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Naphthalene sulphonic acids—new test compounds for characterization of the columns for reversed-phase chromatography

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Abstract

Non-substituted naphthalene sulphonic acids are strong acids, which are completely ionised in aqueous and aqueous-organic solutions. Because of repulsive electrostatic interactions, they are more or less excluded from the pores of the column packing materials commonly used in reversed-phase chromatography. The ionic exclusion can be suppressed by increasing the ionic strength of the mobile phase. In aqueous sodium sulphate solutions, very good selectivity was observed for isomeric naphthalene di- and tri-sulphonic acids, allowing reversed-phase separations of these strongly ionic compounds without addition of ion-pairing reagents to the mobile phase. The retention of the isomeric acids increases proportionally to the dipole moment, which can be explained by its effect on increasing exposure of the naphthalene ring to hydrophobic interactions with the non-polar stationary phases. Chromatographic behaviour of isomeric naphthalene di- and tri-sulphonic acids was investigated on 25 different columns for reversed-phase chromatography. The elution order of the isomers is the same on all the columns, but very strong stationary phase effects were observed on the retention and on the band asymmetry, depending on polar interactions with residual silanol groups and other polar adsorption centres in the stationary phases. These effects are independent of the organic solvents, as the tests are performed in purely aqueous mobile phases and allow classification of the columns into several groups.

Keywords: Naphthalene sulphonic acids; Stationary phases; Separation selectivity; Column characterisation

1. Introduction

Various columns used in contemporary reversed-phase chromatography have different chromatographic properties and even nominally identical columns supplied by the same manufacturer may show different efficiency and reproducibility of the retention and selectivity. Specific polar and non-specific solvophobic interactions between the stationary phase, the analyte and the mobile-phase components, which control the retention, depend on the properties of the chemically bonded ligands (alkyl chains, aryl-, amino groups, etc.). Silanol and other polar groups on the surface of silica gel support affect the retention of non-ionic polar compounds by hydrogen bonding interactions and the retention of ionic compounds, especially the basic ones, by electrostatic interactions causing peak asymmetry and variations in the retention and selectivity [1].

The column performance is usually characterized by simple tests based on the determination of the separation factors and peak asymmetry of several (rather arbitrarily) selected test compounds. Most frequently used tests suggested by Engelhardt et al. [2,3], Tanaka and co-workers [4], Walters [5], Bidlingmeyer et al. [6], or Galushko [7] to characterize column hydrophobicity, shape selectivity and residual silanol activity are usually run in a single mobile phase with 30–80% methanol or acetonitrile in water. However, it is well known that not only the type of the organic modifier, but also its

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concentration in the mobile phase strongly affects the retention and very often the separation selectivity, too. Hence, the mobile phase effects may limit the validity of the simple tests.

Many ionic compounds with bulk non-polar parts of molecules can be separated in reversed-phase systems using mobile phases containing ionic additives in pure water or in mixed aqueous-organic mobile phases with low concentrations of an organic modifier. Even the adsorbents which do not possess ionisable surface groups can form an electrical charge on the surface in contact with an electrolyte solution, as a result of differences in the affinity of the adsorbent and the solution phase for the ions of one charge or the other [8]. The surface of bonded silica gel stationary phases contains a significant proportion of residual hydroxyl groups that cannot be removed or blocked even using the most effective silylation endcapping reactions due to steric hindrance [9]. These silanol groups on the silica surface are partially ionised to $\equiv SiO^-$ groups in pure water or in aqueous salt solutions [9]. In the solution adjacent to the negatively charged surface, there is an equivalent excess of counter ions of opposite charge sign to that of the stationary phase surface, which form a diffuse double layer [10,11]. The electrical double layer interacts by attractive or repulsive forces with ionised sample solutes [12–15] and affects thus their retention. These forces affect significantly the separation selectivity and often enable surprisingly good separations of geometrical isomers. The double layer thickness is usually less than 10 nm and it decreases when the ionic strength of the solution increases [16].

A combination of various attractive and repulsive interactions with different types of adsorption centres may result in significant band tailing. The stochastic theory of chromatography [17,18] explains this behaviour and was employed to describe asymmetrical band shape of enantiomers on some chiral stationary phases [19]. This is also possible reason for strong peak tailing of polar, especially basic compounds on some bonded alkylsilica columns.

In the present work, we investigated possibilities of using ionic test compounds in reversed-phase chromatography for characterisation of stationary phases for reversed-phase HPLC. We focused our attention on naphthalene di- and tri-sulphonic acids, whose earlier observed chromatographic behaviour shows strong dependence on the column type [20,21].

2. Experimental

2.1. Instrument

The liquid chromatograph was comprised of an LC-10AD pump (Shimadzu, Kyoto, Japan), an LCO 101 column thermostat, an LCP 2564 UV detector operated at 254 nm (both from ECOM, Prague, Czech Republic) and a personal computer to process the detector signal using a CSW 1.7 data station for chromatography (Data Apex, Prague, Czech Republic). The solutes were injected with a Rheodyne model 7125 sampling valve with a 20 μl sample loop (Berkeley, CA, USA).

2.2. Materials

The trade names and the manufacturers of 25 column tested are listed in Table 1. Some columns were purchased, other were obtained as a gift or a loan. Methanol for HPLC (Lichrosolv grade) was purchased from Merck, Darmstadt, Germany. Distilled water was purified using a Milli-Q water purification station (Millipore Intertech, Bedford, MA, USA). Sodium sulphate (reagent grade) was obtained from Lachema (Brno, Czech Republic) and dissolved in water in required concentration to prepare the mobile phases, which were filtered using a Millipore 0.45 μm filter and degassed in ultrasonic bath before use. The standard samples of naphthalene sulphonic acids were obtained from Synthesia (Semtín, Czech Republic); the structures and dipole moments are shown in Fig. 1.

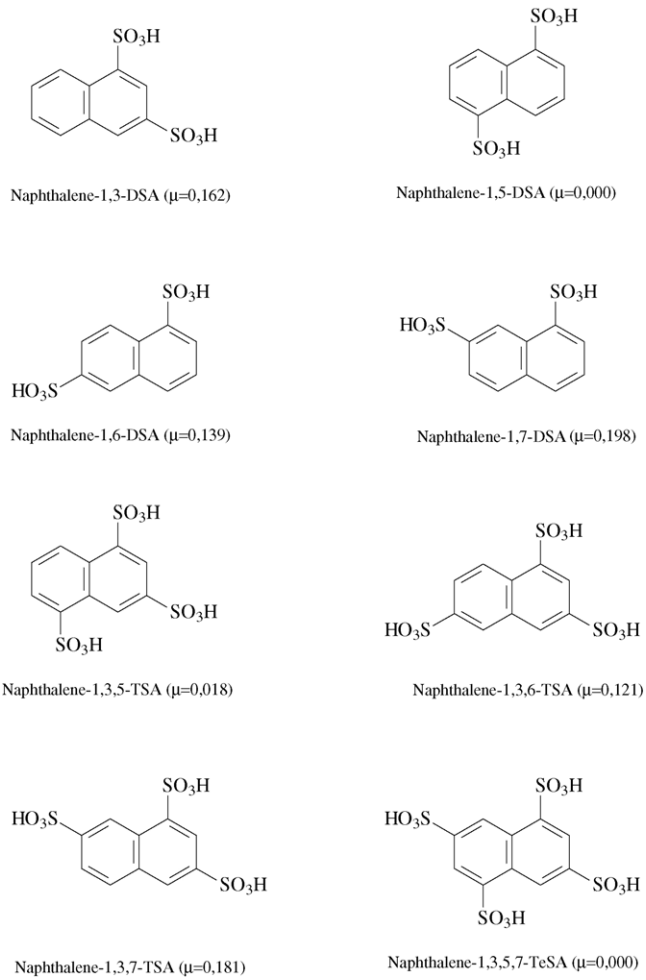

Fig. 1. Structures of naphthalene sulphonic acid test compounds and dipole moments, μ.

Table 1
Properties of the columns tested

Column number	Trade name, dimensions ($L \times$ i.d)	V_M	% C	S	N	F_{as}	Manufacturer
1	Luna C18 (2), 150 × 4.6	1.61	17.3	374	95300	1.0	Phenomenex, Torrance, CA, USA
2	Zorbax 300Extend-C18, 150 × 4.6	1.48	–	180	84700	1.2	Agilent, Palo Alto, CA, USA
3	Luna phenylhexyl, 150 × 4.6	1.69	17.5	386	85300	1.0	Phenomenex, Torrance, CA, USA
4	Hypersil ODS, 60 × 4.6	0.61	10.0	170	108300	1.2	Agilent, Palo Alto, CA, USA
5	XTerra MS C18, 30 × 4.6	0.32	15.0	175	50000	2.0	Waters, Milford, MA, USA
6	Inertsil ODS 2, 250 × 4.6	2.01	18.5	320	75315	1.5	GL Sciences, Tokyo, Japan
7	Zorbax RX C18, 250 × 4.6	2.01	12.0	180	10400	2.0	Agilent, Palo Alto, CA, USA
8	Aqua 5 μ C18125A, 150 × 3.0	0.65	15.0	320	44700	1.3	Phenomenex, Torrance, CA, USA
9	Chromolith Performance RP-18e, 100 × 4.6	1.42	17.0	300	86000	1.2	Merck, Darmstadt, Germany
10	Atlantis column, 150 × 3.9	0.93	12.1	323	54700	2.2	Waters, Milford, MA, USA
11	Alltima C185 μ, 250 × 4.6	2.18	16.1	311	64000	2.4	Alltech, Deerfield, IL, USA
12	Purospher RP-18e, 250 × 4.0	2.55	18.0	350	38000	1.4	Merck, Darmstadt, Germany
13	LiChrosorb RP 8, 250 × 4.0	2.36	9.5	–	78400	1.2	Merck, Darmstadt, Germany
14	Biospher SI C18, 150 × 3.3	1.01	–	–	45000	1.8	Labio, Praha, Czech Republic
15	Luna C18, 150 × 4.6	1.36	19.4	424	80700	1.1	Phenomenex, Torrance, CA, USA
16	Polymer C18, 150 × 4.6	1.37	–	–	66000	2.0	Astec, Whippany, NJ, USA
17	Zorbax SB-Aq, 250 × 4.6	2.47	–	180	88000	2.0	Agilent, Palo Alto, CA, USA
18	LiChrospher 60 RP select B, 250 × 4.0	2.20	11.5	700	65600	1.0	Merck, Darmstadt, Germany
19	Zorbax 300 SB-C18, 150 × 4.6	1.36	10.0	180	81300	1.0	Agilent, Palo Alto, CA, USA
20	Zorbax Eclipse XDB C8, 150 × 4.6	1.29	10.3	180	133300	1.0	Agilent, Palo Alto, CA, USA
21	Separon SGX C8, 150 × 3.0	1.06	–	500	35000	1.0	Tessek, Praha, Czech Republic
22	Separon SGX C18, 150 × 3.0	0.88	18.0	–	40000	1.0	Tessek, Praha, Czech Republic
23	Nova-Pak C18, 150 × 3.9	1.10	7.3	120	68000	1.0	Waters, Milford, MA, USA
24	Luna C8 (2), 150 × 4.6	1.68	14.2	411	92000	1.0	Phenomenex, Torrance, CA, USA
25	Silasorb SPH C18, 250 × 4.0	2.54	–	–	38000	1.0	Lachema, Brno, Czech Republic

L: column length (mm), i.d.: inner diameter (mm), V_M: hold-up volume (ml), S: specific surface area ($m^2\,g^{-1}$), N: number of theoretical plates (toluene, 50%AcN) (m^{-1}), F_{as}: factor of asymmetry (aniline, 50% AcN).

2.3. Methods

The sulphonic acids were dissolved in the mobile phases to yield solutions of approx. concentrations 0.01 mg/ml. Twenty microliters of samples were injected in all experiments. The mobile phase was degassed in the solvent reservoir by continuous stripping by a stream of helium. The flow rate of mobile phase was kept at $1\,ml\,min^{-1}$ and the temperature at 40 °C in all experiments, which were performed in triplicate. The mean values of the retention times were used to calculate the retention factors and the elution ratios. The column hold-up volumes were determined using uracil as non-retained marker with 50% methanol as the mobile phase and are listed in Table 1. The dipole moments of naphthalene sulphonic acids were calculated using PISYSTEM for Windows, version 3.1, obtained from Dr. Rudolf Naef, Im Budler 6, CH-4419 Lupsingen, Switzerland. Microsoft Office software was used for data processing and calculations.

3. Results and discussion

The elution times of naphthalene tetra-, tri- and most disulphonic acids in water or in aqueous-organic mobile phases without ionic additives were lower than the corresponding column hold-up (dead) volumes, i.e., these acids are excluded from the pores of the packing materials. As the molecular weights of the acids tested are in between 146 and 210, it is highly unlikely that this effect could be attributed to steric exclusion from the packing materials with pore size 20–100 Å and the most probable explanation is ionic repulsion by Coulombic forces between the negatively charged stationary phase surface and sulphonic acid anions. The acidity of non-substituted naphthalene sulphonic acids is comparable to strong mineral acids and these compounds are completely ionised to anions in aqueous mobile phases. Hence, they are more or less strongly repulsed from the negatively charged surface of the reversed-phase column packing materials by electrostatic forces, which depend on various factors: (a) the charge of the sample anions corresponding to the number of sulphonate groups in the molecules, (b) the spacing of the sulphonate groups in isomer molecules, (c) the ionic strength and the concentration of organic solvents in the mobile phase (on the other hand, the retention is little affected by pH because of the complete ionisation of samples), and (d) the properties of the column packing material, controlling the surface hydrophobicity, such as the chemistry and the carbon content of the bonded phase, the surface coverage with bonded ligands and the number and spacing of polar groups such as residual silanol groups remaining on the original silica gel surface after the bonding and end-capping chemical reactions.

First, we tested the effect of the concentration of sodium sulphate on the retention of naphthalene di-, tri- and tetrasulphonic acids. For this purpose, we used a Purospher RP-18e column. The retention volumes of the samples increase in

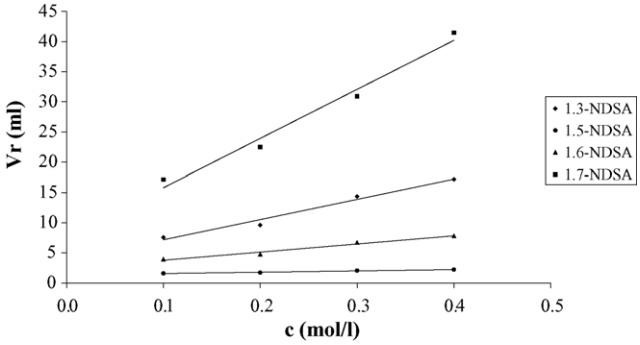

Fig. 2. Effect of the concentration, c, of Na_2SO_4 on the elution volumes, V_R, of isomeric naphthalene disulphonic acids. Column 12, Purospher RP-18e, 250 mm × 4 mm i.d.

approximately linear manner with increasing molar concentration of sodium sulphate in the mobile phase—see plots in Fig. 2 and correlation coefficients in Table 2. Both the slopes p and the intercepts q of the plots increase as the number of sulphonic groups decreases and as the retention of isomers with equal numbers of sulphonic acid groups increases (Table 2). This behaviour is consistent with the earlier described effects of decreasing thickness of the electrical double layer at the adsorbent surface in solutions with a higher ionic strength [16]. At a decreased double-layer thickness, the electrostatic repulsion forces between the ionised silanol groups and the sulphonic acids anions decrease and a larger part of the pore volume is accessible for the acids, enabling a closer contact of non-polar bonded alkyl ligands with the hydrophobic parts of the naphthalene moieties in the acid molecules. In addition, increased ionic strength enhances the differences in the retention enthalpy arising from dipole–dipole and dispersion interactions of the non-ionic parts of the solute molecules, as well as the entropy change associated with a decrease in solvent structuring that occurs when the solute leaves the mobile phase (the salting-out or solvophobic effect) [22].

However, the ionic strength in the mobile phases containing up to 0.4 M sodium sulphate is not sufficient to suppress the ionic exclusion of all sulphonic acids from the pores of the Purospher RP18 phase. The retention volumes of naphthalene-1,3,5,7-tetrasulphonic acid, all naphthalene trisulphonic acids and of naphthalene-1,5-disulphonic acid are lower than the column hold-up volume measured with uracil as non-retained marker in 50% methanol as the mobile phase, $V_M = 2.55$ ml, corresponding to the total column porosity $\varepsilon_T = 0.81$. 1,3,5-NTSA and 1,3,7-NTSA are excluded in mobile phases with less than 0.3 M Na_2SO_4 and 1,5-NDSA in solutions containing less than 0.2 M Na_2SO_4. This means that the column hold-up volume determined using uracil is not suitable for calculation of the retention factors, k, and elution ratios (relative retention) of naphthalene sulphonic acids. Therefore, the relative retention, $\alpha = (t_{Ri}/t_{R1} - 1)$ related to the least retained compound with zero dipole moment and the reference elution time t_{R1} (1,5-NDSA for NDSA isomers and 1,3,5,7-NTeSA for NTSA isomers), was used to characterise the retention instead of k. t_{R1} is the marker of the mobile phase volume accessible for the isomeric compounds with elution times $t_{Ri} > t_{R1}$.

Table 2
Retention data of NSA in mobile phase containing Na_2SO_4

	0.1 M	0.2 M	0.3 M	0.4 M
V_R				
1.3.5.7-NTeSA	1.24	1.31	1.38	1.48
1.3.6-NTSA	1.29	1.38	1.44	1.61
1.3.5-NTSA	1.39	1.53	1.66	1.91
1.3.7-NTSA	1.40	1.54	1.72	1.96
1.5-NDSA	1.59	1.73	2.06	2.21
1.6-NDSA	3.93	4.78	6.71	7.79
1.3-NDSA	7.59	9.64	14.37	17.20
1.7-NDSA	17.16	22.49	30.90	41.48
F_{as}				
1.3.5.7-NTeSA	2.10	2.10	2.18	2.27
1.3.6-NTSA	1.17	1.21	1.33	1.41
1.3.5-NTSA	1.77	1.71	1.55	1.50
1.3.7-NTSA	1.85	1.60	1.50	1.95
1.5-NDSA	1.80	1.75	1.64	1.64
1.6-NDSA	1.59	1.54	1.48	1.48
1.3-NDSA	1.60	1.57	1.54	1.54
1.7-NDSA	1.49	1.42	1.40	1.42
α				
1.3.6/1.3.5.7	1.04	1.05	1.05	1.09
1.3.5/1.3.5.7	1.12	1.17	1.20	1.29
1.3.7/1.3.5.7	1.13	1.17	1.25	1.33
1.6/1.5	1.47	1.75	2.26	2.52
1.3/1.5	3.77	4.56	5.98	6.76
1.7/1.5	9.79	11.96	14.01	17.73

	q	p	R^2
1.3.5.7-NTeSA	1.15	0.79	0.9906
1.3.6-NTSA	1.18	1.02	0.9587
1.3.5-NTSA	1.20	1.69	0.9758
1.3.7-NTSA	1.19	1.86	0.9841
1.5-NDSA	1.35	2.20	0.9754
1.6-NDSA	2.42	13.51	0.9780
1.3-NDSA	3.81	33.54	0.9791
1.7-NDSA	7.66	81.38	0.9794

Column: Purospher RP-18e (250 mm × 4 mm); V_R: retention volume (ml), F_{as}: factor of asymmetry, α: elution ratio [$\alpha = (V_{Rj}/V_{Ri}) - 1$], c_S: concentration of Na_2SO_4 (mol/l) [$V_R = q + pc_S$], R^2: correlation coefficient.

Like the retention volumes, α increase in mobile phases with higher concentrations of sodium sulphate. On the other hand, the asymmetry factors, which can be used a measure of interactions with two or more different adsorption centres (such as non-polar alkyl chains and silanols), slightly decrease at increasing ionic strength of the mobile phase for most compounds tested, probably because of some decrease in the activity of the polar adsorption centres, but this effect is not very significant (Table 3). The greatest asymmetry factors were observed for the earliest eluting 1,3,5,7-NTeSA, but are not very different from F_{as} of the NDSAs and NTSAs. The effect of extra-column band broadening can be ruled out, as the conventional diameter columns were connected to the injector via a short 0.12 mm i.d. capillary and non-retained compounds showed symmetrical bands.

The retention of the naphthalene sulphonic acids decreases as the number of sulphonic groups and hence the negative charge in the molecule increase. The elution order of the acid isomers is the same with all the columns tested in the group of naphthalene disulphonic acids: 1,5-NDSA < 1,6-NDSA < 1,3-NDSA < 1,7-NDSA and in the group of naphthalene sulphonic acids with a higher number of SO_3H groups: 1,3,5,7-NTeSA < 1,3,6-NTSA < 1,3,5-NTSA < 1,3,7-NTSA. The differences in the retention of isomeric acids can be probably explained by the effect of the spacing of sulphonic acid groups on the size of the unshielded part of the molecule that can be exposed to hydrophobic interactions with the non-polar bonded alkyls in the stationary phase. This is supported by the observation that the isomeric acids are eluted in the order of increasing number of adjacent non-substituted carbon atoms (CA) in the naphthalene rings (1,3,5,7-NTeSA with 1 CA, 1,3,6-NTSA with 2 CA, 1,3,5-NTSA and 1,3,7-NTSA with 3 CA; 1,5-NDSA with 3 CA, 1,6-NDSA with 4 CA, 1,3-NDSA and 1,7-NDSA with 5 CA). The elution order of the isomers with the same number of adjacent carbon atoms depends on the localisation of the charge in the molecule, characterized by the dipole moment of the molecules: 1,3-NDSA ($\mu = 0.162$) elutes before 1,7-NDSA ($\mu = 0.198$) and 1,3,5-NTSA ($\mu = 0.018$) before 1,3,7-NTSA ($\mu = 0.181$). Among the NDSA isomers, the size of the part of the naphthalene ring with adjacent non-substituted carbon atoms increases as the charge location is shifted from the centre of the molecule along the central bond in the naphthalene ring, i.e., with increasing dipole moment in the *y*-axis (Fig. 1) In symmetrical molecules, such as 1,5-NDSA and 1,3,5,7-NTeSA, the charge is localized in the centre of the molecules, which posses zero dipole moment, μ.

Fig. 3 illustrates the increase of the retention of isomeric NDSAs with increasing dipole moment, μ, on the logarithmic scale. The plots are similar for most columns, suggesting similar effect of the dipole moment on the isomeric selectivity. The position of the plots on the $\log \alpha$ axis is a measure of increasing effect of the dipole moment on isomeric selectivity for NDSAs, which is high for columns with newer types of silica gel support and high carbon content such as column 11 (Alltima C18) and 6 (Inertsil ODS2) and for columns modified for separation of polar compounds in highly aqueous mobile phases (column 10: Atlantis, or 8: Phenomenex Aqua C18). On the other hand, older columns with high silanol activity, prepared using the silica gel A type show low values of $\log \alpha$ (e.g., Separon, and Nova Pak columns 21–23). The effects of the dipole moment on the retention of isomeric NTSAs are less straightforward, probably because of a smaller hydrophobic part of the naphthalene ring that can be exposed to the interactions with non-polar bonded ligands in the stationary phase.

In Tables 3 and 4, the retention data of the NDSAs and NTSAs in 0.4 M Na_2SO_4 are compared for 25 columns tested. The retention of the acids strongly depends on the column type. The tables list the retention volumes related to the hold-up volumes of uracil, V_R/V_M, the relative reten-

Table 3
Characteristics of NDSA

Column number	V_R/V_M 1.5-NDSA	α		
		1.6/1.5	1.3/1.5	1.7/1.5
1	1.02	0.63	1.71	3.93
2	1.27	2.12	6.03	16.38
3	1.38	2.64	7.32	14.71
4	1.52	2.98	8.46	23.47
5	1.55	2.96	8.25	22.03
6	1.59	3.06	8.20	21.53
7	1.31	1.87	5.20	14.38
8	2.05	3.85	9.36	26.83
9	0.89	0.38	1.07	2.98
10	2.01	3.65	9.11	24.57
11	1.86	3.29	8.59	22.85
12	0.80	2.52	6.78	17.77
13	1.35	1.61	3.99	8.53
14	1.41	1.25	2.82	6.53
15	1.88	1.75	4.71	10.98
16	2.75	0.70	1.45	2.84
17	1.76	1.20	2.10	4.42
18	2.16	1.45	3.41	6.53
19	0.43	0.92	2.24	5.08
20	1.15	0.11	0.32	0.66
21	0.55	0.21	0.47	0.89
22	0.59	*	*	*
23	0.75	0.24	0.67	1.60
24	0.85	0.14	0.41	0.85
25	0.52	2.42	5.55	12.70

	F_{as}			
	1.5-NDSA	1.6-NDSA	1.3-NDSA	1.7-NDSA
1	1.00	1.75	3.60	4.80
2	1.88	1.13	0.91	1.60
3	1.00	1.00	2.75	2.67
4	2.18	1.69	1.48	2.46
5	2.18	1.90	1.63	3.13
6	2.00	1.81	1.52	1.98
7	1.25	1.36	0.99	1.07
8	1.63	0.91	0.82	1.18
9	0.64	1.68	1.66	1.71
10	1.81	1.30	1.15	1.79
11	1.22	1.09	1.09	1.16
12	1.64	1.48	1.54	1.42
13	2.50	2.50	3.20	4.29
14	0.75	1.14	1.00	1.16
15	1.00	3.50	3.50	4.88
16	1.00	1.25	1.43	1.64
17	1.33	1.19	1.13	1.79
18	1.00	1.33	1.75	3.17
19	2.75	4.00	4.80	9.29
20	2.00	2.00	2.25	3.64
21	1.60	1.57	2.83	3.18
22	*	*	*	*
23	3.33	3.00	3.67	8.75
24	1.67	3.75	3.71	5.00
25	2.00	3.00	5.00	17.00

V_R: retention volume (ml), V_M: hold-up volume (ml), α: elution ratio [$\alpha = (V_{Rj}/V_{Ri}) - 1$], F_{as}: factor of asymmetry (*: co-elution).

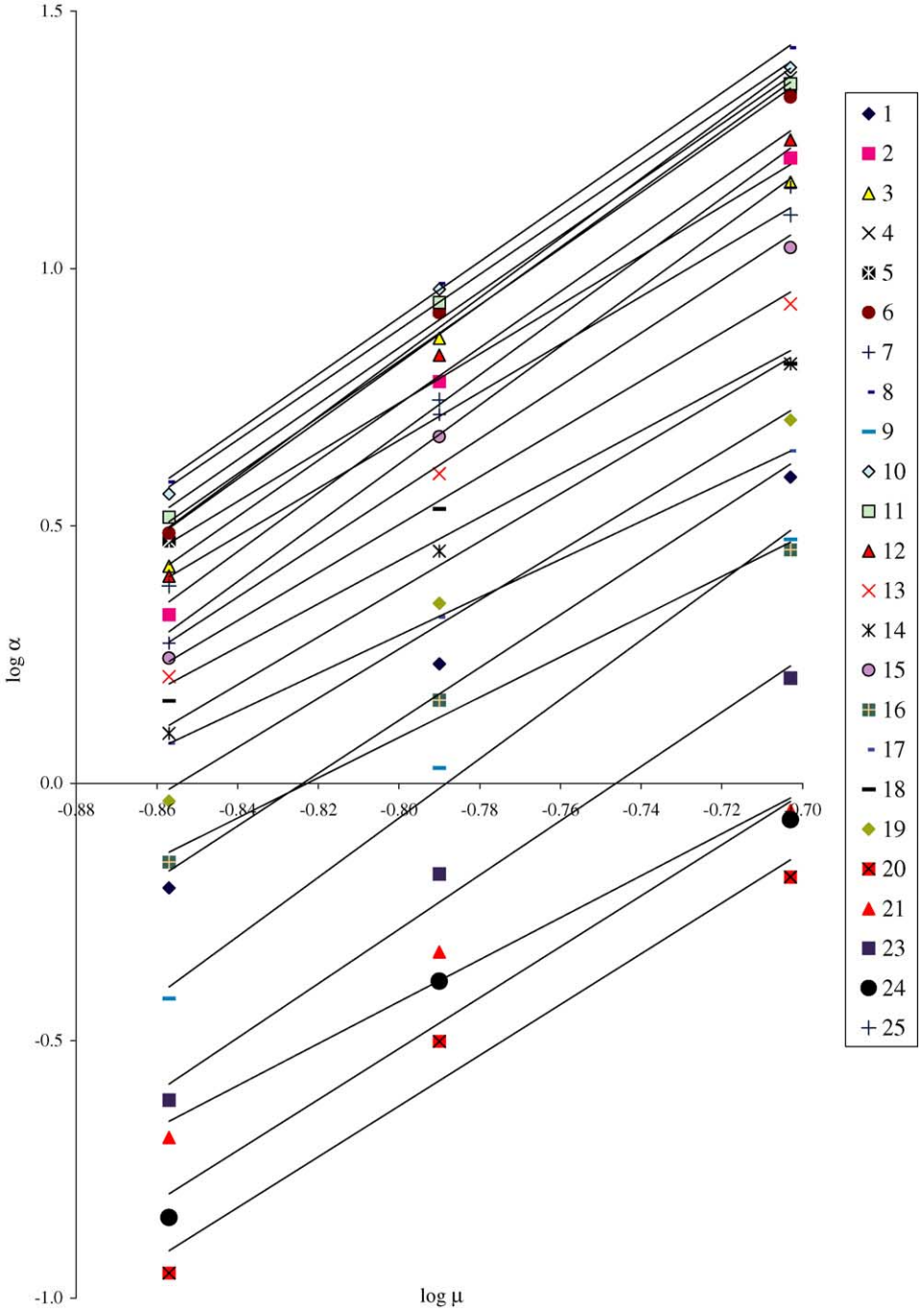

Fig. 3. Effect of the dipole moment, μ, on the relative retention $\alpha = t_{Ri}/t_{R1}$, of isomeric naphthalene 1,6-, 1,3- and 1,7-disulphonic acids (t_{Ri}), related to the retention volume of naphthalene 1,5-disulphonic acid (t_{R1}) in 0.4 M Na_2SO_4. Column numbers as in Table 1.

tion, $\alpha = (t_{Ri}/t_{R1} - 1)$, related to the net retention volume of 1,5-NDSA for NDSAs and to the net retention volume of 1,3,5,7-NTeSA for NTSAs and the asymmetry factors, F_{as}. The values of V_R/V_M of 1,5-NDSA < 1 in Table 3 are characteristic for the stationary phases based on the older type A silica gel as the support material (Separon, Silasorb, Nova-Pak columns), whereas high values of $V_R/V_M > 1.5$ were measured for columns 8, 10 and 17 with polar groups incorporated into the bonded ligands for improved performance in highly aqueous mobile phases, for a hybrid X Terra column 5 with a part of silanols substituted by methyl groups, for columns prepared with newer silica gel supports and a relatively high surface coverage (18: Lichrospher RP select B, 19: Zorbax 300 SB, 11: Alltima C18, 6: Inertsil ODS 2 and 15: Luna C18) and for an Astec polymer column 16 with octadecyl groups incorporated into the (poly)vinyl alcohol matrix (which contains some polar groups giving rise to similar electrostatic interactions as the silanol groups).

Table 4
Characteristics of NTSA and NTeSA

Column number	V_R/V_M 1.3.5.7-NTeSA	α		
		1.3.6/1.3.5.7	1.3.5/1.3.5.7	1.3.7/1.3.5.7
1	0.96	*	*	*
2	0.98	0.07	0.15	0.13
3	1.03	*	*	*
4	1.01	0.07	0.25	0.25
5	1.03	0.07	0.25	0.27
6	1.00	0.12	0.30	0.34
7	1.01	0.05	0.17	0.19
8	1.03	0.14	0.47	0.57
9	0.92	0.01	0.12	0.13
10	0.99	0.18	0.50	0.61
11	1.00	0.14	0.41	0.48
12	0.54	0.09	0.29	0.32
13	1.01	0.16	0.16	0.16
14	1.18	0.00	0.13	0.13
15	1.26	*	*	*
16	1.10	0.15	0.26	0.32
17	1.03	0.20	0.33	0.47
18	1.22	0.14	0.34	0.39
19	0.39	*	*	*
20	0.97	*	*	*
21	0.49	*	*	*
22	0.56	*	*	*
23	0.69	*	*	*
24	0.82	*	*	*
25	0.69	*	*	*

	F_{as}			
	1.3.5.7-NTeSA	1.3.6-NTSA	1.3.5-NTSA	1.3.7-NTSA
1	*	*	*	*
2	0.92	0.78	1.88	1.44
3	*	*	*	*
4	2.56	1.22	2.27	2.18
5	2.89	3.08	2.27	2.17
6	1.92	1.79	1.80	1.93
7	4.10	3.55	3.23	3.23
8	2.80	2.17	1.71	1.57
9	1.14	1.00	0.81	0.79
10	1.80	2.23	1.87	1.93
11	3.27	3.46	3.42	3.08
12	2.27	1.41	1.50	1.95
13	*	*	*	*
14	0.75	1.14	1.00	1.16
15	*	*	*	*
16	*	*	*	*
17	0.58	1.56	1.40	1.46
18	2.00	2.00	*	*
19	*	*	*	*
20	*	*	*	*
21	*	*	*	*
22	*	*	*	*
23	*	*	*	*
24	*	*	*	*
25	*	*	*	*

V_R: retention volume (ml), V_M: hold-up volume (ml), α: elution ratio [$\alpha = (V_{Rj}/V_{Ri}) - 1$], F_{as}: factor of asymmetry (*: co-elution).

Table 5
Classification of columns

V_R/V_M (1.5-NDSA)	α (1.7/1.5)	Class	a [F_{as} (NDSA) < 2]	b [F_{as} (NDSA) > 2]	Co-elution
< 1	≈ 0	I	–	–	22
	< 1	II	–	21, 24	–
	1–10	III	9	19, 23	–
	10–20	IV	12	25	–
1–1.5	< 1	V	–	20	–
	1–10	VI	14	1, 13	–
	10–20	VII	7, 2	3	–
> 1.5	1–10	VIII	16, 17	18	–
	10–20	IX	–	15	–
	> 20	X	6, 8, 10, 11	4, 5	–

F_{as}: factor of asymmetry, V_R: retention volume (ml), V_M: hold-up volume (ml).

The elution ratios of isomeric NDSAs follow approximately the order of the retention of 1,5-NDSA, with some exceptions: the Lichrospher (18), Chromolith (9), Zorbax Aq (17) and Astec polymer (16) columns show relatively low isomer selectivity as compared to other columns with similar retention of 1,5-NDSA; the Silasorb C18 column (25) exhibits high isomer selectivity in comparison to other columns excluding 1,5-NDSA before the hold-up volume (21–24, Separon C8 and C18, Nova-Pak C18, Table 3). This behaviour suggests that the interactions with polar groups may decrease the isomer selectivity.

The retention and the isomer selectivity for naphthalene tri-sulphonic acids is generally much lower in comparison to the naphthalene di-sulphonic acids. A very low or no selectivity (marked with asterisk in Table 4) for isomeric NTSAs was observed with the columns where 1,3,5,7-NTeSA is excluded from the pores (columns 19–25) or elutes close to the column hold-up volume determined using uracil (columns 1–3, 14, 15). Relatively high selectivity for isomer NTAs was observed with columns 8 (Phenomenex Aqua C18), 10 (Atlantis), 11 (Alltima C18) and 17 (Zorbax SB-Aq), i.e., mainly with the columns intended for work in highly aqueous mobile phases.

The asymmetry factors of NDSAs and NTSAs in Tables 3 and 4 also show significant differences between the individual columns. The asymmetry can be probably caused by the heterogeneity of the stationary phase surface and by the presence of groups showing some attractive interactions with the sulphonic acid groups. The surface heterogeneity may affect the local thickness of the electric double layer and consequently a part of the molecules can penetrate deeper into the pores than the other molecules to “feel” stronger interactions with polar groups. The highest asymmetry factors for NDSAs were observed with the Spheron, Silasorb, Nova Pak, X Terra, Zorbax 300 SB, Luna C8(2), Luna phenylhexyl, Luna C18(2) and Hypersil columns. The asymmetry factors of NTSAs were similar or lower than the F_{as} of NDSAs and even “fronting” peaks with $F_{as} < 1$ were observed on Chromolith RP-18e column.

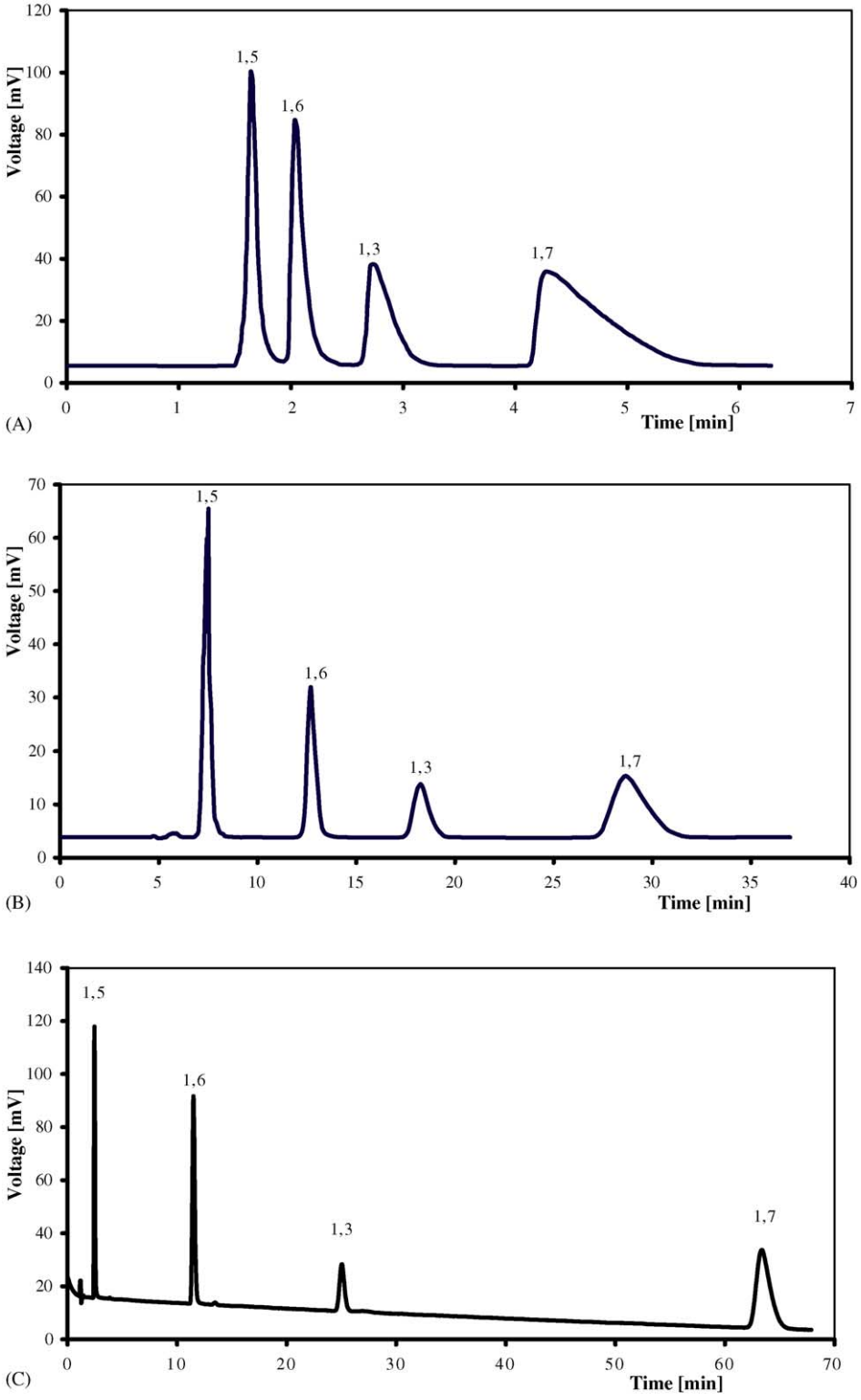

Fig. 4. Separation of 1,5-, 1,6-, 1,3- and 1,7-naphtalene disulphonic acids on a Nova Pak C18 column 23 (A), an organic polymer C18 column 16 (B) and on an Inertsil ODS2 column 6 (C). Mobile phase: 0.4 M Na_2SO_4, flow rate: 1 ml min^{-1}, temperature 40 °C.

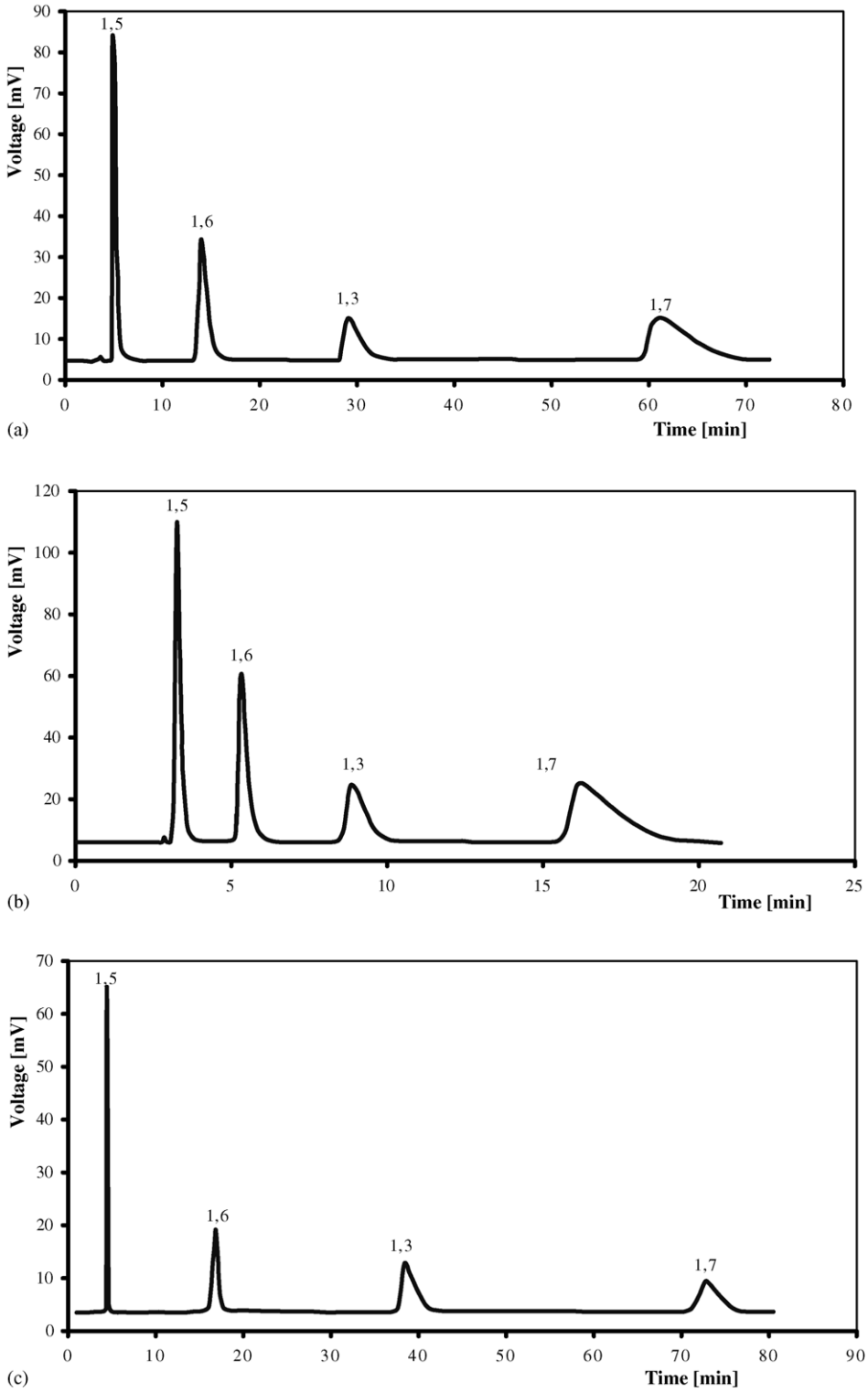

Fig. 5. Separation of 1,5-, 1,6-, 1,3- and 1,7-naphtalene disulphonic acids on a Luna C18 column 15 (A), a Luna C18 (2) column 1 (B) and on a Luna phenyl hexyl column 3 (C). Mobile phase: 0.4 M Na_2SO_4, flow rate: 1 ml min^{-1}, temperature 40 °C.

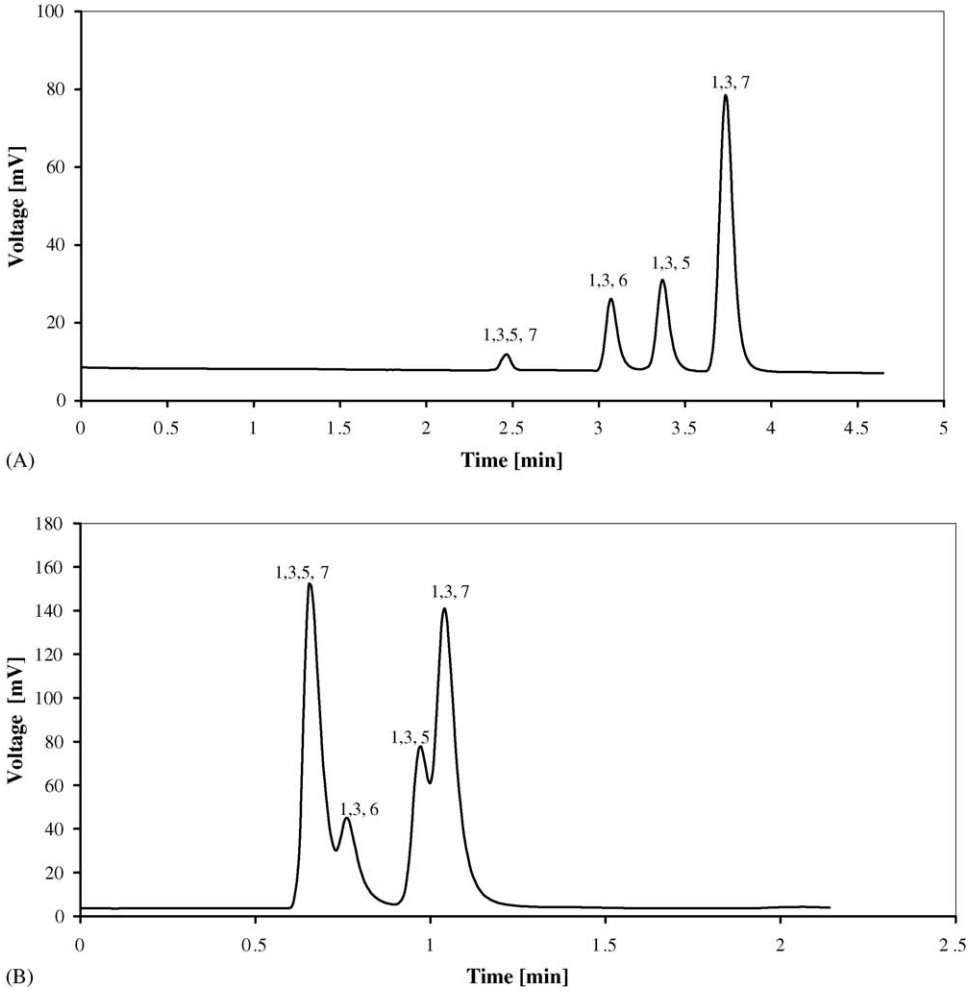

Fig. 6. Separation of 1,3,5,7-naphthalene tetrasulphonic acid, 1,3,6-, 1,3,5- and 1,3,7-naphtalene trisulphonic acids on a Zorbax SB Aq column 17 (A) and on a Phenomenex Aqua column 8 (B). Mobile phase: 0.4 M Na_2SO_4, flow rate: 1 ml min^{-1}, temperature 40 °C.

On some columns with high carbon content and on columns intended for operation in highly aqueous mobile phases, not only isomeric naphthalene disulphonic acids, but also some naphthalene trisulphonic acids can be separated. Fig. 4 compares the separation of four isomeric NDSAs in 0.4 M sodium sulphate on three different column types. The retention strongly increases and the band asymmetry and efficiency improve from the example in Fig. 4A showing separation on a non-end-capped older type silica gel Nova Pak C18 column to the chromatogram B obtained with an organic polymer C18 column (B) and to the separation shown in Fig. 4C for an Inertsil ODS2 column. The separation of isomeric NDSAs strongly depends also on the type of ligand bonded on the support produced by the same manufacturer, see examples for Luna C18, C18(2) and phenylhexyl columns in Fig. 5A–C. Finally, some columns designed for separations in highly aqueous mobile phases enable also separation of isomeric naphthalene tri-sulphonic acids and naphthalene tetrasulphonic acid. Fig. 6A shows the chromatogram of the four acids tested on a Zorbax SB Aqua column, which is significantly better than the separation on a Phenomenex Aqua column (Fig. 6B), at the cost of a slightly longer analysis time.

The retention behaviour of naphthalene di-sulphonic acids can be possibly used for the classification of columns with respect to their hydrophobicity and silanophilic activity. The columns tested can be divided into 10 classes according to the retention of 1,5 NDSA and relative retention of 1,7- and 1,5-NDSA (Table 5). The columns in the classes III, IV, VI–VIII and X can be further divided according to the band symmetry: subclass a with $F_{as} < 2$ and subclass b with $F_{as} > 2$. Separon SGX C18 belongs into class I with very strong Donnan effect resulting into ionic exclusion of all NDSAs in 0.4 M Na_2SO_4. The columns in classes II–IV exclude 1,5-NSDA before the column hold-up volume and the columns show weak (class II), medium (class III) and strong (class IV) selectivity for isomeric NDSAs: class IIb ($\alpha_{1,7/1,5} < 1$, asymmetrical peaks) includes Separon SGX C8 and Luna C8 (2) columns, class III ($\alpha_{1,7/1,5} = 1–10$) Nova Pak C18 column providing asymmetrical peaks and Chromolith C18 column with symmetrical peaks and finally class IV ($\alpha_{1,7/1,5} = 10–20$): Silasorb SPH C18 column showing asymmetrical peaks and

Purospher RP-18e column with symmetrical peaks. Classes V–VII include columns providing relatively weak retention of 1,5-NSDA and weak (Zorbax Eclipse XDB C8, class V), medium (Biospher SiC18, Luna C18(2) and Lichrosorb RP8) and high (Luna phenylhexyl, Zorbax Extend 300 and Rx C18 columns) isomeric selectivity for NDSAs. Columns providing relatively strong retention of 1,5-NDSA in 0.4 M Na_2SO_4 are divided into classes VIII–X. Class VIII includes columns with medium isomeric selectivity ($\alpha_{1,7/1,5}$ = 1–10, Lichrospher 60RP select B: asymmetrical peaks and Polymer C18, Zorbax SB aq: symmetrical peaks). To class IX belong columns with relatively strong isomeric selectivity ($\alpha_{1,7/1,5}$ = 10–20, Luna C18: asymmetrical peaks) and to class X columns with the highest isomeric selectivity ($\alpha_{1,7/1,5}$ > 20, Hypersil ODS, Xterra MS C18: asymmetrical peaks and Inertsil ODS2, Phenomenex Aqua C18, Atlantis, Alltima C18: symmetrical peaks).

4. Conclusions

In aqueous mobile phases containing sodium sulphate, ionic exclusion of completely ionised naphthalene di- and tri-sulphonic acids is suppressed and separation possibilities are improved with respect to mobile phases without a salt addition. The retention of isomeric acids increases proportionally to the dipole moment, which can be explained by its effect on increasing exposure of the naphthalene ring to hydrophobic interactions with the non-polar stationary phases. The properties of the stationary phase, such as the type of the support material, chemistry, coverage density and regularity of localisation of bonded ligands strongly affect the retention, selectivity and the band shape. Very good selectivity allows excellent reversed-phase separations of isomeric naphthalene di- and tri-sulphonic acids on some columns designed for operation in aqueous mobile phases, without ion-pairing reagents in the mobile phase.

Strong stationary phase dependence of chromatographic behaviour of isomeric naphthalene di- and tri-sulphonic acids can be used for testing of columns for reversed-phase chromatography. As the tests are performed in purely aqueous mobile phases, the results are not affected by the nature and concentration of organic solvents, unlike to earlier established chromatographic column tests. The elution order of the isomers was the same on all columns tested so far, but the retention, isomeric selectivity (elution ratios) and band asymmetry factors depend on both hydrophobic and polar interactions with bonded alkyls, residual silanol groups and other polar adsorption centres in the stationary phases. According to these chromatographic characteristics, HPLC columns can be divided into several classes.

Some general trends are apparent in the classification scheme. Zorbax columns prepared using silica gel B support show generally better peak symmetry, higher retention and isomeric selectivity for naphthalene di-sulphonic acids than the columns prepared with older type A silica gel. C18 columns show generally higher retention and selectivity than corresponding C8 columns and new types of columns with high surface coverage and carbon content (Inertsil, Alltima, Polymer C18), or reduced silanol number (X Terra) and (or) bonded ligand chemistry intended for the applications in highly aqueous mobile phases (Atlantis and Aqua columns) show high retention and isomeric selectivity. However, the band asymmetry is not fully correlated with the retention and isomeric selectivity and is probably a measure of the homogeneity of the surface, as asymmetric peaks suggest competitive interactions with various adsorption centres. Symmetrical peaks were observed on Purospher, Chromolith, Inertsil, Alltima, polymer C18, Phenomenex Aqua and most Zorbax columns (Rx, SB, Extend).

In the present work, aqueous 0.4 M sodium sulphate was used as the mobile phase for column tests, as it provides larger retention and satisfactory selectivity differences for isomeric NDSAs, very sensitive to the type of the column. Probably, mobile phases containing other types and concentrations of salts can provide similar useful data for column classification. Further work is in progress to check the validity and limitations of the present column testing method. First, the effects of the individual columns and different column batches of the same types should be determined. However, such tests require purchase of a large number of columns and therefore are limited by the budget possibilities. So far, we compared two Purospher RP-18e, two Nova-Pak C18 and two Chromolith RP-18e columns, with only small differences between the columns of the same type. Further types of columns, including mixed bonded phases, are being investigated and the results will be published soon [23].

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