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ORIGINAL PAPER

Simultaneous chemical fingerprint and quantitative analysis of *Ginkgo biloba* extract by HPLC–DAD

Daoquan Tang · Dongzhi Yang · Anbang Tang · Yuanyuan Gao · Xianglan Jiang · Jie Mou · Xiaoxing Yin

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Abstract A reverse-phase liquid chromatography method with diode array detection was developed to evaluate the quality of *Ginkgo biloba* extract through establishing chromatographic fingerprint and simultaneous determination of eight flavonoid compounds, namely rutin, myricetin, quercitrin, quercetin, luteolin, kaempferol, apigenin, and isorhamnetin. The chromatographic separation was performed on an Agilent SB-C18 column (250×4.6 mm, 5.0 μm) with a gradient elution program using a mixture of methanol and 0.1% formic acid (*v*/*v*) as mobile phase within 55 min at 360-nm wavelength. The correlation coefficients of similarity for different batches of *G. biloba* extract from the same manufacturer and *G. biloba* extract from different manufacturers were determined from the LC fingerprints, and they shared a close similarity. The eight flavonoid compounds showed good regression (R^2>0.9995) within test ranges, and the recovery of the method was in the range of 94.1–101.4%. In addition, the content of those eight flavonoid compounds in *G. biloba* extract prepared by different manufacturers of China was determined to establish the effectiveness of the method. The results indicated that the developed method by having a combination of chromatographic fingerprint and quantification analysis could be readily utilized as a quality control method for *G. biloba* extract and its related traditional Chinese medicinal preparations.

Keywords Column liquid chromatography · Fingerprint analysis · Flavonoids compounds · *Ginkgo biloba* extract

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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. 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 [2]. In 2000, the State Food and Drug Administration (SFDA) of China began to develop the fingerprints of TCM as the standard of quality control [3]. Chromatographic methods were highly recommended for developing fingerprints of TCM and their preparations. Since then, increasing interest in high-performance liquid chromatography (HPLC) fingerprint analysis can be observed, not only in China but also in other countries all over the world [4–7].

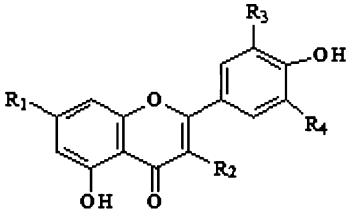
Chromatographic fingerprint analysis by which multiple compounds in single herbal drugs and finished TCM can be identified represents a rational approach for the quality

assessment of TCM. It utilizes chromatographic techniques, such as CE, GC, HPLC, HPTLC, etc. [8], to construct specific patterns of recognition for multiple compounds in herbal drugs. The entire pattern of compounds can then be evaluated to determine not only the absence or presence of desired markers or actives but the complete set of ratios of all detectable analytes [9]. Thus, chromatographic fingerprint analysis of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality, and ensuring the consistency and stability of herbal drugs and their related products.

Plant extracts are complex mixtures whose therapeutic effect is often attributed to the cumulative effects of many components [10] and so it is important to have an overall view of all the components in the extract to evaluate the quality of the plant product as many factors affect their quality and efficacy. Although "chemical fingerprint analysis" can give an overall view of all the components in TCM, it cannot reveal the variation of every ingredient. So, simultaneous quantification of multi-ingredients in TCM also is essential. Combination of chemical fingerprint and quantification of multi-ingredients can be used to control the quality of TCM effectively [11–14].

The extracts of *Ginkgo biloba*, which is among the top ten of sold plant products in the world, possess antioxidant, anti-ischemia, cardiovascular, and cerebrovascular activities [15]. The preparation process of the extracts from *G. 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 [16]. According to the present knowledge, in the *Ginkgo* extract, there are more than 30 different flavonoids, which can chemically represent the character of the *G. biloba* leaves [17–19] as derivatives of the flavonol aglycones, quercetin, kaempferol, and isorhamnetin (see Fig. 1).

In an effort to standardize *Ginkgo* preparations, various analytical techniques have been employed usually using various components as marker compounds or fingerprint chromatographic technology. The current approach to standardization of flavonols in *Ginkgo* extracts is by calculation of the total flavonol glycoside content from the aglycone concentration after acid hydrolysis. Although this procedure is relatively simple and widely accepted, the aglycones already present in extracts and calculations based on the average glycoside mass result in exaggerated reported flavonol glycoside content [16]. The presence of glycosides, which may hydrolyze during extraction and/or incorrect storage, is therefore a useful quality control indicator. An increase in the ratio of aglycones to glycosides in extracts signifies degradation [20]. Although reference standards for all flavonol glycosides are not available, relevant flavonol glycoside markers can be chosen for analytical techniques to ensure comprehensive standardization. Fingerprint chromatographic technology is another approach to control the quality of *G. biloba* extract (GBE) and its preparations [21–25], but it is a qualitative technology and cannot provide the quantity of individual flavonol.



Flavonol	R_1	R_2	R_3	R_4
Quercetin	OH	OH	OH	H
Rutin	OH	Rutinose	OH	H
Quercetrin	OH	Rhamnose	OH	H
Apigenin	OH	H	H	H
Luteolin	OH	H	OH	H
Kaempferol	OH	OH	H	H
Myricetin	OH	OH	OH	OH
Isorhamnetin	OH	OH	OCH_3	H

Fig. 1 Molecular structures of the eight compounds

As mentioned above, there were lots of literature about the quality control of GBE and its preparations, but literature about combination of chromatographic fingerprint and quantification of multi-ingredients for the quality control of GBE has not been published. This paper describes, for the first time, a simple, accurate, and practical HPLC method with photodiode array detection for chromatographic fingerprint analysis and simultaneous quantification of eight compounds (myricetin, quercetin, luteolin, kaempferol, apigenin, and isorhamnetin, two flavonol glycosides, rutin, and quercitrin) in GBE. Chromatographic conditions and method validation were objectives of the research reported. The method was successfully used for analysis of chromatographic fingerprint and amounts of the eight compounds in GBE from different manufacturers.

Materials and methods

Chemicals and reagents

HPLC-grade methanol was obtained from Fisher Scientific (Fisher Scientific, USA). Rutin, myricetin, quercitrin, quercetin, luteolin, kaempferol, apigenin, isorhamnetin (see Fig. 1), and GBE reference standard were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).The purity (99.5%) of these reference standards except GBE reference standard was assumed as provided by the suppliers. Water

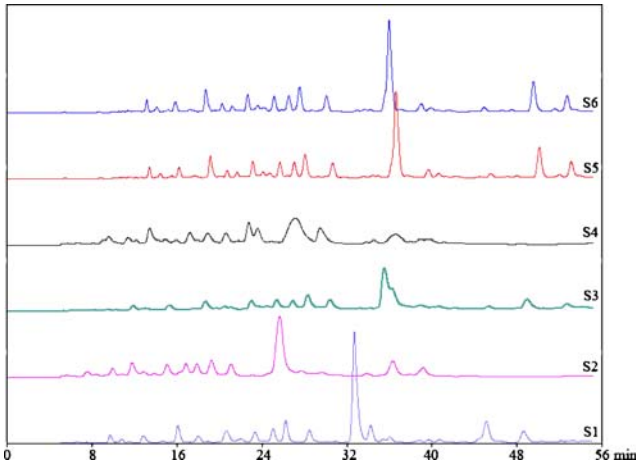


Fig. 2 The chromatogram of GBE separated on different column *S1*: Hypersil ODS_2 (250 mm); *S2*: Kromasil C18 (150 mm); *S3*: Hypersil BDS-C18 (250 mm); *S4*: Shimadzu Chim-pack CLC-ODS (150 mm); *S5*: Kromasil C18 (250 mm); *S6*: Agilent Zorbax SB-C18 (250 mm)

was prepared by automatic double-purified water distilling apparatus (Shanghai Yarong Biochemical Factory, China). The mobile phase was degassed by Ultrasonic Generator (Wuxi Ultrasonic Generator Electronic Equipment Company, China) and filtered by 0.45-μm filter (Autoscience Instrument Co. Ltd., China). Commercial products GBE-A (lot no. 20060725, 20060812, 20060903, 20060930, 20061011, 20061115, 20061129, 20061221, 20070131, 20070212), GBE-B (lot no. 20070213), GBE-C (lot no. 20061218), and GBE-D (lot no. 20070123) were purchased from Enhua pharmaceutical company (Jiangsu, China), Kangenbei Pharmaceutical Company (Zhejiang, China), Yangtze Pharmaceutical Company (Jiangsu, China), and Fuwei Biotechnology Company (Pizhou, China), respectively. All other chemicals were of analytical grade. GBE-A (lot no. 20060725) was selected as the sample for chromatographic conditions and subsequent method validation.

Chromatographic system

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, DGU-20A degasser, FCV-10ALVP, SCL-10AVP system controller, Rheodyne 7725 injector with a 20-μl loop, and a SPD-20AVP diode array detector. System control and data analyses were carried out using LCsolution software (Shimadzu). Separation of these analytes has been done on an Agilent Zorbax SB-C18 column (5-μm particle size, 250×4.6 mm i.d.). The separation was carried out with gradient elution procedure and mobile phase A (methanol) and B (0.1% formic acid) ratios linear changed as follows: 0~5 min, 35~40% A; 5~40 min, 40~50% A; 40~50 min, 50~60% A; 50~55 min, 60~65% A. The total run time was 55 min at a flow rate of 1 ml/min. The eluent was monitored by a diode array detector, and the detection wavelength was set at 360 nm. The sample injection volume was 20 μl, and the column temperature was 35 °C.

Preparation of standard stock solutions

The reference standards of the eight flavonoid compounds (rutin, myricetin, quercitrin, quercetin, luteolin, kaempferol, apigenin, isorhamnetin) were accurately weighed and dissolved in methanol then diluted to appropriate concentration ranges for the establishment of calibration curves. All stock and working standard solutions were stored in brown bottles at 4 °C until used for analysis and were found to be stable for at least 1 month.

Preparation of sample solutions

Dissolve 160 mg of extract of GBE in 5 ml of methanol–water (50:50, *v*/*v*); filter through a 0.45-μm PTFE filter membrane; the filtrate is used as the GBE sample solution.

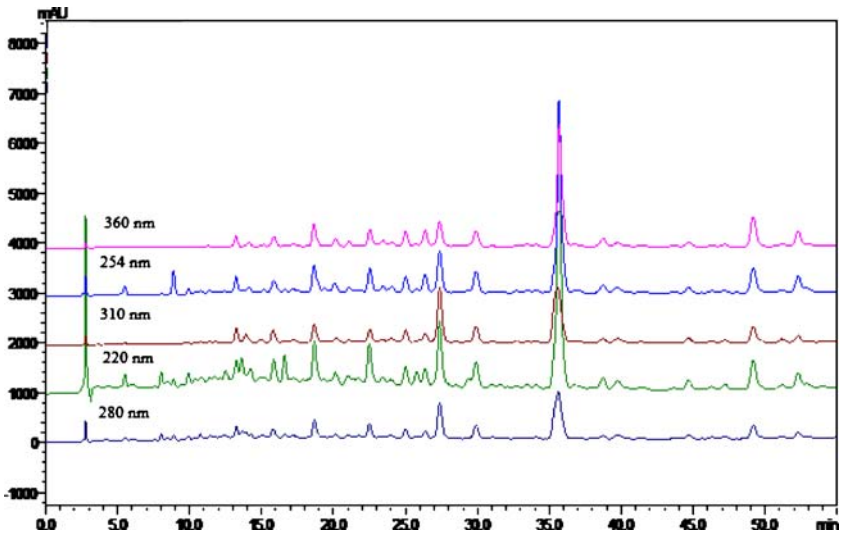


Fig. 3 The chromatogram of GBE detected at different wavelengths

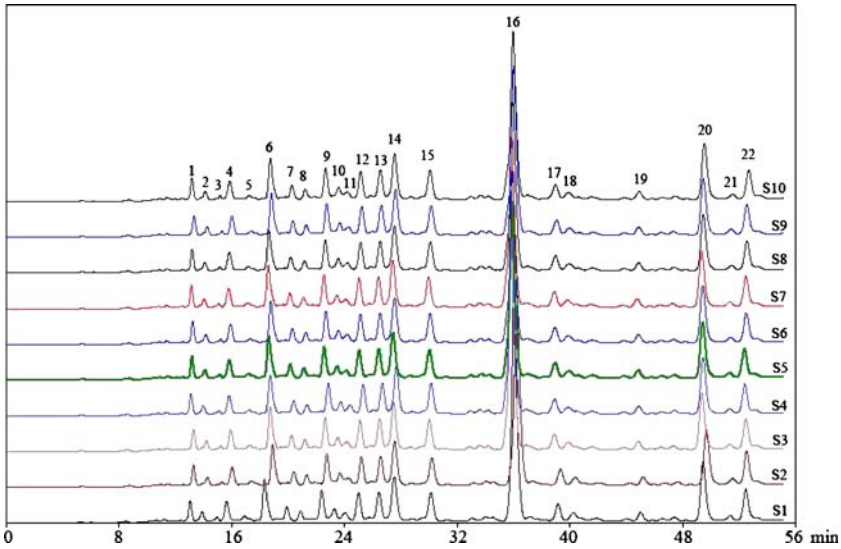


Fig. 4 The chromatogram of ten batches of GBE from the same manufacturer (GBE-A). (*6*: rutin, *10*: myricetin, *11*: quercitrin, *16*: quercetin, *18*: luteolin, *20*: kaempferol, *21*: apigenin, *22*: isorhamnetin)

Data analysis

Similarity analysis was performed by the professional software Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A), which was recommended by SFDA. The software was to employ the correlative coefficient in evaluating the similarities of different chromatograms.

Results and discussion

Selection of a suitable chromatographic system

Reversed-phase liquid chromatography with ODS columns is recommended for separation of quercetin and the corresponding glucoside [26, 27]. Because of the similar interaction with the column which results from their similar chemical structures, it is challenging to develop a chromatograph and separate eight flavonoids simultaneously for GBE. Different types of column were tested such as Kromasil C18 (250 mm×4.6 mm, 5 μm particles), Hypersil BDS-C18 (250 mm×4.6 mm, 5 μm particles), Shimadzu Chim-pack CLC-ODS (150 mm×4.6 mm, 5 μm particles), Hypersil ODS2 (250 mm×4.6 mm, 5 μm particles), Kromasil C18 (150 mm×4.6 mm, 5 μm particles), and Agilent Zorbax SB-C18 (250 mm×4.6 mm, 5 μm particles) and enabled resolution of the all the compounds with different retention behaviors. The Kromasil C18 (250 mm) and Agilent Zorbax SB-C18 gave the best results (see Fig. 2).

Different mobile phases were tried, such as water–acetonitrile–isopropanol–citric acid, acetonitrile–0.01% potassium dihydrogen phosphate (phosphoric acid was used to regulate pH 2.0), methanol–0.1% formic acid, acetonitrile–0.1% formic acid, acetonitrile–0.3% formic acid, etc. Finally, methanol and 0.1% formic acid were selected as an appropriate mobile phase with a step linear gradient, which gave good resolution and acceptable peak parameters for rutin, myricetin, quercitrin, quercetin, luteolin, kaempferol, apigenin, and isorhamnetin (see Fig. 4).

Table 1 The RRT and RPA of these 22 peaks with respect to peak 16 in ten batches of GBE samples from the same manufacturer

Peak no.	RRT	RSD%	RRP	RSD%
1	0.37	1.08	0.07	5.22
2	0.39	1.23	0.05	7.45
3	0.42	1.09	0.02	8.39
4	0.44	1.10	0.09	5.90
5	0.48	1.12	0.04	6.14
6	0.52	1.13	0.18	7.66
7	0.56	0.93	0.07	4.42
8	0.59	0.78	0.05	8.98
9	0.63	0.63	0.14	7.18
10	0.66	0.61	0.07	8.50
11	0.67	0.49	0.05	9.09
12	0.70	0.49	0.13	5.06
13	0.74	0.42	0.15	5.01
14	0.77	0.35	0.24	4.66
15	0.84	0.26	0.16	5.63
16(s)	1.00	0.00	1.00	0.00
17	1.09	0.09	0.09	5.08
18	1.11	0.23	0.05	4.82
19	1.25	0.43	0.04	5.26
20	1.38	0.34	0.31	4.83
21	1.43	0.39	0.03	7.48
22	1.46	0.41	0.16	2.83

Table 2 The similarities of ten batches of GBE samples from the same manufacturer (similarity evaluation system for chromatographic fingerprint of traditional Chinese medicine, version 2004A)

Calculated by the mean of fusion vectors of all samples as the reference fingerprint

No.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1.000	0.999	1.000	1.000	0.996	0.999	0.996	0.995	0.996	0.996
S2	0.999	1.000	1.000	1.000	0.998	1.000	0.998	0.998	0.998	0.998
S3	1.000	1.000	1.000	1.000	0.998	0.999	0.997	0.996	0.997	0.997
S4	1.000	1.000	1.000	1.000	0.997	0.999	0.997	0.996	0.997	0.997
S5	0.996	0.998	0.998	0.997	1.000	0.998	1.000	1.000	1.000	1.000
S6	0.999	1.000	0.999	0.999	0.998	1.000	0.998	0.998	0.998	0.998
S7	0.996	0.998	0.997	0.997	1.000	0.998	1.000	1.000	1.000	1.000
S8	0.995	0.998	0.996	0.996	1.000	0.998	1.000	1.000	1.000	1.000
S9	0.996	0.998	0.997	0.997	1.000	0.998	1.000	1.000	1.000	1.000
S10	0.996	0.998	0.997	0.997	1.000	0.998	1.000	1.000	1.000	1.000

Selection of detection wavelength

Selection of an appropriate detection wavelength was of great importance to ensure precise detection of the eight constituents and to achieve more peaks. The UV spectra of the compounds were detected at 220, 254, 280, 310, and 360 nm by diode array detector with the LCsolution software (Shimadzu) under the chromatography conditions as described in chromatography system. Although more peaks were detected at 220 and 254 nm than other detection wavelengths, more interference was encountered at 220 and 254 nm. Moreover, considering that the condition of chromatography developed should be adapted to quantitative analysis of eight flavonoids, the chromatograms of GBE were obtained by monitoring UV absorption at 360 nm (see Fig. 3)

LC–DAD fingerprint analysis

The fingerprinting analysis were operated through a software Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A), which was recommended by SFDA. To perform it, the chromatograms of different samples have to be standardized. The process of standardization included the selection of "common peaks" in chromatograms and the normalization of retention times of all the common peaks. Furthermore, the total area of the common peaks must be more than 90% of the whole area in one chromatogram which could avoid adulterating common components by manufactures. Here, the extracts of ten batches of GBE samples collected from the same manufacturer (GBE-A) served as the sample set. The liquid chromatographic (LC) fingerprints were shown in Fig. 4. The peak of quercetin (16) at retention time 35.77 min indicated the highest content in all the 22 peaks. Therefore, it was selected as a reference peak. Among all the peaks observed, 22 of them (>5% of total area, denoted from 1 to 22) were defined as common peaks because they showed up in all samples (Fig. 4). The relative retention time (RRT) and relative retention area (RPA) of these 22 peaks with respect to peak 16 in ten samples were shown in Table 1. Based on the comparisons with standard compounds, eight peaks were unambiguously identified rutin (6), myricetin (10), quercitrin (11), quercetin (16), luteolin (18), kaempferol (20), apigenin (21), and isorhamnetin (22). Similarity analysis was performed for the ten chosen GBE from different

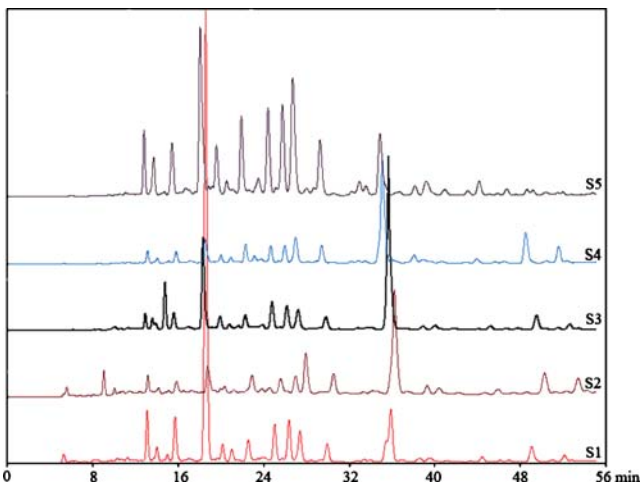


Fig. 5 The chromatogram of five GBE samples from different manufacturer (*S1*: GBE-D; *S2*: GBE-C; *S3*: GBE-B; *S4*: GBE-A (20060725); *S5*: GBE standard)

Table 3 The similarities of five GBE samples from different manufacturer (similarity evaluation system for chromatographic fingerprint of traditional Chinese medicine, version 2004A)

No.	S1	S2	S3	S4	S5
S1	1.000	0.923	0.954	0.967	0.916
S2	0.923	1.000	0.985	0.934	0.998
S3	0.954	0.985	1.000	0.946	0.928
S4	0.967	0.934	0.946	1.000	0.967
S5	0.916	0.998	0.928	0.967	1.000

S1 GBE-D, *S2* GBE-C, *S3* GBE-B, *S4* GBE-A(20060725), *S5* GBE standard

Table 4 Seven concentrations of the eight analyses for linearity

Concentration	1	2	3	4	5	6	7
Rutin	8.11	16.31	32.63	65.25	130.5	261	522
Myricetin	1.63	3.26	6.51	13.03	26.05	52.1	104.2
Quercitrin	0.09	0.19	0.38	1.5	3	6	12
Quercetin	8.08	16.16	32.31	129.25	258.5	517	1,034
Luteolin	0.83	1.65	3.2	6.6	26.4	52.8	105.6
Kaempferol	0.46	0.92	1.84	7.34	29.38	58.76	117.5
Apigenin	0.48	0.96	1.92	3.84	7.68	15.36	30.72
Isorhamnetin	3.06	6.13	12.25	24.5	98	196	392

batches in the same manufacturer. The similarity indexes were calculated by mean fusion vector method. As listed in Table 2, the similarity index of ten samples was higher than 0.995, which suggested that the samples from different batches in the same manufacturer shared the similar chromatographic patterns. Detailed analysis of the LC profile of each sample indicated that the common peak abundance has no significant variation (data not shown), which suggested that the content of eight flavonoids was stable in different batches.

In addition, The LC chromatographic fingerprints of five samples of GBE from different manufacturer was developed and shown in Fig. 5. Similarity analysis was performed for the five samples of GBE from different manufacturer. As listed in Table 3, the similarity index of five samples was higher than 0.916, which suggested that the samples from different manufacturer shared the similar chromatographic patterns. Detailed analysis of the LC profile of each sample indicated that the common peak abundance varies from each other (data not shown), which could be caused by the difference of plant origin, the effect of environment, season of collection, drying process, storage conditions, inclusion of other plants or adulteration, etc.

Identification and purity determination of chromatographic peaks

The identification of the peaks was carried out using the standards and a diode array detector. With a diode array detector and the corresponding computer software (LCsolution software, Shimadzu), the evaluation of peak purity allows checking the singularity of the peak component. The characteristic used for the evaluation of peak purity is that the absorption spectrum of a single component remains invariable at each time point in one peak.

Validation of the method

The HPLC method was validated by defining the linearity, limits of quantification and detection, identification and quantification of the analytes, repeatability, precision, stability, and recovery.

Linearity, limits of quantification, and detection

Calibration working standard solutions was freshly prepared in methanol–water (50:50, *v*/*v*) by appropriate dilution of the stock solutions to yield seven concentrations

Table 5 Calibration plots, LOD, and LOQ for the eight analyses

Compound	Linearity range (μg ml^{-1})	Calibration equation $y=a+bx$ [a]	LOD[b] (μg ml^{-1})	LOQ[b] (μg ml^{-1})	Correlation factor (R^2)
Rutin	8.11~522	y=31,961x−304,571	0.01	0.03	0.9996
Myricetin	1.63~104.2	y=74,581x−184,505	0.03	0.09	0.9996
Quercitrin	0.09~12	y=342,058x−36,917	0.03	0.09	0.9998
Quercetin	8.08~1,034	y=77,064x−836,858	0.002	0.008	0.9996
Luteolin	0.83~105.6	y=47,020x−47,856	0.02	0.08	0.9997
Kaempferol	0.46~117.5	y=191,073x−29,448	0.01	0.05	0.9998
Apigenin	0.48~30.72	y=76,425x−46,114	0.01	0.05	0.9997
Isorhamnetin	3.06~392	y=15,785x−66,342	0.01	0.03	0.9995

[a] y and x are, respectively, the peak areas and concentrations (μg ml^{-1}) of the analytes

[b] The LOD was defined as the concentration for which the signal-to-noise ratio was 3; the LOQ was defined as the concentration for which the signal-to-noise ratio was 10

Table 6 Precision, repeatability, and stability data of eight flavonoids (RSD%, n=6)

Compound	Precision				Repeatability		Stability	
	Interday		Intraday					
	Retention time	Peak area	Retention time	Peak area	Retention time	Content	Retention time	Peak area
Rutin	0.5	0.4	0.6	0.7	0.6	2.4	0.3	1.0
Myricetin	0.6	0.5	0.7	0.8	1.2	2.1	0.4	1.3
Quercitrin	0.5	0.3	1.0	0.8	2.4	1.6	0.4	1.6
Quercetin	0.7	0.6	0.7	0.9	1.2	2.4	0.5	1.0
Luteolin	0.7	0.2	0.3	1.0	2.1	1.6	0.4	1.1
Kaempferol	0.5	0.9	0.4	1.2	1.5	1.4	0.5	1.4
Apigenin	0.5	0.6	0.3	1.1	1.8	2.3	0.4	1.4
Isorhamnetin	0.4	0.4	0.3	1.3	0.7	1.8	0.3	0.9

as shown in Table 4. The calibration curve of the individual flavonoid is based on these seven concentrations of standard. The peak area values were the average values of three replicate injections. The results of calibration are summarized in Table 5, and a good correlation was found between the peak area (y) and the concentrations (x) (R^2> 0.9995) for all the compounds in the range of concentration tested at their detected wavelengths.

Table 7 Recovery of each analyte determined by standard addition method (n=3)

Compound	Original amount (μg)	Spiked amount (μg)	Found amount (μg)	Recovery (%)	RSD(%)
Rutin	92.16	83.52	172.28	98.1	1.4
	92.44	104.40	195.24	99.2	1.9
	92.26	125.28	219.81	101.1	1.7
Myricetin	25.22	16.67	41.10	98.1	2.1
	25.30	20.84	45.41	98.4	1.5
	25.25	25.01	49.64	98.8	2.4
Quercitrin	2.70	2.88	5.59	100.1	1.6
	2.71	3.60	6.23	98.8	2.4
	2.70	4.32	7.04	100.3	2.5
Quercetin	230.85	165.44	385.20	97.2	2.8
	231.54	206.80	422.51	96.4	1.6
	231.08	247.44	485.39	101.4	1.6
Luteolin	24.05	16.90	39.78	97.1	1.3
	24.13	21.12	42.89	94.8	2.5
	24.08	25.34	46.48	94.1	2.7
Kaempferol	35.34	23.50	57.02	96.9	2.5
	35.45	29.38	63.42	97.8	2.7
	35.38	35.25	68.05	96.4	1.8
Apigenin	5.91	4.92	10.64	98.3	1.8
	5.93	6.14	11.89	98.5	1.5
	5.92	7.37	13.33	100.3	2.1
Isorhamnetin	160.62	156.80	307.72	96.9	1.3
	161.10	196.00	358.43	100.4	1.9
	160.78	235.20	400.08	101.0	2.9

The data were presented as average of three determinations

Recovery (%)=100×(amount spiked + original amount)/amount found

The limit of detection (LOD) is defined as the smallest peak detected with a signal height three times that of the baseline while the limit of quantification (LOQ) value is often calculated as ten times the signal height to the baseline. In our work, detection and quantification limits were estimated by successively decreasing the concentration of the prepared standards to the smallest detectable peak. This concentration was multiplied by 3 and 10 to obtain the detection and quantification limits, respectively. Table 5 summarizes LOD and LOQ values of individual compounds and clearly indicates that the analytical method has excellent sensitivity.

Repeatability, precision, and stability

The repeatability of the method was evaluated by measurement of analysis repeatability. The analysis repeatability was examined by the injection of six different samples prepared by the same sample preparation procedure. The relative standard deviation (RSD) of retention time and component content for the eight flavonoids was used to estimate the repeatability. The results for analysis repeatability are shown in Table 6; RSD values for component content and retention time were all <3.0%, which could meet the need of quantitative analysis. The RSD value of retention time and component content for every flavonoid exhibited a difference as shown in Table 6. The former may result from difference of component content for different flavonoid in sample, while the latter may result from error in sample preparation.

The precision of the proposed method is reported as interday and intraday precision that can be determined from RSD for retention time and peak area resulting from the analysis of the studied compound. In our work, the interday and intraday precision was determined for all eight flavonoid standards by repeated analysis for six times within 1 day or on five separate days. The RSD of retention time and peak area was used to estimate the precision. These results are shown in Table 6; RSD values for peak area were all ≤2.0%, and retention time were all ≤1.0%

For the stability test, retention time and peak area of eight flavonoids in sample solution (methanol–water extracts) were analyzed every 8 h within 48 h, and the sample solution was found to be rather stable within 48 h (RSD≤0.6% for retention time and RSD≤1.6% for peak area, see Table 6). There was no decrease in the peak area with time for every flavonoid. The variation of peak area for every flavonoid may be due to the precision of the method.

Accuracy

The accuracy of the method was confirmed by measurement of recovery by the standard addition method, to assess possible positive or negative interferences from other chemical constituents present in the samples. Three different quantities (low, medium, and high) of the authentic standards were added to a previously analyzed real sample for which the concentrations of the compounds of interest were known. The mixtures were extracted by the method described in the section "Preparation of sample solutions," and the extracts were analyzed by the use of the HPLC method described above. The quantity of each component was subsequently obtained by use of the corresponding calibration plots. Each set of additions was repeated three times. The results from determination of accuracy, expressed as the percentage of the analytes recovered by the assay, are listed in Table 7. As shown in the table, recovery of the components ranged from 94.1% to 101.4%, and the RSD values were all <3.0%; this indicates that the method enables highly accurate simultaneous analysis of the eight compounds.

Simultaneous quantification of eight flavonoids in GBE

The developed analytical method was successfully applied to the simultaneous determination of rutin, myricetin,

Table 8 Amounts of the eight flavonoids in GBE

Sample	Amount (mg g^{-1}, mean ± *S*, *n*=3)							
	Rutin	Myricetin	Quercitrin	Quercetin	Luteolin	Kaempferol	Apigenin	Isorhamnetin
GBE-A	9.12±0.26	2.91±0.03	0.30±0.006	21.01±0.4	2.20±0.05	2.52±0.04	0.49±0.009	14.01±0.3
GBE-B	13.50±0.13	1.95±0.05	0.10±0.003	17.67±0.5	1.09±0.02	0.21±0.001	0.06±0.002	1.65±0.03
GBE-C	32.23±0.44	2.93±0.06	0.22±0.003	27.23±0.3	1.23±0.02	1.36±0.03	0.34±0.003	6.13±0.1
GBE-D	114.38±1.35	2.34±0.01	0.22±0.006	7.61±0.09	0.94±0.02	0.69±0.007	0.16±0.001	2.99±0.03
GBE-S	33.87±0.74	6.40±0.15	0.36±0.01	7.29±0.04	3.56±0.05	0.24±0.001	0.47±0.002	1.51±0.04
RSD%	105.1	53.7	41.1	53.6	60.9	96.5	62.3	99.5

GBE ginkgo biloba extract, *A* Enhua (20060725), *B* Kangenbei, *C* Yangtze, *D* Pizhou, *S* standard

quercitrin, quercetin, luteolin, kaempferol, apigenin, and isorhamnetin in five samples of GBE, which were obtained from various provinces and cities in China (Fig. 5). Each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing the retention times and online UV spectra with those of the standards.

The LC–diode array detection profiles are illustrated in Fig. 5. Table 8 shows the content of the eight flavonoid compounds in five samples of GBE. It was found that the content of each analyte varied greatly among the different samples. In the majority of cases, rutin and quercetin were the main component, whose content varied from 9.12 to 114.38 and 7.61 to 27.23 mg g^{-1}, respectively. In product D, content of rutin was more than others exceptionally (RSD%=105.1). Similar variation could also be found for the other flavonoid components (RSD% varied from 41.1 to 99.5). In Chinese Pharmacopeia, standardization of flavonoids in GBE is by calculation of the total flavonol glycoside content from the aglycone concentration after acid hydrolysis. As we know, rutin can be hydrolyzed to quercetin by acid. The other flavonol glycoside can be hydrolyzed to its aglycone. Thus, this method of quality control may lead to the possibility of adulteration. Moreover, the variation in content of constituents could certainly lead to the variation of therapeutic effects. Hence, each procedure involved should be standardized.

Conclusion

Combination of fingerprint with quantitative analysis of several marker compounds for quality control of traditional Chinese herbal medicines is definitely an improvement over the old methodology. The chromatographic fingerprint has predominance in showing the authenticity, quality consistency, and stability of this herb, while the quantification of several marker compounds can better reflect the quality of TCM. In the present study, a simple, accurate, and reliable LC method was developed to evaluate the quality of GBE through establishing chromatographic fingerprint and simultaneous determination of eight flavonoid compounds, namely rutin, myricetin, quercitrin, quercetin, luteolin, kaempferol, apigenin, and isorhamnetin. The results demonstrate that the developed method is accurate and reproducible and could be readily utilized as a suitable quality control method for the quantification of GBE. The method also can be utilized as reference for analysis of GBE preparations or other phytomedicine contained flavonoids compounds. The results of the analysis on the five GBE samples suggested that the content of the eight flavonoid compounds varied significantly from different locations of China. Therefore, the evaluation of data might be useful in quality assurance as well as for determination of adulteration of the crude drug. This method also has the advantage that a hydrolysis step, previously used to for the standardization of flavonols in GBE by calculation of the total flavonol glycoside content from the aglycone concentration in extracts, is not required.

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