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Exploratory analysis of chromatographic fingerprints to distinguish rhizoma chuanxiong and rhizoma ligustici

G. Alaerts[a], M. Merino-Arévalo[b,1], M. Dumarey[a], B. Dejaegher[a], N. Noppe[a], N. Matthijs[a], J. Smeyers-Verbeke[a], Y. Vander Heyden[a,*]

[a] Department of Analytical Chemistry and Pharmaceutical Technology (FABI), Center for Pharmaceutical Research (CePhaR), Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, B-1090 Brussel, Belgium
[b] Koninklijke Militaire School (Royal Military Academy), Renaissancelaan 30, 1000 Brussel, Belgium

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ABSTRACT

Identification and quality control of products of natural origin, used for preventive and therapeutical goals, is required by regulating authorities, as the World Health Organization. This study focuses on the identification and distinction of the rhizomes from two Chinese herbs, rhizoma *Chuanxiong* (from *Ligusticum chuanxiong Hort.*) and rhizoma *Ligustici* (from *Ligusticum jeholense Nakai et Kitag*), by chromatographic fingerprints. A second goal is using the fingerprints to assay ferulic acid, as its concentration provides an additional differentiation feature. Several extraction methods were tested, to obtain the highest number of peaks in the fingerprints. The best results were found using 76:19:5 (v/v/v) methanol/water/formic acid as solvent and extracting the pulverized material on a shaking bath for 15 min. Then fingerprint optimization was done. Most information about the herbs, *i.e.* the highest number of peaks, was observed on a Hypersil ODS column (250 mm × 4.6 mm ID, 5 μm), 1.0% acetic acid in the mobile phase and employing within 50 min linear gradient elution from 5:95 (v/v) to 95:5 (v/v) acetonitrile/water. The final fingerprints were able to distinguish rhizoma *Chuanxiong* and *Ligustici*, based on correlation coefficients combined with exploratory data analysis. The distinction was visualized using Principal Component Analysis, Projection Pursuit and Hierarchical Clustering Analysis techniques. Quantification of ferulic acid was possible in the fingerprints of both rhizomes. The time-different intermediate precisions of the fingerprints and of the ferulic acid quantification were shown to be acceptable.

1. Introduction

Besides synthetic drugs, the use of herbs or herbal extracts, among which Traditional (Chinese) Medicines (TCM), is becoming more and more popular. In contrast to synthetic drugs, containing known and a very limited number of rather pure active compounds, herbs contain a high number of unknown substances. Moreover, their contents vary, because of differences in climate, harvest conditions, preservation, extract preparation, ... [1]. Besides the differences within a species, different species that may look similar can contain different concentrations of compounds or even different compounds. Therefore, to verify their quality, a proper identification of the plant materials is needed. For identification and quality control of herbs, some 'marker' compounds (regardless their therapeutic properties [2]) could be qualitatively and quantitatively tested. However, studying some markers does not necessarily reflect the global activity or property of a herb [3]. To obtain a characteristic profile or 'fingerprint' of the sample, chromatography can be used [4–6]. Analytical separation techniques to develop fingerprints are, for example, gas chromatography (GC) [7], high-performance liquid chromatography (HPLC) [8] and capillary electrophoresis (CE) [9], combined with a suitable detection technique. Additionally, certain spectroscopic techniques also can be used for fingerprint development.

The Chinese State Food and Drug Administration demands identification and quality control of certain herbal medicines [10]. For that purpose, chromatographic fingerprints are acceptable. The American Food and Drug Administration proposes besides chromatographic also spectroscopic techniques, and macroscopic and microscopic identification [11]. Fingerprint chromatography is also presented as a suitable identification test by the European Medicine Evaluation Agency (EMEA) [2]. The EMEA accepts chromatographic data combined with identification techniques as diode array detection (DAD) or mass spectroscopy (MS), which allow specific identification of the compounds. The World Health Organisation recommends that countries should have national standards, technical protocols and methods to control the safety and qual-

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

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ity of traditional medicines, and monographs of therapeutic plant materials [12].

In this paper common identification conditions, based on HPLC fingerprints, are developed for rhizoma *Chuanxiong* (Szechwan Lovage Rhizome origin *Ligusticum chuanxiong* Hort. (*RC*)) and rhizoma *Ligusticum* (Gaoben or Chinese Lovage Rhizome/Jehol Ligusticum Rhizome origin *Ligusticum sinense* Oliv. or *Ligusticum jeholense* Nakai et Kitag. (*RL*)), both used in TCM [13–15]. *RC* is used for menstrual, cardiovascular and cerebrovasculair disorders [16]. Some compounds, like 3,8-dihydrodiligustilide and riligustilide, possess a progestagen activity and cause uterus relaxation [17]. Vasorelaxation is caused by ligustilide and senkyunolide [18]. Ferulic acid (HMCA or 4-hydroxy-3-methoxycinnamic acid) inhibits uterus contraction [19], neutralizes free radicals and forms stable radicals by resonance [20]. The nitrite-scavenging and glutathion-S-transferase inhibition properties of HMCA are responsible for its anti-cancer activities [19]. Prohibition of radicals-based deoxyribonucleic acid damage and peroxidation of lipids by HMCA and other antioxidants is the reason why *RL* is used for cardiovascular disorders [21]. *RL* is also used to treat hyperpigmentation [22].

Former studies using thin layer chromatography (TLC) [15,23], HPLC [1,3,23–29], GC [24,30], CE [25,31] and pressurized capillary electrochromatography (pCEC) [32] focused mainly on given *RC* compounds. Detection was performed by DAD, MS or Ultra-Violet (UV) absorption. The drawback of GC is that the sample is subjected to high temperatures which can degrade thermo-labile compounds (like ligustilide dimers) [23]. Hu et al. [33] optimized a two-dimensional method separating about 120 compounds of methanolic *RC* and *Angelica sinensis* extracts (used together in a TCM combination). Ma et al. [34] identified *RL* compounds with MS and Nuclear Magnetic Resonance. With MS also non-UV absorbing compounds can be detected, but some compounds (like Z-ligustilide) detected by UV are not by MS [23].

In the Chinese Pharmacopoeia (Ch. Ph.), *RC* identification is based on visual evaluation, a colour identification reaction and TLC, while to identify *RL* only TLC is demanded [15]. However, for both Rhizomes different mobile phases are used in the TLC methods. For *RL*, the Ch. Ph. also specifies the HMCA assay, which should not be less than 0.05% (g/g) relative to the dried herb. The assay consists of a reversed phase (RP)-HPLC method using an ODS column with a 40:60 (v/v) mixture of methanol/water (MeOH/H_2O) adjusted to pH 3.5 with phosphoric acid. For *RC*, no assay limits are given. An indication of the expected amount is given by the assay of a TCM, *Chuanxiong Chatiao Wan*, in which *RC* plus radix and rhizoma *Notopterygii* are present in a ratio of 2:1. This TCM should contain not less than 0.015% (g/g), referred to the total mass of RC plus radix and rhizoma Notopterygii [15]. The latter assay proposes an HPLC method using an ODS column with a mobile phase MeOH/H_2O containing 2% acetic acid (20:80, v/v).

As indicated above, published studies concern the analysis of either *Ligusticum sinense* or *Ligusticum jeholense*. None was found describing their simultaneous analysis, although an industrial demand exists to distinguish both with a common method, since macro- and microscopic identification is not always decisive.

In this paper a common method to identify both *RC* and *RL* is developed. The goal is to identify them based on their HPLC/DAD fingerprints. ELS detection also is considered to detect non-UV absorbing compounds. The extraction procedure and the fingerprints were optimized by means of experimental designs. The extraction is validated for repeatability and the fingerprint for time-dependent intermediate precision. The second goal was to assay HMCA from the fingerprints, to meet the requirement of the Ch. Ph. Distinction between *RC* and *RL* was quantified by the correlation coefficients between fingerprints and visualized by Principal Component Analysis (PCA), Projection Pursuit (PP) and Hierarchical Clustering Analysis (HCA).

2. Theory

2.1. Factorial designs

An experimental design is a setup where different factors are varied simultaneously between experiments, allowing to estimate the factor effects on the responses of interest. If f factors are tested on l levels, the setup containing all possible level combinations, is called a full factorial design and requires to perform l^f experiments. A fractional factorial design contains a fraction of the experiments of the full factorial design. For example in a 3^{4-2} design, a 3^{-2} or $1/9^{th}$ fraction of the 3^4 full factorial design is performed.

As response, the number of peaks, representing the peak capacity, was used to evaluate the quality of fingerprints. The estimated effect (E) for changing factor f from a low (-1) to a high ($+1$) level equals to the difference of the average responses obtained at these levels [35]:

$$E_f = \frac{\sum Y(+1) - \sum Y(-1)}{N/l} \quad (1)$$

with E_f the effect of factor f, $\sum Y(-1)$ and $\sum Y(+1)$ the sums of the responses where f is at (-1) or ($+1$) level, respectively, N the number of design experiments, and l the number of levels. From a three-level screening design the effects between (-1) and (0), and between (0) and ($+1$) also can be estimated.

The interpretation of E could be done graphically (*e.g.* by drawing a normal probability plot) or statistically. For small designs the algorithm of Dong is suitable to estimate the experimental error [36]. First an initial error s_0 is estimated based on all E_i (Eq. (2)) and secondly from all E_j for which $|E_i| \leq 2.5$. s_0, the final error $s_1 = \sqrt{m^{-1} \sum E_j^2}$ is estimated. The critical effect (E_{crit}) is given in Eq. (3).

$$s_0 = 1.5 \times \text{median}|E_i| \quad (2)$$

$$E_{crit} = t_{(1-\alpha/2,df)} \times s_1 \quad (3)$$

with E_i the value of an effect i; m the number of absolute effects $|E_j|$, smaller than or equal to $2.5 \times s_0$; α the significance level, and $df = m$ the degrees of freedom. An effect is significant at a given α level (*e.g.* 0.05) when $|E_i|$ is higher than or equal to E_{crit}.

2.2. Data analysis

The data was organized in a $p \times q$ matrix with p fingerprints and q data points per fingerprint. The data exploration methods applied in this study, Principal Component Analysis, Projection Pursuit and Hierarchical Clustering Analysis are briefly overviewed. Prior to the exploratory analysis some pre-processing techniques are applied.

2.2.1. Pre-processing

Some pre-processing techniques are tested to enhance the interpretability of the visualisation methods described below. First a blank chromatogram, obtained by daily analysis of the extraction solvent, is subtracted to remove baseline shift. Secondly, because of shifts in retention times between chromatograms, caused by column ageing or small variations in mobile phase composition, flow rate and temperature [37], aligning corresponding peaks is recommended. In this study, correlation optimized warping was applied [38]. This method aligns two signals by means of piecewise linear stretching and compression of the chromatogram, to match each chromatogram as good as possible with a target chromatogram. The method requires two user-defined input parameters. The chromatogram to be aligned (P) and the target chromatogram (T) are first divided into a user-defined number of sections with equal length (L). Then, the length of each section of P is stretched or

compressed by shifting the position of its section end points by a limited number of data points, *i.e.* the user-defined slack parameter (t), allowing the sections end points to shift from $-t$ to $+t$ points. Finally, the optimal combination of the L warped sections is considered the optimal alignment, resulting in the aligned chromatogram (P') [38]. Thirdly, the warped data was normalized where each element of a row, *i.e.* the fingerprint, is divided by the norm of that row vector [37]. Concentration differences are in this way eliminated, without removing the relative concentration ratios of the compounds in a fingerprint.

Prior to Principal Component Analysis, an additional preprocessing technique, column-centering, where the column mean is subtracted from each element of the corresponding column, is applied [37]. Consequently, the deviations from the mean value emphasise the variation of peaks (variables) between the fingerprints. Prior to Projection Pursuit, the warped and normalized data are sphered. The goal is to set the data origin to the data mean by column-centering and to set the variance of each variable to its unity by dividing the data by its standard deviation [39].

2.2.2. Exploratory data analysis

To distinguish between the fingerprints of both species, besides Similarity Analysis by means of correlation coefficients (r), PCA, PP and Hierarchical Clustering Analysis (HCA) are used to visualize the data [4,40].

PCA and PP are both projection methods which reduce the number of original variables to a smaller number of latent variables while preserving the most important information. In PCA and PP this is done by making linear combinations of the original variables, so-called Principal Components (PC's) or Projection Pursuit Features (PPFs), respectively. In PCA, the orthogonal PC's are constructed in such a way that they maximize the description of the variance in the data matrix. Projections of the objects on a given PC are called scores. Thus, a score plot gives information on the objects, here the fingerprints [37,41]. In PP, the PPFs are constructed by optimizing another objective function, *e.g.* to find clusters in the data with the most informative low dimensional projection or to highlight the presence of objects with atypical properties [42,43]. In this study the Yenyukow index Q is used [42]. This index is the ratio (Q) of the mean of all inter-objects distances (D) and the average nearest neighbour distance (d). Q will be large for clustered data.

HCA is a technique to find (dis)similar objects in a hierarchical way [37]. Objects or clusters which are most similar will merge to a new cluster. This is repeated in a hierarchical way resulting finally in one big cluster. In this study the similarity between fingerprints is measured using the Euclidean distance, which often best detects differences, or the correlation, which often best detects similarities [44]. Normalization as pre-processing is not necessary when representing the similarity by the correlation coefficient as its calculation already includes this step. The criterion to decide which individual objects or clusters should be merged, used in this study, is the complete linkage or furthest neighbour method. The distance between two clusters is considered equal to the largest distance between two individual objects from each cluster. The visualization of the hierarchical clustering is a dendrogram. The highest connected clusters are most dissimilar in the property considered.

3. Experimental

3.1. Instrumentation

The rhizome pieces were pulverized with a Mixer A10 of Janke & Kunkel (Staufen, Germany) and sieved through Prüfsieb DIN 4188 sieves with meshes of 1 mm and 0.5 mm, respectively. The weighing was performed on an analytical balance A200S and a microbalance, both of Sartorius (Göttingen, Germany). The extraction was done on a shaking bath Edmund Bühler Swip KL-2 and an ultrasonic water bath 5210 of Branson Ultrasonic Corporation (Danbury, Connecticut, United States). Samples were filtered through paper filters of Schleicher & Schuell (Dassel, Germany) and then through polypropylene filters with a pore diameter 0.20 μm in a disposable syringe filter holder from VWR International Europe (Leuven, Belgium).

For the fingerprints an HPLC system LaChrom Elite from Merck-Hitachi (San Jose, California) was used. It includes an L-2130 pump with solvent degasser, L-2200 auto sampler, L-2350 column oven and an L-2455 DAD. As second detection system, an Alltech 2000 ELS (Alltech, Deerfield, Illinois) was connected in series, using pressurized air (60 psi, 4.14 bars) as nebulizing gas. The ELS detector is controlled with the ELS 2000 Control software (Alltech). The HPLC system operates with EZChrom Elite Version 3.2.1.31 software (Scientific Software, Agilent, Pleasanton, California). The separations were performed on two coupled Chromolith Performance RP-18e (100 mm × 4.6 mm) endcapped columns in combination with a Chromolith guard column RP-18e (5 mm × 4.6 mm), all from Merck (Darmstadt, Germany). A column coupler for Chromolith columns (Merck) was used. A C18 particle Hypersil ODS C18 column (250 mm × 4.6 mm, 5 μm) (Alltech) was also used. The pH meter Orion model 520A was from Ankersmit (Oosterhout, Nederland).

Microsoft Excel (Microsoft Corporation, 2002), SPSS for Windows 17.0 (SPSS, Chicago, Illinois) and Matlab software 7.0.1 (The MathWorks, 2004, Natick, MA) were used for calculations.

3.2. Herbs, chemicals and reagents

Five samples of *RC* and four of *RL* were kindly donated by Conforma (Destelbergen, Belgium). The samples originated from *Ligusticum chuanxiong Hort* and from *Ligusticum jeholense Nakai et Kita* and were collected in Jiangxi (RC1), Sichuan (RC2-4) and Liaoning (RL1-4) in China. The standards HMCA (purity 99%) and coniferyl alcohol (4-(3-hydroxy-1-propenyl)-2-methoxyphenol) were purchased at Sigma–Aldrich (Steinheim, Germany).

Absolute ethanol analytical reagent grade (EtOH), MeOH (HPLC grade) and acetonitrile (HPLC far UV gradient grade) (ACN) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). Glacial acetic acid 100% (AA) and formic acid 98–100% pro analyse (FA), from Merck, and trifluoroacetic acid reagent grade >98% (TFA), from Sigma–Aldrich, were used in the mobile phase. H_2O was prepared daily with a Milli-Q water purification system (Millipore, Molsheim, France).

3.3. Sample and standard preparation

Before use, the mixed, sieved and homogenated rhizome powder was stored in an exsiccator and protected from light, as compounds like senkyunolide A, coniferylferulaat and Z-ligustilide, are light sensitive [1]. Four extraction solvents, two extraction approaches and two extraction times are tested. For an extraction, 20.0 ml of a solvent (EtOH, MeOH, 80:20 (v/v) EtOH/water or 80:20 (v/v) MeOH/water) was added to 1.000 g rhizome powder in a shaking tube, and placed either in an ultrasonic bath or on a shaking bath at 250 rpm, for either 15 or 30 min. After filtering, washing the residue with the same solvent and collecting the solutions in a flask of 25.0 ml, it is adjusted to volume with the same solvent. The obtained solution represents a 4.00% (m/v) solution. A filter pore diameter of 0.20 μm was chosen to preserve the extracts from particles and microbiotic contamination [45]. The solutions were stored in dark brown glass bottles at 4 °C.

During the extraction optimization, a one hundred times diluted HMCA standard of 1 mg/ml in extraction solvent was used for identification. A HMCA standard stock solution of 200 μg/ml in solvent

was prepared and ten times diluted with the same solvent to reach a test concentration equivalent to the minimal limit of the Ch. Ph. [15]. It was used to identify HMCA in the fingerprints obtained in the design experiments.

Six replicate stock solutions of 400 μg/ml were prepared to test the linearity of a calibration curve between 10 and 100 μg/ml.

All HMCA standards and blanks in extraction solvents were subjected to the same steps as the rhizome powder.

3.4. Chromatographic conditions, detection and integration settings

To test the different extracts, separations were initially performed at 35 °C on a monolithic column with a length of 200 mm (2 coupled Chromolith Performance RP-18e columns of 100 mm), applying a flow rate of 1.0 ml/min and 20 μl injection volume. The mobile phase contained 0.05% (v/v) TFA in both aqueous and organic modifier phases. A linear gradient was run from 95:5 (v/v) H_2O/ACN to 5:95 (v/v) H_2O/ACN in 50 min and maintained isocratically till 60 min. These initial mobile phase conditions are as in [8,46].

During fingerprint optimization, the mobile phase, *i.e.* the organic modifier, the acid and its concentration, were changed. These experiments were also performed on the C18 particle Hypersil ODS C18 column. The pH range of the mobile phases was 2.15–2.80.

The column lengths allowed using all organic modifiers without exceeding the maximum allowed pressures, which is 200 bar for the monolithic and 345 bar for the particle column. The columns were stored in ACN. Before starting gradient runs, start conditions were maintained during 30 min for column equilibration. Between runs equilibration was performed for 15 min.

The DAD detection was performed between 200 and 400 nm. The ELS detector was used in the 'impactor on' mode with a nebulising gas flow of 1.5 l/min, a drift tube temperature of 40.0 °C as recommended by the manual [47] and the gain set at 1 during the extraction method experiments and at 16 during the fingerprint development.

All chromatograms are integrated after subtraction of the solvent blank. The peak integration parameters for DAD and ELS data in the software are set on threshold areas of 4000 and 16,000, respectively, and a baseline window width of 0.2 based on the noise of a blank for both detectors. The threshold area of the ELS data is higher due to a higher noise level.

4. Results and discussion

A chromatographic method to distinguish rhizoma *Chuanxiong* (*RC*) and rhizoma *Ligusticum* (*RL*) is developed. Simultaneously, ferulic acid (HMCA) is assayed to check compliance with the Ch. Ph. In a first step, extraction conditions are investigated in a 2-level full factorial design. Then, the separation conditions are varied according to a 3-level fractional factorial design. The repeatability of the analysis, including the extraction and the HMCA assay, was checked and the time-different intermediate precision estimated. Finally, the fingerprints are used to distinguish between samples of both rhizomes.

4.1. Extraction

A common extraction procedure for *RC* and *RL* had to be defined. The HMCA solubility is to be taken into account as it should be assayed. In the literature, several extraction solvents were used, *e.g.* MeOH or EtOH/H_2O [15,32]. In a former study on several herbs 80:20 (v/v) EtOH/H_2O was used [8]. In preliminary work, less compounds were extracted with 65:35 (v/v) EtOH/H_2O than with the previous solvent ratio. Addition of 5% formic acid was considered necessary to inhibit hydrolysis of coniferylferulate to HMCA [3,48]. Therefore, the rhizome powders were extracted with 95:5 (v/v) EtOH/FA, 95:5 (v/v) MeOH/FA, (76:19:5 (v/v/v) EtOH/H_2O/FA or 76:19:5 (v/v/v) MeOH/H_2O/FA. The influences of the different factors of the extraction, *i.e.* water content in the extraction mixture, solvent type, instrument and extraction time, are evaluated according to a 2-level full factorial design (Table 1).

Table 1
Factors (A–D) and levels (−1,1) investigated during extraction evaluation.

Factors	Levels	
	−1	1
A: % water in organic solvent	0	20
B: Type of solvent	EtOH	MeOH
C: Extraction instrument	Ultrasonic water bath	Shaking bath
D: Extraction time (min)	30	15

The number of peaks detected are a measure for the number of compounds extracted. Detection was performed at 220, 254, 280 and 323 nm, and with ELSD. This selection is motivated as follows. Experimentally HMCA λ_{max} were found to be around 230 and 323 nm (Fig. 1). The first λ_{max} varies depending on the solvent used [28,49]. Aromatic compounds absorb at 254 nm. In former studies on *RC* [3,26,27], 280 nm (λ_{max} for C=O binding) was used and 220 nm to detect compounds absorbing at low λ. As at 220 nm the high baseline noise interfered with the peak detection, only 254, 280 and 323 nm are taken into account during the fingerprint development evaluation. Since 254 nm was selected for fingerprint evaluation, only these results together with the ELS responses are discussed (Table 2).

The estimated effects (*E*) are given in Table 3. They are interpreted statistically as discussed higher. No extremely large effects are observed and only few were considered significant. Some observed tendencies will be discussed. The addition of water (A) results in 7–10 extra peaks at 254 nm. For *RL* the effect was found significant. Probably a higher number of hydrophilic compounds are extracted. The addition of water is less good for ELS detection at low drift tube temperature. The effect of changing the organic solvent (B) from EtOH (−1) to MeOH (+1) is positive with about 7–9 more peaks and was found significant for *RL*. For ELS this factor is unimportant. The interaction effect of water and organic solvent (AB) for ELS detection is negative and important but for the UV detection it is negligible. Consequently, the mixture 76:19:5 (v/v/v/) MeOH/H_2O/FA is further used. The effect of using a shaking instead of an ultrasonic water bath (C) is, although not significant for the UV signals, obviously positive (8 extra UV-peaks and even 9–11 ELS peaks), especially for the *RC*

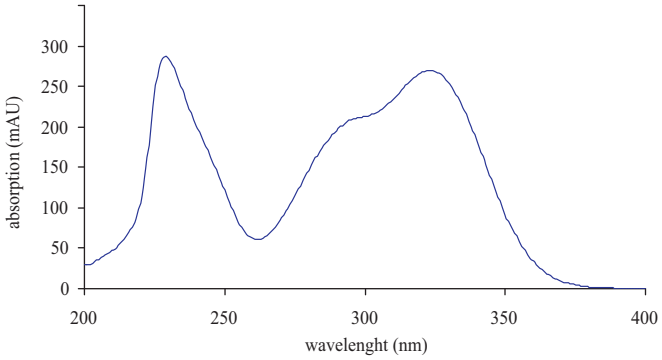

Fig. 1. UV spectrum of HMCA measured with DAD. Sample dissolved in MeOH/H_2O/FA (76:19:5) and using at the time of elution a mobile phase 1.0% AA in H_2O/ACN (81:19).

Table 2
The 2^4 full factorial design evaluating four factors (A–D) at two levels (−1 and 1) during extraction evaluation. Factors and levels: see Table 1. Responses: the number of peaks *n* at 254 nm and with ELS detection for rhizoma *Chuanxiong* (*RC*) and *Ligusticum* (*RL*).

Factors				$n_{254\,nm}$		n_{ELS}	
A	B	C	D	*RC*	*RL*	*RC*	*RL*
−1	−1	1	1	37	52	22	54
−1	−1	1	−1	23	53	40	47
−1	−1	−1	1	39	46	16	32
−1	−1	−1	−1	35	55	8	14
−1	1	1	1	45	65	36	55
−1	1	1	−1	48	47	38	49
−1	1	−1	1	32	59	33	50
−1	1	−1	−1	49	55	38	46
1	−1	1	1	56	52	44	27
1	−1	1	−1	55	56	31	39
1	−1	−1	1	46	59	29	37
1	−1	−1	−1	26	54	22	33
1	1	1	1	62	64	31	30
1	1	1	−1	51	74	22	31
1	1	−1	1	50	65	16	18
1	1	−1	−1	38	69	16	27

extraction. An additional advantage of using a shaking bath is the constant temperature during the extraction which is recommendable for thermo-labile compounds. As a shorter extraction time (D) is economically beneficial, samples will be extracted for 15 min, also because extraction of 30 min does not lead to more peaks observed.

In conclusion, the selected extraction procedure uses a mixture of 76:19:5 (v/v/v) MeOH/H_2O/FA as extraction solvent on a shaking bath for 15 min.

4.2. Fingerprint development

To obtain the fingerprint with maximal capacity, a 3^{4-2} fractional factorial design was executed. The factors and their levels are given in Table 4. Because herbs are complex mixtures with a broad polarity range, dissimilar columns were tested. According to an online column classification system [50], a different selectivity is expected between the Chromolith and Hypersil columns. Other factors studied are the type of organic modifier, the type of acid and its concentration. As organic modifier, either ACN or MeOH is tested. Tetrahydrofuran was not because it provided less good separations in [8]. A third level for the factor modifier is their mixture, *i.e.* ACN/MeOH (50:50, v/v). Three acid types were examined, *i.e.* FA, AA and TFA, chosen for their volatility with ELS detection [47]. FA and AA are tested in concentrations of 0.5; 1.0 and 1.5% (v/v) and TFA of 0.01; 0.05 and 0.10% (v/v). The design was executed on both columns separately. Since only 3 factors are investigated, the fourth factor of the design becomes a dummy factor [36].

As a reference (Exp. 0), the conditions used during the optimization of the extraction procedure are applied. The change of the monolithic to the particle column provided 15–28 extra peaks at the examined λ's. ELS detection also was performed but a high baseline noise interferes with the peak detection. Only the results at 254 nm obtained on the particle column are further discussed (Table 5).

In Table 6, the effects and E_{crit} are given. $E(-1, +1)$ is the sum of $E(-1, 0)$ and $E(0, +1)$ and was not used in the estimations of E_{crit}. The effects of changing from modifier MeOH to ACN (border-

Table 3
The estimated effects of the evaluated factors. Factors A–D: see Table 4. Factor E_{crit} = critical effect estimated with the algorithm of Dong.

Effect	n_{254}		n_{ELS}	
	RC	*RL*	*RC*	*RL*
A	9.5	7.6[a]	−2.5	−13.1[a]
B	7.2	8.9[a]	2.2	2.9
C	7.8	0.1	10.8[a]	9.4
D	5.2	−0.1	1.5	2.1
AB	−2.8	3.9	−12.5[a]	−10.4
AC	8.2	−0.4	0.5	−6.4
AD	5.8	−3.1	5.8	−6.6
BC	1.5	0.4	−4.8	−3.4
BD	−4.5	2.1	−1.0	−2.1
CD	0.5	0.9	−1.0	−2.1
ABC	−5.0	1.9	4.0	8.4
ABD	5.0	−5.9[a]	−1.8	1.6
ACD	−5.5	−4.6	4.8	0.1
BCD	2.8	1.1	4.0	4.6
ABCD	1.8	−0.4	−3.8	1.4
E_{crit}	11.7	5.7	9.2	11.6

[a] Significant effects.

Table 4
Factors (A–C) and their levels (−1, 0 and 1) investigated during fingerprint development.

Factors	Levels		
	−1	0	1
A: Organic modifier	MeOH	ACN	MeOH/ACN (50:50)
B: Acid	FA	TFA	AA
C: Concentration acid (%)			
FA	0.5	1.0	1.5
TFA	0.01	0.05	1.0
AA	0.5	1.0	1.5

Table 5
The 3^{4-2} fractional factorial design evaluating three factors (A–C) at three levels (−1, 0 and 1) executed during fingerprint development factors and levels: see Table 4. Responses: the number of peaks *n* at 254 nm for rhizoma *Chuanxiong* (*RC*) and *Ligusticum* (*RL*) obtained on the Hypersil column.

Exp.	Factors			$n_{254\,nm}$	
	A	B	C	*RC*	*RL*
1	−1	−1	−1	81	106
2	−1	0	0	90	106
3	−1	+1	+1	90	109
4	0	−1	0	98	103
5	0	0	+1	93	115
6	0	+1	−1	88	117
7	+1	−1	+1	93	112
8	+1	0	−1	88	106
9	+1	+1	0	92	119
0	0	0	0	93	120

Table 6
The estimated effects of the evaluated factors. Factors A–C: see Table 4 factor D = dummy factor. E_{crit} = critical effect estimated with the algorithm of Dong.

Effect	*RC*	*RL*
A (−1,0)	6.0[b]	4.7
A (0,+1)	−2.0	0.7
A (−1,+1)	4.0	5.3
B (−1,0)	−0.3	2.0
B (0,+1)	−0.3	6.0
B (−1,+1)	−0.7	8.0[b]
C (−1,0)	7.7[a]	−0.3
C (0,+1)	−1.3	2.7
C (−1,+1)	6.3[a]	2.3
D (−1,0)	1.7	−1.7
D (0,+1)	1.7	−5.7
D (−1,+1)	3.3	−7.3
E_{crit}	6.2	8.3

[a] Significant effect.
[b] Borderline significant effect.

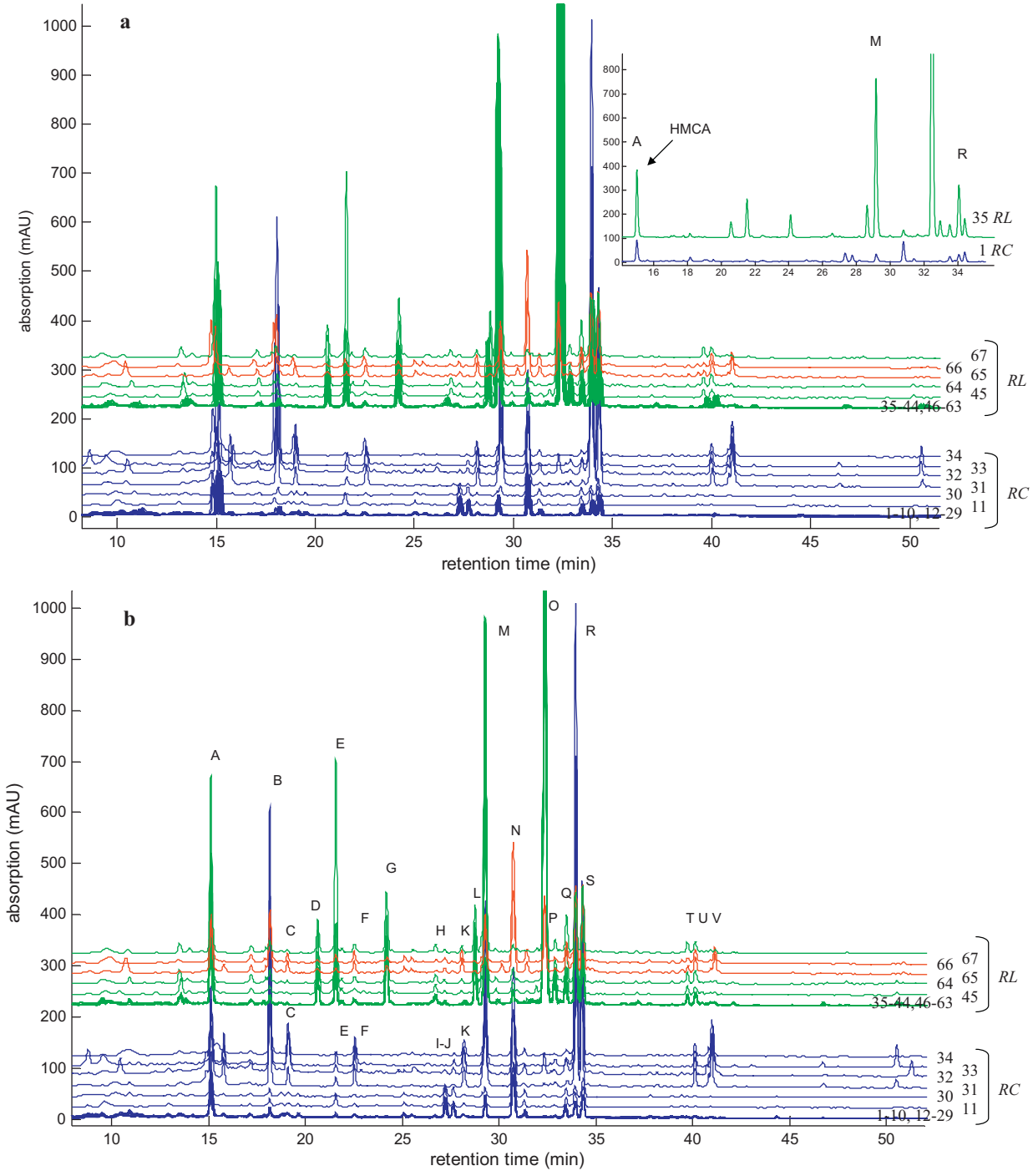

Fig. 2. HPLC fingerprints of rhizoma *Chuanxiong* (*RC*) and rhizoma *Ligusticum* (*RL*) at 254 nm (a) before alignment and (b) after alignment. Insert: zoom of fingerprints 1 and 35. Data set and peaks A–V: see text. Experimental conditions: Hypersil ODS 5 μm 250 mm × 4.6 mm ID, gradient elution from 5% to 95% of ACN within 50 min, then isocratic till 60 min; mobile phase containing1.0% AA.

line significant increase with 6 peaks for *RC*), and from MeOH to 50:50 (v/v) ACN/MeOH were positive. MeOH is consequently less preferred. Between MeOH/ACN (+1) and ACN (0) no pronounced effect is seen and since a single solvent system is preferred, ACN is chosen. For *RC* fingerprints, no influence of the acid type was seen, while for *RL* a positive effect of AA (borderline significant effect of 8 extra peaks) was observed. Moreover, AA caused less interference for ELS detection than TFA. An increase of the acid concentration to medium and high levels reveals increases of 8 and 6 peaks, respectively, for the *RC* fingerprints. As no pronounced difference is seen between the medium and high levels and column ageing may be accelerated by high acid concentrations, the medium level is preferred.

Summarized, the best *RC* and *RL* fingerprints, represented in the insert of Fig. 2a were obtained using the C18 particle-based column and a mobile phase containing 1.0% acetic acid used in a linear gradient from 5 to 95% ACN within 50 min, then isocratic till 60 min. The visual distinction between both herbs is more obvious at 254 nm than at 280 nm or 323 nm, which is confirmed by their mutual correlation coefficients (r) 0.22, 0.93 and 0.78, respectively. Identification is thus preferably done at 254 nm, while 323 nm is more useful to assay HMCA (peak A).

4.3. Precision evaluation of the fingerprints

The time-different intermediate precision ($s^2_{I(t)}$) of the fingerprints was evaluated, analyzing daily during eight days three individually prepared extracts of both rhizomes. The precision measures were estimated from an analysis of variance (ANOVA) [41]. The total variance is decomposed into a within-day component, which is an estimate of the repeatability, and a between-day component. The sum of both estimates the time-different intermediate precision.

For each herb, ANOVA was performed first for the number of peaks obtained at 254 nm, as this was the parameter used during method development. The Grubbs' tests ($\alpha = 0.05$) revealed no outliers [41]. The number of peaks was 82 ± 6 and 105 ± 6 for *RC* and *RL*, respectively. The percent relative standard deviations (% RSD) of *n* were 7.6% and 5.5%, respectively. A remark can be made about estimating the repeatability (s^2_r) of the method by analysing six extracts per herb in one day. The maximum standard deviation for this approach was four peaks and % RSD was between 1.9% and 3.4%, which shows that the repeatability of the method estimated from the latter set-up might be underestimated compared to the repeatability estimated from the evaluation of the time-different intermediate precision.

Concerning $s^2_{I(t)}$ the variances of the number of peaks obtained for both rhizomes at each wavelength are not significantly different (*F* test). The (non-significant) difference in the detected number of peaks is caused by peaks with a concentration around the integration limit. The %RSD is acceptable and shows that the number of peaks can be reliably used as response during method development.

Secondly, $s^2_{I(t)}$ of the fingerprints was checked for the retention time (t_R) and peak area of three randomly chosen, good identifiable peaks in both *RC* and *RL* fingerprints, spread over the chromatogram, *i.e.* peaks A, M and R (insert Fig. 2a). The retention times of the original unaligned chromatograms were evaluated. The areas are corrected with respect to the expected weight of 1.00 g rhizome powder at extraction. The Grubbs' test revealed a slight t_R shift of peak R in the first analysis for both rhizomes (straggler *i.e.* significant at $\alpha = 0.05$ and not at $\alpha = 0.01$) [41]. The % RSD of the t_R and area is between 0.07–0.83% and 2.22–6.19%, respectively. The same remark concerning the estimation of the repeatability (s^2_r) of the method by analysing six extracts per herb in one day can be made since the % RSD of the retention times and peak areas with this approach was only between 0.015–0.020% and 0.92–3.87%, respectively.

Considering that the areas were not evaluated at their λ_{max} but at the wavelength of the fingerprint evaluation, the time-different intermediate precision of the method including the sample preparation was considered acceptable.

4.4. Ferulic acid assay validation

As mentioned before, Ref. [15] specifies that *RL* should contain not less than 0.05% (g/g) of HMCA with reference to the dried herb. Therefore, it would be convenient if the developed fingerprints allowed assaying HMCA simultaneously. The assay of HMCA ($t_R \sim 15.0$ min) is performed at its λ_{max} of 323 nm. To quantify HMCA in both herbs, *a calibration curve* was constructed in the range 10–100 μg/ml in the used solvent 76:19:5 (v/v/v) MeOH/H_2O/FA. To test the linearity, standards of 10, 20, 80 and 100 μg/ml were prepared six times out of six independently prepared stock solutions of 400 μg/ml. Four additional standards of 16, 40, 60 and 72 μg/ml were prepared from the first stock solution. This setup allows checking the lack-of- fit of the regression line by means of ANOVA [41].

All peak areas were corrected with respect to the concentration of the stock solutions and the purity (99%) of the HMCA standard. The replicated measurements of the areas were evaluated for outliers with the Grubbs tests ($\alpha = 0.05$), and none were observed. To check the homoscedasticity, the variances of the areas were compared [51]. Those from the smallest (10 μg/ml) and the highest standard (100 μg/ml) are significantly different (*F* test, $\alpha = 0.05$), but the variances of the smallest and of the 80 μg/ml standard are not. Therefore, the variance is considered constant in this range. Unweighted regression can thus be used to model the calibration line. To check its goodness of fit, a lack-of-fit test is performed using all standards in the range of 10–80 μg/ml. No significant lack of fit of the regression line was revealed (*F* test, $\alpha = 0.05$) [41].

HMCA in both herbal extracts was assayed relative to the above regression line. Sulfamethoxazole was tested as internal standard, as in the analysis of rat plasma samples in [19], but revealed peak splitting. Of both rhizomes, six independent replicated extracts are prepared. Again areas are corrected for weight and checked for outliers. The variances of areas in both rhizomes were compared with those of the closest repeated standard, *i.e.* 20 μg/ml for *RC* and 80 μg/ml for *RL*. No significant difference was seen. The use of an internal standard therefore seems unnecessary. The HMCA concentration in *RC* was $0.045 \pm 0.001\%$ (g/g). Ref. [15] does not specify a minimum concentration for this herb. The HMCA concentration in *RC* is acceptable for use in the *TCM Chuanxiong Chatiao Wan*. *RC* contains less than half of the HMCA concentration in *RL*, which was $0.116 \pm 0.003\%$ (g/g). *RL* is therefore conform to its monograph in [15]. The % RSD of the HMCA concentrations from six independent extracts under repeatability conditions are 2.34% and 2.69% in *RC* and *RL*, respectively, which is acceptable.

The assay was also performed using a *single-point calibration* as in [15] with respect to the standard 20 μg/ml, which represents the Ch. Ph. limit in *RL*. For *RC* and *RL* HMCA concentrations of $0.045 \pm 0.001\%$ (g/g) and $0.113 \pm 0.003\%$ (g/g), respectively, were found. The %RSD was 2.11% and 2.68% in *RC* and *RL*, respectively, which was similar to those reported above.

The assay from the *time-different intermediate precision study with single-point calibration* resulted in HMCA concentrations of $0.045 \pm 0.001\%$ (g/g) and $0.118 \pm 0.005\%$ (g/g) in *RC* and *RL*, respectively. The % RSD for *RC* and *RL* of 1.39% and 4.00%, respectively, were found acceptable (<5%). Summarized, the HMCA assay in both rhizomes is possible with an acceptable time-different intermediate precision.

4.5. Similarity and exploratory analysis of RC and RL fingerprints

To evaluate whether the fingerprints allow distinguishing both rhizomes, a data matrix obtained during validation and from additional rhizome samples was created (Fig. 2a). Fingerprints 1 and 35 (insert Fig. 2a) are measured at the end of the development. The samples, resulting in the fingerprints called *RC*1 and *RL*1, were also used during validation and consist of 27 chromatograms per sample. They are overlaid in Fig. 2a, *i.e.* fingerprints 1–10, 12–29 for *RC*1 and 35–44, 46–63 for *RL*1. To evaluate the stability of the extracts, an extract of *RC*1 and *RL*1 was injected after ten months of storage (fingerprints 11 and 45). After ten months, extracts from *RC*1 and *RL*1 freshly prepared by a second analyst were also analysed (fingerprints 30 and 64). The fingerprints of the ten months old extract and the fresh prepared extract are both quite similar to the fingerprints prepared and analysed ten months earlier. Additionally for *RC*, four (*RC*2–*RC*5) and for *RL* three (*RL*2–*RL*4) other samples were analysed.

Prior to similarity and exploratory analysis, peak alignment is performed [4]. As target chromatogram for each species the one

with the highest mean correlation coefficient to all other chromatograms was determined. First, the target fingerprints were aligned and secondly, the other fingerprints were individually aligned relative to their target chromatogram. In Fig. 2b the aligned fingerprints are shown.

Visual evaluation of the fingerprints of both rhizomes show some similarities and differences between both species (Fig. 2b), but also similarity between batches *RL*2 (65) and *RL*3 (66), plotted in red, with the fingerprints of *RC*. The HMCA peak (A) at 15.0 min is usually higher in *RL* than in *RC* (see also assay). In the samples *RC*2–*RC*5 the same peaks as in *RC*1 are found, but the intensities are usually higher. Based on the visual interpretation of the presence or absence and the height differences of the given peaks A to V (see Fig. 2b), identification of both rhizomes seems not evident. A multivariate analysis is recommended.

Based on the concentration of the marker, ferulic acid, also no distinction could be made. The HMCA assay for the different *RC* samples gave 0.047, 0.085, 0.117, 0.078 and 0.074% (g/g), while for the *RL* samples it was 0.160, 0.075, 0.089 and 0.118% (g/g). Although the Ch. Ph. [15] specification that *RL* should contain not less than 0.05% (g/g) of HMCA with reference to the dried herb is fulfilled, the variation of its concentration is obvious. Moreover, as some *RC* samples also fulfill this requirement, it can not be used as a differentiation criterion. A remark can be made about the ferulic acid concentration in the ten months old *RL* extract. It is more than doubled relative to the original, *i.e.* 0.279% (g/g). This is probably caused

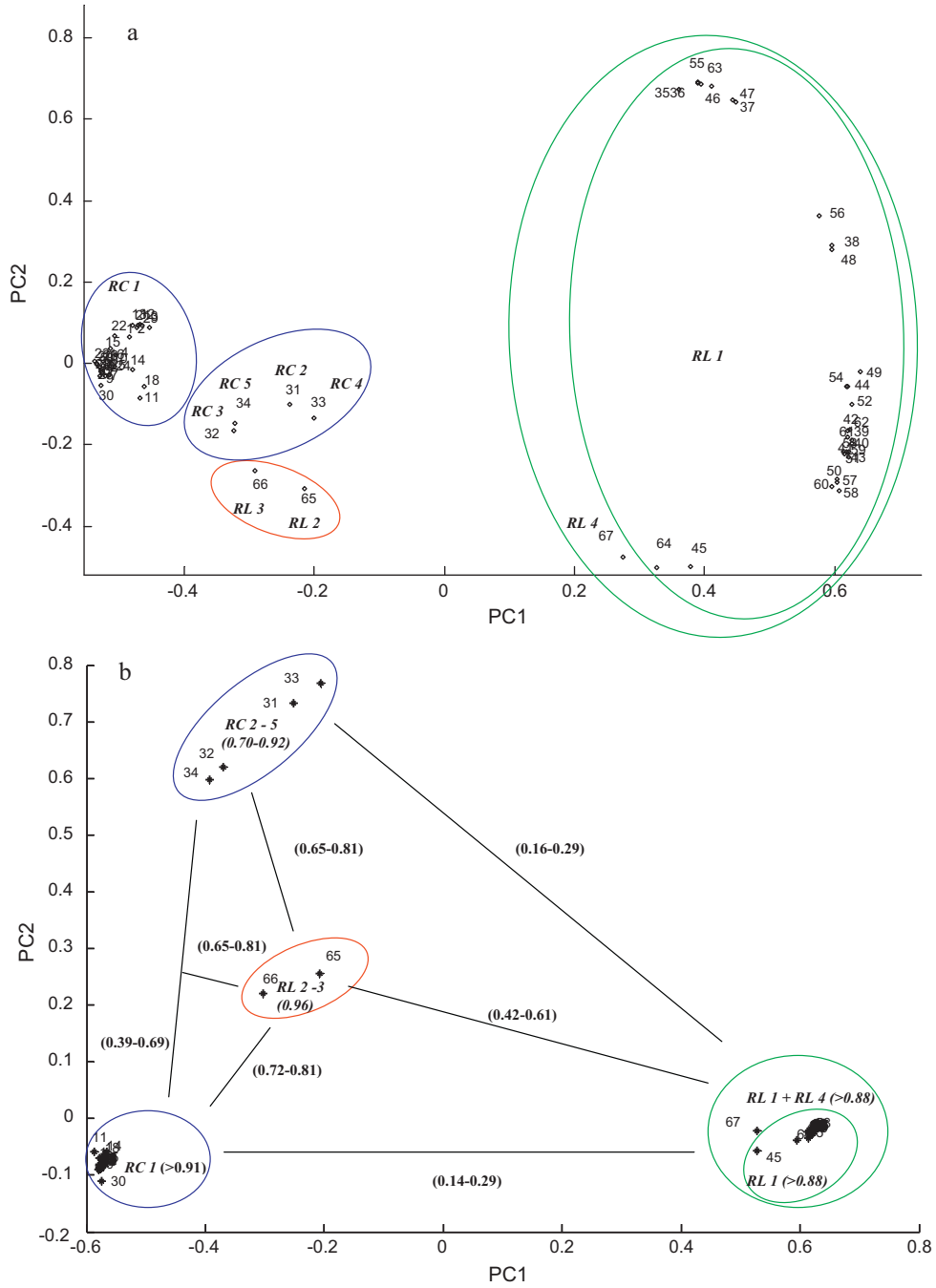

Fig. 3. PC1–PC2 score plot of the normalized, centered, (a) original and (b) aligned fingerprints (brackets: r-range); (c) correlation- and (d) Euclidean-distance-based dendrograms obtained with the complete linkage method. Numbers of fingerprints: see text.

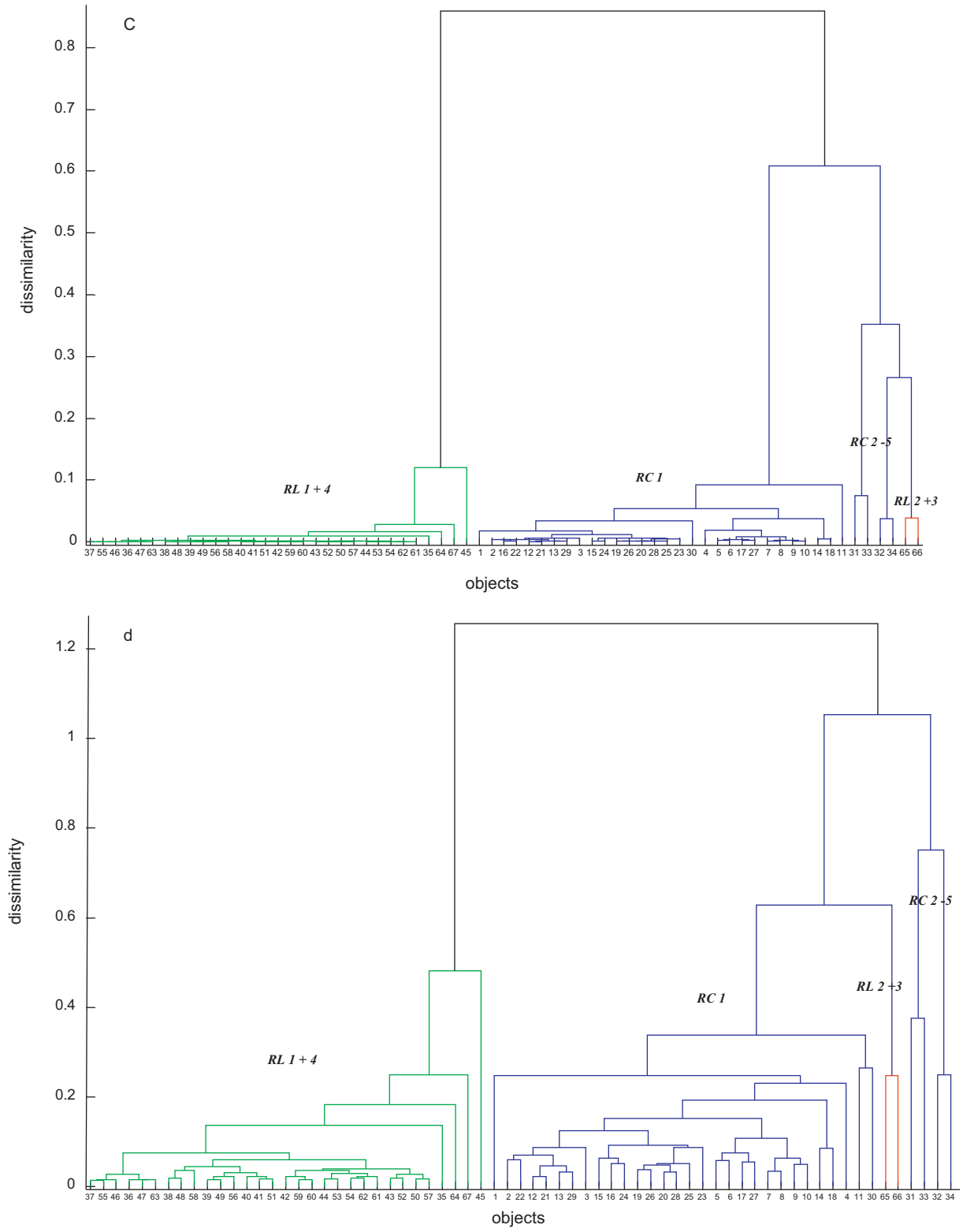

Fig. 3. (*Continued*).

by the degradation of coniferyl ferulate into ferulic acid (peak A) and coniferyl alcohol (Fig. 2b, t_R = 13.5 min).

To distinguish samples of both rhizomes, the correlation coefficients (r) between the fingerprints are evaluated for similarity analysis. Additionally, the data were visualised by the exploratory techniques, PCA, PP and HCA (Fig. 3). In the PCA score plots, the data structure between the fingerprints is visualized, after normalization and column centering of the data. In Fig. 3a and b, the PC1–PC2 score plots of (a) the original and (b) the aligned fingerprints are shown. After aligning the fingerprints, the distinction between the different groups is clearer. Therefore only the PCA results from Fig. 3b are discussed.

For the *RC*1 fingerprints 1–10 and 12–29, *i.e.* all measured within a month after preparation, the range of r is 0.9611–0.9997. Fin-

gerprints 30 (freshly prepared extract by a second analyst) and 11 (ten months old extract) reduce somewhat the *r* values to 0.9457 and 0.9079, respectively. For the *RL*1 fingerprints 35–44 and 46–64, *i.e.* obtained within a month, the range of *r* is 0.9903–0.9999. Fingerprints 64 (freshly prepared extract by a second analyst) and 45 (ten months old extract) again reduce *r* to 0.9834 and 0.8802, respectively. These high correlation coefficients between the fingerprints of a same sample, *i.e. RC*1 (1–30) or *RL*1 (35–64), confirm that the analysis method, including the extraction procedure, within a time frame of 10 months, is producing similar fingerprints.

Between the fingerprints of *RC*1 and *RL*1 low correlations are observed (0.1407–0.2479), which is also clearly visualized in the PCA score plot where the fingerprints *RC*1 and *RL*1 form two well-separated groups (Fig. 3b). The *RC* samples form two distinct clusters. The *RC*1 group is clearly separated from the group with the other four *RC* samples (*RC*2–*RC*5). Fingerprints 31–34, representing these four additional *RC* samples, correlate amongst themselves (0.6988–0.9255), but less to *RC*1 ($r = 0.3916$–0.6901) (Fig. 3b). Nevertheless, their correlation is still higher than that to the fingerprints *RL*1 and *RL*4 (0.1605–0.2896). *RL*1 and *RL*4 form a dense cluster clearly separated from the other fingerprints. Looking at these *RL* samples, as expected, the correlation between *RL*4 and *RL*1 is high (0.8801–0.9882), while it is low compared to *RC*1 (0.1407–0.2904) or to the *RC*2–*RC*5 group (0.1605–0.2896) (Fig. 3b). A third observation is the clearly separated *RL*2–*RL*3 samples. These samples seem to have a higher similarity with the *RC* samples than with *RL*1 and *RL*4, which is also revealed by the correlation coefficients. Samples *RL*2 and *RL*3 (with mutual correlation 0.9609), for which it was already visually observed that they were more similar to *RC*, correlate better with *RC*1 (0.7175–0.8133) and with the *RC*2–*RC*5 group (0.6483–0.8094), than with *RL*1 and *RL*4 (0.4238–0.6110).

The distances between the four groups seem to correspond to the correlation coefficients between the members of different groups, as indicated in Fig. 3b.

The second visualization technique, PP, should emphasise the clustering tendency outliers in the data better than PCA [43]. The PPF1–PPF2 plot of the aligned, normalized and sphered fingerprints is quite similar to the PC1–PC2 plot (figure not shown). For this dataset, PP did not provide additional information.

With HCA the (dis)similarities are visualized in dendrograms. In Fig. 3c and d, the dendrograms from the aligned and normalized fingerprints, with dissimilarities either based on correlation or euclidean distance, respectively, are shown. The higher the clusters are linked, the more dissimilar they are. The cluster containing the *RL*1–*RL*4 fingerprints is well separated from the rest. The rest consists of the *RC*1 fingerprints, the *RC*2–*RC*5 and *RL*2–*RL*3 fingerprints. As observed earlier, the *RL*2–*RL*3 fingerprints are more similar to the *RC* samples than to the *RL*1 and *RL*4 fingerprints. Although the differences between the *RL* samples seemed related to the provider, *i.e. RL*1 and *RL*2–*RL*3 originating from two different companies, this was not the case for the *RC* batches. Further, neither a relation with the Chinese province of origin nor with the harvest time could be made.

From the above, it is observed that fingerprint analysis using correlation coefficients of the entire fingerprints and exploratory data analysis visualization techniques, as Principal Component Analysis, projection pursuit and hierarchical clustering, provide information about the similarities and differences among rhizoma *Chuanxiong* and rhizoma *Ligustici* samples. The *RL*2 and *RL*3 samples showed more similarity with *RC* samples than with other *RL* samples, which raises the question whether the identification of the rhizomes by means of the available monographs is sufficient. Fingerprint analysis on the other hand provides an overall picture on the composition of the rhizomes.

5. Conclusion

This study focused on the simultaneous evaluation of the similarities and differences between two Chinese herbs, rhizoma *Chuanxiong* (from *Ligusticum chuanxiong L.*) and rhizoma *Ligustici* (from *Ligusticum jeholense Nakai et Kitag*) based on their chromatographic fingerprints.

The highest number of compounds was observed using an extraction mixture with 76:19:5 (v/v/v) methanol/water/formic acid as extraction solvent and extracting the powdered plant material on a shaking bath for 15 min. The fingerprints were developed, varying a number of factors. Most information, *i.e.* largest peak capacity in the fingerprint, was acquired on a Hypersil ODS (C18) column (250 mm × 4,6 mm ID, 5 μm particle diameter), employing within 50 min an acetonitrile gradient elution from 5 to 95 (v/v) in 1.0% acetic acid and isocratic elution till 60 min. The fingerprints were evaluated in a time different intermediate precision study of the entire method including the sample preparation, which was acceptable.

The ferulic acid quantification was possible. The repeatability and time-different intermediate precision of the ferulic acid assay were acceptable. The ferulic acid amount in *RL* showed to be in accordance with the demand in the Ch. Ph., *i.e.* higher than 0.05% (g/g) calculated with reference to the dried herb. Differentiation among both species was not possible based on the ferulic acid concentration, as in some *RC* samples it is as high as in *RL*.

The fingerprints at 254 nm reveal some characteristic peaks for the rhizomes but are insufficient for identification. Based on the correlation coefficients of the entire fingerprints, combined with exploratory data analysis visualization techniques, as Principal Component Analysis, projection pursuit and hierarchical clustering, extra information was obtained. It was observed that some RL samples were more similar to the *RC* samples than to other *RL* samples. If these observations are confirmed for a large data set of both rhizomes, fingerprint analysis is a highly potential strategy for identification and distinction between rhizoma *Chuanxiong* and rhizoma *Ligustici* samples.

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