Analysis of flavonoids from lotus (*Nelumbo nucifera*) leaves using high performance liquid chromatography/photodiode array detector tandem electrospray ionization mass spectrometry and an extraction method optimized by orthogonal design

Sha Chen [a,b,c], Ben-Hong Wu [b], Jin-Bao Fang [d], Yan-Ling Liu [a], Hao-Hao Zhang [b], Lin-Chuan Fang [a], Le Guan [b], Shao-Hua Li [a,*]

[a] *Key Laboratory of Plant Germplasm Enhancement and Speciality Agriculture, Wuhan Botanical Garden, The Chinese Academy of Sciences, Wuhan 430074, China*
[b] *Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*
[c] *Graduate School of Chinese Academy of Sciences, Beijing 100049, China*
[d] *Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Zhengzhou 450009, China*

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ABSTRACT

The extraction protocol of flavonoids from lotus (*Nelumbo nucifera*) leaves was optimized through an orthogonal design. The solvent was the most important factor comparing solvent, solvent:tissue ratio, extraction time, and temperature. The highest yield of flavonoids was achieved with 70% methanol–water and a solvent:tissue ratio of 30:1 at 4 °C for 36 h. The optimized analytical method for HPLC was a multi-step gradient elution using 0.5% formic acid (A) and CH_3CN containing 0.1% formic acid (B), at a flow rate of 0.6 mL/min. Using this optimized method, thirteen flavonoids were simultaneously separated and identified by high performance liquid chromatography coupled with photodiode array detection/electrospray ionization mass spectrometry (HPLC/DAD/ESI-MS^n). Five of the bioactive compounds are reported in lotus leaves for the first time. The flavonoid content of the leaves of three representative cultivars was assessed under the optimized extraction and HPLC analytical conditions, and the seed-producing cultivar 'Baijianlian' had the highest flavonoid content compared with rhizome-producing 'Zhimahuoulian' and wild floral cultivar 'Honglian'.

1. Introduction

Lotus (*Nelumbo nucifera* GAERTN.), which is distributed widely throughout East Asia, Australia and North America, is an aquatic plant that has been cultivated for thousands of years and holds particular religious significance [1]. All of the tissues of *N. nucifera*, including the leaves, stamens, flowers, rhizomes, seeds and the embryo of seeds, are commonly used as traditional medicines as well as being common foods. They are known to contain bioactive components such as flavonoids and alkaloids in addition to nutritional ingredients like carbohydrates, proteins and fats [2–6].

Flavonoids have been isolated and characterized from various plants [7], and previous studies have shown that lotus leaves are rich in flavonoids [8,9]. The antioxidant [9,10], antibacterial [8], anti-HIV [11], antimalarial and antifungal [12], anti-obesity [13–15] and potential anti-atherogenic [16] activities of lotus leaves have

been evaluated and reported in recent years. The biological activities of lotus leaves that have led to its use as a traditional medicine have been identified. However, the physiological impacts are strongly dependent on the composition of flavonoids and their contents [17].

Classical separation and identification methods used for flavonoids are high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS) and high-speed counter-current chromatography coupled with nuclear magnetic resonance spectroscopy (HSCCC)-NMR. The former is a fast and reliable method for flavonoid analysis, and it has been widely applied during recent years due to its low limit detection. Compositional analysis of flavonoids by HPLC depends on the development of successful separation protocols. This means that the use of different experimental conditions including the mobile solvent system, elution gradient, column temperature and elution flow rate can have a significant influence on the separation of often closely-related compounds. The mobile solvent system is a particularly important factor in flavonoid separation. Formic acid was added to the mobile phase to allow the separation of flavonoids from lotus leaves by Goo et al. [18] and Deng et al. [19], and five

* Corresponding author at: Wuhan Botanical Garden, the Chinese Academy of Sciences, Wuhan 430074, PR China. Tel.: +86 27 87510599; fax: +86 27 87510251.
E-mail address: shhli@wbgcas.cn (S.-H. Li).

quercetin derivatives (quercetin 3-*O*-glucuronide, quercetin 3-*O*-arabinopyranosyl-(1 → 2)-galactopyranoside, rutin, isoquercitrin and hyperoside) were found [18]. However, in this study, flavonoid identification was performed using only negative mode electrospray ionization (NI) such that some flavonoids may not have been detected. Optimized methods are required to ensure that the complete flavonoid composition of lotus leaves is revealed. HSCCC-NMR was used to identify one further compound-kaempferol 3-*O*-glucoside (astragalin) from lotus leaves [19].

The traditional parameters used to extract flavonoids in plants generally include the solvent, solvent-to-sample ratio, extraction temperature and extraction time, as well as further methods for sample clean-up by liquid–liquid extraction (LLE), usually with ethyl acetate, or solid phase extraction (SPE). Common solvents used to extract flavonoids from lotus leaves at room temperature include ethanol, methanol, or mixtures of these with water [11,18,19]. The solvent-to-sample ratio and the extraction temperature often vary significantly among reported extraction protocols [20–22]. Overnight sample treatment is the most commonly used extraction period for polyphenol, flavonoid and anthocyanin extraction [23–25], although extraction times as long as five days or more have been used for the extraction of flavonoids from wormwood [26]. Previously reported extraction and analysis techniques may not have been the most suitable for lotus tissue extraction or determination [18]. An optimized protocol that allows highly efficient extraction of flavonoids from lotus leaves needs to be investigated.

The objective of the present study was to develop a method for the simultaneous separation and identification of flavonoids from lotus leaves by HPLC-MS. Optimization of flavonoid extraction from lotus leaves was studied via an orthogonal experimental design by considering extraction conditions including solvent, solvent-to-sample ratio, extraction time and extraction temperature. In total, thirteen flavonoids were simultaneously separated and identified by HPLC, five of which have not been reported previously in lotus leaves. This method therefore has potential to aid in the quality control of this important medicinal plant, and could also be useful in the development of high-flavonoid content lotus leaves for use as a food-stuff or medicine.

2. Experimental

2.1. Chemicals and materials

Five flavonoid glycoside standards (quercetin 3-*O*-rutinoside (rutin), quercetin 3-*O*-galactoside (Qc-3-Gal), quercetin 3-*O*-glucoside (Qc-3-Glu), kaempferol 3-*O*-glucoside (Kae-3-Glu), isorhamnetin 3-*O*-glucoside (Iso-3-Glu)) and three aglycone standards (quercetin, kaempferol, isorhamnetin) were obtained from Chromadex (Laguna Hills, CA, USA). Acetonitrile and formic acid (eluent and eluent additive used for HPLC and HPLC-MS analysis) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other analytical grade chemicals were obtained from Beijing Chemistry Factory (Beijing, China). HPLC-grade water was obtained using a Milli-Q System (Millipore, Billerica, MA, USA). Millipore membranes (0.22 μm) were purchased from Alltech Scientific (Beijing, China) and Oasis hydrophilic–lipophilic balance (Oasis HLB) cartridges (used for sample preparation) were obtained from Waters Corporation (Miford, MA, USA).

2.2. Plant materials

Three lotus cultivars, 'Baijianlian' (for seed production), 'Honglian' (wild cultivar for flower production), 'Zhimahuoulian' (for rhizome production) used in this study, were cultivated in the same-sized containers under the same conditions at the Germplasm Repository for lotus, Wuhan Botanical Garden of the Chinese Academy of Sciences, Wuhan, China. Fresh leaves were collected in early July of 2009. The leaves were powdered in liquid nitrogen using an analytical mill (IKA A11 basic machine, Germany) and then stored at −40 °C for later analysis. 'Honglian' was used to optimize the extraction conditions of flavonoids while 'Baijianlian' was used during optimization of the HPLC method. All three cultivars were assessed using the optimized method for chromatographic separation and MS identification of the flavonoid composition.

2.3. HPLC analysis of flavonoids

The analysis of flavonoids was carried out using an Agilent 1290 HPLC consisting of an auto-sampler and binary pump system (Agilent Corporation, CA, USA) coupled with a UV–Vis detector. A 10-μL aliquot of each sample solution was injected and analyzed on a Sunfire C18 column (150 mm × 4.6 mm, 3.5 μm, Waters, MA, USA). Chromatograms were acquired at 350 nm and photodiode array spectra were recorded from 210 to 600 nm. The separation of flavonoids was optimized by varying chromatographic parameters including elution gradient, initial mobile phase concentration, column temperature and flow rate.

2.4. Preparation of standard solutions and method validation

Each standard was accurately weighed, dissolved in MeOH, and the standard solutions were then diluted to generate an appropriate concentration range to establish calibration curves at 350 nm. All calibration curves were constructed using six different concentrations of each standard and measuring each sample in triplicate. Method validated characteristic indices, including linearity, correlation determination (r^2), limit of detection (LOD) and limit of quantitation (LOQ), accuracy and recovery, were assessed for each of the six available standards. Repeatability and intermediate precision for each compound was estimated by sample extraction according to previously published analytical determination methods [23,24,27].

2.5. Acid hydrolysis of flavonoids

Freshly crushed leaves (100 g) were suspended in 3 L 70% MeOH and extracted at 4 °C for 36 h. The supernatant was partially purified using a waters Oasis HLB solid phase, dried in a rotary evaporator (35 °C) and then re-dissolved in 30 mL 2.5 M HCl in a methanol–water solution (50:50, v/v). The solution was heated in a capped tube at 105 °C for 2 h. The hydrolysate obtained was partially purified on the Oasis HLB Cartridge before HPLC and HPLC-MS analysis.

2.6. Identification of flavonoids

Flavonoids in the lotus leaf extracts were identified using an Agilent 1290 HPLC-photodiode array 6460 triple quad mass spectrometry system analysis coupled with a UV–Vis detector (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization (ESI) was applied in both positive (PI) and negative (NI) modes for MS and MS/MS (MS^2) to give fragmentation information on the molecular weights, aglycone groups and glycosylation patterns. The mass spectrometric parameters were optimized using the six flavonoid standards listed in Section 2.1. Positive mode parameters were as follows: HV voltage, 3.5 kV; capillary 7 μA; nozzle voltage 500 V, delta emv 300 V, 5 L/min gas flow, gas temp 350 °C, nebulizer 45 psi, sheath gas temp 350 °C, sheath gas flow 11 L/min, and scan range, *m/z* 100–700 units. The parameters used in negative mode that

were different from those stated above were the following: delta emv at −300 V, and scan range at m/z 150–800 units. A collision energy of 20 V was used in the negative mode during MS^2 analysis. MS data, retention times and UV–Vis spectra were used to identify the flavonoids contained in the lotus leaves. The assignments were validated by co-elution with listed standards and by comparison with published data.

2.7. Optimization of flavonol extraction

'Honglian' leaves were used to optimize the extraction conditions of flavonoids. The solvent, solvent-to-sample ratio, extraction time and extraction temperature were optimized. The frozen powder of 1 g of the leaves was extracted with two solvents, methanol and ethanol, at 100% and at 70% aqueous solution (v:v). Four solvent to sample ratios [5:1, 10:1, 20:1, 30:1, (v/w)] and four different extraction times (12 h, 24 h, 36 h, 48 h) were tested at two temperatures (4 °C and 25 °C). All the factors were investigated using an orthogonal ($L_{16}4^3 \times 2^1$) experimental design, and each extract combination was tested in triplicate.

Using the selected optimal extraction conditions, the fresh leaves of 1 g of each lotus sample were accurately weighed and extracted at 4 °C with 30 mL 70% methanol (v:v) for 36 h. The extraction of each sample was performed in triplicate. The sample was extracted and then centrifuged at 20,000 × g for 10 min. Supernatants were dried at 35 °C using a rotary evaporator (RE52AA, YaRong, Shanghai, China) then re-dissolved in 2 mL methanol. The sample solutions were partially purified using a solid phase extraction cartridge (Oasis HLB). This purification step was carried out by first equilibrating the cartridge with methanol and water. Then, after introduction of the supernatant, the cartridge was eluted with 20% methanol 3 times to eliminate sugars and other strongly polar components. Then, the flavonoids were eluted with 4 mL methanol [28]. The methanol solution was filtered through a 0.22 μm Millipore filter before HPLC analysis.

3. Results and discussion

3.1. Chromatographic separation of flavonoids

The first step in this study was to optimize the analytical separation by HPLC analysis of the lotus leaf flavonoids. The best analytical method must achieve a high separation efficiency allowing target compound identification in a short time. Formic acid was used as an additive with a concentration of 0.1% in the mobile phase, as used in the lotus leaf analytical method developed by Goo et al. [18] and Deng et al. [19]. The mobile phase solvents chosen were 0.1% formic acid in acetonitrile as the B (organic) phase, and water as the A (aqueous) phase. The gradient elution conditions for the separation of the flavonoids extracted from 'Baijianlian' leaves were as follows: 0–10 min at 10% B; 10–40 min from 10 to 20% B; 40–42 min at 20% B; 42–45 min at 60% B; and 45–60 min at 10% B, all at a flow rate of 0.2 mL/min. This gradient led to the separation of only five peaks within 60 min, with the first peak eluting at nearly 40 min (Fig. 1A-0). The formic acid concentration in phase A was optimized within 0.1% to 0.8%, and had a significant impact on the separation of lotus leaf flavonoids. A concentration of 0.5% gave the best separation. The high flow rate used in Deng's HSCCC-NMR method [19] and the low flow rate used in Goo's HPLC method [18] was deemed unsuitable for flavonoid separation with lotus leaves. The column temperature had no significant influence on flavonoid separation, so 30 °C was used. The optimized chromatographic analytical separation conditions of flavonoids from lotus leaves were as described below. A mobile solvent system of water containing 0.5% formic acid and acetonitrile containing 0.1% formic acid as

mobile phases A and B, respectively, was used. Chromatographic separation was accomplished using a Waters Sunfire C$_{18}$ column (150 mm × 4.6 mm, 3.5 μm) at 30 °C with the following gradient elution program: 0–10 min at 12% B; 10–32 min from 12 to 20% B; 32–40 min from 20 to 30% B; and, 40–48 min from 30 to 60% B, at a flow rate of 0.6 mL/min, with re-equilibration of the column at 48–49 min from 60% to 12% B, and 49–53 min at 12% B. Under this optimized analytical method, 11 to 13 flavonoids were separated and detected within 53 min, and the first glycoside eluted at 25 min with samples of all three cultivars (Fig. 1A–C).

3.2. Method validation

3.2.1. Calibration curves, limits of detection and quantification
Table 1 shows the results of method validation for six external standards (rutin, Qc-3-Gal, Qc-3-Glu, Kae-3-Glu, Iso-3-Glu, quercetin). The calibration curves showed good linearity for all standards at 350 nm ($r^2 \geq 0.9986$). The standard solutions were detected by chromatography until the signal-to-noise (S/N) ratio corresponded to 3 and 10, and the corresponding concentrations at these S/N ratios were defined as the LOD and LOQ, respectively. The lowest and highest LOD and LOQ were obtained for quercetin (0.03 and 0.11 μg/mL) and rutin (0.20 and 0.67 μg/mL) (Table 1), respectively.

3.2.2. Precision and accuracy of flavonoid quantification
The precision of flavonoid quantification was studied by examining the repeatability and intermediate precision for all the compounds separated from 'Baijianlian' leaves, since this cultivar contained the largest number of individual flavonoid components. Six samples of 'Baijianlian' leaves were extracted and evaluated on the same day to determine the intra-day precision. Three samples were also extracted and analyzed on three consecutive days to determine the inter-day precision. Sample solutions were made at three concentrations (low, middle and high) with three replications of each concentration in order to validate method precision. Relative standard deviations (RSDs) were calculated to assess repeatability and precision. The results showed that the RSDs of the 13 compounds were less than 2.39% for inter-day precision and less than 4.59% for intra-day precision at the three concentrations (Table 2). The low RSD values obtained for all 13 compounds confirmed the high repeatability and intermediate precision of the method developed here.

3.2.3. Accuracy and recovery of flavonoid quantification
The accuracy of the method was investigated by detecting the recovery, which was assessed by adding three concentration levels (high, middle and low) of standard flavonoid solutions to known amounts of the 'Baijianlian' samples. The mixed samples were extracted and partially purified by solid phase extraction and detected for quantitative analysis as above. Each standard was tested at each concentration in triplicate. The equation used to define the recovery percentage was (detected amount − original amount)/spiked amount × 100. As shown in Table 3, the recoveries obtained in this study ranged from 85.12% to 97.63%. This demonstrated that the analytical method developed in this study showed high accuracy, and the low relative standard deviations (RSDs) of all standards (<3.22%) indicated a good reproducibility.

3.2.4. Identification of flavonoids
Acid hydrolysis of the sample was performed to allow the aglycone form of the isolated flavonoids to be identified. Five aglycones were identified from the acid hydrolysis sample: quercetin, kaempferol, isorhamnetin, myricetin and diosmetin. Quercetin, kaempferol and isorhamnetin could be validated by HPLC co-elution with the corresponding standards. Myricetin, previously
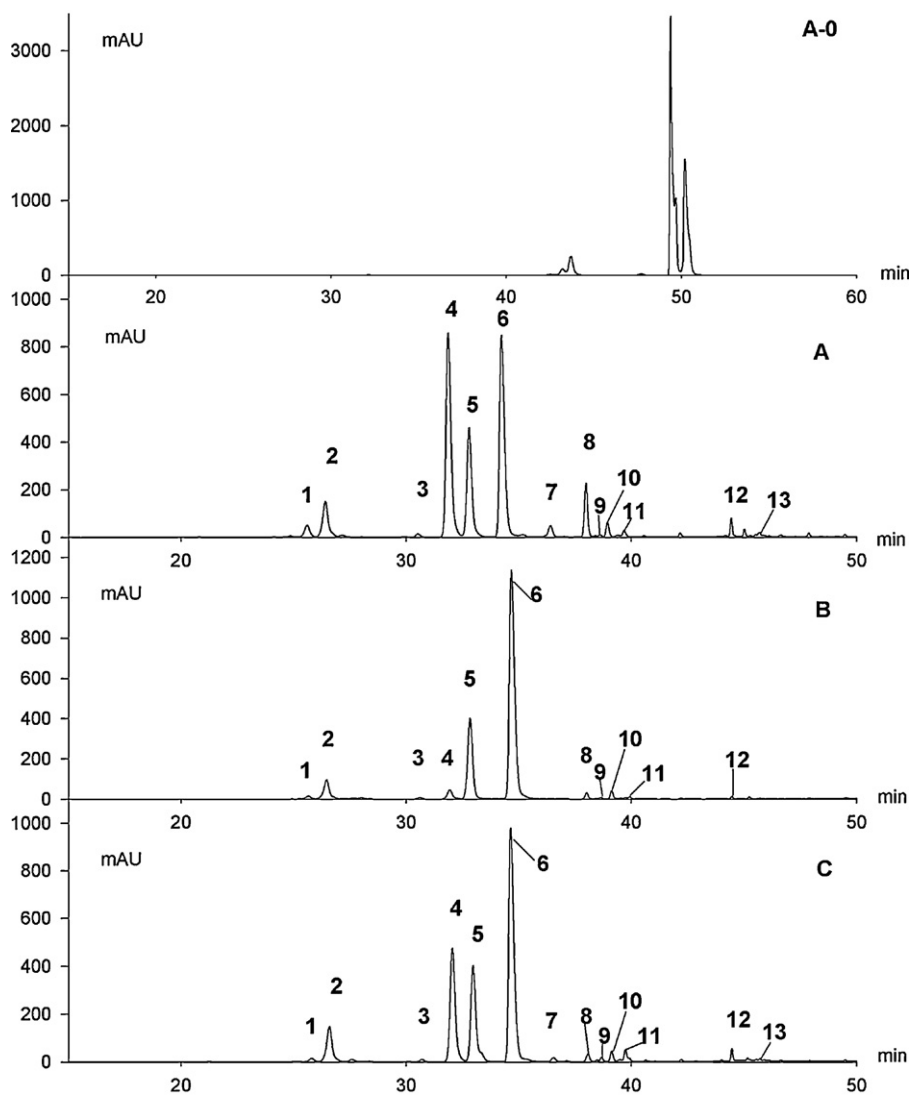
Fig. 1. HPLC chromatograms at 350 nm of flavonoids in 'Baijianlian' leaves obtained using the analytical method reported by Goo et al. [18] (A-0) and the optimized chromatographic analytical method (A) developed in the present study. (B) and (C) presents the chromatograms of flavonoids in 'Honglian' and 'Zhimahuoulian' leaves, respectively, using the optimized analytical method. The peak numbers in this figure correspond to the compound numbers used in Table 4.

isolated from the lotus seed epicarp by Kredy et al. in 2010 [2], was identified by mass spectrometry. The final compound eluted was identified as diosmetin (3′,5,7-trihydroxy-4′-methoxyflavone) by comparison of its mass spectral data (m/z 299 ion observed by NI and m/z 301 ion observed by PI) with published data on the same compound isolated from oregano extracts [29], as well as its UV–Vis spectrum. HPLC tandem MS analysis, in both positive and negative modes, gave valuable information for structural assignment, with the PI mode fragmentation particularly useful in assigning the aglycone from MS analysis. Table 4 lists the chromatographic and MS

data of the flavonoids extracted from lotus leaves, including the UV–Vis spectra, retention times, the MS and MS/MS characteristic spectral data and aglycone and sugar fragmentation data. The structures, MS and MS/MS model of all the 13 glycosides found in lotus leaves are shown in Fig. 2E. For flavonoids glycosylated with monosaccharide glycosides [30], the glycoside was determined to be conjugated at the 3-position if the abundance of the radical aglycone, denoted as $[A-2H]^-$, was significantly higher than that of the aglycone product ion, denoted as $[A-H]^-$. If the opposite abundance trend was observed, the glycoside was determined as being

Table 1
Linearity, LOD and LOQ in the determination of six flavonoid standards.

Compounds	Regression equation	r^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Rutin	$y = 25.65x - 29.18$	0.9992	9.38–250.00	0.20	0.67
Quercetin 3-O-galactoside	$y = 40.57x - 100.23$	0.9994	5.73–183.33	0.10	0.33
Quercetin 3-O-glucoside	$y = 39.28x - 135.00$	0.9994	7.50–240.00	0.07	0.25
Kaempferol 3-O-glucoside	$y = 30.21x + 18.85$	0.9994	3.13–100.00	0.16	0.52
Isorhamnetin 3-O-glucoside	$y = 23.46x + 3.87$	0.9986	4.69–150.00	0.12	0.40
Quercetin	$y = 48.15x - 9.93$	0.9998	0.38–93.33	0.03	0.11

Note: y, peak area; x, compound concentration (μg/mL); LOD = limit of detection, S/N = 3; LOQ = limit of quantitation, S/N = 10.
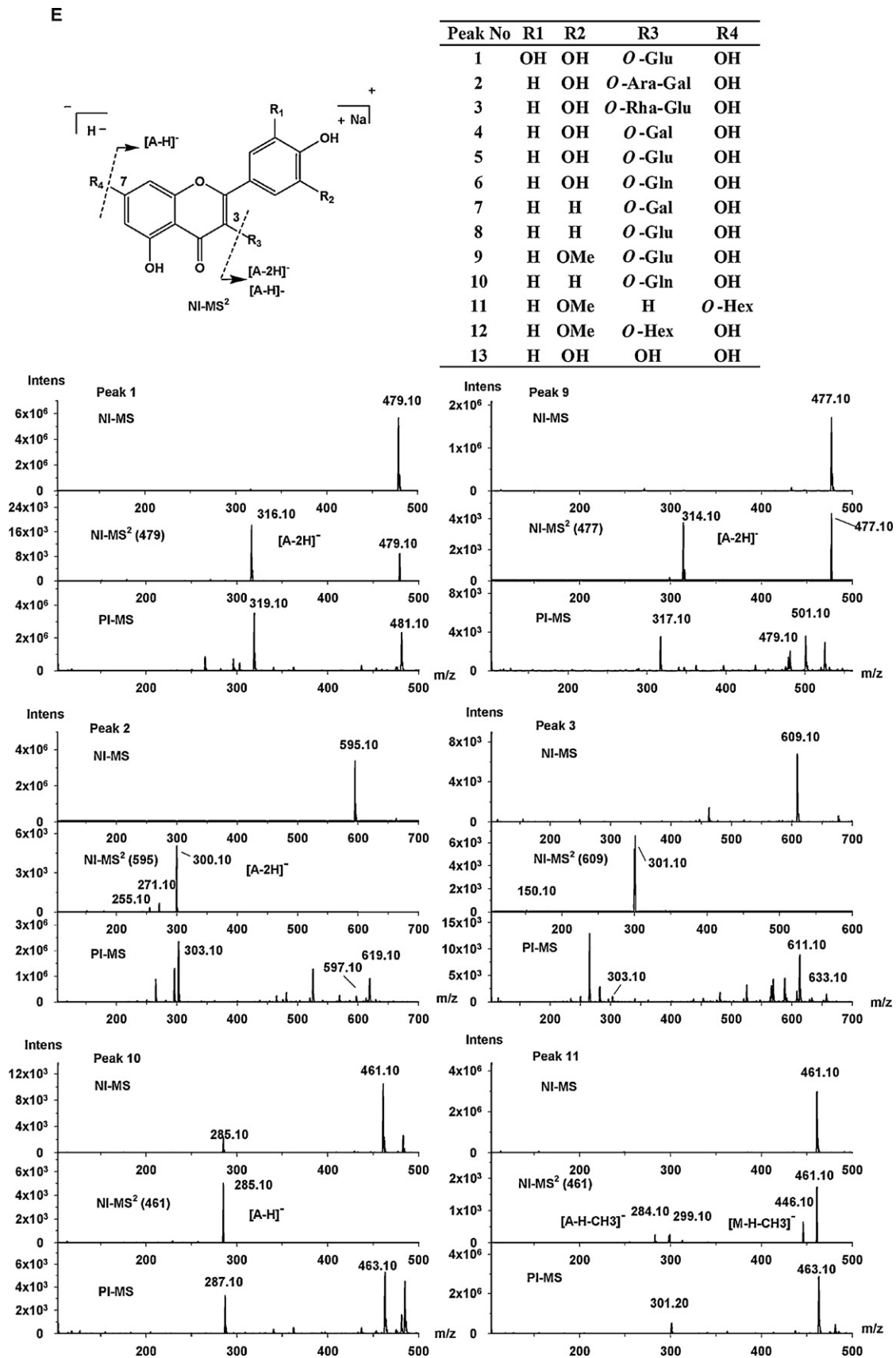
Fig. 2. The structures, MS and MS/MS model of the 13 glycosides found in lotus leaves (Fig. 2E). MS spectra of compounds 1, 2, 3, 9, 10, 11 extracted from the leaves of the 'Baijianlian' lotus. The intensities of MS spectra fragment ions gave information on the molecular weight, the aglycone and the glycosylation position that was used to assign the structure of the flavonoids. Peak numbers in this figure correspond to the compound numbers used in Table 4.

Table 2
Intra- and inter-day precision for each of the lotus leaf flavonoids separated by HPLC.

Compounds	Intra-day (n = 6)		Inter-day (n = 3)	
	Concentration (μg/mL)	RSD[c] (%)	Concentration (μg/mL)	RSD (%)
1[a]	5.7 ± 0.01[b]	0.24	5.7 ± 0.03[b]	0.55
	14.8 ± 0.04	0.26	14.8 ± 0.01	0.04
	26.9 ± 0.10	0.42	27.1 ± 0.60	0.23
2	19.6 ± 0.07	0.37	19.5 ± 0.10	0.49
	49.2 ± 0.04	0.09	49.4 ± 0.05	0.10
	93.3 ± 0.60	0.62	94.3 ± 0.20	0.25
3	2.5 ± 0.01	0.32	2.6 ± 0.02	0.63
	4.5 ± 0.07	1.50	4.5 ± 0.03	0.59
	7.8 ± 0.02	0.25	7.9 ± 0.02	0.25
4	64.6 ± 0.05	0.08	64.6 ± 0.30	0.39
	156.5 ± 0.10	0.08	156.8 ± 0.10	0.09
	312.1 ± 0.50	0.15	312.9 ± 0.20	0.06
5	36.6 ± 0.04	0.10	36.7 ± 0.10	0.37
	85.1 ± 0.30	0.29	85.3 ± 0.06	0.08
	174.4 ± 0.30	0.17	174.9 ± 0.10	0.06
6	99.6 ± 0.70	0.67	99.1 ± 0.40	0.37
	249.1 ± 1.90	0.77	247.9 ± 0.10	0.05
	499.2 ± 0.70	0.15	500.0 ± 0.30	0.07
7	4.5 ± 0.06	1.24	4.6 ± 0.003	0.07
	11.5 ± 0.20	1.61	11.6 ± 0.07	0.58
	24.3 ± 0.06	0.25	24.3 ± 0.05	0.19
8	13.1 ± 0.09	0.70	13.0 ± 0.04	0.28
	33.6 ± 0.03	0.08	33.7 ± 0.001	0.02
	68.3 ± 0.90	0.13	68.4 ± 0.04	0.06
9	0.5 ± 0.02	4.59	0.4 ± 0.01	1.41
	1.5 ± 0.40	2.56	1.5 ± 0.03	2.10
	3.5 ± 0.20	4.20	3.4 ± 0.04	1.04
10	3.7 ± 0.05	1.46	3.7 ± 0.01	0.22
	10.4 ± 0.10	0.91	10.3 ± 0.02	0.18
	21.3 ± 0.20	0.98	21.3 ± 0.06	0.29
11	1.6 ± 0.07	4.31	1.6 ± 0.04	2.39
	4.5 ± 0.10	2.41	4.5 ± 0.03	0.60
	9.9 ± 0.30	2.69	9.9 ± 0.06	0.60
12	3.1 ± 0.01	0.33	3.1 ± 0.004	0.12
	7.8 ± 0.01	0.13	7.8 ± 0.01	0.12
	16.2 ± 0.03	0.20	16.2 ± 0.02	0.14
13	2.39 ± 0.09	3.57	2.38 ± 0.06	2.67
	4.31 ± 1.10	1.11	4.27 ± 0.02	0.36
	6.08 ± 1.20	1.22	6.07 ± 0.07	1.09

[a] The numbers assigned to compounds correspond to those used in Table 4.

[b] Mean concentration ± SD, Concentrations of compounds 3, 4, 5, 8, 9 and 13 were quantified by comparison with external standards, while compounds 1, 2, 6, 7, 10, 11 and 12 are given in μg/mL equivalent of rutin.

[c] RSD = (SD/mean) × 100.

at the 7-position. This evaluation could not be made for glucuronic acid glycosides, as only the [A−H]⁻ ion was observed during the MS² process.

Based on the UV–Vis spectrum (λ_{max} 232.2 and 354.1, Table 4) and MS data of peak 1 (Fig. 2 and Table 4, NI-mode m/z 479 [M−H]⁻, PI-mode m/z 481 [M+H]⁺, NI-MS/MS m/z 316 [A−2H]⁻), its structure was determined to be a myricetin monosaccharide. MS data of the radical aglycone, with m/z 316 [A−2H]⁻ and the loss of a 162 unit fragment produced from the ion 479 [M−H]⁻ in NI-MS² mode, assigned that peak 1 was a glucoside conjugated at the 3-position. Peak 1 in the chromatograms of Fig. 1 was therefore shown to be myricetin 3-O-glucoside, which has been previously found in lotus leaves and seed epicarp [2,10,31].

The compounds corresponding to peaks 4 and 5 produced major ions at m/z 463 [M−H]⁻ in NI mode and the corresponding ions at m/z 487 [M+Na]⁺ ion in PI-mode. This indicated that they have the same molecular weight of 464 (Table 4). According to data for the radical aglycones, m/z 300 [A−2H]⁻ in NI mode and m/z

303 [A+H]⁺ in PI mode, it was revealed that a hexose was conjugated to quercetin at the 3-position in each of these compounds. By comparing the retention time and co-elution with standards, peak 4 was identified as hyperoside and peak 5 was identified as isoquercitrin. Both of these compounds have been previously identified in lotus leaves [18,19]. The molecular weight of the compound corresponding to peak 6 was found to be 478, and a significantly larger aglycone ion was observed at m/z 301 [A−H]⁻ in NI-MS². This indicated that a radical aglycone could not be produced, and that peak 6 therefore contained a glucuronic acid glycoside. Peak 6 was identified as quercetin 3-O-glucuronide (Qc-3-Gln), which has previously been reported to be the dominant flavonoid in lotus leaves [18].

The compounds corresponding to peaks 2 and 3 were identified as quercetin diglycosides by their ions at m/z 609 [M−H]⁻, m/z 301 [A−H]⁻, m/z 595 [M−H]⁻ and m/z 300 [A−2H]⁻ in NI-MS² mode and at m/z 633 [M+Na]⁺ and m/z 303 [A+H]⁺ in PI mode (Table 4, Fig. 2). According to Ablajan et al. [30], the mass spectrometric

Table 3
Recovery of six flavonoid standards in the extraction of lotus leaves ($n = 3$).

Compound[a]	Initial amount (μg)	Added amount (μg)	Total recovered amount[b] (μg)	Recovery[c] (%)	RSD[d] (%)
Rutin	3.38	1.17	4.51 ± 0.03	95.63	2.70
	3.38	2.34	5.52 ± 0.04	92.63	1.95
	3.38	4.69	7.74 ± 0.02	90.73	0.46
Qc-3-Gal	5.39	11.46	16.34 ± 0.06	97.27	0.59
	5.39	5.73	11.06 ± 0.04	96.90	0.73
	5.39	2.86	8.04 ± 0.08	94.07	3.02
Qc-3-Glu	38.10	60.00	92.58 ± 0.26	92.55	0.47
	38.10	30.00	64.26 ± 0.10	88.63	0.38
	38.10	15.00	51.44 ± 0.21	89.95	1.57
Kae-3-Glu	19.24	6.25	25.36 ± 0.10	97.30	1.63
	19.24	3.13	22.35 ± 0.10	97.63	3.22
	19.24	1.56	20.69 ± 0.04	94.00	2.42
Iso-3-Glu	1.57	3.18	4.57 ± 0.01	88.31	0.33
	1.57	1.59	2.95 ± 0.01	86.30	0.72
	1.57	0.79	2.23 ± 0.02	85.12	3.03
Quercetin	1.36	2.51	3.7 ± 0.05	89.35	2.13
	1.36	0.43	1.74 ± 0.01	88.30	2.62
	1.36	0.35	1.66 ± 0.01	87.97	3.32

[a] Qc-3-Gal = quercetin 3-O-galactoside; Qc-3-Glu = quercetin 3-O-glucoside; Kae-3-Glu = kaempferol 3-O-glucoside; Iso-3-Glu = isorhamnetin 3-O-glucoside.
[b] Total recovered amount = mean content ± SD.
[c] Recovery (%) = (detected amount − original amount)/spiked amount × 100.
[d] RSD (%) = (recovery SD/mean) × 100.

behaviors of diglycosides are notably different in NI-mode depending on the linkage between the two monosaccharides. Radical aglycone [A−2H]⁻ ions tend to be generated in the case of a C1 → C2 linkage between the two monosaccharides, while the [A−H]⁻ ion is indicative of a C1 → C6 linkage. The MS² spectrometric behavior in NI mode of peak 2 differed significantly from that of peak 3 (Fig. 2). The presence of the radical aglycone ion at m/z 300 [A−2H]⁻, and characteristic ions at m/z 255, 227 in NI-MS² for peak 2 indicated that the interglycosidic linkage between the two monosaccharides in this compound was C1 → C2. An aglycone ion at m/z 301 [A−H]⁻ and the absence of a characteristic ion in NI mode established that peak 3 contained a C1–C6 linkage. Based on the above information and the fragment ions, peak 2 was identified as quercetin 3-O-arabinopyranosyl-(1 → 2)-galactopyranoside (Qc-3-AraGal). This flavonoid has been found previously in lotus leaves by Jung et al. [6] and Kashiwada et al. [11]. Peak 3 was quercetin 3-O-rhamnopyranosyl-(1 → 6)-glucopyranoside (rutin), confirmed by co-elution with standard.

The molecular weights of the compounds corresponding to peaks 7 and 8 were both 448, as determined by the ions observed at m/z 471 [M+Na]⁺ and the aglycone product ion m/z 284 [A−2H]⁻ in NI mode, and the 162 unit loss fragment ion 448 in NI mode (Table 4). This indicated that both were kaempferol 3-O-hexoses. Peak 8 was identified as kaempferol 3-O-glucoside by co-elution with a standard. By comparing the retention time and the UV–Vis spectrum, peak 7 was identified as kaempferol 3-O-galactoside,

which is in agreement with previous reports on flavonoids in lotus petals [25].

The compounds corresponding to peaks 9, 10, 11 and 12 were determined to be monosaccharide glycosides by examination of their MS data (Table 4 and Fig. 2). The monosaccharide glycosides of peaks 9 and 12 were identified as isorhamnetin derivatives on the basis of aglycone fragment ions at m/z 314 [A−2H]⁻ in NI mode and m/z 317 [A+H]⁺ in PI mode. By co-elution with a standard, the monosaccharide glycoside of peak 9 was identified as isorhamnetin 3-O-glucoside, which has been previously reported in lotus petals [25]. Peak 12 was tentatively identified as isorhamnetin 3-O-hexose, and further work should be done to identify the nature of the hexose. The aglycone fragment ions at m/z 285 [A−H]⁻ and m/z 287 [A+H]⁺ in NI mode and PI mode indicated that peak 10 was a kaempferol derivative. The loss of a 176 unit fragment produced an ion of 461, identifying peak 10 as kaempferol 3-O-glucuronide, which has been previously reported in lotus stamen by Jung [6] and in lotus petals by Yang [25]. Peak 11 was identified as diosmetin monosaccharide by comparison of the UV–Vis spectrum (λ_{max} 238.1 and 346.9) and MS data (NI-mode m/z 461 [M−H]⁻, PI-mode m/z 463 [M+H]⁺ and 301 [A−H]⁺). There were abundant aglycone ions at m/z 299 [A−H]⁻ and m/z 284 [A-H-CH₃]⁻ corresponding to the precursor ion 461 [M−H]⁻ in NI-MS² mode (Fig. 2). This indicates that peak 11 is a hexose linked at the 3-position. The abundance of the ions at m/z 446 [M−H−CH₃]⁻ and m/z 284 [A−H−CH₃]⁻ indicated that the methoxyflavone readily lost a methyl group. Peak

Table 4
UV–Vis absorption maxima in HPLC and main ESI-MSⁿ ions of flavonoids in lotus leaves. The peak numbers correspond to those used in Fig. 1.

Peak no	Identification	Rt (min)	λ_{max} (nm)	NI⁻	MS/MS	PI⁺	MS/MS
1	Myricetin 3-O-glucoside	26.33	232.2, 354.1	479	316	481	319
2	Quercetin 3-O-arabinopyranosyl-(1 → 2)-galactopyranoside	27.16	254.7, 354.1	595	300.1	619, 597	303
3	Quercetin 3-O-rhamnopyranosyl-(1 → 6)-glucopyranoside	31.41	254.7, 352.9	609	301, 150	633, 611	303
4	Quercetin 3-O-galactoside (hyperoside)	32.70	261.9, 354.1	463	300	487	303
5	Quercetin 3-O-glucoside (isoquercitrin)	33.62	254.7, 354.1	463	300	487	303
6	Quercetin 3-O-glucuronide	35.22	254.7, 354.1	477	301	479	303
7	Kaempferol 3-O-galactoside	37.12	264.3, 346.9	447	284	471	287
8	Kaempferol 3-O-glucoside (astragalin)	38.51	264.3, 346.9	447	284	471	287
9	Isorhamnetin 3-O-glucoside	39.11	236.9, 350.5	477	314	479, 501	317
10	Kaempferol 3-O-glucuronide	39.45	264.3, 345.6	461	285	463	287
11	Diosmetin 7-O-hexose	40.21	238.1, 346.9	461	299, 446	463	301
12	Isorhamnetin 3-O-hexose	44.78	241.7, 350.5	477	314	479	317
13	Quercetin	49.13	254.7,369.5	301	301	303	nd

Table 5
Orthogonal ($L_{16}4^3 \times 2^1$) extraction efficiency results.

Test no	A (solvents)	B (solvent to sample ratio)	C (time)	D (temperature)	Yields (mg/100 g)	
					Qc-3-Gln	Other Flavonoids
1	A1 (100%MeOH)	B1 (5:1)	C1 (12 h)	D1 (4 °C)	231.36	121.82
2	A1 (100%MeOH)	B2 (10:1)	C2 (24 h)	D1 (4 °C)	246.52	131.63
3	A1 (100%MeOH)	B3 (20:1)	C3 (36 h)	D2 (25 °C)	279.41	150.96
4	A1 (100%MeOH)	B4 (30:1)	C4 (48 h)	D2 (25 °C)	339.95	148.79
5	A2 (70%MeOH)	B1 (5:1)	C2 (24 h)	D2 (25 °C)	268.20	125.81
6	A2 (70%MeOH)	B2 (10:1)	C1 (12 h)	D2 (25 °C)	330.26	158.40
7	A2 (70%MeOH)	B3 (20:1)	C4 (48 h)	D1 (4 °C)	345.97	163.08
8	A2 (70%MeOH)	B4 (30:1)	C3 (36 h)	D1 (4 °C)	390.66	165.34
9	A3 (100%EtOH)	B1 (5:1)	C3 (36 h)	D1 (4 °C)	173.13	116.58
10	A3 (100%EtOH)	B2 (10:1)	C4 (48 h)	D1 (4 °C)	133.88	133.98
11	A3 (100%EtOH)	B3 (20:1)	C1 (12 h)	D2 (25 °C)	90.33	115.38
12	A3 (100%EtOH)	B4 (30:1)	C2 (24 h)	D2 (25 °C)	69.57	108.49
13	A4 (70%EtOH)	B1 (5:1)	C4 (48 h)	D2 (25 °C)	230.82	113.00
14	A4 (70%EtOH)	B2 (10:1)	C3 (36 h)	D2 (25 °C)	321.45	152.49
15	A4 (70%EtOH)	B3 (20:1)	C2 (24 h)	D1 (4 °C)	361.19	170.44
16	A4 (70%EtOH)	B4 (30:1)	C1 (12 h)	D1 (4 °C)	342.85	164.05

	Yield of Qc-3-Gln (mg/100 g)				Yield of other flavonoids (mg/100 g)			
	A	B	C	D	A	B	C	D
k1[a]	274.3	225.9	248.7	278.2	138.3	119.3	139.9	145.9
k2	333.8	258.0	236.4	241.3	153.2	144.1	134.1	134.2
k3	116.7	269.2	291.2		118.6	150.0	146.3	
k4	314.1	285.8	262.7		150.0	146.7	135.1	
R[b]	217.1	59.9	54.8	36.9	34.6	30.7	12.2	11.7
Important	A > B > C > D				A > B > C > D			
Order optimal level	A2	B4	C3	D1	A2	B3	C3	D1

Note: 'Honglian' leaves were used during optimization of the quantitative extraction of flavonoids by using an orthogonal ($L_{16}4^3 \times 2^1$) test with each combination carried out in triplicate. Qc-3-Gln denotes quercetin 3-*O*-glucuronide; other flavonoids denote all the identified compounds besides Qc-3-Gln.

[a] The $k_i^A = \sum$ Extraction yield at $A_i/3$.

[b] The $R_i^A = \sum max\{k_i^A\} - min\{k_i\}$.

3.3. Optimization of quantitative flavonoid extraction

11 was identified as diosmetin 7-*O*-hexose. Its spectral behavior agreed with the compound isolated from fresh oregano [32]. This is the first report of the identification of these four flavonoids in lotus leaves.

The parameters obtained from the orthogonal ($L_{16}4^3 \times 2^1$) test of the flavonoid extraction were weighted and quantitatively analyzed using evaluation indices k and R (Table 5). Qc-3-Gln was the dominant flavonol found in 'Honglian' leaves, and accounted for more than 70% of the total flavonoid content. Statistical analysis was therefore carried out separately for Qc-3-Gln content and the total amount of the other flavonoids. The R value of factor A was highest for the extraction of both Qc-3-Gln and the other flavonoids, indicating that solvent choice was the most important factor among the four studied parameters for maximum flavonoid extraction from lotus leaves. Extraction time and extraction temperature generally had much less significant effects on the yield of Qc-3-Gln and the other flavonoids. Solvent-to-sample ratio was similar in importance to the solvent for all flavonoids except Qc-3-Gln. Based on the R values, the factors can be ranked by importance for extracting both Qc-3-Gln and the other flavonoids in lotus leaves as follows: extraction solvent > solvent-to-sample ratio > extraction time > extraction temperature.

Organic–water solvent mixtures (70% v/v) gave a higher yield of both Qc-3-Gln and other flavonoids than 100% solvent (Table 5). This difference was much greater for ethanol than for methanol. Methanol–water (70:30) had a higher extraction efficiency than ethanol–water (70:30). The effect of the solvent-to-sample ratio on the extraction of Qc-3-Gln was slightly different from that of the other flavonoids. A ratio of 30:1 (v/w) gave the highest yield of Qc-3-Gln, while a ratio of 20:1 (v/w) was best for extracting the other flavonoids. An extraction time of 36 h and an extraction temperature of 4 °C gave the highest yield of both Qc-3-Gln and the other flavonoids.

3.3.1. Flavonoid content in representative cultivars

We used the optimized extraction and analysis methods to investigate the flavonoid content of three representative lotus cultivars. Leaves (1 g) from 'Baijianlian', 'Honglian' and 'Zhimahuoulian' lotus plants were extracted in 30 mL methanol–water (70:30) for

Table 6
Flavonoid content (mg/100 g FW) of the leaves of three lotus cultivars.

Component no.[a]	Cultivars		
	Honglian	Baijianlian	Zhimahuoulian
1	6.3 ± 0.2a[b]	16.3 ± 0.8b	2.9 ± 0.1c
2	41.5 ± 1.6a	53.6 ± 2.4b	46.9 ± 1.8ab
3	3.6 ± 0.1a	4.5 ± 0.1b	5.0 ± 0.2b
4	11.7 ± 0.2a	179.1 ± 5.4b	69.9 ± 2.2c
5	95.8 ± 2.7a	99.1 ± 3.1a	139.0 ± 5.1b
6	504.2 ± 38.6a	284.0 ± 11.4b	359.4 ± 21.4b
7	–a	13.6 ± 0.4b	2.6 ± 0.1c
8	5.3 ± 0.1a	38.9 ± 1.3b	5.0 ± 0.2a
9	2.3 ± 0.2a	2.3 ± 0.3a	5.2 ± 0.3b
10	10.2 ± 0.5a	12.1 ± 0.4b	6.7 ± 0.3c
11	6.1 ± 0.2a	7.6 ± 0.3a	13.7 ± 0.8b
12	2.6 ± 0.1a	10.2 ± 0.5b	7.9 ± 0.4c
13	–a	5.6 ± 0.3b	4.3 ± 0.3c
Total	689.5 ± 43.6	727.0 ± 26.2	668.5 ± 31.3

– means not detected.

[a] The compound numbers correspond to those used in Table 2. Compounds 3, 4, 5, 8, 9 and 13 were quantified by comparison with external standards, while the content of compounds 1, 2, 6, 7, 10, 11 and 12 are given in mg/100 g FW equivalents of rutin.

[b] Different letters within a row indicate significant content differences between the cultivars at $P < 0.05$ by LSD.

36 h at 4 °C. The flavonoid composition and content was dependent on the genetic background (Table 6). The seed lotus cultivar 'Baijianlian' and the rhizome lotus cultivar 'Zhimahuoulian' contained all 13 flavonoids, while kaempferol 3-O-galactoside and quercetin were not detected in the leaves of the wild flowering lotus 'Honglian'. 'Baijianlian' leaves contained the highest total flavonoid content, with 727.0 mg/100 g FW, compared to 660–690 mg/100 g FW in the other two cultivars. Qc-3-Gln was the dominant flavonoid in all three cultivars, with the highest content found in 'Honglian' leaves (504.2 mg/100 g) and the lowest in 'Baijinglian' leaves (284.0 mg/100 g). Isoquercitrin was also a major component of the 'Honglian' leaves. Isoquercitrin and hyperoside were the main flavonols found in the 'Baijianlian' and 'Zhimahuoulian' leaves. The other flavonoids were only present in trace quantities and their contents also showed significant variance among the three cultivars.

4. Conclusion

The extraction solvent was the most important factor in achieving a high yield of flavonoids from lotus leaves, followed by solvent-to-sample ratio. The optimal conditions for flavonoid extraction from lotus leaves were that 1 g of leaf tissues were extracted in 30 mL methanol–water mixture (70:30) for 36 h at 4 °C and the analytical method for HPLC was a multi-step gradient. Thirteen flavonoids were detected in lotus leaves under the optimized conditions, five among which are reported for the first time in this study. The flavonoid composition and content was found to vary with cultivar. However, Qc-3-Gln, isoquercitrin and hyperoside were the main flavonoids in lotus leaves.

Acknowledgments

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