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Separation of isomeric naphthalenesulphonic acids by micro high-performance liquid chromatography with mobile phases containing cyclodextrin

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Abstract

Aromatic sulphonic acids are important dye intermediates and the determination of the individual isomers after their preparation by sulphonation of the parent aromatic hydrocarbon is important for the monitoring of the dye production process. For this purpose, either reversed-phase chromatography with mobile phases containing strong electrolytes as additives or capillary zone electrophoresis with working electrolytes containing cyclodextrins can be used to separate and determine not only individual sulphonation products with various numbers of sulphonic groups, but also various isomeric di- and trisulphonic acids. However, the separation of some isomers using either of the two techniques is not fully satisfactory. In the present work, HPLC with mobile phases containing cyclodextrins was employed to improve previously achieved separations of aromatic sulphonic acids. Because of the high cost of cyclodextrin, microcolumn HPLC with diode-array detection on the columns prepared in laboratory by supercritical fluid packing technique was employed for this purpose. Capillary columns packed with various octadecyl silica gel materials were compared and their stability and efficiency were found suitable for the separation of the compounds tested. The selectivity of separation of some isomers improved significantly with respect to the previous methods. Procedures were designed for separation and analytical control of technological processes producing dye intermediates. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Mobile phase composition; Naphthalenesulphonic acids

1. Introduction

Aromatic sulphonic acids are important intermediates in the production of a variety of synthetic dyes, optical brighteners and fluorescent whitening agents. High-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) are well suited for the analysis of these anionic compounds.

Liquid–liquid chromatography with *n*-octylamine stationary phase was attempted for the separation of aromatic sulphonic acids, but long time is necessary to attain equilibrium between the adsorbent, the liquid stationary and the mobile phases [1]. Anion-exchange chromatography is occasionally used for their separation [2–6], but this method usually lacks selectivity for separation of isomeric compounds. In reversed-phase systems with pure aqueous–organic mobile phases the acids are usually eluted close to the column dead volume with little separation and often even with strongly asymmetrical peak shape, because of the interactions with residual silanol groups on the surface of the bonded phase [7]. To increase the retention and to achieve successful

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separations, it is necessary to add ionic compounds to the mobile phase.

Sulphonic acids are strong acids, completely dissociated in aqueous or aqueous–organic solutions over a broad pH range, so that diluted acidic buffers cannot be used to suppress their ionization and to improve their chromatographic properties, unlike to reversed-phase separations of weaker carboxylic acids. Aromatic aminosulphonic acids are exception from this rule and their acido–basic properties are similar to those of carboxylic acids. Consequently, separation of these acids in buffered aqueous organic mobile phases is principally possible [8]. This is not the case with other aromatic sulphonic acids, which can be separated using ion-pair reversed-phase chromatography with mobile phases containing tetraalkylammonium salts in concentrations 10^{-3}–10^{-4} mol/l [9–12].

The retention and the separation selectivity depend on the nature of the ion-pairing reagent, on the number of sulphonic groups, on the character of the aromatic ring and on the nature, number and position of the substituents. The retention can be controlled by adjusting the concentration of both the ion-pairing reagent and the organic solvent in the mobile phase [13–16]. This technique was successfully used to separate linear alkylbenzenesulphonic acids, lignosulphonic acids and polyaromatic sulphonic acids in wastewater in a single run [17]. Some non-substituted naphthalene- and anthraquinonesulphonic acids [13,18] and their hydroxy and amino derivatives (letter acids) [19–21] could be separated, but the technique failed to resolve complex mixtures containing various isomers of naphthalenesulphonic acids with different numbers of sulphonic groups [13].

Separation of sulphonic acids can be achieved also by reversed-phase HPLC with mobile phases containing strong electrolytes (salts) in concentrations 0.1–1 mol/l. In the presence of salts ionic interactions in the stationary phase are suppressed, retention is increased and separation selectivity enhanced [7,22]. The retention can be controlled by adjusting the concentrations of either the salt or the organic solvent in mixed mobile phases. This technique makes it possible to separate not only 1- and 2-naphthalenesulphonic acids [23,24], but more than ten isomeric naphthalene mono- to tetrasulphonic acids [25,26], which are eluted in the order of decreasing number of sulphonic groups. Substituted amino- and hydroxynaphthalenesulphonic acids [20,27,28] or isomers of 4,4′-diaminostilbene-2,2′-disulphonic acid [29] can be separated, too. Aqueous–organic mobile phases were used to separate sulphonic acid derivatives of phenol on a β-cyclodextrin bonded phase [30].

Capillary electrophoresis (CE) has been employed for the separations of acidic azo dyes in industrial products [31–33] and in the environment [34], of several substituted benzenesulphonic [35] and anthraquinonesulphonic [16] acids and isomeric 4,4′-diaminostilbene-2,2′-disulphonic acid [27]. Some acidic azo dyes and aromatic sulphonic acids could be separated by micellar electrokinetic capillary chromatography in borate buffers containing cholic acid [36].

A few earlier results published so far [17,28,31] indicate that HPLC and capillary electrophoresis complement each other with respect to the resolution of aromatic sulphonic acids. Cylodextrin additives to working electrolytes are widely used as chiral selectors for separation of optical isomers, but they can be used also for separation of positional isomers [37]. Addition of β-cyclodextrin to the working electrolyte was suggested to improve the selectivity of separation of isomeric sulphonic acid dyes used as artifical food colourants [38–40] or for the separation of naphthalenesulphonic acids and their amino and hydroxy derivatives [28,41]. Best separation of strong unsubstituted isomeric naphthalenesulphonic acids can be achieved in the electrolytes containing a mixture of β- and γ-cyclodextrin [42]. β-Cyclodextrin additive is necessary also for the separation of the aminonaphthalenesulphonic acids present in technological samples of I and H acids [42].

Successful applications of cyclodextrins in CE of aromatic sulphonic acids suggest that cyclodextrins as mobile phase additives could improve the separation of some isomeric acids by HPLC. However, the cost of these chemicals makes impractical their use in conventional analytical HPLC with column I.D.s between 2 and 4.6 mm and flow-rates between 0.5 and 3 ml/min. The reduction of the internal column diameter in microcolumn HPLC, especially when using packed capillary columns with I.D.$<$0.5 mm, results in small volumetric flow-rates and a very

significant decrease of the consumption of the mobile phase, so that the cost of the mobile phase is much less important and expensive mobile phase additives can be used. Other advantages of microcolumn HPLC are the ability to work with minute sample sizes, increased efficiencies of packed capillary columns with respect to the conventional analytical HPLC columns, possibility of packing long efficient columns and strongly reduced chromatographic dilution allowing the use of sensitive detection devices, such as the mass spectrometric detector [43–47]. These characteristics make possible using expensive mobile phases additives without increasing significantly the cost per analysis.

However, the main problems with packed capillary HPLC microcolumns are their high cost and limited mechanical stability. Fused silica capillaries with in-situ prepared porous ceramic frits packed using supercritical fluid slurry technique [48,49], can be prepared with excellent efficiency and good ruggedness and mechanical stability.

In the present work, possibilities of separations of aromatic sulphonic acids used as dye intermediates were studied on packed capillary columns with β-cyclodextrin as the mobile phase additive. Special attention has been paid to the effect of the column end frits on the analytical separation of ionic compounds in aqueous or low organic concentrations mobile phases.

2. Experimental

2.1. Instrumentation

The apparatus for micro-HPLC was assembled using an LC-10AD pump (Shimadzu, Kyoto, Japan), an ACI4W.06 high-pressure sample valve equipped with a 60 nl internal sample loop switched by a DVI pneumatic actuator (all from Valco, Houston, TX, USA) with helium as the pressure gas, a Crystal 240 diode-array UV detector (ATI Unicam, Cambridge, UK) and a personal computer used to process the detector data. The capillary column was connected to the injector valve using a T-shape splitter and to the detector by a piece of silica capillary tubing, 75 μm I.D., which also served as a detector cell with windows burned in the polyimide coating. The connection tubing to the detector was also made of fused-silica capillary, 75 μm I.D., and all connections were made as short as possible to reduce the extra-column contributions to the band broadening.

2.2. HPLC microcolumns

Fused silica capillaries, 120–150 mm×0.32 mm I.D. were obtained from Chrompack (Prague, Czech Republic). First, porous ceramic end frits were prepared by polymerizing solutions containing potassium silicate in-situ within a column according to Cortes et al. [48]. Columns 1 and 2 were equipped with a glass paper column bed support instead of a ceramic frit, but these columns were less stable than the columns with frits. Column with frits were then packed by supercritical fluid slurry technique [49].

2.3. Packing materials and column dimensions

The following columns were prepared with the octadecyl silica materials: Biospher Si C_{18}, obtained from Bio Lab (Prague, Czech Republic) and Zorbax XDB Eclipse, C_{18} (Hewlett-Packard, Palo Alto, CA, USA):

1. Biospher Si C_{18}, 5 μm, 135×0.32 mm I.D.
2. Biospher Si C_{18}, 5 μm, 140×0.32 mm I.D., column hold-up volume V_M=7.3 μl, extra-column contribution V_{ex}=1.4 μl
3. Biospher Si C_{18}, 5 μm, 162×0.32 mm I.D., V_M=8.8 μl, V_{ex}=2.0 μl
4. Biospher Si C_{18}, 5 μm, 166×0.32 mm I.D., V_M=7.6 μl, V_{ex}=1.6 μl
5. Zorbax XDB, Eclipse, C_{18}, 5 μm, 146×0.32 mm I.D., V_M=7.5 μl, V_{ex}=2.0 μl

The standard sample compounds were obtained from Synthesia, Semtín, Czech Republic: (1) Naphthalenesulphonic acids: naphthalene-1-sulphonic acid (1-NSA); naphthalene-2-sulphonic acid (2-NSA); naphthalene-1,3-disulphonic acid (1,3-NDSA); naphthalene-1,5-disulphonic acid (1,5-NDSA); naphthalene-1,6-disulphonic acid (1,6-NDSA); naphthalene-1,7-disulphonic acid (1,7-NDSA); naphthalene-2,6-disulphonic acid (2,6-NDSA); naphthalene-2,7-disulphonic acid (2,7-NDSA); naphthalene-1,3,5-trisulphonic acid (1,3,5-NTSA); naphthalene-1,3,6-trisulphonic acid (1,3,6-NTSA)

naphthalene-1,3,7-trisulphonic acid (1,3,7-NTSA); naphthalene-1,3,5,7-tetrasulphonic acid (1,3,5,7-NTeSA).

(2) Aminonaphthalenesulphonic acids, the structures and abbreviations used are listed in Fig. 1.

2.4. Separation conditions

The mobile phases were comprised of aqueous or aqueous–methanolic solutions containing 0.4 *M* Na_2SO_4 or tetrabutylammonium hydrogensulphate

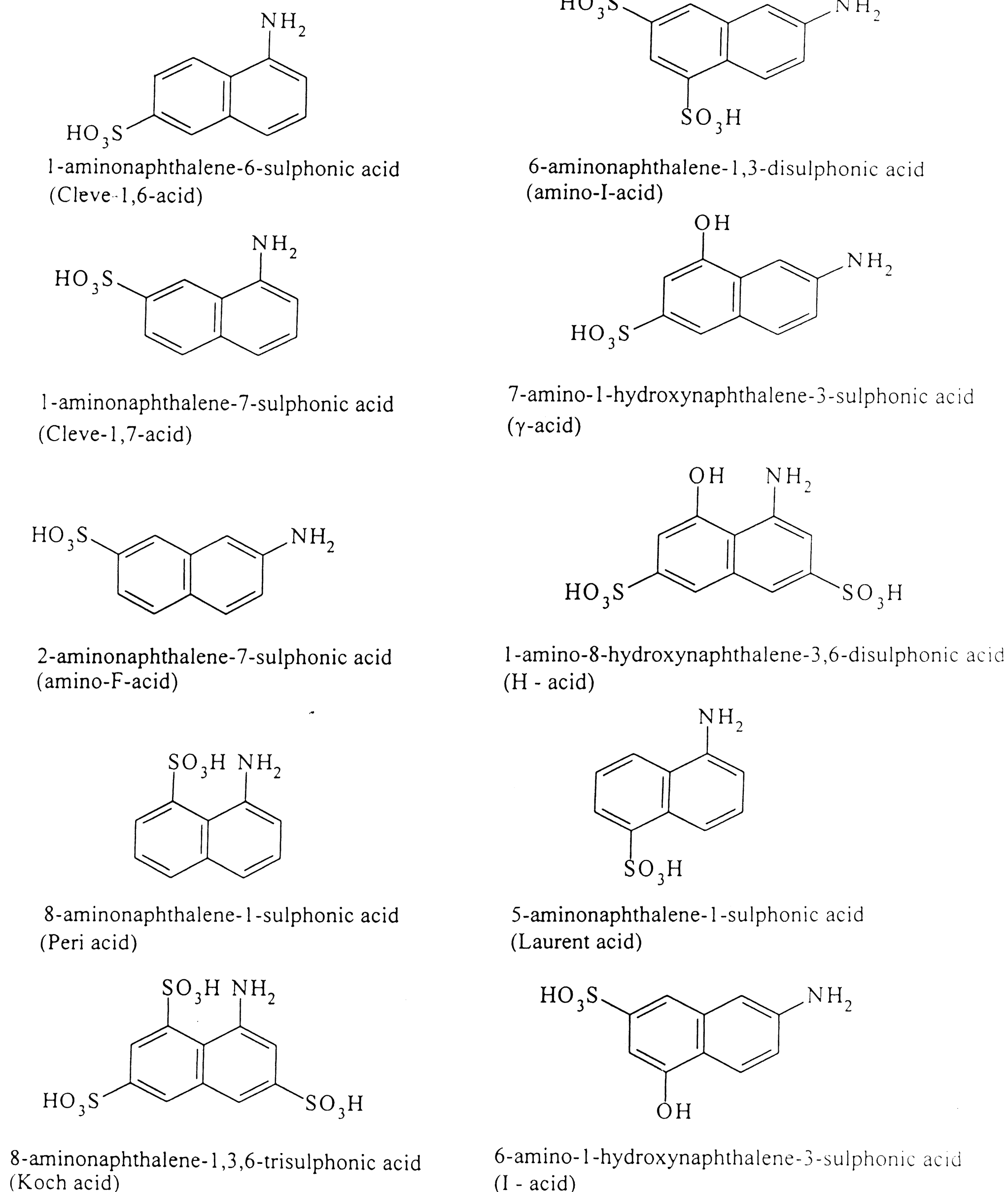

Fig. 1. Structures of aminonaphthalenesulphonic acids.

(TBAS) with or without addition of β-cyclodextrin (0.01 *M*) and triethylamine (TEA), 0.2–2.0%. The mobile phase components were pre-mixed in appropriate volume ratios and degassed by ultrasonication before the use. 60 nl of solutions of samples of sulphonic acids dissolved in the mobile phase were injected in all experiments.

The flow-rate of the mobile phase was kept constant at 3 or 5 μl/min in all separations by using the flow-split ratio 1:5 and was regularly checked during the experiments using a microburette and a stop-watch. The temperature was ambient, 20±1°C. For the diode-array detection, wavelength interval from 220 to 230 nm was used to construct the chromatograms.

3. Results and discussion

3.1. Testing and characterization of HPLC microcolumns

Originally prepared columns contained glass paper bed supports, but these columns were not stable. Columns prepared with bed supports prepared by in-situ polymerizing solutions containing potassium silicate according to the earlier described procedures [48,49], were rugged and mechanically stable over a long period of time.

3.2. Column efficiency

A representative h–u plot for toluene as the test compound on a Biospher Si C_{18} column 1 is shown in Fig. 2. Minimum reduced plate height $h = H/d_p =$ 1.8 is obtained at $u_{min} = 0.8$ mm/s; i.e., at the volumetric flow-rate $F_m = 3$ μl/min. In all experiments, the flow-rate was kept close to the optimum conditions, either at 3 μl/min or at 5 μl/min.

3.3. Column hold-up volume

Exact values of the column hold-up volume, V_M, should be known for calculation of the retention factors of sample compounds, k, of the column porosity and other important characteristics of separation. Direct determination of V_M of capillary columns as the elution volume of an inert compound is subject to positive errors because of significant extra-column contributions of the injector, connecting

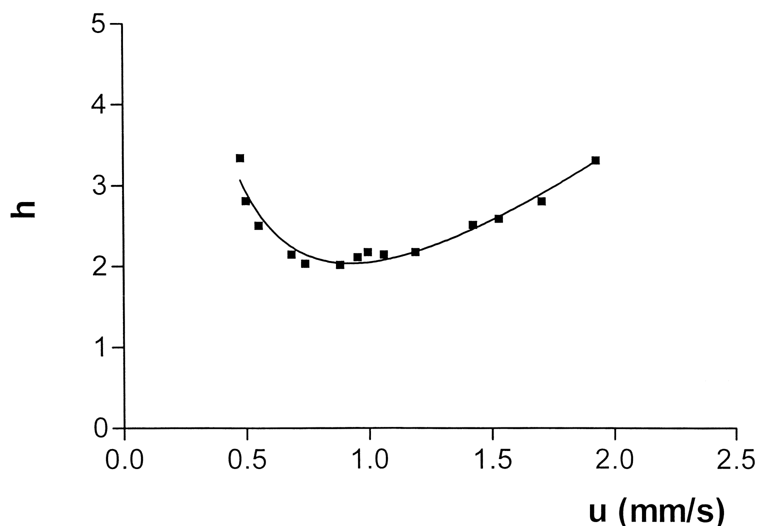

Fig. 2. Efficiency of a Biospher Si C_{18}, 5 μm, microcolumn, 135×0.32 mm I.D., in dependence on the flow-rate of the mobile phase [70% (v/v) methanol in water]. Test compound: toluene, $k = 2.75$, $h = H/d_p$ is the reduced height equivalent to a theoretical plate. Detection: UV, 254 nm. Ambient temperature.

capillaries (liner) and of the frits. Hence, we employed a method based on regular retention behavior of homologous compounds series [50] for the calculation of the column hold-up volume. However, we had to modify the method of calculation of V_M to take into account the extra-column contributions, V_{ex}. Both V_M and V_{ex} can be calculated from the slope and from the intercept of Eq. (1):

$$V_R = V_{ex} + V_M[1 + 10^{(\beta + \alpha.n)}] \quad (1)$$

where V_R is the uncorrected experimental retention volume of homologous n-alkylbenzenes with n carbon atoms in the alkyl chain and α, β are the constants of the dependence

$$\log k = \log \beta + n \log \alpha$$

determined on a conventional analytical column, packed with the same sorbents. The sums of the V_M and V_{ex} were in good agreement with the values of the uncorrected elution volumes of uracil as the inert compound. The values of V_M and V_{ex} are given in the Experimental section.

3.4. Repeatability

The repeatability of the retention volumes was tested in four repeated separations of phenol, acetophenone, 2-bromonitrobenzene and 3-chlorotoluene as the standard compounds for the calibration of retention of a capillary column 1 in methanol–water (70:30) as the mobile phase at the flow-rate of 5 μl/min. The standard deviation of the repeated runs was in the range 0.12% for the least retained phenol ($k = 2.4$) to 0.24% for the most retained 3-chlorotoluene ($k = 16.8$).

3.5. Separation of naphthalenesulphonic acids

In our previous work dealing with the separation of mono-, di-, tri- and tetrasulphonic acids on conventional analytical octadecyl silica columns, we found better separation of the positional isomers in mobile phases containing a strong electrolyte (0.4 *M* Na_2SO_4) than in ion-pair chromatography with mobile phases containing tetrabutylammonium ions [13,22,25]. The retention factors, k, of naphthalene tri- and tetrasulphonic acids on a Biospher C_{18} capillary column 2 with a glass paper bed support in 0.4 *M* Na_2SO_4 mobile phase are similar to, or slightly larger than, k on a conventional Biospher glass cartridge column with ten times larger inner diameter (Table 1). However, the values of k of naphthalenedisulphonic acids, except for 1,5-NDSA, are significantly greater on the conventional column, than on the capillary column. Further, the addition of methanol to the mobile phase has an unexpected effect — the retention factors of all NSAs on the microcolumn increased in mobile phases with 10% methanol with respect to aqueous mobile phases, whereas very significant decrease of k was observed, as expected, on a conventional Biospher column. In our opinion, the retention behavior of NSAs on the microcolumn can be possibly explained by mixed

Table 1
Retention factors, $k = (t_R - t_M)/t_M$, of aromatic sulphonic acids on: a Biospher Si C_{18}, 5 μm (140×0.32 mm I.D.) micro-HPLC column) and a Biospher Si C_{18}, 7 μm (150×3.3 mm I.D.) analytical column in mobile phases: (I) 0.4 *M* Na_2SO_4 in water and (II) 10% (v/v) methanol in 0.4 *M* Na_2SO_4 in water. Chromatographic conditions as in the Experimental section

Sample	Biospher Si C_{18}, 5 μm 140×0.32 mm I.D. micro-HPLC		Biospher Si C_{18}, 7 μm 150×3.3 mm I.D.	
	I	II	I	II
1,3-NDSA	2.32	2.68	5.72	0.94
1,5-NDSA	1.15	1.25	0.88	<0
1,6-NDSA	1.37	1.61	2.09	0.22
1,7-NDSA	4.54	5.25	8.16	2.21
1,3,5-NTSA	0.47	1.09	0.28	<0
1,3,6-NTSA	0.61	0.92	0.49	<0
1,3,7-NTSA	0.60	0.86	0.62	0.24
1,3,5,7-NTeSA	0.11	0.26	0.12	<0

retention mechanism. The ceramic column end frits made of silica gel were used in most experiments (columns 3–5), as the capillary columns with a glass paper bed support showed poor stability. The microcolumns with the ceramic frits contain parts with silica gel in addition to the C_{18} packing material, so that the retention of sample compounds is similar as on two columns in series, one with a C_{18} packing and the other packed with silica gel. On the microcolumns with ceramic frits, all naphthalene di-, tri-, and tetrasulphonic acids were poorly resolved and were eluted close to, or even before the column hold up volume, whereas the two naphthalene monosulphonic acids were strongly retained and 0.4 *M* Na_2SO_4 in 30% methanol was necessary to accomplish their elution in reasonable time ($k=5.29$ for 1-NMSA and $k=8.27$ for 2-NMSA). Possible explanation is in the effect of negatively charged SiO^- groups in the frits, which cause ionic exclusion of the molecules carrying multiple negative charges. These groups are obviously present in higher concentrations in the ceramic frits than in the glass paper bed support. In mobile phases with methanol, the repulsive forces between the acid anions and the negatively charged SiO^- groups in the frits are decreased with respect to aqueous sodium sulphate.

To overcome this effect, triethylamine (TEA) was added to the mobile phase in order to block the free SiO^- groups. The retention of all NDSAs and NTSAs increased with increasing concentration of TEA in 0.4 *M* Na_2SO_4 mobile phases (Fig. 3), which allowed significantly improved separation of four naphthalenedisulphonic acids (Fig. 4C) with respect to the mobile phase containing only sodium sulphate (Fig. 4A). Increasing retention and improved separation of NDSAs was observed also when adding β-cyclodextrin (CD), 0.01 *M*, to the mobile phase (Fig. 4B), but the retention of NTSAs and NTeSA was not affected (Table 2). The effect of CD on the retention of NDSAs may be explained by the formation of complexes with CD by hydrophobic interactions of the naphthalene rings with the hydrophobic cavity of CD and by hydrogen bonding interactions of the sulphonic acid groups with hydroxyl groups at the ''mouth'' of the cyclodextrin cavity. The isomeric NDSAs were better separated in mobile phases containing TEA than in mobile phases with CD. The addition of CD did not affect the

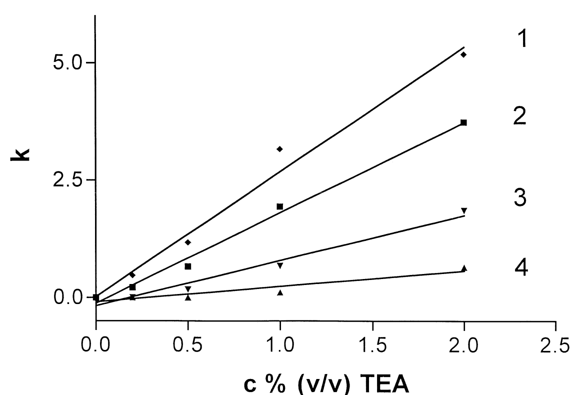

Fig. 3. Retention factors of NDSAs on a Biospher Si C_{18}, 5 μm, 166×0.32 mm I.D. column in mobile phases with various concentrations of triethylamine (TEA) in 0.4 *M* Na_2SO_4. Conditions as in the Experimental section.

behavior of NTSAs, which were excluded and eluted before the column hold-up volume, like in mobile phases containing only sodium sulphate. On the other hand, isomeric NTSAs were not separated in mobile phases containing TEA only, but addition of TEA and CD resulted in improved separation of NTeSA and isomeric NTSAs, except for the 1,3,6- and 1,3,7-isomers, which were poorly resolved (Table 2).

Generally, similar behavior as on the Biospher Si C_{18} microcolumns was observed on microcolumns packed with Superspher ODS (Merck, results are not shown) and with Zorbax XDB, C_{18} sorbents (Table 2 and Fig. 5A–C). However, the character of the column packing material has strong effect on the retention behavior of naphthalenesulphonic acids. On the Zorbax XDB C_{18} column, addition of 2% TEA to the mobile phase was necessary to resolve all four isomeric NDSAs (Fig. 5C). On this column, isomeric NDSAs can be resolved also in mobile phases containing only CD, but the separation takes much longer time than in the mobile phase with 2% TEA. Simultaneous addition of TEA and CD to the mobile phase leads to minor increase of retention and some changes in the separation selectivity with respect to the mobile phases containing only TEA, but the resolution and the time of the analysis do not change dramatically (Table 2).

The effects of the mobile phase additives on the retention behavior of NTSAs were similar as with

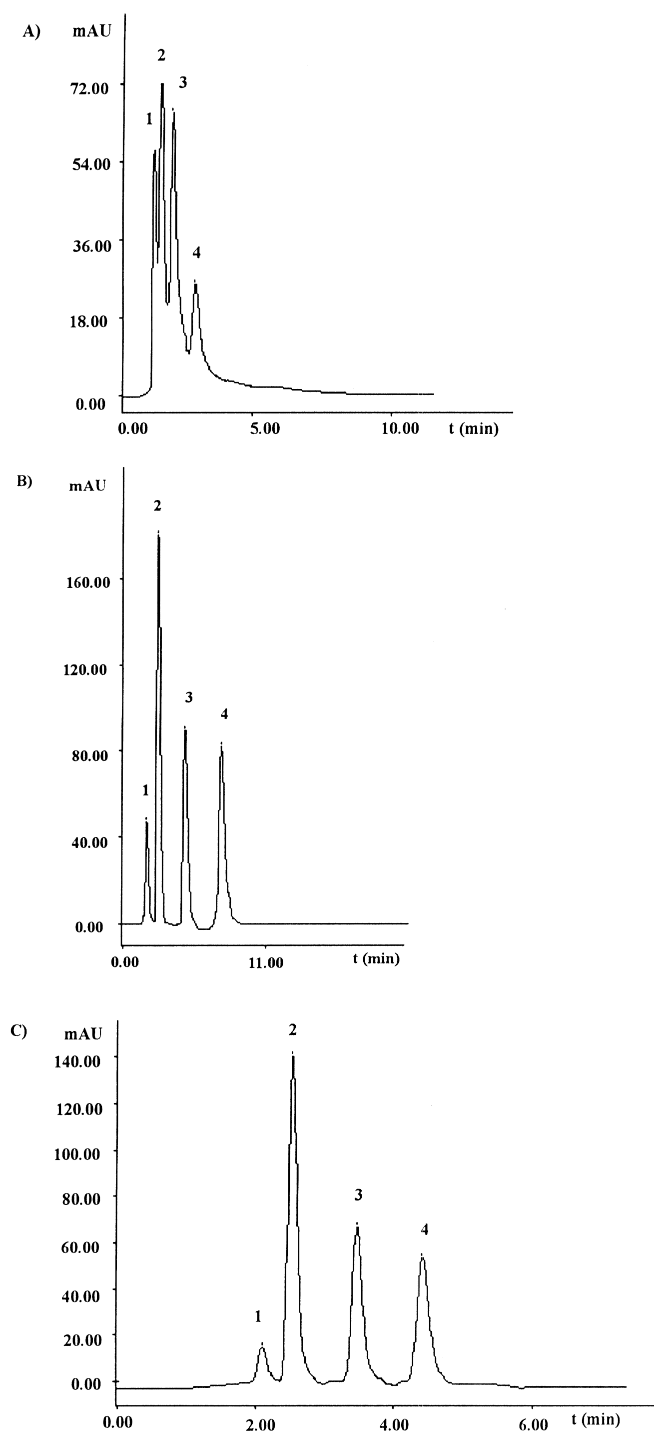

Fig. 4. Comparison of micro-HPLC separations of naphthalenedisulphonic acids: (1) 1,5-NDSA; (2) 1,6-NDSA; (3) 1,3-NDSA and (4) 1,7-NDSA on a Biospher Si C_{18}, 5 μm, 166×0.32 mm I.D. column; with mobile phases: (A) 0.4 *M* Na_2SO_4 in water, (B) 0.4 *M* Na_2SO_4 in water with 0.01 *M* β-cyclodextrin and (C) 0.4 *M* Na_2SO_4 in water with 0.5% triethylamine; flow-rate: 5 μl/min, column temperature: ambient, detection, UV, 220–230 nm.

Table 2
Retention factors, $k=(t_R-t_M)/t_M$, of aromatic sulphonic acids on: a Biospher Si C_{18}, 5 μm (166×0.32 mm I.D.) micro-HPLC column and a Zorbax XDB, Eclipse Si C_{18}, 5 μm (146×0.32 mm I.D.) micro-HPLC column in mobile phases: (I) 0.4 *M* Na_2SO_4 in water with 0.01 *M* β-cyclodextrin, (II) 0.4 *M* Na_2SO_4 in water with 2% TEA and (III) 0.4 *M* Na_2SO_4 in water with 2% TEA with 0.01 *M* β-cyclodextrin. Chromatographic conditions as in the Experimental section

Sample	Biospher Si C_{18}, 5 μm 166×0.32 mm I.D. micro-HPLC			Zorbax XDB, Eclipse Si C_{18}, 5 μm 146×0.32 mm I.D. micro-HPLC		
	I	II	III	I	II	III
1,3-NDSA	1.60	2.36	–	5.19	0.65	1.01
1,5-NDSA	0	0.65	–	1.31	<0	0.14
1,6-NDSA	0.18	1.86	–	2.90	0	0
1,7-NDSA	3.51	4.56	–	7.03	2.06	2.54
1,3,5-NTSA	<0	0.15	1.43	2.22	0	0
1,3,6-NTSA	<0	0.15	1.80	2.22	0.26	0.73
1,3,7-NTSA	<0	0.15	1.94	2.22	1.83	2.18
1,3,5,7-NTeSA	<0	0.15	0.94	2.22	<0	<0

NDSAs. The separation of the three naphthalenetrisulphonic acids and 1,3,5,7-NTeSA on the Zorbax XDB C_{18} column can be accomplished in the mobile phase containing 2% TEA (Fig. 6A), but the retention slightly increased and the resolution significantly improved when β-cyclodextrin was added to the mobile phase containing 2% TEA (Fig. 6B).

The strong effect of the column packing material on the retention behavior of naphthalenesulphonic acids can be explained by different nature of the silica gels used for the production of the octadecyl silica materials and by different bonding and end-capping chemistry, which results in different amount and spacing of residual silanol groups on the surface of the column packing material. This results in different effects on the exclusion of isomeric acids and in different requirements on the suppression of silanophilic interaction by their neutralisation with TEA and by the formation of the inclusion complexes with β-cyclodextrin, so that the optimum composition of the mobile phase strongly depends on the character of the column packing material.

Table 3
Retention factors, *k*, of aromatic sulphonic acids on a Biospher Si C_{18}, 5 μm, column, 162×0.32 mm I.D. (I, III) and a Separon SGX RPS, 7 μm, conventional analytical column, 150×3 mm I.D. (II) with mobile phases containing 0.005 *M* TBAS in 15% methanol in water (I, II) and 0.005 *M* TBAS in 15% methanol in water with β-cyclodextrin (III)

Compound	I	II	III
Laurent acid	1.40	1.19	1.57
Amino-F acid	2.40	2.25	2.55
Cleve-1,6-acid	3.76	3.76	3.89
Peri acid	3.99	4.66	4.61
Cleve-1,7-acid	11.94	11.60	13.58
I-acid	1.36		1.58
Amino-I acid	4.55		5.98
H-acid	4.91		5.61
γ-acid	1.64		2.38
Koch acid	2.01		3.27

3.6. Separation of aminonaphthalenesulphonic acids

The retention factors, *k*, of aminonaphthalenesulphonic acids studied are listed in Table 3. On a conventional analytical column, all acids can be successfully resolved in 0.005 *M* tetrabutylammonium hydrogensulphate in 15% methanol [28]. The values of *k* on a Biospher microcolumn are very close to the data on the analytical column, except for a lower *k* of Peri acid, which results into co-elution with the Cleve-1,6-acid (Fig. 7A). However, the addition of 0.01 *M* β-cyclodextrin to the mobile phase results in increased retention and improved separation of all acids occurring in technological mixtures during the production of Cleve acids (Fig. 7B). The method using the mobile phase with β-cyclodextrin on a Biospher C_{18} microcolumn can be used for quantitative analysis of such mixtures, as the calibration curves are linear (Fig. 8).

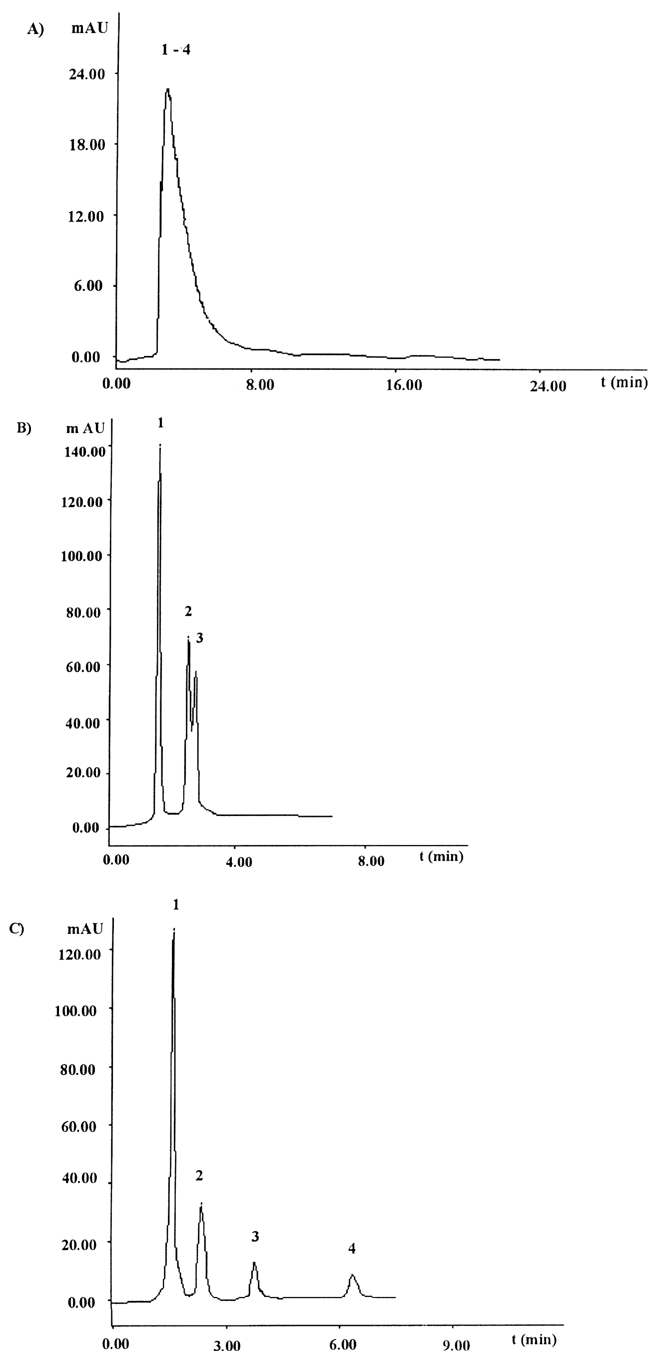

Fig. 5. Comparison of micro-HPLC separations of naphthalenedisulphonic acids: (1) 1,5-NDSA; (2) 1,6-NDSA; (3) 1,3-NDSA and (4) 1,7-NDSA on a Zorbax Eclipse, C_{18}, 5 μm, 146×0.32 mm I.D. column; with different mobile phases: (A) 0.4 *M* Na_2SO_4 in water, (B) 0.4 *M* Na_2SO_4 in water with 1% triethylamine and (C) 0.4 *M* Na_2SO_4 in water with 2% triethylamine; flow-rate: 5 μl/min, column temperature: ambient, detection, UV, 220–230 nm.

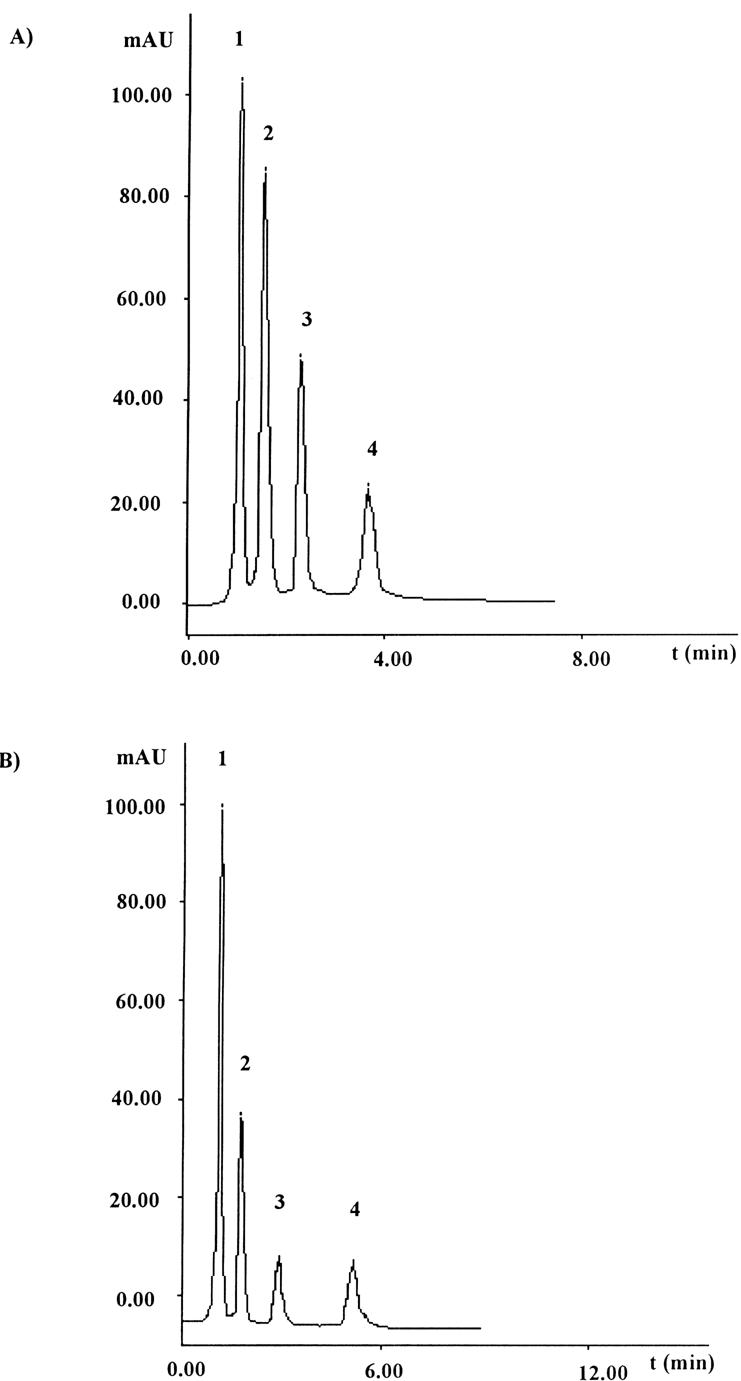

Fig. 6. Comparison of micro-HPLC separations of naphthalene tri- and tetrasulphonic acids: (1) 1,3,5,7-NTeSA; (2) 1,3,5-NTSA; (3) 1,3,6-NTSA and (4) 1,3,7-NTSA on a Zorbax Eclipse, C_{18}, 5 μm, 146×0.32 mm I.D. column; with different mobile phases: (A) 0.4 *M* Na_2SO_4 in water with 2% triethylamine and (B) 0.4 *M* Na_2SO_4 in water with 2% triethylamine and 0.01 *M* β-cyclodextrin; flow-rate: 5 μl/min, column temperature: ambient, detection, UV, 220–230 nm.

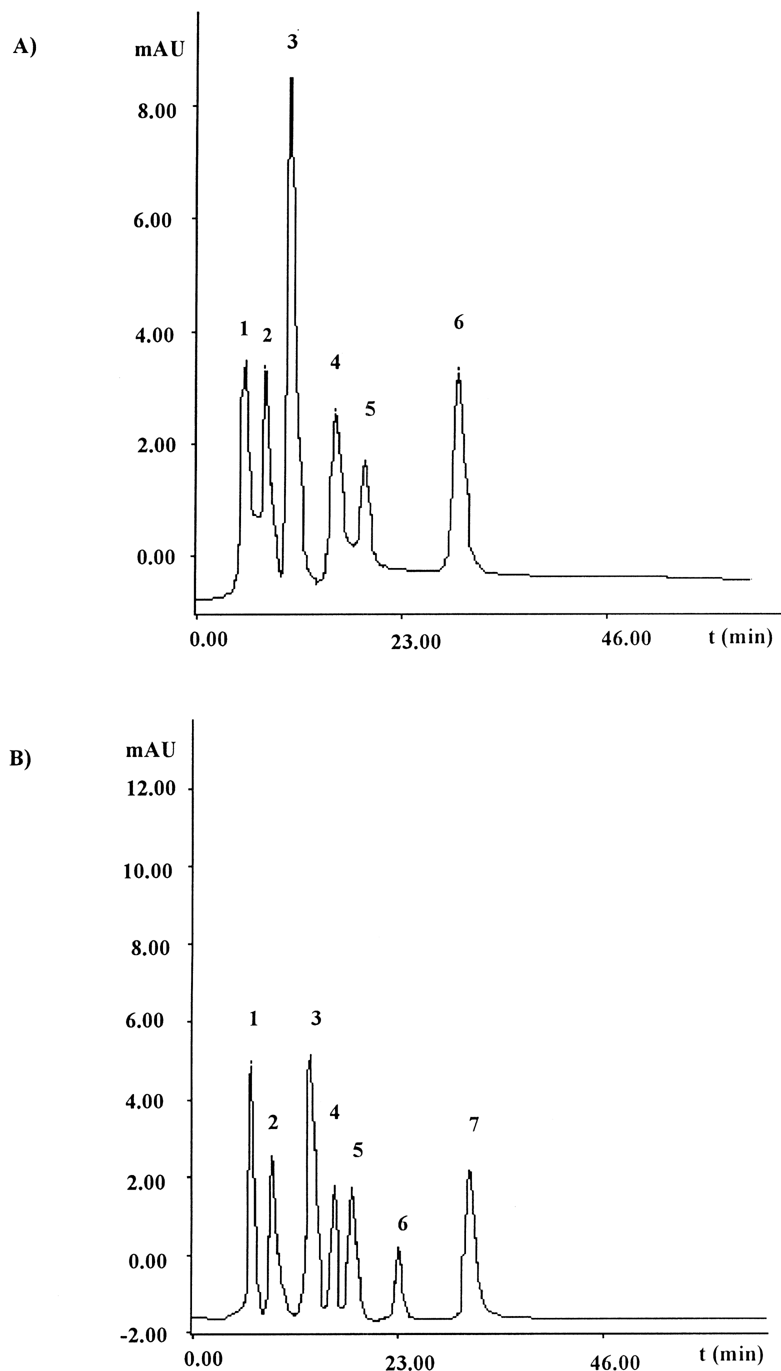

Fig. 7. Comparison of micro-HPLC separations of aromatic sulphonic acids in different mobile phases: (A) 0.005 *M* tetrabutylammonium hydrogensulphate (TBAS) in 15% (v/v) methanol in water; (1) Laurent acid, (2) amino-F-acid, (3) Cleve-1,6- and Peri acids, (4) unidentified impurity, (5) Cleve-1,7-acid and (6) unidentified impurity. (B) 0.005 *M* tetrabutylammonium hydrogensulphate (TBAS) in 15% (v/v) methanol in water with 0.01 *M* β-cyclodextrin (CD): (1) Laurent acid, (2) amino-F-acid, (3) Cleve-1,6-acid, (4) Peri acids, (5) unidentified impurity, (6) Cleve-1,7-acid and (7) unidentified impurity. Column, Biospher Si C_{18}, 5 μm, 162×0.32 mm I.D.; flow-rate: 5 μl/min, column temperature: ambient, detection, UV, 220–230 nm.

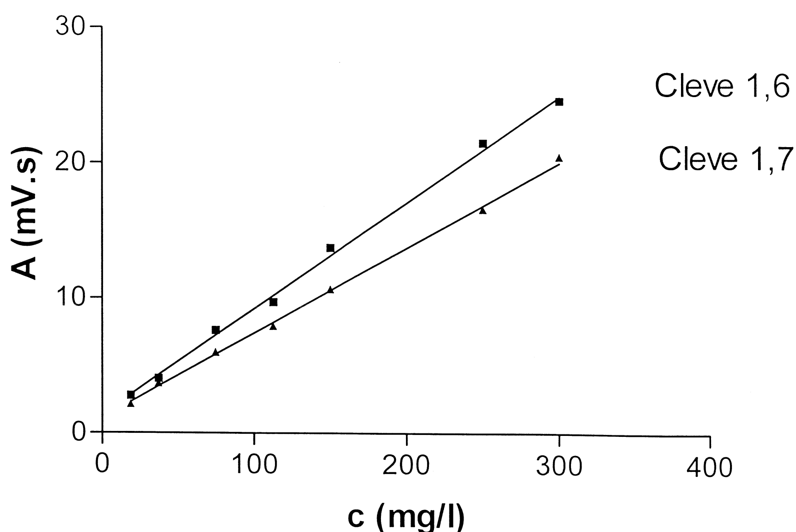

Fig. 8. Calibration curves of (1) Cleve-1,6- and (2) Cleve-1,7-acids on a Biospher Si C_{18}, 5 μm, 162×0.32 mm I.D. column in mobile phase 15% (v/v) methanol in water with 0.005 *M* tetrabutylammonium hydrogensulphate; flow-rate: 5 μl/min, column temperature: ambient, detection, UV, 220–230 nm. Equations of the calibration lines: Cleve 1,6: $A=(0.0792\pm0.0018)c+(1.3485\pm0.3028)$; Cleve 1,7: $A=(0.0638\pm0.0011)c+(1.0830\pm0.1887)$.

4. Conclusions

Stable packed microcolumns (0.32 mm I.D.) for reversed-phase HPLC with $h=1.8$–2.0 were prepared using a supercritical fluid slurry packing technique. HPLC on microcolumns has the advantage of a low consumption of mobile phase, so that expensive mobile phase additives can be used without increasing significantly the costs per analysis. However, the bed supports in the capillary column, especially the ceramic frits, may contain SiO^- groups that interfere with the reversed-phase separations of very hydrophilic or ionic compounds. This effect can be overcome by addition of an aliphatic amine, such as triethylamine (TEA), to the mobile phase. Using this approach, isomeric naphthalenedisulphonic acids could be successfully separated. Complexation of naphthalenesulphonic acids with β-cyclodextrin leads to increased retention and modifies the separation selectivity. Some naphthalenesulphonic acids can be separated in mobile phases containing TEA, other in mobile phases with β-cyclodextrin, but successful separation of some mixtures requires simultaneous addition of the two additives to the mobile phases. The optimum combination and concentrations of the mobile phase additives strongly depends on the character of the column packing material.

On a Zorbax XDB column, the separation of NDSAs was possible using mobile phases containing β-cyclodextrin instead of TEA. On this column, isomeric naphthalenetrisulphonic acids could be separated using mobile phases with addition of TEA and with or without β-cyclodextrin. β-Cyclodextrin was required as a mobile phase additive also for successful separation and analysis of amino-naphthalenesulphonic acids in technological mixtures occurring in production of Cleve acids as dye intermediates.

Using an alkylbenzene homologous series for the calibration of the retention scale, exact determination of the hold-up volume of the capillary columns and of the extra-column contributions of frits, injector, liner and couplings was possible.

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