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Characterisation of reversed-phase liquid chromatographic columns by chromatographic tests. Evaluation of 36 test parameters: repeatability, reproducibility and correlation

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Abstract

The European Pharmacopoeia (Ph. Eur.) or other official compendia give only a general description of the stationary phase in the description of a liquid chromatographic method. Therefore the selection of a column giving suitable selectivity presents difficulties. Earlier, a test procedure was proposed that allows measurement of a number of parameters which are reported to be representative for stationary phase characteristics. This paper describes how the test procedure was applied on 69 RP-LC C_{18} columns. Chromatographic parameters obtained as test results were evaluated, and their repeatability, reproducibility and correlation were examined.

Keywords: Chromatographic test; Test parameters; Column characterization; Stationary phases, LC

1. Introduction

Many liquid chromatographic (LC) methods are described in the Ph. Eur. and most use reversed-phase (RP) C_{18} columns. In the description of a liquid chromatographic method, the Ph. Eur. [1] or other official compendia such as the United States Pharmacopeia (USP) [2] give the precise eluent composition but they do not mention the brand of the stationary phase(s) that can (has to) be used in order to obtain the required selectivity. Thus, monographs do not give precise information about column identity. Instead of mentioning the brand name, which is not allowed to be communicated in an official monograph, the Ph. Eur. prescribes a system suitability test and further refers to a description of the stationary phase in the reagents part with information on particle size, pore size, specific surface area and chain length [3]. This information is

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insufficient to choose a suitable column from a market offering more than 600 different brands.

Engelhardt et al. carried out the determination of impurities of salicylic acid according to the Ph. Eur. on three different commercially available RP columns [4]. With one of the columns, all acidic solutes were coeluted with the solvent, on the other, stationary phases changes in the elution sequence were observed. This example demonstrates the problem that may occur if the column properties are not sufficiently described. Steffeck et al. also drew attention to the difficulties related to RP-LC column selection [5].

A number of factors influence the properties of silica-based reversed phases. The nature of the silica can be characterised by the particle diameter, specific surface area, pore diameter, pore volume, chemical purity and acidity. The silanol derivatization, e.g. length and nature of the alkyl group, the use of mono-, di- or trichlorosilanes for derivatisation, the surface concentration of bonded alkyl groups and the amount of unreacted, accessible silanol groups also affect the properties of the RP stationary phases [6].

Properties of RP-LC columns can be characterised by non-chromatographic and chromatographic methods [7]. Carbon content, amount of metal impurities, particle size, surface area, pore size, packing density and acidity can be determined by non-chromatographic methods. However, these techniques are not easy to perform and cannot be carried out on a packed column without destruction.

Properties such as column efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and the amount of metal impurities can be characterised by chromatographic tests. Many papers proposed different chromatographic tests [4,5,8–35] to characterise commercial columns which have been reviewed recently [36,37]. Until now none of these tests has been widely accepted. It has never been verified sufficiently whether columns having closely related characteristics as determined by these chromatographic tests, are indeed suitable for the same chromatographic separation. Such verification is the subject of a project, which the work described here, belongs to. The final aim of this project is to verify whether it is possible to formulate a chromatographic test procedure for the characterisation of stationary phases in order to facilitate selection of appropriate columns for a particular separation. Such a chromatographic test procedure can also be used to verify the performance of a column at any time of its life cycle.

After a careful study of the literature, eight different methods were chosen which allow the determination of 36 test parameters [38]. The test procedure and output parameters used are summarised in the Experimental section, and for further detailed information, the reader is referred to the literature [4,13,16,26,38,39]. This test procedure allows examination of all important properties of the RP stationary phases such as efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and presence of metal impurities.

This test procedure was now carried out on 69 C_{18} RP-LC columns. A general test procedure needs to use repeatable and reproducible test methods. Because repeatability data and interlaboratory results for chromatographic tests were not found in the literature, several columns were examined in different laboratories. In this paper, the selected general test methods were examined; a critical discussion about the different chromatographic parameters, their repeatability, reproducibility and correlation is made. The study also evaluated whether some of the test parameters allow column classification.

2. Experimental

2.1. Instrumentation

2.1.1. Laboratory 1

HPLC system: Varian (Walnut Creek, CA, USA) 9010 LC pump, a 9100 autosampler and 9050 UV–Vis detector; column-thermostat: water-bath equipped with a Julabo EM thermostat; data acquisition: ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK); pH-meter: Consort C831 (Consort, Turnhout, Belgium) equipped with a Hamilton (Bonaduz, Switzerland) combination glass electrode.

2.1.2. Laboratory 2

HPLC system: Merck–Hitachi (Tokyo, Japan) L-6200 Intelligent pump, L-4000 UV detector and Rheodyne (Cotati, CA, USA) injector; data acquisi-

tion: D-2500 Chromato-Integrator; column-thermostat: T-6300 column thermostat; pH-meter: Ankersmit A520 (Orion, Boston, MA, USA) with Orion combination glass electrode.

2.1.3. Laboratory 3

HPLC system: Waters (Milford, MA, USA) 625 S/N pump with 600E control unit 717plus autosampler and 996 Photodiode array detector; column-thermostat: water-bath equipped with a Haake W19 DC1 thermostat; data acquisition: Millennium 3.0 software; pH meter: Metrohm 713 (Herisau, Switzerland) with automatic temperature correction system, Metrohm 6.0203.100 combination glass electrode.

2.2. Chemical and chromatographic conditions

Solvents were of LC grade, other chemicals were AR grade. Electrodes were calibrated daily according to the Ph. Eur. [40] with 0.05 *M* potassium phthalate (pH 4.01) and 0.05 *M* potassium tetraoxalate (pH 1.78) or 0.01 *M* borax (pH 9.18) buffers. The pH of aqueous buffers of mobile phases was adjusted by mixing 0.2 *M* H_3PO_4, 0.2 *M* KH_2PO_4 and 0.2 *M* K_2HPO_4 solutions. Water and organic solvents were added afterwards. Preparation of mobile phases was carried out by weight (w/w).

The C_{18} RP-LC columns examined are reported in Table 1, all the columns were new. Column temperature was maintained at 40 °C. Stationary phases were flushed with the mobile phase for 90 min before any sample was injected. The mobile phases were not pre-heated. A flow-rate of 1 ml/min was used and 20 μl of sample was injected, UV-detection was performed at 254 nm.

2.3. Test methods and output parameters

Eight chromatographic methods (M1-M8) chosen from the literature are summarized below. The development of this test procedure was described in our previous paper [38]. Concentration dependence of the parameters, the effect of the method sequence and the selection of the dead time marker was also discussed there. The pH of the mobile phase of Method 3 was lowered from 7.6 to 7.3 to protect the columns as much as possible.

When a column property was examined in different tests, the results were discussed together. Some parameters, which were adapted from the originally published methods and which do not represent a well-defined character of the stationary phase, are reported under ''other parameters'' here. The Ph. Eur. or IUPAC nomenclature was used, and calculations were carried out according to the formulas of the Ph. Eur. In some methods the peak order changed depending upon the column examined. Since the selectivity factor is always larger than 1 according to IUPAC nomenclature, the relative retention factor (rk') was used instead of the selectivity factor. Changes in the elution order can be characterised in this way.

2.3.1. Method 1 [26]

Mobile phase: acetonitrile/0.025 *M* CH_3COONH_4 pH 7.05 (26.2:100 w/w).

Sample: 2,3-dihydroxynaphthalene (6 mg), 2,7-dihydroxynaphthalene (3 mg) dissolved in 10.0 ml of acetonitrile.

Output parameter: metal impurities: Dihydroxynaphthalene efficiency ratio test

$$(\mathrm{DERT}) = \frac{n_{2,7}\text{-dihydroxynaphthalene}}{n_{2,3}\text{-dihydroxynaphthalene}}$$

2.3.2. Method 2 [4]

Mobile phase: acetonitrile/water/0.2 *M* potassium phosphate buffer pH 2.3 (312:340:340 w/w).

Sample: uracil (0.1 mg), diphenhydramine (6 mg), *o*-hydroxyhippuric acid (0.7 mg), acetylsalicylic acid (2.5 mg), 5-*p*-methylphenyl-5-phenylhydantoin (MPPH) (4 mg), diazepam (0.5 mg), toluene (15 mg) dissolved in 10.0 ml of mobile phase.

Output parameters: efficiency: n_{MPPH}, hydrophobicity: $rk'_{\mathrm{diazepam/MPPH}}$, $rk'_{\mathrm{toluene/MPPH}}$, silanol activity: $rk'_{\mathrm{diphenhydramine/MPPH}}$, symmetry factor of diphenhydramine, others: $rk'_{\mathrm{acetylsalicylic\ acid/MPPH}}$, $rk'_{o\text{-hydroxyhippuric\ acid/MPPH}}$.

2.3.3. Method 3 [13]

Mobile phase: methanol/0.5% CH_3COONa pH 7.80 (118.5:100 w/w).

Sample: acetylacetone (1 mg) dissolved in 10.0 ml of methanol.

Output parameter: metal impurities: peak area/

Table 1
C_{18} RP-LC columns investigated

Column number	Name of column	Length (mm)[a]	Particle size (μm)	Manufacturer/supplier
1	ACE 3 C18	150	3	Advanced Chrom. Tech./Achrom
2	ACE 5 C18	250	5	Advanced Chrom. Tech./Achrom
3	Alltima C18 3	150	3	Alltech
4	Alltima C18 5	250	5	Alltech
5	Apex Basic	250	5	Jones Chromatography/Sopachem
6	Apex ODS II	250	5	Jones Chromatography/Sopachem
7	Aqua C18	150	5	Phenomenex/Bester
8	μBondapak C18	250	10	Waters
9	Brava BDS C18 3	150	3	Alltech
10	Brava BDS C18 5	250	5	Alltech
11	Chromolith Performance	100	–	Merck
12	Discovery C18	250	5	Supelco
13	Genesis C18 3	100	3	Jones Chromatography/Sopachem
14	Genesis C18 4	250	4	Jones Chromatography/Sopachem
15	Hypersil BDS C18 3	100	3	Alltech
16	Hypersil BDS C18 5	250	5	ThermoQuest
17	Hypersil ODS 3	100	3	Alltech
18	Hypersil ODS 5	250	5	ThermoQuest
19	HyPURITY Elite C18 3	150	3	ThermoQuest, SerCoLab
20	HyPURITY Elite C18 5	150	5	ThermoQuest, SerCoLab
21	Kromasil 100-3 C18	100	3	Alltech
22	Kromasil 100-5 C18	250	5	Macherey-Nagel/Filter Service
23	Kromasil 100-5 C18 EKA	250	5	Akzo Nobel/SerCoLab
24	LiChrosorb RP-18	250	5	Merck
25	LiChrospher 100 RP-18	250	5	Merck
26	Luna C18 (2)	150	5	Phenomenex/Bester
27	Nucleosil 100-3 C18	100	3	Alltech
28	Nucleosil 100-5 C18	250	5	Macherey-Nagel/Filter Service
29	Nucleosil 100-5 HD C18	250	5	Macherey-Nagel/Filter Service
30	Nucleosil 100-5 Nautilus C18	250	5	Macherey-Nagel/Filter Service
31	OmniSpher C18	250	5	Varian
32	Pecospher C18	83	3	Perkin-Elmer
33	Platinum C18 3	150	3	Alltech
34	Platinum C18 5	250	5	Alltech
35	Platinum EPS C18 3	150	3	Alltech
36	Platinum EPS C18 5	250	5	Alltech
37	Prodigy ODS (3)	100	3	Phenomenex/Bester
38	Purospher RP-18	250	5	Merck
39	Purospher RP-18 e	250	5	Merck
40	Purospher STAR RP-18 e	250	5	Merck
41	SPHERI-5 RP C18	250	5	Perkin-Elmer
42	Spherisorb ODS2 3	100	3	Waters
43	Spherisorb ODS2 5	250	5	Waters
44	Supelcosil LC-18	250	5	Supelco
45	Supelcosil LC-18 DB 3	150	3	Supelco
46	Supelcosil LC-18 DB 5	250	5	Supelco
47	Superspher RP-18 e	250	5	Merck
48	Symmetry C18 3.5	100	3.5	Waters
49	Symmetry C18 5	250	5	Waters
50	TracerExcel ODS A-3	150	3	Teknokroma/SerCoLab
51	TracerExcel ODS A-5	250	5	Teknokroma/SerCoLab
52	TSKgel ODS-80TS	150	5	TosoHaas/SerCoLab

Table 1. Continued

Column number	Name of column	Length (mm)[a]	Particle size (μm)	Manufacturer/supplier
53	TSKgel Super ODS	100	2	TosoHaas/SerCoLab
54	Uptisphere 3 HDO C18	100	3	Interchrom/Achrom
55	Uptisphere 5 HDO C18	250	5	Interchrom/Achrom
56	Uptisphere 3 ODB C18	100	3	Interchrom/Achrom
57	Uptisphere 5 ODB C18	250	5	Interchrom/Achrom
58	Validated C18	250	5	Perkin-Elmer
59	Wakosil C18 HG 5–10	100	5	SGE/Achrom
60	Wakosil C18HG 5–25	250	5	SGE/Achrom
61	Wakosil C18 RS 3–10	100	3	SGE/Achrom
62	Wakosil II C18 RS 3–25	250	3	SGE/Achrom
63	X-Terra 3	100	3	Waters
64	YMC-Hydrosphere C18	150	5	YMC Sep. Techn./ThermoQuest
65	YMC-Pack Pro C18-3	150	3	YMC Sep. Techn./ThermoQuest
66	YMC-Pack Pro C18-5	150	5	YMC Sep. Techn./ThermoQuest
67	Zorbax Eclipse XDB-C18	250	5	Agilent Technologies
68	Zorbax Extend C18	250	5	Agilent Technologies
69	Zorbax SB-C18	250	5	Agilent Technologies

[a] The internal diameter is always 4.6 mm.

peak height of acetylacetone, $n_{\text{acetylacetone}}$, symmetry factor of acetylacetone.

2.3.4. Method 4 [26]

Mobile phase: methanol/water/0.2 *M* potassium phosphate buffer pH 2.7 (34:90:10 w/w).

Sample: phenol (5 mg), benzylamine (5 mg) dissolved in 10.0 ml of mobile phase.

Output parameter: ion-exchange capacity at low pH: $rk'_{\text{benzylamine/phenol}}$.

2.3.5. Method 5 [26]

Mobile phase: methanol/water/0.2 *M* potassium phosphate buffer pH 7.3 (34:90:10 w/w).

Sample: phenol (5 mg), benzylamine (5 mg) dissolved in 10.0 ml of mobile phase.

Output parameter: ion-exchange capacity at high pH: $rk'_{\text{benzylamine/phenol}}$.

2.3.6. Method 6 [39]

Mobile phase: methanol/water (34:100 w/w).

Sample: uracil (0.1 mg), caffeine (0.3 mg), theobromine (0.2 mg), theophylline (1 mg), phenol (3.5 mg), pyridine (1 mg), 2,2′-dipyridyl (3 mg), 2,3-dihydroxynaphthalene (3 mg) dissolved in 10.0 ml of mobile phase.

Output parameters: silanol activity: $rk'_{\text{caffeine/phenol}}$, $rk'_{\text{pyridine/caffeine}}$, $rk'_{\text{pyridine/phenol}}$, symmetry factor of pyridine, metal ion impurities: $k'_{\text{2,2′-dipyridyl}}$, $k'_{\text{2,3-dihydroxynaphthalene}}$, $rk'_{\text{2,3-dihydroxynaphthalene/2,2′-dipyridyl}}$, symmetry factor of 2,2′-dipyridyl, symmetry factor of 2,3-dihydroxynaphthalene, other parameters: $rk'_{\text{theophylline/theobromine}}$, $rk'_{\text{caffeine/theophylline}}$.

2.3.7. Method 7 [4]

Mobile phase: methanol/water (50:50 w/w).

Sample: uracil (0.1 mg), phenol (1.3 mg), toluene (10 mg), ethylbenzene (12 mg), *p*-ethylaniline (1 mg) dissolved in 10.0 ml of mobile phase.

Output parameters: efficiency: n_{toluene}, hydrophobicity: k'_{toluene}, $k'_{\text{ethylbenzene}}$, $rk'_{\text{ethylbenzene/toluene}}$, silanol activity: symmetry factor of *p*-ethylaniline, other parameter: $rk'_{\text{toluene/phenol}}$.

2.3.8. Method 8 [39]

Mobile phase: methanol/water (317:100 w/w).

Sample: uracil (0.1 mg), phenol (0.6 mg), toluene (2.5 mg), ethylbenzene (2.5 mg), butylbenzene (7 mg), amylbenzene (7 mg), *o*-terphenyl (0.2 mg), triphenylene (0.02 mg) dissolved in 10.0 ml of mobile phase.

Output parameters: efficiency: $n_{\text{amylbenzene}}$, hydrophobicity: $k'_{\text{amylbenzene}}$, $rk'_{\text{ethylbenzene/toluene}}$, $rk'_{\text{amylbenzene/butylbenzene}}$, steric selectivity: $rk'_{\text{triphenylene/}o\text{-terphenyl}}$, other parameter: $rk'_{\text{toluene/phenol}}$.

All samples described above were diluted with an equal volume of the appropriate solvent, both solutions were used as samples. In each method, three chromatograms were recorded for each of the two samples. Mobile phases in the consecutive methods are sequenced in the way that intermediate column washing between different mobile phases is not necessary. After Method 8, the column was flushed with methanol/water (50:50 w/w) (mobile phase in Method 2) and finally with acetonitrile, both for 90 min [38]. Columns were stored in pure acetonitrile.

The retention time of uracil was used as dead time. The dead time measured with Method 6 was also used for Methods 4 and 5 [38].

3. Results and discussion

The measured parameters are reported and discussed according to the column properties. When a column property was examined by different test methods, the results from these methods are discussed in the same subsection. Repeatability and reproducibility data are examined in detail because only repeatable and reproducible methods can be used in a general test procedure.

Repeatability and reproducibility data are expressed in terms of RSD; these values were calculated from two data sets. It should be mentioned that usually at least three data are required for calculating RSD values.

Repeatability was examined with four columns of different type on the same instrument. The stationary phases were new and the test procedure was performed consecutively twice on the same columns. Repeatability data are reported in terms of RSD values in Table 2.

Reproducibility data were based on data from 29 columns belonging to 14 different types. All were new and columns of the same type were from the same batch. All types but one were tested in two different laboratories; column of type 66 was examined in three laboratories. Reproducibility data are summarised in terms of RSD values in Table 3. The laboratories that tested a given column are also identified in these tables.

Each column was tested once except those, which were involved in the repeatability study. In case of the latter ones, the results of the first test series were used to calculate the reproducibility. The values of the discussed parameters for each column are summarised in Table 4 and are shown in Figs. 1–6. The correlation data between parameters are summarised in Table 5.

3.1. Efficiency

Efficiency can be characterised in terms of theoretical plate numbers. Generally aromatic hydrocarbons are employed for this reason as probes in the literature [4,41,42]. In this work the test compounds were MPPH in Method 2, toluene in Method 7 and amylbenzene in Method 8. The theoretical plate number was measured with buffered (Method 2) and non-buffered (Methods 7 and 8) mobile phases as well.

Repeatability and reproducibility of the parameters were good, RSD values were below 10% in every case, the average RSD values were less than 5%. Fig. 1 shows the theoretical plate number/column of amylbenzene for all columns. The list of the columns contains 13 pairs of longer (250 mm) and shorter (100 or 150 mm) columns, packed with the same stationary phase, but with a smaller particle diameter for the shorter ones. In all but one case, the shorter column is less efficient; columns 33 and 34 show similar efficiency.

Table 5(a) shows that the correlation coefficients between the theoretical plate numbers, obtained according to three different methods, are greater than 0.9. The theoretical plate numbers measured with amylbenzene and toluene are very highly correlated. This means that parameters estimating the efficiency are interchangeable. Claessens et al. observed the same phenomenon in their comparative study [43]. No real classes of columns with similar efficiency can be distinguished from Fig. 1. Column classification by any of these methods provides a similar pattern. Only a few columns (columns 5, 30, 57 and 61) appear at different places.

Although significant differences exist between individual columns, no clear-cut classification can be made based on this parameter only. Columns 62 and 63 are examples of extreme efficiencies. The high value for column 62 can be explained by the combination of small particle diameter (3 μm) and 25 cm length.

Table 2
Repeatability of the parameters

Property	Parameter	Method	Hypersil BDS RSD (%)	Hypersil ODS RSD (%)	Kromasil RSD (%)	Nucleosil RSD (%)
Efficiency	n_{MPPH}	M2	0.70	4.33	2.24	7.39
	$n_{toluene}$	M7	1.60	0.62	3.44	6.22
	$n_{amylbenzene}$	M8	2.12	1.97	0.33	1.09
Hydrophobicity	$rk'_{diazepam/MPPH}$	M2	0.08	0.05	0.75	0.48
	$rk'_{toluene/MPPH}$	M2	0.60	0.24	1.51	0.39
	$k'_{toluene}$	M7	0.67	0.35	0.40	1.48
	$k'_{ethylbenzene}$	M7	0.83	0.41	0.55	1.74
	$rk'_{ethylbenzene/toluene}$	M7	0.09	0.07	0.02	0.59
	$k'_{amylbenzene}$	M8	2.06	1.97	1.20	1.71
	$rk'_{ethylbenzene/toluene}$	M8	0.23	0.18	0.05	0.09
	$rk'_{amylbenzene/butylbenzene}$	M8	0.31	0.10	0.19	0.01
Silanol activity	Symmetry factor of diphenhydramine	M2	10.2	9.78	8.80	14.2
	$rk'_{diphenhydramine/MPPH}$	M2	0.96	3.65	3.95	0.83
	$rk'_{caffeine/phenol}$	M6	0.63	3.12	1.09	9.70
	$rk'_{pyridine/caffeine}$	M6	–	–	6.08	1.84
	$rk'_{pyridine/phenol}$	M6	–	–	5.00	8.01
	Symmetry factor of pyridine	M6	–	–	24.0	6.80
	Symmetry factor of *p*-ethylaniline	M7	11.3	20.2	5.49	5.13
Ion-exchange capacity	$rk'_{benzylamine/phenol}$ pH 2.7	M4	0.24	0.55	1.06	1.92
	$rk'_{benzylamine/phenol}$ pH 7.3	M5	0.56	1.65	2.90	3.47
Steric selectivity	$rk'_{triphenylene/o\text{-}terphenyl}$	M8	0.65	0.01	0.12	0.30
Metal impurity	DERT	M1	1.65	9.04	2.67	1.00
	Peak area/peak height of acetylacetone	M3	1.07	28.9	1.86	6.39
	$n_{acetylacetone}$	M3	8.14	25.9	1.33	5.38
	Symmetry factor of acetylacetone	M3	3.58	40.1	5.09	16.8
	$k'_{2,2'\text{-}dipyridyl}$	M6	–	–	–	17.0
	$k'_{2,3\text{-}dihydroxynaphthalene}$	M6	0.52	27.3	2.94	22.8
	Symmetry factor of 2,2′-dipyridyl	M6	–	–	–	7.91
	Symmetry factor of 2,3-dihydroxynaphthalene	M6	21.3	22.5	2.94	9.28
	$rk'_{2,3\text{-}dihydroxynaphthalene/2,2'\text{-}dipyridyl}$	M6	–	–	–	6.62
Other parameters	$rk'_{acetylsalicylic\ acid/MPPH}$	M2	0.13	0.83	7.41	0.99
	$rk'_{o\text{-}hydroxyhippuric\ acid/MPPH}$	M2	0.13	0.30	8.40	1.05
	$rk'_{theophylline/theobromine}$	M6	0.15	0.73	0.63	0.48
	$rk'_{caffeine/theophylline}$	M6	0.62	2.48	0.76	0.56
	$rk'_{toluene/phenol}$	M7	0.47	0.14	0.12	1.74
	$rk'_{toluene/phenol}$	M8	1.12	0.18	0.93	0.71

Testing laboratory used was Laboratory 1. M refers to the method used to determine the given parameter; *n*, theoretical plate number; rk', relative retention factor; k', retention factor; DERT, dihydroxynaphthalene efficiency ratio test; empty cell, the compound was not eluted.

3.2. Hydrophobicity

Hydrophobicity can be characterised by the retention factor of an aromatic hydrocarbon [4,14,15,44–46] or by the selectivity factor (α) between nonpolar alkylbenzenes in a homologous series, differing by one methylene group [8,15,17,19,21,45]. The determination of $\alpha_{ethylbenzene/toluene}$ or $\alpha_{amylbenzene/butylbenzene}$ is typical for this purpose, using methanol/water or methanol/aqueous buffer mixtures as mobile phases.

The following parameters were examined:

Table 3
Reproducibility of the parameters

Property	Parameter	Method Testing laboratory	Discovery 1, 2 RSD (%)	Genesis 3 1, 2 RSD (%)	Genesis 4 1, 2 RSD (%)	HyPurity Elite 5 1, 2, 3 RSD (%)	Kromasil EKA 1, 2 RSD (%)	Purospher STAR 1, 3 RSD (%)	SPHERI 1, 2 RSD (%)	Symmetry 5 1, 3 RSD (%)
Efficiency	n_{MPPH}	M2	3.26	0.34	3.07	9.06	2.05	0.03	0.89	0.90
	$n_{toluene}$	M7	4.35	5.10	1.86	4.62	6.48	0.36	1.34	8.90
	$n_{amylbenzene}$	M8	10.9	8.54	2.54	3.49	3.94	2.01	3.30	2.77
Hydrophobicity	$rk'_{diazepam/MPPH}$	M2	10.1	8.34	6.90	4.94	14.6	0.47	6.93	1.70
	$rk'_{toluene/MPPH}$	M2	0.51	0.43	0.69	1.90	0.67	1.36	0.75	3.84
	$k'_{toluene}$	M7	1.65	0.33	0.63	2.57	1.30	1.22	1.78	3.92
	$k'_{ethylbenzene}$	M7	1.78	0.17	0.39	2.48	1.35	1.05	1.75	3.98
	$rk'_{ethylbenzene/toluene}$	M7	0.15	0.17	0.33	0.30	0.06	0.17	0.04	0.54
	$k'_{amylbenzene}$	M8	1.29	0.86	0.20	2.14	0.74	0.21	0.94	1.51
	$rk'_{ethylbenzene/toluene}$	M8	0.04	0.23	0.07	0.35	0.13	0.04	0.08	0.63
	$rk'_{amylbenzene/butylbenzene}$	M8	0.09	0.03	0.13	0.26	0.07	0.05	0.04	0.26
Silanol activity	Symmetry factor of diphenhydramine	M2	31.1	9.31	4.53	11.2	5.28	9.49	53.8	11.9
	$rk'_{diphenhydramine/MPPH}$	M2	0.58	0.37	0.11	2.17	1.03	1.01	14.7	3.25
	$rk'_{caffeine/phenol}$	M6	0.68	1.67	0.77	1.02	0.22	1.78	1.33	2.13
	$rk'_{pyridine/caffeine}$	M6	7.70	1.48	0.83	2.25	1.20	1.76	–	10.5
	$rk'_{pyridine/phenol}$	M6	5.61	0.23	0.02	3.25	1.44	0.02	–	12.6
	Symmetry factor of pyridine	M6	27.9	25.3	0.28	17.5	4.48	4.69	–	21.8
	Symmetry factor of *p*-ethylaniline	M7	16.5	27.5	6.32	2.41	26.8	0.14	–	13.3
Ion-exchange capacity	$rk'_{benzylamine/phenol}$ pH 2.7	M4	1.90	3.02	5.13	5.83	0.09	6.52	12.2	14.0
	$rk_{benzylamine/phenol}$ pH 7.3	M5	5.15	18.5	15.2	3.52	0.63	0.13	17.9	7.43
Steric selectivity	$rk'_{triphenylene/o\text{-}terphenyl}$	M8	0.32	0.38	0.98	0.91	0.17	0.35	0.23	1.45
Metal impurity	DERT	M1	31.7	51.2	39.2	57.9	41.2	49.6	–	19.5
	peak area/peak height of acetylacetone	M3	30.2	15.4	16.9	30.1	12.5	21.5	–	19.3
	$n_{acetylacetone}$	M3	58.5	31.3	40.2	45.6	34.6	42.9	–	40.0
	Symmetry factor of acetylacetone	M3	42.4	30.9	12.2	26.2	10.4	5.52	–	10.9
	$k'_{2,2'\text{-}dipyridyl}$	M6	3.05	6.46	3.51	3.10	1.77	1.55	–	11.4
	$k'_{2,3\text{-}dihydroxynaphthalene}$	M6	5.07	6.34	3.45	4.67	2.36	0.34	2.72	17.7
	$rk'_{2,3\text{-}dihydroxynaphthalene/2,2'\text{-}dipyridyl}$	M6	2.02	0.16	0.07	1.57	0.55	1.20	–	5.69
	Symmetry factor of 2,2′-dipyridyl	M6	17.3	24.3	7.13	8.60	2.51	14.1	-	46.3
	Symmetry factor of 2,3-dihydroxynaphthalene	M6	3.07	5.75	8.85	5.67	6.11	26.7	8.99	10.5
Other parameters	$rk'_{acetylsalicylic\ acid/MPPH}$	M2	0.02	1.02	2.15	2.16	0.23	2.35	0.23	4.34
	$rk'_{o\text{-}hydroxyhippuric\ acid/MPPH}$	M2	1.30	2.49	3.34	1.77	1.82	2.24	1.80	2.72
	$rk'_{theophylline/theobromine}$	M6	2.53	8.72	4.03	3.91	0.03	5.27	0.47	5.40
	$rk'_{caffeine/theophylline}$	M6	0.32	6.58	3.80	1.16	0.17	4.44	0.39	3.95
	$rk'_{toluene/phenol}$	M7	1.26	0.27	0.37	0.54	0.05	0.40	0.23	0.40
	$rk'_{toluene/phenol}$	M8	0.28	0.34	0.10	2.13	0.64	1.19	0.43	1.13

		Testing laboratory	Validated 1, 2	YMC-Hydro-sphere 1, 2	YMC Pro Pack 5 1, 3	Zorbax Eclipse 1, 2	Zorbax Extend 1, 2	Zorbax SB 1, 2	Average RSD of the 14 columns
Efficiency	n_{MPPH}	M2	3.27	0.48	5.29	0.52	10.9	5.41	3.24
	$n_{toluene}$	M7	4.14	7.09	1.84	4.20	5.14	4.38	4.26
	$n_{amylbenzene}$	M8	4.50	4.23	0.93	3.63	6.31	4.24	4.38
Hydrophobicity	$rk'_{diazepam/MPPH}$	M2	8.04	6.11	4.40	9.08	1.97	4.94	6.32
	$rk'_{toluene/MPPH}$	M2	0.32	0.45	1.50	0.32	0.12	0.06	0.92
	$k'_{toluene}$	M7	0.97	5.69	4.21	0.52	2.28	0.62	1.98
	$k'_{ethylbenzene}$	M7	0.88	5.61	5.27	0.26	2.43	0.20	0.97
	$rk'_{ethylbenzene/toluene}$	M7	0.09	0.58	1.06	0.30	0.13	0.83	0.34
	$k'_{amylbenzene}$	M8	0.85	5.45	2.29	1.34	1.36	0.51	1.41
	$rk'_{ethylbenzene/toluene}$	M8	0.09	0.34	0.01	0.13	0.01	3.18	0.38
	$rk'_{amylbenzene/butylbenzene}$	M8	0.06	0.71	0.05	0.07	0.05	0.46	0.16
Silanol activity	Symmetry factor of diphenhydramine	M2	33.3	9.63	52.1	16.3	20.4	12.8	20.1
	$rk'_{diphenhydramine/MPPH}$	M2	1.81	11.8	43.3	0.08	4.45	1.50	6.15
	$rk'_{caffeine/phenol}$	M6	1.21	1.56	1.44	2.45	2.59	0.65	1.39
	$rk'_{pyridine/caffeine}$	M6	0.77	0.41	5.62	1.86	1.99	0.48	2.84
	$rk'_{pyridine/phenol}$	M6	0.45	1.17	4.24	0.60	0.72	21.9	4.02
	Symmetry factor of pyridine	M6	3.59	18.3	28.4	4.18	15.3	2.39	13.4
	Symmetry factor of *p*-ethylaniline	M7	24.5	14.0	1.74	29.8	2.62	5.59	13.2
Ion-exchange capacity	$rk'_{benzylamine/phenol}$ pH 2.7	M4	11.2	8.21	15.2	0.43	1.52	0.13	6.10
	$rk'_{benzylamine/phenol}$ pH 7.3	M5	18.5	17.7	3.58	16.2	12.0	5.62	10.2
Steric selectivity	$rk'_{triphenylene/o\text{-}terphenyl}$	M8	0.14	0.8	0.26	0.08	0.07	0.01	0.44
Metal impurity	DERT	M1	53.2	30.2	52.0	30.5	27.9	35.5	40.0
	peak area/peak height of acetylacetone	M3	25.0	10.9	19.4	16.7	13.5	20.1	19.4
	$n_{acetylacetone}$	M3	24.7	28.1	39.3	31.9	27.4	30.8	36.6
	Symmetry factor of acetylacetone	M3	5.00	17.5	9.41	25.1	17.3	24.4	18.2
	$k'_{2,2'\text{-}dipyridyl}$	M6	4.88	10.8	0.24	1.66	1.72	6.37	4.34
	$k'_{2,3\text{-}dihydroxynaphthalene}$	M6	5.31	11.4	0.06	1.05	2.97	3.41	4.77
	$rk'_{2,3\text{-}dihydroxynaphthalene/2,2'\text{-}dipyridyl}$	M6	0.41	0.62	0.31	0.65	1.22	3.14	1.35
	Symmetry factor of 2,2′-dipyridyl	M6	5.99	9.98	15.8	13.1	38.3	13.3	16.7
	Symmetry factor of 2,3-dihydroxynaphthalene	M6	14.1	27.6	35.5	3.91	4.85	9.09	12.2
Other parameters	$rk'_{acetylsalicylic\ acid/MPPH}$	M2	0.23	0.98	1.31	0.93	0.28	0.55	1.19
	$rk'_{o\text{-}hydroxyhippuric\ acid/MPPH}$	M2	1.43	3.65	0.30	2.31	0.99	0.94	1.94
	$rk'_{theophylline/theobromine}$	M6	11.4	8.25	16.7	0.21	5.45	3.17	5.39
	$rk'_{caffeine/theophylline}$	M6	11.8	5.87	14.6	1.33	0.59	0.91	3.99
	$rk'_{toluene/phenol}$	M7	0.16	0.46	1.17	0.53	0.12	0.09	0.43
	$rk'_{toluene/phenol}$	M8	0.21	1.23	0.11	0.75	0.19	3.15	0.85

M refers to the method used to determine the given parameter: n, theoretical plate number; rk', relative retention factor; k', retention factor; DERT, dihydroxynaphthalene efficiency ratio test; empty cell, the compound was not eluted.

Table 4
Measured parameters of the columns

Column number	Peak area/peak height of aa	$rk'_{benzylamine/phenol}$ pH 2.7	pH 7.3	$k'_{2,2'\text{-dipyridyl}}$	$rk'_{caffeine/phenol}$	$k'_{toluene}$	$rk'_{ethylbenzene/toluene}$	SF *p*-ethylaniline	$n_{amylbenzene}$	$rk'_{triphenylene/terphenyl}$
Method	M3	M4	M5	M6	M6	M7	M7	M7	M8	M8
1	11.5	0.082	0.309	6.81	0.37	5.81	1.80	1.36	18 680	1.50
2	14.0	0.085	0.306	6.32	0.36	5.46	1.80	1.25	24 170	1.51
3	11.6	0.058	0.454	12.32	0.47	8.59	1.81	1.33	17 230	1.45
4	17.4	0.073	0.461	11.98	0.44	8.86	1.81	1.35	20 010	1.46
5	23.1	0.001	0.953	7.77	0.80	3.13	1.65	1.13	16 240	2.32
6	148				0.51	4.86	1.76		15 100	1.40
7	13.0	0.065	0.314	9.24	0.51	6.56	1.83	1.25	12 940	1.27
8	42.8	0.090	0.771	7.48	0.65	3.56	1.70	1.27	10 980	1.22
9	11.3	0.090	0.441	5.96	0.47	3.86	1.78	2.25	16 070	1.37
10	12.7	0.118	0.928	6.10	0.47	3.79	1.76	2.10	14 890	1.50
11	11.5	0.073	0.472	3.63	0.41	2.82	1.79	1.55	7330	1.46
12	12.6	0.086	0.301	4.36	0.39	3.67	1.78	1.15	21 120	1.50
13	11.8	0.082	0.334	8.86	0.44	7.47	1.83	1.33	8640	1.37
14	15.9	0.079	0.302	9.91	0.44	8.07	1.85	1.46	23 840	1.32
15	8.02	0.122	0.271	6.08	0.36	4.70	1.79	1.97	7670	1.53
16	12.0	0.136	0.332	6.60	0.35	4.33	1.79	4.14	18 900	1.56
17	12.5	0.692	1.361		0.43	4.59	1.40	1.83	7840	1.28
18	20.5	0.680	1.318		0.43	4.43	1.79		17 410	1.31
19	10.7	0.086	0.298	4.43	0.37	3.76	1.79	1.28	14 410	1.55
20	12.3	0.081	0.295	4.35	0.36	3.79	1.79	1.35	10 730	1.58
21	10.9	0.077	0.279	11.22	0.38	9.74	1.82	2.27	7510	1.52
22	12.3	0.065	1.971	9.66	0.41	7.67	1.77	2.44	18 810	1.63
23	16.3	0.076	0.260	9.40	0.37	8.77	1.82	1.78	22 560	1.56
24	17.7	0.187	2.717		0.81	4.26	1.72	2.36	19 105	1.78
25	194	0.176			0.63	8.39	1.77	2.32	16 630	1.77
26	13.8	0.031	0.249	8.63	0.41	7.10	1.84	1.27	13 100	1.15
27	7.33	0.068	1.984	16.42	0.82	5.60	1.74	1.87	8590	1.72
28	13.1	0.114	1.573	13.01	0.74	5.78	1.74	1.79	20 050	1.66
29	32.4	0.081	0.331	8.39	0.41	7.11	1.81	1.45	20 780	1.45
30	11.0	0.012	0.393	5.47	0.34	4.42	1.69	1.18	18 140	1.95
31	18.7	0.081	0.305	10.36	0.34	9.09	1.81	1.24	22 630	1.67
32	215	0.133	0.516	14.24	0.48	7.21	1.84	2.93	7390	1.33
33	12.9	0.178	1.085	7.26	0.74	3.01	1.73	2.71	13 440	1.21

34	24.6	0.196	1.963	9.15	0.83	2.95	1.70	1.64	12 320	1.23
35	10.4	0.386	8.163	9.34	0.45	1.44	1.58		6920	1.87
36	12.2	0.401	8.837	10.20	0.43	1.48	1.57	1.41	11 390	1.89
37	11.3	0.057	0.260	8.61	0.41	7.08	1.84	1.44	11 510	1.20
38	8.89	0.019	1.000	11.27	0.57	6.01	1.72	1.19	22 620	1.90
39	19.4	0.059	0.324	14.21	0.44	10.0	1.79	1.15	11 580	1.71
40	18.3	0.062	0.310	12.39	0.44	9.08	1.79	1.18	20 160	1.54
41	175	0.238	0.904		0.50	8.58	1.83	5.53	17 270	1.42
42	143	0.222	1.477		0.63	7.01	1.79	1.95	9340	1.56
43	180	0.263	1.615		0.65	7.09	1.79	2.53	23 340	1.55
44	51.2				0.41	4.97	1.81		20 960	1.43
45		0.134	0.350	6.96	0.43	4.83	1.81	2.52	17 420	1.40
46		0.157	0.418	7.07	0.43	4.72	1.81	3.47	20 960	1.37
47	33.9	0.107	0.374	9.21	0.39	7.96	1.79	2.51	22 690	1.59
48	6.94	0.024	0.281	8.62	0.37	7.47	1.80	1.56	9470	1.57
49	16.0	0.022	0.318	9.01	0.38	7.90	1.81	1.17	22 310	1.56
50	12.4	0.073	0.332	9.28	0.44	7.37	1.84	1.40	19 240	1.35
51	13.4	0.068	0.381	8.82	0.46	7.00	1.82	1.40	21 930	1.37
52	14.9	0.059	0.328	9.06	0.47	6.74	1.82	1.33	13 120	1.28
53	11.3	0.072	0.457	4.24	0.44	2.88	1.78	1.49	7830	1.46
54	10.9	0.065	0.355	9.17	0.46	6.49	1.83	1.37	8160	1.28
55	14.0	0.082	0.528	9.64	0.50	6.46	1.82	1.53	16 910	1.30
56	9.90	0.096	1.000	8.69	0.47	6.51	1.80	2.14	9770	1.43
57	14.1	0.070	0.383	9.25	0.44	7.32	1.82	1.26	17 570	1.37
58	26.1	0.068	0.785	9.86	0.51	6.83	1.81	1.14	21 620	1.41
59	11.6	0.057	0.370	7.33	0.40	6.38	1.83	1.31	6820	1.41
60	14.8	0.070	0.387	7.70	0.43	6.43	1.83	1.21	14 130	1.41
61	13.8	0.051	0.350	10.22	0.26	8.00	1.84	1.78	8130	1.27
62	15.2	0.044	0.385	10.41	0.43	8.17	1.84	1.72	27 860	1.30
63	7.43	0.045	0.157	3.94	0.32	3.45	1.61	2.00	5840	1.82
64	11.4	0.032	0.309	7.93	0.52	5.10	1.81	1.19	14 090	1.19
65	11.8	0.048	0.292	7.95	0.44	6.63	1.83	1.29	19 910	1.32
66	12.2	0.035	0.282	8.34	0.44	6.77	1.86	1.25	16 350	1.26
67	12.7	0.072	0.304	7.99	0.41	7.01	1.87	1.37	22 120	1.30
68	12.4	0.067	0.430	7.83	0.37	7.32	1.85	1.38	19 200	1.48
69	11.5	0.067	0.613	9.27	0.57	5.97	1.85	1.35	21 000	1.22

Empty cells, parameter cannot be measured because the compound(s) is not eluted.

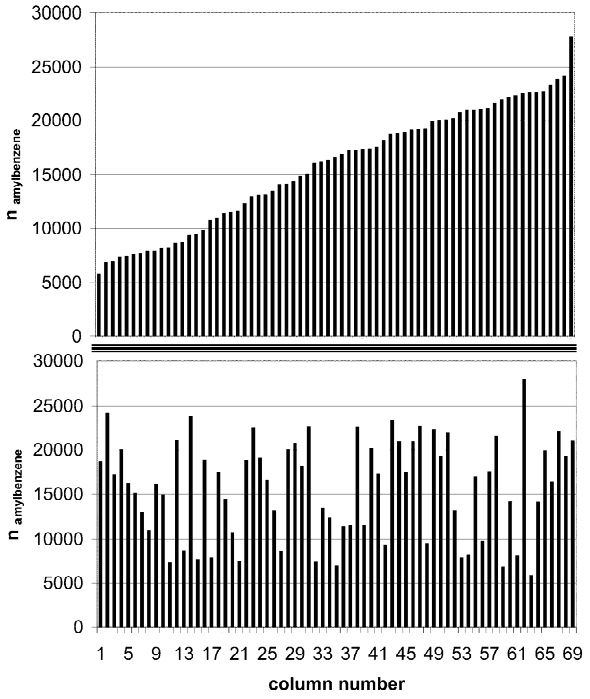


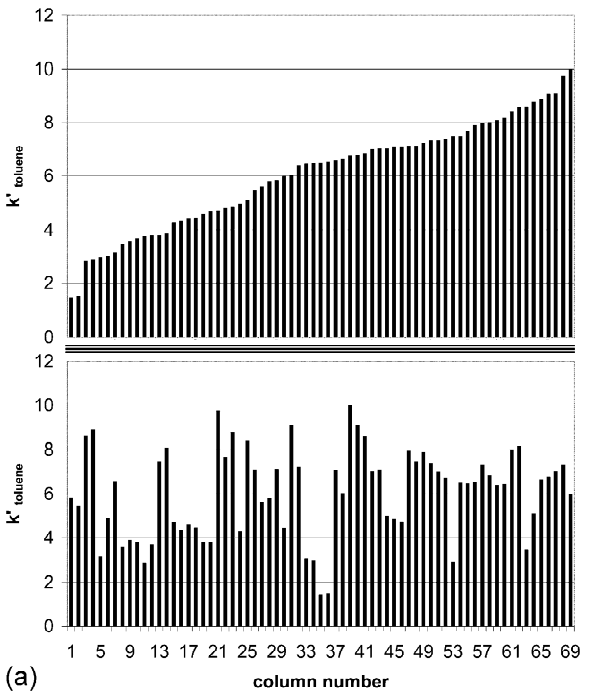
Fig. 1. Efficiency of RP-LC columns: theoretical plate number of amylbenzene determined according to Method 8. Bottom: columns arranged as in Table 1; top: columns arranged according to ascending efficiency.

$rk'_{\text{diazepam/MPPH}}$ and $rk'_{\text{toluene/MPPH}}$ (Method 2); k'_{toluene} and $k'_{\text{ethylbenzene}}$ (Method 7); $rk'_{\text{ethylbenzene/toluene}}$ (Methods 7 and 8), $k'_{\text{amylbenzene}}$ (Method 8) and $rk'_{\text{amylbenzene/butylbenzene}}$ (Method 8). No practical problems were observed during their determination.

Repeatability and reproducibility of these parameters were excellent, the average RSD values between laboratories were less than 2% except in the case of $rk'_{\text{diazepam/MPPH}}$, which showed the poorest reproducibility.

The retention factors of toluene, ethylbenzene and amylbenzene measured with Methods 7 and 8 are highly correlated ($r>0.99$, see Table 5(b)).

The correlation coefficients between $rk'_{\text{ethylbenzene/toluene}}$ (Methods 7 and 8) and $rk'_{\text{amylbenzene/butylbenzene}}$ (Method 8) were larger than 0.94. The relative retention factors evaluated from Method 2 ($rk'_{\text{diazepam/MPPH}}$, and $rk'_{\text{toluene/MPPH}}$), do not correlate well ($r=0.63$). They do not provide the same information as the relative retention factors from Methods 7 and 8, the correlation between them



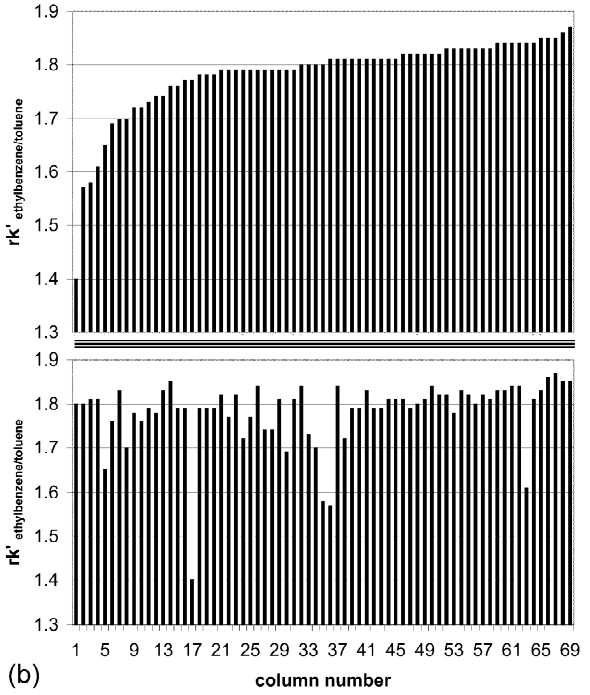
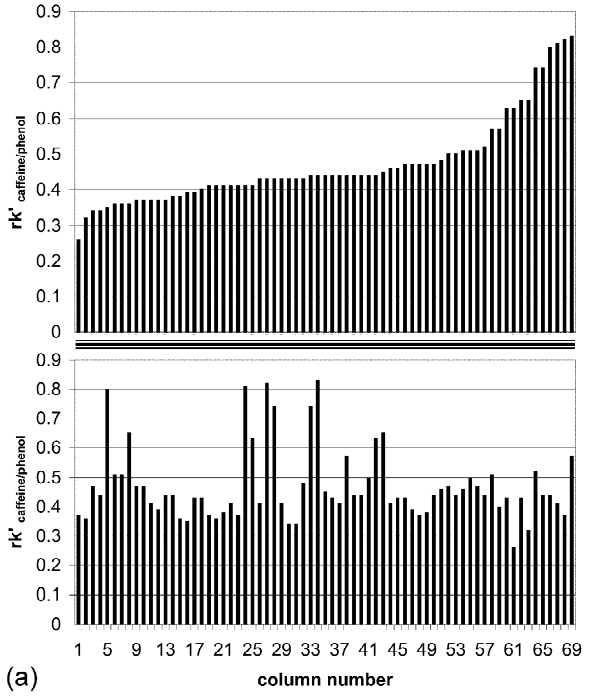


Fig. 2. Hydrophobicity of RP-LC columns: (a) retention factor of toluene, (b) relative retention factor of ethylbenzene/toluene, both determined according to Method 7. Bottom: columns arranged as in Table 1; top: columns arranged according to ascending hydrophobicity.



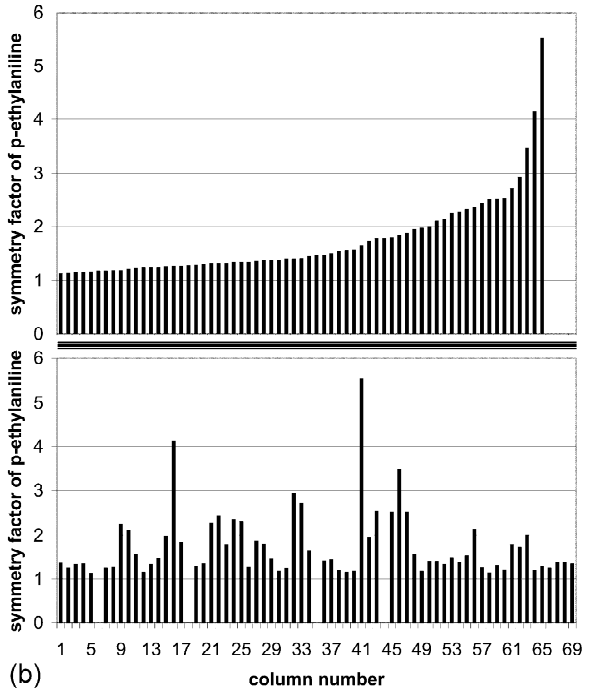
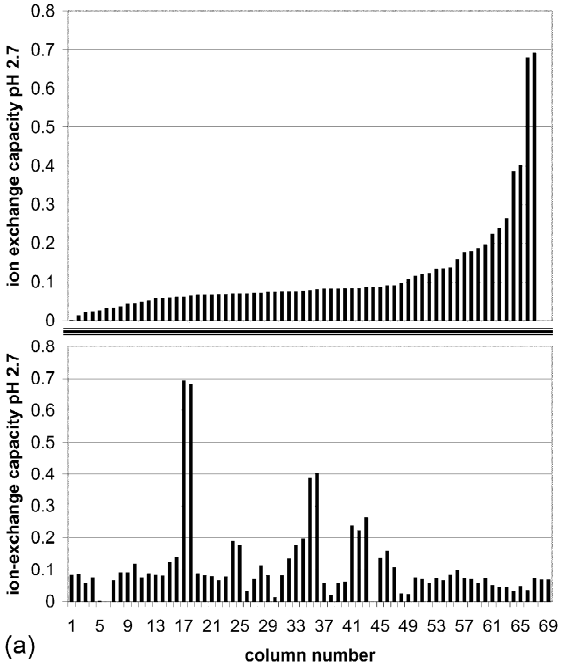


Fig. 3. Silanol activity of RP-LC columns: (a) relative retention factor of caffeine/phenol determined with Method 6, (b) symmetry factor of *p*-ethylaniline, determined according to Method 7. Bottom: columns arranged as in Table 1; top: columns arranged according to ascending silanol activity.



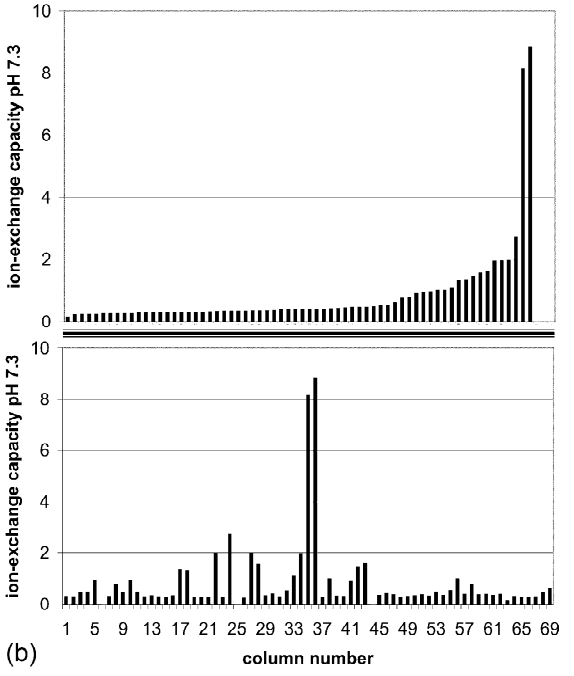


Fig. 4. Ion-exchange capacity of RP-LC columns: (a) relative retention factor of benzylamine/phenol at pH 2.7 according to Method 4, (b) relative retention factor of benzylamine/phenol at pH 7.3 according to Method 5. Bottom: columns arranged as in Table 1; top: columns arranged according to ascending ion-exchange capacity.

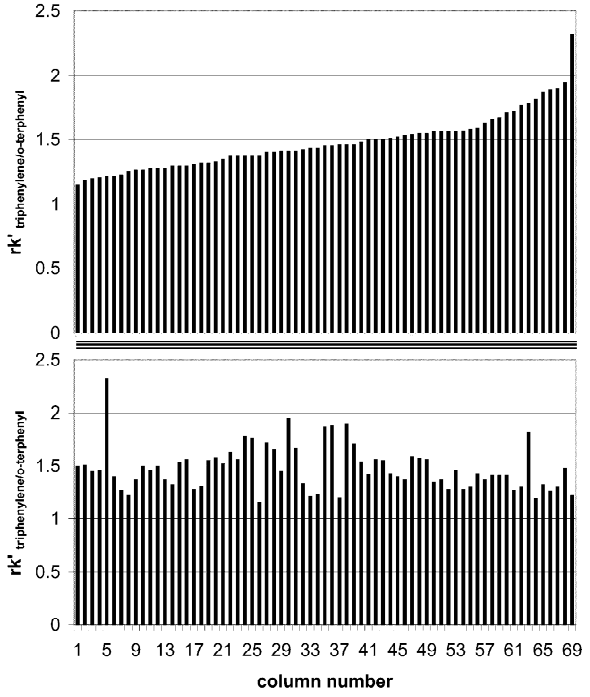
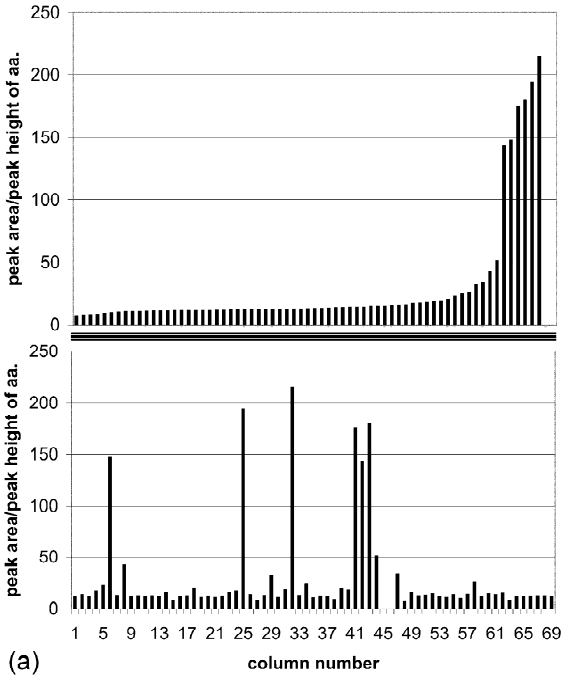


Fig. 5. Steric selectivity of RP-LC columns: relative retention factor of triphenylene/*o*-terphenyl, determined according to Method 8. Bottom: columns arranged as in Table 1; top: columns arranged according to ascending steric selectivity.

is rather poor ($0.51<r<0.76$). It should be mentioned that the analytes in Method 2 are not purely aromatic and that the mobile phase of Method 2 is buffered, while Methods 7 and 8 use unbuffered eluent.

Retention factors and relative retention factors have correlation coefficients between 0.36 and 0.77. Better correlation ($0.58<r<0.77$) can be found if only non-buffered data are taken into account, although the probes employed are neutral.

The hydrophobicity parameters, which were measured with non-buffered mobile phase, all gave similar information and therefore any of them can be used for column classification. In Fig. 2, the hydrophobicity of the columns, obtained with two different methods, is reported. The pattern of columns remains the same if the different retention factors are considered (exception: column 52) and very similar if retention factors and relative retention factors are examined (exceptions: columns 11, 21, 25, 39, 52 and 53). These figures are not shown. Because the



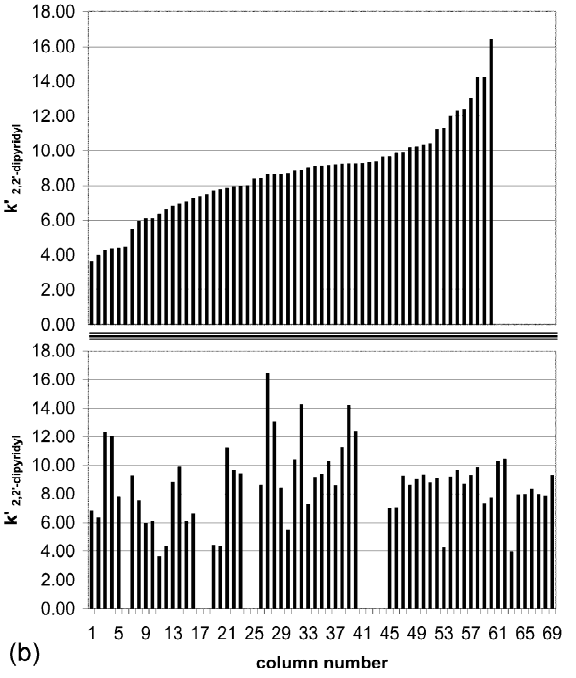


Fig. 6. Metal impurity of RP-LC columns: (a) peak area/peak height of acetylacetone (a.a.), determined with Method 3, (b) relative retention factor of 2,3-dihydroxynaphthalene/2,2′-dipyridyl, determined with Method 6. Bottom: columns arranged as in Table 1; top: columns arranged according to ascending metal contamination value.

Table 5
Correlation coefficients (r) between parameters estimating the same column property

Parameter	Method	1	2	3	4	5	6	7	8	9
(a) Efficiency										
1. n_{MPPH}	M2	1.000	0.903	0.917						
2. $n_{toluene}$	M7		1.000	0.982						
3. $n_{amylbenzene}$	M8			1.000						
(b) Hydrophobicity										
1. $rk'_{diazepam/MPPH}$	M2	1.000	0.629	0.364	0.377	0.505	0.383	0.523	0.561	
2. $rk'_{toluene/MPPH}$	M2		1.000	0.692	0.707	0.672	0.713	0.657	0.764	
3. $k'_{toluene}$	M7			1.000	0.999	0.667	0.989	0.579	0.669	
4. $k'_{ethylbenzene}$	M7				1.000	0.700	0.994	0.615	0.726	
5. $rk'_{ethylbenzene/toluene}$	M7					1.000	0.734	0.976	0.950	
6. $k'_{amylbenzene}$	M8						1.000	0.663	0.768	
7. $rk'_{ethylbenzene/toluene}$	M8							1.000	0.943	
8. $rk'_{amylbenzene/butylbenzene}$	M8								1.000	
(c) Silanol activity										
1. Symmetry factor of diphenhydramine	M2	1.000	0.816	0.212	0.630	0.650	0.545	0.637		
2. $rk'_{diphenhydramine/MPPH}$	M2		1.000	0.180	0.710	0.663	0.335	0.463		
3. $rk'_{caffeine/phenol}$	M6			1.000	0.067	0.681	0.388	0.107		
4. $rk'_{pyridine/caffeine}$	M6				1.000	0.815	0.150	0.349		
5. $rk'_{pyridine/phenol}$	M6					1.000	0.547	0.482		
6. Symmetry factor of pyridine	M6						1.000	0.465		
7. Symmetry factor of *p*-ethylaniline	M7							1.000		
(d) Metal impurity										
1. DERT	M1	1.000	0.800	−0.327	0.211	−0.116	0.061	0.400	−0.072	0.518
2. Peak area/peak height of acetylacetone	M3		1.000	−0.453	0.510	0.300	0.118	−0.092	0.343	0.631
3. $n_{acetylacetone}$	M3			1.000	−0.294	0.006	−0.270	−0.146	0.189	−0.145
4. Symmetry factor of acetylacetone	M3				1.000	0.113	−0.028	−0.076	0.522	0.364
5. $k'_{2,2'\text{-dipyridyl}}$	M6					1.000	0.362	−0.461	0.259	0.018
6. $k'_{2,3\text{-dihydroxynaphthalene}}$	M6						1.000	0.484	0.028	−0.013
7. $rk'_{2,3\text{-dihydroxynaphthalene}/2,2'\text{-dipyridyl}}$	M6							1.000	−0.171	0.000
8. Symmetry factor of 2,2′-dipyridyl	M6								1.000	0.382
9. Symmetry factor of 2,3-dihydroxynaphthalene	M6									1.000

retention factor can be determined with most precision, and only one substance is required to measure it, this parameter can be recommended to characterise hydrophobicity. These observations are in accordance with those in the literature [43].

Columns 35 and 36 clearly show lower hydrophobicity with both methods (Fig. 2a,b) but no classification of the columns is possible on the basis of hydrophobicity alone.

3.3. Silanol activity

It was an early observation that free silanol groups on RP-LC columns can be evaluated with basic compounds using normal-phase chromatography. Strong retention of nitrobenzene [47,48], pyridine and 2,6-dimethylpyridine [45] or aniline [14] is a sign of high silanol activity. Later, basic compounds and reversed-phase chromatography were generally

used to examine silanol activity. Poor peak symmetry [12,21,42] and strong retention of basic compounds [19,41,49] indicate the activity and accessibility of free silanols on the silica surface. Pyridine [39,45], aniline [18,21], aniline derivatives [18,27,42] and basic drugs (e.g. diphenhydramine, amytriptiline) [12,31] are applied for these measurements. Free silanol groups can be evaluated by a selectivity factor of two compounds, which have different acid/base characters: α of caffeine/phenol [15,28], pyridine/phenol [50], caffeine/theophylline [8] or diphenhydramine/5-*p*-methylphenyl-5-phenylhydantoin [12] are frequently used. Separation of *ortho*-, *meta*- and *para*-toluidine is also a sign of high silanol activity [17,23,25]. Characterisation of silanol activity was reviewed a few years ago [51].

Some practical problems were observed during examination of the silanol activity. On some columns, diphenhydramine, pyridine and *p*-ethylaniline peaks could not be detected because they were too strongly retained. The repeatability and reproducibility of all the symmetry factors was poor (Tables 2 and 3). The relative retention factors can be measured with good repeatability (RSD: 1–10%) and reproducibility (average RDS: 1.4–6.1%). The $rk'_{\text{caffeine/phenol}}$ results for the columns are shown in Fig. 3a.

Table 5(c) contains the correlation data between parameters regarding silanol activity. Good correlation was found between the symmetry factor of diphenhydramine and $rk'_{\text{diphenhydramine/MPPH}}$ ($r=0.82$), both measured in Method 2. Less correlation ($r=$ 0.63 and 0.71) exists between these two parameters and $rk'_{\text{pyridine/caffeine}}$ (Method 6). These three parameters also show some correlation ($r=0.35$–0.64) with the symmetry factor of *p*-ethylaniline. The correlation coefficients of $rk'_{\text{pyridine/phenol}}$ and all the other parameters are between 0.48 and 0.82. Poor correlation was observed between the symmetry factor of pyridine and *p*-ethylaniline ($r=0.47$). No correlation was found between the latter parameter and $rk'_{\text{caffeine/phenol}}$ ($r=0.11$). These results clearly indicate that the parameters do not represent the same column property. This can be due to the fact that the silanol activity may not be the same at buffered (M2) or non-buffered conditions (M7 and M8). It is also possible that some of the parameters are less suitable for characterising silanol activity.

It was observed that there is no method in this protocol in which a symmetry factor of a base is measured in neutral mobile phase. Therefore additional data on the symmetry factor of benzylamine were obtained with Method 5 for 60 columns (data not provided). The correlation between the symmetry factor of benzylamine determined at pH 7.3 and the other parameters related to silanol activity was calculated. The following correlation coefficients were obtained: symmetry factor of diphenhydramine ($r=0.750$), $rk'_{\text{diphenhydramine/MPPH}}$ ($r=0.720$), $rk'_{\text{benzylamine/phenol}}$ pH 2.7 ($r=7.190$) and $rk'_{\text{pyridine/phenol}}$ ($r=0.713$). According to these results, which are not discussed further, it was concluded that no information was lost due to the fact that the determination of the symmetry factor of benzylamine was not included in the protocol.

The symmetry factor of *p*-ethylaniline is a frequently used parameter for silanol activity. Considering the precision problems related to its determination, it is not recommended as a test parameter. Classification of columns according to $rk'_{\text{caffeine/phenol}}$ (Fig. 3a) yields totally different results than classification using the symmetry factor of *p*-ethylaniline (Fig. 3b) as could be expected from their correlation coefficients. The above results confirm the claim of Claessens et al. [55] that different silanol activity test results often are not in mutual agreement and not interchangeable. Column classification on silanol activity will depend on the test method applied.

In Fig. 3a, a small group of columns with high silanol activity can be distinguished ($rk'=0.5$). There is a large group of columns with medium silanol activity ($0.3<rk'<0.5$) and only one column (Column 61) with low silanol activity ($rk'=0.3$). In Fig. 3b, which shows the symmetry factor of *p*-ethylaniline, again a small group with high silanol activity can be recognised with the symmetry factor of *p*-ethylaniline>2.5 or ∞ (empty cases where no peak of *p*-ethylaniline can be detected). Columns with distinctly low silanol activity do not form a well-separated group.

3.4. Ion-exchange capacity

The ion-exchange capacity can be characterised by measuring the relative retention factors between a base and a neutral or a slightly acidic compound at

low and relatively high pH values [32,33]. Low ion-exchange capacity is characterised by small values. The selectivity factor of benzylamine/phenol is widely used in the literature [15,26,28,35], recently bretylium tosylate [52] was employed for this purpose.

In this test procedure, the ion-exchange capacity was measured as the $rk'_{\text{benzylamine/phenol}}$ determined at acidic (Method 4) and neutral pH (Method 5). The retention time and peak symmetry of benzylamine in Method 5 depended on the amount injected, therefore a well-defined concentration has to be used. The repeatability was good at both pH values, and the reproducibility was acceptable at pH 2.7 but poor at pH 7.3.

Fig. 4a and 4b show the ion-exchange capacity of the stationary phases examined at pH 2.7 and 7.3, respectively. No benzylamine peak could be detected for column 6 at both pH values and therefore it is not possible to determine its ion-exchange capacity with this method.

At pH 2.7, there is a small group of columns with high ion-exchange capacity ($rk'_{\text{benzylamine/phenol}}$ 2.7$>$0.3) and a large group with low capacity ($rk'_{\text{benzylamine/phenol}}$ 2.7$<$0.10). At pH 7.3, few columns show high ion-exchange capacity ($rk'_{\text{benzylamine/phenol}}$ 7.3$>$2.0) while several columns have low ion-exchange capacity ($rk'_{\text{benzylamine/phenol}}$ 7.3$<$0.75). In total, 85% of the columns having low ion-exchange capacity at pH 2.7 also show this property at pH 7.3 but several stationary phases have high ion-exchange capacity at only one pH value.

No good correlation was noted between parameters characterising silanol activity and ion-exchange capacity.

3.5. Steric selectivity

Steric selectivity can be determined using two aromatic hydrocarbons: one twisted and one planar, with mobile phases containing methanol/water [32–34]. The selectivity factor of the compounds is a measure for the steric selectivity. Tetrabenzonaphthalene/benzo[*a*]pyrene [25], phenantrophenantrene/tetrabenzo-naphthalene [54,55] and other polycyclic aromatic hydrocarbons [41,53,54] are generally used. These compounds are toxic, and most of them are carcinogenic. The selectivity factor of triphenylene/*ortho*-terphenyl is also widely applied to characterise steric selectivity [15,26,28,33].

In this work, steric selectivity was calculated using $rk'_{\text{triphenylene/}ortho\text{-terphenyl}}$ because the two compounds used are not carcinogenic. There was no practical problem during the determination of this parameter. Repeatability and reproducibility were excellent, RSD values were below 1.5% in every case. Results for the columns tested can be seen in Fig. 5. Classification of the columns cannot be made.

The highest steric selectivity is observed for columns 5, 30, 33, 34, 38 and 63; most of these columns are polar-embedded. All the other columns are in the range 1.0–2.0 and most show values in the range 1.0–1.5. There are no stationary phases with extremely low steric selectivity.

3.6. Metal impurity

Metal impurity on the silica surface can be examined using chelating agents. Beer bitter acids [56], acetylacetone [13,25], 2,2′-dipyridyl [39,57,58] 2,3-dihydroxynaphthalene [26,39,58] or hinokitiol [49,50] have been used to characterise metal contamination. Small theoretical plate numbers, strong retention and poor peak symmetry of these compounds are indications for metal ions on the silica surface [58]. Measurements have to be carried out at optimum pH, where these compounds can form complexes with metal ions. It is also recommended to carry out these measurements on a metal-free instrument to avoid metal–ion accumulation on the packing.

Nine parameters were introduced in the test procedure to characterise metal contamination of the supports. Each method gave problems because the compounds were not eluted from some stationary phases. Repeatability was poor, reproducibility was even worse (Tables 2 and 3). Only the $rk'_{\text{2,3-dihydroxynaphthalene/2,2′-dipyridyl}}$, $k'_{\text{2,3-dihydroxynaphthalene}}$ and $k'_{\text{2,2′-dipyridyl}}$ could be reproduced easily (Tables 2 and 3).

Only one high correlation can be found in Table 5(d): the dihydroxynaphthalene efficiency ratio test (DERT) and peak area/peak height of acetylacetone ($r=0.80$). These two parameters give the same information. Poor correlation ($r=0.48$) was found between $k'_{\text{2,3-dihydroxynaphthalene}}$ and

$rk'_{2,3\text{-dihydroxynaphthalene}/2,2'\text{-dipyridyl}}$ measured with Method 6. Very different correlation coefficients were found between the parameters, indicating that they do not measure the same column property. Correlation factors >0.75 were observed between $k'_{2,3\text{-dihydroxynaphthalene}}$ (representing metal impurity) and k'_{toluene} or $k'_{\text{ethylbenzene}}$ (representing hydrophobicity). This indicates that the retention of this aromatic metal complexing agents also depends on the hydrophobicity of the columns or that this parameter measures hydrophobicity rather than metal contamination.

Peak area/peak height ratio of acetylacetone may be considered as a standard method to characterise metal contamination [13] (Fig. 6a). A cluster of columns with a high amount of metal impurity can be distinguished when this parameter is above 100. It has to be noted that this parameter also depends on the column dimension and the particle size. If one compares the columns from the same brand but having different length and/or particle size (Columns 1–2, 3–4, 9–10, 13–14, 19–20, 33–34, 35–36, 50–51, 54–55, 56–57, 61–62 and 65–66), it can be seen that in most of the cases these latter effects are smaller than the differences between columns from different brands.

One of the most repeatable and reproducible metal impurity parameters is $k'_{2,2'\text{-dipyridyl}}$ (Fig. 6b). It can be regarded as a sign of high metal contamination when this parameter is above 12. Empty cases in Fig. 6 mean that the compound was not eluted. No columns with extremely low values can be found with any of these methods. As can be expected from the poor correlation in Table 5(d) ($r=0.30$), the column ranking in Fig. 6a,b is different.

It can be concluded that classification of RP-columns based on metal impurity depends on the parameter used.

3.7. Other parameters

Further correlation was examined between the parameters measuring the above properties and the so-called other parameters (Tables 2 and 3) that were proposed in the original articles.

No examples of high correlation were found between other parameters and one that refers to a particular property (e.g. silanol activity). An additional parameter for a particular property was therefore not detected.

3.8. Comparison of short and long columns

Parameters of columns, which were made from the same material but have different particle size and column length, showed similar parameters. Only results concerning the efficiency were different. This means that these columns have the same selectivity but their efficiency depends on the particle size and column length.

4. Conclusion

After careful study of literature data, a test procedure was proposed to characterise RP-LC columns. This test procedure was applied to 69 C_{18} RP-LC columns in order to characterise them on the basis of the parameters measured. Several columns were examined in different laboratories in order to check the reproducibility of the results.

Each property of the RP stationary phases was characterised by different parameters, which were compared. At least one parameter regarding the efficiency, hydrophobicity, silanol activity, steric selectivity and metal impurity can be determined with good precision. For the ion-exchange capacity, the precision is poorer. It is therefore more difficult to examine the latter property.

Parameters characterising efficiency are highly correlated. Classification based on any of these parameters provides a similar pattern, independent of the parameter applied. Hydrophobicity parameters were also well-correlated and all of them give similar classification. Correlation between parameters describing silanol activity is usually poor, therefore the classification pattern highly depends on the parameter used. This statement is also true for the parameters concerning metal impurity. It can be concluded that not all the parameters measure the properties they are claimed to do in the literature.

For each of the parameters, the columns showed at least slightly different results, which means that these parameters allow characterising the columns. It was observed that one parameter is insufficient to classify stationary phases in distinct groups. There-

fore in a next step, classification of the columns based on several test parameters needs to be evaluated. This will be done using chemometric methods. Such chemometric methods have already been applied in the classification of columns, but only a restricted number of testing results was used [18,23,25,26,28,30,34,48,59–61].

Later, a classification (clustering) of columns based on a limited (minimal) number of test parameters will be made and representative separations will be carried out on these stationary phases. The correlation between the results of the test procedure and the chromatographic behaviour of these columns in the compendial analyses will be examined by chemometric methods.

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