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Orthogonality and similarity within silica-based reversed-phased chromatographic systems

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Abstract

The starting point of this study was a current set of 32 chromatographic systems used to select initial conditions for method development to determine the impurity profile of a drug. The system exhibiting the best selectivity is then selected for further method development. In this current set eight silica-based phases are applied in conjunction with four mobile phases at different pH. In order to save time and resources, the possibilities for a meaningful subset selection were investigated. The most differing systems in terms of selectivity, in other words only the most orthogonal systems, need to be selected. Since the stationary phases are all silica-based, the selectivity differences are examined within a more homogeneous group than if, for instance, also zirconia- or polymer-based columns would be involved. To select the subset of systems also the best overall separation performances are taken into account. The selection is based both on the HPLC–DAD data of a generic set of 68 drugs, and on the LC–MS–DAD results for a mixture of 15 drugs, less different in structure. The orthogonality is evaluated using weighted-average-linkage dendrograms and color maps, both created from the Pearson-correlation coefficients r between normalized retention times τ. The Derringer's desirability functions are applied to define the systems with the best overall separation performances. Proposals for different representative subsets of the initial 32 systems are made.

Keywords: Orthogonal chromatographic systems; Correlation coefficients color map; Weighted-average-linkage dendrogram; Derringer's desirability function; Method development

1. Introduction

The presence of impurities in drugs must be evaluated qualitatively and quantitatively, because of the potential risk for negative side effects. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [1] has defined guidelines to characterize impurities. Analytical methods are required to separate, identify and quantify the main compound and all important related substances (impurities). When using HPLC as separation technique, a proper stationary and mobile phase have to be chosen. The type of stationary phase and the mobile phase pH are the two most important parameters influencing selectivity in gradient-elution HPLC [2–4].

In order to select appropriate columns from the continuously expanding group of potentially suitable ones, a test that evaluates stationary phase properties (hydrophobicity, efficiency, steric selectivity, silanol activity, ion-exchange capacity, hydrogen-bonding capacity) by measuring chromatographic parameters was performed earlier [5]. Based on these results, at Johnson & Johnson Pharmaceutical Research and

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Development (J&J-PRD), a division of Janssen Pharmaceutica N.V., a set of eight silica-based reversed-phase columns was selected, and is now systematically screened when HPLC methods have to be developed to clarify the impurity profile of new drugs.

In a screening module of 32 systems, each of the eight stationary phases is tested at four different mobile phase pH-values (2.5, 4.8, 7.0 and 9.0) using a standard gradient elution method. Of these 32 systems applied for screening, only 27 are considered as potential starting points for further method development, since five columns (Zorbax Bonus-RP, YMC-Pack C4, SymmetryShield RP_{18}, YMC-Pack Pro C18 and XTerra Phenyl) are not certified at the highest pH tested (pH 9.0). However, the latter pH is used on all columns during screening, because these conditions may help reveal the complete impurity profile of a new drug.

In this study it is evaluated whether the number of stationary phases and/or pH-values in the current set of systems can be reduced to an optimized set with only highly orthogonal systems.

It can be noticed that the term orthogonal or orthogonality is not used in its strict mathematical sense here. In chemometrics two parameters are orthogonal when they are uncorrelated ($r = 0$) and they are either orthogonal or not. In comprehensive two-dimensional chromatography two methods are called orthogonal if the constituent dimensions operate independently and synentropy across the dimensions is zero [6]. However, in situations as ours where various systems are compared, e.g. as potential starting points for method development, often a less strict definition is applied. Orthogonal systems are then defined as systems "that differ significantly in chromatographic selectivity" [7]. This means that systems for which r between the retention data is low are also considered or called orthogonal. It also means that, e.g. while comparing pairs of systems, terms as more orthogonal (or more dissimilar, or with more selectivity differences) and rather orthogonal can be applied. For reasons of analogy with previous publications [3–5,7] usually the term orthogonal is used, rather than dissimilar. Orthogonal systems differ in selectivity [3,4], because the retention of the solutes is caused by different substance properties and interactions. Application of a set of orthogonal systems allows obtaining separations that are as diverse as possible, implying that the chromatographic systems complement each other in the information provided. Two sets of test compounds were used to make a thorough evaluation. One consisted of 68 diverse marketed drugs (from different sources), whereas the other consisted of 15 structurally relatively similar substances obtained from J&J-PRD. The normalized retention times τ on each system are determined under gradient conditions for every compound of both test sets.

The substances in the first set differed in structure (functional groups, ring structures), molecular weight, pK_a, $\log P$, and pharmacological class, so they can potentially reveal generic orthogonality between systems. To increase the throughput, mixtures of three or four components were injected [4]. The normalized retention times were determined on an HPLC–DAD instrument. The second set of test compounds consisted of 15 in-house drug substances, with related chemical structures, and thus providing retention data for similar molecules. This allows determining the orthogonality of the systems for similar structures, a situation that is representative for impurity profiling of drugs. A mixture of all substances was injected and LC–MS–DAD was applied to track each compound. This mixture was also used to determine the overall separation performance of the systems, in terms of separation and analysis time.

The aim of this study is to select subsets from both the 32 screening systems and the 27 method development ones. The selected systems should exhibit a high degree of orthogonality and good overall separation performances. The subsets will be applied during the search for initial separation conditions for a new drug in early development or to find starting conditions to develop a final separation method in late drug development. The orthogonality between systems was evaluated using visualization methods already applied to define orthogonal and similar systems, i.e. weighted-average-linkage dendrograms [3,4,8–13], and correlationcoefficients color maps [4]. To evaluate the overallseparation performance of each system, a multicriteria decision-making method based on Derringer's desirability functions was used [5,14–18]. Multicriteria decision approaches allow selecting the systems with the best compromise for a number of chromatographic responses (i.e. minimal and maximal normalized retention time, and minimal, median and maximalselectivity).

2. Experimental

2.1. Drugs and reagents

The determination of orthogonality (or similarity) was performed using two sets of test compounds. The first set consisted of 68 drugs of which the majority were basic (55) (typical for most pharmaceuticals), some were neutral (4) and the rest acidic (9) in nature. To increase the experimental throughput, the test substances were injected as mixtures. The 68 drugs, the stock solution concentrations (in 50:50% (v/v) methanol/Milli-Q water) and the mixtures injected are summarized in Table 1. Each mixture was prepared by equivolumetrical addition of the stock solutions. The concentration of a given substance was chosen based on its absorbance at 254 nm. To prepare the stock solutions, methanol, Hypersolv for HPLC (BDH, Poole, England) was used.

The multidimensional data from HPLC–DAD are used to determine the elution time of each component. The composition of the mixtures was chosen such that the probability for co-eluting peaks is minimized. Mixtures were composed of compounds with different UV-spectra, acidic–basic, and pharmacological properties. For strongly overlapping peaks, checking the UV-spectra from the spectrochromatograms al-

Table 1
Summary of the 68 substances used, their stock solution concentrations and distributors

Mixture	Substance (concentration (mg/1))	Distributed by
1	Cocaine hydrochloride (1000)	Bios Coutelier (Brussels, Belgium)
	Naphazoline hydrochloride (2000)	Sigma–Aldrich (Steinheim, Germany)
	Ranitidine hydrochloride (2000)	Sigma (St. Louis, Missouri)
2	Acebutolol hydrochloride (1000)	Sigma (St. Louis, Missouri)
	Codeine base (1000)	Bios Coutelier (Brussels, Belgium)
	Pentoxifylline (1000)	Sigma–Aldrich (Steinheim, Germany)
	Pizotifen (5000)	Novartis Pharma (Wehr, Austria) (gift)
3	Dimetindene maleate (1000)	Novartis (Basel, Switzerland) (gift)
	Flurazepam (1000)	Dolorgiet Arzneimittel (Bonn, Germany)
	Morphine hydrochloride (2000)	Bios Coutelier (Brussels, Belgium)
4	Caffeine (1000)	Fluka (Neu-Ulm, Switzerland)
	Chloropyramine hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
	Fenfluramine hydrochloride (1000)	Technologie Servier (Orleans, France)
	Lidocaine hydrochloride (1000)	Bios Coutelier (Brussels, Belgium)
5	4-Benzylphenol (1000)	Aldrich (Milwaukee, WI)
	Oxeladin citrate (2000)	Sigma–Aldrich (Steinheim, Germany)
	Prenalterol hydrochloride (1000)	Ciba-Geigy (Basel, Switzerland)
	Pyrilamine maleate (1000)	Sigma–Aldrich (Steinheim, Germany)
6	Ketotifen fumarate (1000)	Sigma–Aldrich (Steinheim, Germany)
	Pindolol (1000)	Sigma–Aldrich (Steinheim, Germany)
	Sulfapyridine (1000)	Bios Coutelier (Brussels, Belgium)
	Thiothixene (USP grade) (2000)	Sigma–Aldrich (Steinheim, Germany)
7	Bupranolol hydrochloride (1000)	Schwarz Pharma (Monheim, Germany)
	Cimetidine (10,000)	Penn Chemicals (Pennsylvania, PA) (gift)
	Famotidine (2000)	Sigma–Aldrich (Steinheim, Germany)
	Tetrahydrozolin hydrochloride (4000)	U.S.P.C. (Rockville, MD)
8	Antazoline hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
	Digitoxine (1000)	Mann Research Laboratories (New York, NY)
	Phenol (1000)	Merck (Darmstadt, Germany)
9	(±)-Camphor (5000)	Sigma–Aldrich (Steinheim, Germany)
	Propiomazine maleate (1000)	Sanofi (Paris, France) (gift)
	Tolazoline hydrochloride (5000)	Sigma–Aldrich (Steinheim, Germany)
10	Diphenhydramine hydrochloride (5000)	Sigma–Aldrich (Steinheim, Germany)
	L-(+)-Ascorbic acid (1000)	Merck (Darmstadt, Germany)
	Miconazol nitrate (1000)	Certa (Braine-1'Alleud, Belgium)
11	α-Lobeline hydrochloride (1500)	Carl Roth (Karlsrhue, Germany)
	Isothipendyl hydrochloride (1000)	Novartis Pharma (Wehr, Austria) (gift)
	Oxprenolol hydrochloride (500)	Sigma–Aldrich (Steinheim, Germany)
	Terbutaline sulphate (1000)	Astra Draco (Lund, Sweden)
12	Cirazoline hydrochloride (400)	Research Biochemicals International (Natick, MA)
	Desipramine hydrochloride (5000)	Sigma–Aldrich (Steinheim, Germany)
	Promethazine hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
	Resorcine (1000)	Merck (Darmstadt, Germany)
13	Diclofenac sodium (5000)	Sigma–Aldrich (Steinheim, Germany)
	Prazosin hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
	Strychnine base (1000)	Bios Coutelier (Brussels, Belgium)
14	5-Hydroxytryptamine hydrochloride (500)	Sigma–Aldrich (Steinheim, Germany)
	Carbamazepine (1000)	Sigma–Aldrich (Steinheim, Germany)
	Nadolol (1000)	Sigma–Aldrich (Steinheim, Germany)
	Sotalol (1000)	Merck (Darmstadt, Germany)
15	Betaxolol hydrochloride (1000)	Synthelabo (Paris, France) (gift)
	Fluphenazine dihydrochloride (USP grade) (2000)	Sigma–Aldrich (Steinheim, Germany)
	Procaine hydrochloride (1000)	Merck (Darmstadt, Germany)
16	5-Sulfosalicylic acid dihydrate (2000)	Merck (Darmstadt, Germany)
	Lorazepam (1000)	MSD (Haarlem, The Netherlands)
	Terazosin hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)

Table 1 (*Continued*)

Mixture	Substance (concentration (mg/1))	Distributed by
17	Dopamine hydrochloride (2000)	Sigma–Aldrich (Steinheim, Germany)
	β-Estradiol (500)	Sigma–Aldrich (Steinheim, Germany)
	Efedrine hydrochloride (2000)	Vel (Leuven, Belgium)
18	1,1-Dimethylbiguanide hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
	Celiprolol (1000)	Rhône-Poulenc-Rorer (Madrid, Spain) (gift)
	Nizatidine (2000)	Norgine (Marburg, Germany) (gift)
	Timolol maleate (1000)	Sigma–Aldrich (Steinheim, Germany)
19	Digitoxigenine (500)	Fluka (Neu-Ulm, Switzerland)
	Histamine dihydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
	Nicardipine hydrochloride (1000)	UCB (Leuven, Belgium)
20	Amiodarone hydrochloride (5000)	Clin-Midy groupe Sanofi (Montpellier, France)
	Ibuprofen (5000)	Sigma–Aldrich (Steinheim, Germany)

ready might reveal the identity of the individual substances. The different pH-values and the use of gradient elution imply also that the UV-spectra might be affected. However, reference spectra in acidic, basic and alcoholic environment were available [19,20]. When necessary, chemometric techniques, e.g. orthogonal projection approach (OPA), can be applied to track co-eluting peaks [4].

The second set consisted of 15 active compounds, injected as one mixture, and LC–MS–DAD was used to track components and their elution times. The 15 drug substances (all J&J-PRD, Beerse, Belgium), forming the second set, were: astemizole, azaconazole, cinnarizine, domperidone, droperidol, flubendazole, inazalil, isoconazole nitrate, itraconazole, ketanserin tartrate, ketoconazole, levamisole hydrochloride, liarozole hydrochloride, risperidone and sabeluzole. All compounds were used at a concentration of 0.10 mg/ml. The solvent for this test mixture was methanol/tetrahydrofuran/dimethyl formamide 40:40:20% (v/v/v). Here, methanol for HPLC, tetrahydrofuran and dimethyl formamide (Acros Organics, Geel, Belgium) were used.

The mobile phases were prepared with acetonitrile, for HPLC far UV (Acros Organics), ammonium carbonate extra pure (Riedel-de Haën, Seelze, Germany), ammonium acetate Microselect, acetic acid 50% puriss. pro analysi (GR) for HPLC, trifluoroacetic acid for protein sequence analysis, and diethylamine puriss. plus (GC) (all from Fluka Chemie, Buchs, Switzerland). In buffers, stock solutions and samples, Milli-Q water is used, prepared with the Millipore purification system (Millipore, Molsheim, France).

2.2. Chromatographic conditions

The HPLC–DAD experiments were executed using four instruments, each consisting of an autosampler, a diode array detector, a vacuum degasser and a pump, all from Hewlett Packard Series 1100 (Agilent, Waldbronn, Germany) and a column switcher (VICI AG, Schenkon, Switzerland). The chromatographic methods were created and the data collected and treated with the Chemstation Rev. A.08.03 software (Agilent).

The LC–MS–DAD analyses were performed on an on-line coupling of four high performance liquid chromatographs, each consisting of a Waters 2695 Separations Module (=alliance) HPLC compartment (Waters, Milford, MA), a Mistral column oven (Spark Holland, Emmen, The Netherlands), a column switcher (VICI AG) and a Waters 996 Photodiode Array Detector, linked with a single quadrupole mass spectrometer, Waters micromass ZQ, using multi-plexed electrospray ionization, Waters micromass MUX mass spectrometer with rotor (a four-channel MUX interface), applied in the positive ionization mode. Injection was performed simultaneously on a four-channel CTC PAL injector (CTC Analytics, Zwingen, Switzerland). The effluent from the HPLC's was splitted prior to diode array detection using a zero-dead-volume T-piece so that about 100 μl/min per channel enters the interface. Typical parameters of the ion source are: capillary voltage: 3.6 kV; cone voltage: 20 V; source temperature: 150 °C; desolvation temperature: 100 °C; and cone gas flow: 113 l/h N_2. The specifications for the mass spectrometer are: scan range: 165–750 (M_r); scan time: 0.23 s; interscan time: 0.1 s; photomultiplier voltage: 550 V; resolution: for low mass, 13.0 U, and for high mass, 13.5 U. The chromatographic methods were created and the data treated using both Millennium^{32} Version 4.0 software (Waters) for the spectral data, and MassLynx Version 3.5 software (Micromass, Cary, North Carolina) for the mass spectrometry data.

The eight stationary phases tested were: (a) Zorbax Extend-C18, (100 mm × 4.6 mm i.d., 3.5 μm) (Agilent) (ZE), a bidentate bonded and double-endcapped ultrapure C_{18}-silica stationary phase [21]; (b) Zorbax Bonus-RP, (100 mm × 4.6 mm i.d., 3.5 μm) (Agilent) (ZB), a triple-endcapped ultrapure C_{14}-silica with embedded polar amide group and sterically protecting diisopropyl group [21]; (c) XTerra MS C_{18}, (100 mm × 4.6 mm i.d., 3.5 μm) (Waters) (XMS), a hybrid C_{18}-silica with trifunctional bonding and embedded polar group [22]; (d) XTerra RP_{18}, (100 mm × 4.6 mm i.d., 3.5 μm) (Waters) (XRP), a hybrid C_{18}-silica shielded through embedding a polar group [22]; (e) YMC-Pack C4, (100 mm × 4.6 mm i.d., 5 μm) (YMC c/o Waters, Milford, MA) (YC4), a fully-endcapped C_4-silica with high-coverage monomeric bonding [23]; (f) Symme-

Table 2
(a) Composition of the mobile phases and (b) conditions for the gradient runs

(a)

pH	Mobile phase A	Mobile phase B
2.5	Ammonium acetate in water–acetonitrile 950:50% (v/v) + trifluoroacetic acid	Ammonium acetate in water–acetonitrile 300:700% (v/v) + trifluoroacetic acid
4.8	Ammonium acetate in water–acetonitrile 950:50% (v/v) + acetic acid	Ammonium acetate in water–acetonitrile 300:700% (v/v) + acetic acid
7.0	Ammonium acetate in water–acetonitrile 950:50% (v/v)	Ammonium acetate in water–acetonitrile 300:700% (v/v)
9.0	Ammonium acetate in water–acetonitrile 950:50% (v/v) + diethylamine (HPLC–DAD) or + ammonium carbonate (LC–MS–DAD)	Ammonium acetate in water–acetonitrile 300:700% (v/v) + diethylamine (HPLC–DAD) or + ammonium carbonate (LC–MS–DAD)

(b)

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	100	0
20	0	100
25	0	100

tryShield RP_{18}, (100 mm × 4.6 mm i.d., 3.5 μm) (Waters) (WSS), a C_{18}-silica shielded through an embedded polar group [22]; (g) YMC-Pack Pro C18, (100 mm × 4.6 mm i.d., 3 μm) (YMC c/o Waters) (YC18), a C_{18}-silica with high-coverage carbon bonding and an endcapping procedure utilizing Lewis acid–base chemistry [23]; and (h) XTerra Phenyl, (100 mm × 4.6 mm i.d., 3.5 μm) (Waters) (WXP), a hybrid phenyl-silica with difunctional bonding [22].

Mobile phases with four different pH-values (2.5, 4.8, 7.0 and 9.0) were applied. Their composition is given in Table 2a. Each mobile phase runs on a separate HPLC instrument, each equipped with a column switcher to which the eight stationary phases are coupled. Thus, in total 32 columns are used to create the 32 systems. The systems are identified by the stationary phase abbreviations, extended with an index referring to the mobile phase pH, e.g. $ZE_{2.5}$ stands for Zorbax Extend-C18 applied at pH 2.5.

The same gradient elution scheme was used, at a flow of 1.0 ml/min, for all mobile phases and on all columns (Table 2b). The injection volume was 5 μl. After each run, the stationary phase was equilibrated at starting conditions for 5 min. All experiments, performed at the HPLC–DAD instrument, were carried out at ambient temperature; on the LC–MS–DAD equipment, they were thermostated at 35 °C. The normalized retention times τ measured at a wavelength of 225 nm were used. The normalized retention time is defined as the difference between the retention time and the dead time, divided by the dead time, measured under gradient conditions.

3. Results and discussion

Since the goal of this study is to reduce the number of systems to be tested for screening and method development purposes, while maintaining as much information as possible about impurity profiles, the most orthogonal of the chromatographic systems in the current screening and method development modules need to be selected.

A distinction is made between screening for initial separation conditions for new drugs, and finding a starting method for a drug in late development. In the first situation the impurity profile is not yet known. Therefore, highly selective methods with maximal orthogonality and high efficiency are needed. In late development, the impurity profile is usually known, therefore highly selective methods along with high efficiency may be sufficient to separate the main compound and its impurities in the drug. Although some columns are not certified to be applied at high pH, they were all screened at buffer pH 9.0. For the purpose of screening, analysis at high pH may generate maximal information about the unknown impurity profile (of a new drug), as the pH plays an important role in establishing selectivity differences [2–4]. The application of stationary phases that are not certified at high pH, is feasible for a limited number of runs, may help to clarify the impurity profile and can be used in early development.

However, in late drug development, only columns certified at pH 9.0 can be applied in starting systems for method development, because robust separation conditions are preferred. Only three of the eight stationary phases in the screening module are certified at this pH, i.e. Zorbax Extend-C18 ($ZE_{9.0}$), XTerra MS C_{18} ($XMS_{9.0}$) and XTerra RP_{18} ($XRP_{9.0}$). Therefore, the module for late development actually consists of fewer systems (27) than the screening module for early drug development (32).

3.1. Relationships between the systems

The relationships between the chromatographic systems were evaluated from the Pearson-correlation coefficients r between the normalized retention times τ of the substances [3,4,24]. In general, a lower r-value reveals larger selectivity differences, and thus more orthogonality. A high correlation coefficient reflects a high degree of linear association between the normalized retention times for all substances on the com-

pared systems, i.e. the solutes are eluting in a similar order and at elution times that are proportional. Such systems are not orthogonal, but have a high level of similarity, i.e. they are interchangeable [3,4].

However, since the number of evaluated systems is rather high, retrieving relationships between all systems is difficult. Therefore, two visualization techniques were used, i.e. the weighted-average-linkage hierarchical clustering method [3,4,8–13] and correlation coefficients color maps [4]. First, dendrograms were constructed applying the weighted-average-linkage technique [3,4,8–13]. Average linkage is an agglomerative method as it starts with clusters each containing one object and successively merges the two clusters for which the dissimilarity value is smallest, until only one cluster remains. The dissimilarity between two clusters is defined as the average dissimilarity between all pairs of objects in them. In the weighted method, objects of smaller clusters carry a larger weight than those from larger ones. As a consequence, each cluster weighs the same. This technique is also called Weighted Pair Group Method using arithmetic Averages (WPGMA) [25]. Fig. 1 shows a WPGMA-dendrogram of 32 systems, obtained using the normalized retention times of the 68 substances (Fig. 1a) and of the 15 compounds (Fig. 1b). The dissimilarity criterion applied is $1 - |r|$. The (dis)similarity between clusters is visualized in the dendrogram by the height at which they are connected. The higher two clusters are connected, the more dissimilar they are.

Secondly, the matrix of correlation coefficients between chromatographic systems was visualized as a color map (Fig. 2). In such a map, the *r*-values are represented by colors. The bar next to the map links the value of the correlation coefficient to a color. The systems in the color map can be ranked in different ways. It has been shown [4] that color maps with the systems ranked according to either increasing or decreasing dissimilarities in the WPGMA-clustering are useful to visualize their relationships. In Fig. 2, the *r*-color maps of 32 systems are shown applying the normalized retention times of the 68 (Fig. 2a) and of the 15 substances (Fig. 2b), ranking the systems according to increasing dissimilarities observed in the dendrograms of Fig. 1.

The selectivity differences can also pair-wise be visualized by plotting the normalized retention times on Cartesian axes [3,4], as is for instance shown in Fig. 3 for the pairs $ZB_{2.5}$–$WSS_{2.5}$, $ZB_{2.5}$–$YC4_{9.0}$ and $WSS_{2.5}$–$YC4_{9.0}$. In Fig. 3a, the elution order is quite similar on both systems, with only minor changes in the elution order, which results in the high correlation coefficient of 0.994. This implies that mostly similar information will be gathered when these systems would be applied in parallel, and therefore they are considered interchangeable. Fig. 3b and c show dissimilarity, resulting in a cloud of points and a low *r*-value. Both $ZB_{2.5}$ and $WSS_{2.5}$ are thus considered rather orthogonal towards $YC4_{9.0}$. This means that the elution order on the compared systems is different. The latter figures also indicate that the selectivity differences are general. A low *r*-value namely also can be obtained when a small number of substances shows extremely different normalized retention times on the compared systems, whereas the majority is eluting in a similar order [3]. Visualizing the τ-values as in Fig. 3 is therefore useful to detect whether the selectivity differences are generic.

In Fig. 1a, $YC4_{9.0}$ and $ZB_{4.8}$ are interesting, as they are situated at high dissimilarity values from each other and from several other systems. It means that $YC4_{9.0}$ and $ZB_{4.8}$ will play an important role in selecting orthogonal systems. Fig. 2a confirms that this pair is indeed the most orthogonal one, as the lowest *r*-value is encountered for them, and that they both are interesting to compare with others for selectivity differences, since a number of low *r*-values are observed for these systems in comparison with several other systems. Fig. 2a shows that low correlation coefficients are obtained for the combination of $YC4_{9.0}$ with either $ZB_{2.5}$, $WSS_{2.5}$ and $ZB_{4.8}$, and for the pair $ZB_{4.8}$–$WSS_{9.0}$. The *r*-values of the most orthogonal pairs are summarized in Table 3a. It can be seen that the lowest correlation coefficients are obtained comparing systems at the highest pH (9.0) on the one hand with one at the lower ones (2.5 or 4.8) on the other. The 10 most orthogonal systems contain only five columns from the eight in the screening module: YMC-Pack C4, Zorbax Bonus-RP, Waters SymmetryShield RP_{18}, YMC-Pack Pro C18 and Zorbax Extend-C18.

Besides detecting the most orthogonal systems, Figs. 1a and 2a can also be used to derive similar ones. Several groups of similar systems can be distinguished. It is observed that they are, in general, clustered according to pH, which, for instance, clearly can be seen when using in Fig. 1a an arbitrary limit of $1 - |r| = 0.05$. From Fig. 2a it is shown that all systems of pH 2.5 (numbers 1–8) are situated in the same cluster (group I). For those at pH 4.8 (numbers 9–16), all are also clustered (group II), except for $ZB_{4.8}$ (indicated as IIa), which is isolated, meaning it is found more dissimilar. Further on, it can be observed in Fig. 1a that the systems at pH 7.0 (numbers 17–24; group III) exhibit somewhat higher dissimilarities towards those at pH 2.5 and 4.8, as the $1 - |r|$-value at which the clusters are branched, is about 0.11, which means that more selectivity differences are expected when comparing a system of group III with one of groups I or II, than if systems from the latter clusters would be compared. Analogously, Fig. 2a shows lower correlation coefficient values when comparing a system of group III with one of groups I or II. The systems at pH 7.0 (group III) and 9.0 (group IV) are also situated in separate clusters, except for $WSS_{7.0}$ and $YC4_{7.0}$ (pH 7.0), which are in the cluster of the systems at pH 9.0, and $YC4_{9.0}$, which is separated from the rest. Within group III, $ZB_{7.0}$ is remarkable as a higher dissimilarity (Fig. 1a) and a lower *r*-value (Fig. 2a) are observed towards the other systems. All systems of group IV are connected with those of groups I–III at the highest dissimilarity value (Fig. 1a) and exhibit the lowest correlation coefficients when compared with the systems from the latter groups (Fig. 2a).

In Fig. 1b, the dendrogram for the 32 systems, using the set of 15 substances, is given. The clustering of the systems

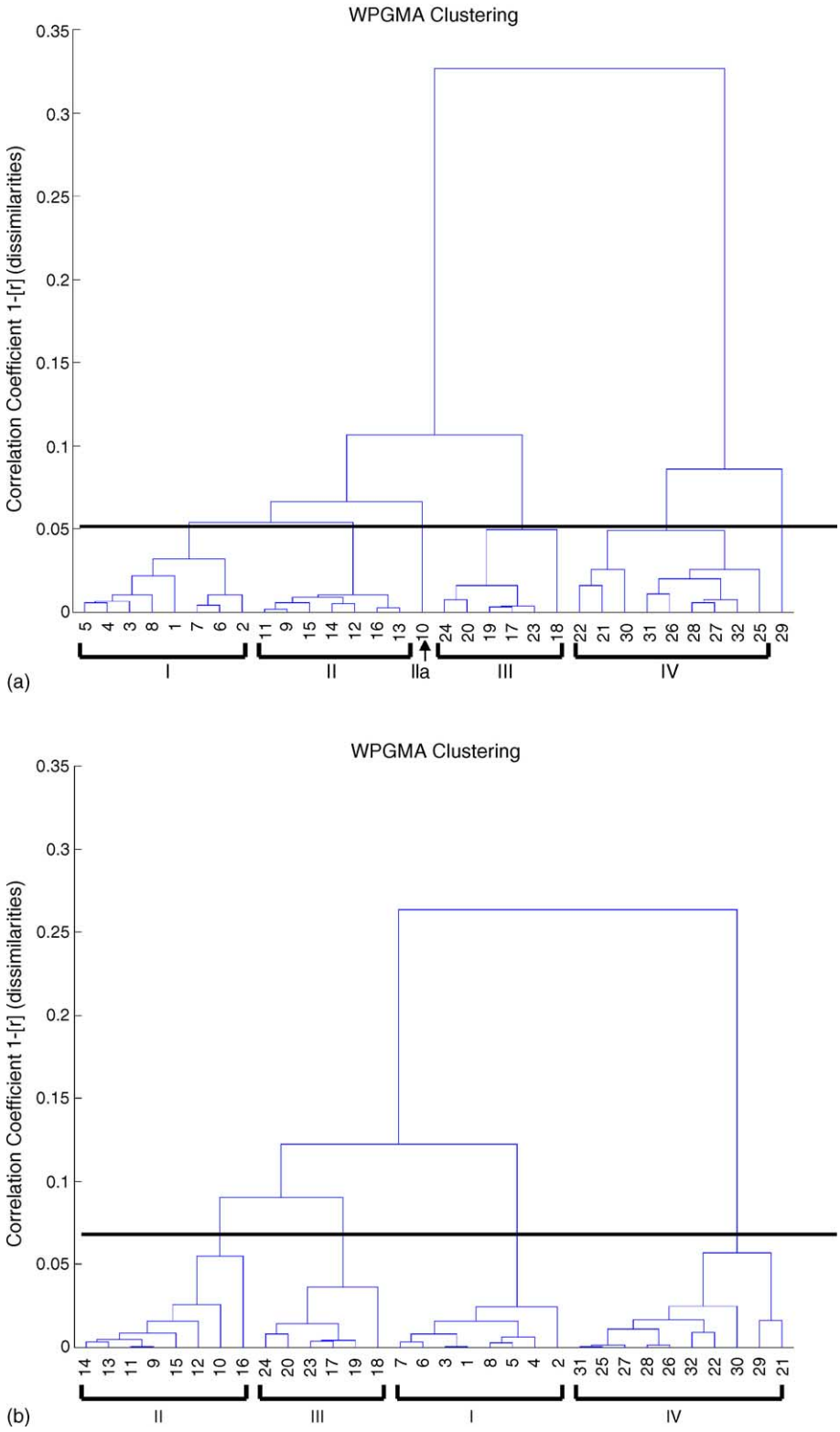

Fig. 1. Dendrogram of 32 chromatographic systems resulting from the hierarchical weighted-average-linkage technique on τ of (a) 68 substances, and (b) 15 substances. The abscissa shows the system numbers and groups of similar systems (roman numbers). The system numbers represent (1) $ZE_{2.5}$, (2) $ZB_{2.5}$, (3) $XMS_{2.5}$, (4) $XRP_{2.5}$, (5) $YC4_{2.5}$, (6) $WSS_{2.5}$, (7) $YC18_{2.5}$, (8) $WXP_{2.5}$, (9) $ZE_{4.8}$, (10) $ZB_{4.8}$, (11) $XMS_{4.8}$, (12) $XRP_{4.8}$, (13) $YC4_{4.8}$, (14) $WSS_{4.8}$, (15) $YC18_{4.8}$, (16) $WXP_{4.8}$, (17) $ZE_{7.0}$, (18) $ZB_{7.0}$, (19) $XMS_{7.0}$, (20) $XRP_{7.0}$, (21) $YC4_{7.0}$, (22) $WSS_{7.0}$, (23) $YC18_{7.0}$, (24) $WXP_{7.0}$, (25) $ZE_{9.0}$, (26) $ZB_{9.0}$, (27) $XMS_{9.0}$, (28) $XRP_{9.0}$, (29) $YC4_{9.0}$, (30) $WSS_{9.0}$, (31) $YC18_{9.0}$, and (32) $WXP_{9.0}$.

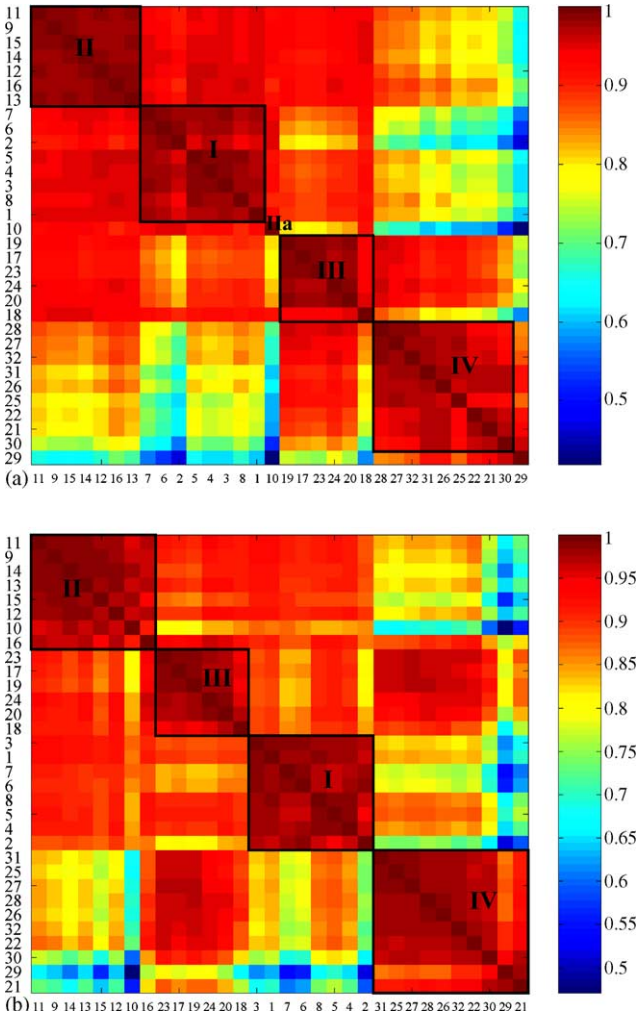

Fig. 2. Color map of correlation coefficients for the 32 systems ranked according to increasing dissimilarities in the weighted-average-linkage dendrogram using the sets of (a) 68 substances and (b) 15 substances. For the system numbers, see Fig. 1.

is very similar to that of Fig. 1a. However, the order of the groups is switched, which is due to rotational freedom of the branches in the dendrogram, because the algorithm consists of comparing dissimilarities between pairs of systems [26]. The same trend of orthogonal and interchangeable (similar) systems is present in the *r*-color map (Fig. 2b) as when interpreting the 68 substances data set. The systems are in general again clustered according to the buffer pH.

The same systems to achieve selectivity differences are selected from Fig. 1b: $ZB_{4.8}$, $WXP_{4.8}$, $ZB_{7.0}$, $ZB_{2.5}$, $WSS_{9.0}$, $YC4_{9.0}$, and $YC4_{7.0}$. Fig. 2b also indicates those, except for $WXP_{4.8}$. Moreover, Fig. 2b shows that $WSS_{2.5}$, $YC18_{2.5}$, $WSS_{4.8}$, and $YC18_{4.8}$ have important selectivity differences compared to $YC4_{9.0}$.

The most orthogonal pairs of systems based on the use of the second test set and their correlation coefficients are listed in Table 3b. For a subset of 10 systems from the set of 32, only five columns instead of eight are applied, being the YMC-Pack C4, the Zorbax Bonus-RP, the YMC-Pack Pro C18, the Waters SymmetryShield RP_{18} and the Waters XTerra MS C_{18}. Four of the five stationary phases are the same for both sets of substances, except for the Zorbax Extend-C18, which is replaced here with the Waters XTerra MS C_{18}. Considering the buffer pH to use, it is obvious that pH-values 2.5 or 4.8, and 9.0 are most different, whereas pH 7.0 adds much less information.

The pH turned out to be the most important factor in achieving selectivity differences, since the systems still remained largely grouped according to that parameter. Concerning the stationary phases, the Zorbax Bonus-RP, the YMC-Pack C4, the Waters SymmetryShield RP_{18}, the YMC-Pack Pro C18, the Zorbax Extend-C18, and the Waters XTerra MS C_{18} column are very interesting to obtain differences in selectivity, since they are involved in rather orthogonal systems at several pH-values for both sets of substances.

For the set of 68 substances, WSS and YC4 are most influenced by changing the pH, as the lowest correlation coefficients when only differing this parameter are encountered for these stationary phases (Fig. 2a). For the set of 15 substances, the same columns were found most important (Fig. 2b).

It was also considered interesting to determine which multiplets of systems are as different as possible, i.e. which triplet, quartet, quintet and sextet demonstrated the largest selectivity differences. This set then could be used in case a further reduction in systems would be required. The aim is thus to find, e.g. the triplet of systems (A, B, C) for which the correlation coefficients between all pairs (A–B, A–C and B–C) are as low as possible. One therefore could consider all conceivable combinations of three systems. However, here an alternative approach was used. A chemometric tool that enables to find such multiplets is the Kennard and Stone algorithm. It is a uniform mapping algorithm allowing to select a subset of systems that are both uniformly distributed in the experimental data space and as far as possible from each other [24,27]. The multiplets should demonstrate large selectivity changes, and therefore they should be chosen from dissimilar locations in the retention data space. The algorithm is based on maximizing the minimal (squared [27]) Euclidean distance between each of the earlier selected objects and all the others. Thereby, it can be executed starting from the object that is situated either closest or furthest from the mean, and each consecutively added object is at a maximal distance of those already included [24]. As a consequence, the Kennard and Stone algorithm might enable to select an orthogonal subset, when starting with the object furthest from the mean. Since the algorithm selects the systems one-by-one according to decreasing distances, a given subset can easily be extended or reduced.

To obtain the most dissimilar multiplets, the autoscaled retention matrix was submitted to the algorithm, starting furthest from the mean. For screening purposes, for the set of 68 substances, systems $YC4_{9.0}$, $ZB_{4.8}$ and $ZE_{7.0}$, respectively, were selected as the most diverse triplet. This selection turned out to be optimal as in general lower correlation coefficients were obtained for all three possible pairs

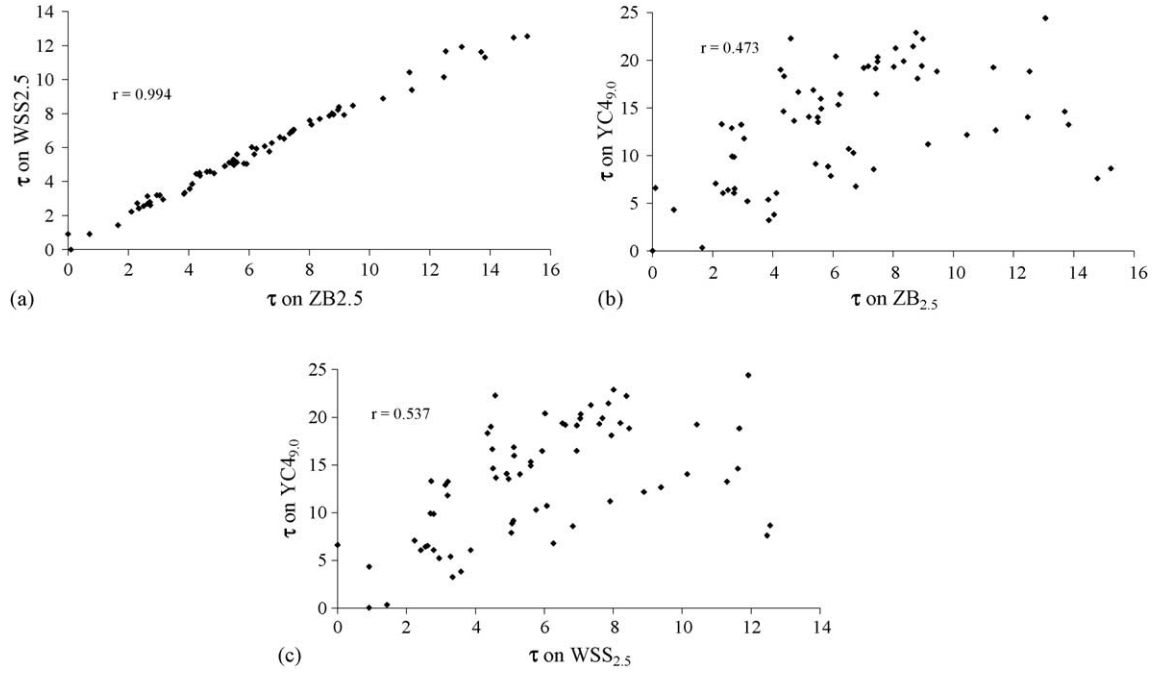

Fig. 3. Normalized retention times of the 68 substances on (a) $WSS_{2.5}$ vs. $ZB_{2.5}$, (b) $YC4_{9.0}$ vs. $ZB_{2.5}$, and (c) $YC4_{9.0}$ vs. $WSS_{2.5}$.

of systems (r($YC4_{9.0}$, $ZB_{4.8}$) = 0.418; r($YC4_{9.0}$, $ZE_{7.0}$) = 0.739; r($ZB_{4.8}$, $ZE_{7.0}$) = 0.794) than if the most orthogonal pair $YC4_{9.0}$ and $ZB_{4.8}$ would be compared to, e.g. $ZB_{2.5}$ (r($YC4_{9.0}$, $ZB_{4.8}$) = 0.418; r($YC4_{9.0}$, $ZB_{2.5}$) = 0.473; but r($ZB_{4.8}$, $ZB_{2.5}$) = 0.962) or $WSS_{9.0}$ (r($YC4_{9.0}$, $ZB_{4.8}$) = 0.418; r($ZB_{4.8}$, $WSS_{9.0}$) = 0.521; but r($YC4_{9.0}$, $WSS_{9.0}$) = 0.964) (Fig. 2a), i.e. systems that give rise to the other lowest r-values in Table 4a. To extend the multiplet further, $WXP_{2.5}$, $ZB_{9.0}$ and $ZE_{4.8}$, respectively, would consecutively be added.

One could also suggest to reduce the number of different columns involved, and for instance select the triplet with $YC4_{9.0}$, $ZB_{4.8}$ and $ZB_{9.0}$. However, it has to be said that this change would be somewhat at the expense of

Table 3
Correlation coefficients of the most orthogonal pairs of screening systems from (a) the set of 68 substances and (b) the set of 15 substances

(a)

Pair of systems	r (τ)	Description of the systems
$YC4_{9.0}$–$ZB_{4.8}$	0.418	YMC-Pack C4, pH 9.0–Zorbax Bonus-RP, pH 4.8
$YC4_{9.0}$–$ZB_{2.5}$	0.473	YMC-Pack C4, pH 9.0–Zorbax Bonus-RP, pH 2.5
$WSS_{9.0}$–$ZB_{4.8}$	0.521	Waters SymmetryShield RP_{18}, pH 9.0–Zorbax Bonus-RP, pH 4.8
$YC4_{9.0}$–$WSS_{2.5}$	0.537	YMC-Pack C4, pH 9.0–Waters SymmetryShield RP_{18}, pH 2.5
$YC4_{9.0}$–$YC18_{2.5}$	0.570	YMC-Pack C4, pH 9.0–YMC-Pack Pro C18, pH 2.5
$WSS_{9.0}$–$ZB_{2.5}$	0.573	Waters SymmetryShield RP_{18}, pH 9.0–Zorbax Bonus-RP, pH 2.5
$YC4_{9.0}$–$ZB_{7.0}$	0.584	YMC-Pack C4, pH 9.0–Zorbax Bonus-RP, pH 7.0
$ZB_{4.8}$–$YC4_{7.0}$	0.586	Zorbax Bonus-RP, pH 4.8–YMC-Pack C4, pH 7.0
$ZB_{4.8}$–$WSS_{7.0}$	0.597	Zorbax Bonus-RP, pH 4.8–Waters SymmetryShield RP_{18}, pH 7.0
$ZB_{4.8}$–$ZE_{9.0}$	0.597	Zorbax Bonus-RP, pH 4.8–Zorbax Extend-C18, pH 9.0

(b)

Pair of systems	r (τ)	Description of the systems
$YC4_{9.0}$–$ZB_{4.8}$	0.471	YMC-Pack C4, pH 9.0–Zorbax Bonus-RP, pH 4.8
$YC4_{9.0}$–$ZB_{2.5}$	0.517	YMC-Pack C4, pH 9.0–Zorbax Bonus-RP, pH 2.5
$YC4_{9.0}$–$YC18_{2.5}$	0.545	YMC-Pack C4, pH 9.0–YMC-Pack Pro C18, pH 2.5
$ZB_{4.8}$–$YC4_{7.0}$	0.558	Zorbax Bonus-RP, pH 4.8–YMC-Pack C4, pH 7.0
$YC4_{9.0}$–$WSS_{2.5}$	0.559	YMC-Pack C4, pH 9.0–Waters SymmetryShield RP_{18}, pH 2.5
$YC4_{9.0}$–$YC18_{4.8}$	0.566	YMC-Pack C4, pH 9.0–YMC-Pack Pro C18, pH 4.8
$WSS_{9.0}$–$ZB_{4.8}$	0.601	Waters SymmetryShield RP_{18}, pH 9.0–Zorbax Bonus-RP, pH 4.8
$YC4_{9.0}$–$WSS_{4.8}$	0.606	YMC-Pack C4, pH 9.0–Waters SymmetryShield RP_{18}, pH 4.8
$YC4_{7.0}$–$YC18_{2.5}$	0.613	YMC-Pack C4, pH 7.0–YMC-Pack Pro C18, pH 2.5
$YC4_{9.0}$–$XMS_{2.5}$	0.617	YMC-Pack C4, pH 9.0–Waters XTerra MS C_{18}, pH 2.5

Table 4
Description of 13 rather orthogonal screening systems, selected using both data sets

Columns at pH 2.5
Zorbax Bonus-RP ($ZB_{2.5}$)
Waters XTerra MS C_{18} ($XMS_{2.5}$)
Waters SymmetryShield RP_{18} ($WSS_{2.5}$)
YMC-Pack Pro C18 ($YC18_{2.5}$)
Columns at pH 4.8
Zorbax Bonus-RP ($ZB_{4.8}$)
Waters SymmetryShield RP_{18} ($WSS_{4.8}$)
YMC-Pack Pro C18 ($YC18_{4.8}$)
Columns at pH 7.0
Zorbax Bonus-RP ($ZB_{7.0}$)
YMC-Pack C4 ($YC4_{7.0}$)
Waters SymmetryShield RP_{18} ($WSS_{7.0}$)
Columns at pH 9.0
Zorbax Extend-C18 ($ZE_{9.0}$)
YMC-Pack C4 ($YC4_{9.0}$)
Waters SymmetryShield RP_{18} ($WSS_{9.0}$)

selectivity differences. For the set of 15 substances the optimum triplet for screening would consist of $YC4_{9.0}$, $ZB_{4.8}$ and $XMS_{7.0}$, the quartet would be obtained by adding $ZB_{2.5}$, the quintet by $WXP_{4.8}$ and the sextet by $YC4_{2.5}$. Again this selection is optimal, since, e.g. for the triplet, in general lower correlation coefficients are obtained comparing all three possible pairs of the systems $YC4_{9.0}$, $ZB_{4.8}$ and $XMS_{7.0}$ ($r(YC4_{9.0}, ZB_{4.8}) = 0.471$; $r(YC4_{9.0}, XMS_{7.0}) = 0.804$; $r(ZB_{4.8}, XMS_{7.0}) = 0.798$) than if extending the most orthogonal pair $YC4_{9.0}$, $ZB_{4.8}$ with either $ZB_{2.5}$ ($r(YC4_{9.0}, ZB_{4.8}) = 0.471$; $r(YC4_{9.0}, ZB_{2.5}) = 0.517$; but $r(ZB_{4.8}, ZB_{2.5}) = 0.863$) or $YC18_{2.5}$ ($r(YC4_{9.0}, ZB_{4.8}) = 0.471$; $r(YC4_{9.0}, YC18_{2.5}) = 0.545$; but $r(ZB_{4.8}, YC18_{2.5}) = 0.874$) (Fig. 2b), which are both systems that lead to the other lowest *r*-values in Table 4b.

In method development, the triplet for the set of 68 substances would, respectively, contain $YC4_{7.0}$, $ZB_{4.8}$ and $YC18_{7.0}$, the quartet will additionally consist of $WXP_{2.5}$, the quintet will be obtained by increasing the formerly selected set of systems with $ZE_{4.8}$ and the sextet if also $ZE_{9.0}$ would be added; for the set of 15 compounds, the triplet would, respectively, consist of $YC4_{7.0}$, $ZB_{4.8}$ and $YC4_{2.5}$, the quartet would be obtained by increasing the system set with $ZB_{7.0}$, the quintet by additionally selecting $ZE_{9.0}$ and the sextet by $WXP_{4.8}$.

3.1.1. A subset of orthogonal systems to be applied in early pharmaceutical development

When the impurity profile of a new drug has to be determined, highly selective separation methods are needed to make sure that all potentially occurring related compounds can be separated from the main substance and from each other. The major issue at this stage of development is that the number of potentially occurring related compounds is not yet known. Highly orthogonal chromatographic systems applied for this purpose increase the probability for success to determine the impurity profile completely [3]. From the current set of screening systems, we wanted to select a subset of only the most orthogonal ones. The results from both sets of test compounds allow defining a subset of systems with the broadest range of selectivities. Merging those of Table 3 leads to a reduction in the number of systems from 32 to only 13, a summary of which is given in Table 4. The 13 systems consist of six columns instead of eight (YMC-Pack C4, Zorbax Bonus-RP, Waters SymmetryShield RP_{18}, YMC-Pack Pro C18, Zorbax Extend-C18 and Waters XTerra MS C_{18}) that are to be applied at the specified pH conditions. Only three stationary phases have to be used at each pH, except at pH 2.5, where four columns are to be tested.

The new subset allows evaluating all pairs of specified systems, but stricto senso only the two most orthogonal ones might be sufficient to determine an impurity profile. The most important pairs of systems regarding selectivity differences are obtained by comparing systems at pH 9.0 with those at pH 2.5 or 4.8. Although at pH 7.0 also three systems are indicated, they are involved in fewer pairs of orthogonal ones.

In some cases it may be needed to replace systems from the subset for similar ones, e.g. to further improve the separation obtained with a specific system. In Section 3.2, the Derringer's desirability functions approach [14–18] is applied to rank the systems according to overall separation performances. The best performing ones at each of the four pH-values can then be used as alternative similar systems.

3.1.2. A subset of orthogonal systems to be applied in late pharmaceutical development

At this stage of drug development the impurity profile is typically known and the challenge is to develop final methods that are robust, reliable, and transferable from one site to the other. The stability of the applied chromatographic system is crucial for optimal performance. The screening is used here to find starting conditions for final method development. Columns that are not certified to be applied in a certain pH-region are excluded from use at such pH-values. Therefore, the initial set of 32 systems is reduced to 27 systems, since Zorbax Bonus-RP ($ZB_{9.0}$), YMC-Pack C4 ($YC4_{9.0}$), Waters SymmetryShield RP_{18} ($WSS_{9.0}$), YMC-Pack Pro C18 ($YC18_{9.0}$) and Waters XTerra Phenyl ($WXP_{9.0}$) are not certified at pH 9.0.

To select orthogonal subsets for final method development, the dendrograms and *r*-color maps were redrawn for the 27 systems. They were similar to Figs. 1 and 2, except that group IV contained fewer systems. Since in the above, the most orthogonal pairs were obtained comparing systems at pH 2.5 or 4.8 with those at pH 9.0, especially with $YC4_{9.0}$ and $WSS_{9.0}$, the elimination of five systems at pH 9.0 (among which $YC4_{9.0}$ and $WSS_{9.0}$) decreased the range of *r*-values. This means that in the new correlation coefficient color maps a new color scale is used compared to the old maps (Fig. 2), even when the *r*-values of a given pair remained the same. For the reduced set of systems, $ZB_{2.5}$, $WSS_{2.5}$, $YC18_{2.5}$, $ZB_{4.8}$,

Table 5
Description of 12 rather orthogonal method development systems, selected using both data sets

Columns at pH 2.5
Zorbax Bonus-RP ($ZB_{2.5}$)
Waters XTerra MS C_{18} ($XMS_{2.5}$)
Waters SymmetryShield RP_{18} ($WSS_{2.5}$)
YMC-Pack Pro C18 ($YC18_{2.5}$)
Columns at pH 4.8
Zorbax Bonus-RP ($ZB_{4.8}$)
Waters SymmetryShield RP_{18} ($WSS_{4.8}$)
YMC-Pack Pro C18 ($YC18_{4.8}$)
Columns at pH 7.0
YMC-Pack C4 ($YC4_{7.0}$)
Waters SymmetryShield RP_{18} ($WSS_{7.0}$)
Columns at pH 9.0
Zorbax Extend-C18 ($ZE_{9.0}$)
Waters XTerra MS C_{18} ($XMS_{9.0}$)
Waters XTerra RP_{18} ($XRP_{9.0}$)

$YC4_{7.0}$, $WSS_{7.0}$, $ZE_{9.0}$, $XMS_{9.0}$ and $XRP_{9.0}$ lead to considerable selectivity differences for the set of 68 test compounds. For the set of 15 substances, $ZE_{2.5}$, $ZB_{2.5}$, $XMS_{2.5}$, $WSS_{2.5}$, $YC18_{2.5}$, $ZB_{4.8}$, $WSS_{4.8}$, $YC18_{4.8}$ and $YC4_{7.0}$ were found to be important. Merging both subsets of systems leads to a set of 13. Although the elimination of the five systems did not change the correlation coefficients between the remaining ones, $YC4_{7.0}$ and $WSS_{7.0}$ become more frequently involved in the selection of rather orthogonal systems. As a result, pH 7.0 becomes relatively more important in the late-development system set than in the early-development system set. At pH 2.5, five columns are included. For practical reasons, it was decided to select at the most four systems at a given pH. Because $ZE_{2.5}$ and $XMS_{2.5}$ are similar ($r = 0.999$) and $XMS_{2.5}$ was also already involved in the screening subset of Table 4, it was selected as fourth system at pH 2.5 (Table 5). Application of the 12 systems covers the selectivity differences, observed for both sets of substances.

3.2. Taking into account the separation performance

When a pair of systems is rather orthogonal, it means that the retention is based on different mechanisms, in other words the systems complement each other in the information they provide about the substances and have different selectivities. However, this does not imply that individually they exhibit a good overall separation performance. It was considered important to include systems with good overall separation performance in our final subsets. Five parameters were used to describe the separation performance of a system: the minimal and maximal normalized retention time (τ_{min} and τ_{max}), the minimal and maximal selectivity (α_{min} and α_{max}), and the median selectivity (α_{med}). The normalized retention times reflect appropriate retention, taking into account both sample throughput (τ_{min}) and analysis time (τ_{max}). All selectivities between successive peaks were calculated. The selectivity responses were included to characterize the separation power of the systems.

To interpret these five responses simultaneously, a multicriteria decision-making method is applied. Derringer's desirability functions [14–18] were used to rank the 32 systems. A linear desirability function was defined for the five responses and a one-sided transformation performed (Fig. 4). The obtained *d*-values range between 0 for a completely undesirable response to 1 for a fully desired one [17]. All transformations were defined following some rules, so that none of the transformed values becomes either zero or one. To do so, the measured interval for a response was extended to [lowest value −10% of interval range; highest value +10% of interval range], and these extremes were assigned a d-value of either 0 or 1 [5]. After calculating the desirability (*d*) values, the geometric mean (*D*) is determined, expressing the overall separation performance of a system. The system for which the combination of the different responses is globally best is represented by the highest *D*-value [14].

In the context of this study, the approach was applied using the set of 15 substances. They consist of related structures and therefore are expected to reflect the impurity profiles of interest better than the larger and more diverse set of 68 solutes. In Fig. 4a, the desirability function is visualized for τ_{min}. A low τ-value means that substances are hardly or not retained. The minimum normalized retention time is preferred to be as high as possible, and low τ_{min}-values get low desirability values. Large minimal normalized retention times can imply that all substances elute slow, which causes long analysis times. To take this into account, a response τ_{max} was also considered, but it is aimed at being as small as possible (Fig. 4b).

For α_{min}, the desirability function is defined linearly increasing, i.e. the better the worst separated peaks are separated, the more desired (Fig. 4c). Higher values of α_{med} imply that peaks "on average" will be better separated, and thus an increasing desirability function is constructed (Fig. 4d). For α_{max}, a linearly decreasing function was defined (Fig. 4e), since it is not desired that peaks be separated too much (at the expense of analysis time). Table 6 gives an overview of the individual *d*- and the resulting *D*-values, and displays the systems sorted by decreasing desirability *D*. On several systems, co-elution occurred at least once (i.e. $d(\alpha_{min})$ is 0.1, see Fig. 4c). Therefore, the *D*-value was also calculated without taking into account this response (called *D*(without α_{min})). The systems that should have the best separation performances are situated in the upper part of Table 6. The systems ranked highest in Table 6 for *D*(without α_{min}) also belong to the upper part of the ordering according to *D*. Only four systems ranked high for *D* appear low for *D*(without α_{min}), and vice versa.

The results for *D* are first discussed. It can be concluded that $XMS_{9.0}$, $YC4_{7.0}$, $XMS_{7.0}$, $YC18_{2.5}$, $YC18_{7.0}$, $WXP_{4.8}$ and $XMS_{2.5}$ will have the best chances to determine an impurity profile of a new drug in an acceptable analysis

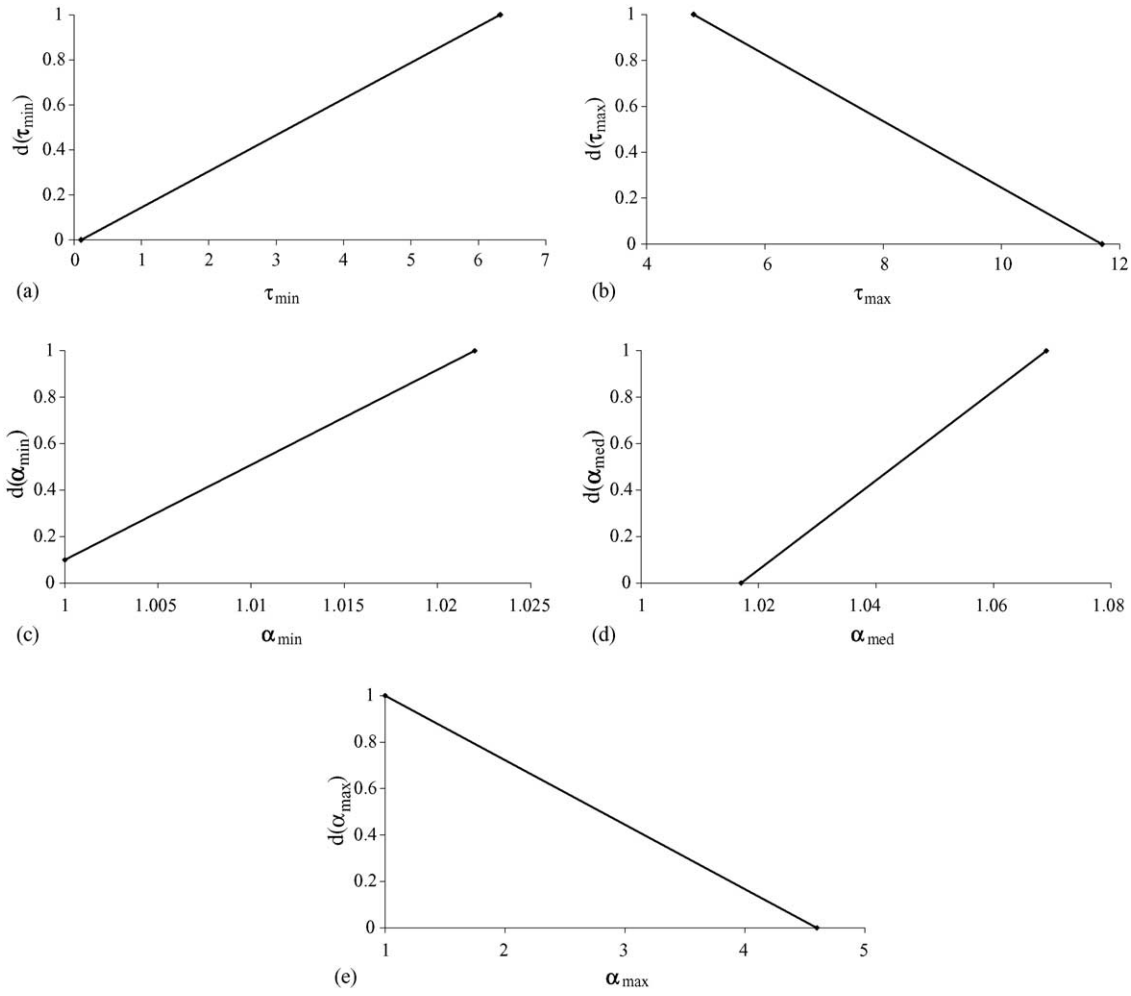

Fig. 4. Derringer's desirability functions for (a) τ_{min}, (b) τ_{max}, (c) α_{min}, (d) α_{med}, and (e) α_{max} to rank the 32 systems according to their overall separation performances, using the set of 15 substances.

time. As a consequence, these systems are potentially interesting to be used for development of final separation methods. It has to be noticed that in gradient-elution chromatography, the analysis time is mainly determined by the gradient program. However, if in further method development, conditions would be changed to isocratic ones, then a smaller τ_{max}- and α_{max}-value could lead to a shorter analysis time while still maintaining appropriate separation of the substances.

It was evaluated whether the best-performing systems could complement or be an alternative for those in Tables 4 and 5. As can be observed, most of them are already included and thus should perform well regarding separation performance. Exceptions are $ZB_{2.5}$, $ZB_{4.8}$, $WSS_{4.8}$, $YC18_{4.8}$, $ZB_{7.0}$ and $YC4_{9.0}$ in Table 4 and $ZB_{2.5}$, $ZB_{4.8}$, $WSS_{4.8}$ and $YC18_{4.8}$ in Table 5. The Derringer desirability-function proved to be useful for ranking chromatographic systems according to their separation performance. From our own experience it was, for instance, known that $WXP_{4.8}$, in general, shows a good performance. This was indeed confirmed from our Derringer's approach, both when *D* and *D*(without α_{min}) were considered. Although this system initially was not selected when evaluating orthogonality, it is certainly worthwhile to extend the subset with it. It was concluded that it is best to increase the orthogonal set with those exhibiting a good separation performance to come to four columns tested per pH. Using the *D*-ranking, Table 4 is thus extended with $WXP_{4.8}$, $XMS_{7.0}$ and $XMS_{9.0}$, and Table 5 with $WXP_{4.8}$, $XMS_{7.0}$ and $YC18_{7.0}$. Applying Derringer's desirability functions, it was also shown that $XMS_{2.5}$ is performing better than $ZE_{2.5}$, which justifies the selection of $XMS_{2.5}$ in Table 5. The final subsets of screening and method development systems are summarized in Table 7. If the *D*(without α_{min})-ranking was followed, the same systems would be selected to increase Tables 4 and 5 with, except that $XRP_{9.0}$ would be added instead of $XMS_{9.0}$ in Table 4, and $WXP_{7.0}$ instead of $YC18_{7.0}$ in Table 5.

Both for the optimum multiplets, and for the subsets in Tables 4 and 5, it could be considered to exchange a member by a similar one exhibiting better overall separation properties. However, each change to improve the latter performances will be at the expense of selectivity differences.

Table 6
Systems ordered according to a decreasing D- and D(without α_{min})-value as a result of the Derringer's desirability functions for five (τ_{min}, τ_{max}, α_{min}, α_{med} and α_{max}) and four parameters (τ_{min}, τ_{max}, α_{med} and α_{max})

System	$d(\tau_{min})$	$d(\tau_{max})$	$d(\alpha_{min})$	$d(\alpha_{med})$	$d(\alpha_{max})$	D
$XMS_{9.0}$	0.640	0.255	0.918	0.769	0.963	0.644
$YC4_{7.0}$	0.640	0.450	0.427	0.615	0.963	0.592
$XMS_{7.0}$	0.395	0.355	0.673	0.923	0.825	0.591
$YC18_{2.5}$	0.309	0.799	0.632	0.442	0.906	0.574
$YC18_{7.0}$	0.518	0.252	0.632	0.442	0.871	0.502
$WXP_{4.8}$	0.290	0.628	0.345	0.577	0.745	0.486
$XMS_{2.5}$	0.315	0.765	0.468	0.250	0.904	0.480
$XRP_{7.0}$	0.339	0.411	0.305	0.577	0.730	0.447
$XRP_{9.0}$	0.595	0.377	0.100	0.788	0.948	0.441
$WSS_{2.5}$	0.335	0.707	0.223	0.327	0.909	0.436
$ZE_{9.0}$	0.693	0.083	0.468	0.577	0.951	0.431
$WXP_{9.0}$	0.624	0.505	0.100	0.481	0.959	0.429
$WSS_{9.0}$	0.788	0.194	0.100	0.904	0.963	0.422
$WSS_{7.0}$	0.739	0.367	0.100	0.500	0.967	0.420
$ZE_{2.5}$	0.335	0.732	0.100	0.519	0.906	0.410
$ZB_{9.0}$	0.700	0.259	0.100	0.654	0.955	0.408
$YC4_{9.0}$	0.917	0.258	0.100	0.442	0.973	0.399
$ZB_{7.0}$	0.270	0.497	0.305	0.288	0.733	0.387
$YC18_{9.0}$	0.719	0.174	0.100	0.712	0.959	0.386
$WXP_{7.0}$	0.372	0.616	0.100	0.423	0.815	0.380
$WXP_{2.5}$	0.284	0.863	0.100	0.346	0.886	0.376
$YC4_{4.8}$	0.260	0.638	0.100	0.519	0.718	0.362
$YC18_{4.8}$	0.279	0.331	0.100	0.885	0.716	0.358
$ZB_{2.5}$	0.173	0.917	0.100	0.423	0.869	0.357
$WSS_{4.8}$	0.225	0.548	0.100	0.519	0.639	0.333
$XMS_{4.8}$	0.244	0.561	0.100	0.404	0.691	0.328
$ZE_{7.0}$	0.442	0.153	0.100	0.673	0.830	0.328
$ZE_{4.8}$	0.259	0.406	0.100	0.481	0.681	0.322
$XRP_{4.8}$	0.180	0.583	0.100	0.442	0.496	0.297
$YC4_{2.5}$	0.262	0.861	0.100	0.115	0.867	0.295
$XRP_{2.5}$	0.262	0.789	0.100	0.077	0.891	0.269
$ZB_{4.8}$	0.083	0.552	0.182	0.673	0.089	0.219

D(without α_{min})	System
0.643	$YC4_{7.0}$
0.640	$XRP_{9.0}$
0.617	$WXP_{9.0}$
0.604	$WSS_{9.0}$
0.602	$WSS_{7.0}$
0.590	$XMS_{9.0}$
0.583	$ZE_{2.5}$
0.580	$ZB_{9.0}$
0.572	$XMS_{7.0}$
0.565	$YC4_{9.0}$
0.561	$YC18_{2.5}$
0.541	$YC18_{9.0}$
0.530	$WXP_{7.0}$
0.529	$WXP_{4.8}$
0.523	$WXP_{2.5}$
0.515	$WSS_{2.5}$
0.499	$YC4_{4.8}$
0.492	$XRP_{7.0}$
0.492	$YC18_{4.8}$
0.491	$ZB_{2.5}$
0.483	$XMS_{2.5}$
0.474	$YC18_{7.0}$
0.450	$WSS_{4.8}$
0.442	$XMS_{4.8}$
0.441	$ZE_{7.0}$
0.431	$ZE_{4.8}$
0.422	$ZE_{9.0}$
0.410	$ZB_{7.0}$
0.389	$XRP_{4.8}$
0.387	$YC4_{2.5}$
0.345	$XRP_{2.5}$
0.229	$ZB_{4.8}$

Table 7
Finally selected subsets of systems to be applied for (a) screening, and (b) method development

(a) Screening
Columns at pH 2.5
Zorbax Bonus-RP ($ZB_{2.5}$)
Waters XTerra MS C_{18} ($XMS_{2.5}$)
Waters SymmetryShield RP_{18} ($WSS_{2.5}$)
YMC-Pack Pro C18 ($YC18_{2.5}$)
Columns at pH 4.8
Zorbax Bonus-RP ($ZB_{4.8}$)
Waters SymmetryShield RP_{18} ($WSS_{4.8}$)
YMC-Pack Pro C18 ($YC18_{4.8}$)
Waters XTerra Phenyl ($WXP_{4.8}$)
Columns at pH 7.0
Zorbax Bonus-RP ($ZB_{7.0}$)
Waters XTerra MS C_{18} ($XMS_{7.0}$)
YMC-Pack C4 ($YC4_{7.0}$)
Waters SymmetryShield RP_{18} ($WSS_{7.0}$)
Columns at pH 9.0
Zorbax Extend-C18 ($ZE_{9.0}$)
Waters XTerra MS C_{18} ($XMS_{9.0}$)
YMC-Pack C4 ($YC4_{9.0}$)
Waters SymmetryShield RP_{18} ($WSS_{9.0}$)
(b) Method development
Columns at pH 2.5
Zorbax Bonus-RP ($ZB_{2.5}$)
Waters XTerra MS C_{18} ($XMS_{2.5}$)
Waters SymmetryShield RP_{18} ($WSS_{2.5}$)
YMC-Pack Pro C18 ($YC18_{2.5}$)
Columns at pH 4.8
Zorbax Bonus-RP ($ZB_{4.8}$)
Waters SymmetryShield RP_{18} ($WSS_{4.8}$)
YMC-Pack Pro C18 ($YC18_{4.8}$)
Waters XTerra Phenyl ($WXP_{4.8}$)
Columns at pH 7.0
Waters XTerra MS C_{18} ($XMS_{7.0}$)
YMC-Pack C4 ($YC4_{7.0}$)
Waters SymmetryShield RP_{18} ($WSS_{7.0}$)
YMC-Pack Pro C18 ($YC18_{7.0}$)
Columns at pH 9.0
Zorbax Extend-C18 ($ZE_{9.0}$)
Waters XTerra MS C_{18} ($XMS_{9.0}$)
Waters XTerra RP_{18} ($XRP_{9.0}$)

3.3. Conclusion

Based on the weighted-average-linkage dendrogram and the *r*-color map constructed from applying normalized retention times, orthogonal subsets of systems for both a generic set of 68 test compounds and one containing 15 more related substances were selected. Both sets of test components led to similar results and the number of columns selected for the reduced subsets decreased, while only 12 or 13 systems from the 32 had to be used. A distinction was made between systems to be applied for screening purposes in early pharmaceutical development and those to be used for the selection of starting conditions for final methods in late pharmaceutical development. The subsets are, in general, comparable, but include a number of different systems at pH 9.0. The overall separation performances of the systems, evaluated using Derringer's desirability functions, were also considered during selection of the final subset of systems and as a consequence, the orthogonal subset was extended with some well-performing ones.

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References

[1] The European Agency for the Evaluation of Medicinal Products—Evaluation of Medicines for Human Use, ICH Topic Q3A(R)—Impurities Testing Guideline: Impurities in New Drug Substances (ICH Step 5), Note for guidance on Impurities Testing: Impurities in New Drug Substances (Revision), February 2002, http://www.emea.eu.int/pdfs/human/ich/273799en.pdf.
[2] W. Steuer, I. Grant, F. Erni, J. Chromatogr. 507 (1990) 125.
[3] E. Van Gyseghem, S. Van Hemelryck, M. Daszykowski, F. Questier, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 988 (2003) 77.
[4] E. Van Gyseghem, I. Crosiers, S. Gourvénec, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 1026 (2004) 117.
[5] E. Van Gyseghem, M. Jimidar, R. Sneyers, D. Redlich, E. Verhoeven, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 1042 (2004) 69.
[6] C.J. Venkatramani, J. Xu, J.B. Phillips, Anal. Chem. 68 (1996) 1486.
[7] G. Xue, A.D. Bendick, R. Chen, S.S. Sekulic, J. Chromatogr. A 1050 (2) (2004) 159.
[8] B.G.M. Vandeginste, D.L. Massart, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics: Part B, Elsevier, Amsterdam, 1998.
[9] A. Smolinski, B. Walczak, J.W. Einax, Chemom. Intell. Lab. Syst. 64 (2002) 45.
[10] D.L. Massart, L. Kaufman, The Interpretation of Analytical Chemical Data by the Use of Cluster Analysis, Wiley, New York, 1983.
[11] W. Vogt, D. Nagel, H. Sator, Cluster Analysis in Clinical Chemistry: A Model, Wiley, Chichester, 1987.
[12] L. Kaufman, P.J. Rousseeuw, Finding Groups in Data—An Introduction to Cluster Analysis, Wiley, New York, 1990.
[13] H.C. Romesburg, Cluster Analysis for Researchers, Lifetime Learning Publications, Belmont, 1984.
[14] M. Jimidar, B. Bourguignon, D.L. Massart, J. Chromatogr. A 740 (1996) 109.
[15] B. Bourguignon, D.L. Massart, J. Chromatogr. 586 (1991) 11.
[16] E.C. Harrington, Ind. Qual. Control 21 (1965) 494.
[17] G. Derringer, R. Suich, J. Qual. Technol. 12 (1980) 214.
[18] G.C. Derringer, Qual. Progr. (June) (1994) 51.
[19] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdot (Eds.), Clarke's isolation and identification of drugs in pharmaceuticals, body fluids, and post-mortem material, second ed., The Pharmaceutical Press, London, 1986.
[20] UV and IR Spectra of Some Important Drugs, fifth supplement, Editio Cantor Verlag, Aulendorf, 1998.
[21] Agilent Technologies Consumables and Accessories Catalog 2000/2001.

[22] Waters Chromatography Columns and Supplies Catalog 2003–2004.
[23] YMC website, http://www.ymc.co.jp/hplc/index_e.html.
[24] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics: Part A, Elsevier, Amsterdam, 1997.
[25] P. Sneath, R. Sokal, Numerical Taxonomy. The Principles and Practice of Numerical Classification, Freeman, San Francisco, 1973.
[26] M. Daszykowski, B. Walczak, D.L. Massart, J. Chem. Inf. Comput. Sci. 42 (2002) 500.
[27] M. Daszykowski, B. Walczak, D.L. Massart, Anal. Chim. Acta 468 (2002) 91.