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Development, optimization and validation of a fingerprint of *Ginkgo biloba* extracts by high-performance liquid chromatography

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Abstract

A four-step development, optimization and validation strategy for high-performance liquid chromatography (HPLC) fingerprints of *Ginkgo biloba* extract is described. A suitable chromatographic system was selected first. The following step was performing a screening design to select important parameters. After selecting some controllable parameters and their range to further optimize, gradient optimization with uniform design was done. At last, method validation including determination of injection precision, repeatability, and a sample stability test, was performed. Through this effective and integrated four-step method, a feasible and reliable HPLC fingerprint to identify and assess the *Ginkgo biloba* quality can easily be established using a linear gradient elution with acetonitrile/0.1% phosphoric acid (from 14/86 to 30/70, v/v, in 40 min) as mobile phase, a column temperature of 30 °C and a detection wavelength of 350 nm. The strategy can also be applied for the development of fingerprints in the quality control of other herbal medicines.

Keywords: HPLC fingerprint; Method development; Uniform design; *Ginkgo biloba*; Herbal medicines; Quality control

1. Introduction

Herbal medicines have been widely used for health needs over many centuries, and become more and more popular worldwide during the last decade [1]. However, due to the fact that in those herbs there may be hundreds of complex active components of which we have limited knowledge, it is almost impossible to identify all these substances and to carry on quantitative analysis. Generally, only a few effective components are determined by high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) for evaluating the quality of herbal medicines [2–5]. According to the theory of traditional Chinese medicine (TCM), the therapeutic actions of herbal medicines are based on integral interaction of many kinds of ingredients combined rationally. The fingerprint chromatographic technology was introduced and accepted by the WHO as a strategy for identification and quality evaluation of herbal medicine [6]. In 2000, the State Drug Administration (SDA) of China began to develop the fingerprints of TCM as the standard of quality control [7]. Chromatographic methods were highly recommended for developing fingerprints of TCMs and their preparations. Since then, increasing interest in HPLC fingerprint analysis can be observed, not only in China but also in other countries all over the world [8–11]. Moreover, CE also seemed to be a potential technique in the analysis of herbal drugs [12,13].

The obtained fingerprints are usually complex chromatograms (or electropherograms), and their application relies on a comparison of profiles. Hence, fingerprint development has a stringent demand on resolution and peak capacity in the separation process. However, it is really difficult to separate all active components of herbal medicines in a single chromatographic run. Therefore, the development and optimization of a methodology to create fingerprints seems to be very important. It should be pointed out that the obtained

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chromatographic fingerprints would be used to authenticate and identify herbal medicines by the verification institute or by quality control laboratories. Hence, the developed fingerprint should feature good selectivity, reproducibility and feasibility.

The extracts of *Ginkgo biloba*, which is among the top 10 of sold plant products in the world, possess antioxidant, anti-ischemia, cardiovascular and cerebrovascular activities [14]. The preparation process of the extracts from *Ginkgo biloba* leaves may vary among different manufacturers. However, almost all of the final extracts contain flavonoids and terpene trilactones, which are considered the two pharmacologically most important groups [15]. According to the present knowledge, in the Ginkgo extract there are more than 30 different flavonoids, which can chemically represent the character of the *Ginkgo biloba* leaves. Hasler et al. [16] reported the reversed-phase separation of 33 flavonol glycosides, flavonols and biflavones using a complex ternary gradient elution. A similar but binary gradient HPLC separation method for fifteen Ginkgo flavonoids was reported by Pietta et al. [17]. You et al. [18] published a multi-step binary gradient HPLC method for a fingerprint of the Ginkgo injection.

However, among the published methods to separate and identify flavonoids, there are no integrated experimental design based fingerprint development studies, which include development, optimization and validation of the method. In addition, for the purpose of improving resolution, too complex HPLC gradient methods were used in the literature mentioned above, which might affect the feasibility and reproducibility of the fingerprints. It thus is necessary to establish a universal and systematized approach to conveniently develop reliable chromatographic fingerprints of herbal medicines.

In this paper, we describe a four-step strategy for method development, optimization and validation of an HPLC fingerprint of *Ginkgo biloba* extracts. A relatively simple and feasible system, including separation mode, stationary phase and mobile phase, was selected first. Then, using a factorial screening design and main effect plots, some controllable parameters and the range of the factors, which need to be further optimized, can be selected. Thirdly, further gradient optimization was performed using a uniform design, which is very useful when a large number of levels is evaluated [19]. An effective linear gradient elution method was obtained. The last step was method validation consisting of injection precision, repeatability, reproducibility and a sample stability test. The aim of this study was to create a universal process to establish HPLC fingerprints, which is applicable on diverse herbal medicines.

2. Theory

2.1. Screening design and main effect plot

Screening design refers to an experimental plan that is intended to find the relatively few significant factors from a list of many potential ones [20]. The common families of screening designs are the Plackett-Burman designs, named after their inventors, and fractional factorial design with resolution three (sometimes four), which permit to explore the effects of many factors in a limited number of runs. A fractional factorial design was used in this study. The specification of this design can be seen in Table 1.

Table 1
Two-level fractional factorial design for 7 factors (2^{7-4}_{III})

No.	Factors						
	X1	X2	X3	X4	X5	X6	X7
1	−1	−1	−1	+1	+1	+1	−1
2	+1	−1	−1	−1	−1	+1	+1
3	−1	+1	−1	−1	+1	−1	−1
4	+1	+1	−1	+1	−1	−1	−1
5	−1	−1	+1	+1	−1	−1	+1
6	+1	−1	+1	−1	+1	−1	−1
7	−1	+1	+1	−1	−1	+1	−1
8	+1	+1	+1	+1	+1	+1	+1

−1 and +1 represent the factor levels.

The main effect plot, used to provide insight into the data, is a visual approach to analyze the data from a designed experiment [20]. The vertical axis of such a plot represents the mean response for a given setting of the factor, while the horizontal axis represents the factor levels examined. A factor when varied from one to the other level can be important if it leads to a significant change in the response. A large difference in the two means implies the factor is important while a small difference indicates the factor is not. A factor setting is best (in average) if it results in a response that is closest in location to the desired response.

2.2. Uniform design

Too many experimental runs are needed using factorial design when the number of factors or the number of levels increases. Therefore, factorial design is generally used for two or three levels. On the other hand, in chemical experiments, the number of levels for a factor is often more than 2 or 3. In these cases, uniform design can be a good choice. Uniform design is an experimental design, which allocates experimental points uniformly scattered on the domain, proposed by K.T. Fang and Y. Wang in 1980s, based on quasi-Monte Carlo method or number-theoretic method [21,22]. It becomes more popular recently because of some theoretical and application reports [23–25]. The key idea of uniform design is to choose the most representative points in the experimental domain to obtain as much information as possible by relatively few experiments.

The most widely used structure, the U-type design, which provides an *n*-run experimental design for *s* factors, each having n levels, is denoted as U_n (n^s) [23]. Like other experimental designs, uniform designs offer lots of experimental possibilities [26]. For instance, U_7 (7^2) and U_8 (8^6) are shown in Tables 2 and 3. One can see that a large number of lev-

Table 2
Uniform design with seven experiments for two factors at seven levels, U_7 (7^2)

No.	Factor	
	X1	X2
1	4	4
2	3	7
3	5	1
4	2	2
5	1	5
6	7	3
7	6	6

Table 3
Uniform design with eight experiments for six factors at eight levels, U_8 (8^6)

No.	Factor					
	X1	X2	X3	X4	X5	X6
1	4	6	7	3	7	1
2	8	2	5	7	6	3
3	3	4	2	6	1	2
4	1	1	4	1	5	5
5	5	5	1	5	8	8
6	7	8	3	2	3	4
7	2	7	6	8	4	6
8	6	3	8	4	2	7

els, which is equal to the number of experimental runs, are allowed in the uniform design. The U_7 (7^2) design of Table 2 was used in this study.

Compared to factorial designs, an outstanding advantage of uniform design is that it offers as many levels as one needs for the factors within a small number of experiments. For example, suppose the experimenter wants to consider two factors and choose seven levels for each factor. The total number of level-combinations is $7^2 = 49$, which is usually experimentally not feasible. However, according to the application table U_7 (7^2) (Table 2), only 7 runs are needed using the uniform design method.

3. Experimental

3.1. Instrumentation

Experiments were performed on a D-7000 Merck-Hitachi system (Hitachi Instruments, Tokyo, Japan) equipped with a low pressure L-7100 HPLC pump, an L-7612 degasser, an L-7400 UV detector, an L-7250 autosampler and a Model 7956 column oven (Jones chromatography, Mid Glamorgan, UK), operated with the Merk-Hitachi D-7000 HPLC data station software. Peak purity determination was operated on the HPLC instrument combining a Merck Hitachi L-6200 Intelligent pump (Hitachi, Japan), a SPD-M10A diode-array UV detector and a CLASS-LC10A workstation (Shimadzu, Japan).

3.2. HPLC columns

An Alltima C18, 150 mm × 4.6 mm i.d., 5 μm (Alltech, Lokeren, Belgium), a Nucleosil C18, 150 mm × 4.6 mm i.d., 3 μm (Alltech), and a Lichrosorb® RP-18, 250 × 4.6 mm i.d., 5 μm (Merck, Darmstadt, Germany) columns were used.

3.3. Chemicals and reagents

A *Ginkgo biloba* extract (GBE 20030106) was provided by the TSI Natural products company (Xuzhou, China). Another standard *Ginkgo biloba* extract GK501 was purchased from Finzelberg Company (Andernach, Germany). The standards, including 3-O-(6-O-(α-L-rhamnosyl)-β-D-glucosyl)quercertin (rutin) and quercetin, were from SIGMA (St. Louis, USA), 3-O-(β-D-glucoside)kaempferol, 3-O-(6-O-(α-L-rhamnosyl)-β-D-glucosyl)kaempferol and 3-O-(α-L-rhamnosyl)quercetin from Extrasynthese (Genay Cedex, France) were used. The mobile phases were prepared using acetonitrile (ACN) and methanol, both HPLC grade (Fisher scientific, Leicester, UK). Tetrahydrofuran (THF), isopropanol, phosphoric acid, all pro analysis quality, were from Merck. Milli-Q water was obtained with the Milli-Q water purification system (Millipore, Molsheim, France). Bakerbond C18 (500 mg) disposable extraction columns (Mallinckrodt Baker, Phillipsburg, NJ, USA) were used for sample clean-up.

3.4. Sample preparation

The dried extracts were exactly weighed (ca. 0.5 g) into a 100 ml-flask, 50 ml 80% ethanol was added, the mixture was shaken for a while, sonicated at room temperature for 30 min, and then diluted to 100 ml with 80% ethanol in a volumetric flask. A 5 ml volume of this solution was filtered through a Bakerbond C18 cartridge equilibrated with 80% ethanol. The cartridge was then eluted with 3 ml 80% ethanol and the solution was diluted to 10 ml with 80% ethanol in a volumetric flask.

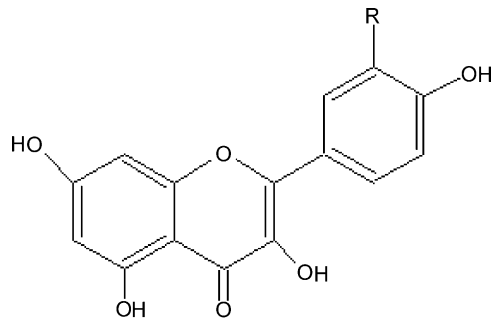
3.5. Chromatographic conditions

At general conditions, the chromatographic runs were performed at a flow rate of 1 ml/min, a column temperature of 30 °C, a detection wavelength of 350 nm, and an injection volume of 10 μl, unless specified in the text.

4. Results and discussion

4.1. Selection of a suitable chromatographic system

In this study, most compounds of interest are glycoside derivatives of quercetin, kaempferol and isorhamnetin of which the structures are given in Fig. 1. Reversed-phase liquid chromatography methods with ODS columns and



Kaempferol: R=H
Quercetin: R=OH
Isorhamnetin: R=O-Me

Fig. 1. The structures of the three aglycones, quercetin, kaempferol and isorhamnetin.

acetonitrile–water mobile phases are recommended to separate this kind of substances [15]. A low concentration phosphoric acid is added to restrain ionization of flavonoids and other carboxylic acids. Three columns were screened with acetonitrile/0.1% phosphoric acid (B/A) as mobile phase. Considering the complexity of the Ginkgo extract, a 30 min-linear gradient elution, where the initial and the final B% are 15 and 30%, respectively, was run for each column. Fig. 2 illustrates the chromatograms of sample solution using three different columns. The best selectivity and resolution can be observed on the Alltima C18 column (C). Over 30 sharp and symmetrical peaks were obtained. Therefore, this column was selected. It was suggested that isopropanol and tetrahydrofuran could be used as organic modifier to improve the selectivity [16]. A mobile phase including methanol was also examined. However, no progress in resolution using these organic solvents was observed. Additionally, it is beneficial

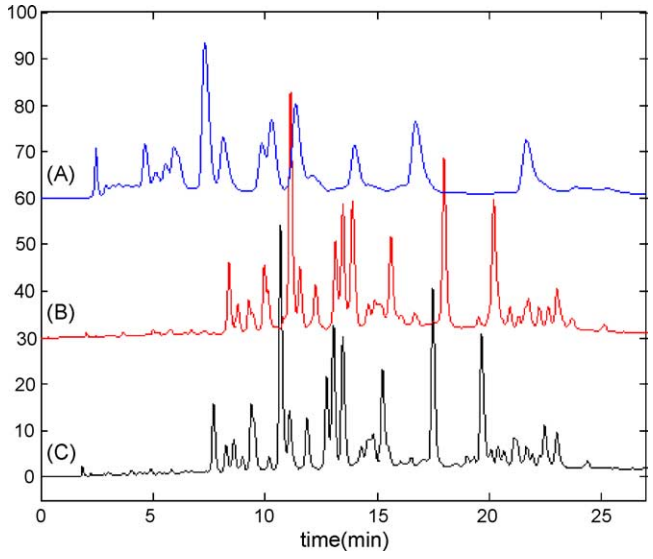


Fig. 2. Chromatograms of Ginkgo sample on three different columns. (A) Lichrosorb RPC18, 5 μ, 250 mm × 4.6 mm, (B) Nucleosil C18, 3 μ, 150 mm × 4.6 mm, (C) Alltima C18, 5 μ, 150 mm × 4.6 mm. Other experimental conditions as in text.

for the robustness of the method to use a relatively simple mobile phase. Thus, it was decided to use the chromatographic system consisting of the Alltima C18 column and an acetonitrile–0.1%phosphoric acid mobile phase combination, to proceed to the next optimization step.

4.2. Screening design

It is not likely that a satisfactory separation can be obtained only through multiple-column/mobile phase screening, due to the fact there are too many components present in herbal medicines. Some critical experimental settings, including controllable factors such as column temperature, detection wavelength, injection volume, and gradient elution parameters, e.g. initial organic solvent concentration and gradient slope should be considered prior to further optimization. Frequently, the choice of the proper parameter levels is a time-consuming process through trail-and-error experiments, from which the optimal parameter setting possibly is not obtained.

We selected a fractional factorial design as screening design since its primary purpose is to identify significant main effects. In the study of fingerprints, more information can be collected when more peaks are observed in the output chromatogram. Therefore, the peak number was used as response and seven factors were examined (Table 4). A feasible wide range of experimental conditions was chosen for each factor. Column temperature is recognized as an important parameter for its influence on retention, selectivity, system pressure, and column stability [27]. However, it is possible that some laboratories lack temperature control. On the other hand, under the acidic conditions and high temperatures, Ginkgo flavonoids possibly hydrolyze to the aglycones [15]. Therefore, a range just above room temperature (30–50 °C) was chosen to establish fingerprints method that can be transferable from one laboratory to another. Maximum absorbance values around the wavelengths of 260 and 350 nm were observed on the UV spectra of Ginkgo extract and the flavonoid standards. Comparing the chromatograms detected at these two wavelengths, more peaks could be obtained at 350 nm. Moreover, the wavelength of 350 or 370 nm was selected in the references [16–18]. Hence, the wavelength range from 350 to 370 nm was examined. According to similar practical considerations, the ranges of the other parameters were also selected

Table 4
Factors and levels investigated in the screening design

Factor	Units	Level (−1)	Level (+1)
X1: concentration of phosphoric acid	%	0.1	0.5
X2: detector wavelength	nm	350	370
X3: column temperature	°C	30	50
X4: initial concentration of acetonitrile (B%)	%	12	18
X5: gradient time	min	30	50
X6: injection volume	μl	5	10
X7: concentration of isopropanol in mobile phase	%	0	10

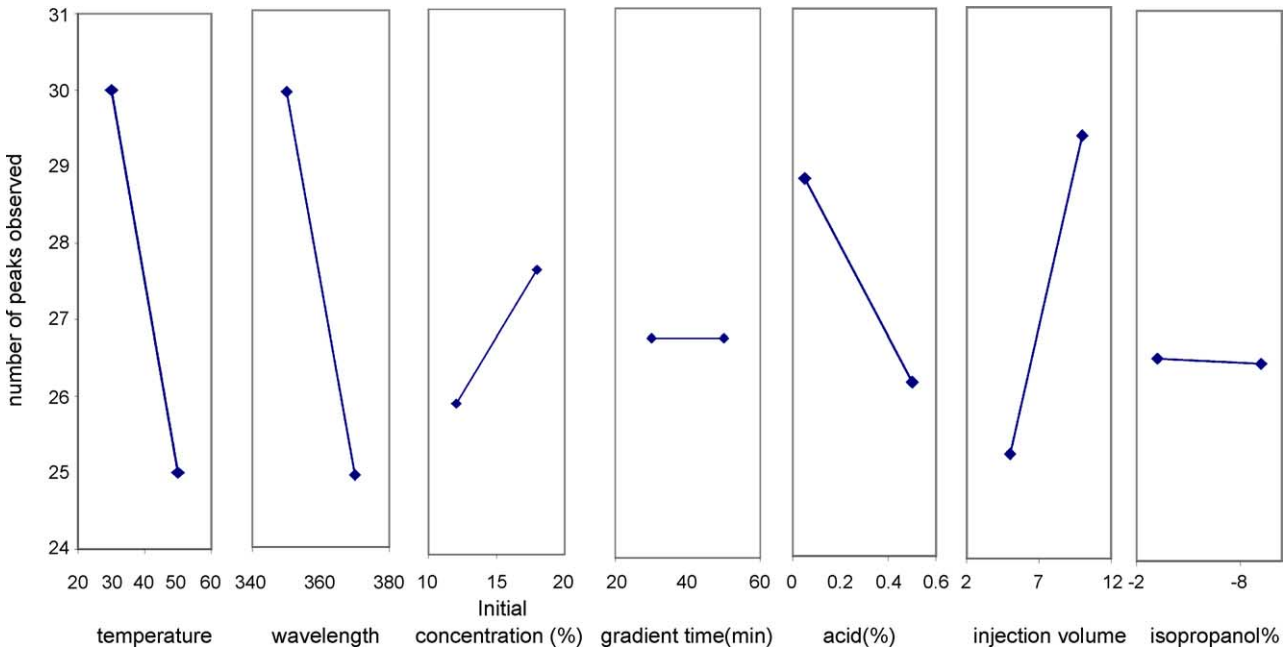


Fig. 3. Main effect plots from the screening design.

(Table 4). A schedule of 8 runs was carried out according to the 2^{7-4}_{III} fractional factorial design (Table 1). Instead of traditional statistical effect evaluation, the main effect plot was applied as tool for determining the important factors and the best factor setting [20].

The main effect plots (Fig. 3) revealed that detection wavelength and column temperature have significant effects on the peak capacity (the total number of peaks observed). Most of flavonoids in Ginkgo have maximum UV absorbance around 350 nm at which the better characterization can be attributed [17]. Fig. 3 also illustrated that both higher temperature and acid concentration were not beneficial for the separation and thus the number of peaks observed. Neither the longer gradient time nor using isopropanol as organic additive improved the separation. Finally, it is reasonable that more information can be obtained using a higher injection volume.

According to the screening result, the values of some important controllable parameters can be selected. Detection wavelength, column temperature, acid concentration and injection volume were set at 350 nm, 30 °C, 0.1% and 10 μl, respectively, while no isopropanol was needed in the mobile phase. Comparing to the separation of the previous step, a higher total peak number (39 versus 36) was observed from the chromatogram of a screening run. However, the separations in the fingerprint still might to be improved by further optimization. From the results of the screening experiments, two factors, the initial concentration of organic solvent and the gradient time did not seem very important. However, as mentioned above, a screening design is mainly for identification of significant main effects, rather than of interaction effects. The later can not always be considered negligible for these two parameters. Moreover, the gradient optimization can be considered as a point of a general strategy to improve the HPLC separation [28–30]. Therefore, it was selected in the next step.

4.3. Gradient optimization

HPLC Gradient optimization methods, which usually include two steps, modeling of retention and optimization, have been discussed in the literature [28–30]. In order to simulate the chromatographic process, the retention time and peak shape of every solute must be determined by running several isocratic or linear gradient experiments. Some computer software packages, such as Drylab [31], ChromSword [32], and Osiris [33] include gradient optimization facilities. However, it is difficult to separate and track every compound of a complex herbal extract in which too many compounds are unknown and reference standards are unavailable. Additionally, it is not necessary (nor practically feasible) to baseline separate all components in one run, due to the identification objectives of fingerprints of herbal medicines. Therefore, we prefer an optimization approach without modelling [26]. Uniform design can be a good choice for this purpose.

In the performance of gradient optimization, gradient time, gradient shape and initial composition of the mobile phase can be considered [34]. As mentioned above, the feasibility and reproducibility of the final fingerprints is very important for the method development. For this purpose, a linear gradient shape was applied in this work. Two parameters were chosen as optimization factors, the gradient time (t_G) and the concentration of organic solvent at the beginning of the gradient (B%). The optimization region selected was 28–52 min for t_G and 11 to 17% for B% considering the

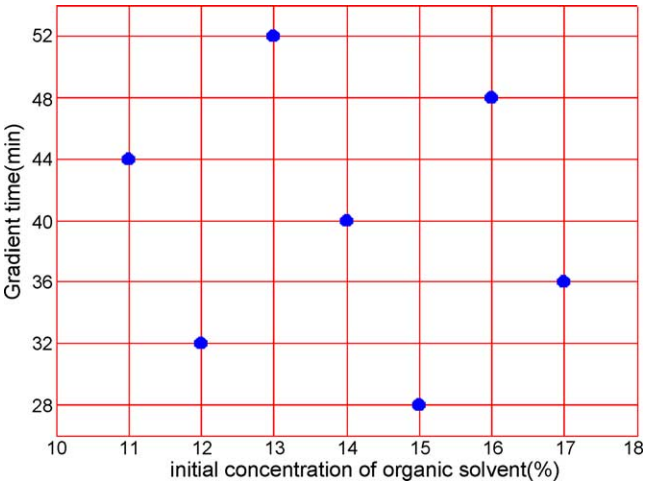


Fig. 4. Uniform design setting of the seven experimental runs in a U_7 (7^2) design.

result of the screening experiment of step two. The final concentration of B was set at 30% to ensure that every solute can elute in one run. Using the uniform table U_7 (7^2), seven experiments were designed. As shown in Fig. 4, the designed points are scattered uniformly in the variables region.

Another point to be considered in the optimization, is the chromatographic criterion that can evaluate the quality of fingerprints. The selection of suitable criteria to achieve an optimal conclusion can vary according to the different goals of an optimization process. Some evaluation parameters based on R_s, the resolution between two neighboring peaks, such as the sum of all resolutions $\left(\sum R_s\right)$, is sometimes used as criteria to evaluate the separation quality [35]. A normalized resolution factor was defined as:

$$r^* = \prod_{i=0}^{n-1} \left(\frac{R_{si,i+1}}{\overline{R_s}} \right) \tag{1}$$

to gain uniform distribution of detected peaks. However, $\left(\sum R_s\right)$ is usually determined by the largest resolution values, which probably are less relevant for the total quality of fingerprints. As for r^*, due to normalization, a very short chromatogram with all components evenly distributed will still be heavily favored, even if the maximum observed resolution is small [36]. Information theory was applied to evaluate the performance of the chromatographic fingerprint [37]. An information content ϕ, which is calculated based on:

$$\phi = -\int \frac{p_x}{\mathrm{sum}(p_x)} \frac{\log\, p_x}{\mathrm{sum}(p_x)} \mathrm{d}x \tag{2}$$

where p_x is the real chromatographic response of all chemical components involved in the fingerprint, reaches its maximum when all the peaks of the fingerprints were separated completely and distributed equally in the chromatogram.

It is considered most important that the chromatographic fingerprints selected, which contain more peaks of pharmacologically active components and have relatively shorter analysis time, will be preferred over less well-separated or longer ones. Therefore, it is necessary to consider simultaneously the number of peaks that may be detected, the resolution and time of analysis. A hierarchical chromatographic response function (HCRF) can fit for this purpose [38],

$$\mathrm{HCRF} = 1,000,000n + 100,000R_{\min} + (t_m - t_l) \tag{3}$$

where n is the number of peaks in the chromatogram, $R_{\min}$ is the resolution of the least separated pair of peaks, t_m and t_l are the maximum acceptable analysis time (100 min can be accepted in this study) and the retention time of the last peak, respectively. From the output value of HCRF, the number of peaks, the worst resolution and time of analysis are immediately apparent. For example, from the HCRF value 10,120,066.35, one sees from the first two digits that the number of peaks is 10. The 3–5th digits represent the resolution factor while the resolution of the least-separated pair of peaks being 1.20. And the last four digits represent the time factor while the analysis time being 100 − 66.35 = 33.65 min. The values of $\left(\sum R_s\right)$, r^*, ϕ and HCRF of the uniform design results are shown in Table 5. The values of these criteria are not consistent with each other due to the different objectives mentioned above. In our opinion, HCRF, which is conveniently acquired from the chromatogram, is a reasonable criterion for evaluating the separation quality in fingerprint chromatograms. According to the results of Table 5, the conditions of Experiment Nos.1 and 7 were selected (Fig. 5a and b). As seen from Fig. 5, these two chromatograms are very similar. However, shorter analysis time can be obtained in Fig. 5a, and the resolution of the peaks around 10 min is better. Therefore, the conditions of Experiment No. 1, which was performed at a linear gradient elution with acetonitrile/0.1% phosphoric acid (from 14/86 to 30/70 in 40 min) as mobile phase, a column temperature of 30 °C, a detection wavelength of 350 nm, and an injection volume of 10 μl, were chosen as optimized method conditions.

Table 5
The result of the uniform design

No.	B_0 (%)	t_G (min)	r^* ($\times 10^{-3}$)	$\sum R_s$	ϕ	HCRF
1	14	40	8.80	67.10	8.127	43,068,057
2	13	52	2.62	65.25	8.008	41,068,045
3	15	28	9.02	55.54	7.893	39,056,069
4	12	32	5.92	52.67	7.635	39,034,066
5	11	44	4.78	58.37	7.720	40,067,053
6	17	36	5.02	60.95	7.916	41,080,057
7	16	48	6.16	65.79	8.075	46,068,049

Compared to the best separation after the screening design (HCRF is 39070047), the separation of the fingerprint was obviously improved after gradient optimization (Table 5). Therefore, this step was considered and demonstrated necessary in developing a fingerprint even though the two gradient elution parameters, initial concentration of organic solvent and gradient time, were not found very important factors from the two-level screening design.

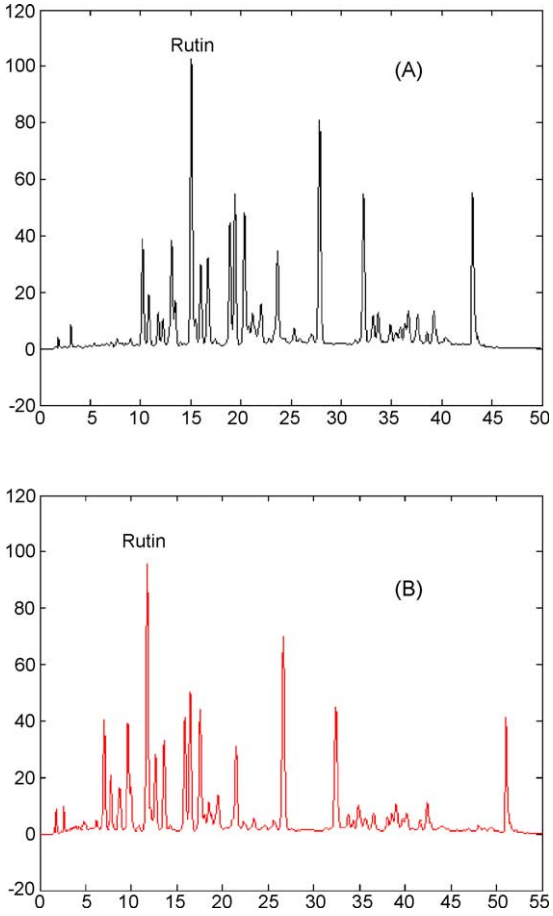


Fig. 5. HPLC fingerprints of Ginkgo extract in two different experiment conditions. (A) No. 1 (B) No. 7 experimental runs in a U_7 (7^2) design, HPLC conditions as in text.

4.4. Methodology validation

HPLC fingerprint determination is not a quantitative method. The parameters evaluated and validation aspects are different from general assaying methods. The authentication and identification of a herbal medicine can be accurately carried out using the chromatographic fingerprints obtained, even if the batches or the concentrations are not the same in different samples of this drug [37]. Considering these characteristics of fingerprints, the relative retention time (the ratio of retention time of sample constituents to an internal standard) and the relative peak area (the ratio of peak area of sample constituents to an internal standard) of main peaks (>1% of total peak area) detected were used to evaluate the quality of fingerprints.

4.4.1. Identification and purity determination of chromatographic peaks

The identification of the peaks was carried out using the standards and a diode-array detector. The highest peak in the chromatogram is belonging to rutin (Fig. 5), which was used as internal standard for method validation. With a diode-array detector and the corresponding computer software, the evaluation of peak purity allows checking the singularity of the peak component. The characteristic used is that the absorption spectrum of a single component remains invariable at each time point in one peak. In the chromatographic fingerprint of *Ginkgo biloba* extract, the peaks with larger area (>5% of total area) have high purity according to the normalized match factor value (>0.99). However, the normalized match factors sometimes give an imprecise result for the peak purity estimation because the UV spectra of components are too similar, and it will be dealt with further later.

4.4.2. Injection precision

The injection precision was determined by replicate injection of the same sample solution for six times in a day. The relative standard deviation (RSD) of relative retention time and relative peak area found were not exceeding 0.27 and 4.96%, respectively.

4.4.3. Repeatability

The repeatability was assessed by analyzing six independently prepared samples of Ginkgo extract. The relative standard deviation of relative retention time and relative peak area were below 0.39 and 5.75%, respectively.

4.4.4. Sample stability test

The sample stability test precision was determined with one sample during 6 days. During this period, the solution was stored at room temperature. The RSD of relative retention time and relative peak area found were less than 0.40 and 5.89% respectively. The similarity of these results with those from the repeatability and the injection precision indicate that the sample remained stable during this period.

5. Conclusion

The process of development, optimization and validation of HPLC fingerprints of *Ginkgo biloba* extract was introduced in detail. From the selection of a suitable chromatographic system, the screening for important parameters, gradient optimization to method validation, an integrated and universal HPLC fingerprint approach was performed using both fractional factorial and uniform designs. With each optimization step, except for the validation, the separation quality of the fingerprint improved. The results of the method validation, based on the relative standard deviation of relative retention times and relative peak areas, were acceptable. This four-step strategy can not only be utilized for the *Ginkgo biloba* extract but also for other herbal medicines to identify and assess their quality.

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