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Impact of the probe solutes set on orthogonality evaluation in reverse phase chromatographic systems

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ABSTRACT

Two-dimensional liquid chromatography (2DLC) is a very attractive technique for the characterization of complex samples due to its separation power obtained *via* the coupling of two separation modes exhibiting different mechanisms, *i.e.* orthogonality. In reverse phase, orthogonality is mainly governed by three factors: the pH of the mobile phase, the structure of the stationary phase and the nature of the organic modifier. In the present paper, we studied the impact of the nature of the probe solutes on orthogonality evaluation. A set of 63 compounds with various physicochemical properties was used to evaluate 32 reverse phase chromatographic systems (2 pH × 8 stationary phases × 2 organic modifiers). Principle component analysis revealed that the solutes could be split into three subsets according to their charge in the experimental conditions. The factors affecting orthogonality and the magnitude of their effect were shown to depend on the charge of the compounds. For positively charged (basic) compounds, the pH was the most important factor, followed by the nature of the stationary phase. For negatively charged (acidic) compounds, the nature of the stationary phase had the highest impact. For neutral compounds, only the nature of the stationary phase and, to a smaller extent, the organic modifier had an influence. The present study also showed that a reduced set of only 9 test compounds instead of whole set of the 63 could enable an appropriate orthogonality evaluation.

1. Introduction

The increase in resolution and selectivity obtained in two dimensional chromatography depends on the degree of orthogonality of the coupled separation mechanisms [1]. In liquid chromatography (LC), dissimilar separation mechanisms are obtained when the retention of the solutes results from different interactions between solute, stationary phase and mobile phase. Hence, coupling systems with different stationary phases increases the selectivity in LC. In reverse phase (RP), the mobile phase's characteristics, *i.e.* the organic modifier and the pH of the mobile phase have a dramatic effect on column behaviors in isocratic mode [2–4]. In a previous study [5], a set of 32 chromatographic systems was evaluated in a gradient mode, each system being defined by: the stationary phase (8 different were used), the pH value of the aqueous fraction of the mobile phase (2.5 or 7.0) and the organic modifiers (acetonitrile or methanol) (appendices 1 and 2). The orthogonality of 496 couples of systems was evaluated and ranked using seven different criteria: the three classical correlation coefficients (Pearson's, Spearman's and Kendall's), two geometric criteria characterizing the coverage of the 2D separation space and two χ^2 statistics of independence. On one hand, the results revealed that Kendall's coefficient showed the greatest sensitivity to the three factors: difference in pH, difference in stationary phase and difference in organic modifier. On the other hand, they showed that changing the pH had the highest impact on orthogonality, followed by changing the stationary phase and with the least impact, changing the type of the organic modifier. These results were obtained for a set of 63 probe solutes covering a wide range of physicochemical properties [5]; they differed by their pK_a values (between 0.6 and 14.0), their molecular mass (between 76.12 and 1485.71 g mol^{-1}), their hydrophobicity (log *P*-values between −1.08 and 7.72) and the presence/absence of heteroatoms.

However, one can expect that the nature of the set of probe solutes may have also a striking impact on orthogonality evaluation of couples of chromatographic systems. Nevertheless, to the best of our knowledge, little attention has been paid to this point in the literature [6].

In the present study, we will first get a general overview of the solute behavior when used to probe 32 RP chromatographic

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systems. To this aim, principal component analysis (PCA) will be used. Then, from the observed pattern, subsets of solutes will be defined, and an orthogonality evaluation will be carried out on each subset. Finally, a reduced set of probe solutes will be proposed, the aim being to have similar orthogonality evaluation with this reduced set than with the whole set of 63 compounds.

2. Experimental conditions

2.1. Instrumentation

Gradient separation was carried out using a Dionex HPLC system-Germany (UltimateTM 3000 Nano HPLC) equipped with a UV detector (UltimateTM 3000 variable wavelength) operated at 3 detection wavelengths: 220, 230 and 250 nm depending on the solute (rate of data acquisition was 2.5 Hz, time constant was 0.60 s, conventional 2.5 μl cell with 7.5 mm path length), two pumps (Ultimate 3000), a degasser (LPG-3000), a thermostatic automated autosampler (UltimateTM 3000 series Nano/Cap) and a column oven (Ultimate 3000 column compartment). PEEK (polyether ether ketone) tubes were used for connections.

2.2. Chemicals and reagents

Acetonitrile (MeCN) and methanol (MeOH) (HPLC ultra gradient grade) originated from Carlo Erba Reactifs (Val de Reuil-France). Ultrapure water for HPLC mobile phases was produced by a Milli-Q Plus purification system (Millipore, Molsheim, France). Phosphoric acid (85%) and potassium phosphate were obtained from PROLABO, whereas hydrochloric acid was from Carlo Erba. Tris base [tris(hydroxymethyl)amino-methane] was supplied by Sigma.

2.3. Preparation of samples solutions

Stock solutions of the 63 test compounds (Table 1 and appendix 3) were prepared at the concentration of ≈1000 mg L^{-1} in pure MeOH. The injection solutions for the chromatographic runs were diluted from the stock solutions in MeOH/water 50/50, (v/v) in order to provide an UV absorbance around 200 mAU (milli-absorbance unit) and a concentration range of 10–500 mg L^{-1}. Samples were stored at 4 °C or less to avoid degradation.

2.4. Buffer preparation

The mobile phases were buffered [7–10] in order to improve the peak shape and reliability [11]. The concentration of the buffer was 5 mM of KH_2PO_4 for pH 2.5 and 5 mM of Tris base for pH 7.0. The choice of these buffers was guided by their buffer capacity at the chosen pH rather than by their volatility. In fact, coupling with mass spectrometry was not the aim of the present study. They were prepared by dissolving the accurate quantity of each salt in pure water separately. After making up to 2 L in a volumetric flask with pure water, the pH value of 2.5 was adjusted with phosphoric acid, while for the value of pH 7.0, hydrochloric acid was used. All buffers were filtered through 0.45 μm HA type filters (Millipore, Moleshiem, France) before mixing with pure MeCN or MeOH in the desired volume ratio. Then the mixtures were degassed by ultrasonication for 20 min immediately before use at room temperature.

2.5. Stationary phases

The testing procedure has been applied to eight different stationary phases (see Fig. 1). The detailed physical and chemical properties of these phases can be found in Table 2 [5]. These columns are among those commonly used for RP liquid chromatography. In all cases, column dimensions were 150 mm × 4.6 mm I.D. with 5 μm particle size, except for Zorbax SB-CN filled with 3.5 μm particles. The chosen RP columns differed from each other in the grafting and protection against residual silanol groups. They were chosen because they are structurally very different from each other.

2.6. Running conditions

A linear generic binary gradient was systematically implemented with a mobile phase A made of 10% MeCN: 90% H_2O and a mobile phase B made of 90% MeCN: 10% H_2O. It increased from 0 to 100% B in 30 min, and then 100% of B was maintained for 10 min. With a few columns, some compounds were not eluted using this gradient (see appendix 4). In that case, the plateau at 100% B was maintained until 200 min. The plateau was required for the elution of the most retained compounds on the HSF5 column. This solution could appear as not fully satisfactory if our final objective had been to model the chromatographic behavior of the probe solutes. Our objective here consisting only in orthogonality evaluation, it was the best compromise possible. After the end of each gradient run, the composition of the mobile phase was gradually set back to the starting conditions and 15 column volumes were pumped for equilibration before starting the next analysis. Mobile phases were freshly prepared just before use in order to avoid any degradation. To ensure a stable baseline, at least 1 h of equilibration was performed for each mobile phase before the injection of 1.0 μl of adequate mixtures of tested compounds. Two consecutive repeated injections were done, and the mean of the retention times was registered. In addition, each solute was injected individually for identification purposes. Three wavelengths 220, 230 and 250 nm were used depending on the compound. The column hold-up volumes were measured as the elution volumes of non-retained compounds.

System back pressure without column was 30 bars with MeCN/buffer, and it was 37 bars for MeOH/buffer. Column temperature was kept constant at 35 °C *via* the oven during the overall tests. All runs were operated at a flow rate of 1.0 ml min^{-1}, checked daily by using a burette and a stopwatch.

2.7. Dwell volume

The dwell volume (also called "gradient delay volume") of a gradient or on-line mixing system is the volume of liquid contained in the system between the point where the gradient is formed and the point where the mobile phase enters the column. This volume includes the mixer, transfer lines, and any swept volume (including the sample loop) in the injection system. In a low-pressure-mixing ("one pump") system, the dwell volume also includes the proportioning valve and pump liquid-end components. Measuring dwell volume is important to verify the functions of the pumps.

The dwell volume of the system was measured by replacing the column with zero dead volume connector. The mobile phase used consists of 10% acetone in a non UV absorbent mobile phase (*e.g.* water). Gradient elution was implemented. For our system, the dwell volume was 0.70 ml.

2.8. Data processing

All chromatographic data acquisition and processing were conducted using Chromeleon (6.8 chromatography data system) software. The statistical analysis was performed with MATLAB (Mathworks, Natick, MA, USA) software v. 7.7.0.471 (2008b) and the

Table 1
Characteristics of the compounds of the test set.

No	Compound	Manufacturer	Molecular structure	Molar-mass	p*Ka*	Log *P*
1.	Phenanthrene	Jansen	$C_{14}H_{10}$	178.23		4.46
2.	Naphthalene	Aldrich	$C_{10}H_8$	128.17		3.30
3.	Anthracene	Prolabo	$C_{14}H_{10}$	178.23		4.45
4.	Triphenylene	Fluka	$C_{18}H_{12}$	228.3		5.49
5.	Salicylic acid	Prolabo	$C_7H_6O_3$	138.12	2.97	2.26
6.	4-Hydroxybenzoic acid	Aldrich	$HOC_6H_4CO_2H$	138.12	4.54	1.58
7.	4-Hydroxy-3-methylbenzoic acid	Aldrich	$HOC_6H_3(CH_3)CO_2H$	152.15	4.26/9.78	1.84
8.	Benzoic acid	Merck	C_6H_5COOH	122.12	4.19	1.87
9.	Mandelic acid	Touzart & Matignon	$C_6H_5CH(OH)CO_2H$	152.15	3.41	0.62
10.	(S)-(+)-Ibuprofen	Aldrich	$(CH_3)_2CHCH_2C_6H_4CH(CH_3)CO_2H$	206.28	4.51	3.6
11.	Phenylacetic acid	Ega	$C_6H_5CH_2CO_2H$	136.15	4.31	1.41
12.	p-Toluamide	Lancaster	$CH_3C_6H_4CONH_2$	135.16		1.18
13.	Loperamide hydrochloride	Sigma	$C_{29}H_{33}ClN_2O_2{\cdot}HCl$	513.5	N.F	5.15
14.	Benzamide	Sigma	$C_6H_5CONH_2$	121.14	1.82	0.64
15.	Phenol	Acros	C_6H_5OH	94.11	9.99	1.46
16.	1-Naphthol	Fluka	$C_{10}H_7OH$	144.17	9.34	2.85
17.	4-Isopropylphenol	Aldrich	$(CH_3)_2CHC_6H_4OH$	136.19	10.2	2.9
18.	4-Dodecylresorcinol	Aldrich	$C_{18}H_{30}O_2$	278.43	11.61/9.2	6.77
19.	Piperonal	Aldrich	$C_8H_6O_3$	150.13		0.64
20.	3-Hydroxybenzaldehyde	Aldrich	HOC_6H_4CHO	122.12	8.98	1.29
21.	3.4-Dichloroaniline	Aldrich	$Cl_2C_6H_3NH_2$	162.02	2.97	2.69
22.	2.4.6-Trichloroaniline	Aldrich	$Cl_3C_6H_2NH_2$	196.46		3.52
23.	Bromacil	Dr Ehrenstorfer	$C_9H_{13}BrN_2O_2$	261.12	9.30	2.11
24.	Napropamid	Dr Ehrenstorfer	$C_{10}H_7OCH(CH_3)CON(CH_2CH_3)_2$	271.35		3.36
25.	Vinclozolin	Dr Ehrenstorfer/Fluka	$C_{12}H_9Cl_2NO_3$	286.11		3.1
26.	Carbaryl	Fluka	$C_{10}H_7OCONHCH_3$	201.22		2.36
27.	Diuron	Dr Ehrenstorfer	$C_9H_{10}Cl_2N_2O$	233.09		2.68
28.	Monuron	Dr Ehrenstorfer	$ClC_6H_4NHCON(CH_3)_2$	198.65		1.94
29.	Linuron	Fluka	$C_9H_{10}Cl_2N_2O_2$	249.09		3.20
30.	Atrazine-desisopropyl	Dr Ehrenstorfer	$C_5H_8ClN_5$	173.6		1.15
31.	Prometryn	Dr Ehrenstorfer	$C_{10}H_{19}N_5S$	241.36	4.1	3.51
32.	Atraton	Dr Ehrenstorfer	$C_9H_{17}N_5O$	211.26		2.69
33.	Toluene	Aldrich	$C_6H_5CH_3$	92.14		2.73
34.	Ethylbenzene	Aldrich	$C_6H_5C_2H_5$	106.17		3.15
35.	Propylbenzene	Fluka	$C_6H_5CH_2CH_2CH_3$	120.19		3.69
36.	Butylbenzene	Aldrich	$C_6H_5(CH_2)_3CH_3$	134.22		4.38
37.	Pentylbenzene	Aldrich	$C_6H_5(CH_2)_4CH_3$	148.24		4.9
38.	Imipramine hydrochloride	Sigma	$C_{19}H_{24}N_2{\cdot}HCl$	316.87	9.4	4.8
39.	Caffeine	Fluka	$C_8H_{10}N_4O_2$	194.19	0.6/14.0	−0.07
40.	Phenothiazine	Aldrich	$C_{12}H_9NS$	199.27	2.52	4.15
41.	Carbazole	Aldrich	$C_{12}H_9N$	167.21		3.72
42.	Umbelliferone	Sigma	$C_9H_6O_3$	162.14		1.03
43.	Nicotine	Sigma	$C_{10}H_{14}N_2$	162.23	3.10/8.02	1.17
44.	1.2-Phenylenediamine	Merck	$C_6H_4(NH_2)_2$	108.14	4.47	0.15
45.	Methyl 4-hydroxybenzoate	Merck	$HOC_6H_4CO_2CH_3$	152.15		1.96
46.	Propyl 4-hydroxybenzoate	Merck	$HOC_6H_4CO_2CH_2CH_2CH_3$	180.21	7.91	3.04
47.	Bis(2.4.6-trichlorophenyl) oxalate	Fluka	$C_{14}H_4Cl_6O_4$	448.9		7.72
48.	Estrone	Sigma	$C_{18}H_{22}O_2$	270.37		3.13
49.	Cortisone	Merck	$C_{21}H_{28}O_5$	360.44		1.47
50.	Estriol	Sigma	$C_{18}H_{24}O_3$	288.38		2.45
51.	Benzylamine	Fluka	$C_6H_5CH_2NH_2$	107.15	9.33	1.09
52.	Clofazimine	Sigma	$C_{27}H_{22}Cl_2N_4$	473.4	7.57	7.66
53.	Strychnine hemisulfate salt	Sigma	$C_{21}H_{22}N_2O_2{\cdot}1/2H_2SO_4$	383.45	8.26	1.93
54.	o-Terphenyl	Fluka	$C_6H_5C_6H_4C_6H_5$	230.3		5.52
55.	Digitoxin	Sigma	$C_{41}H_{64}O_{13}$	764.94		1.85
56.	Thiourea	Aldrich	NH_2CSNH_2	76.12	2.03	−1.08
57.	Ampicillin	Sigma	$C_{16}H_{18}N_3NaO_4S$	371.39	3.7/7.3	1.35
58.	Vancomycin	Sigma	$C_{66}H_{75}Cl_2N_9O_{24}$	1485.71	3.6/8.2/9/9.2/10.3/10.8	N.F
59.	Amiodarone hydrochloride	Sigma	$C_{25}H_{29}I_2NO_3{\cdot}HCl$	681.77	8.73	7.57
60.	(+)-Tubocurarine chloride- hydrate	Sigma	$C_{37}H_{42}Cl_2N_2O_6$	681.65	8.1/9.1	N.F
61.	Atropine	Sigma	$C_{17}H_{23}NO_3$	289.37	9.43	1.83
62.	Phloroglucinol	Merck	$C_6H_6O_3$	126.11	8.45	0.16
63.	Cyanocobalamin	Sigma	$C_{63}H_{88}CoN_{14}O_{14}P$	1355.37	7.64	3.57

statistics toolbox (Version 7.0). The retention times of the solutes were normalized in the interval [0,1] according to:

$$\mathrm{Rt}_{i(\mathrm{norm})} = \frac{\mathrm{Rt}_i - \mathrm{Rt}_0}{\mathrm{Rt}_{\max} - \mathrm{Rt}_0}$$

where Rt_i is the solute retention time, $Rt_{\max}$ is the retention time of the longest-eluting solute and Rt_0 is the retention time of the less retained solute.

3. Results and discussion

3.1. Principal component analysis

Principal component analysis (PCA) is a powerful tool for the interpretation of large data tables [12–14]. It is a projection method that is able to extract the main information from the original data set while projecting it onto a lower dimension space. This space is defined by linear combination of variables, called principal

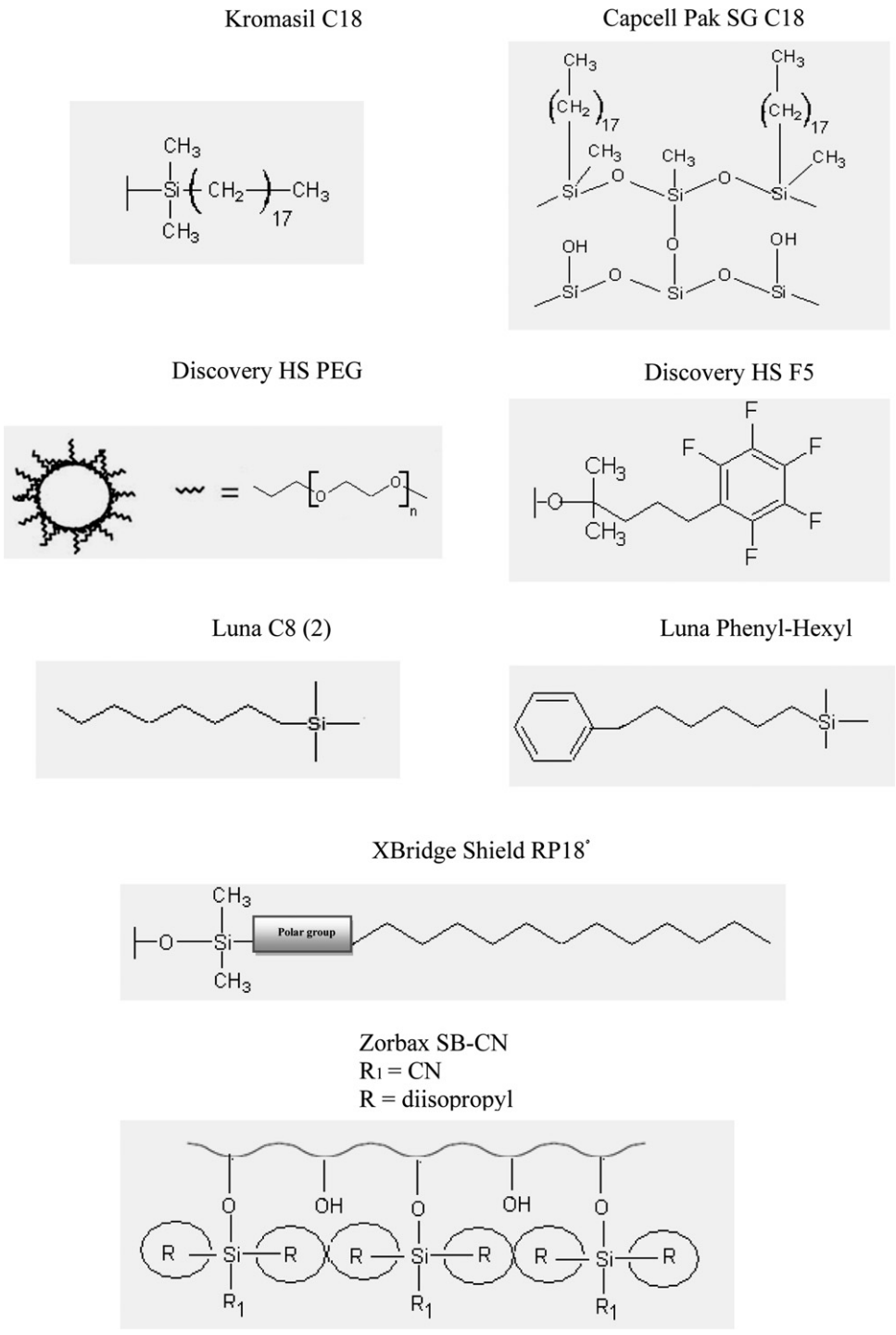

Fig. 1. Structures of the different stationary phases.

Table 2
Characteristics of the 8 stationary phases used in this study.

Stationary phase	Endcapping	Manufacturer	pH range	*C* (%)	Surface area (m^2/g)	Pore size (A°)	Grafting
XBridge shield RP18	Yes	Waters, Ireland	2–11	17	185	135	Polar embedded-Octadecyl
Kromasil C18	Yes	Macherey-Nagel, Germany	1–10	20	330	110	Octadecyl
Zorbax SB-CN	No	Agilent, USA	1.8–8	4	180	80	Cyanopropyl
Luna C8(2)	Yes	Phenomenex, USA	1.5–10	13.5	400	100	Octylsilane
Luna Phenyl–Hexyl	Yes	Phenomenex, USA	1.5–10	17.5	400	100	Phenyl–Hexyl
Discovery HS PEG	No	Supelco, USA	2–8	12	300	120	Polyethyleneglycol
Discovery HS F5	Yes	Supelco, USA	2–8	12	300	120	Pentafluorophenylpropyl
Capcell Pak SG C18		Shiseido, Japan	2–9	14	300	120	Octadecyl

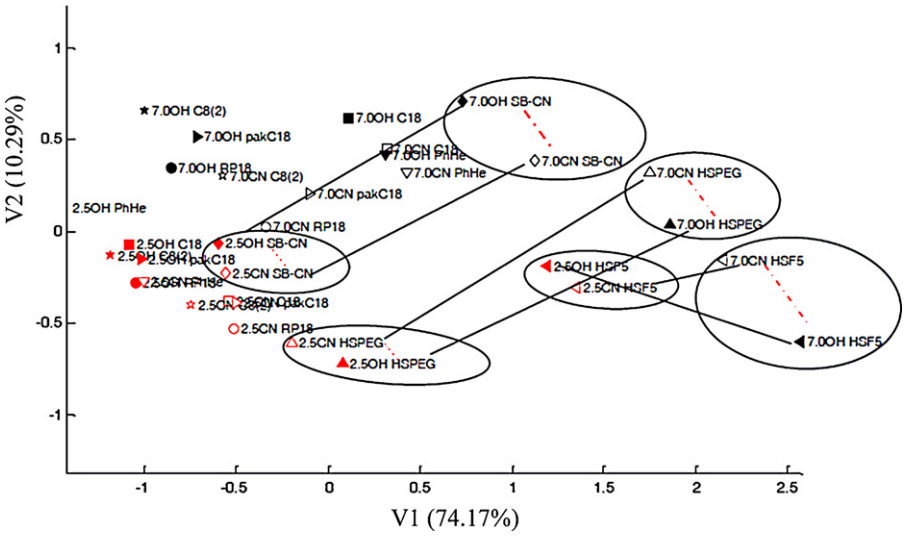

Fig. 2. PCA of the 32 chromatographic systems. The number indicates the pH value (red = 2.5, black = 7.0). The letters indicate the organic modifier (OH & full marker = MeOH, CN & empty marker = MeCN). There is one marker for each stationary phase. The numbers in parenthesis on the axes indicates the percentage of the total variance accounted for by the corresponding principal component. The distance between chromatographic systems corresponding to the same column at different pH values with the same organic modifier are linked with a continuous black line, whereas those corresponding to the same pH but with different organic modifier are linked with a dotted red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

components (PCs). The last ones are computed in such a way that the successive PCs convey less and less information while being orthogonal and maximizing their information load.

PCA was implemented in many studies in the literature [4,12–24] in order to identify the criteria affecting retention, the possible correlations between these criteria, and to group the stationary phases in two or three-dimensional spaces [17–26].

3.1.1. PCA of the 32 chromatographic systems

Fig. 2 shows the projection of the 32 chromatographic systems (described by the 63 normalized retention times) in the space of the two first principal axes. This projection is clearly structured: there is a fairly good separation according to the pH along the horizontal axis, except for HS F5 column. It can also be noticed that for the same columns, differences induced by the change in pH value are more important than those induced by the change of the organic modifier. For example, on HS PEG and Zorbax SB-CN, the distance between chromatographic systems corresponding to the same column at different pH values (linked with a continuous black line) with the same organic modifier is about four times larger in comparison with those corresponding to the same pH but with different organic modifier (linked with a dotted red line).

These behaviors are illustrated with chromatograms in Fig. 3. It can be noticed that, surprisingly, the retention of phenanthrene is affected by the change of pH. This phenomenon was observed only on polar stationary phases (HS PEG and Zorbax SB-CN) (see appendix 5). It could be attributed to the nature and characteristics of the buffer [27] (appendix 6). Tris is likely to induce either a modification of the stationary phase and/or a shift of the mobile phase eluent strength which could affect hydrophobic effect and retention.

It must be kept in mind that these considerations are valid only for the whole set of 63 compounds.

The coordinates of the two first principle axes on the 63 compounds are presented in Fig. 4. It appears clearly that the largest negative contributions to the second principal axis are obtained for the acidic compounds, whereas the largest positive contributions are obtained for the basic compounds (thiourea is an exception since it is a void volume marker).

HS F5 phase with MeOH at pH 7.0 is located at the extreme right of the plot in Fig. 2. In general, this column showed higher retention times for the 63 compounds as compared to the seven other columns, especially for some basic compounds (*i.e.* amines) (see appendix 4). It even necessitated an extended gradient (in this case, the plateau at 100% B was maintained until 200 min) since these compounds did not elute with the usual gradient used for the other compounds on other columns. These characteristics allow very polar compounds, usually eluting close to the void volume on other columns, to be sufficiently retained by HS F5 phase.

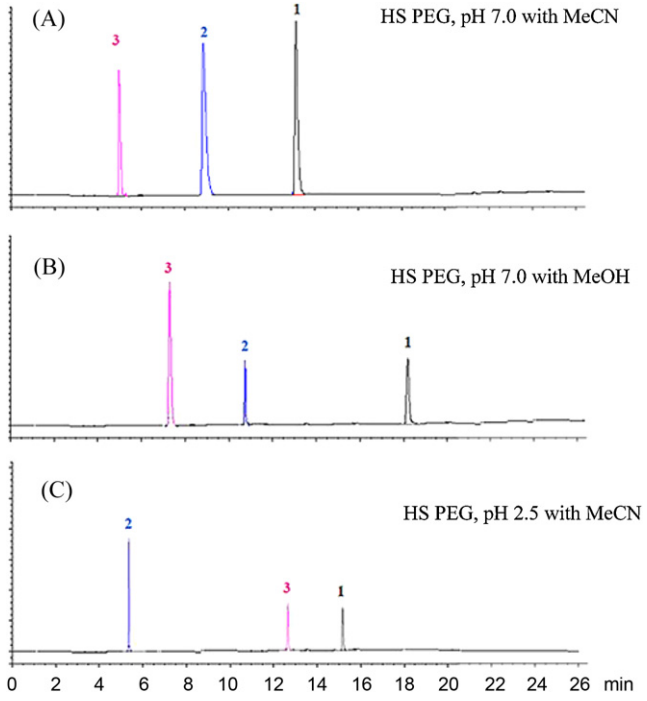

Fig. 3. Chromatograms illustrating the effect of changing the pH value and the organic modifier on the selectivity. (A) HS PEG, pH 7.0 with MeCN, (B) HS PEG, pH 7.0 with MeOH and (C) HS PEG, pH 2.5 with MeCN. Representative compounds are: (1) phenanthrene, (2) prometryn and (3) ibuprofen.

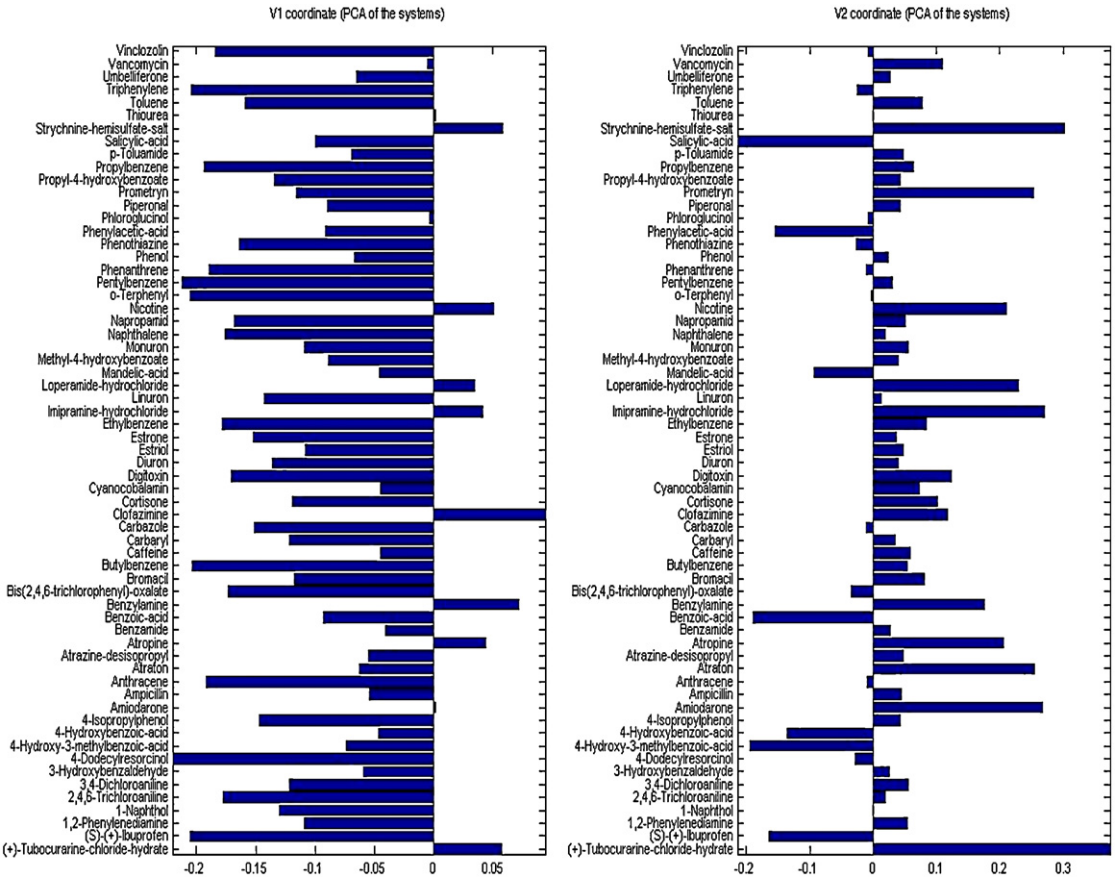

Fig. 4. Coordinates of the two first principle axes on the compounds for the PCA of the chromatographic systems.

But HS F5 was not only extreme for retention; it also behaved quite differently from the other columns. Actually, the difference in behavior between the 2 pH values with HS F5 is less pronounced. This indicates a lower sensitivity to the ionization degree of the solutes at HS F5. Moreover, unlike the other columns with which decreasing the pH from 7.0 to 2.5 induced a large shift along both principal axes, for HS F5 the shift is smaller and along the first axis only. As suggested by the coordinates of the first two principal axes of PCA given in Fig. 4, it seems to indicate a global decrease of retention when decreasing pH rather than with the other columns, a

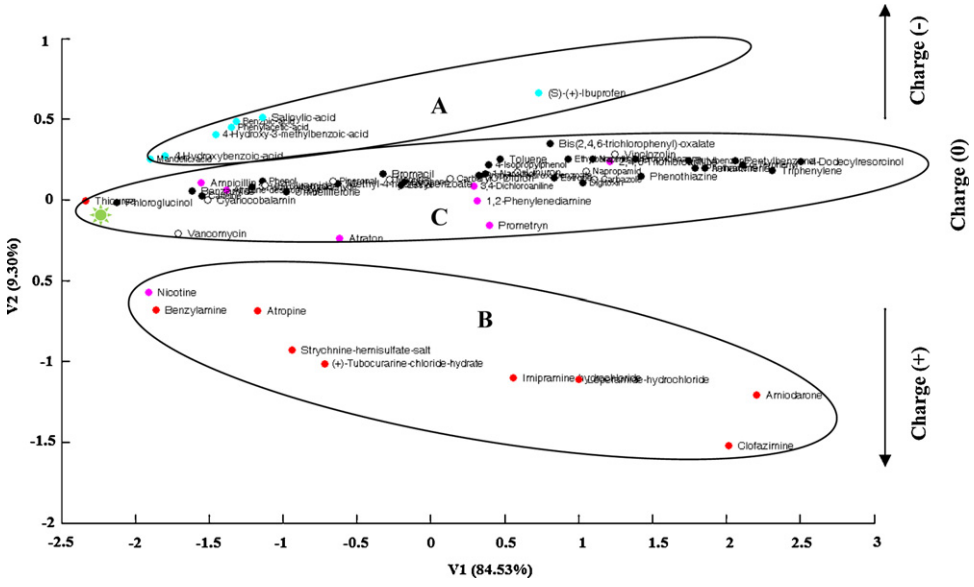

Fig. 5. PCA of the 63 test compounds. Marker color: compounds with negative charge (blue), neutral compounds (black), compounds weekly positively charged according to the pH (pink), compounds with positive charge (red), and non classified compounds (white). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

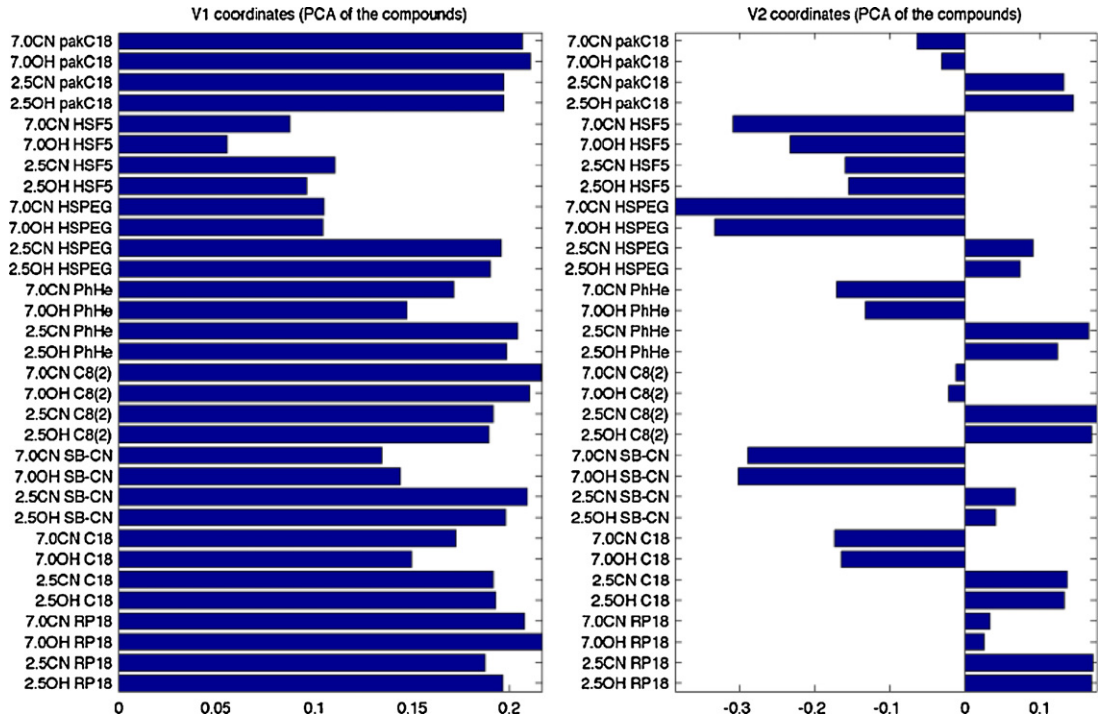

Fig. 6. Coordinates of the first 2 principal axes on the chromatographic systems for the PCA of the solutes.

combined effect of hydrophobic retention and interaction related to the sign and degree of charge of the solutes. Concerning the effect of the organic modifier at pH 7.0, HS F5 was the column presenting the highest sensitivity to this parameter. What is different with HS F5 is that when the pH is decreased with the same organic modifier, the effect is not the same as that observed on other columns: lines symbolizing these changes are not parallel on HS F5, whereas they are parallel for the other columns. Also, changing pH from 7.0 to 2.5 with HS F5 has a more pronounced effect with MeOH than with MeCN. Furthermore, with MeOH, this effect is positive for the second principal component.

This singular behavior of HS F5 could be related to the retention mechanisms proposed in the literature for fluorinated aromatic columns, with pentafluorophenyl (PFP) phases. These stationary phases are difficult to classify accurately on a global polarity scale, but they are convenient to place between C18 and silica, since they show some retention characteristics of both C18 and silica [28–31]. The unusual selectivity of fluoro columns compared to alkyl-silica can be rationalized by large differences in bonded phase polarizability, leading to differential dispersion interactions of solutes and column [28]. There were many attempts to explain the mechanism of this phase. Sadek and Carr [32] studied the retention properties of the PFP phase and concluded that it could be useful for the separation of molecules containing aromatic groups, but they were cautious about proposing a mechanism because of the possibility of silanophilic interaction. Przybyciel and Santangelo [33] suggested that the retention behavior of PFP phase for the separation might involve some π–π interaction, and mechanisms such as charge transfer or electrostatics mode. Marin and Barbas [34] mentioned that HS F5 had higher retention, especially for basic compounds, a phenomenon that we also observed (see appendix 4). The phenyl ring confers an aromatic character to the surface, but the electronegative fluorine tends to withdraw electron density, resulting in a ring system that is very electron deficient. Indeed, PFP behaves as a strong Lewis acid by attracting compounds that can act as Lewis bases by donating electrons, *i.e.* amines. In addition to forming π–π complexes, PFP phase can retain solutes by dipolar and H-bonding forces at the very polar C–F bonds. Strong π–π interactions and column characteristics make PFP column attractive for the separation of aromatic and hydrophobic molecules in RP. So this study confirmed that PFP stationary phase presented original selectivity for our set of probe solutes and appeared as an interesting complement to traditional C8 and C18 phases in the perspective of a LC × LC system [35–37].

3.1.2. PCA of the 63 probe solutes

Fig. 5 shows the projection of the 63 solutes (described by their normalized retention times for the 32 chromatographic systems) in the space of two first principal axes. It clearly appears that the location along the second principal axis reflects the charge of the solutes in the experimental conditions, *i.e.* their acido-basic properties. As can be seen in Fig. 6, the contributions to the second principal axis of chromatographic systems at pH 2.5 are mainly positive, while those of systems at pH 7.0 are mainly negative, and this is independent from the organic modifier. It could be interpreted in relation to the coordinates given in Fig. 6 as a retention for acidic compounds favored in acidic conditions by a lower ionization degree and a retention for basic compounds favored in

Table 3
ANOVA reparametrization.

	om=		om ≠	
	sφ =	sφ ≠	sφ=	sφ ≠
pH=	m	m + b=	m + c	m + b = +c
pH ≠	m + a	m + a + b ≠	m + a + c	m + a + b ≠ +c

Table 4
ANOVA parameter values obtained with LOO cross-validation for the three correlation coefficients. a, $b=$, $b\neq$, and c are the mean effects obtained on the LOO subsets, and σ_a, $\sigma_{b=}$, $\sigma_{b\neq}$ and σ_c are the corresponding standard deviations. f_a, $f_{b=}$, $f_{b\neq}$ and f_c denote the frequency of significantly non zero values of a, $b=$, $b\neq$ and c, respectively on the LOO subsets.

	Criterion	a	σ_a	f_a	$b=$	$\sigma_{b=}$	$f_{b=}$	$b\neq$	$\sigma_{b\neq}$	$f_{b\neq}$	c	σ_c	f_c	δ	σ_δ
Whole set of 63 compounds	r_P	0.45	0.005	100.0	0.37	0.005	100.0	0.12	0.004	98.4	0.03	0.001	0.0	0.60	0.006
	r_S	0.54	0.007	100.0	0.34	0.007	100.0	0.09	0.006	38.1	0.03	0.002	0.0	0.66	0.006
	r_K	0.52	0.006	100.0	0.32	0.006	100.0	0.13	0.006	100.0	0.06	0.003	100.0	0.70	0.005
Group A = 7 acid compounds	r_P	0.37	0.074	100.0	0.24	0.032	100.0	0.09	0.039	28.6	0.01	0.006	0.0	0.48	0.040
	r_S	0.17	0.045	28.6	0.27	0.029	100.0	0.15	0.039	85.7	0.01	0.005	0.0	0.33	0.021
	r_K	0.17	0.046	57.1	0.27	0.031	100.0	0.16	0.041	85.7	0.01	0.005	0.0	0.34	0.021
Group B = 9 basic compounds	r_P	0.29	0.036	100.0	0.25	0.021	100.0	0.01	0.020	0.0	0.03	0.009	0.0	0.34	0.014
	r_S	0.31	0.060	100.0	0.23	0.037	100.0	0.00	0.039	0.0	0.03	0.013	0.0	0.35	0.034
	r_K	0.31	0.067	100.0	0.23	0.032	100.0	0.03	0.036	0.0	0.03	0.012	0.0	0.37	0.046
Group C = 47 compounds	r_P	0.15	0.011	6.4	0.39	0.016	100.0	0.32	0.016	100.0	0.13	0.007	100.0	0.60	0.011
	r_S	0.17	0.014	80.9	0.40	0.015	100.0	0.30	0.018	100.0	0.11	0.008	100.0	0.58	0.011
	r_K	0.13	0.012	0.0	0.36	0.011	100.0	0.32	0.011	100.0	0.15	0.007	100.0	0.59	0.012
28 Neutral compounds	r_P	−0.06	0.005	0.0	0.19	0.005	100.0	0.26	0.004	100.0	0.22	0.007	100.0	0.42	0.006
	r_S	−0.06	0.008	0.0	0.18	0.012	100.0	0.25	0.010	100.0	0.15	0.017	100.0	0.34	0.012
	r_K	−0.08	0.008	0.0	0.19	0.011	100.0	0.28	0.007	100.0	0.14	0.012	100.0	0.34	0.010

neutral conditions by a higher molecular form proportion. All the contributions to the first axis are positive and rather balanced. Thus, the first principal component could be interpreted as global retention.

Three groups of solutes can be distinguished in Fig. 5: 7 solutes with negative charge (acidic) on the upper part of the plot (group A), 9 solutes with positive charge (basic) on the lower part (group B), and 47 [neutral (28) or weekly positively charged (19)] solutes on the intermediate region, which all behaved like neutral solutes in our operating conditions (group C).

3.2. Orthogonality evaluation as a function of the probe solutes set

The criteria considered for orthogonality evaluation were Pearson's (r_P), Spearman's (r_S) and Kendall's (r_K) correlation coefficients. These criteria have the advantage not to require the discretization of the separation space [5].

In order to study how the nature of the probe solutes set acts on orthogonality evaluation, the strategy described in [5] was applied. We have chosen to evaluate the effect on the orthogonality criteria of three factors: pH, stationary phase and organic modifier. We first conducted a classic three-way analysis of variance (ANOVA), the tests being performed with a type I error risk of 5%. For all criteria, there was never a significant interaction between the difference in pH or in stationary phase and the difference in organic modifier. Thus, we performed the ANOVA without the two non-significant interactions with the organic modifier difference, hence with only five parameters. However, we chose a parameterization which is more suited to our problem than the classic (centered) one. As shown in Table 3, it allows to characterize the mean score increase, *i.e.* the mean orthogonality increase, due to the factor modalities of interest, *i.e.* the difference in stationary phase ($s\varphi=$ or $s\varphi\neq$), the difference in organic modifier ($om=$ or $om\neq$) and the difference in pH value ($pH=$ or $pH\neq$), as opposed to identity of the latter. The meaning of these parameters is the following:

- m represents the mean score when all three factors are identical
- a represents the mean score increase due to different pHs (whatever the organic modifiers),
- $b=$ represents the mean score increase due to different stationary phases when the pH values are equal (whatever the organic modifiers),
- $b\neq$ represents the mean score increase due to different stationary phases when the pHs are different (whatever the organic modifiers),
- c represents the mean score increase due to different organic modifiers (whatever the pHs and the stationary phases).

Finally, in order to account for the finite character of the probe sets (of $n = 63$ solutes or less), we have performed leave-one out (LOO) cross-validation for the whole procedure, *i.e.* the ANOVA was performed on n different sets of solutes, the ith set being obtained by removing the ith solute from the whole probe set. The means and standard deviations of the effects are given, as well as the frequencies of rejection of the associated null hypotheses (*i.e.* of the significance of the effects) with a type I error risk of $\alpha = 5\%$.

Table 4 displays the ANOVA results obtained on the whole probe set, on the three subsets identified by PCA, and on the 28 neutral solutes only. The sum $\delta = a + b\neq + c$ is also given: it represents the mean score increase between chromatographic systems that differ with respect to all the three factors (pH, stationary phase and organic modifier).

For the whole set of 63 compounds, the pH appears as the most significant factor in 100% of the cases with an a value around 0.5 whatever the criterion considered. The effect of the stationary phase for the same pH was also significant in 100% of the cases

Table 5
ANOVA parameter values obtained with LOO cross-validation for the two subsets.

	Criterion	a	σ_a	f_a	$b=$	$\sigma_{b=}$	$f_{b=}$	$b\neq$	$\sigma_{b\neq}$	$f_{b\neq}$	c	σ_c	f_c	δ	σ_δ
Group I	r_P	0.04	0.036	0.0	0.19	0.022	100.0	0.20	0.027	100.0	0.07	0.011	88.9	0.31	0.030
	r_S	0.02	0.043	0.0	0.18	0.035	77.8	0.18	0.020	100.0	0.04	0.006	0.0	0.24	0.042
	r_K	0.01	0.040	0.0	0.18	0.036	77.8	0.19	0.019	100.0	0.04	0.007	0.0	0.24	0.040
Group II	r_P	0.45	0.032	100.0	0.29	0.018	100.0	0.08	0.022	33.3	0.01	0.003	0.0	0.54	0.016
	r_S	0.58	0.044	100.0	0.27	0.029	100.0	0.03	0.015	0.0	0.00	0.000	0.0	0.60	0.037
	r_K	0.57	0.038	100.0	0.27	0.025	100.0	0.03	0.007	0.0	0.00	0.000	0.0	0.60	0.035

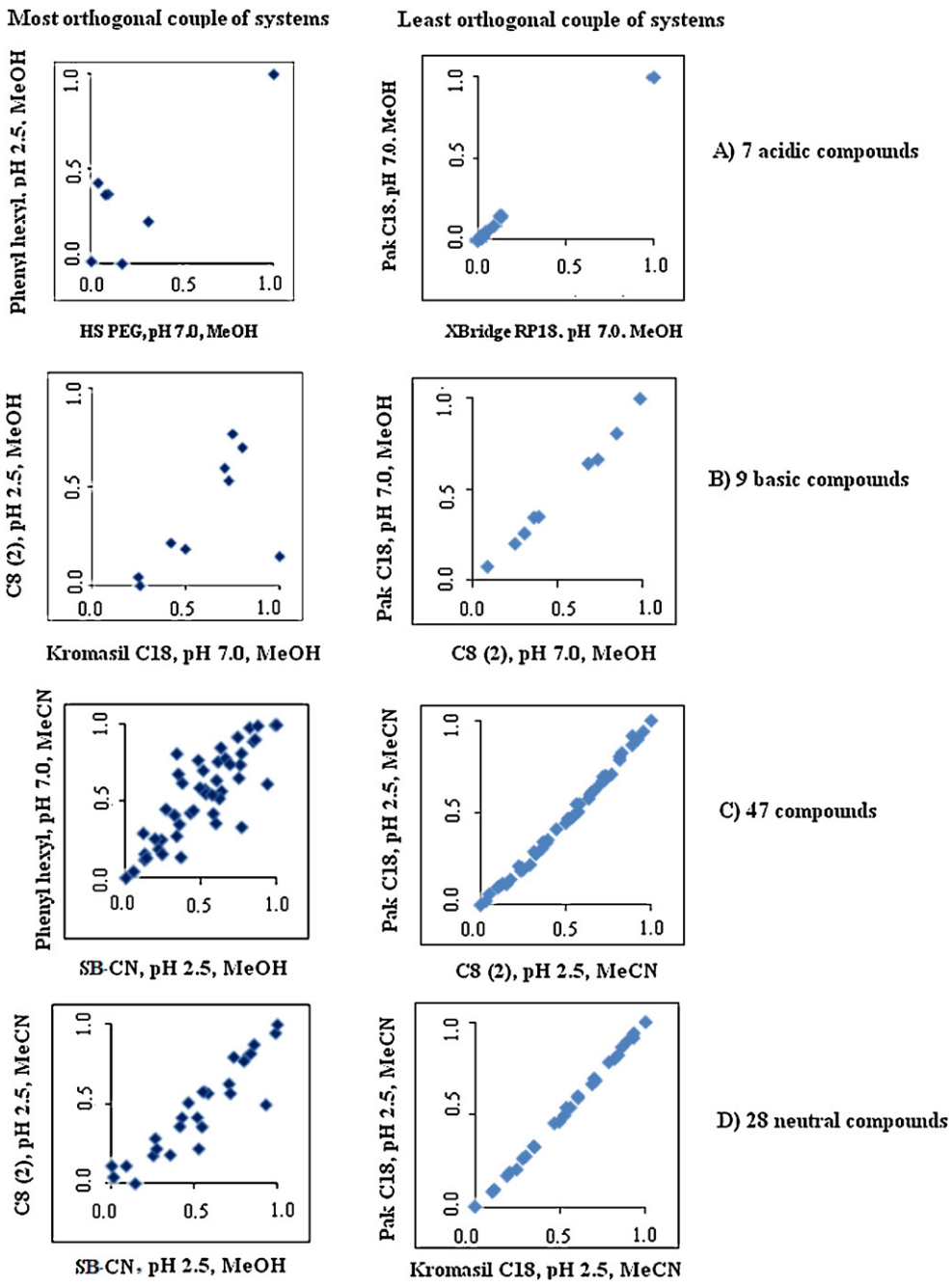

Fig. 7. 2D plots representing the most (left) and the least (right) orthogonal couple of system with the subsets of probe solutes: (A) 7 acidic, (B) 9 basic and (C) 47 solutes include 28 neutral solutes in (D). The coordinates of each compound in a plot are its normalized retention times in the two systems.

with a *b*= value around 0.3 whatever the considered criterion. The effect of the stationary phase at different pH was significant in 100% of the cases with Kendall's correlation coefficient only, with a $b \neq$ value around 0.13. The same behavior was observed for the effect of organic modifier with a *c* value of 0.06. Being less parametric (and hence less affected by non Gaussian data) than for example Pearson's coefficient, Kendall's coefficient has a higher capacity to exhibit the effect of the factors, and hence to differentiate orthogonal and non orthogonal system couples (the discriminating power).

For the set of 7 acidic compounds (group A), the stationary phase appeared as the only significant factor in 100% of the cases with a *b*= value around 0.25 whatever the considered criterion. Only Pearson correlation coefficient indicated a significant effect of the pH in 100% of the cases with an *a* value of 0.37, however, this mean value is to be considered with caution given its large standard deviation. It indicates that for acidic compounds, likely to be negatively charged, the best option to find orthogonal separation conditions consists in varying the stationary phase. The pH of the mobile phase had a rather limited influence and the organic modifier nearly none. The nature of the stationary phase governs the kind and intensity of the main interaction of the compounds, *i.e.* the hydrophobic effect. The pH of the mobile phase has mainly an influence on the ionization degree of the solutes, modifying so their hydrophobicity. The most and the least orthogonal couple of systems obtained for this acidic subset are given in Fig. 7-A.

For the subset of 9 basic compounds (group B), the pH and the stationary phase appeared as the only two significant factors

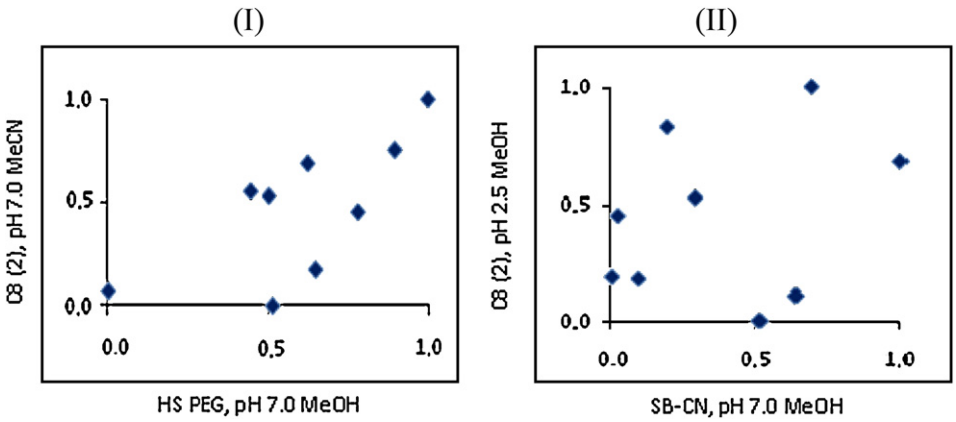

Fig. 8. 2D plots representing the most orthogonal couples of systems with the subsets of probe solutes: (I) 9 compounds from group C, (II) 9 compounds, 3 from each PCA group.

in 100% of the cases, whatever the considered criterion, with *a* and *b*= values around 0.30 and 0.24, respectively. The effect of the difference in the organic modifier never appeared significant. Here, the pH is the most important factor not only because it affects on the ionization degree of the solutes, modifying so their hydrophobicity, but also because it acts on the ionization of the silanol groups of the stationary phase. Thus, the high-energy electrostatic interaction between ionized silanol groups and positively charged basic compounds explains the strongest magnitude of the pH effect. The nature of the stationary phase is naturally also significant, but its effect is a little smaller than that of the pH. Thus, for basic compounds, orthogonality benefits first from the difference in pH, and then from the difference in stationary phase. It indicates that for basic compounds, likely to be positively charged, the best option to find orthogonal separation conditions consists in varying the pH and the stationary phase. The most and the least orthogonal couples of systems for this subset are given in Fig. 7-B.

Results for both the subset of the 28 neutral compounds, or for the subset of 47 compounds behaving like neutral compounds in our conditions (group C), are rather similar. In both cases, only the stationary phase and the organic modifier have a significant effect in 100% of the cases, whatever the criterion considered. For the 28 neutral compounds, the magnitude of the effects due to the difference in stationary phase and to organic modifier is rather similar, with a value around 0.20–0.25. For the 47 compounds (group C) behaving like neutrals (there are some weakly basic compounds), the effect of the stationary phase is stronger than the effect of the organic modifier with values around 0.35 and 0.13, respectively. Once again, these conclusions are logical since, for neutral compounds, the pH of the mobile phase cannot affect pure hydrophobic effect. However, even for neutral compounds, if they present some polar groups, some interactions between these polar groups and the residual silanol groups (ionized or not) could modulate the retention through dipole–dipole or ion–dipole interactions. The pH intervenes here on the degree of ionization of the silanol groups. These effects are all the more pronounced as the set of probe solutes includes some weakly basic compounds. In practice, it means that for neutral compounds, or behaving as neutral, the best option to find orthogonal separation conditions consists in varying both the stationary phase and the organic modifier. The most and the least orthogonal couples of systems for these subsets are given in Fig. 7-C and D.

To summarize, orthogonality and the factors affecting it are highly dependent on the nature of the solutes used as probes. Orthogonality for RP systems is not absolute; it is a function of the nature of the compounds.

3.3. How to choose the probe solutes set for orthogonality evaluation?

Is it possible to use a reduced set of solutes while preserving the quality of orthogonality evaluation? To answer this question, two extreme subsets of probe solutes were chosen on the basis of the PCA of the solutes (Fig. 5):

- The first subset (I) consisted of 9 compounds belonging to group C (solutes behaving like neutral ones): toluene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene, 3,4-dichloroaniline, 2.4.6 trichloroaniline, methyl-4-hydroxybenzoate, and bis (2,4,6-trichlorophenyl)-oxalate.
- The second subset (II) consisted of 9 compounds as different as possible, three of each PCA group: ibuprofen, salicylic acid, mandelic acid (group A), clofazimine, tubocurarine, and benzylamine (group B), benzamide, bromasil, dodecylresocinol (group C).

The results of Table 5 show that the choice of the probe solute set can dramatically change the evaluation. With subset I, the stationary phase had the highest impact. Pearson coefficient is the most sensitive criterion, because of the regular distribution of the retention times of the solutes of subset I, whatever the system. With subset II, results similar to those obtained with the whole set of the 63 solutes [5] are observed: the pH has the highest impact factor on orthogonality followed by the stationary phase. In addition, Kendall's correlation coefficient is the most sensitive criterion, as for the whole set of 63 compounds [5]. The 2D plots of the most orthogonal couples of chromatographic systems for subsets I and II are shown in Fig. 8.

Thus, a reduced but appropriately chosen set of compounds, can work as well as a large set, (*e.g.* 9 compounds instead of 63) in order to achieve orthogonality evaluation of RP systems.

4. Conclusion

The results presented here clearly showed how the choice of probe solutes could influence orthogonality evaluation. Different subsets of probe solutes obtained from a PCA analysis and corresponding to different acido-basic properties were examined. The results obtained were:

- For the basic compounds, the difference in pH and stationary phase had the highest impact on orthogonality.
- For the acidic compounds, the difference in stationary phase had the highest impact on orthogonality.

- For neutral compounds, both the difference in stationary phase and the difference in organic modifier increased orthogonality.

All the experimental results obtained in the present study and their interpretations are in the direct line of Giddings [38] principles about sample dimensionality. In practice, during orthogonality evaluation, the nature of the compounds the sample is made of should be taken into account. Effectively, orthogonal conditions for neutral compounds could not be suited for a sample consisting mainly of ionic compounds. In addition, a reduced set of appropriately chosen solutes could provide similar results for orthogonality evaluation as a whole set.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.056.

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