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Evaluation of chemometric techniques to select orthogonal chromatographic systems

E. Van Gyseghem [a], B. Dejaegher [a], R. Put [a], P. Forlay-Frick [a], A. Elkihel [a], M. Daszykowski [a], K. Héberger [b], D.L. Massart [a], Y. Vander Heyden [a,*]

[a] *Department of Analytical Chemistry and Pharmaceutical Technology, A VICIM Partner, Vrije Universiteit Brussel-VUB, Laarbeeklaan 103, B-1090 Brussels, Belgium*

[b] *Institute of Biomolecular Chemistry, Chemical Research Center, Hungarian Academy of Sciences, H-1525 Budapest, P.O. Box 17, Hungary*

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Abstract

Several chemometric techniques were compared for their performance to determine the orthogonality and similarity between chromatographic systems. Pearson's correlation coefficient (r) based color maps earlier were used to indicate selectivity differences between systems. These maps, in which the systems were ranked according to decreasing or increasing dissimilarities observed in the weighted-average-linkage dendrogram, were now applied as reference method. A number of chemometric techniques were evaluated as potential alternative (visualization) methods for the same purpose. They include hierarchical clustering techniques (single, complete, unweighted-average-linkage, centroid and Ward's method), the Kennard and Stone algorithm, auto-associative multivariate regression trees (AAMRT), and the generalized pairwise correlation method (GPCM) with McNemar's statistical test. After all, the reference method remained our preferred technique to select orthogonal and identify similar systems.

Keywords: Orthogonal chromatographic systems; Hierarchical clustering techniques; Correlation coefficients color map; Kennard and Stone algorithm; Auto-associative multivariate regression trees; Generalized pairwise correlation method with McNemar's test

1. Introduction

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) demands that all impurities in pharmaceuticals, exceeding a certain threshold, should be characterized [1], as they can cause undesired side effects. The Food and Drug Administration (FDA) requires methods in which all components are resolved. For instance, a separation method, usually chromatographic, is necessary to separate, identify and quantify all impurities. The development of chromatographic methods is laborious, costly and not evident. Therefore defining a strategy to rapidly find initial separation conditions, which then occasionally can be used as a starting point for further method development, is very interesting [2–6].

Orthogonal chromatographic systems have a strongly differing selectivity, because retention is caused by different mechanisms or solute properties. Such systems are helpful tools in developing methods to separate impurities from the active substance and from each other in drugs with unknown impurity profile. Therefore, orthogonal or dissimilar systems are desired as potential starting points for method development [2–6]. In our context, the term orthogonal or orthogonality is not used in its strict mathematical sense [5]. In mathematics two parameters are orthogonal when they are uncorrelated ($r = 0$), and they are either orthogonal or not. In comprehensive two-dimensional chromatography (LC × LC) two methods are called orthogonal when the constituent dimensions operate independently and synentropy across the dimensions is zero [7,8]. In our situation, orthogonality was evaluated for various individual systems (i.e. containing one column) that can be used as potential starting points for classic method development. In our case, but also in LC × LC studies, often a less strict definition for orthogonality is applied. Orthogonal systems are then defined as systems

* Corresponding author. Tel.: +32 2 477 47 34; fax: +32 2 477 47 35.
E-mail address: yvanvdh@vub.ac.be (Y.V. Heyden).

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"that differ significantly in chromatographic selectivity" [6]. This means that systems for which r between the retention data is low are also considered or called orthogonal. It means too that, e.g. when 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 [2–6] usually the term orthogonal, rather than dissimilar is used.

To study orthogonality between systems, both classical silica-based and more recently developed reversed-phase columns (e.g. polar-endcapped silica-based, or zirconia-based stationary phases) have been examined. Also the influence of the buffer pH, the organic modifier type or the column temperature on the selectivity was evaluated. Fourty-six systems were examined by injecting a generic set of 68 drug substances. The compounds chosen differ in structure (functional groups, various ring structures), molecular weight, pK_a, log P and pharmacological class [2,3].

The orthogonality/similarity between systems earlier was determined applying visualization techniques [2,3,5]. Color maps and weighted-average-linkage (weighted pair group method using arithmetic averages or WPGMA) dendrograms [9–12], both based on the correlation coefficients r between the normalized retention times τ of the substances on the different systems, were used. The parameter τ is defined as the difference between the retention time and the dead time, divided by the dead time, measured under gradient conditions. The r-color maps with the systems ranked according to decreasing or increasing dissimilarities in the WPGMA-dendrogram showed to be easily and straightforwardly interpretable to select orthogonal as well as groups of similar systems [3,5] for the studied data sets. It is therefore used here as a reference method.

To select orthogonal systems, Put et al. [13] found that both from univariate regression trees and auto-associative multivariate regression trees (AAMRT) analogous orthogonal selections as those generated with the reference method are obtained. Forlay-Frick et al. [14] applied the generalized pairwise correlation method (GPCM) with different statistical tests (William's t, conditional Fisher's, McNemar's and χ^2-tests). Several correlation measures (Spearman's-ρ and Kendall's-τ besides Pearson's correlation coefficient) were also considered. They moreover defined an orthogonality ratio to rank the systems based on their orthogonality towards the rest. The GPCM with McNemar's test was found best performing to select orthogonal systems. All methods in [13,14] provide a ranking of the systems according to orthogonality, which is not the case with our reference method. However, the latter allows defining groups of similar systems, which is not the case with those applied in [13,14].

The aim of this study is to evaluate several chemometric techniques as potential alternative (visualization) methods for the reference. The performances of five hierarchical clustering techniques, i.e. single, complete and unweighted-average-linkage (unweighted pair group method using arithmetic averages or UPGMA), the centroid and Ward's methods [9–12,15,16] to determine a ranking for the color map, are compared to that of the reference. The use of the Euclidean distance [9,15] in the construction of the dendrograms was compared to that of the correlation coefficient. Also other, ranking, techniques, i.e. the Kennard and Stone algorithm [17–19], auto-associative multivariate regression trees (AAMRT) [13] and the generalized pairwise correlation method (GPCM) with McNemar's statistical test [14], were evaluated. The outcome of all techniques was intended to rank the systems in the color map, and the obtained ranking was then compared with that from the reference method for its ability to select orthogonal and distinguish similar systems.

2. Experimental

2.1. Drugs and reagents

The 68 drug substances used and their stock-solution concentrations are listed in Table 1. The concentrations depended on the UV absorbances at 254 nm. The solutions were prepared in 1:1 (v/v) organic modifier/Milli-Q water. The organic modifier used was either acetonitrile or methanol, both Hypersolv for HPLC (BDH, Poole, England).

The mobile phase preparation for the first 38 chromatographic systems (CS1-CS38; Table 2) was already described elsewhere [2,3]. For the mobile phases of the remaining systems (CS39-CS46; Table 2) either acetonitrile for HPLC (Acros, Geel, Belgium) or methanol for LC (Merck, Darmstadt, Germany), both pro analysi (GR quality), were used. Buffers were prepared with phosphoric acid solution min. 85% (Carlo Erba, Milan, Italy), disodium hydrogenium phosphate dihydrate, sodium dihydrogenium phosphate monohydrate, and sodium hydroxide pellets, all pro analysi (all from Merck).

2.2. Chromatographic conditions

The chromatographic conditions in systems CS1–CS38 were described earlier [2,3]. The other experiments were executed on an HPLC-instrument consisting 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). The methods were created and the data treated with the Class-M10A LC workstation software (Shimadzu). The column oven was kept at 40 °C.

For systems CS39–CS46, five stationary phases were applied: (a) Shodex RSpak DE-413, (150 mm × 4.6 mm i.d., 4 μm) (Showa Denko, Tokyo, Japan), a polymethacrylate-packed column, (b) Discovery RP-AmideC16 (100 mm × 4.6 mm i.d., 5 μm) (Supelco, Bellefonte, PA), a high-purity hexadecylsilica with a polar-embedded amide function bonded to the silica surface with a propyl group, (c) Fluophase PFP, (100 mm × 4.6 mm i.d., 5 μm) (Thermo Hypersil Keystone, Cheshire, UK), a high-purity, base-deactivated silica stationary phase with perfluorophenyl bonding, (d) Platinum C18 100 Å Rocket, (53 mm × 7 mm i.d., 3 μm) (Alltech, Deerfield, IL), a base-deactivated octadecylsilica, (e) Fluophase RP, (100 mm × 4.6 mm i.d., 5 μm) (Thermo Hypersil Keystone), a high-purity, base-deactivated silica stationary phase with straight-chain perfluorohexyl bonding. Because the set of sub-

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

Substance (concentration in mg/ml)	Distributed by
(±)-Camphor (5)	Sigma–Aldrich (Steinheim, Germany)
1,1-Dimethylbiguanide hydrochloride (1)	Sigma–Aldrich (Steinheim, Germany)
4-Benzylphenol (1)	Aldrich (Milwaukee, WI)
5-Hydroxytryptamine hydrochloride (0.5)	Sigma–Aldrich (Steinheim, Germany)
5-Sulfosalicylic acid dihydrate (2)	Merck (Darmstadt, Germany)
Acebutolol hydrochloride (1)	Sigma (St. Louis, Missouri)
Amiodarone hydrochloride (5)	Clin-Midy groupe Sanofi (Montpellier, France)
Antazoline hydrochloride (1)	Sigma–Aldrich (Steinheim, Germany)
Betaxolol hydrochloride (1)	Synthelabo (Paris, France) (gift)
Bupranolol hydrochloride (1)	Schwarz Pharma (Monheim, Germany)
Caffeine (1)	Fluka (Neu-Ulm, Switzerland)
Carbamazepine (1)	Sigma–Aldrich (Steinheim, Germany)
Celiprolol (1)	Rhône-Poulenc-Rorer (Madrid, Spain) (gift)
Chloropyramine hydrochloride (1)	Sigma–Aldrich (Steinheim, Germany)
Cimetidine (10)	Penn Chemicals (Pennsylvania, PA) (gift)
Cirazoline hydrochloride (0.4)	Research Biochemicals International (Natick, MA)
Cocaine hydrochloride (1)	Bios Coutelier (Brussels, Belgium)
Codeine base (1)	Bios Coutelier (Brussels, Belgium)
Desipramine hydrochloride (5)	Sigma–Aldrich (Steinheim, Germany)
Diclofenac sodium (5)	Sigma–Aldrich (Steinheim, Germany)
Digitoxigenine (0.5)	Fluka (Neu-Ulm, Switzerland)
Digitoxine (1)	Mann Research Laboratories (New York, NY)
Dimetindene maleate (1)	Novartis (Basel, Switzerland) (gift)
Diphenhydramine hydrochloride (5)	Sigma–Aldrich (Steinheim, Germany)
Dopamine hydrochloride (2)	Sigma–Aldrich (Steinheim, Germany)
Efedrine hydrochloride (2)	Vel (Leuven, Belgium)
Famotidine (2)	Sigma–Aldrich (Steinheim, Germany)
Fenfluramine hydrochloride (1)	Technologie Servier (Orleans, France)
Fluphenazine dihydrochloride (USP grade) (2)	Sigma–Aldrich (Steinheim, Germany)
Flurazepam (1)	Dolorgiet Arzneimittel (Bonn, Germany)
Histamine dihydrochloride (1)	Sigma–Aldrich (Steinheim, Germany)
Ibuprofen (5)	Sigma–Aldrich (Steinheim, Germany)
Isothipendyl hydrochloride (1)	Novartis Pharma (Wehr, Austria) (gift)
Ketotifen fumarate (1)	Sigma–Aldrich (Steinheim, Germany)
L-(+)-ascorbic acid (1)	Merck (Darmstadt, Germany)
Lidocaine hydrochloride (1)	Bios Coutelier (Brussels, Belgium)
Lorazepam (1)	MSD (Haarlem, The Netherlands)
Miconazol nitrate (1)	Certa (Braine-l'Alleud, Belgium)
Morphine hydrochloride (2)	Bios Coutelier (Brussels, Belgium)
Nadolol (1)	Sigma–Aldrich (Steinheim, Germany)
Naphazoline hydrochloride (2)	Sigma–Aldrich (Steinheim, Germany)
Nicardipine hydrochloride (1)	UCB (Leuven, Belgium)
Nizatidine (2)	Norgine (Marburg, Germany) (gift)
Oxeladin citrate (2)	Sigma–Aldrich (Steinheim, Germany)
Oxprenolol hydrochloride (0.5)	Sigma–Aldrich (Steinheim, Germany)
Pentoxifylline (1)	Sigma–Aldrich (Steinheim, Germany)
Phenol (1)	Merck (Darmstadt, Germany)
Pindolol (1)	Sigma–Aldrich (Steinheim, Germany)
Pizotifen (5)	Novartis Pharma (Wehr, Austria) (gift)
Prazosin hydrochloride (1)	Sigma–Aldrich (Steinheim, Germany)
Prenalterol hydrochloride (1)	Ciba-Geigy (Basel, Switzerland)
Procaine hydrochloride (1)	Merck (Darmstadt, Germany)
Promethazine hydrochloride (1)	Sigma–Aldrich (Steinheim, Germany)
Propiomazine maleate (1)	Sanofi (Paris, France) (gift)
Pyrilamine maleate (1)	Sigma–Aldrich (Steinheim, Germany)
Ranitidine hydrochloride (2)	Sigma (St. Louis, Missouri)
Resorcine (1)	Merck (Darmstadt, Germany)
Sotalol (1)	Merck (Darmstadt, Germany)
Strychnine base (1)	Bios Coutelier (Brussels, Belgium)
Sulfapyridine (1)	Bios Coutelier (Brussels, Belgium)
Terazosin hydrochloride (1)	Sigma–Aldrich (Steinheim, Germany)
Terbutaline sulphate (1)	Astra Draco (Lund, Sweden)
Tetrahydrozolin hydrochloride (4)	U.S.P.C. (Rockville, MD)
Thiothixene (USP grade) (2)	Sigma–Aldrich (Steinheim, Germany)
Timolol maleate (1)	Sigma–Aldrich (Steinheim, Germany)
Tolazoline hydrochloride (5)	Sigma–Aldrich (Steinheim, Germany)
α-Lobeline hydrochloride (1.5)	Carl Roth (Karlsrhue, Germany)
β-Estradiol (0.5)	Sigma–Aldrich (Steinheim, Germany)

stances is diverse, gradient elution was used to limit analysis time. The gradients applied are summarized in Table 2.

The buffer pH was measured on a daily-calibrated Orion 520A (Orion Research, Boston, MA) pH-meter, and the buffers were filtered through a 0.2 μm membrane filter (Schleicher & Schuell, Dassel, Germany). The substances were injected as 20 mixtures containing three or four components, the composition of which is described in [3]. In all buffers, stock solutions and samples, Milli-Q water (Millipore Purification System, Molsheim, France) was used.

For all substances on each system, the normalized retention time τ was calculated. On all systems, the normalized retention

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 M 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-C_8	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-C_8	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-C_8	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-C_8	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/0.04M 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/0.04M 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 pKb-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 pKb-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 pKb-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 pKb-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
39	Shodex RSpak DE-413	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 1.5 ml/min; 40 °C
40	Shodex RSpak DE-413	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 5 min; flow rate 1.5 ml/min; 40 °C
41	Shodex RSpak DE-413	Methanol/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 1.2 ml/min; 40 °C
42	Discovery RP-AmideC16	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 5 min; flow rate 1.5 ml/min; 40 °C
43	Fluophase PFP	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 2.5 ml/min; 40 °C
44	Platinum C18	Acetonitrile/0.04 M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 4 min; flow rate 2.5 ml/min; 40 °C
45	Discovery RP-AmideC16	Acetonitrile/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 1.5 ml/min; 40 °C
46	Fluophase RP	Methanol/0.04 M sodium phosphate buffer pH 3.0 from 10:90 to 80:20% (v/v) in 5 min; flow rate 1.5 ml/min; 40 °C

time measurement was preceded by a search for optimal peak form by adaptation of the gradient, the flow rate and/or the buffer concentration.

3. Results and discussion

In Refs. [2,3], the orthogonality and similarity between chromatographic systems was determined from interpreting Pearson's correlation coefficients between the normalized retention times τ of the 68 substances. In [2], where only 11 systems were considered, the relationships were deduced from a direct interpretation of the correlation coefficients matrix. Additionally several visualization methods, i.e. principal component analysis (PCA) [17], the density-based cluster technique ordering points to investigate the clustering structure (OPTICS) [20,21] and weighted-average-linkage clustering, were performed to facilitate the selection of similar and dissimilar systems. PCA and OPTICS did not allow defining orthogonal nor similar ones. The WPGMA-dendrogram on the other hand grouped the similar and separated the orthogonal systems [2,3].

For a data set of 38 systems, the correlation matrix was transformed into a color map to visually evaluate the relationships [3]. This map is obtained by replacing each correlation coefficient by a color. The systems were ranked according to increasing or

decreasing dissimilarities in the dendrogram, resulting in a color map that allowed defining orthogonal and groups of similar systems.

For the actually studied set of 46 systems, the color map based on the above-mentioned procedure is shown in Fig. 1a. It is used as a reference to evaluate the results of the other techniques studied. The systems considered orthogonal from this approach are given in Table 3.

Several chemometric methods were researched for their performance to select orthogonal and groups of similar systems. A color map was drawn, whenever possible, ranking the systems according to the results obtained from these techniques, and the selection was compared to that of the reference.

3.1. Hierarchical clustering techniques

The methods are called hierarchical because smaller clusters are included in larger, or vice versa [9]. The result of such classification is visualized as a dendrogram. When objects are sequentially merged, the technique is called agglomerative; when clusters are sequentially split until they all contain

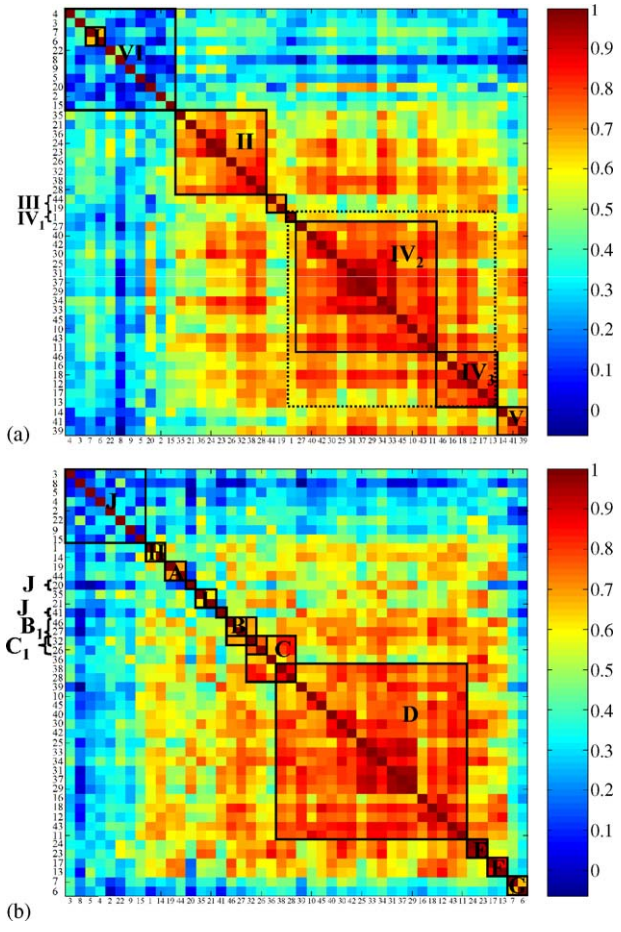

Fig. 1. Color map of Pearson's correlation coefficients between the normalized retention times τ of 68 substances on 46 systems, with the systems ranked according to decreasing dissimilarities $(1 - |r|)$ in the: (a) WPGMA, (b) single linkage, (c) complete linkage, and (d) UPGMA dendrograms. I–V, similar classes; VI, orthogonal systems; A–J, classes defined from techniques other than the reference and not corresponding to I–VI.

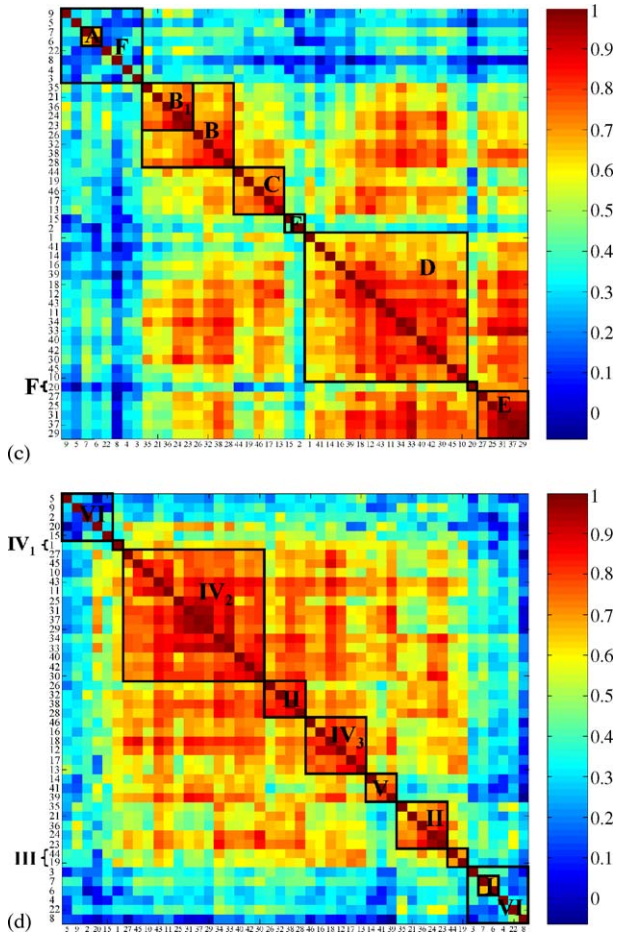

Fig. 1. (*Continued*).

single objects, it is called divisive [9–11,15]. Since the latter methods are much less applied [9], only the agglomerative were examined. These all are based on the same procedure, i.e. classification of m objects in $m - 1$ steps. In each consecutive step, the two most similar objects (clusters) are merged [9]. The objects (or clusters) to join are derived from the (dis)similarity matrix, representing the (dis)similarities between each pair of objects (clusters) [10]. Dissimilarities or dissimilarity coefficients are nonnegative numbers that are small when objects or clusters are closely related, and large if they are very different [11,22]. The two least dissimilar objects (or clusters) are merged, and the dissimilarity matrix is recalculated for the new situation [9].

The agglomerative techniques differ in the calculation of the dissimilarities [11]. The weighted and unweighted-average-linkage, single and complete linkage, the centroid and Ward's method were evaluated in this study. In the average linkage technique, the dissimilarity between two clusters is defined as the average of all dissimilarities calculated between any object in both clusters [11]. Two variants exist, the weighted and unweighted. The former, also called weighted pair group method using arithmetic averages (WPGMA), considers every object in the cluster equally important, i.e. every object weighs the same. Clusters consisting of a larger number of objects carry a larger weight. In the latter, the unweighted pair group method

Table 3
Systems defined as orthogonal from the different methods

Reference method	Single linkage	Complete linkage	UPGMA	Kennard and Stone	AAMRT	GPCM
4	3	9	5	8	5	2
3	8	5	9	39	2	3
6	5	6	2	3	4	8
22	4	22	20	9	3	5
8	2	8	15	5	8	6
9	22	4	1	15	7	4
5	9	3	27 or 45	6	6	7
20	15	21 or 35	26	4	22	22
2	1 or 14	26	13 or 16	2	15	9
15	19 or 44	19 or 44	41	21	9	15
35	20	15	21	22	1	36
19	21 or 35	2	19	19	41	41
1	41	1 or 41	3	20	19	20
27 or 45	24	20	6	35	36	1
13 or 16	13 or 17	25 or 27	4	1		14
41	6		22	7		19
			8			35

using arithmetic averages (UPGMA), every cluster weighs the same [10,11], meaning the weight of every object in the cluster is adjusted to keep the total weight of the cluster constant. In single linkage, the dissimilarity between two clusters is the minimum dissimilarity between the objects in the clusters; while for complete linkage, the maximum dissimilarity is considered. In the centroid method, clusters are merged based on the minimal squared Euclidean distance between their centroids. Ward's method, also called the error sum of squares method, joins clusters that give rise to a minimal increase in the total within-groups error sum of squares.

For interval-scaled variables [11] such as the normalized retention times, several possibilities to determine dissimilarity exist. Earlier, $1 - |r|$ was used as dissimilarity criterion [2,3]. In this study, it is compared to the Euclidean distance [9,15] for WPGMA, UPGMA, single and complete linkage, and to the squared Euclidean metric [10] for the centroid and Ward's methods. In the dendrograms, the dissimilarity of the objects (systems) is represented by the height at which the branches are connected.

In the color maps drawn, the systems were ranked according to decreasing dissimilarities in the trees. The results obtained with the reference method are shown in Fig. 1a, the corresponding dendrogram in Fig. 2a. Considering a dissimilarity of about 0.4 the same groups as in the color map are observed.

The groups of similar systems are denoted as I–V (with for IV three subgroups of more similar ones); the systems orthogonal to the rest are in VI (Figs. 1a and 2a). An orthogonal set can be selected taking every system in VI, and one from each group I–V, which results in 16 systems (Table 3). From the groups I–V, a system splitting in the dendrogram with group VI at (one of) the highest dissimilarity values is selected. If two systems are connected equally high in the tree, e.g. CS6 and CS7 (group I), it is derived from the *r*-color map which of those has the lowest correlation coefficients relative to most other systems. In Table 3, the systems are ranked as they were selected according to the above rules.

For single linkage clustering, the results are shown in Figs. 1b and 2b. The *r*-color map was built, ranking the systems according to decreasing dissimilarities in the tree. Several groups can be noticed, with A–G containing similar ($r \geq 0.6$), H and I merging intermediately orthogonal ($0.4 < r < 0.6$), and J orthogonal ($r \leq 0.4$) systems. The groups are much less pronounced and exclusive than in Fig. 1a. In the dendrogram, no dissimilarity value allows discriminating the orthogonal and similar systems as was the case above. This is a consequence of the dendrogram construction where objects are sequentially linked at a higher dissimilarity level, i.e. nearest neighbours are considered. The orthogonal set selection from the groups in the *r*-color map (Fig. 1b) is given in Table 3. It can be concluded that single linkage clustering results in building a color map in which the orthogonal systems are grouped and can be selected, yet that lacks in the clustering of similar ones. Hence, this technique seems less suitable than the reference for these data.

For complete linkage clustering (Figs. 1c and 2c), the division of the systems into groups is improved. When sorting them in the *r*-color map according to decreasing dissimilarities in the dendrogram, classes A–F can be distinguished. Classes A–E group similar systems, while F classifies those orthogonal to the rest. However, in Fig. 1c, group F is not completely clustered (e.g. CS20) as was the case in Fig. 1a for group VI. The orthogonal subset from Figs. 1c and 2c selected in analogy with the above techniques is given in Table 3. The selection is largely similar to that obtained applying WPGMA. Though the groups defined in Fig. 1c can be retrieved in the dendrogram, they cannot be isolated using one dissimilarity value as was the case in Fig. 2a. Therefore, we consider the complete linkage clustering somewhat less preferable for this data set.

For the UPGMA technique (Figs. 1d and 2d), from the *r*-color map built on decreasing dissimilarities in the dendrogram, the same groups as in Fig. 1a can be distinguished, except for II, which is split. Besides, the classes gathering the similar systems in IV are separated. The orthogonal subset chosen by selecting all systems of the orthogonal group, which also is split in the

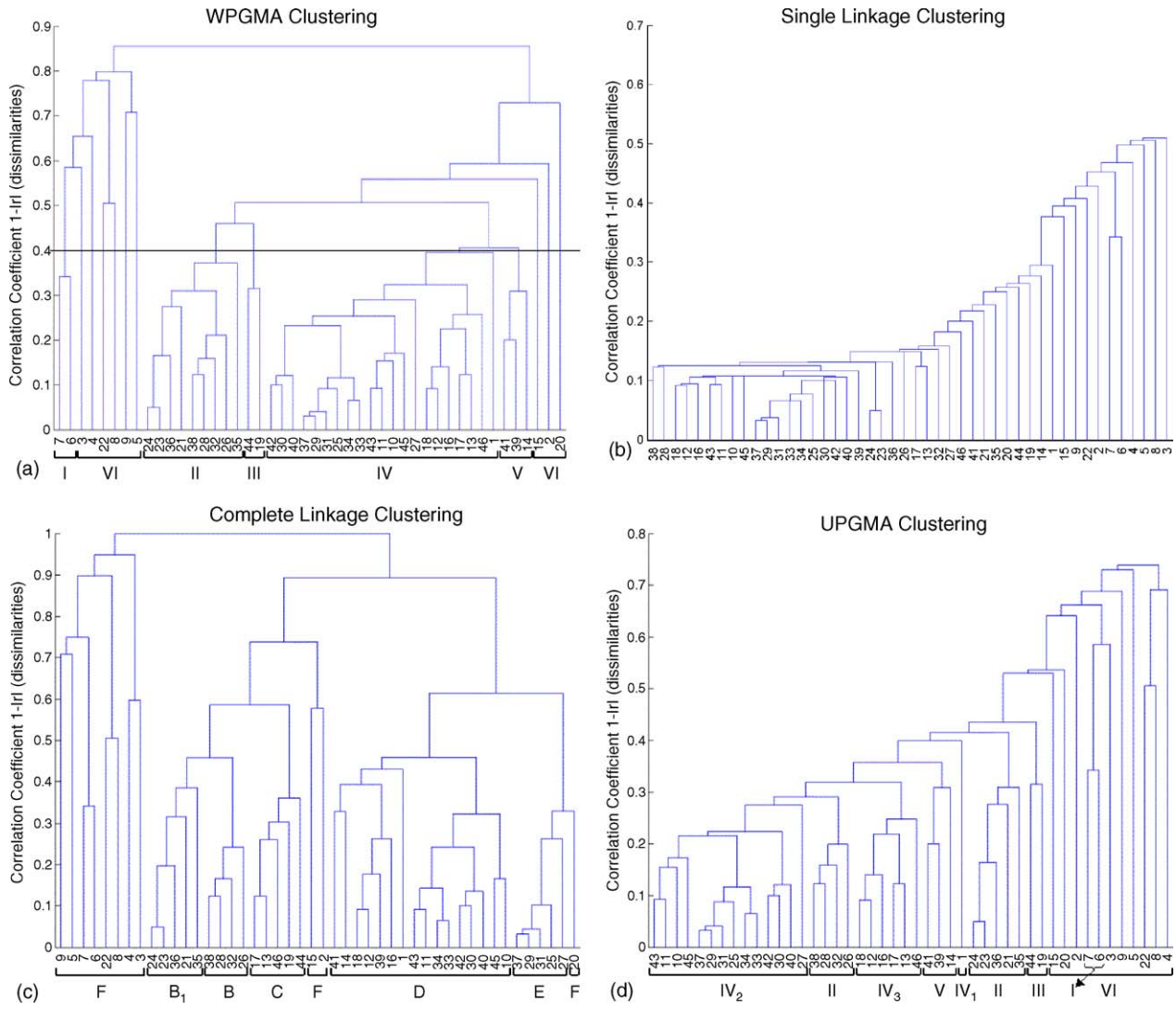

Fig. 2. Dendrogram of 46 systems based on the $1 - |r|$ dissimilarity criterion, using: (a) WPGMA, (b) single linkage, (c) complete linkage, and (d) UPGMA. Abscissa: system numbers and groups.

r-color map, and one with one of the highest individual dissimilarities from each similar class, is shown in Table 3. The selection is largely analogous to that obtained using WPGMA. As for the complete linkage, no dissimilarity value separating the groups of similar and orthogonal systems could be selected neither. Thus, the UPGMA technique also is considered somewhat less preferred for these data than WPGMA. From the above, it can be stated that a preferred clustering method allows selecting a dissimilarity value separating the dissimilar systems from groups of similar, which facilitates the interpretation of both the tree and the color map.

When applying the Euclidean distance as dissimilarity criterion in the hierarchical clustering techniques, both the similar and the orthogonal systems were classified differently, which influenced the selection of the orthogonal subset. No method delivered a dendrogram (and a dissimilarity level) that showed straightforwardly orthogonal and similar relationships. Using *r*-limits to categorize systems as orthogonal, intermediately orthogonal or similar, those considered analogous were found in different groups when using Ward's method, UPGMA, complete linkage and WPGMA. Application of the centroid method even showed several reversals [9] of the dendrogram branches, i.e. in successive linkages the dissimilarity drops again, which complicated the interpretation. The single linkage tree exhibited chaining tendency, as each consecutive node is made at a successively higher dissimilarity, and emphasized on systems that are not the most orthogonal, i.e. these systems exhibited the highest dissimilarities. The color maps of all methods showed dissimilar and analogous systems mixed up, making the definition of groups impossible. As a consequence, the use of the Euclidean distance was found a badly performing dissimilarity criterion for this data set.

3.2. Kennard and Stone algorithm

The Kennard and Stone algorithm [17–19], a uniform mapping algorithm, also was tested to select an orthogonal subset of systems. The technique is based on the fact that

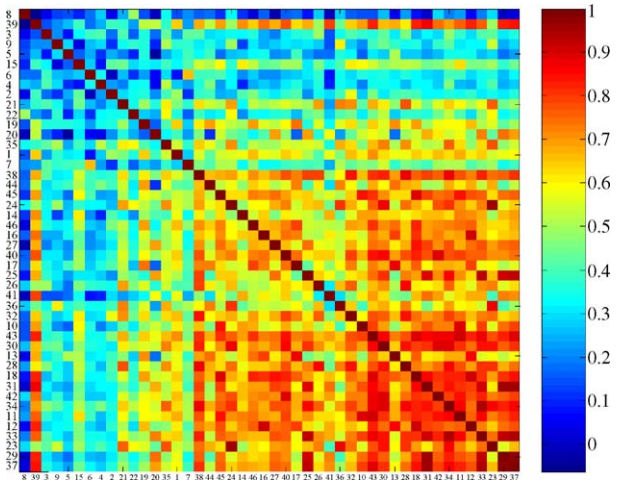

Fig. 3. Color map of Pearson's correlation coefficients between the normalized retention times τ of the 68 substances on 46 systems, with the systems ranked according to the Kennard and Stone algorithm, starting furthest from the mean, using the autoscaled normalized retention times as selection criterion.

objects (systems) leading to different outcomes should not occupy similar locations in the multidimensional space [17]. Accordingly, orthogonal systems should be chosen from dissimilar places, thereby covering this space as uniformly as possible. The algorithm is based on maximizing the minimal (squared) Euclidean distance between each selected object and all previously chosen. It can be executed starting from the object that is situated either closest or furthest from the mean, and each consecutively selected one is at maximal distance of those already included [17]. As a consequence, the Kennard and Stone algorithm might enable selecting an orthogonal subset. It ranks the systems according to decreasing distances, and thus allows choosing subsets with different size.

Fig. 3 shows the color map with the systems ranked according to the selection obtained from the Kennard and Stone algorithm performed on the autoscaled normalized retention times, starting furthest from the mean (Table 3). From Fig. 3 and Table 3, it can be seen that indeed the most dissimilar systems are ranked first. Twelve out of the 16 selected correspond to that in the reference method: i.e. all systems from VI, and half of the remaining. It can be concluded that the Kennard and Stone algorithm, starting furthest from the mean, executed on the autoscaled normalized retention times, allows selecting the most orthogonal systems for the studied data set.

3.3. Auto-associative multivariate regression trees (AAMRT)

The auto-associative multivariate regression trees (AAMRT) technique allowed for two sets of chromatographic systems, using the normalized retention times from the 68 substances, a very similar subset selection of orthogonal systems [13] as that found with the reference method. Therefore, this technique was also applied here.

AAMRT [13,23] allows the simultaneous description of several responses in a decision tree. It is an unsupervised technique. In our approach the variables are applied both as explanatory and as response variables. The data are divided using consecutive binary splits, thus creating nodes that group the objects exhibiting an analogous multivariate response profile, i.e. in this case the substances that show similar retention behaviours on the systems. The starting point in the tree-building procedure is called the parent node; the subgroups are denominated child nodes. A split is defined by a single explanatory variable for which the best split value is selected. The latter is defined as the value that minimizes the impurity of the two child nodes, the goodness of a split as the impurity decrease between the parent and resulting child nodes. The splitting process is repeated considering each child node as a parent node. The decision tree is grown so that the homogeneity is maximized and the impurity is minimized within each node, and splitting is continued until homogenisation of all child nodes, i.e. a maximal tree, is obtained. The importance of an explanatory variable to introduce a split is detected by the variable ranking method [13]. The explanatory variable with the largest impurity decrease is the most important and is given an importance value of 1, while all other get a score on the importance scale relative to that of the most important [13].

In terms of selecting an orthogonal set, it is supposed that systems involved in the splits (i.e. with the highest importance) allow describing the retention on several systems, whereas those being least important to grow the tree can be looked upon as most orthogonal. In Fig. 4, the importance plot for all systems is shown, and the most orthogonal ones, having the smallest importance, can be found to the right [13]. The importance slowly decreases and is relatively similar for the first 32 systems. For the last 14 (starting with CS36 in Fig. 4), lower importance values are observed. Therefore, it could be suggested to select these 14 systems as orthogonal subset, the composition of which is described in Table 3. Twelve of the 14 systems correspond to those obtained with the reference method. The *r*-color map with the systems ranked according to increasing importance is shown in Fig. 5. A gradual decrease in dissimilarity is observed, and the orthogonal systems can clearly be derived. From this color map, the same orthogonal subset would be selected as from Fig. 4. All systems obtained with the reference method (group VI in Figs. 1a and 2a), except CS20, are present in the AAMRT selection, including also some from the similar groups.

It can be concluded for the studied data that the AAMRT technique indeed could be useful to select an orthogonal subset. The difference with the reference method is that AAMRT only focuses on the selection of the most orthogonal systems, while groups of similar systems are not found.

3.4. Generalized pairwise correlation method (GPCM) with McNemar's statistical test

The generalized pairwise correlation method (GPCM) with McNemar's statistical test already demonstrated for a set of 38 systems examined with the 68 substances to result in an analogous orthogonal subset selection as the reference method [14]. Therefore, this technique also was evaluated here.

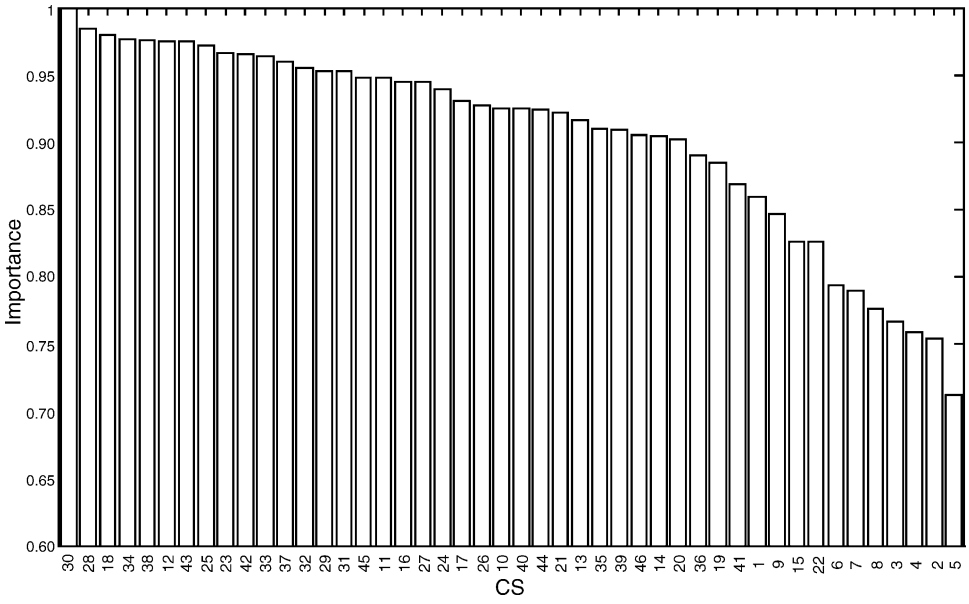

Fig. 4. Importance plot for the explanatory variables (46 chromatographic systems) in the AAMRT.

GPCM [24–26], a non-parametric technique, takes into account the pairwise relations between the systems. A number of superiority is determined comparing all possible independent variable pairs, and is defined as the number of wins, i.e. the number of times the considered independent variable was found superior. Analogously, a number of inferiority, i.e. of losses, can be determined. Each system was once considered as dependent variable (supervisor) and the remaining 45 were then ranked with GPCM. The superiority was calculated using the non-parametric statistical McNemar's test [17,27], and the ranking of the systems was based on the number of wins minus the number of losses. This approach leads to 46 rankings.

To construct a color map based on the results of this approach (Fig. 6), several actions had to be taken. The color map ($n \times m$) is asymmetrical (contrary to previous maps) and contains the reference systems (supervisors) in the n direction, and the results relative to those in the m direction. The asymmetry can be explained from the fact that elements x_{ij} and x_{ji} are obtained using different systems as reference. The systems in the color map are considered orthogonal when the number of wins minus the number of losses is beneath or equal to −19. This value is the consequence of two measures: (a) in real-life situations, orthogonality with $r = 0$ is rare [5,6]. Stated that also statistically non-significant correlation between systems can be considered as orthogonality, a limit value of correlation is defined below which a non-zero value can be considered originating from orthogonal systems. It amounts 0.291 at the 5% significance level for $n = 46$. The threshold was derived from the linear correlation coefficients table of Bevington [28]. This implies that pairs of systems for which the correlation coefficient is lower than 0.291 are considered orthogonal; (b) for 46 systems, the difference between number of wins and number of losses can range from −45 to +45, i.e. 90 possibilities. Combining measures (a)

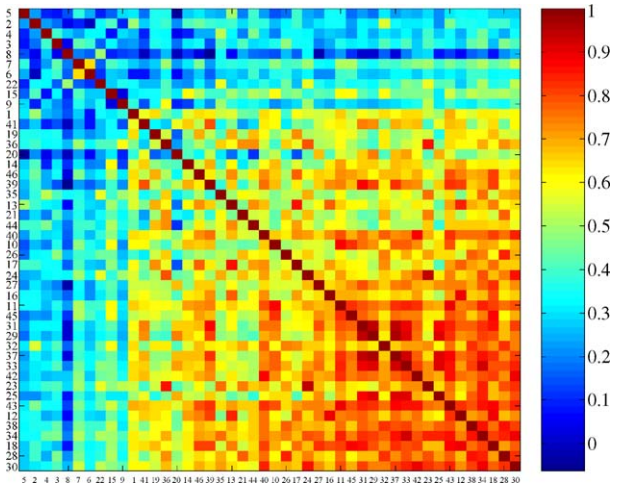

Fig. 5. Color map of Pearson's correlation coefficients between the normalized retention times τ of the 68 substances on 46 systems, with the systems ranked according to their importance in the AAMRT method (Fig. 4).

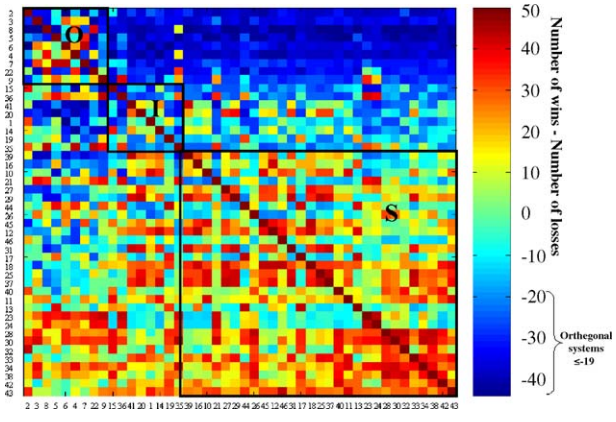

Fig. 6. Color map based on the number of wins minus the number of losses obtained using the GPCM method with McNemar's test as selection criterion; systems ranked according to decreasing orthogonality ratios. O, I, S = orthogonal, intermediately orthogonal and similar systems, respectively.

and (b) leads to the definition of −19 (=−45 + 0.291 × 90) as the threshold below which systems are considered orthogonal. When acting as supervisor, a system does not obtain a number. Since no system can be more equal than to itself, it is arbitrarily given the highest number, i.e. above 45, and 50 was applied.

Another criterion to express the orthogonality between systems is the orthogonality ratio (OR) [14]. To calculate this ratio for a given system, the values below or equal to −19 were replaced by −1, and those above by +1 (referring to orthogonality and similarity, respectively). Then, the number of −1 occurrences was calculated for each system, and its orthogonality ratio was obtained dividing this number by 45 (=46 − 1), and multiplying with 100%. The systems in the color map of Fig. 6 were ranked based on decreasing orthogonality ratios. High ratios refer to systems exhibiting orthogonality to most other systems, while low values indicate similarity to most (Table 4). These OR-values allow selecting an orthogonal subset (Table 3).

Comparing the selection to that of the reference method (Table 3), it can be concluded that the orthogonal subset formed with the first nine systems, i.e. those having orthogonality ratios of ≥60% in Table 4, contains all of group VI of Fig. 1a, except for CS15 and 20. In case also the following eight systems, exhibiting OR-values between 30 and 60% (Table 4) are included, CS15 and CS20 are selected as well as some of the other systems appearing in the reference method selection. The latter originate from the similar groups (II–V). Fourteen of the 17 systems thus also appeared in the selection of the reference method.

To conclude, it can be stated that the GPCM method applying McNemar's statistical test and the related OR-values give a comparable orthogonal subset selection as the reference for the studied data. Using the orthogonality ratio as a criterion to select systems makes the color map less important. This is because the ranking method provides an ordering of the systems from most orthogonal to most similar. However, as in the Kennard and Stone or the AAMRT approach, no similar systems are derived.

Table 4
Orthogonality ratios (OR) with the 46 systems

CS	OR
2	91.1
3	91.1
8	91.1
5	88.9
6	88.9
4	86.7
7	84.4
22	80.0
9	68.9
15	57.8
36	53.3
41	51.1
20	44.4
1	33.3
14	33.3
19	31.1
35	31.1

Orthogonal (≥60%) and intermediately orthogonal (30–60%) systems. Intermediately orthogonal values are given in italics.

4. Conclusions

The applied reference method to select an orthogonal subset, i.e. WPGMA with dissimilarity criterion $1 - |r|$, and the resulting *r*-color map with the systems ranked according to decreasing dissimilarities in the dendrogram, remains our preferred approach for selecting orthogonal and similar systems. Both the tree and the color map indicate the orthogonal and groups of similar systems. It was possible to define a uniform dissimilarity level in the dendrogram, allowing a distinction between the same groups as observed in the color map. Within a group of similar systems that with the highest individual dissimilarity in general also has the lowest correlation coefficient with the other systems, i.e. is most orthogonal. All other hierarchical clustering methods were found somewhat less good for one or more of these criteria. The results of the UPGMA technique correspond best with the conclusions of the reference method.

The three ranking techniques, i.e. the Kennard and Stone algorithm, AAMRT and the GPCM method with McNemar's test allowed selecting the most orthogonal systems. These techniques, which focus most on orthogonal system selection, resulted in a quite similar subset. Their disadvantage is that groups of similar systems are not considered. Therefore the construction of a color map for these methods is less relevant.

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