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SEPARATION OF PROTON-DONATING SOLUTES BY LIQUID CHROMATOGRAPHY WITH A STRONG PROTON ACCEPTOR, TRI-*n*-OCTYLPHOSPHINE OXIDE, IN THE LIQUID STATIONARY PHASE

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SUMMARY

Proton-donating solutes, such as aromatic and aliphatic carboxylic acids, are separated in a reversed-phase liquid–liquid chromatographic system with a stationary phase consisting of tri-*n*-octylphosphine oxide (TOPO) in *n*-decane. The retention is based on the formation of a complex between the sample and TOPO, the stoichiometry depending on the number of proton-donating groups in the sample. The retention and separation selectivity are governed by the pH of the mobile phase and the concentration of TOPO in the stationary phase. The latter can be regulated by the addition of competing proton donors to the mobile phase. An example of the separation of several aromatic carboxylic acids and phenol by gradient elution is given.

INTRODUCTION

Reversed-phase liquid–liquid chromatography offers interesting possibilities for the separation of hydrophilic organic acids when a strong proton acceptor such as tri-*n*-octylphosphine oxide (TOPO) is added to the liquid stationary phase. A retention model and some results obtained with such systems have been given in a previous paper[1].

The system contains a solution of TOPO in *n*-decane as stationary phase, coated on a microparticulate support. The mobile phase is an aqueous buffer solution. The concentration of free TOPO in the organic stationary phase determines the retention and the separation selectivity. The concentration can be varied by changing the stationary phase[1], but a more convenient way is to add a competing proton-donating solute to the mobile phase. This makes it possible to perform, *e.g.*, gradient elution to separate both hydrophobic and hydrophilic samples, and also protolytes of different acidity.

The aim of this study was to elucidate the regulation of the retaining properties of the system by changing the composition of the mobile phase.

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EXPERIMENTAL

Apparatus

The pump was a Model 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.), equipped with a pulse damper and a pressure transducer (Touzart et Matignon, Vitry, France). A Valco CV-6-UHPa-N60 injection valve (Valco, Houston, TX, U.S.A.) with a 30.6-μl loop was used for injection of the samples. The UV detectors (LDC, Riviera Beach, FL, U.S.A.) were a UV III Monitor Model 1203, wavelength 254 nm, with a cell volume of 10 μl, and a Spectromonitor III Model 1204, with a variable wavelength and a cell volume of 12 μl. The time constant of both detectors was set to 0.5 sec.

The columns (150 × 4.6 mm I.D.) were made of stainless steel with a polished inner surface, equipped with modified Swagelok connectors and Altex stainless-steel frits (2 μm). The volume of the empty columns, V_0, was 2.46 ml as measured volumetrically.

A HETO waterbath (Birkerød, Denmark) Type 02 PT 923 was used to thermostat the system.

For the preparation of the columns a Haskel (Burbank, CA, U.S.A.) air-driven fluid pump, Type DST-150A was used.

Chemicals and reagents

TOPO (for extraction analysis) was obtained from E. Merck (Darmstadt, G.F.R.) and *n*-decane (for synthesis grade) (99%) and *n*-valeric acid (p.a. grade) were obtained from Schuchardt (Hohenbrunn, G.F.R.).

All other substances were of analytical or reagent grade and were used without further purification.

Column preparation

The support material, LiChrosorb RP-8, mean particle diameter (d_p) 7 μm, research sample KE 5679, was kindly supplied by E. Merck. It was packed upwards into the column by a slurry packing technique at a pressure of 37.5 MPa, using cyclohexane, isopropanol or mixtures of these with *n*-hexane as suspending liquid (1.3 g of support in 10–20 ml of liquid). After packing, the filling was washed with *n*-hexane (100–200 ml upwards and 100–150 ml downwards), and tested with 0.5% (v/v) *n*-butanol in *n*-hexane as eluent. Toluene, 1,4-dinitrobenzene and 2,3,5-trimethylphenol were used as test solutes. In this study columns were used that had a reduced plate height ($h = H/d_p$) of less than 7 at a flow rate of 1 mm/sec and asymmetry factors (measured at 10% of the peak height) between 0.85 and 1.6.

Coating technique

After testing, the column was washed with *n*-hexane. The stationary phase (*n*-decane with varying amounts of TOPO) was applied on the support by the pumping method, as described previously[1].

Chromatographic technique

The reservoir and the column were immersed in the water-bath at 25.0°C.

The mobile phases were aqueous buffer solutions of different pH, ionic

strength 0.1, if not stated otherwise. The mobile and stationary phases were usually not equilibrated with each other.

The volume of the mobile phase, V_m, was determined from the hold-up volume of sodium nitrate or water, after substraction of the dead volume. The dead volume was determined by measuring the elution volume with the injector directly connected to the detector at a flow-rate of 0.08 ml/min. It was largely due to the volume of the connecting tubings and the heat exchanger in the detector and corresponded to 127 μl with the Model 1203 detector and 110 μl with the Model 1204 detector, *i.e.*, *ca.* 10% of V_m.

The porosity, ε_m, was calculated from the equation

$$\varepsilon_m = V_m/V_0 \quad (1)$$

RESULTS AND DISCUSSION

Choice of support and diluent

The proton-accepting properties of the complexing agent TOPO are best utilized if TOPO is dissolved in an organic solvent which has a very low proton-donating tendency. To obtain good stability the support material should be better wetted by the stationary liquid than by the mobile liquid, *i.e.*, it should be hydrophobic[2]. A totally porous silica support derivatized with octyl chains (LiChrosorb RP-8) was used in these studies.

Several organic solvents were tested as diluents for TOPO. A chloroform coating stripped off very rapidly. *n*-Dodecane and *n*-hexadecane required very long equilibration times, and *n*-hexane showed some instability (stripping). *n*-Decane, showing good stability and a not too long equilibration time, was chosen as diluent in these studies.

Stability and reproducibility of n-decane/TOPO-coated columns

The stability of the *n*-decane-coated columns was studied for periods of 2

TABLE I

STABILITY OF COATED COLUMNS

Stationary phase: 0.1 *M* TOPO in *n*-decane on LiChrosorb RP-8. Mobile phases: days 7–24, phosphate buffer solutions (pH 1.8), containing 0–0.0025 M valeric acid; days 24–55, formate buffer solution (pH 3.9). Formate buffer solution (pH 3.9) was used as the mobile phase for all measurements of k'.

Solute	k'		
	Day 7	*Day 24*	*Day 55*
Phenol	42.9	38.7	37.0
Benzoic acid	53.4	48.1	44.5
3-Hydroxybenzoic acid	39.0	33.7	32.2
3-Hydroxy-4-methoxybenzoic acid	5.45	4.88	4.63
4-Hydroxyphenylacetic acid	4.50	4.01	3.76
5-Hydroxyindole-3-acetic acid	1.91	1.76	1.69

months at most. The retention decreased by less than 1% per day under conditions that implied frequent changes of the composition of the mobile phase (Table I). As the capacity ratios change by nearly the same factor in spite of differences in the stoichiometry of the complexes[1], the decrease in k' cannot be due to a change in the TOPO concentration. A change in the volume of the stationary phase, V_s, seems to be more plausible, but no significant changes in the porosity could be observed.

Changes in the pH of the mobile phase and pre-saturation of the mobile phase with stationary phase had no significant influence on the stability, probably owing to the very low solubility of the organic phase in the mobile phase.

With the pumping technique, the porosity reached a value of 0.425–0.430 (15 columns), which indicates that the pores of the support are almost completely filled[2]. The pore filling is obviously highly reproducible.

On three columns prepared in the same way, the retention of solutes with a capacity ratio between 4 and 40 could be reproduced to within 15%.

Regulation of the retention

The capacity factor of a proton-donating solute HX in the liquid–liquid system with the strong proton acceptor TOPO in the stationary phase can be expressed by

$$k'_{HX} = \frac{V_s}{V_m} \cdot \frac{K_{D(HX)} \cdot K_{HXTOPO_n} \cdot [TOPO]^n}{1 + K'_{a(HX)} \cdot (a_{H^+})^{-1}} \quad (2)$$

where the subscript m refers to the mobile phase; concentrations in the stationary liquid phase are given without a subscript; $K_{D(HX)} = [HX]/[HX]_m$ is the distribution constant and $K_{HXTOPO_n} = [HXTOPO_n]/[HX] \cdot [TOPO]^n$ is the formation constant between the solute and TOPO.

A demonstration of the effect of the number of proton-donating groups in the solute (n) is given in Figs. 1 and 2. With 0.15 M TOPO in the stationary phase, mandelic acid and its 3-hydroxy derivative are not separated (Fig. 1). According to eqn. 2 the separation factor of these compounds will change with the TOPO concentration, because the n values are different. On decreasing the TOPO concentration to 0.10 M. 3-hydroxymandelic acid ($n = 2$) becomes less retained than mandelic acid ($n = 1$), giving a good separation (Fig. 2). The separation factor between 3-hydroxymandelic acid and 3,4-dihydroxymandelic acid remains almost constant, indicating that n is the same for these compounds. 3,4-Dihydroxymandelic acid has three proton-donating groups but the second phenolic hydroxy group might be occupied by internal hydrogen bonding.

Retention with formate buffer solutions

The retention of phenol ($pK'_a = 9.9$) should be independent of the composition of the mobile phase at a pH lower than 8 (see eqn. 2). Constant capacity factors at pH 2–7 were obtained with phosphate buffer solutions and sodium perchlorate solution as mobile phases, but with a formate buffer solution the retention at pH 4 was *ca.* 25% lower (Table II). This effect might be due to complex formation between formic acid (HFo) and TOPO in the stationary phase. The concentration of free TOPO, $[TOPO]_f$, in the formate system decreases:

$$[TOPO]_f = C_{TOPO} - n[HFoTOPO]_n \quad (3)$$

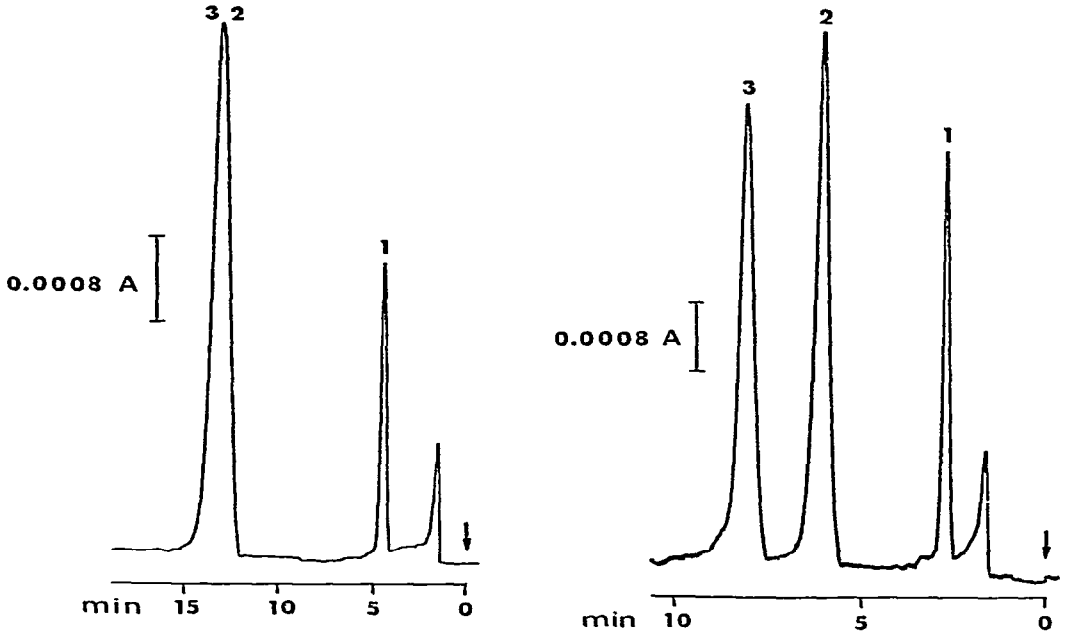

Fig. 1. Separation of mandelic acid derivatives. Stationary phase: 0.15 *M* TOPO in *n*-decane. Mobile phase: phosphate buffer solution (pH 1.8); 0.8 ml/min. Detection wavelength: 254 nm. Solutes: 1 = 3,4-dihydroxymandelic acid; 2 = 3-hydroxymandelic acid; 3 = mandelic acid.

Fig. 2. Separation of mandelic acid derivatives. Stationary phase: 0.10 *M* TOPO in *n*-decane. Mobile phase, detection wavelength and solutes as in Fig. 1.

where C_{TOPO} is the total concentration of TOPO.

If we assume that phenol (ph) and formic acid have $n = 1$, and that the complex formation between TOPO and perchlorate is negligible, the following relationship is obtained between the capacity factors in perchlorate solution, $k'_{ph(p)}$, and in formate buffer solution, $k'_{ph(f)}$, (*cf.*, eqn. 2):

$$\frac{k'_{ph(p)}}{k'_{ph(f)}} = \frac{C_{TOPO}}{[TOPO]_f} \tag{4}$$

TABLE II

INFLUENCE OF MOBILE PHASE COMPOSITION ON RETENTION

C_{TOPO} (*M*)	k'_{phenol}		*k'* ratio	$K_{D(HFo)} \cdot K_{HFoTOPO}$
	0.1 M $NaClO_4$	*Formate pH 4*		
0.075	39.1	30.6	1.28	6.37
0.100	57.0	45.0	1.28	6.38
0.150	104.7	81.4	1.29	6.56

V_s/V_m and C_{TOPO} have to be kept constant. The ratios $k'_{ph(p)}/k'_{ph(f)}$ are presented in Table II.

The ratio is independent of the total concentration of TOPO, which supports the assumption of $n = 1$ for formic acid. Phenol almost had the same retention with 0.1 *M* sodium perchlorate solution and with phosphate buffer solutions at pH 2, 3, 6 and 7, which indicates that the concentration of free TOPO was not influenced by phosphoric acid, phosphates or perchlorate. Sekine and co-workers[3,4] have made the same observations in batch experiments with similar systems.

The distribution of formic acid to the stationary phase is governed by $K_{D(HFo)} \cdot K_{HFoTOPO}$, defined by

$$K_{D(HFo)} \cdot K_{HFoTOPO} = \frac{[HFoTOPO]}{[TOPO] \cdot [HFo]_m} \tag{5}$$

An expression that allows the calculation of this constant can be obtained from eqn. 3:

$$K_{D(HFo)} \cdot K_{HFoTOPO} = \left(\frac{C_{TOPO}}{[TOPO]_f} - 1\right) \cdot \frac{1}{[HFo]_m} \tag{6}$$

The concentration of formic acid in the mobile phase can be calculated from the pH of the mobile phase and the K'_a value of formic acid. The values of $K_{D(HFo)} \times K_{HFoTOPO}$ calculated according to eqn. 6 are given in Table II.

Regulation of the concentration of free TOPO

Eqn. 6 can be transformed into a general expression for the concentration of free TOPO when a competing monobasic acid HY is added to the mobile phase:

$$[TOPO] = C_{TOPO} \left\{1 + K_{D(HY)} \cdot K_{HYTOPO} \cdot \left[\frac{C_{HY_m}}{1 + K'_{a(HY)} \cdot (a_{H^+})^{-1}}\right]\right\}^{-1} \tag{7}$$

This equation shows that the concentration of free TOPO at a given C_{TOPO} depends on the properties of the competing acid HY, expressed by the product of the distribution constant and the complex formation constant, $K_{D(HY)} \cdot K_{HYTOPO}$, and by the acid dissociation constant, $K'_{a(HY)}$. The concentration of free TOPO can be regulated by the total concentration of the competitor in the mobile phase, C_{HY_m}, and the pH of the mobile phase.

Eqn. 7 can be used to calculate the amount of competing acid that is needed to obtain a given suppression of [TOPO]. If, for example, a reduction by 90% is required, then the product $K_{D(HY)} \cdot K_{HYTOPO} \cdot C_{HY_m}$ has to be 9, if HY is undissociated at the pH of the mobile phase $[K'_{a(HY)}/a_{H^+} \ll 1]$.

From a practical point of view, it is favourable if the competing acid has a low detector response. A number of aliphatic organic acids were tested. They have a $\lambda_{max.}$ at *ca.* 210 nm, whereas the samples, aromatic acids, were detected at 254 nm. The study was made at pH 1.85 where all competing acids, except formic acid, are undissociated. Their complexing ability can be determined by chromatography. According to eqn. 2, $K_{D(HY)} \cdot K_{HYTOPO}$ is proportional to k'_{HY}, and can be calculated if V_s/V_m, [TOPO], n and $pK'_{a(HY)}$ are known.

TABLE III

RETENTION OF ALIPHATIC ACIDS

Stationary phase: 0.1 *M* TOPO in *n*-decane. Mobile phase: phosphate buffer solution (pH 1.85), ionic strength 0.1. $V_s/V_m = 0.7$.

Acid	k'	$K_{D(HY)} \cdot K_{HYTOPO}$
Formic	0.43	6.2
Acetic	0.38	5.4
Propionic	1.77	25.2
Butyric	7.3	103
Valeric	30.5	433

The capacity factors of some hydrophilic aliphatic acids, intended as competing acids, are given in Table III, together with the calculated $K_{D(HY)} \cdot K_{HY\,TOPO}$ values; *n* was taken as equal to unity. It can be seen that, with the exception of formic acid, $K_{D(HY)} \cdot K_{HY\,TOPO}$ increases with increasing number of carbon atoms in the molecule ($\Delta \log k' = 0.63$ per CH_2 group). At the same time however, the solubility of the acids in water decreases, and the product $K_{D(HY)} \cdot K_{HY\,TOPO} \cdot C_{HY_{m,max}}$ (*cf.*, eqn. 7) is virtually constant for the more hydrophobic acids, which means that the maximal suppression of free TOPO cannot be increased by increasing the number of carbon atoms in the competitor.

In the following experiment we used *n*-valeric acid as competitor. A concentration of 0.0025 *M* in the mobile phase was sufficient to decrease the concentration of free TOPO by 50%.

Regulation of retention by competition

A number of samples were chromatographed with different concentrations of valeric acid in the mobile phase. The retention of the sample is described by eqn. 2. When the concentration of free TOPO is regulated by the addition of a competing acid HY, [TOPO] can be calculated from eqn. 7. $K_{D(HY)} \cdot K_{HY\,TOPO}$ for valeric acid was taken from Table III. A linear relationship was obtained between $\log k'_{HY}$ and $\log$

TABLE IV

REGULATION OF RETENTION BY COMPETITION

$C_{TOPO} = 0.1$ *M*. Mobile phase: phosphate buffer solution (pH 1.85). Competitor: *n*-valeric acid, 0–0.0025 *M*, $V_s/V_m = 0.7$. Evaluation methods: logarithmic form of eqn. 2 (log) and non-linear regression using eqn. 8 (non-lin).

Solutes	$\log K_{D(HX)} \cdot K_{HX\,TOPO}$		*Number of TOPO molecules in complex (n)*			k'_0
	Log	*Non-lin*	*Log*	*Non-lin*	*Expected*	
Mandelic acid	1.95	2.05	1.2	1.3	1	0.3
Phenol	2.91	2.99	1.0	1.1	1	3.8
3-Hydroxy-4-methoxybenzoic acid	2.75	3.00	1.5	1.8	2	1.0
3-Hydroxybenzoic acid	4.06	4.21	1.8	2.0	2	4.4

[TOPO], which is in accordance with eqn. 2 (*cf.*, ref. 1). The constants n and log $K_{D(HX)} \cdot K_{HXTOPO_n}$, which can be calculated from the slope and the intercept of the line, are presented in Table IV.

The agreement between these values and the previously published constants (Table I in ref. 1) is not satisfactory. There are a number of possible reasons for these deviations. It is assumed that retention of the sample is zero in the absence of TOPO, *i.e.*, the sample is not retained by the support and the magnitude of the distribution constant $K_{D(HX)}$ is negligible in comparison with $K_{D(HX)} \cdot K_{HXTOPO_n} \cdot [TOPO]^n$. Batch experiments have shown that this condition is fulfilled for 3-hydroxybenzoic acid in the [TOPO] range used chromatographically. However, it was found that all samples are retained on the column even in the absence of TOPO, indicating that adsorption to some surface is taking place. Further details about this side-effect will be given in a forthcoming paper. A slight modification of eqn. 2 will describe the retention better:

$$k'_{HX} = k'_0 + \left[\frac{V_s}{V_m} \cdot \frac{K_{D(HX)} \cdot K_{HXTOPO_n}}{1 + K'_{a(HX)} \cdot (a_{H^+})^{-1}}\right] \cdot [TOPO]^n \qquad (8)$$

where k'_0 represents the retention of the sample in absence of TOPO. The constants k'_0, n and $K_{D(HX)} \cdot K_{HXTOPO_n}$ cannot be evaluated by simple linear or logarithmic plots, and a non-linear curve-fitting procedure using eqn. 8 was applied to the data (*cf.*, ref. 5). The constants found are presented in Table IV.

The data from ref. 1 should be treated in the same way in order to make comparison possible, but the constants evaluated in this way are very uncertain owing, for example, to the fact that different columns had to be used. A further

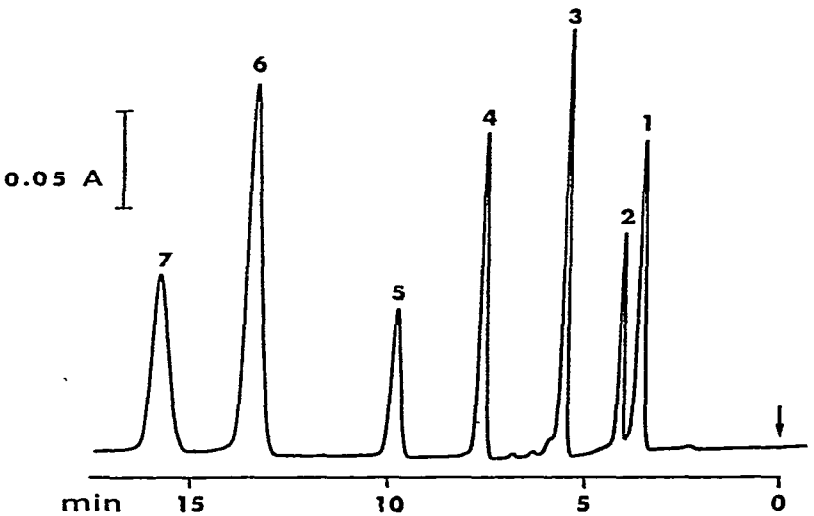

Fig. 3. Separation of some aromatic acids by gradient elution. Stationary phase: 0.165 *M* TOPO in *n*-decane. Mobile phase: step gradient from phosphate buffer solution of pH 1.9 (0–4 min) to acetate buffer solution of pH 2.9 (4–20 min); 0.6 ml/min. Detection wavelength: 270 nm. Solutes: 1 = 4-hydroxy-3-methoxymandelic acid; 2 = 3,4-dihydroxymandelic acid; 3 = 4-hydroxyphenylacetic acid; 4 = 4-hydroxybenzoic acid; 5 = phenylacetic acid; 6 = phenol; 7 = benzoic acid.

discussion on the differences between the values of n and $K_{D(HX)} \cdot K_{HXTOPO_2}$ will be given in a forthcoming paper. In the meantime, we can conclude that addition of a competing acid to the mobile phase can be used to regulate the retention and the selectivity of proton-donating samples.

Gradient elution

The regulation of the retaining properties of the system by the composition of the mobile phase can be easily utilized for gradient elution. An example is given in Fig. 3. The first two solutes, 4-hydroxy-3-methoxymandelic acid and 3,4-dihydroxymandelic acid, are so hydrophilic that they can be separated in the phase system only if the stationary phase contains a high concentration of free TOPO and if the pH of the mobile phase is low. Under these conditions phenol has a very high capacity factor. An increase in the pH of the mobile phase to 7 does not influence the retention, owing to its pK_a' value of 9.9. Only a decrease in [TOPO] will greatly reduce the retention of phenol. This was done by changing to an acetate buffer solution as the mobile phase. Under these conditions a high separation factor between benzoic acid and 4-hydroxybenzoic acid is also obtained.

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