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Characterisation of reversed-phase liquid-chromatographic columns by chromatographic tests
Comparing column classification based on chromatographic parameters and column performance for the separation of acetylsalicylic acid and related compounds

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Abstract

Selection of RP-LC columns with suitable selectivity for a given analysis is difficult. For example, the European Pharmacopoeia (Ph. Eur.) and other official compendia for drug analysis only give a general description of the stationary phase in the operating procedure of a liquid chromatographic method. The need for a general test method to characterise RP-LC columns has been rising since the 1970s. A project to define a chromatographic procedure characterising RP-LC columns was started earlier. A procedure to measure test parameters was introduced and a classification of the columns, based on a minimal number of parameters, was obtained. This paper focuses on correlating the column classification with the selectivity obtained for a real separation. The separation of acetylsalicylic acid (aspirin) and related compounds was performed according to the Ph. Eur. monograph on the stationary phases previously characterised chromatographically. It was examined whether the classes of columns, determined using test parameter results, contain either suitable or unsuitable supports for the aspirin separation. The system suitability test prescribed by the Ph. Eur. in order to distinguish between suitable or unsuitable columns for this separation was also evaluated.

Keywords: Stationary phases, LC; Chromatographic tests; Principal component analysis; Column characterisation; Selectivity; Column performance; Chemometrics; Acetylsalicylic acid

1. Introduction

Official compendia, such as the European Pharmacopoeia (Ph. Eur.) or the United States Pharmacopeia (USP) contain numerous liquid chromatographic methods, mainly under reversed-phase conditions [1,2]. In the description of these methods, exact eluent composition and other experimental conditions are provided. Contrary to that, only very general information is given about the stationary phase to be used. Since the brand name of a suitable stationary phase is not allowed to be communicated in the monograph, the Ph. Eur. describes the type of the stationary phase in terms of chain length, end-capping, base-deactivation, particle size and sometimes pore size and specific surface area. This information is usually insufficient to select a suitable column with the required selectivity from a market offering more than 600 brands. Moreover, manufacturers provide only limited information about their columns. Earlier Steffeck and Engelhardt already drew the attention to the difficulties related to RP-LC column selection [3,4].

This problem can be solved if a general test method is available to characterise RP-LC columns. Several

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chromatographic column tests were published [5–37]. Properties of RP-LC columns, such as efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and the level of metal impurity are determined, without column destruction. However, none of the tests is generally accepted. Also, it has never been proven that columns with similar test characteristics indeed give similar chromatographic selectivity. Such verification is the goal of a project, to which the work described here belongs.

Earlier, after a study of the literature, eight different methods were selected and a test procedure was developed which allowed determining 36 parameters [38,39]. The test procedure was carried out on 69 RP-LC columns. A critical examination of the test parameters, their repeatability, reproducibility and correlation was made. Principal component analysis (PCA) was employed to deal with the column classification [40,41]. The column classification initially was performed with 24 parameters (of the 36 measured), which could be determined in a reproducible way. Rational classification was achieved and the number of parameters was reduced. It was shown that the classification could be maintained employing only four parameters [41]. It was also shown that column classification using three parameters was similar to a great extent, while the use of one or two parameters was not meaningful [41].

In this paper, the correlation between the test results to characterise or classify columns and the performance in a real separation is examined. The separation of acetylsalicylic acid (ASA) and its impurities, as prescribed by the Ph. Eur., was selected as a case study and carried out on the columns tested earlier. The Ph. Eur. system suitability test (SST), which requires a resolution between salicylic acid (SA) and ASA of at least 6.0, was performed in order to examine whether this test can be used to differentiate between suitable and non-suitable columns. Another method was also performed to determine the suitability of stationary phases by calculating the chromatographic response function (CRF), which is a measure for the overall selectivity [42,43]. Although this method gives more complete information, it needs the availability of all the components as a reference substance. Finally, it was examined whether column classes, based on the four test parameters, can be related with good or poor aspirin separations. In other words it was examined whether stationary phases, which have closely related test characteristics, show similar separations for ASA and its impurities.

In a recent paper of Gilroy et al., it is suggested that samples which have no base functions, as is the case for the ASA sample, show much less variability in column selectivity than samples which contain both acid and base functions [35]. Therefore, it can be remarked that this study of the ASA separation alone is insufficient to prove the ability of the column classification to select suitable columns. Indeed, other separations with different molecules and with differing chromatographic conditions (mobile phase composition, mobile phase pH, organic modifier, etc.) have to be studied.

2. Experimental

2.1. Chromatographic tests and columns tested

All columns were donations of manufacturers or distributors. They are reported in Table 1. Information concerning the test methods, the chromatographic conditions applied, the measured parameters and the column properties was published earlier [38,39,41].

2.2. Separation of ASA and its related compounds

2.2.1. Samples and reagents

Acetonitrile from Biosolve (Valkenswaard, The Netherlands) was of LC grade, other chemicals were of AR grade. Phosphoric acid, acetylsalicylic acid (ASA), salicylsalicylic acid (SSA), 4-hydroxybenzoic acid (HBA), 4-hydroxyisophthalic acid (HIPA) and acetylsalicylsalicylic acid (ASSA) were from Acros Organics (Beerse, Belgium), salicylic acid (SA) was purchased from Merck (Darmstadt, Germany). Acetylsalicylic anhydride (ASAN) was prepared in the laboratory according to a previously described method [44].

The sample solution contained 0.3 mg of HBA, 0.1 mg of HIPA, 0.2 mg of SA, 2.5 mg of ASA, 0.5 mg of ASSA, 0.7 mg of SSA and 0.3 mg of ASAN in 50.0 ml of acetonitrile. A good chromatographic system should separate all these potential impurities. The sample was prepared daily because some compounds are unstable in solution.

2.2.2. Chromatographic conditions

Analyses were carried out using a Varian (Walnut Creek, CA, USA) 9010 LC pump, 9100 autosampler and 9050 UV-Vis detector, with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK) for data acquisition. Column temperature was maintained by immersion in a water bath at 30 ± 0.1 °C, the laboratory was air-conditioned at 25 °C.

The separation of ASA and related compounds was performed according to the Ph. Eur. monograph [1] on the columns (Table 1) tested before with the chromatographic test procedure. Some earlier tested stationary phases (14, 15, 17, 21, 24, 27, 42, 48, 62 and 63) were no longer included in the study. However, the original numbering employed in previous papers [38,39,41] was kept. The mobile phase for the separation of ASA and related compounds was acetonitrile/water/phosphoric acid, 400/600/2 (v/v/v). Helium was used to degas the mobile phase. Columns were equilibrated for 30–90 min depending on their length. The flow rate was 1 ml/min. The injection volume was 20 μl and the detection wavelength 237 nm. A typical chromatogram is shown in Fig. 1a.

Table 1
Stationary phases involved in this project and their characteristics provided by the manufacturer

Column number	Name of the column	Length (mm)[a]	Particle size (μm)	Manufacturer/supplier	End-capped	Base deactivation	Polar embedded	Pore size (Å)
1	ACE 3 C18	150	3	Advanced Chrom. Tech./Achrom	+	+	−	100
2	ACE 5 C18	250	5	Advanced Chrom. Tech./Achrom	+	+	−	100
3	Alltima C18 3	150	3	Alltech	+	+	−	120
4	Alltima C18 5	250	5	Alltech	+	+	−	120
5	Apex Basic	250	5	Jones Chromatography/Sopachem	+	+	−	100
6	Apex ODS II	250	5	Jones Chromatography/Sopachem	+	−	−	100
7	Aqua	150	5	Phenomenex/Bester	+	−	−	125
8	μBondapak	250	10	Waters	+	−	−	125
9	Brava BDS 3	150	3	Alltech	+	+	−	145
10	Brava BDS 5	250	5	Alltech	+	+	−	145
11	Chromolith	100	–	Merck	+	−	−	–
12	Discovery C18	250	5	Supelco	+	−	−	180
13	Genesis C18 3	100	3	Jones Chromatography/Sopachem	+	+	−	100
16	Hypersil BDS 5	250	5	ThermoQuest	+	+	−	130
18	Hypersil ODS 5	250	5	ThermoQuest	+	−	−	120
19	HyPURITY Elite 3	150	3	ThermoQuest, SerCoLab	+	+		200
20	HyPURITY Elite 5	150	5	ThermoQuest, SerCoLab	+	+		200
22	Kromasil (MN)	250	5	Macherey-Nagel/Filter Service	+	−	−	100
23	Kromasil (EKA)	250	5	Akzo Nobel/ SerCoLab	+	−	−	100
25	LiChrospher	250	5	Merck	−	+	−	100
26	Luna	150	5	Phenomenex/Bester	+	−	−	100
28	Nucleosil 5	250	5	Macherey-Nagel/Filter Service	+	−	−	100
29	Nucleosil HD	250	5	Macherey-Nagel/Filter Service	+	−	−	100
30	Nucleosil Nautilus	250	5	Macherey-Nagel/Filter Service	−	−	+	100
31	OmniSpher	250	5	Varian	−	−	−	110
32	Pecospher C18	83	3	Perkin-Elmer	+	−	−	80
33	Platinum C18 3	150	3	Alltech	+	+	−	100
34	Platinum C18 5	250	5	Alltech	+	+	−	100
35	Platinum EPS C18 3	150	3	Alltech	−	+	−	100
36	Platinum EPS C18 5	250	5	Alltech	−	+	−	100
37	Prodigy	100	3	Phenomenex/Bester	+	−	−	100
38	Purospher	250	5	Merck	+	−	−	80
39	Purospher endcapped	250	5	Merck	+	+	−	80
40	Purospher STAR e	250	5	Merck	+	+	−	80
41	SPHERI-5	250	5	Perkin-Elmer	−	−	−	80
43	Spherisorb ODS2 5	250	5	Waters	+	−	−	80
44	Supelcosil LC-18	250	5	Supelco	−	−	−	120
45	Supelcosil LC-18 DB 3	150	3	Supelco	−	+	−	120
46	Supelcosil LC-18 DB 5	250	5	Supelco	−	+	−	120
47	Superspher	250	4	Merck	+	−	−	100
49	Symmetry 5	250	5	Waters	+	−	−	100
50	TracerExcel ODS A-3	150	3	Teknokroma/SerCoLab	NA	NA	NA	120
51	TracerExcel ODS A-5	250	5	Teknokroma/SerCoLab	NA	NA	NA	120
52	TSKgel ODS-80TS	150	5	TosoHaas/SerCoLab	+	−	−	80
53	TSKgel Super ODS	100	2	TosoHaas/SerCoLab	+	−	−	110
54	Uptisphere 3 HDOC18	100	3	Interchrom/Achrom	+	−	−	120
55	Uptisphere 5 HDOC18	250	5	Interchrom/Achrom	+	−	−	120
56	Uptisphere 3 ODB	100	3	Interchrom/Achrom	+	−	−	120
57	Uptisphere 5 ODB	250	5	Interchrom/Achrom	+	−	−	120
58	Validated C18	250	5	Perkin-Elmer	+	−	−	100
59	Wakosil C18 HG 5–10	100	5	SGE/Achrom	+	−	−	120
60	Wakosil C18HG 5–25	250	5	SGE/Achrom	+	−	−	120
61	Wakosil C18 RS 3–10	100	3	SGE/Achrom	+	−	−	125
64	YMC-Hydrosphere C18	150	5	YMC Sep. Techn./ThermoQuest	+	−	−	120
65	YMC-Pack Pro C18-3	150	3	YMC Sep. Techn./ThermoQuest	+	+	−	120
66	YMC-Pack Pro C18-5	150	5	YMC Sep. Techn./ThermoQuest	+	+	−	120
67	Zorbax Eclipse XDB-C18	250	5	Agilent Technologies	+	−	−	80
68	Zorbax Extend C18	250	5	Agilent Technologies	+	−	−	80
69	Zorbax SB-C18	250	5	Agilent Technologies	+	−	−	80

[a] The internal diameter is always 4.6 mm.

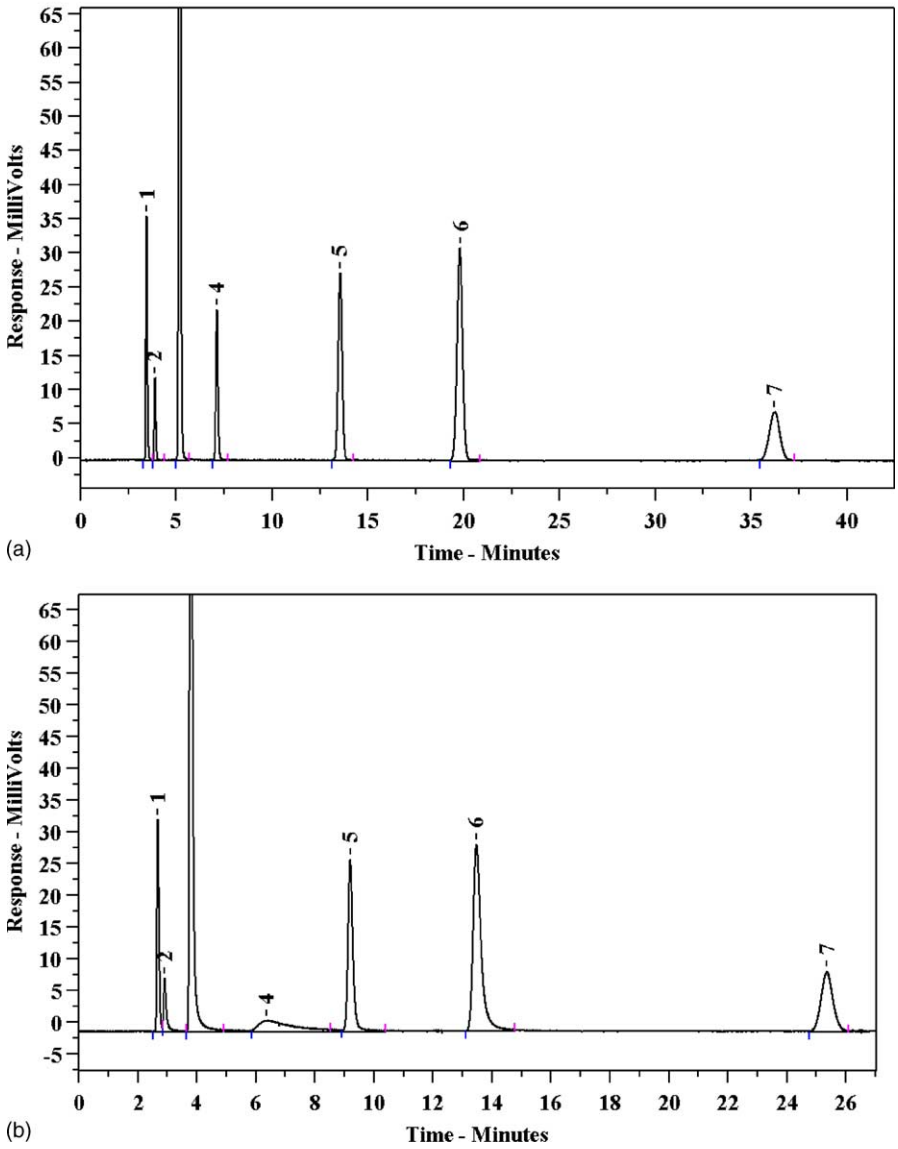

Fig. 1. Separation of ASA and its related compounds. (1) 4-OH benzoic acid (HBA), (2) 4-OH isophthalic acid (HIPA), (3) acetylsalicylic acid (ASA), (4) salicylic acid (SA), (5) acetylsalicylsalicylic acid (ASSA), (6) salicylsalicylic acid (SSA), (7) acetylsalicylic anhydride (ASAN). Columns: (a) TracerExcel 5 (no. 51), (b) Zorbax Extend (no. 68).

The separation was performed three times on each column. The resolution between SA (peak 4) and ASA (peak 3) and the symmetry factor for SA were calculated with ChromPerfect 4.4.0. software. Ph. Eur. equations were used [1].

The symmetry factor (SF) was calculated as:

$$\mathrm{SF} = \frac{w_{0.05}}{2d}$$

with $w_{0.05}$ the width of the peak at 5% of the peak height and d the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height.

The CRF was calculated as:

$$\mathrm{CRF} = \prod_{i=1}^{n-1} \frac{f_i}{g_i} \quad (1)$$

where n is the total number of solutes, g the interpolated peak height, i.e. the distance between the baseline and the line connecting the two peak tops, at the location of the valley, and f the depth of the valley, measured from the line connecting two peak tops [42,43]. This means that a baseline separated peak pair has an f/g ratio of 1, a non-separated pair has a value of 0, while a partly separated peak pair has an intermediate value. The use of these values is described

for thin layer chromatographic methods [43], but they can be used in LC as well [45].

2.2.3. Principal component analysis

The PCA calculations were executed with the Statistica 6.0 software (StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Column classification

In a previous study [41] the 24 reproducible test parameters were classified and seven classes were obtained. A representative parameter was chosen from each cluster. These seven test parameters and the property they are supposed to represent are: the theoretical plate number of amylbenzene, $n_{\text{amylbenzene}}$ (efficiency), the retention factor of amylbenzene, $k'_{\text{amylbenzene}}$ (hydrophobicity), the relative retention factor benzylamine/phenol, $rk'_{\text{benzylamine/phenol}}$, at pH 2.7 (silanol activity), the relative retention factor triphenylene/*o*-terphenyl, $rk'_{\text{triphenylene/}o\text{-terphenyl}}$ (steric selectivity), the retention factor of 2,2′-dipyridyl, $k'_{2,2'\text{-dipyridyl}}$ (silanol activity and metal impurity), the relative retention factor 2,3-dihydroxynaphtalene/2,2′-dipyridyl, $rk'_{2,3\text{-dihydroxynaphthalene/}2,2'\text{-dipyridyl}}$ (metal impurity) and the relative retention factor acetylsalicylic acid/5-*p*-methylphenyl-5-phenylhydantoin (MPPH), $rk'_{\text{acetylsalicylic acid/MPPH}}$ (non-defined property). A classification of the RP-LC columns was made from PCA plots, based on all 24 parameters. Three different major groups were distinguished: a main group with the majority of columns, a group of columns with high silanol activity and a group of columns suitable for the analysis of polar compounds. For more information the reader is referred to reference [41]. The classification, based on 24 parameters, could be maintained with the 7 parameters or even with only 4 out of the 7 [41]. For each column, the values of the four finally selected parameters, $k'_{\text{amylbenzene}}$, $rk'_{o\text{-terphenyl/triphenylene}}$, $rk'_{\text{benzylamine/phenol pH 2.7}}$ and $rk'_{2,2'\text{-dipyridyl}}$ are shown in Table 2. These parameters can be determined with three simple, fast, repeatable and reproducible methods (Table 3) [41]. The PCA score plot derived with these four parameters is shown in Fig. 2a. The PC1–2 loading plot (Fig. 2b) shows the position of the four parameters.

3.2. Separation of ASA and its related compounds

The Ph. Eur. monograph of ASA prescribes an LC method as a limit test for related substances [1]. A column (0.25 m long, 4.6 mm i.d.) packed with 5 μm octadecylsilyl silica gel for chromatography has to be used. The SST requires a resolution between ASA and SA of at least 6.0. The chromatographic conditions given in the monographs may be adjusted. The Ph. Eur. allows to adapt the stationary phase column length by ±70%, the internal diameter by ±25% and the particle size (reduction only) by 50%. Columns 8 (containing 10 μm particles), 11 (a monolithic column), and 53 (containing 2 μm particles) do not meet these Ph. Eur. requirements, and were removed from the data set. The mobile phase ratio was never adjusted.

As the prescription of the stationary phase given by the Ph. Eur. does not mention a brand name, analysts have to select a suitable column. The separation of ASA and related compounds was performed on 56 columns, which comply with the requirements given by the Ph. Eur., and thus may be selected by chromatographers, who want to analyse ASA according to the Ph. Eur. monograph.

3.2.1. Column differentiation based on the SST

Once a chromatographer has selected a column, it is to be checked for compliance with the SST requirement. Columns complying with the SST should be suitable for the analysis. However, one may wonder whether the SST results provide the correct information regarding the suitability of a stationary phase for this analysis. The SST results for all 56 stationary phases, grouped according to the clusters defined in Fig. 2a, are shown in Table 4. Columns shorter than 0.25 m are in italics.

According to the Ph. Eur. SST requirements, 25 columns have a resolution below 6.0 and have to be ranked as “not suitable”. Columns 5, 30, 38, 45 and 46, which show a different selectivity with a changed elution order, must also be qualified as “not suitable”. It should be noted that none of the 10 cm columns passes the SST. Although the adjustment of the mobile phase may solve this problem, it was not adjusted during this study because the aim was to compare RP columns. Finally, 26 columns were ranked as “suitable” for this analysis according to the SST requirements: 1, 2, 3, 4, 7, 12, 16, 22, 23, 26, 28, 31, 39, 40, 49, 50, 51, 52, 55, 57, 60, 64, 65, 66, 67 and 69. When the results are more closely examined, it can be observed that the SST criterion does not always give the correct/required information. Five columns (1, 3, 12, 16 and 52), suitable according to the SST, do not give a baseline separation, while seven columns (25, 29, 43, 47, 54, 58 and 68), not suitable according to the SST, do give a baseline separation. Note however, that the SST makes use of the resolution, which is influenced by the peak width. Indeed, even if a tailing peak is completely separated from all other peaks, its resolution will considerably decrease. The symmetry factor (SF) for SA on the 56 columns is given in Table 4. Columns with a high SF for SA, i.e. above 2.5, may have SST values <6 while all peaks are baseline separated. The tailing of the SA peak has important consequences for the analysis, as it leads to a decreased sensitivity for SA. The SST has the advantage that besides the selectivity, also the symmetry for SA is examined. However, the SST does not always provide the right information concerning the overall selectivity.

Table 2
Four test parameters, determined for RP-LC stationary phases

Column number	Column name	k'_{amb}	$k'_{ba/ph\,pH\,2.7}$	$rk'_{tri/ter}$	$k'_{2,2'\text{-}d}$
1	ACE 3 C18	4.88	0.079	1.50	6.80
2	ACE 5 C18	4.56	0.082	1.51	6.32
3	Alltima C18 3	7.04	0.056	1.45	12.2
4	Alltima C18 5	7.28	0.071	1.46	11.9
5	Apex Basic	2.15	0.001	2.32	7.66
6	Apex ODS II	3.89	0.760	1.40	18.0
7	Aqua	5.71	0.063	1.26	9.22
8	μBondapak	2.50	0.087	1.22	7.41
9	Brava BDS 3	3.10	0.086	1.37	5.93
10	Brava BDS 5	3.01	0.114	1.50	6.04
11	Chromolith	2.28	0.073	1.46	3.59
12	Discovery C18	3.04	0.083	1.50	4.36
13	Genesis C18 3	6.37	0.079	1.37	9.00
16	HyperBDS 5	3.56	0.131	1.56	6.59
18	HyperODS 5	3.56	0.607	1.31	18.0
19	HyPurity Elite 3	3.11	0.083	1.55	4.43
20	HyPurity Elite 5	3.16	0.079	1.58	4.35
22	Kromasil (MN)	6.20	0.063	1.63	9.37
23	Kromas (EKA)	7.46	0.074	1.56	9.40
25	LiChrospher	6.44	0.157	1.77	18.0
26	Luna	5.72	0.031	1.15	8.65
28	Nucleosil 5	4.40	0.111	1.66	13.47
29	Nucleos HD	6.03	0.081	1.45	8.38
30	Nucleosil Nautilus	3.22	0.014	1.95	6.38
31	OmniSpher	7.56	0.078	1.67	9.55
32	Pecospher C18	6.21	0.125	1.33	14.24
33	Platinum C18 3	2.18	0.174	1.22	7.47
34	Platinum C18 5	2.00	0.190	1.23	8.98
35	Platinum EPS C18 3	0.95	0.379	1.87	9.33
36	Platinum EPS C18 5	0.97	0.401	1.88	10.0
37	Prodigy	5.92	0.055	1.20	8.60
38	Purospher	4.50	0.001	1.90	11.3
39	Purospher endcapped	7.75	0.058	1.71	14.1
40	Purospher STAR e	6.92	0.060	1.54	12.3
41	SPHERI-5	7.10	0.230	1.42	18.0
43	Spherisorb ODS2 5	5.53	0.252	1.55	18.0
44	Supelcosil LC-18	4.16	0.760	1.43	18.0
45	Supelcosil LC-18 DB 3	4.10	0.155	1.40	6.36
46	Supelcosil LC-18 DB 5	3.98	0.164	1.37	6.51
47	Superspher	6.40	0.103	1.59	9.21
49	Symmetry 5	6.50	0.041	1.56	8.94
50	TracerExcel ODS A-3	6.40	0.071	1.35	9.25
51	TracerExcel ODS A-5	6.00	0.066	1.37	8.83
52	TSKgel ODS-80TS	5.66	0.059	1.28	9.06
53	TSKgel Super ODS	2.28	0.067	1.46	4.24
54	Uptisphere 3 HDOC18	5.53	0.062	1.28	9.14
55	Uptisphere 5 HDOC18	5.76	0.080	1.30	6.87
56	Uptisphere 3 ODB	5.39	0.099	1.42	8.95
57	Uptisphere 5 ODB	6.31	0.068	1.37	9.18
58	Validated C18	5.69	0.067	1.41	9.86
59	Wakosil C18 HG 5–10	5.31	0.057	1.41	7.33
60	Wakosil C18HG 5–25	5.34	0.069	1.41	7.70
61	Wakosil C18 RS 3–10	6.65	0.049	1.27	10.2
64	YMC-Hydrosphere C18	4.20	0.032	1.19	7.95
65	YMC-Pack Pro C18-3	5.71	0.048	1.32	8.03
66	YMC-Pack Pro C18-5	5.96	0.034	1.27	8.29
67	Zorbax Eclipse XDB-C18	6.02	0.069	1.30	7.96
68	Zorbax Extend C18	6.16	0.065	1.48	7.80
69	Zorbax SB-C18	5.06	0.065	1.22	9.22

k'_{amb}: retention factor of amylbenzene, $k'_{ba/ph\,pH\,2.7}$: relative retention factor of benzylamine/phenol at pH 2.7, $rk'_{tri/ter}$: relative retention factor of triphenylene/*o*-terphenyl, $k'_{2,2'\text{-}d}$: retention factor of 2,2′-dipyridyl.

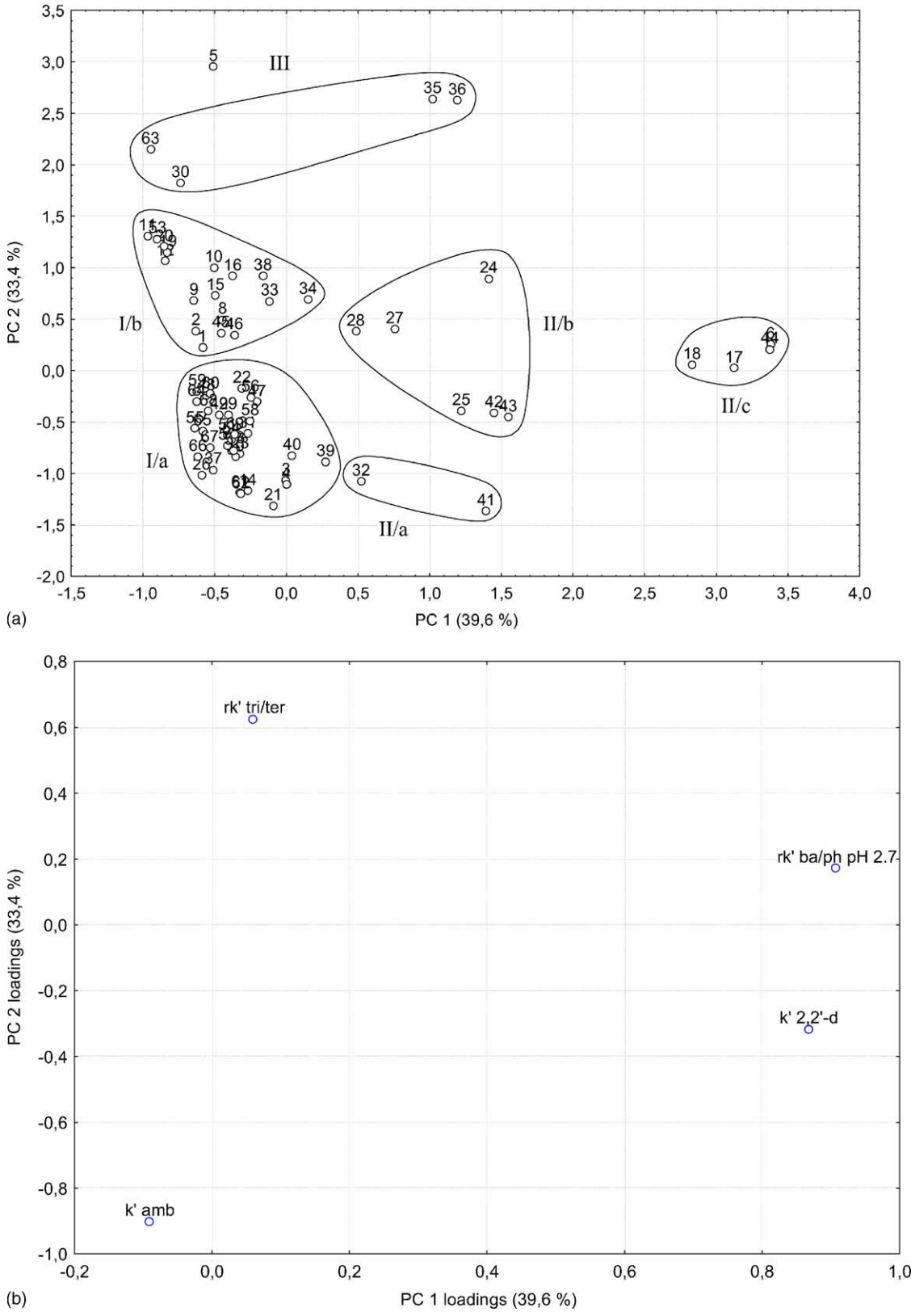

Fig. 2. (a) Score plot for 69 RP-LC columns employing 4 chromatographic parameters: $k'_{\text{amylbenzene}}$, $rk'_{o\text{-terphenyl/triphenylene}}$, $rk'_{\text{benzylamine/phenol pH 2.7}}$ and $k'_{2,2'\text{-dipyridyl}}$ (see details in the text). (b) PC1–2 loading plot. Abbr: k'_{amb}: retention factor of amylbenzene, $rk'_{\text{ba/ph pH 2.7}}$: relative retention factor of benzylamine/phenol at pH 2.7, $rk'_{\text{tri/ter}}$: relative retention factor of triphenylene/*o*-terhpenyl, $k'_{2,2'\text{-d}}$: retention factor of 2,2′-dipyridyl.

Table 3
The four final parameters, the chromatographic property they represent and the mobile phase composition, in order of execution

Parameter	Chromatographic property	Mobile phase
$rk'_{\text{benzylamine/phenol pH 2.7}}$	Silanol activity	Methanol–water–0.2 M KH_2PO_4 pH 2.7 (34/90/10, w/w)
$k'_{2,2'\text{-dipyridyl}}$	Silanol activity and metal impurity	Methanol–water (34/100, w/w)
$k'_{\text{amylbenzene}}$	Hydrophobicity	Methanol–water
$rk'_{\text{triphenylene/}o\text{-terphenyl}}$	Steric selectivity	(317/100, w/w)

3.2.2. Column differentiation based on the CRF

The suitability of stationary phases can also be evaluated by calculation of the CRF values. CRF values are always situated between 0 (two ore more peaks are coeluted) and 1 (all peaks are baseline separated). The CRF values for all 56 stationary phases are shown in Table 4. We have introduced the CRF as criterion to evaluate the quality of separations on the different columns studied. The CRF is a measure of the completeness of separation and does not take into account the peak shape directly (while resolution does). This might explain why some columns have CRF values of 1, but at the same time SST values <6. Usually these columns have a high SF for SA, i.e. above 2.5 (Table 4). Anyway, this peak was still baseline separated from all other peaks. The CRF can be used to evaluate the separations in this study but can never be used in practice as an SST. Measuring the CRF requires the availability of all the potential impurities as a reference substance and therefore would be too cumbersome.

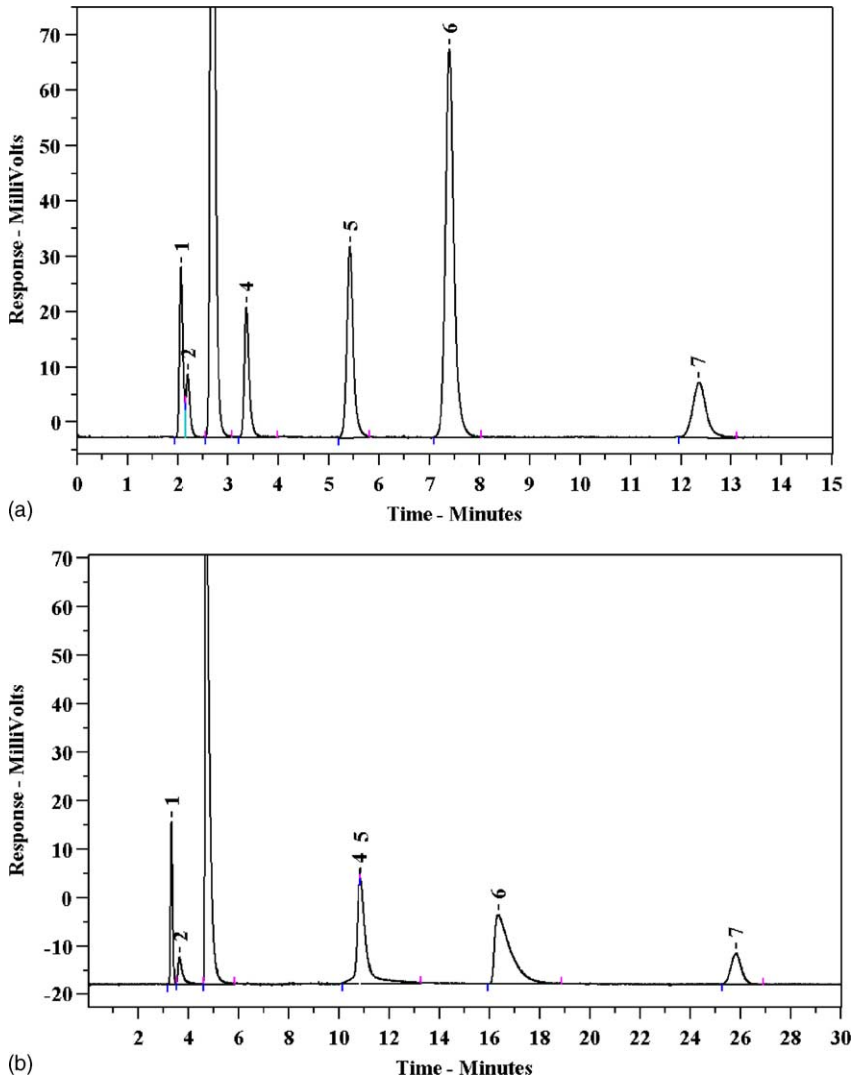

Fig. 3. Separation of ASA and its related compounds. Columns: (a) HyPurity Elite 5 (no. 20), (b) Supelcosil DB 5 (no. 46). Peak identification: see Fig. 1.

Table 4
Results for the CRF, SST and the symmetry factor for SA (SF) on the RP-LC stationary phases tested

Group	No	Column	SST	CRF	SF
Ia	*3*	*Alltima 3*	*7.8*	*0.00*	*2.5*
	4	Alltima 5	9.6	1.00	1.5
	7	*Aqua 5*	*7.1*	*1.00*	*1.2*
	13	*Genesis C18-3*	*4.9*	*0.74*	*1.3*
	22	Kromasil NM	10.0	1.00	1.3
	23	Kromasil EKA	7.0	1.00	4.2
	26	*Luna 5*	*6.9*	*1.00*	*1.5*
	29	Nucleosil HD	3.2	1.00	3.2
	31	OmniSpher	6.0	1.00	3.9
	37	*Prodigy 3*	*5.4*	*0.81*	*1.4*
	39	Purospher endcapped	6.9	1.00	1.1
	40	Purospher Star	10.6	1.00	1.2
	47	Superspher	5.1	1.00	4.4
	49	Symmetry	8.8	1.00	2.3
	50	*TracerExcel 3*	*8.0*	*1.00*	*1.3*
	51	TracerExcel 5	10.4	1.00	1.2
	52	*TSKgel ODS-80TS*	*6.1*	*0.84*	*1.4*
	54	*Uptispher HDO3*	*5.8*	*1.00*	*1.2*
	55	Uptispher HDO5	9.8	1.00	1.1
	56	*Uptispher ODB3*	*5.9*	*0.94*	*1.2*
	57	Uptispher ODB5	8.3	1.00	1.1
	58	Validated C18	4.5	1.00	3.4
	59	*Wakosil HG 5_10*	*4.2*	*0.66*	*1.6*
	60	*Wakosil HG 5_25*	*7.8*	*1.00*	*1.7*
	61	*Wakosil HG 3_10*	*5.3*	*0.89*	*1.5*
	64	YMC Hydrospher C18	7.3	1.00	1.1
	65	*YMC-Pack-ProC18-3*	*7.6*	*1.00*	*1.4*
	66	YMC-Pack-Pro C18-5	7.7	1.00	1.3
	67	Zorbax Eclipes XDB	8.6	1.00	1.4
	68	Zorbax Extend C18	2.4	1.00	2.6
	69	Zorbax SB-C18	6.6	1.00	3.1
Ib	*1*	*ACE C18-3*	*7.1*	*0.93*	*1.4*
	2	ACE C18-5	9.0	1.00	1.2
	9	*Brava BDS 3*	*5.5*	*0.63*	*1.4*
	10	Brava BDS 5	5.8	0.67	1.4
	12	Discovery	6.6	0.96	1.3
	16	Hypersil BDS	6.6	0.91	1.3
	19	*HyPurity Elite 3*	*5.1*	*0.80*	*1.4*
	20	*HyPurity Elite 5*	*4.4*	*0.66*	*1.4*
	33	*Platinum 3*	*4.6*	*0.40*	*1.5*
	34	Platinum 5	3.0	0.24	3.0
	38	Purospher	a	0.00	b
	45	*Supelcosil LC-18 DB 3*	*a*	*0.00*	*b*
	46	Supelcosil LC-18 DB 5	a	0.00	b
IIa	*32*	*Pecosphere*	*3.0*	*0.50*	*2.8*
	41	Spheri	3.4	0.96	1.4
IIb	25	LiChrospher	5.2	1.00	3.8
	28	Nucleosil NM	6.4	1.00	1.4
	43	Spherisorb ODS2	3.8	1.00	3.5
IIc	6	Apex ODS	5.1	0.67	2.8
	18	Hypersil ODS	5.2	0.88	3.8
	44	Supelcosil LC18	4.2	0.00	4.9
III	30	Nucleosil C18 Nautilus	a	0.00	1.0
	35	*Platinum EPS 3*	*2.8*	*0.09*	*1.5*
	36	Platinum EPS 5	3.5	0.42	1.3
Outlier	5	Apex Basic	a	0.00	b

(a) Changed selectivity and (b) peak coeluted or not observed. Italics are used for columns shorter than 0.25 m.

This is the reason why compendia prescribe an SST with only a few impurities (mostly a critical pair).

3.2.3. Column differentiation based on the column classification

As both the SST and the CRF show shortcomings, another test procedure is needed to predict the suitability of columns for the separation. A comparison was made between the above mentioned column classification and the separation data for ASA. The similarity of the separations was evaluated by the CRF values.

It can be observed that in Group I/a all the 0.25 m columns give a CRF value of one, i.e. they give complete baseline separation for ASA and its related substances. Several of the short columns show a CRF value of one as well, but not all, as some give no baseline separation for the pair HBA–HIPA (peaks 1–2). Two chromatograms from Group I/a are shown in Fig. 1. A representative chromatogram (column 51) can be seen in Fig. 1a. Very similar results (CRF = 1, SF < 2.5) were obtained on columns 4, 7, 22, 26, 39, 40, 49, 50, 51, 54, 55, 57, 60, 64, 65, 66 and 67. There are three stationary phases (columns 23, 31 and 69) in this group on which SA shows strong peak tailing (SF > 2.5) while the resolution between SA and ASA is still above 6.0. Four other columns (29, 47, 58 and 68), which also show strong peak tailing, are not suitable according to the SST. Although all seven compounds are separated from each other, SA shows strong tailing and therefore the resolution drops below 6.0. Fig. 1b shows the separation on column 68. It should be mentioned that the latter column is recommended for use with basic mobile phases. It can be concluded that 0.25 m columns from Group I/a give suitable selectivity for the analysis of ASA and related substances. The SST does not always provide the right information.

Only one column from Group I/b has a CRF value of one. Three columns give coelution of two or more peaks. In general, columns from Group I/b are not suitable for this separation. Group I/b contains several types of columns and therefore the chromatograms obtained are of variable quality. A common characteristic of stationary phases belonging to this class is that they have a low hydrophobicity [41]. In Fig. 3a, an example, obtained with column 20, is shown. Peaks 1 and 2 are only partly separated and the SST is less than 6.0. Columns 9, 10, 19, 33 and 34 show similar chromatograms. The SST is above 6.0 for columns 1, 12 and 16, but peaks 1 and 2 are still poorly separated. On columns 45 and 46, SA and ASSA are coeluted. Therefore, these columns are not suitable (Fig. 3b). Column 2 is an exception in this group, as it gives a good separation, similar to the supports in Group I/a. Note that this column shifted from Groups I/a to I/b on the score plot when the number of parameters was reduced from 24 to 4 [41]. It can be concluded that in general, supports belonging to Group I/b are not suitable, although some columns fulfil the SST limit.

In Group II, only the columns from Group II/b give baseline separations. Columns from Group II have a higher

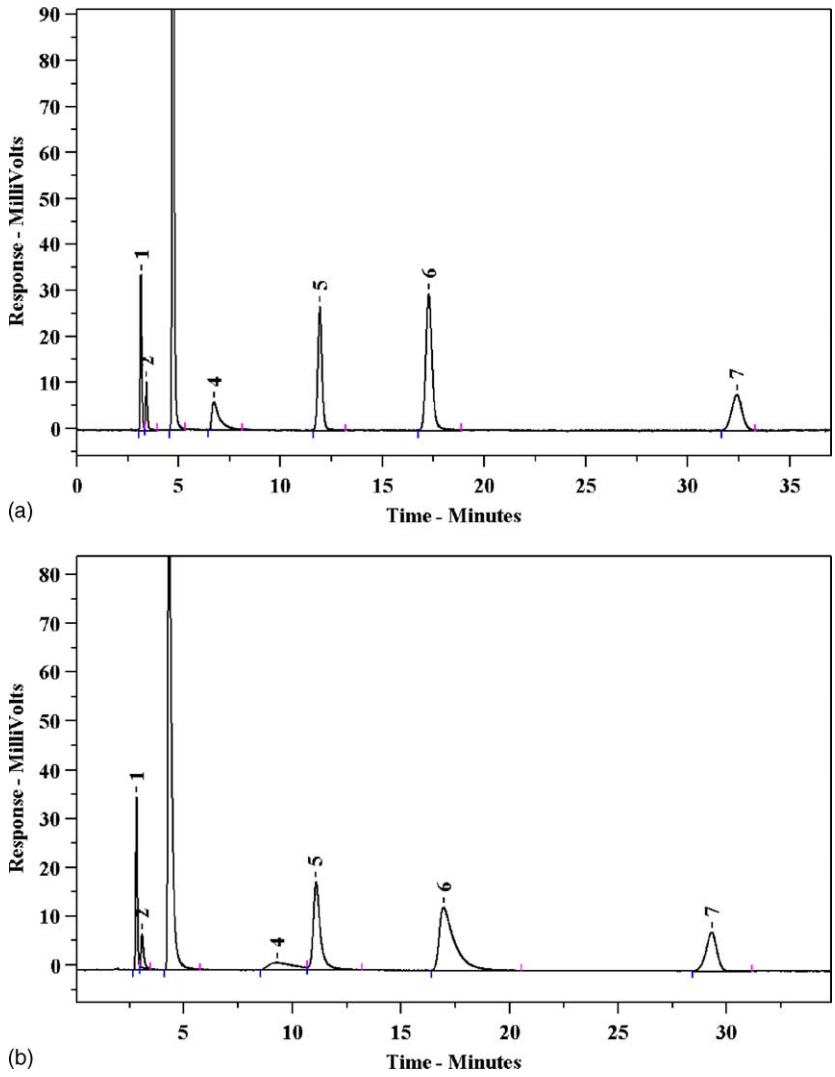

Fig. 4. Separation of ASA and its related compounds. Columns: (a) LiChrospher (no. 25), (b) Spheri (no. 41). Peak identification: see Fig. 1.

silanol activity than those from Group I/a. The silanol activity increases along the *x*-axis of the PCA plot (Fig. 2a and b). The hydrophobicity in this group is similar or below that in Group I/a. All columns from Group II/b give baseline separation of all compounds. On columns 25 (Fig. 4a) and 43, peaks 1 and 2 are completely separated but the salicylic acid shows tailing. Therefore, the SST is just below 6.0. Columns belonging to Group II/a or Group II/c do not give baseline separations. SA, which forms strong complexes with metal ions but is atypical in this context, shows both fronting and strong tailing on column 41 from Group II/a (Fig. 4b). All columns from Group II/c and columns 25 (Fig. 4a) and 43 from Group II/b show strong tailing of the SA peak as well. This can be correlated with the position on the PCA loading plot (Fig. 2a and b) of parameter $k'_{2,2'\text{-dipyridyl}}$, which characterises metal impurity. Although a correlation can be found between parameter $k'_{2,2'\text{-dipyridyl}}$ and tailing of the SA peak on the columns 6, 18, 25, 32, 41, 43 and 44, some columns of Groups I/a and I/b show tailing of SA as well. For these columns no correlation between their classification and the SA peak tailing can be found. During a previous part of this study the parameters $k'_{2,2'\text{-dipyridyl}}$ and $rk'_{2,3\text{-dihydroxynaphthalene}/2,2'\text{-dipyridyl}}$, which both are reported to characterise metal impurity, were found at different positions in the PCA loading plot [41]. This suggests that they do not represent exactly the same chromatographic characteristic and therefore may characterise metal impurity only partly. For $k'_{2,2'\text{-dipyridyl}}$ it was found that it may represent silanol activity as well. The results for the ASA separation, where some columns show a correlation between parameter $k'_{2,2'\text{-dipyridyl}}$ and tailing of SA, while for other columns no correlation can be found, seem to confirm this. However, further research is needed. It can be concluded that supports from Group II/b are suitable, while supports from Groups II/a and II/c are not. In general, for Group II/a and II/c the SST provides the right information. Although columns 25 and 43 give a baseline separation of all peaks, they were found unsuit-

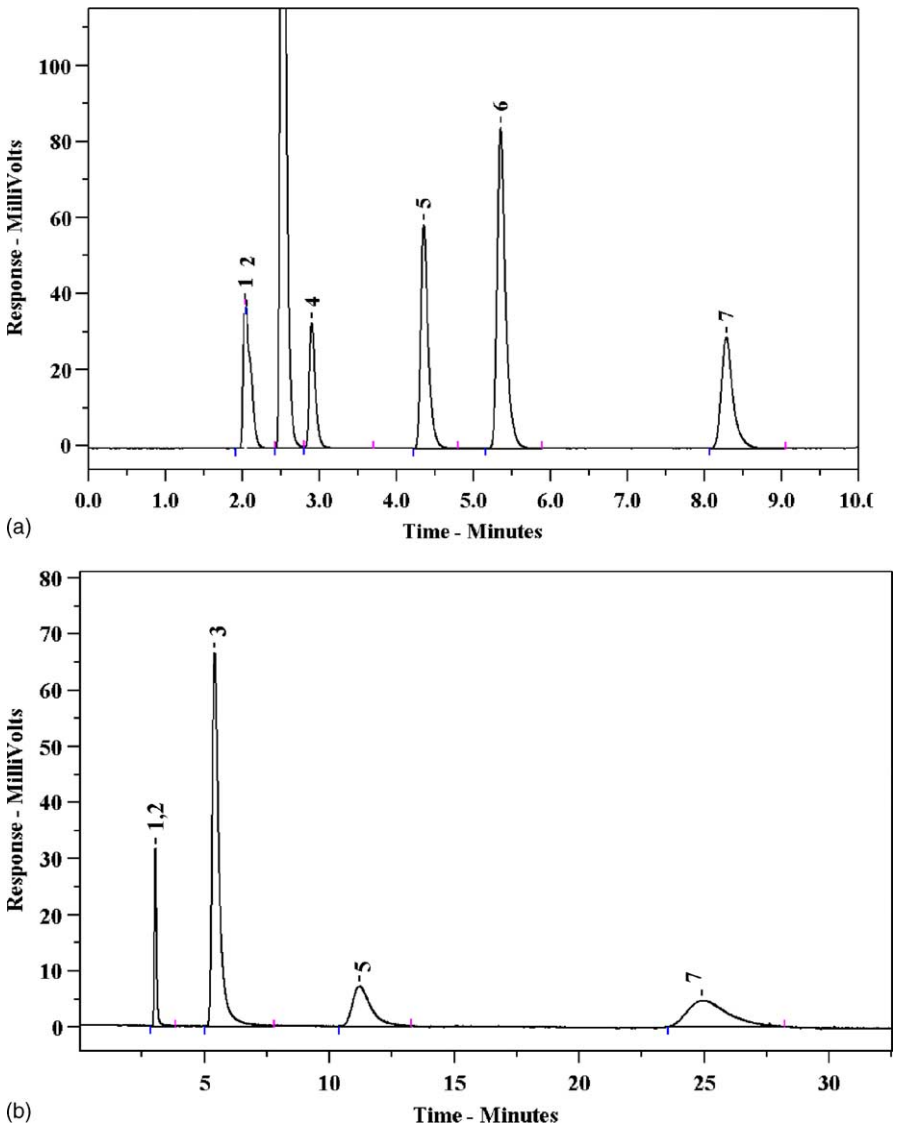

Fig. 5. Separation of ASA and its related compounds. Columns: (a) Nucleosil Nautilus (no. 30), (b) Apex Basic (no. 5). Peak identification: see Fig. 1.

able according to the SST, because of their tailing peak for SA.

Columns from Group III and the outlying column 5 are not suitable either. The polar embedded column 30, columns 35 and 36 with an extended polar selectivity (Group III) and column 5 (outlying column) have a lower hydrophobicity but a higher steric selectivity than the stationary phases in Groups I/a and I/b. The selectivity of these columns is different. The elution order is different on column 30. On columns 35 (Fig. 5a) and 36, peaks 1 and 2 are coeluted and the resolution between SA and ASA is less than 6.0. One of the poorest chromatograms was obtained with column 5 (Fig. 5b), from which SA and SSA are not eluted. It can be concluded that supports belonging to Group III and the outlying column 5 are not suitable. The SST provides the right information.

These results show that the proposed classification of the columns can help in the selection of a suitable column for the separation of ASA and its related substances. Columns with similar column selectivity for this separation are situated in the same group. An analyst preferably should select a 0.25 m column from Groups I/a or II/b in order to achieve a baseline separation.

4. Conclusion

Correlation between a column classification based on chromatographic test parameters and the selectivity of the separation of ASA and its related compounds was studied. To determine whether a separation was good or poor, the overall selectivity was evaluated using the CRF. The classification based on the four parameters ($k'_{\text{amylbenzene}}$, $rk'_{o\text{-terphenyl/triphenylene}}$, $rk'_{\text{benzylamine/phenol pH 2.7}}$ and $k'_{2,2'\text{-dipyridyl}}$) was used and the CRF values of columns belonging to the same class were compared.

The predicting value of the SST was examined. It was found that this test not always could predict the suitability of the column. Some columns failing the SST were found to be suitable while other columns, suitable according to the SST, did not give a baseline separation of all compounds. An advantage of the SST is that it takes into account the peak shape and the symmetry of SA.

The column classification in a PCA score plot based on the chromatographic parameters allows the selection of stationary phases with similar selectivity for the separation of aspirin and related substances. In general, the 0.25 m columns belonging to Group I/a or Group II/b were found to be suitable.

Future case studies are needed to study the correlation between test parameters and their separation characteristics. The final aim of this study is to provide a simple column test procedure, which can predict the suitability of a column for real separations.

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