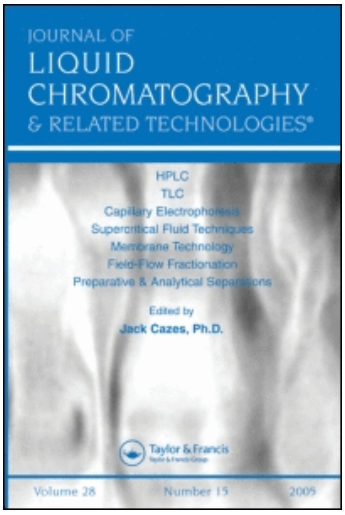

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Tributyl Phosphate as Stationary Phase in Reversed Phase Liquid Chromatographic Separations of Hydrophilic Carboxylic Acids, Amino Acids and Dipeptides

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TRIBUTYL PHOSPHATE AS STATIONARY PHASE IN REVERSED PHASE LIQUID CHROMATOGRAPHIC SEPARATIONS OF HYDROPHILIC CARBOXYLIC ACIDS, AMINO ACIDS AND DIPEPTIDES

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ABSTRACT

Tributyl phosphate (TBP) has been used as stationary liquid phase in reversed phase liquid column chromatography with aqueous buffers or aqueous buffers + methanol as the mobile phase. Hydrophobic microparticulate (5 μm) alkyl-derivatized silica was used as support. The stability of the columns is very high as is their separating efficiency.

The strong hydrogen accepting properties of TBP makes retention possible of highly polar solutes containing hydrogen donating groups. Retention of carboxylic acids is obtained by the distribution of the uncharged form into TBP and it is regulated by the pH of the mobile phase. No influence from the support on the retention of carboxylic acids has been seen. Benzoic, phenylacetic, mandelic, indole-3-acetic and glucuronic acid derivatives, amino acids and dipeptides have been chromatographed and the influence of structural effects on the separation factors is discussed.

INTRODUCTION

In reversed phase partition chromatography, where an organic liquid is used as the stationary phase in combination with an aqueous mobile phase, the retention of highly polar organic molecules may require a stationary phase that can form strong hydrogen

bonds to these molecules. A few liquids have been studied for use as stationary phases in modern liquid chromatography, namely 1-pentanol [1, 2] and butyronitrile [3]. They have been used in systems for separations of nonionic compounds as well as ion-pairs of ionic compounds. Many compounds, e.g. carboxylic acids of biological origin, were very slightly retained in these systems. We therefore investigated the properties of tributyl phosphate (TBP) as a stationary liquid phase. Being a strong hydrogen acceptor it should bind hydrogen donating samples, like carboxylic acids, strongly.

TBP is a well-known and powerful extractant for metal ions and inorganic acids but the extraction of organic acids has also been noticed [4]. TBP has been extensively used as stationary phase in reversed phase liquid chromatographic separations of metal ions [5], but its use for the separation of organic compounds in similar systems seems not to have been studied. Some work has been done using TBP as additive in a mobile organic phase in paper chromatography [6] and column chromatography [7].

This paper introduces TBP as a stationary phase on modern microparticulate alkyl-modified silica as support. The method of coating is discussed and the use of the system is illustrated by separations of very polar carboxylic acids of biological importance.

EXPERIMENTAL

Materials

The chromatographic system consisted of an LDC Solvent Delivery System 711-26, a sample valve injector, either a Valco CV-6UHPa or a Rheodyne 7120, with 10 and 20 μL loops respectively, a Cecil 212 variable wavelength UV-detector with 10 μL cell and set at 254 nm. A Heto waterbath 02 PT 923 (HETO, Birkerød,

Denmark) with external circulation was used to thermostate the chromatograph.

Stainless steel columns of 100 mm length and 4.5 mm inner diameter were used with a 2 μm Altex filter in each end fitting. The columns were slurry packed with 5 μm particles of either LiChrosorb RP-8 or LiChrosorb RP-18 (Merck).

Tributyl phosphate (TBP) and methanol were analytical grades from Merck. Sodium dihydrogenphosphate, disodium hydrogenphosphate and phosphoric acid were used to prepare the buffers and were analytical grades from Merck. Water was purified in a Milli-Q apparatus (Millipore). All chemicals were used without purification.

Batch Distribution Experiments

Batch distribution studies were performed in centrifuge tubes with equal volumes of TBP and aqueous phase. Equilibrium was attained by mechanical shaking for 20 min at 25.0 °C. The TBP phase was pre-saturated twice with an equal volume of water + methanol (9:1). The distribution ratio of carboxylic acids was determined with buffer pH 6.98 + methanol (9:1) as aqueous phase. The initial and the equilibrium concentration in the aqueous phase was determined by UV photometry.

The distribution ratio of methanol was measured in the pre-saturation step by a gas chromatographic determination of methanol in the aqueous phase. Compensation was made for a change in the phase volume ratio.

Chromatographic Technique

The eluent reservoir, immersed in a water-bath, and the column, which was jacketed, were thermostated at 25.0 °C by circu-

lating water.

The eluent contained aqueous phosphate buffers of ionic strength 0.1. In most cases methanol was added to the buffer in the ratio 1:9.

To ensure column stability the eluent was partly saturated with TBP. This was achieved by gently mixing a large volume of pre-thermostated solvent (usually 3 L) with 10 mL of TBP. After phase separation, which usually required 48 hours, partially saturated eluents were prepared by mixing the TBP-saturated solvent with an appropriate amount of unsaturated solvent of the same composition.

Coating of the columns with the stationary liquid phase (TBP) was achieved by two methods: 1. The equilibration method. The eluent was pumped through the column until constant retention volumes were obtained for sodium nitrate (unretained compound) and a retained compound. At completed coating the column porosity, $\varepsilon_m = V_m/V_o$, is ≤ 0.44. V_m is the hold-up volume and V_o is the volume of the empty column calculated from its length and inner diameter. 2. With the injection method TBP is injected directly into the column by the injection valve. Increments of 10 - 50 µL of TBP were injected at a frequency of ca. 10 µL/min. The total volume of stationary phase, V_s, that should be injected is calculated by

$$V_s = V_{m,1} - V_{m,2} = V_{m,1} - \varepsilon_{m,2} \times V_o \qquad (1)$$

$V_{m,1}$ is the hold-up volume before injections have started while $V_{m,2}$ and $\varepsilon_{m,2}$ are the hold-up volume and the desired porosity after injections of stationary phase. After the loading, the columns were conditioned over-night with a recirculating eluent.

Columns once loaded can be stored if filled with an eluent free from salts.

No precolumn needs to be used.

Capacity ratios, k', were calculated from the retention

volume, V_R, and the hold-up volume by $k' = (V_R - V_m)/V_m$.

RESULTS AND DISCUSSION

Solvent Properties

Tributyl phosphate is slightly soluble in water. The solubility at 25.0 °C in phosphate buffer pH 6.05 was determined, by gas chromatography, to be 0.034 % w/v. In pure water the solubility is 0.039 % w/v [8]. It is important to note that the solubility decreases with increasing temperature [8] since this can influence the stability of the chromatographic columns.

The solubility of water in the TBP phase is 4.67 % w/v [8], which corresponds to 3.58 M [9].

TBP, equilibrated with aqueous buffers containing 10 % methanol, contains 2 % v/v methanol since the distribution ratio of methanol was found to be 0.2.

Coating with TBP

Alkyl-modified silica supports can be coated with organic liquids as stationary phases by the equilibration method [1]. In this method the stationary liquid is adsorbed from an eluent which is saturated or partly saturated with the liquid. Fig. 1 gives results for the coating of LiChrosorb RP-18 with TBP. As TBP occupies the pores of the support the porosity decreases while the retention increases. The results indicate that TBP has filled the pores completely since the porosity reaches 0.39 which equals the normal value for the interparticle porosity of 0.4 $\pm$ 0.03. Due to the low solubility of TBP the coating procedure becomes very time-consuming.

Much faster coating was obtained by the injection method. The

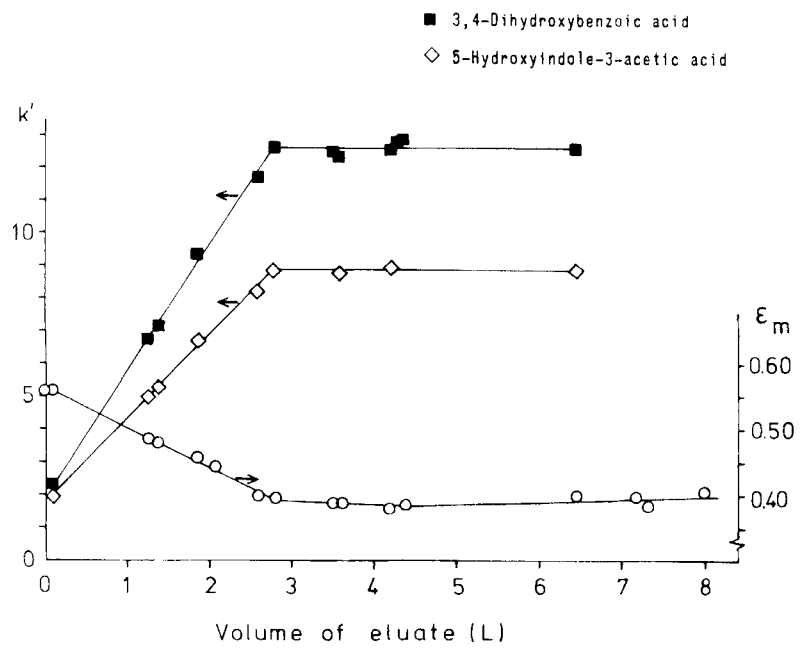

FIGURE 1. Change of retention (k') and porosity (ε_m) during coating by the equilibration method. At the start of this experiment the column was already partly coated with TBP. Column: LiChrosorb RP-8, 5 μm, 100x4.5 mm. Eluent: phosphate buffer pH 6.05, 95 % relative saturation of TBP, 0.5 ml/min.

columns could be coated within one hour but conditioning overnight was necessary in order to get good efficiency. The actual volume of injected TBP was not more than 20 % higher than that calculated from eq. (1), which shows that the uptake is rather efficient. No droplets were observed in the eluate during the coating. However, care should be taken not to over-load the column since an excess is very time-consuming to get rid of.

Variation of the amount of stationary phase can be obtained by adjusting the saturation degree of the eluent [1]. In the present work, however, only maximally loaded columns were used.

The phase-ratio, V_s/V_m, in the columns can be calculated from measured porosity values [1] and was found to be 0.6 based on an ε_m-value of 0.43 and a total porosity of 0.7 for LiChrosorb RP-18 [1].

Stability and Column Efficiency

The TBP-coated columns have a high long-term stability. One column has been in continuous use for 9 months with several changes of the pH of the buffer. The retention volumes changed less than 5 %.

It is suitable to use eluents of 90 % relative saturation with TBP since they gave stable columns with a porosity of 0.42 - 0.44 at equilibrium.

The use of completely saturated eluents always caused detection disturbances in the UV detector due to the presence of microdrops of TBP in the eluate. It seems that the solubility of TBP was lower in the column than in the eluent. Similar effects have been seen in other cases [1].

The presence of methanol in the eluent was necessary in order to avoid a drastic increase in the flow resistance which otherwise occurred, probably caused by particles produced in the sample injection valve and a break-down of the column.

The column efficiency is illustrated in Fig. 2. The plate height tends to decrease with increasing capacity ratios.

Retention Model

Samples which are retained by distribution to the TBP-layer on the columns will get capacity ratios according to

$$k' = (V_s/V_m) \times D \qquad (2)$$

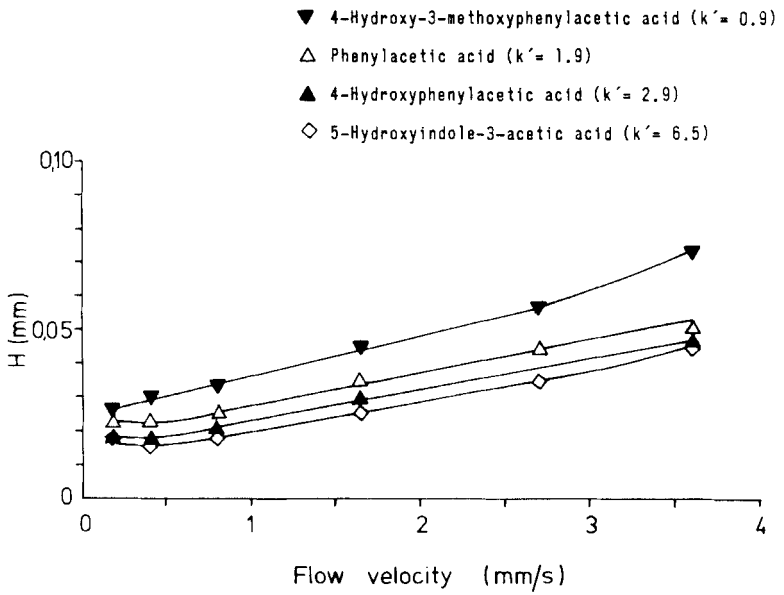

FIGURE 2. Dependence of column efficiency on flow velocity. Column: LiChrosorb RP-18, 5 µm, coated with TBP by the equilibration method, ε_m = 0.42 - 0.43. Eluent: phosphate buffer pH 5.72 + + methanol (9:1), 90 % relative saturation of TBP.

where D is the distribution ratio of the sample. For an acid, HA, its distribution ratio will be given by

$$D = \frac{C_{org}}{C_{aq}} = \frac{[HA]_{org}}{[HA]_{aq} + [A^-]_{aq}} = \frac{K_D}{1 + \frac{K'_a}{a_{H^+,m}}} \qquad (3)$$

which takes into account the distribution of the acid into TBP and its protolysis in the mobile phase. C_{org} and C_{aq} are the total concentrations of the acid in the stationary and mobile phases respectively, and K_D is the distribution constant of the acid. K'_a is the apparent acid dissociation constant and $a_{H^+,m}$ is the hydronium ion activity in the mobile phase. Accordingly, the capacity ratio can be regulated by the pH of the mobile phase. As

the pH was measured in the buffer before the addition of methanol, the relationship between the logarithm of the capacity ratio and the pH in the buffer of the eluent can be expressed by the following function after combination of equations (2) and (3)

$$\log k' = \log (K_D \times V_s/V_m) - \log (1 + K_a^*/10^{-pH}) \qquad (4)$$

K_a^* is an apparent acid dissociation constant which includes the medium factor for the hydronium ion (cf. [11]). The function has two asymptotes, one with a slope of 0 and the other with a slope of -1, which intersect at a pH which equals pK_a^* for the acid. Some examples of these relations are given in Fig. 3 where

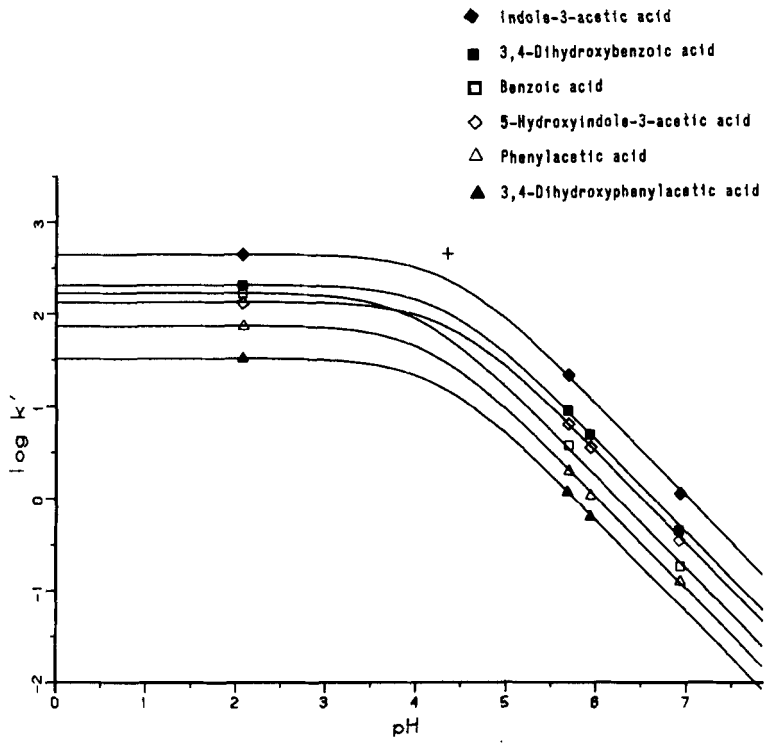

FIGURE 3. Dependence of the retention of acids on the pH of the buffer. Conditions as in Fig. 2. + = intersection point for the asymptotes of indole-3-acetic acid.

the curves have been obtained by non-linear regression according to eq. (4). The experimental points fit well to the curves.

Plots of this kind would be obtained also when the acids interact with the support. A comparison of the found k'-values with those calculated from known distribution ratios between the free liquid phases shows, however, that the influence of the support on the retention is almost negligible. Results are shown in Table 1. The found k'-values were smaller than the calculated by a factor of 0.8. The deviation from unity may be due to errors in the phase-ratio and to a different composition between the chromatographic stationary phase and the TBP-phase used in the batch experiments. Furthermore, the separation factors, α, obtained in chromatography and in distribution between free phases, agree rather closely.

TABLE 1

Comparison of Chromatographic Retention with Batch Distribution

α, the separation factor = quotient between the D-value or k'_{found}-value of an acid and the corresponding data for benzoic acid. Chromatographic conditions; Eluent: phosphate buffer pH 6.94 + + methanol (9:1), 90 % relative saturation with TBP. Porosity (ε_m): 0.42-0.44. Stationary phase: TBP. Support: LiChrosorb RP-18.

Acid	Batch		Chromatogr.		Comparison chrom.-batch
	D	α	k'_{found}	α	$k'_{found}/k'^{*)}_{calc}$
	(pH 6.98)		(pH 6.94)		(pH 6.94)
5-Hydroxyindole-3--acetic acid	0.78	1.77	0.35	1.94	0.83
Indole-3-acetic acid	2.45	5.58	1.12	6.22	0.85
Benzoic acid	0.44	1.00	0.18	1.00	0.76
3-Hydroxybenzoic acid	0.98	2.24	0.42	2.33	0.79
4-Hydroxybenzoic acid	2.60	5.93	1.09	6.06	0.78

*) $k'_{calc} = (V_s/V_m) \times D \times 0.9$ is the capacity ratio calculated from the batch extraction experiments. The phase-ratio is taken to be 0.6. The factor 0.9 compensates for the pH difference between batch and chromatographic experiments.

Retention and Separation of Various Compounds

The strong electron donating ability of the phosphate oxygen in TBP enables the formation of strong hydrogen bonds to acidic solutes and a liquid chromatographic system with TBP as the stationary phase can then give considerable retention of hydrophilic acids and a separation selectivity which is different from that obtained in the conventional reversed phase systems with alkyl--bonded phases.

A survey of the retention obtained for acidic compounds is given in Table 2. It includes derivatives of benzoic, phenylacetic, mandelic and indoleacetic acids. Also included are some glycine and glucuronic acid conjugates, one amino acid and some dipeptides. All peaks had good symmetry except for compounds that contain amino groups i.e. the amino acids and the dipeptides.

TABLE 2

Retention of Acids

Chromatographic conditions; Eluent: phosphate buffer + methanol (9:1), other conditions as in Table 1.
pK_a^o = thermodynamic acid dissociation constant from ref [13].

Sample	pK_a^o	k'		
		pH of the buffer		
		2.08	5.70	6.94
Benzoic acid derivatives				
Benzoic acid	4.20	164	3.72	0.18
Salicylic acid	3.03	700-800	1.36	
3-Hydroxybenzoic acid	4.16	415	8.35	0.42
4-Hydroxybenzoic acid	4.58	420-430	22.1	1.09
3,4-Dihydroxybenzoic acid		203	9.06	0.44
3,5-Dihydroxybenzoic acid		680	11.6	0.57
2,5-Dihydroxybenzoic acid		900-950		
3,4,5-Trihydroxybenzoic acid	4.33	86	3.42	0.14
4-Aminobenzoic acid	2.38, 4.89	21.6	5.30	0.29
4-Hydroxy-3-methoxybenzoic acid			5.12	0.30
3-Hydroxy-4-methoxybenzoic acid		107	4.07	0.21

TABLE 2 (continued)

Sample	pK_a^o	k'		
		pH of the buffer		
		2.08	5.70	6.94
Phenylacetic acid derivatives				
Phenylacetic acid	4.32	73.7	1.98	0.13
2-Hydroxyphenylacetic acid		79.4	2.84	0.20
3-Hydroxyphenylacetic acid		93.6	2.73	0.17
4-Hydroxyphenylacetic acid		80.4	2.88	0.22
3,4-Dihydroxyphenylacetic acid		32.5	1.16	0.03
4-Hydroxy-3-methoxy-phenylacetic acid		26.8	0.92	
Mandelic acid derivatives				
Mandelic acid	3.41	14.9	0.05	
3-Hydroxymandelic acid		18.7	0.07	
4-Hydroxymandelic acid		14.7	0.07	
3,4-Dihydroxymandelic acid		5.76	0.03	
4-Hydroxy-3-methoxy-mandelic acid		5.31		
3-Hydroxy-4-methoxy-mandelic acid		4.87		
Indoleacetic acid derivatives				
Indole-3-acetic acid		439	21.5	1.12
5-Hydroxyindole-3-acetic acid		133	6.56	0.35
Miscellaneous carboxylic acids				
Phtalic acid	2.95, 5.41	29.6	0	
Hippuric acid		9.77	3.73	
Salicyluric acid		156		
Fumaric acid	3.02, 4.38	23.1		0
Maleic acid	1.94, 6.23	0.63		
Glucuronic acid derivatives				
4-Nitrophenyl-β-D-glucuronic acid		9.77		
2-Naphtyl-β-D-glucuronic acid		40.6		
Amino acids and dipeptides				
Phenylalanine	2.16, 9.12	0.87		
Phenylalanyl-Alanine		1.57		
Phenylalanyl-Leucine		23.5		
Leucyl-Phenylalanine		21.9		
Phenylalanyl-Serine		0.55		
Phenylalanyl-Phenylalanine		44.7		
Valyl-Phenylalanine		5.99		
Lysyl-Phenylalanine		0.09		

Their peaks showed pronounced tailing. The influence of pH on retention is in accordance with eq. (4). A typical chromatogram is shown in Fig. 4.

Retention data obtained at acidic pH have been used to calculate the separation factors for some simple structural changes. Increase in retention was obtained for hydroxylation (log α = 0.12, phenol/benzene) and carboxylation (log α = 0.14, benzoic acid/benzene) in benzene while hydroxylations in the benzoic, phenylacetic, mandelic and indoleacetic acids gave either an increase or a decrease depending on the position for hydroxylation.

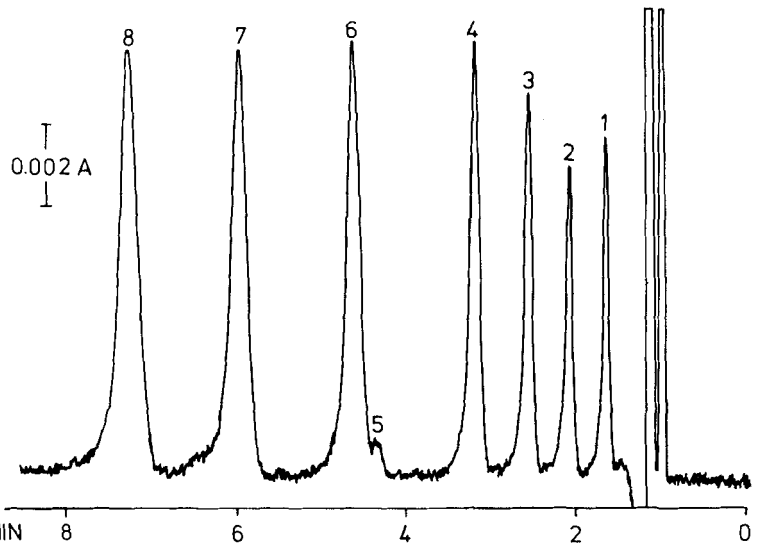

FIGURE 4. Separation of a mixture of carboxylic acids. Conditions as in Fig. 2 but pH = 6.02.
Samples: 1. 3,4-Dihydroxyphenylacetic acid
2. Phenylacetic acid
3. 2-Hydroxyphenylacetic acid
4. 3-Hydroxy-4-methoxybenzoic acid
5. Impurity
6. 5-Hydroxyindole-3-acetic acid
7. 3,4-Dihydroxybenzoic acid
8. 3,5-Dihydroxybenzoic acid

Increase of the carbon chain length increased the retention (log α = 0.47, methylpropylketone/methylethylketone).

Even very hydrophilic acids like fumaric acid and maleic acid are retained. A very high separation factor, 37, is obtained between these cis-trans isomers.

Phenylalanine and the dipeptides were only studied with pH 2 buffer in the eluent. In such a medium they will have a net positive charge and they might be retained as the phosphate ion-pairs.

The phase system can also be used for separations of ion--pairs of hydrophilic amines, like the catecholamines, which will be discussed in forthcoming publications [13].

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