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Ganoderma species discrimination by dual-mode chromatographic fingerprinting: A study on stationary phase effects in hydrophilic interaction chromatography and reduction of sample misclassification rate by additional use of reversed-phase chromatography

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ABSTRACT

Acetonitrile–water extracts of several *Ganoderma* species – a mushroom being used in Traditional Chinese Medicine – were analysed by liquid chromatography–UV detection in hydrophilic interaction chromatography (HILIC) and reversed-phase (RP) elution modes. A set of six polar stationary phases was used for HILIC runs. These columns had remarkably different separation properties under binary gradient conditions as evinced by hierarchical cluster analysis on retention patterns of seven test compounds. Complementary measurements of RP chromatograms were carried out on a C_{18} packing. Injection precision ($n = 5$) and intra-day precision ($n = 5$) were each <2.0% RSD (HILIC) and <0.7% RSD (RP) for relative retention times of main characteristic peaks of a sample extract while for relative peak areas RSD values were max. 6.8%. Repetitive analysis ($n = 7$) of a processed sample stored in the autosampler tray for 48 h was used to confirm within-sequence sample stability. Eleven *Ganoderma lucidum* samples served as training set for the construction of column-specific simulated mean chromatograms. Validation with twelve samples comprising *G. lucidum*, *Ganoderma sinense*, *Ganoderma atrum*, and *Ganoderma tsugae* by correlation coefficient based similarity evaluation of peak patterns showed that a discrimination of *G. lucidum* from other *Ganoderma* species by means of chromatographic fingerprints is conceptually possible on all columns, except of a bare silica packing. The importance of the combined use of RP and HILIC fingerprints to improve the rate of correct sample classification was demonstrated by the fact that each one *G. sinense* specimen was wrongly assigned being *G. lucidum* by all HILIC fingerprints but not the RP fingerprint and *vice versa*. The present data revealed that (i) the analysis of complex biological materials by quasi orthogonal chromatographic modes such as HILIC and RP may deliver more discriminative information than single-mode approaches which strengthens the reliability of fingerprint-based sample classification and (ii) different retention and selectivity characteristics of polar bonded silica packings in the HILIC elution mode may only have a minor impact on chemometric sample discrimination capabilities in such kind of pattern-oriented metabolomics separation problems.

1. Introduction

Positive effects on human health exerted by the intake of certain plant and mushroom preparations are known since ancient times and this knowledge forms the basis for many health-promoting and therapeutic concepts in the framework of Traditional Chinese Medicine (TCM). Today, TCM is popular throughout the world [1]. Identification, standardisation, and quality control of biological materials and extracts prepared thereof are major tasks faced by manufacturers and health authorities as well [2,3]. Mixture-effects often seem to constitute the pharmacological (but also toxicologically relevant) properties of TCM preparations and therefore,

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one particular analytical challenge in the adequate characterisation of traditional remedies resides in the need for a quasi-holistic approach.

To circumvent comprehensive compound identification and absolute quantification in routine work, which would for instance require information-rich detection methods such as MS^n or the availability of reference standards, while still coping with the complexity of biological materials, a "fingerprint analysis" is of viable means [3–13]. Thereby, neat or processed samples are characterised either directly by spectroscopic methods (e.g. IR, NMR) or by separation techniques (chromatography, capillary electrophoresis) hyphenated with selective (e.g. UV/VIS, MS) or almost non-selective (e.g. ELSD) detectors. Chemometric tools are then being applied on raw data in order to discriminate samples by comparison with a reference fingerprint (pattern approach). Meanwhile fingerprint analysis is internationally largely accepted for (batch-)quality control of herbal and fungal preparations and it can also be used for the differentiation of samples by species or geographical origin.

Separation techniques reduce sample complexity by spreading the information content in the time domain and therefore they are particularly valuable for fingerprint analysis. Yet, very polar but also non-polar as well as charged and non-charged compounds are usually present in biological specimens and, intrinsically, the analyte profile actually coverable by a single separation method is limited. For example, the use of reversed-phase (RP) columns in liquid chromatography (LC) is suitable for the separation of moderately polar to non-polar compounds, but very hydrophilic solutes will (co-)elute in the early part of the chromatogram. Consequently, fingerprint information may likely get lost for such compounds and the RP peak profile basically reflects the sample composition with respect to hydrophobic solutes. The selectivity window of RP chromatography may be increased by using stationary phases which have an ionisable or permanently charged group incorporated in the hydrophobic ligand [14–17]. This allows for the addition of strong electrostatic molecular distinction to the base RP separation process. Compared to single-mode RP packings such kind of 'mixed-mode' columns have their application range extended to polar charged compounds. However, under typical RP elution conditions low retention is still obtained for non-charged polar solutes.

Complementary, one may use these mixed-mode materials [14,18,19] or any type of polar packings [20–22] in the hydrophilic interaction chromatography (HILIC) elution mode. HILIC is targeted towards the retention of polar compounds in general. In contrast to RP eluents, HILIC mobile phases are characterised by being rich in (non-protic) organic solvent (usually acetonitrile), they have water (or other polar protic solvents [23]) added as the strong eluting modifier, and they usually contain mM amounts of organic salts (e.g. ammonium salts of formic or acetic acid). The mechanisms that generate retention on polar chromatographic materials when being operated under such elution conditions are not yet fully elucidated. A plausible model is that analytes partition between a dynamically adsorbed polar protic layer and the less-polar mobile phase and that, additionally, adsorptive interactions (e.g. hydrogen bonding) develop between analytes and ligand functionalities or the sorbent surface.

Thus, the compound spectrum available for the characterisation of complex biological samples may be considerably extended by using a dual chromatographic approach in which (quasi-) orthogonal separation modes such as RP and HILIC are combined (on-line or off-line) [22,24–30]. Surprisingly, chromatographic fingerprinting of plant/fungal samples by LC methods is almost exclusively based on the use of RP columns, and only very recently the characterisation of an herbal extract by both RP and HILIC was reported [31]. In the course of the present study it was regarded necessary to explore besides RP in some more detail the potential of HILIC for being applicable for such kind of analytical problem.

In contrast to the stationary phases typically used for RP separations, there is much greater diversity in the phase chemistry of chromatographic materials applicable in the HILIC elution mode. Polar column packings range from non-functionalised silica to silicas bonded with different polar non-chargeable, or chargeable, or permanently charged ligands (anionic, cationic, amphoteric). This diversity is reflected in widely varying retention and selectivity characteristics, especially when charged solutes and charged chromatographic ligands are considered [14]. While such inter-column differences provide flexibility in target-orientated method development their impact on pattern recognition-based non-targeted analytical problems such as chromatographic fingerprinting is difficult to estimate. One particular aim of the present work was therefore to investigate HILIC stationary phase effects in the framework of fingerprint-based sample discrimination capability and to compare the results to a chemometric column classification obtained with a small set of compounds. For this purpose six polar columns were comparatively tested and the analysis of *Ganoderma* extracts served as a real-world example.

The genus *Ganoderma*, a mushroom, comprises more than 50 species to some of which health-promoting and therapeutic properties are attributed [32–36]. In particular, preparations made of strains of *Ganoderma lucidum* and related species have a long history as dietary supplements and curing agents in Asian folk medicine. Positive action on disease states such as migraine, diabetes, asthma, hypertension, and hepatitis are reported. Recently, researchers became strongly interested in the pro-apoptotic and anti-proliferative effects exerted by *Ganoderma* preparations which may hold promise in cancer therapy.

Fingerprint analysis of *Ganoderma* for the purpose of quality control or the discrimination of samples by geographical origin, species or strain was in the scope of a few earlier studies [37–41]. In case LC was employed it was limited to the use of RP packings [38,39,41]. Main compound classes identified in mycelia and fruiting bodies of *Ganoderma* include non-/less polar (e.g. triterpenoids) but also very polar substances (e.g. polysaccharides, nucleosides). Thus, a combined sample characterisation by HILIC and RP seems to be particularly advantageous in order to cope better with this high degree of sample complexity. In the present work, acetonitrile–water extracts of *Ganoderma* specimens were analysed by LC–UV separately in HILIC and RP elution modes to evaluate orthogonality of the obtained fingerprints from a practical point of view. The peak profiles were input data for the development of a chemometric approach to probe the differentiation of *G. lucidum* from some other *Ganoderma* species by similarity analysis of chromatograms.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

HPLC grade acetonitrile (ACN) was purchased from VWR International (Vienna, Austria). Ammonium acetate (analytical grade) and chromatographic test compounds for HILIC column characterisation (benzyltrimethylammonium chloride, cytidine, guanosine, 4-hydroxybenzoic acid, 4-hydroxybenzenesulfonic acid, tyramine, tyrosine) were supplied by Sigma–Aldrich (Vienna, Austria). Water was bidistilled in-house.

2.1.2. Chromatographic set-up

All chromatographic runs were carried out on a 1200 Rapid Resolution LC system from Agilent Technologies (Waldbronn, Germany) equipped with a diode array detector. Instrument control

Table 1
Physico-chemical properties of the evaluated silica-based polar column packings.

Brand name	Particle size (μm)	Pore size (Å)	Polar bonding[a]
Daisogel	5	120	– (bare)
TGO (home-made)	5	120	C_5 ligand with sulfoxide and diol group
Luna HILIC	5	200	Ethylene-bridged diol ligand
TSKGel Amide-80	5	80	Carbamoyl-type ligand
ZIC-HILIC	5	200	Sulfobetaine-type ligand
Polysulfoethyl A	5	300	Poly(2-sulfoethyl aspartamide) ligand

[a] According to respective manufacturer, for description of home-made TGO packing, see Refs. [23,42].

Table 2
Ganoderma samples analysed in the present study.

Species	Commercial name (China)	Origin (all China)	Sample no.
Training set			
G. lucidum	Golden Lingzhi	Yinshan, Hubei	8
G. lucidum	Golden Lingzhi	Taishan, Shandong	9, 10
G. lucidum	Lingzhi	Longquan, Zhejiang	4, 7
G. lucidum	Lingzhi No. 6	Huangshan, Anhui	6
G. lucidum	Lingzhi G8	Jinzhai, Anhui	2
G. lucidum	Korean Lingzhi	Jiaxiang, Shandong	1, 5
G. lucidum	Korean Lingzhi	Jingdangpu, Shandong	11
G. lucidum	Taishan Lingzhi	Wangdangpu, Shandong	3
Validation set			
G. lucidum[a]	Lingzhi	Lijiang, Yunnan	21, 22
G. sinense[a]	Zizhi	Jinzhai, Anhui	19
G. sinense[a]	Zizhi	Lijiang, Yunnan	13, 14
G. sinense	Zizhi	Wuyishan, Fujian	12, 18
G. atrum[b]	Bao-zi-fen	Ganzhou, Jiangxi	15
G. atrum	Black Lingzhi	Ganzhou, Jiangxi	16, 17, 20
G. tsugae	Songshan Lingzhi	Wangdangpu, Shandong	23

[a] Wild samples.
[b] Broken spores.

and data acquisition were carried out with the Chemstation software (version B.04.01).

Commercially available 150 mm × 4.6 mm ID columns packed with Luna HILIC (Phenomenex, Aschaffenburg, Germany), Polysulfoethyl A (PolyLC, Columbia, MD, USA), TSKGel Amide-80 (Tosoh Bioscience, Stuttgart, Germany), and ZIC-HILIC (SeQuant, Umea, Sweden), as well as 150 mm × 4.0 mm ID stainless steel columns packed in-house with commercial bare silica (Daisogel supplied by Daiso Chemical, Osaka, Japan) and a home-made polar functionalised Daisogel material named TGO [23,42] were used for runs in the HILIC elution mode. Some characteristic properties of these polar phases are summarised in Table 1.

The columns were protected by a 10 mm × 2.1 mm ID precolumn from Thermo Fisher Scientific (Waltham, MA, USA) containing a C_4-RP material. Chromatographic runs in the RP elution mode were conducted on a Zorbax SB-C_{18} column (100 mm × 2.1 mm ID, 1.8 μm particle size, 80 Å pore size) from Agilent Technologies which was protected by an in-line filter (2.0 mm ID, 0.2 μm pore size) from the same manufacturer.

2.1.3. *Ganoderma* samples

The fruiting bodies of 23 *Ganoderma* batches comprising *G. lucidum*, *Ganoderma atrum*, *Ganoderma sinense*, and *Ganoderma tsugae* were collected from different locations of China in the period of May 2007–May 2008 (Table 2).

Morphological species identification was carried out by Dr. Zhihong Fu (Jiangxi University of Traditional Chinese Medicine, China). Samples for chromatographic analysis were collected from the pileus of cultivated fruiting bodies, except of specimen nos. 13, 14, 19, 21, and 22 which were taken directly from *Ganoderma* fruiting bodies grown in nature. Sample no. 15 consisted of broken spores. The collected specimens were dried at 60 °C for 12 h and cut into small pieces.

2.2. Methods

2.2.1. Characterisation of polar column packings in the HILIC elution mode

Separation properties of various polar columns (Table 1) in the HILIC elution mode were evaluated using a set of seven test solutes (for structural formulas see Fig. 1).

Elution was carried out using a binary gradient with 10 mM ammonium acetate in ACN/water (50:50, v/v) (solvent channel A) and ACN/water (95:5, v/v) (solvent channel B). The time program was as follows: 0 min: 100% B, 5 min: 100% B, 40 min: 0% B, 45 min: 0% B, 45.1 min: 100% B, 60 min: 100% B. In order to standardise the gradient conditions for the individual columns (column hold-up volume ranging from 1.41 to 1.81 mL as determined with the elution time of toluene using 100% B) flow rates between 1.00 and 1.29 mL min^{-1} were adjusted to end up with an equivalent flow velocity of 1.8 mm s^{-1} for each column. To speed up column re-equilibration the flow rate was each increased by a factor of two between 45 and 55 min. The column compartment temperature was kept at 30 °C and the injection volume was 2 μL. Chromatograms were recorded at 254 nm (8 nm bandwidth) using 450 nm (100 nm bandwidth) as reference wavelength.

Hierarchical cluster analysis (HCA) on retention times was performed using Number Cruncher Statistical System software version 07.1.13 (NCSS, Kaysville, UT, USA). The group average technique (non-weighted pair group) was applied for data agglomeration and Euclidean distances were used for similarity measurements.

2.2.2. *Ganoderma* sample extract preparation

1.0 g of sample was transferred to a 30 mL volumetric flask. 20 mL of ACN/water (50:50, v/v) were added and the mixture was extracted twice at 50 °C for each 60 min. Subsequently, suspensions were filtered through filter paper and the whole filtrate was

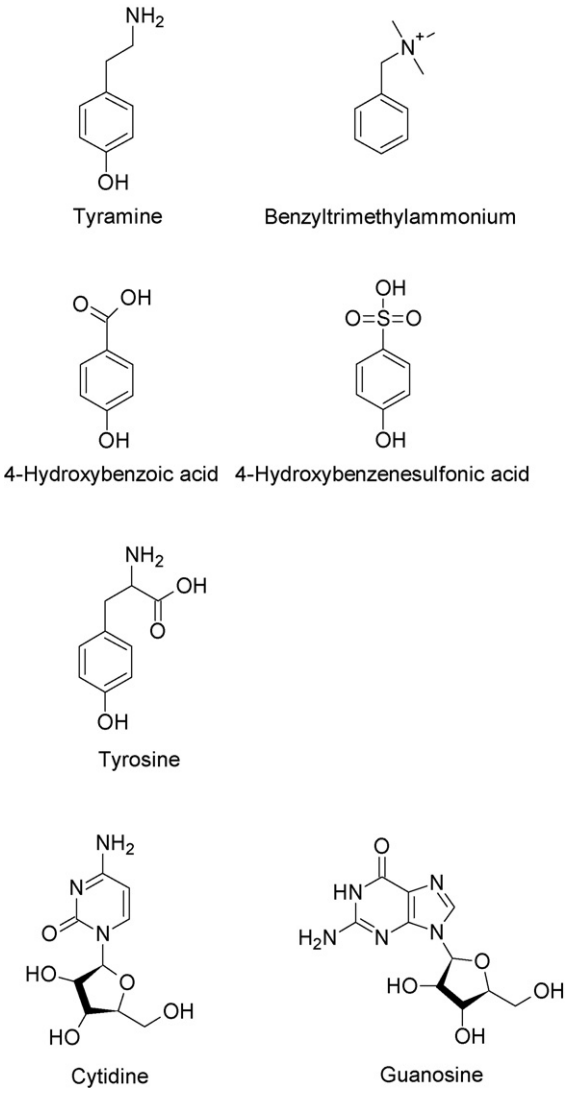

Fig. 1. Structural formulas of the compounds used for column classification in the HILIC elution mode.

evaporated to dryness at 50 °C and 50 mbar. The dried extract was re-dissolved in 1.0 mL ACN/water (50:50, v/v) and the resulting solution was passed through a 0.20 μm nylon membrane filter (Sigma–Aldrich) prior transfer to a glass autosampler vial.

2.2.3. Chromatographic analysis of Ganoderma extracts

Ganoderma sample extracts, prepared according to Section 2.2.2, were analysed in HILIC and RP elution modes. HILIC elution conditions were fully according to those detailed in Section 2.2.1 and each sample extract was run on each polar column. RP runs were conducted on the C_{18} column using a binary gradient with 10 mM ammonium acetate in ACN/water (1:99, v/v) (solvent channel A) and 10 mM ammonium acetate in ACN/water (95:5, v/v) (solvent channel B) as mobile phases. The time program was as follows: 0 min: 10% B, 1.5 min: 10% B, 2 min: 30% B, 15 min: 33% B, 20 min: 35% B, 25 min: 100% B, 45 min: 100% B, 45.1 min: 10% B, 60 min: 10% B. The flow rate was kept at 0.20 mL min^{-1} for the first 15 min, then increased to 0.30 mL min^{-1} within 5 min and kept at 0.30 mL min^{-1} for 25 min before re-equilibrating for 15 min at 0.20 mL min^{-1}. The column compartment temperature was kept at 50 °C and the injection volume was 2 μL. Chromatograms were recorded at 254 nm (8 nm bandwidth) using 450 nm (100 nm bandwidth) as reference wavelength.

2.2.4. Method validation

Ganoderma sample no. 14 (cf. Table 2) was used to validate the HILIC and RP methods with respect to the relative standard deviation (%RSD) of relative retention times (RRT) and relative peak areas (RPA) of some characteristic peaks. In the HILIC elution mode, the ZIC-HILIC column was used a representative for the other polar columns packings. Injection precision was determined by replicate injection ($n = 5$) of a processed sample. Injection of five different sample solutions prepared independently was used to assess intra-day method precision. Stability of processed sample solutions during storage in the autosampler was checked by repetitive injection over a period of 48 h (0, 2, 4, 6, 8, 24, 48 h post-extraction).

2.2.5. Chemometric species differentiation

Similarity analysis of chromatograms was carried out using the software "Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine" (Version 2004 A), which is recommended by the National Commission of the Chinese Pharmacopoeia. The raw chromatographic data were imported in the form of *.cdf-files into the software. In order to focus on the most discriminative information time windows of 0–32 min (HILIC) and 5–42 min (RP), respectively, were used for chemometric analysis. Variations in retention times were corrected by the target peak automatic alignment procedure of the software. A mean chromatogram of *G. lucidum* (chromatographic fingerprint consisting of the "common" peak pattern) was constructed from sample nos. 1–11 (training set; see Table 2). Sample nos. 12–23 comprised different *Ganoderma* species and served as validation set for chromatographic fingerprint-based species discrimination. Correlation coefficients of individual sample chromatograms relative to the simulated mean chromatogram were calculated according to Eq. (1):

$$r = \frac{\sum_{i=1}^{n}(x_i - x_m)(y_i - y_m)}{\sqrt{\sum_{i=1}^{n}(x_i - x_m)^2 \sum_{i=1}^{n}(y_i - y_m)^2}} \quad (1)$$

where x_i and y_i represent the peak areas of the ith peak in the individual chromatogram x and the calculated reference chromatogram y, and n is the total number of peaks in the chromatograms. x_m and y_m are median values of peak areas for n peaks in chromatograms x and y, respectively. Correlation coefficients were used as descriptor for similarity of the chemical character of the various *Ganoderma* samples.

3. Results and discussion

3.1. Characterisation of polar column packings in the HILIC elution mode

The dominant interactions in the HILIC elution mode (partitioning/adsorption, weak/strong electrostatic interactions) may strongly depend on the type of analyte, the type of stationary phase, and the chosen elution conditions. This complexity sometimes hampers straightforward method development. The design of a HILIC column classification test able to adequately characterise the whole spectrum of interactive mechanisms becomes a particularly difficult task as well. Not least due to this reason and despite the still growing interest in HILIC, systematic column classifications did not yet appear in the literature.

In the framework of the present study it was first of interest to get an idea about the separation properties of the selected polar columns towards a small set of compounds. The elution conditions were identical to those used for *Ganoderma* sample extract analysis (10 mM ammonium acetate in acetonitrile–water gradient, no pH adjustment) in order to fulfil one of the main aims

of the present study, i.e. comparing separation properties of polar columns obtained by this targeted approach with non-targeted fingerprint data while keeping other experimental variables constant.

Retention times of weak/strong bases (tyramine, benzyltrimethylammonium chloride), weak/strong acids (4-hydroxybenzoic acid, 4-hydroxybenzenesulfonic acid), an amphoteric compound (tyrosine), and two essentially non-charged solutes (cytidine, guanosine) were measured. Structural formulas of the test compounds are depicted in Fig. 1. This set is characterised by considerable physico-chemical heterogeneity, which was deemed necessary to adequately cover ionic and non-ionic retention increments that may potentially develop between stationary phase and the solutes.

The investigated chromatographic materials involved bare silica and five silicas bonded with different polar functionalities (Table 1). One packing, named TGO, was prepared in-house. This stationary phase is characterised as silica functionalised with a C_5 ligand which carries a diol motif and a sulfoxide functionality [23,42]. Two of the commercial bonded packings are described by the manufacturers to be of ethylene-bridged diol-type (Luna HILIC) and carbamoyl-type (TSKGel Amide-80), respectively. Like the TGO bonding, also the two latter modifications are considered to be non-charged. One column contains a zwitterionic sulfobetaine-type ligand bonded to silica (ZIC-HILIC). As a representative for the considerable number of commercially available chargeable HILIC materials a poly(2-sulfoethyl aspartamide)-type bonded phase (Polysulfoethyl A), i.e. a hydrophilic strong cation exchanger, was selected.

Tyrosine ($\log D = -2.4$ to -2.1 calculated at 25 °C in a range of pH 5–7; data taken from SciFinder scholar database whereby all values were calculated with ACD/Labs Software V8.14, Advanced Chemistry Development Inc., Toronto, Canada) was eluted latest on all columns (retention time t_R window 16–21 min). Cytidine ($\log D^{pH\,5-7} = -1.9$) typically eluted 0.3–2 min before guanosine ($\log D^{pH\,5-7} = -1.7$; t_R window 13–20 min) except of the bare silica material where reversal of the elution order was observed (cf. our earlier results on this analyte pair [42]). If the interpretation of the retention patterns on the set of polar columns is limited to these three analytes it appears that compound polarity in terms of the calculated $\log D$ value is a suitable descriptor for the extent of retention which may thus indicate a partitioning-based mechanism. However, a pronounced scatter in the retention pattern of anionic and cationic solutes, which overall delivered a specific elution order for each column, showed that such a concept is far too simple to adequately describe the HILIC retention process.

To visualise similarities in the separation patterns of the various columns chemometric data treatment by HCA was performed on retention times (Fig. 2).

Several clusters were obtained whereby the bare silica column was found to be very dissimilar from the other columns. For example, the retention of benzyltrimethylammonium cation was 17.0 min on bare silica but 3.4–12.8 min on the polar bonded packings. Benzyltrimethylammonium cation does not possess any polar functionality except the permanent positively charged ammonium group. On bare silica strong electrostatic attractive interactions occurring between the cationic moiety and dissociated surface silanols may likely be the dominant factor for retention. On the other hand, the exceptional low retention of benzyltrimethylammonium cation on non-charged bonded Luna HILIC ($t_R = 3.4$ min) indicates not only that hydrophilic interactions are largely absent on this packing but also that its cross-linking phase chemistry [43] is an effective shielding to prevent background silanophilic interactions. Such an interpretation agrees well with the retention trend of the quaternary amine thiamine on a larger set of non-charged bonded packings, which could be correlated with the 'silanol activity at pH 7' of these phases measured in the RP elution mode [42]. Opposed to benzyltrimethylammonium cation the weak base tyramine was considerably stronger retained on Luna HILIC ($t_R = 10.5$ min) which is explained by a hydrophilic interaction retention increment superimposing the (weak) silanol–solute interactions on this bonded phase. Due to the fact that on bare silica retention times of these two analytes differed by just 0.6 min a pronounced effect of the bonding on chromatographic selectivity was revealed as well.

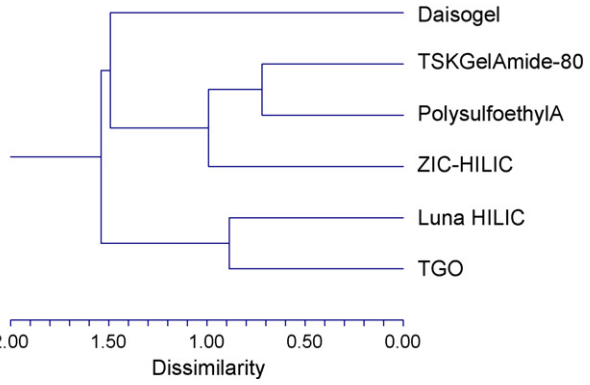

Fig. 2. Classification of polar column packings by hierarchical cluster analysis (HCA). Retention times of the analyte test set (cf. Fig. 1) were used as input parameters. Chromatographic conditions are specified in Section 2.

Opposed to the retention trend of basic solutes, 4-hydroxybenzenesulfonic acid and 4-hydroxybenzoic acid both eluted earlier on bare silica than on the bonded packings. This finding was ascribed to the presence of a larger degree of negative charge on bare silica which causes ion exclusion, i.e. repulsion, and as a consequence antagonism to hydrophilic interactions. Overall, for both cationic and anionic solutes strong electrostatic forces seem to be a prime factor in determining the extent of retention on bare silica under 'typical' HILIC elution conditions.

The most similar columns as delivered by HCA were TSKGel Amide-80 and Polysulfoethyl A. This finding is somehow unexpected as TSKGel Amide-80 is considered to be a non-charged bonded polar phase while Polysulfoethyl A is a hydrophilic strong cation exchanger, although with a non-specified density of $R–SO_3^-$ groups. However, in accordance with earlier interpretations [14,44–46] also the present results indicate that TSKGel Amide-80 has a certain net acidic character (probably stemming from the ligand bonding approach). Ionic forces may thus complement the hydrophilic interaction separation mechanisms provided by the adsorbed water layer and polar ligand functionalities, respectively. A certain relationship of ZIC-HILIC to the two aforementioned packings may be explained by the presence of charged groups in the ligand which may exert strong attractive or repulsive electrostatic interactions with charged analytes. On the other hand, the significant chemometric dissimilarity of these three packings from bare silica demonstrated that a phase with a high degree of available silanols still delivers a different retention profile. This finding points towards the general importance of solute–ligand interactions in the HILIC elution mode (with respect to both strong and weak adsorptive forces) and highlights that a distinct contribution of solute–silanol interactions should not be overlooked in interpreting the retention and selectivity characteristics of bonded phases (*vide infra*).

A distinct grouping from TSKGel Amide-80, Polysulfoethyl A, and ZIC-HILIC was found for the TGO column and Luna HILIC and these two 'diol-type' phases form a separate cluster. Tyrosine, guanosine, cytidine and tyramine were each 1.5–2.5 min stronger retained on TGO, while benzyltrimethylammonium cation eluted

Table 3
HILIC (using the ZIC-HILIC column) and RP elution modes precision (injection, intra-day) and 48 h post-preparation sample stability of sample no. 14 (*G. sinsense*).

Peak no.[a]	Injection precision[b]		Intra-day precision[b]		48 h stability[c]	
	%RSD RRT[d]	%RSD RPA[e]	%RSD RRT	%RSD RPA	%RSD RRT	%RSD RPA
HILIC						
1	1.78	4.12	1.95	3.84	0.34	1.79
2	1.32	5.79	0.23	6.95	0.16	3.94
3	0.65	2.36	1.56	2.78	0.38	3.21
5	0.53	2.48	0.67	2.96	0.12	2.05
RP						
1	0.66	1.09	0.68	3.59	1.23	3.65
2	0.51	2.44	0.43	3.43	1.16	1.53
3	0.40	3.1	0.53	5.18	0.73	1.87
5	0.54	2.03	0.56	4.85	0.79	4.76
6	0.79	2.65	0.69	6.78	1.87	4.23

[a] Peak no. 4 was each used for normalisation.
[b] $n = 5$.
[c] Seven time points.
[d] Relative retention time.
[e] Relative peak area.

later by more than 6 min. On the other hand, 4-hydroxybenzoic acid was only slightly stronger retained on TGO and for 4-hydroxybenzenesulfonic acid stronger retention was observed on the Luna HILIC column. In case retention on the two phases is mainly determined by polarity-related hydrophilic interactions one would expect the much more polar 4-hydroxybenzenesulfonic acid ($\log D^{\mathrm{pH\,5-7}} = -5.2$) to elute considerably later than 4-hydroxybenzoic acid ($\log D^{\mathrm{pH\,5-7}} = 0.85$ to -0.94). Ligand-selective retention increments may in part be responsible for the deviation observed on TGO but, overall, the retention behaviour of this strong acid but also of the strong base benzyltrimethylammonium cation is quite plausibly explained by a different background of accessible silanols on TGO and Luna HILIC (earlier characterised to be significantly higher for TGO [42]). Although the large fraction of ACN as well as the non-adjusted pH of the mobile phase renders an estimation of the degree of ionisation of both silanols and acidic solutes difficult one can assume that at least 4-hydroxybenzenesulfonic acid is almost fully dissociated under the chosen conditions and thus its retention will respond strongly to the presence of residual negative surface charge. Consequently, it appears that a systematic study of retention patterns of charged solutes may deliver descriptors for silanol–solute interactions in the HILIC elution mode, at least for non-charged bonded phases. Parallel work is devoted to this problem and the results will be reported in due time.

At this point, the reader should bear in mind that no data are yet available to which extent a change of elution conditions (e.g. switch to isocratic elution mode; effect of pH, type/amount of salt, type/amount of protic modifier; temperature) will affect the chemometric column classification plotted in Fig. 2. Whether the retention profile of the chosen compound mixture is able to adequately reflect the plurality of interactions between solute and adsorbed water layer/chromatographic ligand/sorbent surface is currently unknown as well. However, for the purpose of the present study valuable information was obtained on the different separation properties of the selected columns. Based on this data set it was interesting to study whether the observed column dissimilarities in this targeted approach are reflected in the abilities of the individual columns to differentiate samples by means of chemometric similarity analysis of chromatograms (non-targeted approach).

3.2. Extraction procedure

To allow for a comprehensive characterisation of *Ganoderma* specimens by both HILIC and RP a sample preparation protocol was required which was suitable to extract compounds of a broad polarity range. A preliminary experimental series with extraction solvents composed of different mixing ratios of ACN and water revealed that the gross peak pattern was not significantly affected when the volume fraction of water was varied within 25–75%. The final method was developed using a mixture of ACN and water (50:50, v/v) in a 1:20 (w/v) sample-solvent ratio (60 min extraction, 50 °C). ACN/water (50:50, v/v) sample solutions concentrated by a factor of 40 compared to the original extract volume were used for injection. These solutions had higher elution strength compared to the starting gradient conditions in both HILIC (mobile phase consisting of 5%, v/v, water at 0 min) and RP (mobile phase

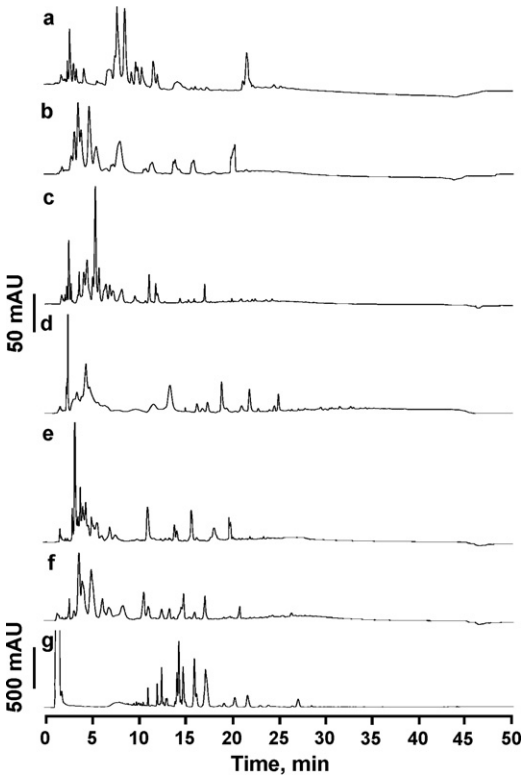

Fig. 3. Separation of *G. lucidum* sample no. 3 on the set of stationary phases investigated in the present study: (a) Daisogel, (b) TGO, (c) Luna HILIC, (d) TSKGel Amide-80, (e) Polysulfoethyl A, (f) ZIC-HILIC and (g) C_{18} packing. Chromatographic conditions are specified in Section 2.

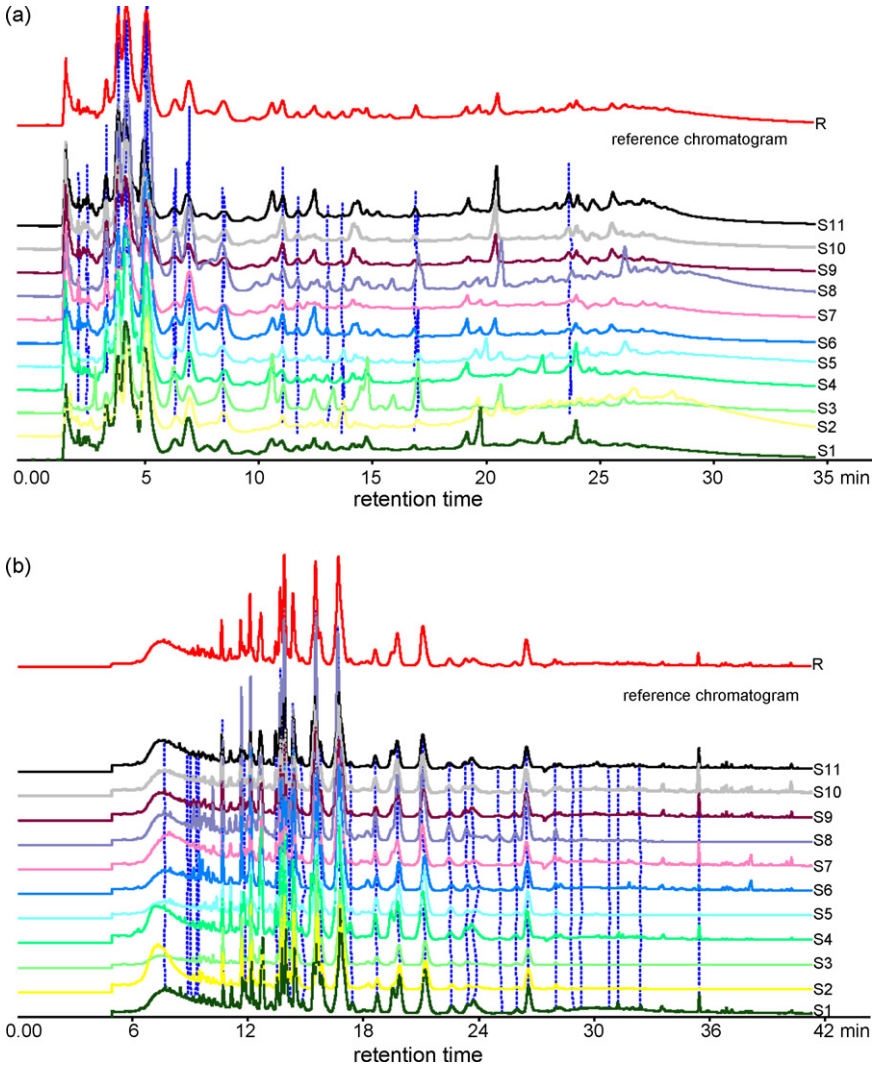

Fig. 4. Individual chromatograms of *G. lucidum* specimens (training set, i.e. sample nos. 1–11) and reference chromatogram as calculated by computer-aided similarity evaluation: (a) HILIC elution mode (ZIC-HILIC column), (b) RP elution mode. Chromatographic conditions are specified in Section 2. Dashed lines indicate peaks matched by the software.

consisting of 10%, v/v, ACN at 0 min). However, the selection of 2 μL as injection volume secured that no significant effects on peak shape or retention time of early eluting solutes occurred as a result of this eluotropic mismatch and the injection volume was already sufficiently high to deliver informative chromatograms in both elution modes.

3.3. Method validation

Validation of the developed HILIC and RP methods focused on assessing the repeatability of the gross peak pattern in terms of RSD values of RRT and RPA for some characteristic peaks. In order to keep the work load in a reasonable range sample no. 14 was selected as a representative for the other specimens and only the ZIC-HILIC column was used for HILIC validation runs. Five (HILIC; t_R-range 4–21 min) and six (RP, t_R-range 14–29 min), respectively, peaks were selected and from each peak no. 4 ($t_R^{\text{ZIC-HILIC}} = 12.5$ min, $t_R^{\text{RP}} = 18.0$ min) RRTs and RPAs were calculated for the other peaks. Injection precision ($n = 5$), intra-day precision ($n = 5$), and 48 h autosampler tray stability were investigated. Table 3 summarises the obtained data.

Injection precision ($n = 5$) was found to be ≤1.78% RSD (HILIC) and ≤0.79% RSD (RP) for RRT. Corresponding values for RPA were maximal 5.79% RSD (HILIC) and 2.65% RSD (RP), thus indicating a slightly lower run-to-run repeatability for HILIC in terms of RRT and RPA. Intra-day precision ($n = 5$) values were for both elution modes in a similar range for both RRT (0.23–1.95% RSD for HILIC, 0.43–0.69% RSD for RP) and RPA (2.78–6.95% RSD for HILIC, 3.43–6.78% RSD for RP) and an acceptable repeatability of the sample extraction procedure was thereby confirmed. Stability testing of post-extraction sample solutions was carried out within a 48 h period in which a processed solution of sample no. 14 was injected repetitively while stored in the autosampler. RSD values of RRTs below 2% and below 5% for RPAs were not significantly different from the figures obtained for the aforementioned injection precision and intra-day precision and thus acceptable chemical stability of the selected compound peaks was assumed. Overall, the experimental data suggested that the developed extraction procedure and the LC methods were applicable for fingerprint analysis of *Ganoderma* specimens.

3.4. Ganoderma species discrimination by chromatographic fingerprinting

The analysis of *G. lucidum* samples in the HILIC elution mode delivered quite different peak patterns on the various polar

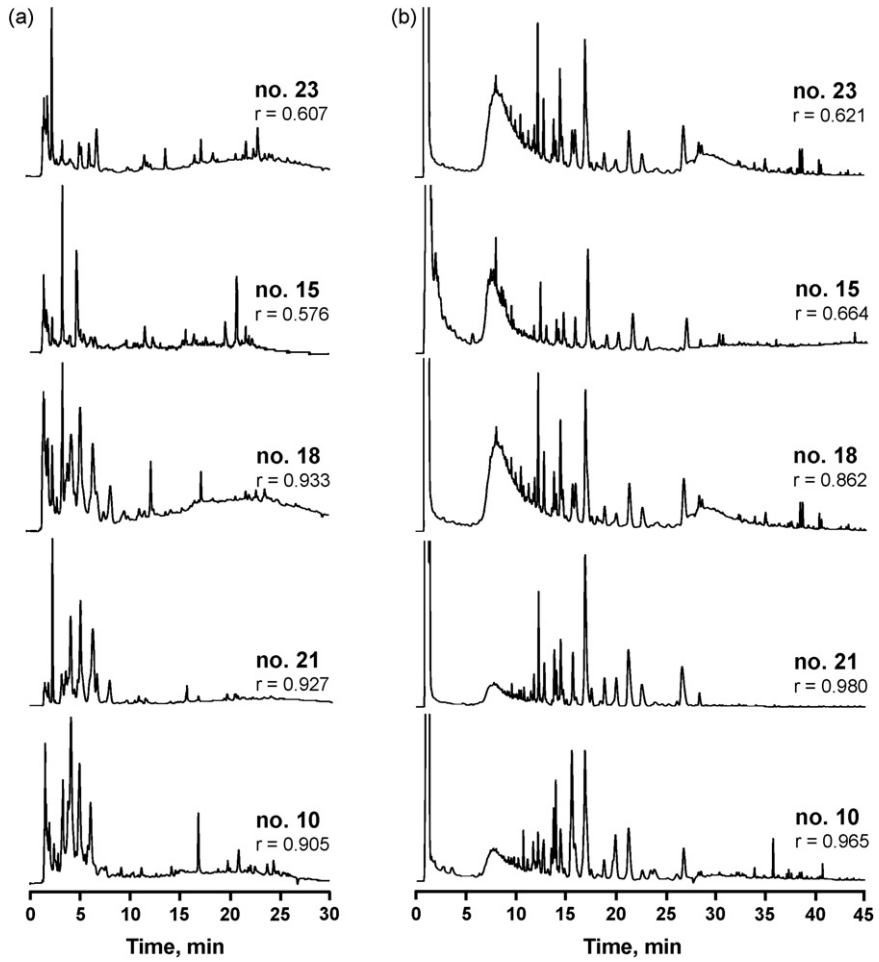

Fig. 5. Chromatograms of sample extracts of *G. lucidum* (sample nos. 10 and 21), *G. sinense* (sample no. 18), *G. atrum* (sample no. 15), and *G. tsuage* (sample no. 23) obtained in the (a) HILIC elution mode (Luna HILIC column) and (b) RP elution mode. *r* = correlation coefficient with respect to the reference chromatogram calculated on the basis of the *G. lucidum* training set chromatograms. All chromatograms are base-peak normalised. Chromatographic conditions are specified in Section 2.

packings (Fig. 3). This finding corroborates the above described column-specific separation properties.

Over the 45 min time scale of the binary gradient the chromatograms of each HILIC phase contained a number of well-retained peaks. Some of them were found to be of critical peak shape, which was one indication for the challenges associated with finding adequate chromatographic conditions for "all" components of complex biological samples. The situation may be improved by a column-specific optimisation of elution parameters, but this was not part of the objectives of the present work. Nevertheless, the gross peak profiles obtained under the selected elution conditions already allowed chemometric fingerprint analysis for all columns.

All chromatograms were also characterised by a bulk of peaks eluting close to the void volume. This was indicative for the presence of less-polar compounds in the sample extracts for which the HILIC elution mode did not provide sufficient retention and selectivity. Such poorly separated peaks are less suitable for chromatographic fingerprinting. Quality control or sample discrimination solely based on HILIC data may thus leave out information about less hydrophilic compounds and, consequently, samples which mainly differ by their composition in less to non-polar solutes may not be adequately characterised by such a single-mode approach. On the other hand, the RP method was of limited selectivity for polar solutes as demonstrated by a large peak eluting at the void time (Fig. 3g). Thus, a combined analysis of the samples in HILIC and RP elution modes was regarded appealing to broaden the accessible compound polarity window in order to end up with more discriminative information for *Ganoderma* sample classification.

Chromatograms of *G. lucidum* sample nos. 1–11 which were collected from some different locations in China (cf. Table 2) were used to construct column-specific simulative mean chromatograms. Fig. 4 exemplarily shows measured and calculated chromatograms as obtained on the ZIC-HILIC column and the RP packing.

Correlation coefficients of each experimental chromatogram to the reference chromatogram were calculated according to Eq. (1) as detailed in Section 2.2.5. With the exception of the bare silica column, correlation coefficients were better than 0.80, typically better than 0.90. Variations of correlation coefficients in terms of %RSD values were 1.3–4.3, while the bare silica column delivered 21.4% RSD (range 0.36–0.88).

It might be argued that the exceptional high variation on bare silica originated from (cationic) sample extract constituents being accumulated on the non-modified silica surface. Such compounds may then either elute in subsequent runs thereby adulterating peak profiles of later injected extracts or, if being irreversibly adsorbed on the surface, they may change the separation properties of the column itself. Supporting evidence for the latter comes from the fact that after less than thirty runs of real samples retention times of some of the test compounds on the bare silica column differed by more than 10% to the original values while such a large deviation was not observed for the bonded phases.

To validate the species selectivity of the developed reference chromatograms two wild *G. lucidum* specimens (both independent

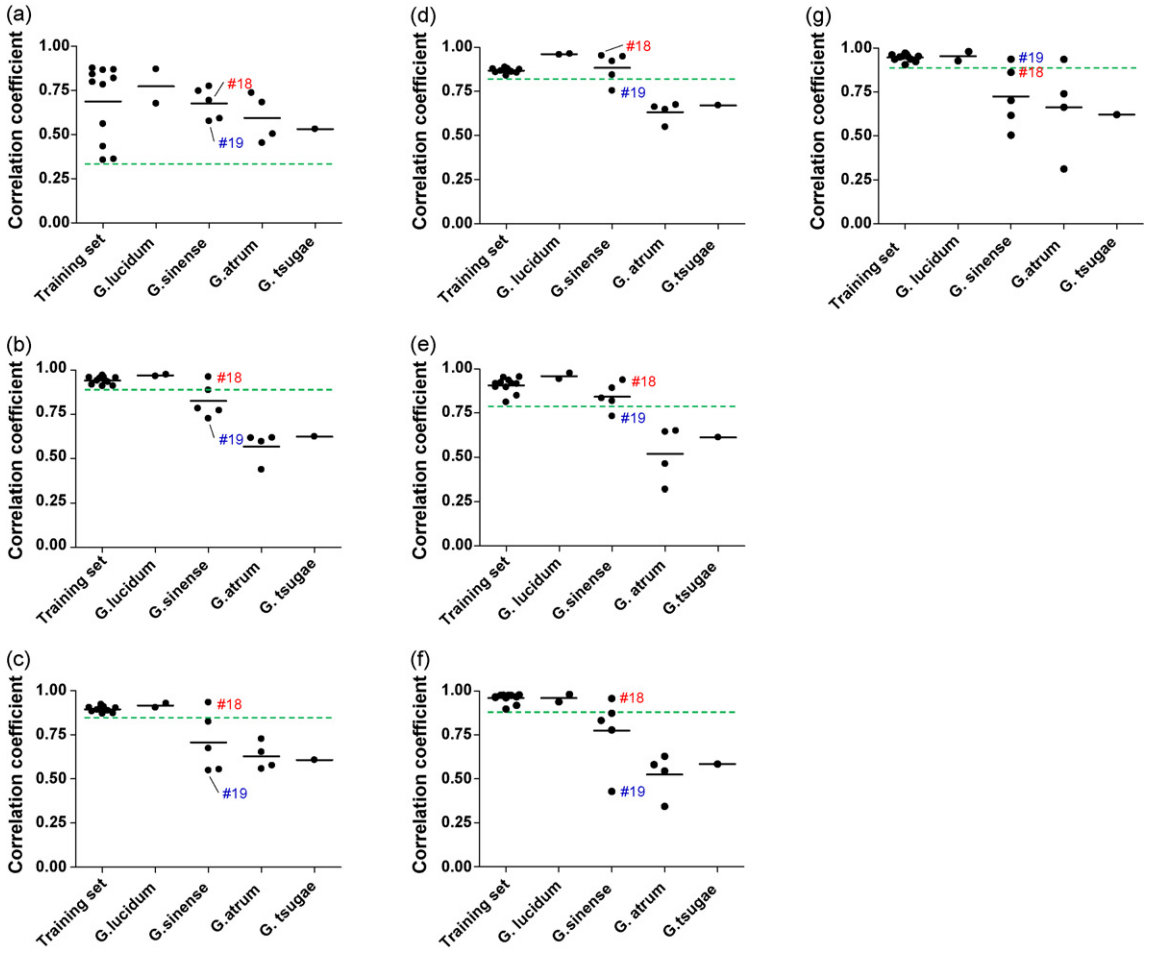

Fig. 6. Scatter plots of column-specific correlation coefficients (i.e. similarity of measured chromatograms to the calculated reference chromatogram of the training set) used for species discrimination: (a) Daisogel, (b) TGO, (c) Luna HILIC, (d) TSKGel Amide-80, (e) Polysulfoethyl A, (f) ZIC-HILIC and (g) C_{18} packing. Solid lines represent the mean correlation coefficient for each *Ganoderma* species and dashed lines represent the value of the lowest correlation coefficient within the *G. lucidum* training set. Points lying below the dashed lines were classified as being "different" from *G. lucidum*.

from the training set) as well as 10 samples stemming from *G. sinense*, *G. atrum*, and *G. tsuage* were measured as well (validation set). As depicted in Fig. 5 for Luna HILIC and the RP column, the extracts of the various *Ganoderma* species gave to some extent different peak patterns. A certain capability for species discrimination by chemometric similarity analysis of chromatograms was therefore anticipated. For this purpose, the lowest correlation coefficient obtained from the *G. lucidum* training set was set as a column-specific cut-off and samples from the validation set having a correlation coefficient to the calculated reference chromatogram below this cut-off were classified as being "different" from *G. lucidum*.

No capabilities to differentiate *G. lucidum* from other *Ganoderma* species could be recognised for the bare silica column, basically due to the aforementioned wide range of correlation coefficients already obtained for the training set (Fig. 6a). On the other hand, a successful discrimination of the four samples of *G. atrum* and the one *G. tsuage* specimen from *G. lucidum* could be achieved on all bonded packings. Additionally, the two independent *G. lucidum* samples were correctly classified because the calculated correlation coefficients were each higher than that of the column-specific cut-off values.

Some limitations were, however, found for *G. sinense* samples, for which correlation coefficients were, except of the Luna HILIC column, higher than that of *G. atrum* and *G. tsuage*. Specifically, *G. sinense* sample no. 18 was wrongly classified as *G. lucidum* by all HILIC fingerprints (Fig. 6). For TSKGel Amide-80 and Polysulfoethyl A results were even worse as each four out of the five *G. sinense* specimens could not be distinguished from the *G. lucidum* reference. This similar behaviour of the two latter columns is somehow a reflection of their chromatographic relationship as has been determined by HCA (*vide supra*). However, overall the HILIC data demonstrated that the type of polar bonded stationary phase had only a minor effect on the capability to discriminate *Ganoderma* samples by means of chromatographic fingerprinting. In other words, a change of the column in HILIC, although potentially leading to quite different peak patterns (cf. Fig. 3), was insufficient to avoid sample misclassification, probably owing to limited orthogonality of the chosen polar packings.

Complementary analysis of the *Ganoderma* sample extracts in the RP elution mode on the high-efficiency C_{18} packing allowed a differentiation of *G. tsuage* and three out of four *G. atrum* samples from *G. lucidum* (Fig. 6g) and the two independent *G. lucidum* samples were correctly identified as well. Sample no. 19 had the highest correlation coefficient within the set of *G. sinense* samples and it was wrongly assigned to be *G. lucidum*. Interestingly, this sample had lowest correlation coefficients on all HILIC columns which indicated that sample no. 19 differs from *G. lucidum* basically by its composition in hydrophilic compounds.

Lumping together these results unveils some limitations of the single-mode chromatographic fingerprint approaches: (i) when

using the bare silica column it was not at all possible to differentiate *G. lucidum* from other *Ganoderma* species, (ii) *G. sinense* sample no. 18 was misclassified by all polar bonded phases in the HILIC elution mode, (iii) *G. sinense* sample nos. 12, 13 and 14 were misclassified by the HILIC fingerprints when using TSKGel Amide-80 or Polysulfoethyl A, (iv) samples nos. 19 (*G. sinense*) and 20 (*G. atrum*) were misclassified in the RP elution mode.

In an attempt to decrease the rate of misclassification observed in the single-mode approaches one may now combine HILIC and RP data. In such a multi-modal concept only samples are assigned being *G. lucidum* when the correlation coefficients in HILIC and RP are each above the cut-off values in the respective chromatographic mode. Following this idea a 100% correct sample discrimination rate was obtained for the present sample set because the critical *G. sinense* specimen no. 18 (misclassification on all polar columns) was correctly classified by the RP fingerprint. Moreover, the *G. sinense* samples which were misclassified on TSKGel Amide-80 and Polysulfoethyl A were correctly differentiated from *G. lucidum* in the RP elution mode. *Vice versa*, *G. sinense* sample no. 19 and the *G. atrum* sample no. 20, which were both assigned to be *G. lucidum* in the RP elution mode, were correctly classified being different from *G. lucidum* by the chromatographic results of all bonded HILIC phases. Thus, irrespectively of the type of polar bonded stationary phase sufficiently orthogonal information to the HILIC fingerprint was always obtained when RP data were additionally taken into account.

From a biological point of view, the results of the chemometric similarity analysis indicated a closer relationship of the chemical composition of *G. lucidum* and *G. sinense* compared to that of *G. lucidum* and *G. atrum* as well as to that of *G. lucidum* and *G. tsugae*, at least with regards to the compounds accessible by the selected LC–UV conditions. However, the remarkable scatter of correlation coefficients obtained for *G. sinense* and *G. atrum* samples (cf. Fig. 6) demonstrated the need for analysing a larger number of specimens in order to cover within-species variation more adequately and, consequently, to obtain more valid criteria for species discrimination when it comes to routine use.

4. Conclusions

Two prime parameters that potentially affect the information content of chromatographic fingerprints were investigated in the present study, viz. 'type of stationary phase' and 'chromatographic mode'.

The chromatograms obtained on the various polar bonded packings were largely similar in terms of their usability for chemometric discrimination of some *Ganoderma* species from *G. lucidum*. In contrast, bare silica turned out to be unsuitable for analysing such complex biological samples under the selected elution conditions. The peak patterns of the real samples differed remarkably between the different polar columns. These findings, along with the column classification based on HCA, indicated a certain degree of orthogonality, which, however, did not strongly affect the fingerprint-based sample discrimination capability. Most samples were correctly classified already in the HILIC elution mode while the additional consideration of RP data was a viable means to end up with a 100% correct sample classification rate in the present application. Thus, instead of using different columns in one chromatographic mode multi-modal fingerprinting in largely orthogonal chromatographic modes is regarded an attractive concept for reaching more comprehensive and objective information about sample composition for the purpose of identification, characterisation, or quality control of TCM-relevant samples and complex biological specimens in general.

As concerns the still barely understood mechanisms that generate retention in the HILIC elution mode the data obtained for the small set of charged and non-charged test compounds provided valuable preliminary information. The results allowed concluding that besides 'hydrophilic interactions' (irrespectively if being based on partitioning of compounds between mobile phase and water-enriched surface layer or weak electrostatic solute–ligand/solute–surface adsorption) also strong electrostatic interactions between charged entities (solute–ligand, solute–silanol) are often of relevance and they may act synergistically (ion exchange/attraction) or antagonistically (ion exclusion/repulsion) to the hydrophilic interaction mechanism. It is hoped that future work will allow gaining deeper insight in the processes being actually at work in so-called HILIC separations in order to exploit more straightforwardly and comprehensively this promising chromatographic mode.

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