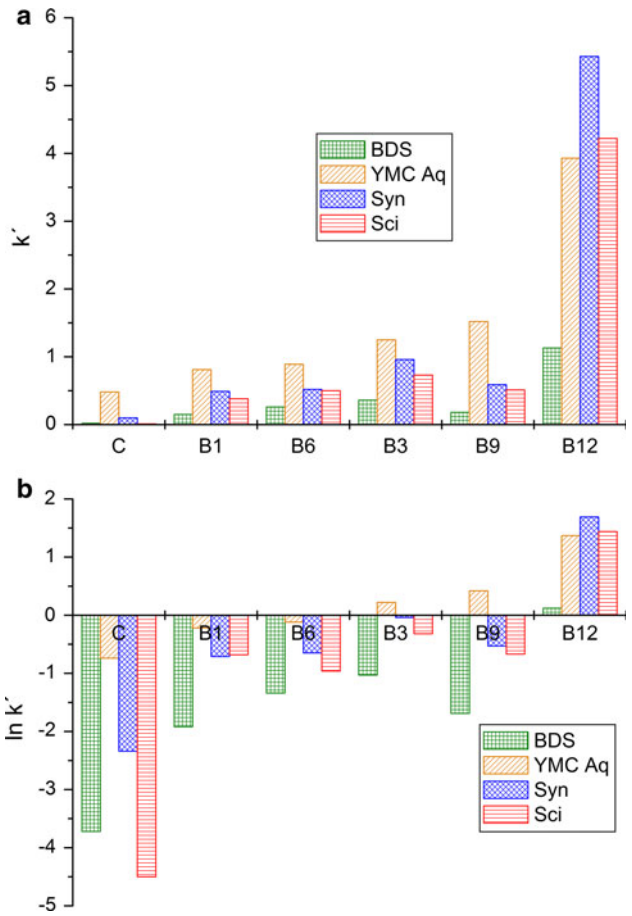


Fig. 3 Comparison of retention between the tested phases (pH 4.2) Conditions: see Fig. 2

stationary phases). Moreover, Xu et al. [22] showed that *p-tert*-butylcalix[4]arene forms a 1:1 inclusion compound with acetonitrile as guest.

Chromatographic performance of tested columns was studied at three pH levels (3.0, 4.2 and 6.0) and different proportions of buffer/methanol. Some vitamins will be ionized or non-ionized based on the pH value of the mobile phase. It would be yet better to have a wider range. However, the Synergi RP-Polar column can be used only to pH of 7.0. The used three points should bracket the pKa values of most of the used water-soluble vitamins in this study and give as a result different retention behaviors as shown in the study.

The chromatographic conditions that give higher selectivity between the tested compounds and reasonable analysis time were in most cases 25 mM potassium dihydrogen phosphate/methanol, 75/25, (v/v), which are employed for the comparison. Results are shown in Figs. 2, 3 and 4.

Uracil typically is used as void volume marker because it is not retained with most reversed-phase columns. However, uracil was retained on the tested ether-linked phenyl polar-endcapped phase. Therefore, methanol was used to determine t_0 of the tested phases.

The pH value of the mobile phase is a major factor influencing the chromatographic behavior of water-soluble vitamins. Since these vitamins are very different in their chemical properties, their retention behavior is influenced by the presence of positively charged, negatively charged or neutral polar groups that can interact with polar binding sites of the stationary phase. Basic molecules (vitamins B_1 and B_6) and acidic molecules (vitamins C and B_9) are dramatically influenced even by small changes in the pH of the mobile phase. As the increase of pH in mobile phases, the retention values of vitamins C and B_9 decreased, the retention of B_1 and B_6 increased and the retention of B_3 and B_{12} slightly changed, which correspond to their different ionization properties, and the fact that the main retention mechanism in all phases is based more or less mainly on hydrophobic interaction. On the other hand, the

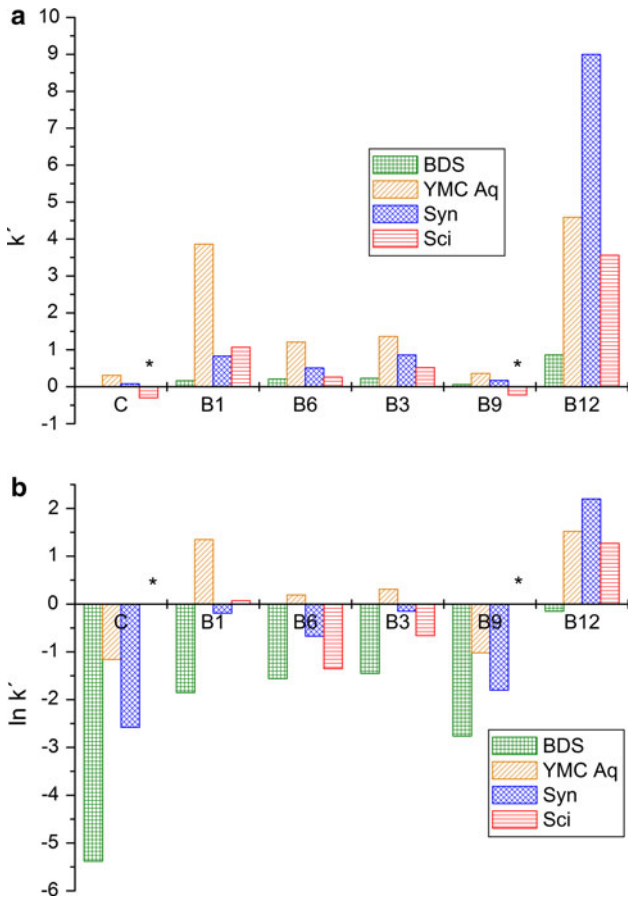


Fig. 4 Comparison of retention between the tested phases (pH 6). Conditions: see Fig. 2

retention values of B_3 and B_{12} were largely dependent on the methanol content in the mobile phases.

The reproducibility of the retention time for the water-soluble vitamins was tested with the injection of the standard solution of single analyte, to check the elution order of the vitamins in the mixture, for every new condition. The retention time deviation was not more than ±0.06 min. That reason could be that, the methanol proportion in the mobile phase was not less than 15 % in this study.

Chromatographic Behavior of Water-Soluble Vitamins at pH 3

Comparison of the capacity factors k′ and the ln k′ for the tested water-soluble vitamins under the low pH conditions shows very interesting trends (Fig. 2a, b). For basic vitamins (B_1 and B_6), the ODS polar-endcapped phase (YMC ODS-AQ) displayed significantly higher capacity factors than the other phases, especially for B_1. Presumably, the enhanced retention on the polar-endcapped phase is due to the relatively high silanol activity and hydrogen bonding capacity of these phases.

On the conventional C_{18} phase (BDS Hypersil), vitamin B_1 showed, as expected, no retention, what could be interpreted by the fact that the silica of this phase is based deactivated with minimal residual silanol activity. The retention of the basic vitamins on the ether-linked phenyl polar-endcapped phase (Synergi polar) was considerably less than on the ODS polar-endcapped phase, despite the polar-endcapping. A possible explanation for the low activity of these polar groups on the ether-linked phenyl phase in comparison with those on the ODS polar-endcapped phase may be that the ether-linked phenyl groups play a steric hindrance role and partially shield the polar groups on the silica. This explanation is also supported by more symmetrical peaks of B_1 and B_6 on ether-linked phenyl polar-endcapped phase (peak symmetry: $B_1 = 0.99$ and $B_6 = 1.31$) in comparison with those on the ODS polar-endcapped phase ($B_1 = 0.70$ and $B_6 = 0.52$). The symmetry of peaks was defined by a symmetry factor equal to one for absolutely symmetrical chromatographic peak. Additionally, the selectivity of polar-endcapped phases varies with the nature of the polar group. However, there is unfortunately no detailed information for the exact nature of these polar or hydrophilic endcapping reagents. The calixarene-bonded phase (Caltrex Science), which based on a conventional endcapped silica, retained B_1 more than the ether-linked phenyl polar-endcapped phase and B_6 almost the same (Fig. 2a, b). The reason for this is more likely to be due to the host–guest complexes between calixarene and analyte molecules, which are stabilized by hydrogen bonds, π–π and van der Waals interactions between the host and guest [23].

The retention behavior and the elution order of the other vitamins in the mixture at pH 3 are similar with two exceptions, which are as follows:

1. The retention and the overall analysis time of all vitamins on the conventional C_{18} phase (BDS Hypersil) are very short due to the use of sub 3 μm particles, which lead to fast elution. On the other hand, the use of "base deactivated silica" with silanols, which are less acidic and are less likely to be available for ion-exchange interaction with ionized basic analytes and are also less likely to hydrogen bond with polar analytes, minimizes the secondary interactions at this low pH.
2. Vitamin B_{12} is remarkably long retained on the ether-linked phenyl polar-endcapped phase (Fig. 2a, b). The reason could be the additional π–π interactions between the analyte and the phenyl functional group of the phase, and the hydrogen bonding interaction. As the structure of B_{12} contains seven free amido groups, which arranged in the out of its molecule, and these amido groups could easily form hydrogen bonds with

the ether group of the phase, a strong retention of B_{12} on the ether-linked phenyl polar-endcapped phase can be observed. On the ODS polar-endcapped phase and the calixarene-bonded phase, capacity factors of B_{12} are almost the same. The relatively small ring size of calix[4]arene and *p-tert*-butylcalix[4]arene makes it unfavorable to make a host–guest complex with a large molecule such as vitamin B_{12}. Favorable inclusion into *p-tert*-butylcalix[8]arene relative to calix[4]arenes might contribute to a better selectivity of silica gels with calixarenes of larger ring size, since the interaction of the B_{12} with calix[4]arene is hindered by the small ring size. These could explain the changes in selectivity by changes in ring size of the calixarenes [24].

Chromatographic Behavior of Water-Soluble Vitamins at pH 4.2

The retention behavior of water-soluble vitamins in this relatively weak acidic medium is noticeably different from that at pH 3 (Fig. 3a, b). Under these conditions, the best chromatographic separation of the water-soluble vitamins was obtained on the ODS phases (YMC ODS-AQ) and (BDS Hypersil). On the ODS polar-endcapped phase, well-separated symmetric peaks of the tested vitamins with exception of vitamins B_1 and B_6 were obtained when we applied the mixture of 25 mM potassium dihydrogen phosphate at pH 4.2 and 22 % methanol as a mobile phase (Fig. 5). This separation with a satisfactory retention of the mixture, as can be seen in Fig. 3 from the capacity factors, was achieved using isocratic mobile phase without using any ion-pair reagent. It is also remarkable that vitamin B_9 eluted after vitamin B_3 under these conditions only on this phase, while the other phases showed lower retention of vitamin B_9, which eluted before vitamin B_3. That could be interpreted with the additional interaction with the polar-endcapping groups of this phase. The same is noticed with vitamin C, which also much more retained on this phase compared to its almost "zero" retention on the other phases (Fig. 3a, b).

The obtained separation on the conventional C_{18} phase (BDS Hypersil) was relatively good despite the short retention times of all vitamins (Fig. 6). Moreover, the resolution between vitamins B_1 and B_6 on this phase was even better than on the ODS polar-endcapped phase (Table 2). This C_{18} phase "packed with 2.4 µm particles" gives always faster analysis. The advantage of using 2.4 µm particle size columns is that high speed and high efficiency separations are achievable using conventional HPLC systems. However, the overall resolution of vitamins was superior on the ODS polar-endcapped phase.

The behavior of the ether-linked phenyl polar-endcapped phase and the calixarene-bonded phase is also similar under

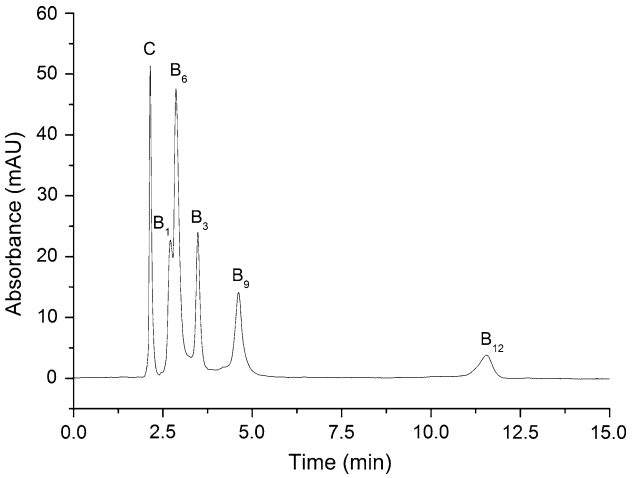


Fig. 5 Representative chromatogram for the separation of water-soluble vitamins Conditions: Stat. phase: C_{18} polar-endcapped phase (YMC-Pack ODS-AQ, 150 × 4.6 mm I.D., 3 µm). Mob. phase: 25 mM potassium dihydrogen phosphate/methanol, 78/22 (v/v). pH 4.2, flow rate 0.8 mL min^{-1}

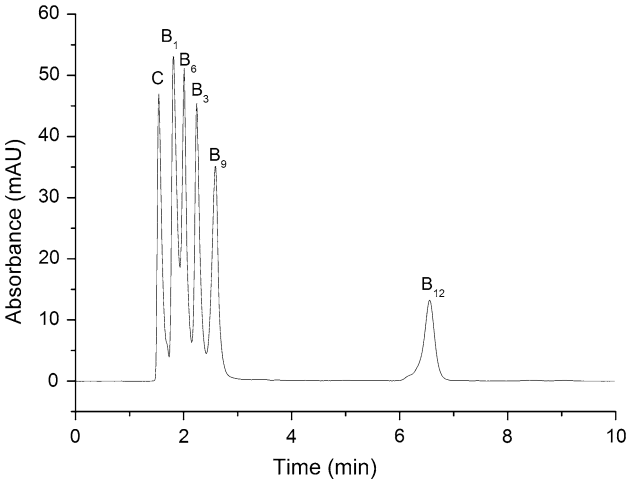


Fig. 6 Representative chromatogram for the separation of water-soluble vitamins Conditions: Stat. phase: C_{18} phase (Thermo Scientific BDS Hypersil® C_{18}, 100 × 4.6 mm I.D., 2.4 µm). Mob. phase: 25 mM potassium dihydrogen phosphate/methanol, 80/20 (v/v). pH 4.2, flow rate 0.7 mL min^{-1}

Table 2 Comparison of the selectivity and resolution factors of Figs. 5 and 6

	Figure 5		Figure 6	
	Selectivity α	Resolution Rs	Selectivity α	Resolution Rs
C				
B1	1.80	3.30	8.00	1.63
B6	1.13	0.66	1.71	1.25
B3	1.43	2.58	1.46	1.38
B9	1.56	3.97	1.49	1.82
B12	3.20	15.32	4.65	13.29

these conditions. Vitamins B_1 and B_6 were better retained on these phases compared to their retention at pH 3. However, vitamins B_1, B_6 and B_9 were almost coeluted, and thus, no separation of the mixture was possible.

The retention behavior of vitamin B_{12} is almost the same as at pH 3 on all phases due to the minimal role of pH on its retention. The exception is the ether-linked phenyl polar-endcapped phase, as the retention of vitamin B_{12} decreased and is more similar to that on the ODS polar-endcapped phase and calixarene-bonded phase (Fig. 3a, b).

The conventional C_{18} phase "packed with 2.4 µm particles" gives always faster analysis. The advantage of using 2.4 µm particle size columns is that high speed and high efficiency separations are achievable using conventional HPLC systems.

Chromatographic Behavior of Water-Soluble Vitamins at pH 6

At neutral pH conditions, the residual silanol groups of base silica will be ionized and should interact relatively strongly with basic compounds. The tested conventional C_{18} phase has a "base deactivated silica" and that might be used for the explanation of the behavior of this phase, especially with the basic molecules (vitamins B_1 and B_6) at pH 6. The capacity factor of these basic molecules is almost the same as at pH 4.2, and the elution order is also the same (Fig. 5a, b). On the other hand, the behavior of vitamins B_1 and B_6 on the polar-endcapped phases (YMC ODS-AQ and Synergi polar) is as expected. At this pH, both molecules displayed a longer retention, especially vitamin B_1, which eluted on the both phases after vitamins B_6, is retained very strongly on the ODS polar-endcapped phase (YMC ODS-AQ) (Fig. 5a, b). The presence of a polar group as an endcapping agent might have at this pH a strong influence on the selectivity of the polar-endcapped phases. However, the different behavior between them might be due to the fact that one of them is an ODS phase and the other is an ether-linked phenyl phase. On the other hand, as manufacturers of polar-endcapped phases provide neither the exact nature of these polar or hydrophilic endcapping reagents nor the endcapping process, we cannot know exactly if the endcapping reagent is the same in all of these polar-endcapped phases.

The calixarene-bonded phase shows very interesting behavior with the basic molecules, as well as with the acidic ones. The retention behavior of vitamins B_1 and B_6 is similar to that of the polar-endcapped phases, and the retention value of B_1 largely increased, which can be explained as follows: on the one hand, the retention value of B_1 increased for its deprotonation. On the other hand, the electron density of the pyrimidine ring of B_1 increased with its deprotonation, which led to stronger π–π interactions between the pyrimidine ring and the moiety of the calix[4]arene. The synergistic effects contribute to the remarkable increase in the retention values of B_1.

At the same time, as the pH of mobile phases was over 4.5, the retention of the acids (vitamins C and B_9) dramatically decreased. This is due to the ionization of residual phenolic hydroxyl groups of the calix[4]arene with increasing pH of mobile phases [25]. The anions of the analytes were repulsed by the anions on the stationary phase, which led to decreasing retention of the acids, what give negative retention values of these molecules (Fig. 5a, b).

Conclusion

After a systematic study to characterize and compare the behavior of a conventional C_{18} phase, a C_{18} polar-endcapped phase, an ether-linked phenyl polar-endcapped phase and a calixarene-bonded phase using a mixture of water-soluble vitamins as a real test mixture, some interesting points have arisen. It is likely then that the polar-endcapped phase is going to display fairly similar retention behavior to conventional C_{18} phases. However, these phases can provide an alternative and complementary separation for many problematic analyses performed on C_{18} columns. On the other hand, the ether-linked phenyl polar-endcapped phase should be expected to behave quite differently due to their reduced hydrophobicity. The ether-linked phenyl polar-endcapped phase showed different behavior and due to π–π interactions between the analyte and the phenyl functional group of the phase, and hydrogen bonding interactions with the ether group of the phase.

Interestingly, the tested calixarene-bonded phase showed a unique behavior, which was somehow similar to that of the ether-linked phenyl polar-endcapped phase. However, the ionization of residual phenolic hydroxyl groups of the calix[4]arene with increasing pH of mobile phases makes theses phases tend to behave totally different at high pH values, especially toward acidic compounds. These data could help both to advance the understanding of the mechanisms of retention and to develop new methods with different selectivity.

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