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Determining orthogonal and similar chromatographic systems from the injection of mixtures in liquid chromatography–diode array detection and the interpretation of correlation coefficients color maps

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Abstract

Generic orthogonal chromatographic systems might be helpful tools as potential starting points in the development of methods to separate impurities and the active substance in drugs with unknown impurity profiles. The orthogonality of 38 chromatographic systems was evaluated from weighted-average-linkage dendrograms and color maps, both based on the correlation coefficients between the retention factors on the different systems. On each chromatographic system, 68 drug substances were injected as mixtures of three or four components to increase the throughput. The (overlapping) peaks were identified and resolved with a peak purity algorithm, orthogonal projection approach (OPA). The visualization techniques applied allowed a simple evaluation of orthogonal and (groups of) similar systems.

Keywords: Orthogonal chromatographic systems; Weighted-average-linkage dendrogram; Correlation coefficients color map; Orthogonal projection approach; Method development

1. Introduction

Impurities in drug substances can cause undesired side effects and their amounts need to be limited. Therefore, it is important that they can be quantified and/or identified, as prescribed by ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) [1]. In the pharmaceutical industry, the Food and Drug Administration (FDA), for instance, also demands the development of methods to separate, identify and quantify them. In that context, it might be useful to create/select a set of orthogonal chromatographic systems. A system is defined as a given combination of stationary and mobile phase. Orthogonal systems have strongly different selectivities, because retention is caused by different mechanisms or based on different charges of the eluted substances. The application of a (new) drug-impurities mixture on such systems might reveal those that could be used as starting point for further method development. In reversed-phase chromatography, the stationary phases [2–5], the mobile phase characteristics, e.g. buffer pH [2] and organic modifier type [6,7], and the column temperature [8] can cause or improve orthogonality between systems.

The orthogonality of chromatographic systems can be determined in different ways. Neue et al. [9] used correlation coefficients and cluster analysis on relative retention data to obtain a classification of orthogonal and similar silica-based reversed-phase packings. Fields et al. [10] applied the correlation coefficient between the retention factor ratios with testosterone as reference substance. Neue et al. [11] also described the use of $s^2 = 1 - r^2$, with s^2 the selectivity parameter and r^2 the coefficient of determination, as a quantitative measure of selectivity difference.

In two-dimensional chromatographic systems, high informational similarity results in solute crowding. The lower the informational similarity (leading to minimal solute crowding), the more orthogonal the systems [12]. Orthogonality can be reached by application of the same technique in the two dimensions, e.g. LC × LC [12,13]. It can also be achieved between different separation techniques (not necessarily coupled), like, for instance,

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pressure-driven ones, e.g. high-performance liquid chromatography (HPLC) or supercritical-fluid chromatography (SFC), and electro-driven ones, e.g. capillary electrophoresis (CE), since their separation mechanisms are different [14].

In this study, orthogonality is evaluated comparing one-dimensional reversed-phase chromatographic systems [15]. A set of 68 drugs was selected to examine the orthogonality/similarity of the systems. The substances chosen differed in structure (functional groups, ring structures), molecular weight, pK_a, log P and pharmacological class, in order to be representative for a broad range of drug molecules and to reveal the generic orthogonality of systems. Initially, individual substances were injected [15]. This approach is time-consuming to investigate large numbers of systems. Therefore, the throughput was increased injecting mixtures of three or four components. However, this may result in peak overlap, which, in severe cases, causes problems to determine the elution times. As a consequence, a hyphenated technique (e.g. HPLC-DAD) delivering multidimensional data is needed to identify the mixture compounds, while peak purity techniques [16–19], e.g. orthogonal projection approach (OPA) [19], are to resolve occasional overlapping peaks. OPA is a multivariate, self-modeling chemometrical tool that enables determining whether a chromatographic peak is pure. In unknown mixtures, the number of substances and their spectrum also can be estimated. OPA first determines the number of components using a dissimilarity criterion [16,18,19]. In our situation, the number of substances is known. Secondly, OPA calculates the pure compound spectra, to identify the components, and the concentration profiles, to determine their elution time, applying a multivariate curve resolution alternating least squares (MCR-ALS) algorithm [19].

The goals of this study are two-fold: (a) evaluation of the use of OPA when mixtures were injected, and (b) evaluation of visual techniques to select orthogonal systems and orthogonal classes of similar systems. Earlier in Ref. [15], several chemometric methods to detect orthogonality already were examined. Pearson's correlation coefficient matrices, dendrograms from the hierarchical weighted-average-linkage clustering technique or weighted pair group method using arithmetic averages (WPGMA) method [15,16,20–22], and color maps from the ordering points to investigate the clustering structure (OPTICS) technique were considered. The OPTICS color map was found less appropriate to determine the orthogonality and similarity of chromatographic systems [15]. In the present paper, Pearson's correlation coefficient color maps are evaluated as an additional visualization method. These maps are constructed by replacing each correlation coefficient matrix element with a color. The systems in the color maps were ranked based on the WPGMA-dendrogram results.

Seven stationary phases creating nineteen chromatographic systems were evaluated by the injection of mixtures. The data set was augmented with nineteen systems examined injecting individual compounds.

2. Experimental

2.1. Drugs and reagents

The 68 substances and their stock-solution concentrations (prepared in 50:50% (v/v) organic modifier/Milli-Q water) are summarized in Table 1. The organic modifier was either acetonitrile or methanol, both Hypersolv for HPLC (BDH, Poole, England). The applied concentration of a substance depended on its absorbance at 254 nm.

Phosphoric acid solution min. 85% (Carlo Erba, Milan, Italy), anhydrous disodium tetraborate, boric acid, disodium hydrogenium phosphate dihydrate, sodium dihydrogenium phosphate monohydrate, sodium hydroxide pellets, all pro analysi (GR quality) (all from Merck, Darmstadt, Germany) also were used in the mobile phases.

2.2. Chromatographic conditions

The HPLC-instrument consisted of a Model 5000 Liquid Chromatograph pump (Varian, Palo Alto, California), a 20 μl loop, a CTO-10A column oven and an SPD-M10A diode array detector (both Shimadzu, Kyoto, Japan). Method creation and data treatment was done with the Class-M10A LC workstation software (Shimadzu). The column oven was kept at either 40 or 75 °C.

Seven stationary phases were used when mixtures were injected: (a) Chromolith Performance, RP-18e (100 mm × 4.6 mm i.d.) (Merck), a monolithic silica phase [23–25], (b) Zorbax Extend-C18, (150 mm × 4.6 mm i.d., 3.5 μm) (Agilent, Palo Alto, California), a bidentate bonded [4] and double-endcapped octadecylsilica phase, (c) ZirChrom-PS, (100 mm × 4.6 mm i.d., 3 μm) (ZirChrom Separations, Anoka, MN), a zirconia-based phase coated with polystyrene [26], (d) Platinum C18 100 Å Rocket, (53 mm × 7 mm i.d., 3 μm) (Alltech, Deerfield, IL), a base-deactivated octadecylsilica, (e) Platinum EPS C18 100 Å Rocket, (53 mm × 7 mm i.d., 3 μm) (Alltech), a base-deactivated octadecylsilica with extended polar selectivity (EPS), (f) Zorbax Eclipse XDB-C8, (150 mm × 4.6 mm i.d., 5 μm) (Agilent), a densely-bonded, double-endcapped C8-silica, and (g) Betasil Phenyl Hexyl, (100 mm × 4.6 mm i.d., 5 μm) (Thermo Hypersil Keystone, Cheshire, UK), a phenyl-hexyl-silica column [27]. The chromatographic systems (CS) created are shown in Table 2 (CS1-CS19). The nineteen systems evaluated from injection of individual compounds [15] are also included (CS20-CS38). They contain three additional stationary phases (CS31–CS36): (i) PLRP-S (150 mm × 4.6 mm i.d., 5 μm) (Polymer Laboratories, Shropshire, UK), a polystyrene-divinylbenzene copolymer-based phase [4], (ii) Luna CN (100 mm × 4.6 mm i.d., 3 μm) (Phenomenex, Torrance, CA), a silica with high cyanopropyl surface coverage, and (iii) ZirChrom-PBD, (100 mm × 4.6 mm i.d., 3 μm) (ZirChrom Separations), a zirconia-based phase coated with polybutadiene [26]. Gradient elution was used to limit the

Table 1
The 68 substances, their stock-solution concentrations and distributors

Substance (concentration in mg/l)	Distributed by
(±)-Camphor (5000)	Sigma–Aldrich (Steinheim, Germany)
1,1-Dimethylbiguanide hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
4-Benzylphenol (1000)	Aldrich (Milwaukee, WI)
5-Hydroxytryptamine hydrochloride (500)	Sigma–Aldrich (Steinheim, Germany)
5-Sulfosalicylic acid dihydrate (2000)	Merck (Darmstadt, Germany)
Acebutolol hydrochloride (1000)	Sigma (St. Louis, Missouri)
Amiodarone hydrochloride (5000)	Clin-Midy groupe Sanofi (Montpellier, France)
Antazoline hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
Betaxolol hydrochloride (1000)	Synthelabo (Paris, France) (gift)
Bupranolol hydrochloride (1000)	Schwarz Pharma (Monheim, Germany)
Caffeine (1000)	Fluka (Neu-Ulm, Switzerland)
Carbamazepine (1000)	Sigma–Aldrich (Steinheim, Germany)
Celiprolol (1000)	Rhône-Poulenc-Rorer (Madrid, Spain) (gift)
Chloropyramine hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
Cimetidine (10000)	Penn Chemicals (Pennsylvania, PA) (gift)
Cirazoline hydrochloride (400)	Research Biochemicals International (Natick, MA)
Cocaine hydrochloride (1000)	Bios Coutelier (Brussels, Belgium)
Codeine base (1000)	Bios Coutelier (Brussels, Belgium)
Desipramine hydrochloride (5000)	Sigma–Aldrich (Steinheim, Germany)
Diclofenac sodium (5000)	Sigma–Aldrich (Steinheim, Germany)
Digitoxigenine (500)	Fluka (Neu-Ulm, Switzerland)
Digitoxine (1000)	Mann Research Laboratories (New York, NY)
Dimetindene maleate (1000)	Novartis (Basel, Switzerland) (gift)
Diphenhydramine hydrochloride (5000)	Sigma–Aldrich (Steinheim, Germany)
Dopamine hydrochloride (2000)	Sigma–Aldrich (Steinheim, Germany)
Efedrine hydrochloride (2000)	Vel (Leuven, Belgium)
Famotidine (2000)	Sigma–Aldrich (Steinheim, Germany)
Fenfluramine hydrochloride (1000)	Technologie Servier (Orleans, France)
Fluphenazine dihydrochloride (USP grade) (2000)	Sigma–Aldrich (Steinheim, Germany)
Flurazepam (1000)	Dolorgiet Arzneimittel (Bonn, Germany)
Histamine dihydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
Ibuprofen (5000)	Sigma–Aldrich (Steinheim, Germany)
Isothipendyl hydrochloride (1000)	Novartis Pharma (Wehr, Austria) (gift)
Ketotifen fumarate (1000)	Sigma–Aldrich (Steinheim, Germany)
L-(+)-Ascorbic acid (1000)	Merck (Darmstadt, Germany)
Lidocaine hydrochloride (1000)	Bios Coutelier (Brussels, Belgium)
Lorazepam (1000)	MSD (Haarlem, The Netherlands)
Miconazol nitrate (1000)	Certa (Braine-l'Alleud, Belgium)
Morphine hydrochloride (2000)	Bios Coutelier (Brussels, Belgium)
Nadolol (1000)	Sigma–Aldrich (Steinheim, Germany)
Naphazoline hydrochloride (2000)	Sigma–Aldrich (Steinheim, Germany)
Nicardipine hydrochloride (1000)	UCB (Leuven, Belgium)
Nizatidine (2000)	Norgine (Marburg, Germany) (gift)
Oxeladin citrate (2000)	Sigma–Aldrich (Steinheim, Germany)
Oxprenolol hydrochloride (500)	Sigma–Aldrich (Steinheim, Germany)
Pentoxifylline (1000)	Sigma–Aldrich (Steinheim, Germany)
Phenol (1000)	Merck (Darmstadt, Germany)
Pindolol (1000)	Sigma–Aldrich (Steinheim, Germany)
Pizotifen (5000)	Novartis Pharma (Wehr, Austria) (gift)
Prazosin hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
Prenalterol hydrochloride (1000)	Ciba-Geigy (Basel, Switzerland)
Procaine hydrochloride (1000)	Merck (Darmstadt, Germany)
Promethazine hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
Propiomazine maleate (1000)	Sanofi (Paris, France) (gift)
Pyrilamine maleate (1000)	Sigma–Aldrich (Steinheim, Germany)
Ranitidine hydrochloride (2000)	Sigma (St. Louis, Missouri)
Resorcine (1000)	Merck (Darmstadt, Germany)
Sotalol (1000)	Merck (Darmstadt, Germany)
Strychnine base (1000)	Bios Coutelier (Brussels, Belgium)
Sulfapyridine (1000)	Bios Coutelier (Brussels, Belgium)
Terazosin hydrochloride (1000)	Sigma–Aldrich (Steinheim, Germany)
Terbutaline sulphate (1000)	Astra Draco (Lund, Sweden)
Tetrahydrozolin hydrochloride (4000)	U.S.P.C. (Rockville, MD)

Table 1 (*Continued*)

Substance (concentration in mg/l)	Distributed by
Thiothixene (USP grade) (2000)	Sigma–Aldrich (Steinheim, Germany)
Timolol maleate (1000)	Sigma–Aldrich (Steinheim, Germany)
Tolazoline hydrochloride (5000)	Sigma–Aldrich (Steinheim, Germany)
α-Lobeline hydrochloride (1500)	Carl Roth (Karlsrhue, Germany)
β-Estradiol (500)	Sigma–Aldrich (Steinheim, Germany)

analysis time and the gradients applied are described in Table 2.

When mixtures were injected both methanol and acetonitrile were used as organic modifier, except on Platinum C18 and Platinum EPS C18 (only acetonitrile). The ZirChrom-PS phase was investigated both at 40 and 75 °C, all other columns only at 40 °C, as the silica-basis cannot withstand temperatures above 60 °C. All columns were investigated at pH 3.0 and 6.8 with phosphate buffers of either 0.04 or 0.08 M (exception Platinum, only pH 3.0). ZirChrom-PS and Zorbax Extend-C18 were also examined at pH 10.0 (0.08 M borate buffer), as their design allows such high pH-values.

Buffer pH was measured on a daily-calibrated Orion 520A (Orion Research, Boston, MA) pH-meter. Buffers were filtered through a 0.2 μm membrane filter (Schleicher & Schuell, Dassel, Germany). Milli-Q water (Millipore purification system, Molsheim, France) is used in all buffers, stock solutions and samples.

Each drug mixture was prepared by equivolumetric mixing of the stock solutions. Afterwards, the 50:50% (v/v) modifier/water solvent was diluted to 10:90% (v/v)

Table 2
Description of the chromatographic systems (CS)

CS	Stationary phase	Mobile phase conditions and column temperature
1	Chromolith Performance	Methanol/0.08 M sodium phosphate buffer pH 3.0 from 10:90 to 75:25% (v/v) in 4 min; flow rate 2.0 ml/min; 40 °C
2	Chromolith Performance	Methanol/0.08 sodium phosphate buffer pH 6.8 from 10:90 to 75:25% (v/v) in 3 min; flow rate 2.0 ml/min; 40 °C
3	Zorbax Extend-C18	Methanol/0.08 M sodium borate buffer pH 10.0 from 10:90 to 75:25% (v/v) in 6 min; flow rate 1.0 ml/min; 40 °C
4	ZirChrom-PS	Methanol/0.08 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 6 min; flow rate 1.5 ml/min; 40 °C
5	ZirChrom-PS	Methanol/0.08 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 4 min; flow rate 1.5 ml/min; 40 °C
6	ZirChrom-PS	Methanol/0.08 M sodium borate buffer pH 10.0 from 10:90 to 70:30% (v/v) in 4 min; flow rate 1.5 ml/min; 40 °C
7	ZirChrom-PS	Methanol/0.08 M sodium borate buffer pH 10.0 from 10:90 to 70:30% (v/v) in 4 min; flow rate 1.2 ml/min; 75 °C
8	ZirChrom-PS	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
9	ZirChrom-PS	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
10	Platinum C18	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 3.0 ml/min; 40 °C
11	Platinum EPS C18	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 3.0 ml/min; 40 °C
12	Zorbax Eclipse XDB-C8	Methanol/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
13	Zorbax Eclipse XDB-C8	Methanol/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
14	Zorbax Eclipse XDB-C8	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
15	Zorbax Eclipse XDB-C8	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
16	Betasil Phenyl Hexyl	Methanol/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
17	Betasil Phenyl Hexyl	Methanol/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
18	Betasil Phenyl Hexyl	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
19	Betasil Phenyl Hexyl	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
20	Suplex pK_b-100	Methanol/Britton–Robinson buffer pH 2.5 from 30:70 to 75:25% (v/v) in 20 min; flow rate 1.0 ml/min; 40 °C
21	Suplex pK_b-100	Methanol/Britton–Robinson buffer pH 7.5 from 30:70 to 70:30% (v/v) in 10 min; flow rate 2.0 ml/min; 40 °C
22	ZirChrom-PBD	Methanol/Britton–Robinson buffer pH 2.5 from 30:70 to 75:25% (v/v) in 20 min; flow rate 1.0 ml/min; 40 °C
23	ZirChrom-PBD	Methanol/Britton–Robinson buffer pH 7.5 from 30:70 to 70:30% (v/v) in 20 min; flow rate 1.0 ml/min; 40 °C
24	ZirChrom-PBD	Methanol/0.016 M borate buffer pH 10.0 from 30:70 to 75:25% (v/v) in 8 min; flow rate 1.5 ml/min; 40 °C
25	Chromolith Performance	Acetonitrile/0.08 M sodium phosphate buffer pH 3.0 from 10:90 to 60:40% (v/v) in 6 min; flow rate 2.0 ml/min; 40 °C
26	Chromolith Performance	Acetonitrile/0.08 M sodium phosphate buffer pH 7.5 from 10:90 to 60:40% (v/v) in 6 min; flow rate 2.0 ml/min; 40 °C
27	Aqua	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
28	Aqua	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 75:25% (v/v) in 4 min; flow rate 2.0 ml/min; 40 °C
29	Suplex pK_b-100	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
30	Suplex pK_b-100	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
31	PLRP-S	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
32	PLRP-S	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
33	Luna CN	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
34	Luna CN	Acetonitrile/0.08 M sodium phosphate buffer pH 5.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
35	ZirChrom-PBD	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 2.0 ml/min; 75 °C
36	ZirChrom-PBD	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 5 min; flow rate 2.0 ml/min; 75 °C
37	Zorbax Extend-C18	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C
38	Zorbax Extend-C18	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 ml/min; 40 °C

modifier/water, i.e. the mobile phase ratio at the beginning of the gradient, and injected on CS1–CS19.

3. Results and discussion

3.1. Injection of mixtures and evaluation with OPA

Initially, orthogonality of systems was evaluated from the individual injection of substances with UV-detection at 254 nm [15]. However, the throughput increases considerably when mixing substances and applying DAD-detection. Mixtures were composed of acidic and basic substances, because, due to different ionization at a given pH, the probability of peak overlap decreases. The mixed substances also originated from different pharmacological classes, and their spectrum was different. Twenty mixtures were created (Table 3). When overlap occurs, the UV-absorbance spectrum will reveal impure peaks. Since gradient elution and different pH values imply that the spectrum changes, reference spectra in acidic, basic and alcoholic environment [28,29] were used.

The elution sequence of the individual mixture substances on a given system is unknown. Therefore, the data matrices (absorbance as a function of time and wavelength) of the HPLC-DAD spectrochromatograms were interpreted with OPA [16–19]. OPA uses a two step approach. First, a dissimilarity criterion is applied. The mean spectrum from the data matrix, i.e. the mean absorbance at each wavelength, is calculated. Starting from this mean spectrum, the number of compounds occurring in the mixture is determined in a sequential approach. This is done by comparing all spectra, measured at the different times, either with the mean spectrum (to identify the first substance) or with all earlier selected spectra (for consecutive substances), and selecting the one with the highest dissimilarity. The dissimilarity plots obtained, which allow such selection, are shown in Fig. 1a–e. For more theoretical background we refer to Refs. [16–19]. In Fig. 1e, a random dissimilarity pattern and considerably lower dissimilarity values are obtained. This implies the plot only shows noise, meaning that only four compounds occur in the mixture.

In a second step, multivariate curve resolution alternating least squares (MCR-ALS) is applied to calculate both the pure compound spectra (Fig. 1f), used to identify the solutes, and the corresponding individual concentration profiles (absorbance versus time) (Fig. 1g), used to determine their elution times [19].

Comparing the obtained spectra with the reference spectra allowed identifying each substance and determining its retention time. The number of peaks determined by OPA was equal to the number of mixture compounds or one more. From the pure compound spectra, the solute peaks easily could be identified while the additional “compound” reflected the spectrum of the mobile phase and was due to the injection peak. Although severe overlap occurred on several systems, and in different mixtures, OPA each time was capable to select the correct number of components, and to retrieve the corresponding retention times.

3.2. Defining orthogonality and similarity between different systems

The retention results of the 68 substances were used to define orthogonal sets of systems. Such sets consist of systems with a similar selectivity within their group, and with different selectivity towards other groups. To select these sets, the correlation coefficients between retention factors on two

Table 3
Composition of the 20 mixtures

Mixture	Substances			
1	Cocaine hydrochloride	Naphazoline hydrochloride	Ranitidine hydrochloride	
2	Acebutolol hydrochloride	Codeine base	Pizotifen	Pentoxifylline
3	Flurazepam	Dimetindene maleate	Morphine hydrochloride	
4	Chloropyramine hydrochloride	Lidocaine hydrochloride	Fenfluramine hydrochloride	Caffeine
5	Pyrilamine maleate	Prenalterol hydrochloride	Oxeladin citrate	4-Benzylphenol
6	Sulfapyridine	Ketotifen fumarate	Pindolol	Thiothixene
7	Tetrahydrozolin hydrochloride	Famotidine	Cimetidine	Bupranolol hydrochloride
8	Antazoline hydrochloride	Phenol	Digitoxine	
9	Tolazoline hydrochloride	(±)-Camphor	Propiomazine maleate	
10	L-(+)-Ascorbic acid	Diphenhydramine hydrochloride	Miconazol nitrate	
11	Terbutaline sulphate	Isothipendyl hydrochloride	Oxprenolol hydrochloride	α-Lobeline hydrochloride
12	Promethazine hydrochloride	Resorcine	Desipramine hydrochloride	Cirazoline hydrochloride
13	Prazosin hydrochloride	Diclofenac sodium	Strychnine base	
14	Carbamazepine	5-Hydroxytryptamine hydrochloride	Sotalol	Nadolol
15	Fluphenazine dihydrochloride	Betaxolol hydrochloride	Procaine hydrochloride	
16	Lorazepam	Terazosin hydrochloride	5-Sulfosalicylic acid dihydrate	
17	Efedrine hydrochloride	Dopamine hydrochloride	β-Estradiol	
18	Timolol maleate	1,1-Dimethylbiguanide hydrochloride	Nizatidine	Celiprolol
19	Histamine dihydrochloride	Nicardipine hydrochloride	Digitoxigenine	
20	Ibuprofen	Amiodarone hydrochloride		

systems can be interpreted [15]. A high correlation coefficient (close to +1) indicates that the elution order of the substances is similar on both systems, i.e. only few selectivity differences occur. A pair of systems with a low correlation coefficient (close to 0) is considered orthogonal; plotting their *k*-values on Cartesian axes leads to a non-structured cloud of points [15]. The above is demonstrated in Fig. 2.

However, it is not evident to visualize the relationships between all systems, especially not when their number becomes large. The weighted-average-linkage based dendrograms (Fig. 3) [15,16,20–22] were used to classify systems. The (dis)similarity between systems is visualized by the height at which the branches of the tree are connected. The higher two objects or clusters are connected, the more dissimilar they are. The dissimilarity criterion applied is $1-|r|$, where r represents Pearson's correlation coefficient. Systems exhibiting the highest dissimilarity values are thus most orthogonal.

An arbitrary limit of dissimilarity beneath which the systems are considered similar was defined as 0.40 (see horizontal line in Fig. 3). It was derived from the knowledge that systems with $r \geq 0.60$ have a similar selectivity. This limit defines five groups of similar systems (*I*–*V* on Fig. 3). All other systems are individually situated in the dendrogram, and are marked with VI.

The systems CS7, CS6, CS3, CS4, CS22 and CS8, CS2, CS9 and CS5 are most orthogonal, as they are connected at the highest dissimilarities. All pairs of the above systems, except CS7 and CS6 (group I), might be considered orthogonal.

The weighted-average-linkage dendrogram facilitates the grouping of similar and the selection of orthogonal systems. The classification obtained required the selection of an arbitrary limit. Therefore, some other visualization methods that might lead to similar conclusions without handling decision limits were evaluated.

The correlation coefficients matrix for the 38 systems was transformed into a color map that represents the degree of

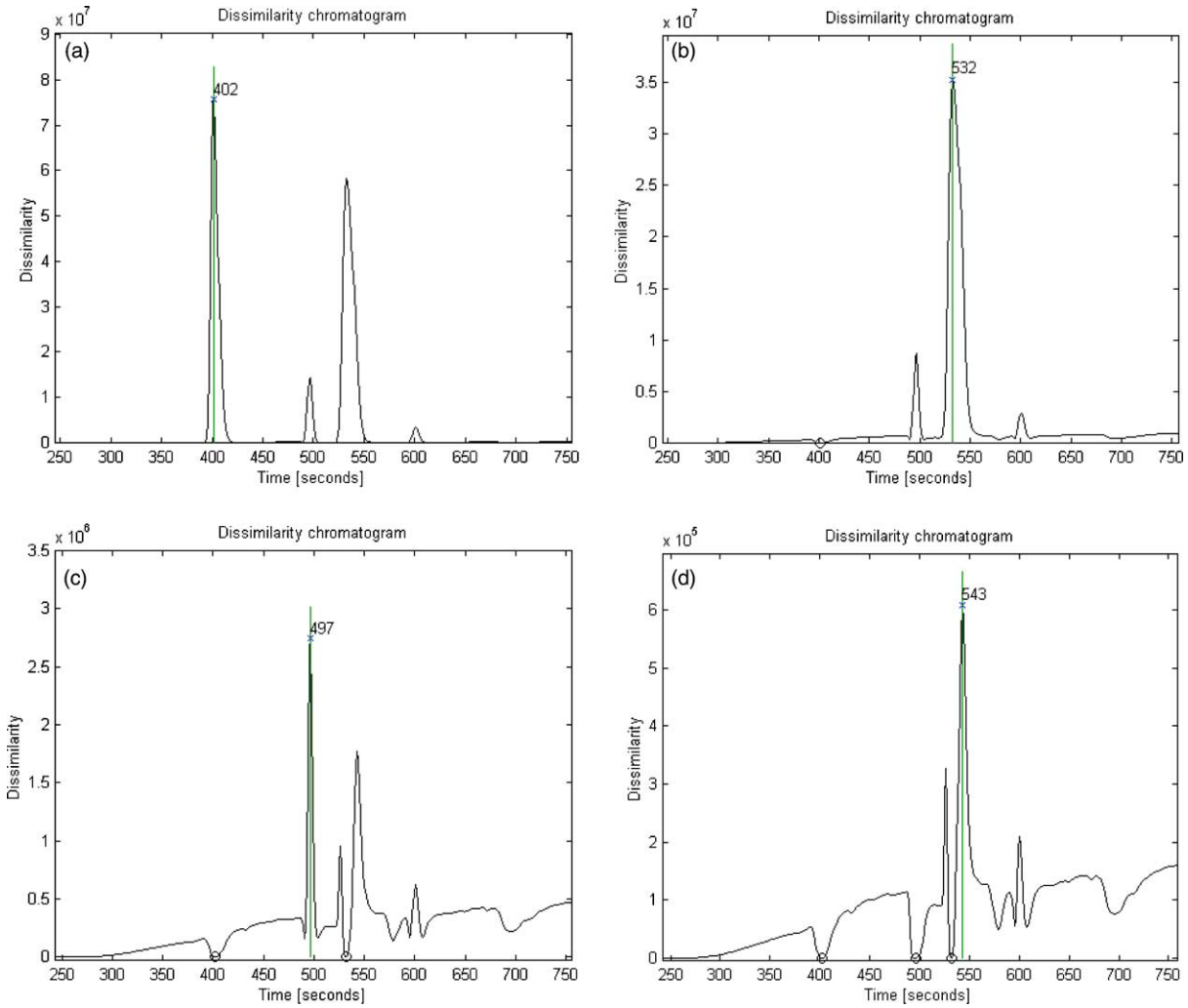


Fig. 1. First (a) to fifth (e) dissimilarity plot calculated by OPA. Values next to the highest peaks indicate elution times of a compound, (f) pure compound spectra and (g) individual concentration profiles for a mixture of ranitidine hydrochloride (A), cocaine hydrochloride (B), naphazoline hydrochloride (C) and oxeladin citrate (D) on CS9.

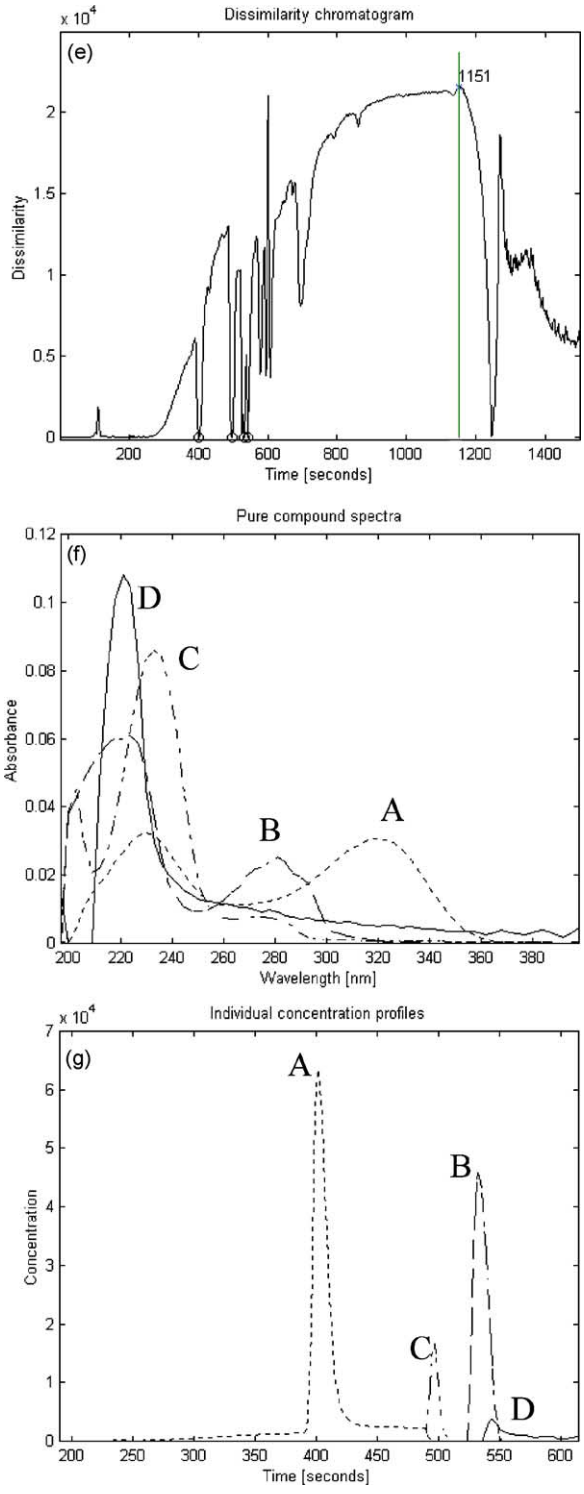


Fig. 1. (*Continued*).

correlation between the retention factors by a color (Fig. 4). The colors scale from dark blue for low correlation coefficients to brown for high *r*-values.

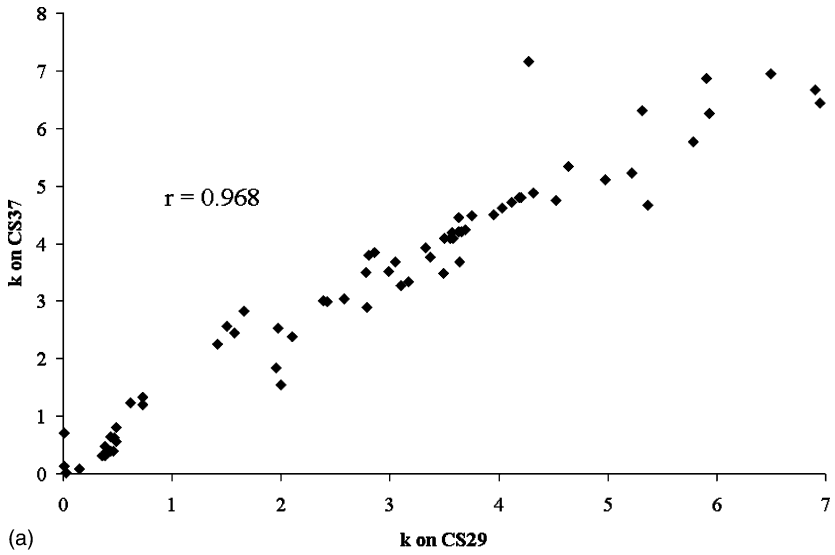
The color map can be represented in several ways, i.e. the sequence of the systems can be varied. One could, for instance, respect the order given in Table 2. However, then, the systems would be ordered randomly, and little information about sets of orthogonal and similar systems would be obtained. Therefore, it is better to define the sequence based on given criteria that promote the clustering of similar systems. A possibility is to respect the sequence in the dendrogram (Fig. 4a). Other possibilities are to rank the systems based on increasing or decreasing dissimilarities in the dendrogram. Only the former is shown (Fig. 4b) as the latter resulted in a color map with analogous information. Color maps are thus created placing mainly blue colors (low correlations) in one corner and brown colors (high correlations) in the diagonally opposite corner. Roman numbers indicate groups of systems, similarly as in the dendrogram (Fig. 3). To draw conclusions about similarity and orthogonality of systems, Fig. 4b is preferred to Fig. 4a since it shows information about similar and orthogonal systems more logically. Further on, only Fig. 4b is discussed.

The systems in VI_1, i.e. CS2–CS9 and CS22, in general (except for the pair CS6–CS7/zone I) show low correlation coefficients (blue squares) when compared to each other, and are considered orthogonal. Zone VI_2 indicates that these systems, usually, also are rather orthogonal to all other systems. Besides zone I, different zones of (relatively) highly correlated systems can be defined. The systems situated in zone V are most correlated. Within this zone, two subunits (V_1 and V_2) with a higher correlation can be identified. The systems from zones II, III and IV are intermediately high correlated within their zone, and intermediately to very low to the other systems. The color map also indicates that systems CS26, CS28, CS32 and CS38 (members of group II), CS12, CS16, CS18 (group III), and CS10, CS11 (group IV) are intermediately high correlated with those from group V, except with CS20. The latter shows relatively high correlations with the systems in group V, but is orthogonal towards all others. This means CS20 might be of interest in a set of orthogonal systems. However, it is orthogonal to fewer systems than those are from group VI.

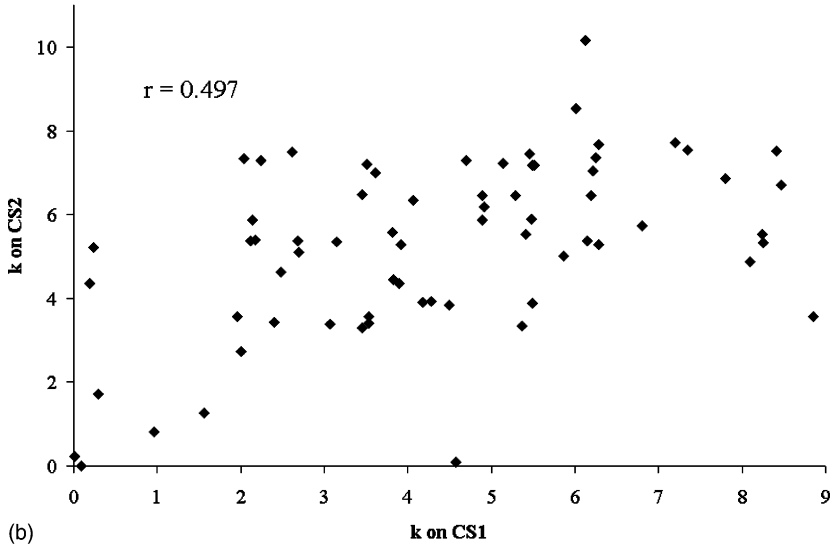
In summary, the information from the dendrogram can be translated in color maps of correlation coefficients, whose interpretation allows deciding on orthogonal or similar systems. The color maps in which the systems are ranked according to either increasing or decreasing dissimilarity were found best. The systems CS2, CS3, CS4, CS5, CS6 and CS7, CS8, CS9, CS22, and some from group V (e.g. one from each subgroup and CS20) could be considered as a set of (rather) orthogonal systems. Selection of a system from the "intermediate" groups II–IV also might be of interest because some are (relatively) low correlated to those of zones V and VI. A reduction in the number of orthogonal systems, or the decision which to select from a group of similar ones, might depend on other criteria, such as column efficiency or other stationary phase properties [30].

3.3. Discussion on the orthogonal systems

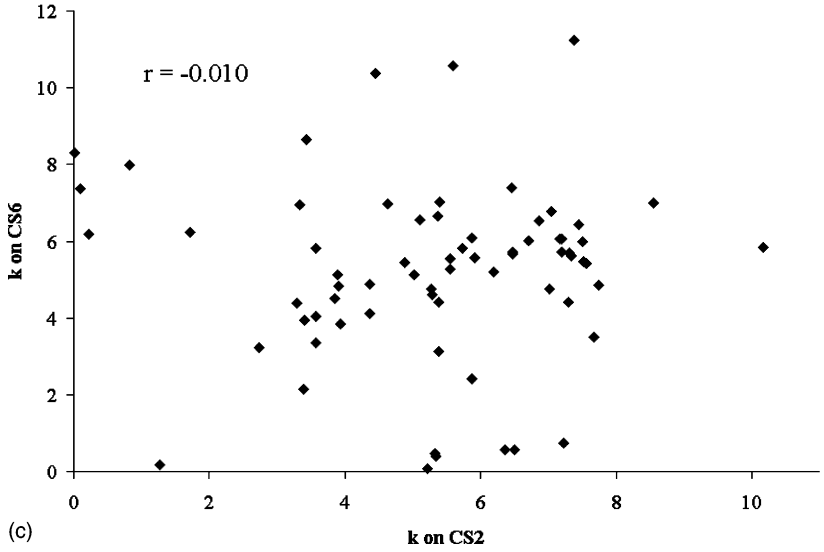
The stationary phases tested were chosen because of special modifications or properties, providing potentially



(a)



(b)



(c)

Fig. 2. Retention factors of the 68 substances on (a) CS37 vs. CS29 ($r = 0.968$), (b) CS2 vs. CS1 ($r = 0.497$), and (c) CS6 vs. CS2 ($r = -0.010$).

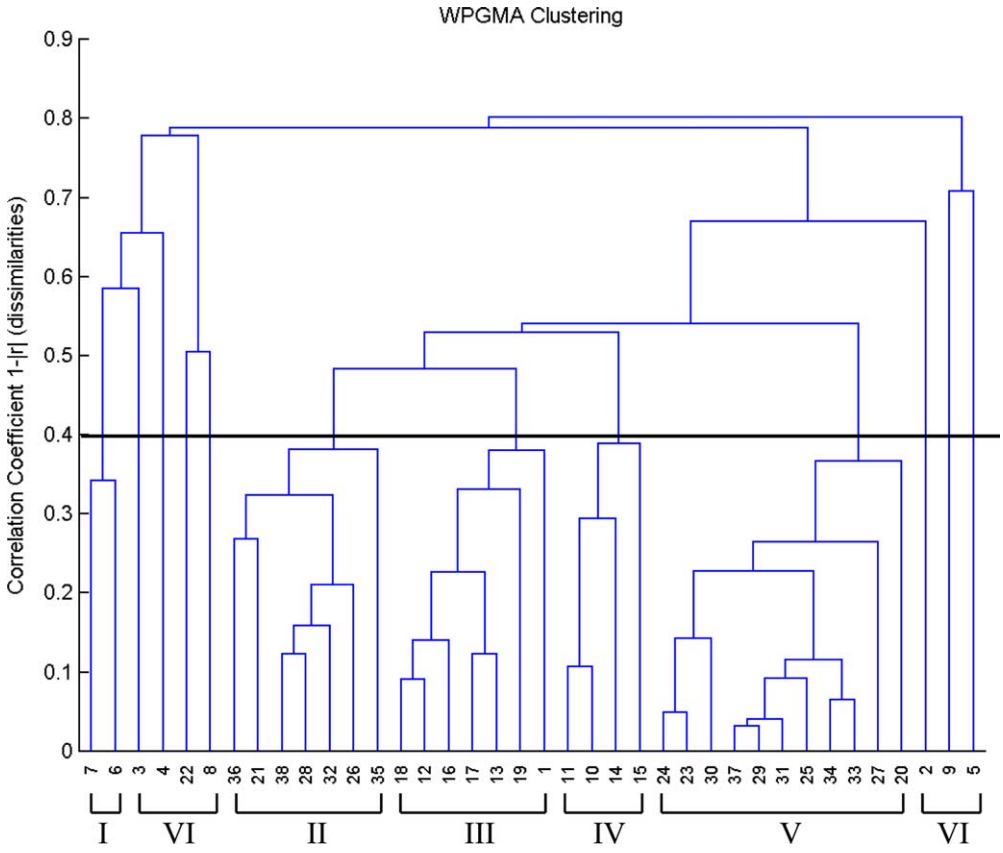


Fig. 3. Hierarchical weighted-average-linkage based dendrogram for the 38 chromatographic systems. Abscissa: system numbers and group numbers (roman numbers).

different selectivity, and because of good chemical or thermal stability [2–5,26]. All systems containing the zirconia-polystyrene (ZirChrom-PS) stationary phase (CS4 till CS9) are orthogonal to almost any other system (Figs. 3 and 4). This is also valid for CS2 (monolithic C18-silica, methanol, pH 6.8, 40 °C), CS3 (bidentate octadecylsilica (Zorbax Extend-C18), methanol, pH 10.0, 40 °C) and CS22 (zirconia-polybutadiene (ZirChrom-PBD), methanol, pH 2.5, 40 °C). The base-deactivated C16-silica (Suplex pK_b-100), methanol, pH 2.5, 40 °C system (CS20) shows considerable selectivity differences compared to several systems. Systems CS20 and CS22 earlier were found most orthogonal in a set of 11 systems [15]. In the new set of 38 systems they still remain interesting, since correlation coefficients below 0.240 were encountered, for instance, when comparing CS20 with CS2-CS9, CS13, CS17 and CS19. For CS22, orthogonal combinations were seen with CS3, CS5-CS7 and CS14, while towards the other systems mostly r-values below 0.4 and never above 0.6 were observed.

The most orthogonal pairs of systems (r between −0.038 and 0.1) are summarized in Table 4. They are obtained when a zirconia-polystyrene phase is compared with a silica-based or a polystyrene-divinylbenzene phase (PLRP-S).

The stationary phase is clearly found to be the most important factor determining orthogonality between systems. The fact that the zirconia-based phases relative to silica-based columns show orthogonality might be due to the fact that the former are able to exchange anions, cations and ligands, whereas on the latter, only cation-exchange is possible [26]. The additional possibility of π-interactions on the zirconia-polystyrene phase [4] might be another explanation.

Systems for which only the buffer pH is different show that this factor also can lead to relatively large selectivity changes, for instance, between CS20 and CS21 ($r = 0.363$), or CS1 and CS2 ($r = 0.497$). In the latter, the monolithic phase, methanol, 40 °C combination is used either at pH 3.0 or at pH 6.8. Selectivity differences are even more pronounced when the pH is changed on a zirconia-polystyrene phase, e.g. CS4-CS5 ($r = 0.104$) or CS8–CS9 ($r = 0.128$). Changing both buffer pH and stationary phase can enhance the orthogonality induced by each factor separately. Consider, for instance, CS5–CS6, where only the buffer pH is changed ($r = 0.250$), and CS2–CS5 ($r = 0.240$), where only the stationary phase is changed, versus CS2–CS6 ($r = -0.010$), where both factors are varied.

A change in the organic modifier only had consequences on the selectivity when the zirconia-polystyrene phase was used, e.g. CS4–CS8 ($r = 0.283$). In other cases, r remains rather high, for instance CS1–CS25 ($r = 0.501$). Temperature changes turned out to be least important. When the temperature was changed from 40 to 75 °C, e.g. for CS6–CS7, a high correlation coefficient ($r = 0.658$) was obtained. The

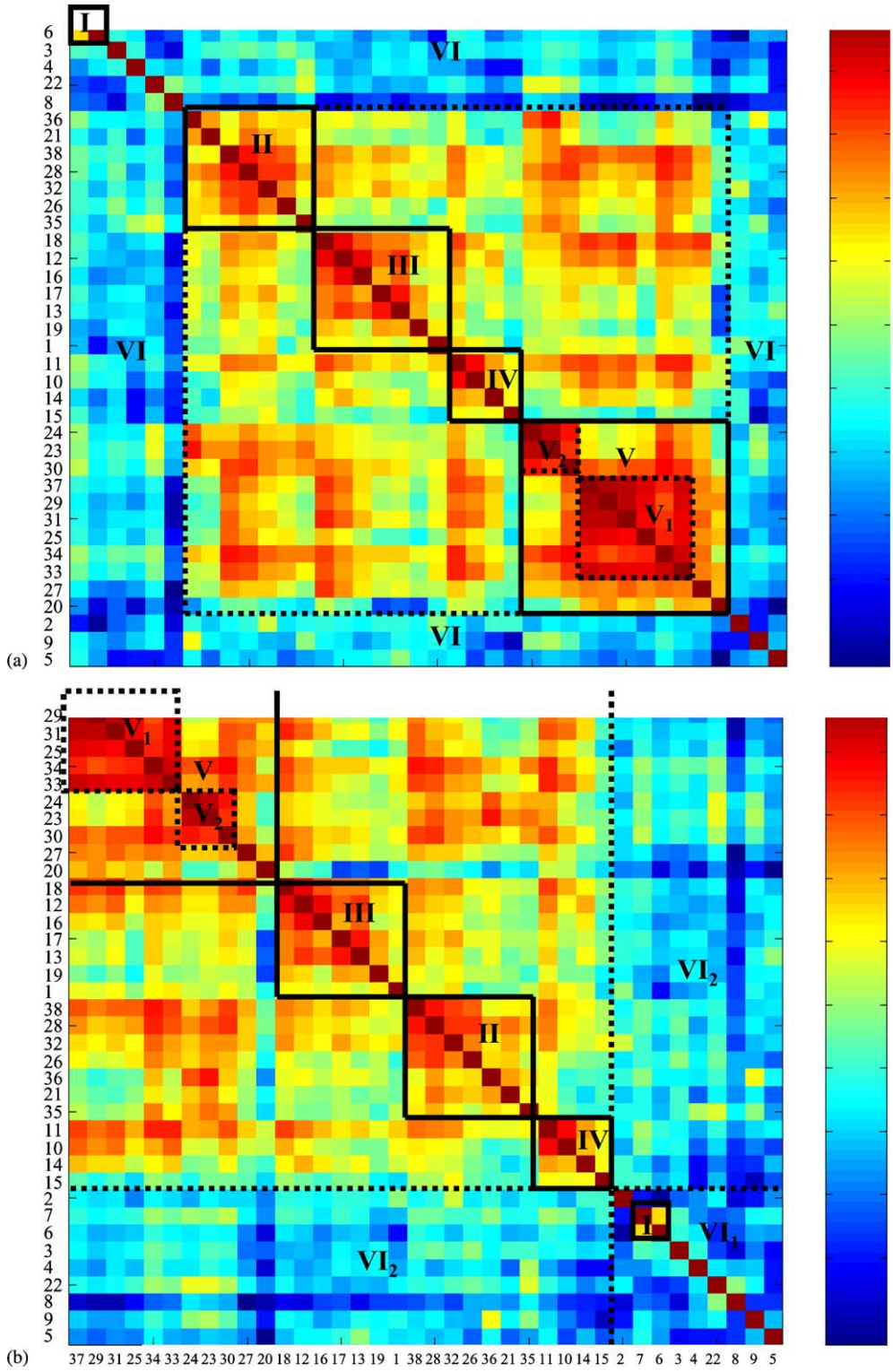


Fig. 4. Color map of the correlation coefficients between the retention factors, measured on the 38 systems from the 68 drugs, (a) sequence of systems as in the dendrogram of Fig. 3, and (b) sequence of systems based on increasing dissimilarities in the dendrogram. Brown: high correlation; blue: low correlation.

Table 4
The most orthogonal pairs of systems ($-0.038 \leq r \leq 0.1$)

Pair of systems	r	Description of system parameters
CS5–CS20	−0.038	CS5: ZirChrom-PS; methanol; pH 6.8; 40 °C CS20: Suplex pK_b-100; methanol; pH 2.5; 40 °C
CS8–CS27	−0.026	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS27: Aqua; acetonitrile; pH 3.0; 40 °C
CS2–CS6	−0.010	CS2: Chromolith Performance; methanol; pH 6.8; 40 °C CS6: ZirChrom-PS; methanol; pH 10.0; 40 °C
CS8–CS20	0.001	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS20: Suplex pK_b-100; methanol; pH 2.5; 40 °C
CS8–CS29	0.008	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS29: Suplex pK_b-100; acetonitrile; pH 3.0; 40 °C
CS8–CS31	0.009	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS31: PLRP-S; acetonitrile; pH 3.0; 40 °C
CS9–CS15	0.023	CS9: ZirChrom-PS; acetonitrile; pH 6.8; 40 °C CS15: Zorbax Eclipse XDB-C8; acetonitrile; pH 6.8; 40 °C
CS8–CS37	0.031	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40°C CS37: Zorbax Extend-C18; acetonitrile; pH 3.0; 40 °C
CS4–CS20	0.039	CS4: ZirChrom-PS; methanol; pH 3.0; 40 °C CS20: Suplex pK_b-100; methanol; pH 2.5; 40 °C
CS2–CS8	0.046	CS2: Chromolith Performance; methanol; pH 6.8; 40 °C CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C
CS3–CS8	0.052	CS3: Zorbax Extend-C18; methanol; pH 10.0; 40 °C CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C
CS6–CS20	0.059	CS6: ZirChrom-PS; methanol; pH 10.0; 40 °C CS20: Suplex pK_b-100; methanol; pH 2.5; 40 °C
CS8–CS12	0.059	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS12: Zorbax Eclipse XDB-C8; methanol; pH 3.0; 40 °C
CS8–CS13	0.067	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS13: Zorbax Eclipse XDB-C8; methanol; pH 6.8; 40 °C
CS8–CS18	0.074	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS18: Betasil Phenyl Hexyl; acetonitrile; pH 3.0; 40 °C
CS3–CS5	0.079	CS3: Zorbax Extend-C18; methanol; pH 10.0; 40 °C CS5: ZirChrom-PS; methanol; pH 6.8; 40 °C
CS1–CS6	0.080	CS1: Chromolith Performance; methanol; pH 3.0; 40 °C CS6: ZirChrom-PS; methanol; pH 10.0; 40 °C
CS8–CS25	0.082	CS8: ZirChrom-PS; acetonitrile; pH 3.0; 40 °C CS25: Chromolith Performance; acetonitrile; pH 3.0; 40 °C
CS4–CS15	0.084	CS4: ZirChrom-PS; methanol; pH 3.0; 40 °C CS15: Zorbax Eclipse XDB-C8; acetonitrile; pH 6.8; 40 °C
CS4–CS14	0.094	CS4: ZirChrom-PS; methanol; pH 3.0; 40 °C CS14: Zorbax Eclipse XDB-C8; acetonitrile; pH 3.0; 40 °C
CS9–CS20	0.097	CS9: ZirChrom-PS; acetonitrile; pH 6.8; 40 °C CS20: Suplex pK_b-100; methanol; pH 2.5; 40 °C

above results about the importance of the factors are in accordance with Steuer et al. [14].

3.4. Conclusion

HPLC-DAD data interpreted with OPA allow in injected mixtures identifying the compounds and their retention times, even in case of severe overlap. This approach considerably increased the throughput of systems during orthogonality testing.

The weighted-average-linkage-based dendrograms are useful to visualize both the groups of similar and of orthogonal systems. The color maps of correlation coefficients could be ordered logically, based on the dendrogram results.

The maps created according to either increasing or decreasing dissimilarities in the dendrogram were found best to define the orthogonal and (groups of) similar systems in the data set studied. From the set of 38 systems, an orthogonal subset can be selected. However, it remains to be seen which of these systems also are useful to develop a separation method for a drug substance and its impurities.

The set of orthogonal systems is relatively high and it might be desirable to limit the number of systems to be tested as a first step in method development to, for instance, three or four well-performing ones. Selection might be made after ranking the set according to some additional column characteristics, a feature that still remains to be studied.

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