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Research Article

Development and validation of an ultra high performance liquid chromatography electrospray ionization tandem mass spectrometry method for the simultaneous determination of selected flavonoids in *Ginkgo biloba*†

A rapid and sensitive ultra high performance liquid chromatography electrospray ionization tandem mass spectrometry method was developed and validated for the simultaneous determination of 13 flavonoids in leaf, stem, and fruit extracts of male and female trees of *Ginkgo biloba* to investigate gender- and age-related variations of flavonoids content. Chromatographic separation was accomplished on an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm id, 1.7 μm) in 5 min. Quantitation was performed using negative electrospray ionization mass spectrometry in multiple reaction monitoring mode. The calibration curves of all analytes showed a good linear relationship ($r^2 \geq 0.9977$) over the concentration range of 1–1000 ng/mL. The precision evaluated by an intra- and interday study showed RSD ≤ 1.98% and good accuracy with overall recovery in the range from 97.90 to 101.09% (RSD ≤ 1.67%) for all analytes. The method sensitivity expressed as the limit of quantitation was typically 0.25–3.57 ng/mL. The results showed that the total content of 13 flavonoids was higher in the leaf extract of an old male *Ginkgo* tree compared to young female *Ginkgo* trees.

Keywords: Flavonoids / *Ginkgo biloba* / Multiple reaction monitoring / Simultaneous determination
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1 Introduction

Ginkgo biloba, a valuable ancient Chinese tree, is dioecious and the only surviving member of Ginkgoaceae family, therefore, regarded as "living fossil" [1]. The standardized extract of green leaves of *G. biloba* provides a wide range of beneficial medicinal and therapeutic effects and is one of the most widely prescribed herbal/dietary supplements in the world and very popular in the United States and Europe [2, 3]. The therapeutic benefits of *Ginkgo* extract are thought to result from the synergistic action of flavonoids and terpene lactones present in *Ginkgo* extract [4]. *Ginkgo* leaves extract contain about 24% flavonoid glycosides and 6% terpene lactones, reported to be clinically effective in the treatment of Alzheimer's disease [5, 6], senile dementia [7, 8], depression, diabetic neuropathy, impotency, memory impairment, peripheral vascular disease, intermittent claudication, vertigo, and tinnitus [9]. The flavonoids are also responsible for *Ginkgo's* antioxidant and free radical scavenger effects, whereas the terpene lactones increase circulation and inhibit platelet-activating factor [9–11]. The biflavones of *G. biloba* exhibited antifungal, antiviral, anti-inflammatory activities [12–14], and form a stoichiometrically defined complex (Phytosome®) with phospholipids and inhibit cAMP phosphodiesterase activity to promote vasorelaxation, with lipolytic and soothing activity [15–18]. Variations of flavonoids and terpene lactones in leaves significantly affect quality of the *Ginkgo* extract. Therefore, it is of paramount importance to have reliable quantitative data on the composition of leaf extract for quality and efficacy.

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Abbreviations: CDRI, Central Drug Research Institute; **ELSD**, evaporative light scattering detection; **IS**, internal standard; **MRM**, multiple reaction monitoring; **PDA**, photodiode array detection; **RDA**, retro-Diels–Alder

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Colour Online: See the article online to view Figs. 1–3 in colour.

Various analytical methods including HPLC with photodiode array detection (PDA), HPLC with evaporative light scattering detection (ELSD), LC–UV–MS, HPLC–MS/MS, UHPLC–PDA, RP high-performance TLC, ^{1}H NMR, GC with flame ionization detection, mixed micellar EKC, CZE, HILIC-UHPLC–ESI-MS/MS, and UHPLC–ESI-MS/MS have been reported for the quantitative determination of flavonoids, terpene lactones, and underivatized amino acids in *Ginkgo* leaf extract and marketed herbal formulations [4, 19–49]. Although HPLC–ELSD and HPLC–PDA methods [44] have been reported to study variations of the flavonol glycosides at different ages, different cultivation sources, and genders of *G. biloba* leaves but variations of flavans, flavonol glycosides, flavonols, flavones, isoflavone, and biflavones in the leaf, stem, and fruit extracts of male and female trees of *G. biloba* using UHPLC–ESI-MS/MS in multiple reaction monitoring (MRM) mode have still not been reported. All these reported methods (HPLC–PDA, HPLC–ELSD, and RP-HPLC with refractive index detection) used for analysis contributed significantly to the current state of knowledge of constituents in the leaf but have drawbacks of long analysis time, high solvent consumption, and low sensitivity. The UHPLC–ESI-MS/MS method in MRM mode is a more powerful approach for simultaneous determination of multicomponents due to its rapid separation power, low solvent consumption, high capacity, high resolution, high sensitivity, high specificity, high selectivity, and multiple ion detection based on selective ion fragmentation.

In this communication, we have reported a rapid, sensitive, and efficient UHPLC–ESI-MS/MS method in MRM mode for the simultaneous determination of selected flavonoids including flavans (epicatechin, catechin), flavonol glycosides (quercetin-3,4′-diglucoside, rutin, and kaempferol-3-*O*-rutinoside), flavonols (quercetin, kaempferol, and isorhamnetin), flavones (luteolin and apigenin), isoflavone (genistein), and biflavones (amentoflavone and sciadopitysin) in leaf, stem, and fruit extracts of male and female trees of *G. biloba* to investigate gender- and age-related variations of flavonoids. The developed method was validated as per International Conference on Harmonization (Q2R1) guidelines in terms of linearity, LOD, LOQ, precision, stability, and recovery.

2 Materials and methods

2.1 Reagents, chemicals, and materials

Acetonitrile with 0.1% formic acid (LC–MS grade) and formic acid (analytical grade) were purchased from Fluka, Sigma–Aldrich (St. Louis, MO, USA) and water was purified using a Milli-Q system (Millipore, Milford, MA, USA). The analytical standards (purity ≥ 90%) of epicatechin, catechin, quercetin-3,4′-diglucoside, rutin, kaempferol-3-*O*-rutinoside, quercetin, luteolin, genistein, amentoflavone, sciadopitysin, and internal standard (IS) daidzein were purchased from Extrasynthese (Z.I Lyon Nord, Genay Cedex, France). The analytical standards (purity ≥ 95%) of apigenin, kaempferol, and isorhamnetin were purchased from Sigma–Aldrich.

The plant materials (leaf, stem of *G. biloba* male tree (175 years old) from Nainital (Uttarakhand, India) and leaf, stem, and fruit of female trees of two different ages (84 and 8 years old) from Ranikhet (Uttarakhand, India)) were provided by Dr. K. R. Arya, Botany division of CSIR-Central Drug Research Institute (CDRI), Lucknow, India. Voucher specimens number of the *G. biloba* male tree (175 years old)-KRA24485 (1), female tree (84 years old)-KRA24445 (2), and female tree (8 years old)-KRA24486 (3) have been deposited in the Botany Department of CSIR-CDRI, Lucknow, India.

2.2 Extraction and sample preparation

The flavonoids were extracted from the *Ginkgo* samples using previously published methods and ethanol was selected for extraction process because it gives a high yield of flavonoids and is nontoxic for dietary purpose as reported in the literature [50, 51]. Each plant part (leaf, stem, and fruit) of male and female trees of *G. biloba* were washed thoroughly under running tap water, dried, and ground into powder with 40 mesh, respectively. The dried powder of each part (0.5 g) was weighed precisely and sonicated with 50 mL of ethanol for 30 min at room temperature using ultrasonic water bath (53 KHz) and left for 24 h at room temperature. Three replicates of the extraction process were carried out on each individual sample. The solution was filtered through Whatman filter paper and evaporated to dryness under reduced pressure using a rotary evaporator (Buchi Rotavapor-R2, Flawil, Switzerland) at 40°C. Dried residues (1 mg) were weighed accurately, dissolved in 1 mL of acetonitrile and sonicated using ultrasonicator (Bandelin SONOREX, Berlin). The solutions were filtered through 0.22 μm syringe filter (Millex-GV, PVDF, Merck Millipore, Darmstadt, Germany). The filtrates were diluted with acetonitrile to final working concentration. 50 μL of IS was spiked in final working solution, vortexed for 30 s, and 1 μL aliquot was injected into the UHPLC–MS/MS system for analysis.

2.3 Preparation of standard solution

A mixed standard stock solution (1 mg/mL) of selected analytes (epicatechin, catechin, quercetin-3,4′-diglucoside, rutin, kaempferol-3-*O*-rutinoside, quercetin, luteolin, apigenin, genistein, kaempferol, isorhamnetin, amentoflavone, and sciadopitysin) was prepared in acetonitrile. The working standard solutions were prepared by diluting the mixed standard solution with acetonitrile and spiking with 50 μL of IS diadzein to a series of concentrations within the ranges from 1 to 1000 ng/mL used for plotting the calibration curve.

The standard stock and working solutions were all stored at −20°C until use and vortexed prior to injection.

2.4 Instrumentation and analytical conditions

The UHPLC–ESI-MS/MS analysis was performed on Waters Acquity UPLC™ system (Waters, Milford, MA, USA) interfaced with hybrid linear ion trap triple-quadrupole mass spectrometer (API 4000 QTRAP™ MS/MS system from AB Sciex, Concord, ON, Canada) equipped with an electrospray (Turbo V) ion source. The Waters Acquity UPLC™ system was equipped with binary solvent manager, sample manager, column oven, and PDA. AB Sciex Analyst software version 1.5.1 was used to control the LC–MS/MS system and for data acquisition and processing. All the statistical calculations related to quantitative analysis were performed on Graph Pad Prism software version 5.

2.4.1 UHPLC conditions

The chromatographic separation of selected flavonoids and IS was achieved on an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm id, 1.7 μm) at a column temperature of 50°C. Analysis was completed with gradient elution of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as mobile phase. The 5 min UHPLC gradient system was as follows: 0–1 min, 10–12% B; 1–2.6 min, 12–30% B; 2.6–3 min, 30–45% B; 3–4 min, 45–100% B; 4–4.8 min, 100–100% B; 4.9–5 min, 10–10% B. Sharp and symmetrical peaks were obtained at a flow rate of 0.35 mL/min with a sample injection volume of 1 μL.

2.4.2 MS conditions

Precursor ion scan was used for the screening and MRM acquisition mode was used for quantitation of flavonoids in *G. biloba*. All the analytes with IS were detected in negative ESI using precursor ion scan and mass spectra were recorded by scanning in the range of m/z 100–1000 at a cycle time of 9 s with a step size of 0.1 Da. Nitrogen was used as the nebulizer, heater, and curtain gas as well as the collision activated dissociation gas. Optimized source parameters were as follows: Ion spray voltage set at −4200 V, curtain gas, nebulizer gas (GS1), and heater gas (GS2) set at 20, 20, and 20 psi, respectively, and source temperature set at 550°C. The collision activated dissociation gas was set at medium and the interface heater was on. Simultaneous quantitation of analytes was carried out using MRM acquisition mode at unit resolution, and its conditions were optimized for each compound during infusion. The transitions and optimized compound-dependent MRM parameters: declustering potential, entrance potential, collision energy, and cell exit potential for each analyte and IS are listed in Supporting Information Table S1.

3 Results and discussion

3.1 Optimization of LC conditions

Gradient and isocratic LC methods were tested to optimize the conditions for separation of all the flavonoids and the IS. The chromatographic conditions such as column, mobile phase, and gradient program were optimized systematically in the preliminary test to improve the separation of the analytes in a short analysis time. Two different analytical columns, Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm id, 1.7 μm) and Acquity UPLC CSH C_{18} column (100 mm × 2.1 mm id, 1.7 μm) were compared. The results showed that although all compounds could be separated satisfactorily with the Acquity UPLC CSH C_{18} column (100 mm × 2.1 mm id, 1.7 μm) but the Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm id, 1.7 μm) produced chromatograms with better peak shape in shorter analysis time. Therefore, the Acquity UPLC BEH C_{18} column was selected as the analytical column. Different mobile phase (water/methanol, water/acetonitrile, 0.1% formic acid in water/methanol, 0.1% formic acid in water/acetonitrile, 0.1% formic acid in water/0.1% formic acid in methanol, and 0.1% formic acid in water/0.1% formic acid in acetonitrile) at different flow rates (0.1, 0.15, 0.2, 0.25, 0.30, 0.35, and 0.4) as well as column temperatures (25, 30, 40, and 50°C) were examined and compared for better chromatographic behavior and appropriate ionization. Accordingly, 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 0.35 mL/min with the column temperature of 50°C resulted in a satisfactory separation in a short analysis time. Results of optimization of LC conditions are shown in Supporting Information Fig. S1.

3.2 Optimization of MS conditions

Each investigated analyte was infused into the mass spectrometer, the precursor ion and at least two product ions were preliminarily selected in both positive ion and negative ion modes. Due to phenolic hydroxyl group in the chemical structure of flavonoids, all compounds exhibited excellent signal sensitivity in negative ionization mode. The compound-dependent MRM parameters—declustering potential, entrance potential, collision energy, and cell exit potential—were optimized by injecting the individual standard solution into the mass spectrometer with FIA to achieve the most abundant, specific, and stable MRM transition for each investigated compound (shown in Supporting Information Table S1). The source parameters including the curtain gas, GS1, GS2, and ion source temperature were further optimized to get the highest abundance of precursor-to-product ions. The optimized compound-dependent parameters and source parameters were combined and finally the optimized UHPLC–ESI-MS/MS method in MRM acquisition mode was applied to quantify 13 flavonoids in the seven extracts of *G. biloba* using daidzein as an IS.

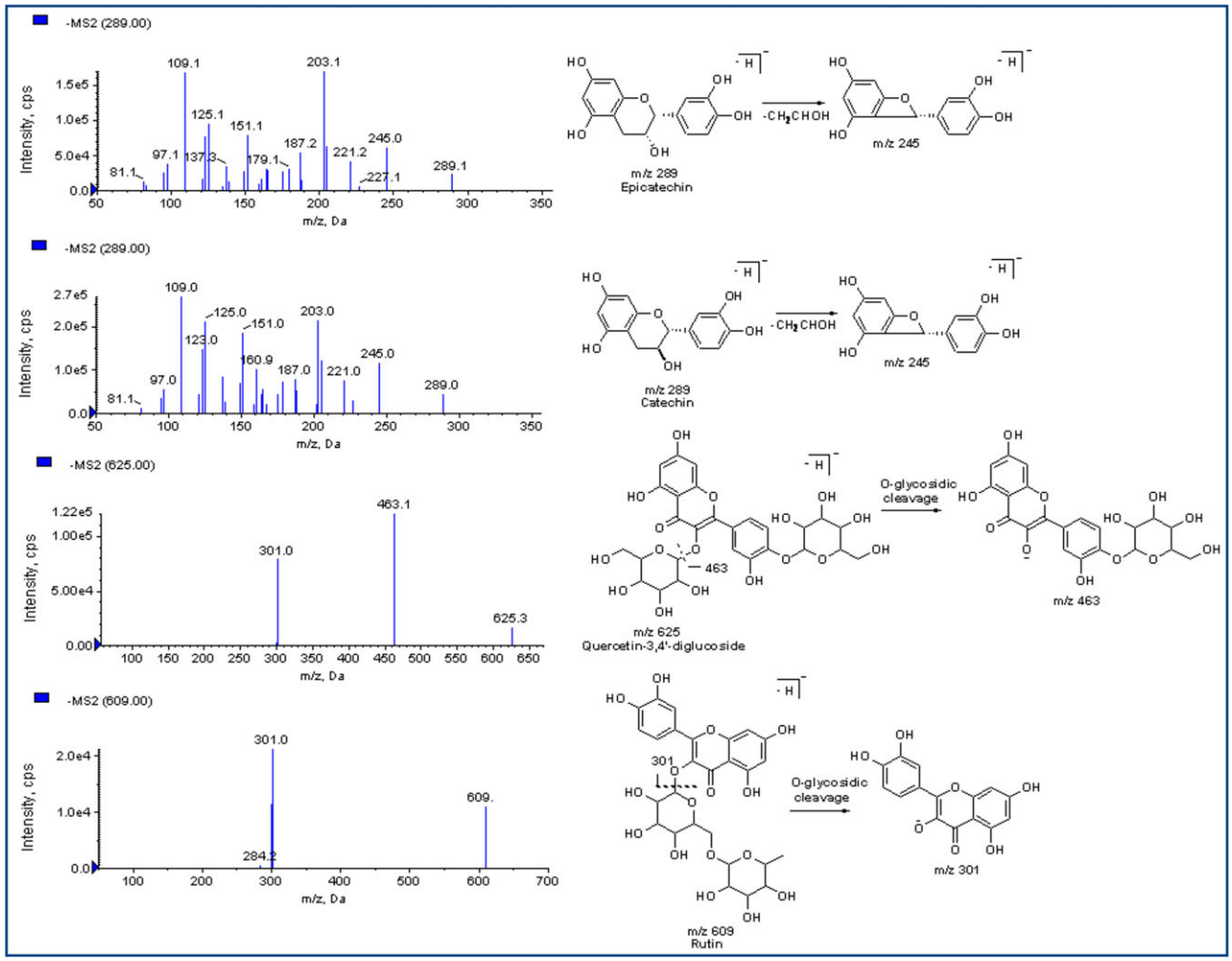

Figure 1. MS/MS spectra and fragmentation scheme of epicatechin, catechin, quercetin-3,4′-diglucoside, and rutin.

3.3 Identification of flavonoids

In the present study, 13 flavonoids including flavans (epicatechin, catechin), flavonol glycosides (quercetin-3,4′-diglucoside, rutin, and kaempferol-3-*O*-rutinoside), flavonols (quercetin, kaempferol, and isorhamnetin) flavones (luteolin and apigenin), isoflavones (genistein), and biflavones (amentoflavone and sciadopitysin) were identified using authentic standards and simultaneously quantified in the seven crude extracts of *G. biloba*. The MS spectra generated for all the flavonoids by ESI-MS in the negative ion mode gave the deprotonated molecule $[M–H]^-$. Peaks 1 and 2 were identified as epicatechin, *m/z* 289 $[M–H]^-$ and catechin, *m/z* 289 $[M–H]^-$, both yielded common fragment ion at *m/z* 245 corresponding to $[M\text{-}CH_2CHOH]^-$ and these fragment ions were selected for the MRM transitions. Peaks 3, 4, and 5 were identified as quercetin-3,4′-diglucoside, *m/z* 625 $[M–H]^-$, rutin, *m/z* 609 $[M–H]^-$, and kaempferol-3-*O*-rutinoside, *m/z* 593 $[M–H]^-$, respectively, yielded major fragment ion at *m/z* 463, 301, and 285, respectively, due to *O*-glycosidic cleavage, and these fragment ions were selected for the MRM transitions. Peaks 7, 11, and 12 were identified as quercetin, *m/z* 301 $[M–H]^-$, kaempferol, *m/z* 285 $[M–H]^-$, and isorhamnetin, 315 $[M–H]^-$, respectively. Quercetin yielded a major fragment ion at *m/z* 151 due to a retro-Diels–Alder (RDA) reaction, kaempferol yielded fragment ion at *m/z* 239 corresponding to $[M–H_2O–CO]^-$ [40] and isorhamnetin yielded a major fragment ion at *m/z* 300 due to loss of methyl radical and these fragment ions were selected for MRM transitions. Peaks 8 and 9 were identified as luteolin, *m/z* 285 $[M–H]^-$ and apigenin, *m/z* 269 $[M–H]^-$, respectively, yielded major fragment ions at *m/z* 133 and 117 by RDA and these fragment ions were selected for MRM transitions. Peak 10 was identified as genistein, *m/z* 269 $[M–H]^-$, yielded major fragment ion at *m/z* 133 due to ^{0,3}B fragmentation selected for MRM transition. Peaks 13 and 14 were identified as amentoflavone, *m/z* 537 $[M–H]^-$ and sciadopitysin, *m/z* 579 $[M–H]^-$, respectively. Amentoflavone yielded major fragment ion at 375 due to RDA and sciadoptysin yielded major fragment ion at *m/z* 547 due to loss of methanol arising from the presence of adjacent hydroxy and methoxy group, these fragment ions were selected for MRM transitions. The IS daidzein, *m/z*

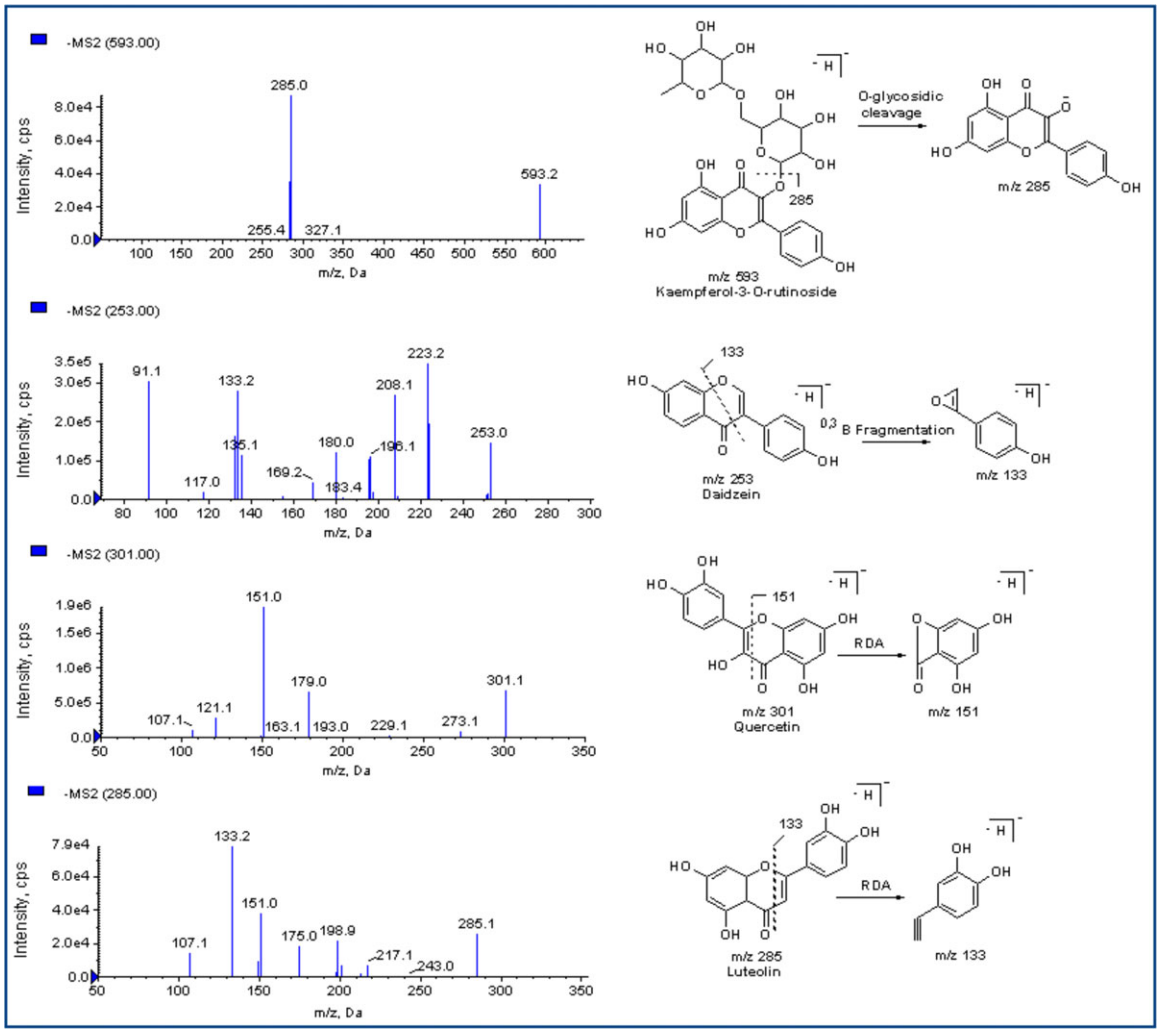

Figure 2. MS/MS spectra and fragmentation scheme of kaempferol-3-*O*-rutinoside, daidzein (IS), quercetin, and luteolin.

253 $[M–H]^-$, yielded fragment ion at m/z 133 due to $^{0,3}B$ fragmentation and this was selected for MRM transition. The MS/MS spectra of the 13 flavonoids, IS daidzein, and the fragmentation schemes selected for MRM method are shown in Figs. 1 and 2 and Supporting Information Figs. S2 and S3.

3.4 Analytical method validation

The proposed UPHLC–ESI-MS/MS method for quantitative analysis was validated according to the guidelines of the International Conference on Harmonization (Q2R1) by determining linearity, LOD, LOQ, precision, stability, and recovery. MRM extracted ion chromatograms of analytes and IS are shown in Fig. 3.

3.4.1 Linearity, LOD, and LOQ

The IS method was employed to calculate the contents of 13 flavonoids in *G. biloba*. The stock solution was diluted with acetonitrile to nine different concentrations for the construction of calibration curves. The linearity of calibration was performed by the analytes-to-IS peak area ratios versus the nominal concentration and the calibration curves were constructed with a weight $(1/x^2)$ factor by least-squares linear regression. The applied calibration model for all curves was $y = ax + b$, where y is the peak area ratio (analyte/IS), x is the concentration of the analyte, a is the slope of the curve, and b is the intercept. The LODs and LOQs were measured with S/N of 3 and 10, respectively. The results are listed in Table 1. All the calibration curves indicated good linearity with correlation coefficients (r^2) from 0.9977 to 0.9998 within the test ranges. The LOD for each analyte varied from 0.08 to 1.18 ng/mL and LOQ from 0.25 to 3.57 ng/mL.

3.4.2 Precision, stability, and recovery

The intraday and interday variations, which were chosen to determine the precision of the developed method, were investigated by determining 13 analytes with IS in six

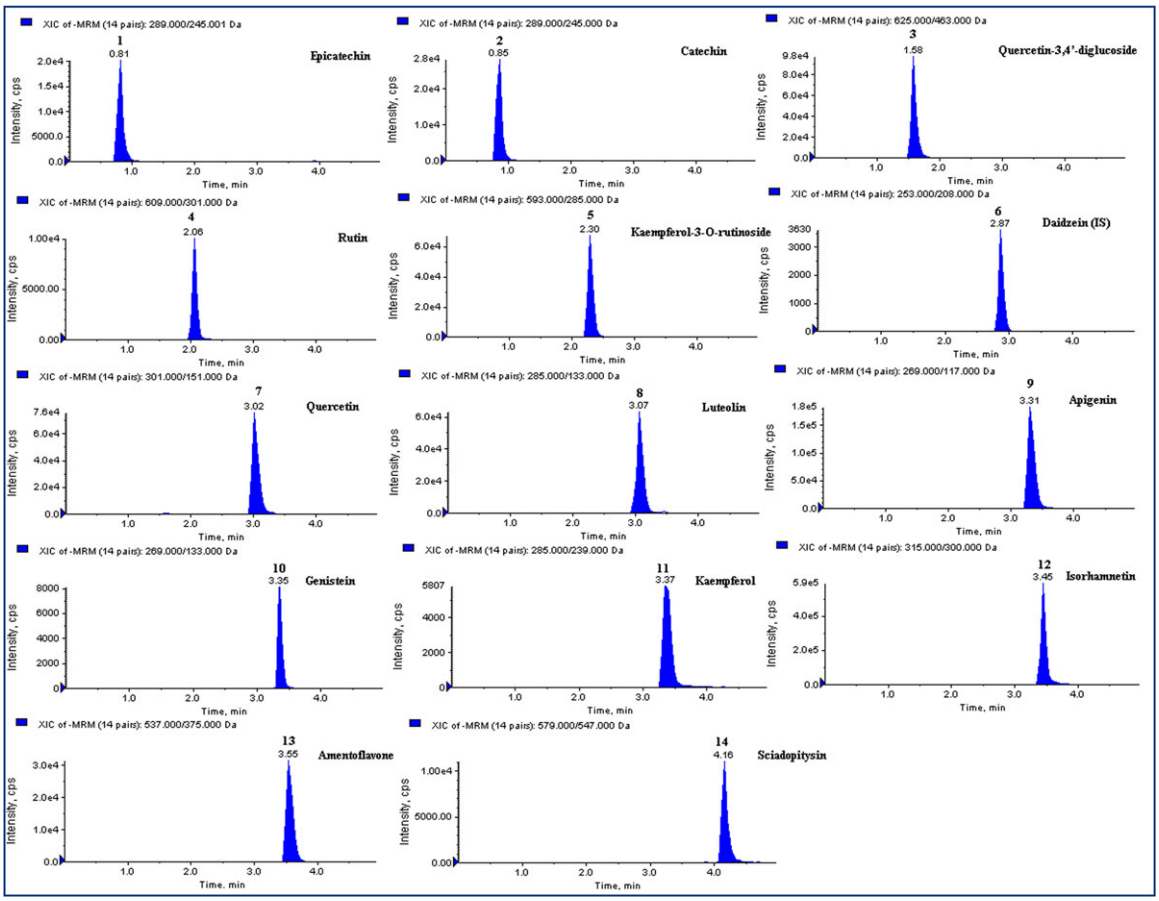

Figure 3. UHPLC–MRM extracted ion chromatogram of analytes and internal standard (IS) daidzein.

replicates during a single day and by duplicating the experiments on three consecutive days. Variations of the peak area were taken as the measures of precision and expressed as percentage RSDs. The overall intraday and interday precisions were not more than 1.98%. Stability of sample solutions stored at room temperature was investigated by replicate injections of the sample solution at 0, 2, 4, 8, 12, and 24 h. The RSD values of stability of the 13 analytes ≤3.25%.

A recovery test was applied to evaluate the accuracy of this method. Three different concentration levels (high, middle, and low) of the analytical standards were added into the samples in triplicate and average recoveries were determined. The analytical method developed had good accuracy with overall recovery in the range from 97.90 to 101.09% (RSD ≤ 1.67%) for all analytes (Table 1).

3.5 Quantitative analysis of samples

The developed UHPLC–ESI-MS/MS method in MRM acquisition mode was applied to investigate gender- and age-related variations in the content of flavonoids in seven samples (leaf, stem, and fruit extracts of male and female trees) of *G. biloba*. The contents of 13 flavonoids are summarized in Table 2 and graphical representations of distribution of 13 flavonoids in seven samples of *G. biloba* are shown in Supporting Information Fig. S4a and b, where it can be observed that the content of the 13 flavonoids varied greatly among seven samples of *G. biloba*. Quantitative analysis of these flavonoids showed that kaempferol-3-*O*-rutinoside, quercetin, luteolin, apigenin, kaempferol, isorhamnetin, amentoflavone, and sciadopitysin were detected in all the analyzed samples whereas epicatechin and catechin were found below detection level in the stem of female tree (84 years old), quercetin-3,4′-diglucoside was found below detection level in the leaf and stem of female tree (8 years old), fruit of female tree (84 years old), and stem of male tree (175 years old). Rutin was found below detection level in the leaf and stem of female tree (8 years old), stem and fruit of female tree (84 years old) and genistein was found below detection level in the leaf of female trees (8 and 84 years old). Sciadopitysin was found the most abundant component in all the analyzed samples and found to be highest in male leaf (71 200 μg/g). The total content of 13 flavonoids in all the seven analyzed samples were found in this decreasing order: male leaf (175 years old) > male stem (175 years old) > female leaf (84 years old) > fruit (84 years old) > female stem (84 years old) > female stem (8 years old) > female leaf (8 years old).

Table 1. Regression equations, correlation coefficients, linearity ranges, LOD, LOQ, intraday, interday precisions, stability, and recovery for the 13 reference flavonoids

Analyte	Regression equation	r^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Precision RSD (%)		Stability RSD (%) ($n = 6$)	Recovery ($n = 3$)	
						Intraday ($n = 6$)	Interday ($n = 6$)		Mean	RSD (%)
Epicatechin	$0.694x - 0.012$	0.9998	10–500	0.16	0.50	1.70	1.98	2.30	98.80	1.34
Catechin	$0.654x + 0.023$	0.9994	10–500	0.30	0.92	1.69	1.95	2.90	101.09	0.55
Quercetin-3, 4′-diglucoside	$1.987x + 0.037$	0.9998	10–500	0.18	0.54	1.39	1.57	2.30	97.90	1.67
Rutin	$0.195x + 0.137$	0.9982	25–1000	1.09	3.30	0.67	1.54	1.93	98.46	1.45
Kaempferol-3-*O*-rutinoside	$1.949x + 0.113$	0.9996	10–500	0.23	0.71	1.28	1.57	1.96	99.33	0.56
Quercetin	$1.968x + 0.135$	0.9997	10–500	0.20	0.60	1.22	1.53	2.93	99.58	1.65
Luteolin	$1.958x + 0.196$	0.9998	10–500	0.20	0.59	1.51	1.94	3.25	99.72	0.95
Apigenin	$6.920x + 0.227$	0.9994	1–200	0.12	0.38	1.52	1.83	3.10	98.93	0.57
Genistein	$0.192x + 0.008$	0.9998	10–500	0.17	0.52	1.90	1.83	3.10	99.65	1.39
Kaempferol	$0.204x + 0.006$	0.9998	10–500	0.16	0.49	1.49	1.78	1.98	98.70	0.55
Isorhamnetin	$15.98x + 0.244$	0.9997	1–200	0.08	0.25	1.59	1.75	2.01	98.22	0.81
Amentoflavone	$0.909x + 0.163$	0.9977	10–500	0.60	1.81	1.72	1.79	2.83	98.44	0.53
Sciadopitysin	$0.121x + 0.012$	0.9982	50–1000	1.18	3.57	1.67	1.74	2.92	99.44	1.02

Table 2. The content (μg/g) of 13 flavonoids in the seven analyzed samples of *G. biloba*

Flavonoids (μg/g)	*G. biloba* samples						
	Leaf Fa	Stem Fa	Leaf Fb	Stem Fb	Fruit Fb	Leaf Mc	Stem Mc
Epicatechin	321.0	322.0	142.0	bdl	170.0	287.0	249.0
Catechin	139.0	104.0	140.0	bdl	130.0	282.0	197.0
Quercetin-3, 4′-diglucoside	bdl	bdl	253.0	251.0	bdl	254.0	Bdl
Rutin	bdl	bdl	651.0	bdl	bdl	2450.0	748.0
Kaempferol-3-*O*-rutinoside	224.0	249.0	2630.0	250.0	327.0	3610.0	2140.0
Quercetin	179.0	145.0	167.0	148.0	138.0	184.0	149.0
Luteolin	245.0	256.0	239.0	236.0	249.0	266.0	243.0
Apigenin	127.0	154.0	144.0	141.0	157.0	233.0	165.0
Genistein	bdl	274.0	bdl	228.0	283.0	257.0	273.0
Kaempferol	325.0	358.0	429.0	226.0	255.0	712.0	377.0
Isorhamnetin	347.0	326.0	324.0	325.0	343.0	328.0	349.0
Amentoflavone	250.0	260.0	269.0	244.0	299.0	465.0	364.0
Sciadoptysin	13 200.0	14 800.0	42 900.0	15 900.0	20 300.0	71 200.0	43 200.0
Total (μg/g)	15 357.0	17 248.0	48 288.0	17 949.0	22 651.0	80 528.0	48 454.0
Total (%)	1.54	1.72	4.83	1.79	2.27	8.05	4.85

M, male tree; F, female tree; a, 8 years old; b, 84 years old; c, 175 years old; bdl, below detection level.

3.5.1 Variation of flavonoids with gender

Considering gender as the only variable, regardless of age, the total content of 13 flavonoids in the male leaf (8.05%) and stem (4.85%) was higher than that in female (84 and 8 years old) leaf (4.83, 1.54%) and stem (1.79, 1.72%). Male leaf and stem contained significantly higher content of sciadopitysin than that of the female leaf and stem whereas the amount of catechin was higher in the stem of female trees than that of male tree.

3.5.2 Variation of flavonoids with age

Regardless of gender effect, the total content of 13 flavonoids in the older tree (175 years old) leaf (8.05%) and stem (4.85%) was found than those in younger trees (84 and 8 years old) leaf (4.83, 1.54%) and stem (1.79, 1.72%). The content of sciadopitysin in the old tree leaf and stem was significantly higher than the young trees leaf and stem. The overall quantitative analysis result indicated that the male *Ginkgo* leaf has the highest total contents of 13 flavonoids,

which quantified in the present study compared to other samples.

4 Concluding remarks

The proposed UHPLC–ESI-MS/MS method using the negative ionization mode and MRM quantitation of flavonoids in *G. biloba* demonstrated good accuracy and reproducibility. The sample preparation and assay procedure involved is simple and offers simultaneous quantitation of 13 flavonoids including flavans, flavonol glycosides, flavonols, flavones, isoflavone, and biflavones in a single run. The content of flavonoids in seven *G. biloba* extracts varied markedly by gender and the age of the tree and results indicated that the total content of 13 flavonoids was found higher in the leaf extract of an old *Ginkgo* male tree compared to young *Ginkgo* female trees. Therefore, the UHPLC–ESI-MS/MS method has been proven to be an excellent approach for quality assessment of *G. biloba* due to its high capacity, high sensitivity, high selectivity, and short analysis time.

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