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Tissue-specific metabolites profiling and quantitative analyses of flavonoids in the rhizome of *Belamcanda chinensis* by combining laser-microdissection with UHPLC-Q/TOF-MS and UHPLC–QqQ-MS

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ABSTRACT

The rhizome of *Belamcanda chinensis* (L.) DC. is a traditionally used medicinal material in China. Due to increasing demand, *B. chinensis* has been cultivated widely, and thus the study on its rational utilization of medicinal part and guidelines for the optimal cultivation and harvest is an important issue. Considering flavonoids were the main bioactive secondary metabolites of *B. chinensis*, fluorescence microscopy, laser microdissection (LMD), ultra-high performance liquid chromatography-quadrupole/time-of-flight-mass spectrometry (UHPLC-Q/TOF-MS), and UHPLC coupled with triple quadrupole mass spectrometer (UHPLC–QqQ-MS) were applied to profile and determine flavonoids in various tissues in this study. Consequently, 43 peaks were detected by UHPLC-Q/TOF-MS, and 26 flavonoid compounds combined with seven triterpene compounds were identified or tentatively identified in the tissue extractions. The results indicated that the hydrophobic compounds, especially flavonoid or isoflavonoid aglycones and xanthone mainly accumulated in the cork, whereas the hydrophilic compounds, namely the flavonoid and isoflavonoid glycosides were usually found in the cortex or center (the part inside of endodermis). Samples of rhizomes from different growth ages and origins were simultaneously analyzed. It was shown that the bulb or lateral part of the rhizome generally possessed more total flavonoids than the vertical part or the primordium. The present study established a new practical method to evaluate the quality of the rhizome of *B. chinensis* and to explore the relationship between distribution patterns of secondary metabolites and growth years of plants, thus important information for cultivation and processing was provided.

1. Introduction

As we all know, resources of Chinese medicinal materials (CMMs) are fundamental for production, application, research and sustainable development of traditional Chinese medicine. Nevertheless, due to an increasing demand for CMMs in the market, the wild resources are being depleted, and cultivation is being attempted in many places. To effectively use the resources, quality assessment is necessary to carry out. The bioactive components, which are often plant secondary metabolites that have accumulated in tissues or cells, are generally responsible for the pharmacological effects of CMMs [1,2]. Hence, the qualitative and quantitative studies on the tissue- or cell-specific chemicals are important for evaluating quality and for determining where active components accumulate, so that the medicinal resources and cultivation techniques can be optimized [3,4].

Among the existing histochemical analysis methods, the combination of fluorescence microscopy, laser microdissection (LMD), and ultra high performance liquid chromatography-quadrupole/time-of-flight-mass spectrometry (UHPLC-Q/TOF-MS) has been demonstrated to be a simple, accurate way to explore how bioactive components are distributed and thus to correlate the quality of CMMs with the morphological characteristics [3,4]. Besides, UHPLC coupled with triple quadrupole mass spectrometer

Abbreviations: LMD, laser microdissection; UHPLC-Q/TOF-MS, ultra-high performance liquid chromatography-quadrupole/time-of-flight-mass spectrometry; UHPLC–QqQ-MS, ultra-high performance liquid chromatography–triple quadrupole mass spectrometer

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(UHPLC–QqQ-MS) has already been reported to do quantitative analyses and bioanalyses of complex mixtures precisely with low detection limits [5–7]. Here, the techniques of UHPLC-Q/TOF-MS and UHPLC–QqQ-MS are used for qualitative and quantitative studies respectively on metabolite profiling of *Belamcanda chinensis* (L.) DC.

B. chinensis, a perennial herb belonging to the family of Iridaceae, is widely distributed in China, Korea, Japan, India, and eastern Russia [8]. The rhizome of *B. chinensis*, called as "She-gan" in Chinese, is listed for curing coughing and asthma, swelling and pain in throat, as well as phlegm in the lung in the Chinese Pharmacopoeia (version 2010) [9]. Phytochemical and pharmacological studies have illustrated that the phenolic compounds, namely flavonoid or isoflavonoid aglycones and their corresponding glycosides are the major bioactive constituents [10,11]. Until now, some research on the flavonoid analyses of the herb have been reported [8,12]; however, there are few reports on the distribution and accumulation patterns of chemical components on the level of tissue or cell.

The rhizome of *B. chinensis* usually develops as follows [13,14] (Fig. 1): In the first year, the primordium of the rhizome differentiates when a seedling appearing the seventh to eighth true leaves, and then the rhizome begins to enlarge, gradually form the vertical part, forms, develops numerous fibrous roots, and germinates new sprouts (Fig. 1A). In the second year, the sprouts on the vertical part grow, amplify, form new buds, and evolve into the lateral parts (Fig. 1B). In the third year, the buds on the lateral parts expand, mature, and eventually become the bulb parts with new primordia (Fig. 1C). Thus, in general, after 3 years of development, the vertical part lives for 3 years, the lateral parts 2 years, the bulb parts 1 year, and the latest primordia less than 1 year.

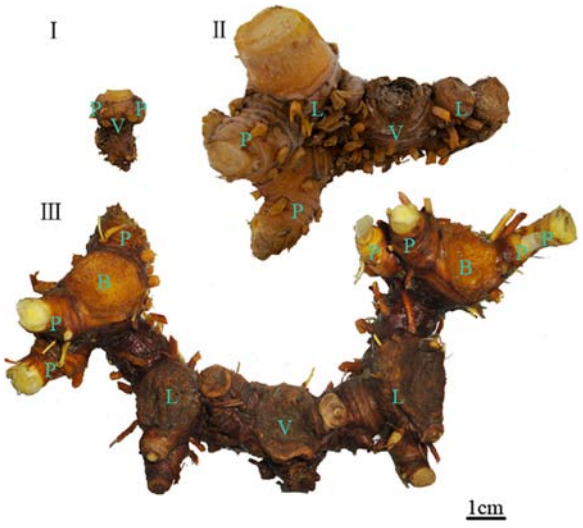


Fig. 1. Development of rhizomes of *Belamcanda chinensis* (L.) DC. I, II, III respectively represents the rhizomes of *B. chinensis* harvested in the first, second and third year after seeding. V: vertical part; L: lateral part; B:bulb part; P: primordium of sprout.

This paper presents spatial chemical profiles of *B. chinensis* at different growth ages from various origins through qualitative and quantitative analyses by using fluorescence microscopy, LMD, combined with UHPLC-Q/TOF-MS and UHPLC–QqQ-MS comprehensively. The results revealed the distribution patterns of flavonoids in tissues and cells. The relationship between quality and growing duration was also discussed. This study would provide valuable information for optimal cultivation and processing of *B. chinensis.*

2. Experimental

2.1. Plant materials

Plants were collected from different habitats in China and transplanted into the Medicinal Botanical Garden of China Pharmaceutical University. They were all identified as *B. chinensis* (L.) DC. by Prof. Minjian Qin from the Department of Resources Science of Traditional Chinese Medicines of China Pharmaceutical University. Details are given in Table 1.

2.2. Chemical and reagents

Nine reference compounds, namely mangiferin, tectoridin, iristectorin B, iridin, tectorigenin, iristectorigenin A, irigenin, irisflorentin and irilone, were isolated in our laboratory before, and were identified by ^{1}H NMR,^{13}C NMR, ESI–MS and other spectroscopic methods [15–17]. All standard compounds possessed purity above 98% as determined by HPLC. Acetonitrile and methanol of HPLC grade were purchased from E. Merck (Darmstadt, Germany). Formic acid of HPLC grade with a purity of 96% was purchased from Tedia, USA. Water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Sample and standard preparation

Fresh material of each plant was cut into small sections, embedded in cryomatrix™ (Thermo Shandon Limited, UK), and placed on a cutting platform in the cryobar of a cryostat (Thermo Shandon As620 Cryotome, UK) at −20 °C. After the sample was frozen, serial slices with the thickness of 40 μm were cut at −20 °C and put on a nonfluorescent polyethylene terephthalate (PET) slide with steel frames (76 mm × 26 mm, 1.4 μm thick, Leica Microsystems, Germany). Then the slides were observed under a

Table 1
Sample information in this study.

Sample no.	Species	Growth year	Harvest area	Collected date
1	*Belamcanda chinensis* (L.) DC.	Fresh, 1 year	Anguo City, Hebei province, China	2013.08.25
2	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Anguo City, Hebei province, China	2013.08.25
3	*Belamcanda chinensis* (L.) DC.	Fresh, 3 years	Anguo City, Hebei province, China	2013.08.25
4	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Anguo City, Hebei province, China	2013.08.25
5	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Anguo City, Hebei province, China	2013.08.25
6	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Tangshan City, Hebei province, China	2013.08.25
7	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Baoding City, Hebei province, China	2013.08.25
8	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Qixia District, Nanjing City, Jiangsu province, China	2013.08.25
9	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Qixia District, Nanjing City, Jiangsu province, China	2013.08.25
10	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Xuanwu District, Nanjing City, Jiangsu province, China	2013.08.25
11	*Belamcanda chinensis* (L.) DC.	Fresh, 2 years	Fuyang City, Anhui province, China	2013.08.25
12	*Belamcanda chinensis* (L.) DC.	Fresh, 3 years	Tuanfeng County, Huanggang City of Hubei, Hubei province, China	2012.10.22

Leica LMD 7000 microscope (Leica, Benshein, Germany) in fluorescence mode with a dichromatic mirror. Microdissection was measured by a DPSS laser beam at 349 nm wavelength, aperture of 15, speed of 5 and power of 50–60 μJ under a Leica LMD-BGR fluorescence filter system at 6.3× magnification. Tissue parts, each with an area about 2.5×10^6 μm^2, were cut separately under fluorescence inspection mode, and collected in the caps of 500 μL microcentrifuge tubes (Leica, Germany). In addition, for each sample, one whole transverse section was reserved for extraction.

The literature was reviewed to determine the optimal extraction method [18]. On the basis of this, the tissue extraction approach used here was as follows: after centrifugation (Centrifuge 5415R, Eppendorf, Hamburg, Germany) at 12,000 rpm for 5 min, tissue parts were transferred from the cap to the bottom of the tube. Then 50 μL 75% methanol was added into each tube and subjected to ultrasonicate for 30 min (CREST 1875HTAG ultrasonic processor, USA) for twice. Tubes were centrifugated again for 10 min at 12,000 rpm. The supernatant was transferred to a flat-bottom glass and insert (400 μL, Grace, USA) in a 1.5 mL brown HPLC vial (Grace, USA). Finally, the sample solution was stored at 4 °C for later analysis. For UHPLC–QqQ-MS analyses, each solution was diluted 50, 100–500 times to obtain 2 or 3 solutions at appropriate concentrations for examination.

Each standard compound was accurately weighed by an analytical balance (Sartorius MSE125P-000-DU, Germany), dissolved, mixed to obtain a mixed standard stock solution.

Table 2
Scan segments of UPLC–QqQ-MS method.

Compound name	Precursor ion	Quantitative ion	Fragmentor voltage (V)	Collision energy (V)	Polarity
Mangiferin	421.1	421.1	145	15	Negative
	331	301	145	15	Negative
Tectoridin	461.1	461.1	170	15	Negative
	299	283	170	30	Negative
Iristectorin B	491	491	180	15	Negative
	329.1	313	180	30	Negative
Iridin	521.1	521.1	180	15	Negative
	359	328	180	45	Negative
Tectorigenin	299	299	115	10	Negative
	284	240	115	20	Negative
Iristectorigenin A	329	329	120	10	Negative
	314	299	120	15	Negative
Irigenin	359	359	130	10	Negative
	344	329	130	15	Negative
Irisflorentin	387	387	155	45	Positive
	357.1	271	155	45	Positive

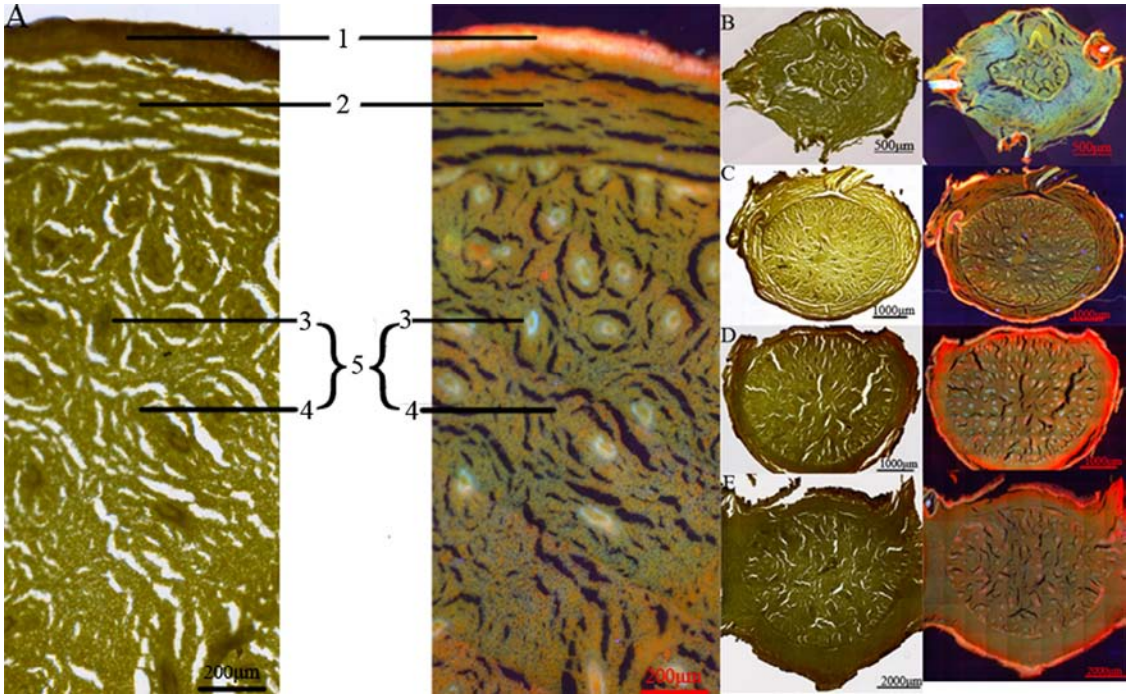


Fig. 2. Characteristics of different parts of the *B. chinensis* rhizome under light and fluorescence microscopy: (A) partial maginified pictures under light and fluorescence microscopy; (B, C, D, E) photos of transverse sections of the spread primordium, bulb part, lateral part, and vertical part under light and fluorescence microscopy respectively. (1) Cork; (2) cortex; (3) vascular bundle; (4) parenchyma tissue; (5) center part which includes vascular bundles and parenchyma cells inside of the cortex. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

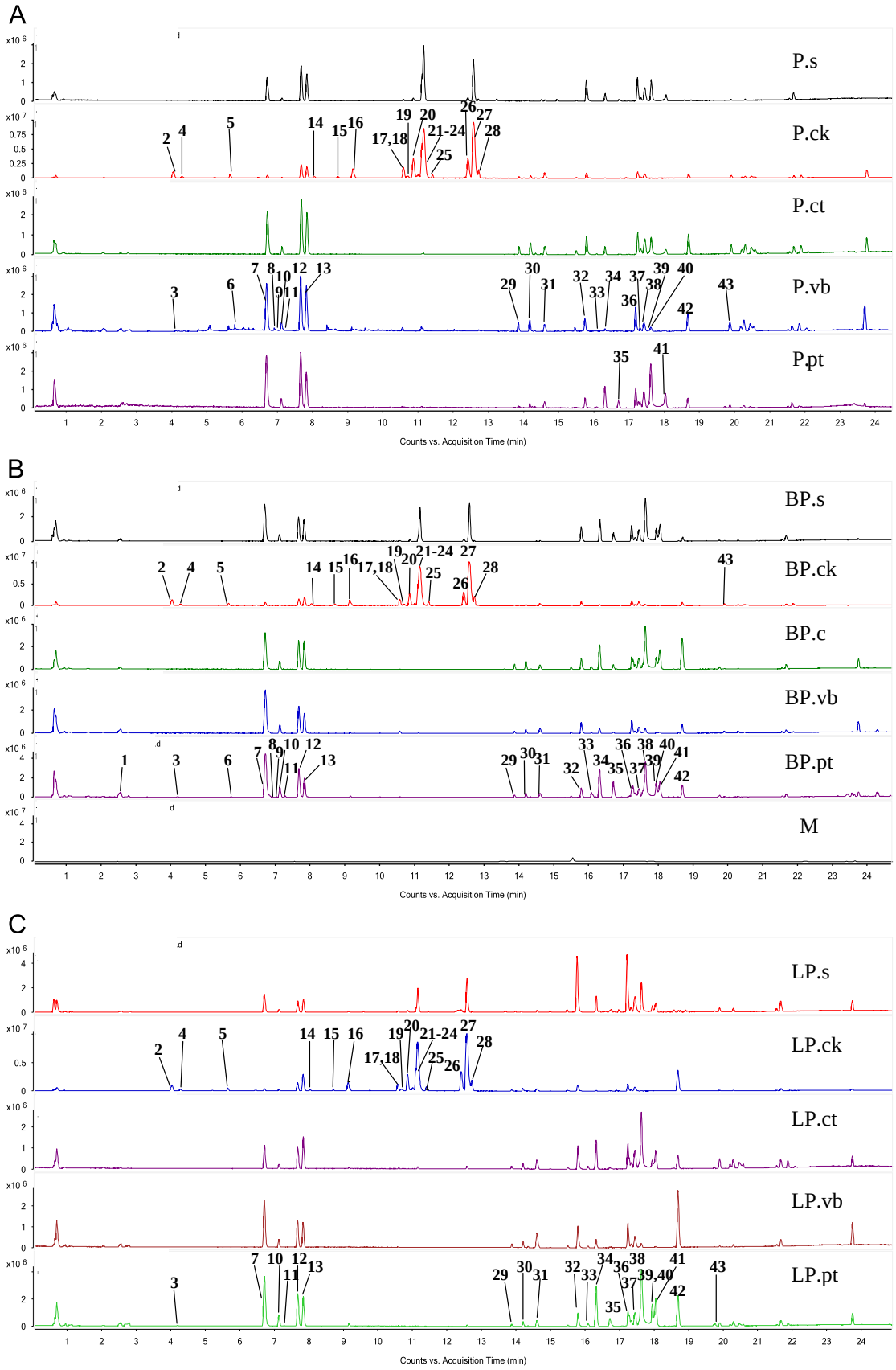


Fig. 3. Tissue-specific chemical profiling of *B. chinensis*. P, BP, LP means primordium, bulb part, and lateral part separately; s, ck, ct, vb and pt respectively represents the extractions of the whole section, cork, cortex, vascular bundle, and parenchymal tissue. M represents a blank solution of 75% methanol.

2.4. UHPLC-Q/TOF-MS method

The UHPLC-Q/TOF-MS analysis was conducted on an Agilent 6540 ultra-high definition accurate mass quadrupole time-of-flight spectrometer with UHPLC (UHPLC-QTOF-MS, Agilent Technologies, USA). A UHPLC C_{18} analytical column (2.1 mm × 100 mm, I.D. 1.7 μm, ACQUITY UHPLC® BEH, Waters, USA) coupled with a C_{18} pre-column (2.1 mm × 5 mm, I.D. 1.7 μm, VanGuardTM BEH, Waters, USA) were used at room temperature of 20 °C. The mobile phase was consisted of (A) 0.1% formic acid–water and (B) 0.1% formic acid–acetonitrile, and the gradient program was optimized as follows: 0–8 min, 5–25% B; 8–18 min, 25–75% B; 18–25 min, 75–100% B. The injection volume was 3 μL for tissue sample and 2 μL for plant section sample. The flow rate was set at 0.4 mL/min. The mass spectra were acquired in positive mode with mass to charge ratio (*m*/*z*) ranging from 100 to 1700. The operation parameters of the mass spectrometer were set as follows: dry gas temperature, 300 °C; dry gas (N_2) flow rate, 8 L/min; nebulizer pressure, 40 psi; capillary voltage, 3500 V; nozzle voltage, 500 V; and fragmentor voltage, 120 V. The energies for collision-induced dissociation (CID) were set at 30 and 45 eV, for fragmentation.

Agilent MassHunter Workstation software-Qualitative Analysis (version B.04.00, Build 4.0.479.5, Service Pack 3, Agilent Technologies, Inc. 2011) was used for qualitative analysis. Results were shown by base peak chromatogram (BPC with *m*/*z* ranged from 150 to 950).

2.5. UHPLC–QqQ-MS method

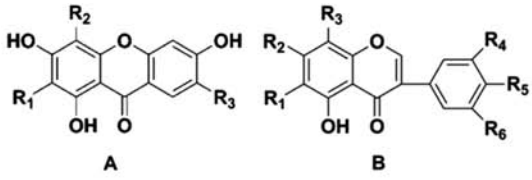
The UHPLC–QqQ-MS analysis was performed on an Agilent 6460 ultra-high performance liquid chromatograph with triple quadrupole mass spectrometer (UHPLC–QqQ-MS, Agilent Technologies, USA) in electrospray ionization (ESI(+)) mode. Separations were obtained at 40 °C with the same analytical column, mobile phase, and flow rate as above. Injection volume was 2 μL for tissue samples and plant section samples. The step gradient was: 0–1.5 min, 10–15% B; 1.5–15 min, 15–48% B; 15–17 min, 48–100% B. The acquisition conditions were gas temperature 300 °C, dry gas (N_2) flow rate 8 L/min, nebulizer 45 psi, sheath gas heater 350 °C, sheath gas flow 8 °C, 1000 V charging, capillary voltage +3500 V for ESI(+), and with a dwell time of 20 ms for each ion pair. Other details are shown in Table 2.

Data were processed by Agilent MassHunter Workstation software-Qualitative Analysis (version B.04.00, Build 4.0.225.19, Service Pack 3, Agilent Technologies, Inc. 2011).

2.6. Method validation

Analyses of linear regression curve, limit of detection (LOD), limit of quantification (LOQ), repeatability, intra-day and inter-day stability as well as recovery for each analyte were performed. The mixed standard solution was diluted with methanol to yield a series of standard solutions at appropriate concentrations to construct the calibration curves. LOD and LOQ were determined with signal-to-noise (*S*/*N*) ratios of 3 and 10 respectively.

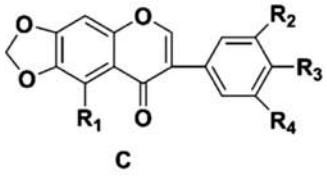
Sample 7 was chosen for experiments of repeatability, stability and recovery. Repeatability was conducted by extracting and analyzing three tissue samples from the same section of sample 7 simultaneously. The intra-day and inter-day stability was determined using an extraction of sample 7 tested at 0, 2, 4, 8, 12, 18, 24, 48 h. As for the recovery validation, six amounts of approximately

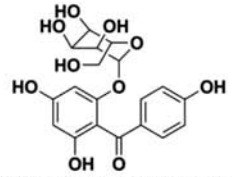


A	R_1	R_2	R_3
Mangiferin (2)	Glc	H	OH
Isomangiferin (4)	H	Glc	OH
7-*O*-methylisomangiferin (5)	H	Glc	OCH_3

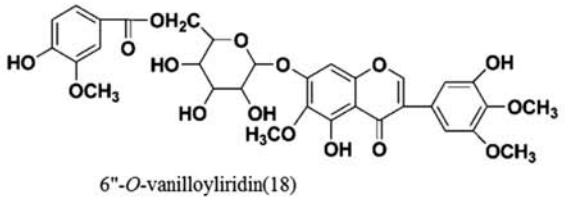
C	R_1	R_2	R_3	R_4
3',5'-Dimethoxyirisolone-4'-O-*β*-D-glucoside (16)	OCH_3	OCH_3	OGlc	OCH_3
Irifloside (19)	OH	OCH_3	OGlc	H
Noririsflorentin (25)	OH	OCH_3	OCH_3	OCH_3
Irilone (26)	OH	H	OH	H
Irisflorentin (27)	OCH_3	OCH_3	OCH_3	OCH_3
Dichotomitin (28)	OH	OH	OCH_3	OCH_3

B	R_1	R_2	R_3	R_4	R_5	R_6
Tectorigenin-7-*O*-glucosyl-4'-*O*-glucoside (3)	OCH_3	OGlc	H	H	OGlc	H
Tectorigenin-7-O-*β*-glucosyl (1→6) glucoside (6)	OCH_3	OGlc-Glc	H	H	OH	H
Tectoridin (7)	OCH_3	OGlc	H	H	OH	H
Iristectorigenin A-7-*O*-*β*-glucosyl(1→6)glucoside (9)	OCH_3	OGlc-Glc	H	OH	OCH_3	H
Iristectorin B (10)	OCH_3	OGlc	H	OCH_3	OH	H
3'-Hydorxytectoridin (11)	OCH_3	OGlc	H	OH	OH	H
Iristectorin A (12)	OCH_3	OGlc	H	OCH_3	OH	H
Iridin (13)	OCH_3	OGlc	H	OH	OCH_3	OCH_3
Tectorigenin (17)	OCH_3	OH	H	H	OH	H
Isotectorigenin (19)	OH	OCH_3	H	H	OH	H
Iristectorigenin A (20)	OCH_3	OH	H	OH	OCH_3	H
Iristectorigenin B (21)	OCH_3	OH	H	OCH_3	OH	H
5,7,4'-trihydroxy-6,3',5'-trimethoxyisoflavone (22)	OCH_3	OH	H	OCH_3	OH	OCH_3
Irigenin (23)	OCH_3	OH	H	OH	OCH_3	OCH_3
Isoirigenin (24)	H	OH	OCH_3	OH	OCH_3	OCH_3





Iriflophenone 2-*O*-*β*-glucoside (1)



6"-*O*-vanilloyliridin(18)

Fig. 4. Chemical structures of identified flavonoids in the tissues of *B. chinensis*.

Table 3
Chemical characterization of cross section extractions of *B. chinensis* by UHPLC-Q-TOF-MS/MS.

Peaks	Retention time (min)	Experimental mass (m/z) $[M+H]^+$	Theoretical mass (m/z) $[M+H]^+$	Error (ppm)	Formula	MS/MS fragments	Identification	Reference	Source
1	2.56	409.1199	409.1129	−17.11	$C_{19}H_{20}O_{10}$	368, 318, 272, 216, 188	Iriflophenone 2-*O*-*β*-glucoside	20	BP.vb, BP.pt
2	4.04	423.0958	423.0922	−8.51	$C_{19}H_{18}O_{11}$	405, 387, 339, 327, 303, 273, 229	Mangiferin[a]	8, 19, 20, 21	P.ck, BP.ck, LP.ck
3	4.21	625.1762	625.1763	0.16	$C_{28}H_{32}O_{16}$	463, 343, 301, 241, 145	Tectorigenin-7-*O*-glucosyl-4′-*O*-glucoside	8, 19, 22, 23	P.vb, BP.ct, BP.vb, BP.pt, LP.pt
4	4.28	423.0934	423.0922	−2.84	$C_{19}H_{18}O_{11}$	405, 387, 369, 357, 351, 339, 327, 313, 303, 285, 273, 261, 243, 225	Isomangiferin	8, 20, 24	P.ck, BP.ck, LP.ck
5	5.66	437.1103	437.1078	−5.72	$C_{20}H_{20}O_{11}$	419, 401, 383, 371, 365, 353, 341, 327, 317, 299, 287, 275, 259, 165	7-*O*-methylisomangiferin	8	P.ck, BP.ck, LP.ck
6	5.74	625.1749	625.1763	2.24	$C_{28}H_{32}O_{16}$	545, 463, 301, 145	Tectorigenin-7-*O*-*β*-glucosyl (1→6) glucoside	8, 17, 20, 23	P.vb, BP.vb, BP.pt
7	6.70	463.1280	463.1235	−9.72	$C_{22}H_{22}O_{11}$	343, 301, 286, 168	Tectoridin[a]	8, 19, 20, 25	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
8	6.91	523.1435	523.1446	2.10	$C_{24}H_{26}O_{13}$	361, 346, 286, 258, 215, 183	Iridin isomer	8, 19, 20, 26, 27, 28	P.vb, BP.vb, BP.pt, LP.pt
9	7.02	655.1827	655.1869	6.41	$C_{29}H_{34}O_{17}$	584, 420, 331, 145	Iristectorigenin A-7-*O*-*β*-glucosyl (1→6) glucoside	19, 29	P.vb, P.pt, BP.pt
10	7.12	493.1360	493.1341	−3.85	$C_{23}H_{24}O_{12}$	331, 145	Iristectorin B[a]	8, 19, 20, 29, 30	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
11	7.26	479.1186	479.1184	−0.42	$C_{22}H_{22}O_{12}$	411, 331, 177	3′-Hydroxytectoridin	8, 20, 26, 31	P.vb, BP.vb, BP.pt, LP.pt
12	7.67	493.1387	493.1341	−9.33	$C_{23}H_{24}O_{12}$	331, 316, 301, 273, 168	Iristectorin A	8, 20, 26, 32	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
13	7.83	523.1491	523.1491	0	$C_{24}H_{26}O_{13}$	361, 232, 145	Iridin[a]	8, 20, 21	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
14	8.05	–	–	–	–	229	Unknown	–	P.ck, BP.ck, LP.ck
15	8.70	–	–	–	–	259	Unknown	–	P.ck, BP.ck, LP.ck
16	9.14	535.1445	535.1446	0.19	$C_{25}H_{26}O_{13}$	415, 373, 127	3′,5′-Dimethoxyirisolone-4′-*O*-*β*-D-glucoside	8, 20, 33	P.ck, P.vb, BP.s, BP.ck, BP.vb, BP.pt, LP.ck, LP.ct, LP.vb, LP.pt, LP.pt
17	10.57	301.0711	301.0707	−1.33	$C_{16}H_{12}O_6$	286, 258, 212, 168, 140, 102	Tectorigenin[a]	8, 19, 20	P.s, P.ck, P.vb, P.pt, BP.s, BP.ck, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
18	10.57	673.1754	673.1763	1.34	$C_{32}H_{32}O_{16}$	524, 403, 361, 313, 151	6″-*O*-vanilloyliridin	8, 10	P.s, P.ck, P.vb, BP.s, BP.ck, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
19	10.68	301.0707	301.0707	0	$C_{16}H_{12}O_6$	301, 286, 269, 168, 121	Isotectorigenin	8	P.ck, BP.ck, BP.vb, LP.ck
20	10.85	331.0842	331.0812	−9.06	$C_{17}H_{14}O_7$	316, 301, 287, 273, 245, 200, 168, 133, 107	Iristectorigenin A[a]	8, 19, 20, 27, 34	P.s, P.ck, BP.ck, BP.vb, LP.s, LP.ck
21	11.09	331.0841	331.0812	−8.76	$C_{17}H_{14}O_7$	316, 301, 273, 245, 213, 194, 168, 131, 105	Iristectorigenin B	8, 20	P.s, P.ck, P.vb, BP.s, BP.ck, LP.s, LP.ck
22	11.13	361.0964	361.0918	−12.74	$C_{18}H_{16}O_8$	331, 313, 303, 286, 275, 258, 240, 227, 213	5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone	8, 33	P.s, P.ck, P.vb, BP.s, BP.ck, LP.s, LP.ck
23	11.14	361.0967	361.0918	−13.57	$C_{18}H_{16}O_8$	346, 301, 258, 232, 208, 183, 147, 119	Irigenin[a]	8, 20	P.s, P.ck, P.vb, BP.s, BP.ck, LP.s, LP.ck, LP.ct, LP.pt
24	11.18	361.0959	361.0918	−11.35	$C_{18}H_{16}O_8$	346, 301, 258, 232, 208, 183, 147, 119	Isoirigenin	8, 33	P.s, P.ck, BP.s, BP.ck, LP.s, LP.ck
25	11.40	373.0941	373.0918	−6.16	$C_{19}H_{16}O_8$	358, 312, 256, 220, 195, 166, 118	Noririsflorentin	8, 35	P.ck, BP.ck, LP. ck
26	12.41	299.0576	299.0550	−8.69	$C_{16}H_{10}O_6$	281	Irilone[a]	8, 19	P.s, P.ck, BP.s, BP.ck, LP.s, LP.ck
27	12.57	387.1110	387.1074	−9.30	$C_{20}H_{18}O_8$	371, 357, 342, 329, 314, 271, 225, 181, 149	Irisflorentin[a]	8, 19, 20	P.s, P.ck, BP.s, BP.ck, LP.s, LP.ck, LP.ct, LP.pt
28	12.71	359.0784	359.0761	−7.24	$C_{18}H_{14}O_8$	329, 295, 249, 227, 185, 158, 121	Dichotomitin	8, 19, 20, 36	P.s, P.ck, BP.s, BP.ck, LP.s, LP.ck
29	13.86	–	–	–	–	288	Unknown	–	P.ck, P.ct, P.vb, P.pt, BP.ck, BP.ct, BP.vb, BP.pt, LP.ck, LP.ct, LP.vb, LP.pt
30	14.19	–	–	–	–	288	Unknown	–	P.ck, P.ct, P.vb, P.pt, BP.ck, BP.ct, BP.vb, BP.pt, LP.ck, LP.ct, LP.vb, LP.pt
31	14.60	–	–	–	–	473, 453, 437, 415	Unknown	–	

32	15.77	–	–	–	–	316, 304	Unknown	–	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
33	16.07	497.3596 ($[M+Na]^+$)	497.3607	2.21	$C_{30}H_{50}O_4$	359, 273, 178	Iridobelamal A/iso-iridogermanal/ isomer	37	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
34	16.31	497.3628 ($[M+Na]^+$)	497.3607	−4.22	$C_{30}H_{50}O_4$	439, 359, 262, 171	Iridobelamal A/iso-iridogermanal/ isomer	37	P.ck, P.ct, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
35	16.70	509.3236 ($[M+Na]^+$)	509.3243	1.37	$C_{30}H_{46}O_5$	449, 381, 289, 184, 131	Iridotectorals A/Iridotectorals B/ Spiroiridal/28-deacetylbelamcandal	37, 38, 39, 40	P.ck, P.pt, BP.s, BP.ct, BP.vb, BP.pt, LP.ct, LP.pt
36	17.22	–	–	–	–	332	Unknown	–	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
37	17.43	–	–	–	–	579→301; 301→286; 279	Unknown	–	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
38	17.62	509.3247 ($[M+Na]^+$)	509.3243	−0.79	$C_{30}H_{46}O_5$	491, 445, 401, 370, 319, 275, 248, 219, 449, 381, 289, 184, 131	Iridotectorals A/Iridotectorals B/ Spiroiridal-1/28-deacetylbelamcandal	37, 38, 39, 40	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
39	17.93	551.3378 ($[M+Na]^+$)	551.3349	−5.26	$C_{32}H_{48}O_6$	491, 357, 109	Belamcandal/Spiroiridal-2	38, 41	P.s, P.ck, P.ct, P.vb, LP.s, LP.ct, LP.pt
40	18.03	–	–	–	–	469→433, 405, 375, 333, 299, 261, 229, 215, 175	Unknown	–	BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ct, LP.pt
41	18.33	–	–	–	–	469→433, 405, 375, 333, 293, 261, 229, 215, 175	Unknown	–	P.s, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP. pt, LP.s, LP.ct, LP.vb, LP.pt
42	18.68	471.3478	471.3474	−0.85	$C_{30}H_{46}O_4$	453, 435, 417, 377, 329, 293, 245, 195, 177, 135	Spiroiridal-2	41	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt
43	19.73	495.3424 ($[M+Na]^+$)	495.3451	5.45	$C_{30}H_{48}O_4$	477, 435, 359, 237, 175, 133	Iridal	41	P.s, P.ck, P.ct, P.vb, P.pt, BP.s, BP.ck, BP.ct, BP.vb, BP.pt, LP.s, LP.ck, LP.ct, LP.vb, LP.pt

–: Not clear.

[a] Authentic standards; P, BP, LP means primordium, bulb part, and lateral part separately; s, ck, ct, vb and pt respectively represents the extractions of the whole section, cork, cortex, vascular bundle, and parenchymal tissue.

half size of cork tissue (1,250,000 μm^2) from more than three spots of the same section of sample 7 were added with certain amounts of standard compounds for extraction and recovery assessment [3].

3. Results and discussion

3.1. Characteristics under fluorescence microscopy and dissection by LMD

Under the fluorescence mode, the rhizome transverse section could be divided into three portions: cork, cortex, and center (the part inside of endodermis). In the center, vascular bundles were scattered among the parenchyma cells (Fig. 2A). Meanwhile, a root section was composed of cork, parenchymatous tissue, and vascular bundles.

Sections of different parts emitted various auto-fluorescence colors. The section of sprout primordium showed colorless, yellow or red fluorescence at the cork, yellow and blue at the cortex, strong blue at the vessels of vascular bundles, and blue at the parenchyma (Fig. 2B). While bulb section showed strong colorless or red fluorescence at the cork; light blue, and yellow to red fluorescence in the cortex; vascular bundles, which were scattered, showed strong blue color among red parenchyma cells (Fig. 2C). Most sections of lateral and vertical parts looked like those of the bulb one, but with stronger fluorescence at the cork and more red color in the cortex and parenchyma tissues (Fig. 2D and E). Nevertheless, some lateral sections of the rhizome aged 2, such as those of sample 9, looked similar with the primordium section. Therefore, various tissues possessed distinct features and could be recognized under fluorescence mode. Each separated tissue was dissected at the size of 2,500,000 μm^2 by LMD.

3.2. Tissue-specific chemical profiling

Considering the similarity in characteristics of lateral and vertical sections of 3 year-old samples under fluorescence microscope, three portions of primordium, bulb part, and lateral part of sample 12 were sectioned for qualitative diagnosis. To give tissue-specific chemical profiling of *B. chinensis*, each section was separated into 4 portions, namely, cork, cortex, vascular bundles and parenchyma tissue. Their mass spectra were obtained using the UHPLC-Q/TOF-MS method (Fig. 3). A total of 43 peaks were detected in all the tissue extractions. Among them, nine constituents, namely mangiferin, tectoridin, iristectorin B, iridin, tectorigenin, iristectorigenin A, irigenin, irisflorentin and irilone, were unambiguously identified by comparing the retention times, accurate mass weights, and mass ions with those of the reference compounds. Twenty-six peaks were tentatively characterized as flavonoid compounds by matching those data with the literatures [8,17,19–36]. The authenticated chemical structures of flavonoids are shown in Fig. 4. Peaks **29–43** were firstly detected in the rhizome of this plant by UHPLC-MS. Seven peaks were tentatively identified as triterpene compounds for the first time by comparing with the literatures [37–41]. Details are given in Table 3.

The chromatograms of various tissues from different rhizome parts revealed that their chemical profiles were quite similar. Total chromatographic peaks of the cork from the primordium, bulb part and lateral part of the rhizome were 31, 32 and 30 respectively. Similar total chromatographic peaks of cortex, vascular bundles and parenchyma tissue from different rhizome parts were also detected. In detail, peaks **2, 4, 5, 14–28** were basically present in the cork; peaks **3, 29, 35, 40** and **41** were mainly contained in the cortex or tissues within it; peaks **1, 6, 8, 9, 11** and **33** occurred exclusively in the vascular bundles and parenchymal

cells; while peaks **7, 10, 12, 13, 29–32, 34, 36–38**, **42, 43** were common in all the tissues. Distinctly, peaks at retention time between 10.5 min and 12.8 min were often detected in the cork extraction, while some other peaks like **7–13** and **32–43** tended to appear in the cortex, vascular bundles and parenchymal cells. The results indicated that the hydrophobic compounds, including flavonoid or isoflavonoid aglycones and xanthone were mainly distributed in the cork, while they were rarely found in the cortex, vascular bundles or parenchyma tissue. At the same time, the hydrophilic compounds, namely the flavonoid and isoflavonoid glycosides usually accumulated in the cortex or center part.

Such secondary metabolite storage diversity among tissues of plants had been reported before [3,4,42]. Various reasons were given to try to explain this phenomenon. Recently, Rogers et al. provided cell type-specific transcriptome profiles and proved that complex gene regulatory networks occur at the cellular level, which led to cell type-specific expression of many proteins involved in secondary metabolism [43]. Groenenboom et al. investigated the flavonoid pathway in tomato seedlings, and found that the variation in transcript abundance resulted in enzymatic variation, which could affect metabolite accumulation [44]. It is no doubt that the metabolite production is regulated by many enzymes and their genes. As for *B. chinensis*, the cells in the cortex, vascular bundles and parenchymal cells might produce more enzymes enabling glycosylic bonds added to aglycones to form glycosides; while those relative enzymes' transcription or expression were inhibited in the cork cells due to gene regulation, transcript abundance variation, or interactions among enzymes. The exact reasons for this phenomenon need to be further studied.

Flavonoid compounds had different functions in different tissues. Since cork was generally thought to serve as defense against external threats, the peripheral hydrophobic constituents might protect the plant from environmental stresses such as UV light, temperature and hydric fluctuation, resist herbivory including pathogens and insects, as well as act as antioxidants. The internal hydrophilic constituents, which could be transported by water through vessels quickly, might play a role as signal compounds involved in allelopathy and growth regulation [45–49]. In addition, flavonoids might be also responsible for the yellow color of the rhizome.

To further explore the phenomenon, eight flavonoid compounds were chosen as analytes for quantitative analyses by UHPLC–QqQ-MS method with various tissue samples at different ages. Since chemical profiles of vascular bundles and parenchymal tissues were similar, and the same compounds were distributed in both tissues, the vascular bundle and the parenchymal tissue were combined as “center” (the part inside of endodermis) for the subsequent determination. Thus, a section was divided as the cork, cortex and center for the following quantitative analyses.

3.3. Method validation

The results of method validation are present in Table 4. Each calibration curve, which was constructed by plotting the peak areas of the flavonoids at different levels versus the concentration (ng/mL), possessed good linearity with correlation coefficients (R^2) ≥ 0.9944 within the selected range. The LODs, calculated by a signal-to-noise (S/N) of 3, were 1.02, 0.51, 0.23, 0.08, 0.07, 0.14, 0.25 and 0.10 ng mL^{-1} for mangiferin, tectoridin, iristectorin B, iridin, tectorigenin, iristectorigenin A, irigenin and irisflrentin respectively. The LOQs, with a S/N of 10, were 4.48, 1.55, 0.90, 0.41, 0.52, 0.55, 1.01 and 0.48 for those analytes separately. The repeatbility ranged from 0.17% to 10.97%, indicating that the precison of this method was acceptable. Since the stability RSD was less than 8.68% within 48 h, all the prepared samples were analyzed within 2 days. In view of the tiny sampling and trcace amounts of flavonoids in the plant, the method showed satisfactory accuracy as the recovery varied from 90.67% to 133.42%. Hence, the method developed was shown to be precise, sensitive, and accurate for tissue-specific determination of 8 analytes in *B. chinensis* samples.

3.4. Quantification of flavonoids in various tissues

As mentioned, eight flavonoid compounds in the laser-dissected tissues of various rhizome parts of *B. chinensis* from different origins were assessed by UHPLC–QqQ-MS technique, and the results are summarized in Table 5 and Figs. 5 and 6. By measuring the major and minor axes of the extracted transverse section and calculating their areas as an ellipse, the relationship between flavonoid concentrations and areas of each sample section are given in Table 6 and Fig. 7.

The results of quantitative analysis showed that the flavonoid and isoflavonoid glycosides, namely tectoridin, iristectorin B, and iridin, usually accumulated in the cortex or center rather than in the cork. Whereas, the xanthone, flavonoid or isoflavonoid aglycones such as mangiferin, tectorigenin, iristectorigenin A, irigenin and irisflorentin showed the highest quantity in the cork, and often slightly higher quantity in the cortex than in the center. The results of quantitative analysis were consistent with those of qualitative analysis.

Samples 1, 2, and 3 were harvested at the first, second and third year after seeding respectively, and were thus selected to explore changes in flavonoid distribution as plants aged. Each rhizome of the samples above was divided according to age, sectioned and dissected by LMD for determination. The top of rhizome of sample 1 had more mangiferin, tectoridin, iristectorin B, iridin, tectorigenin, iristectorigenin A, irigenin and irisflorentin in the cross section than the bottom section. Because rhizome of sample 1 incorporated two sprout primordiums on the both sides of the top portion, the top was found

Table 4
Linear regression data, LODs and LOQs of 8 detected chemical compounds.

Analyte	Calibration curve	R^2	Linear range (ng/mL)	LODs (ng mL^{-1})	LOQs (ng mL^{-1})	Repeatability (n=3, RSD, %)	Stability RSD (%)		Recovery (n=6, %)	
							Intra-day (n=6)	Inter-day (n=3)	Mean	RSD
Mangiferin	y=220.427362x−44.024976	0.9998	0.01016–1016	1.02	4.48	8.23	3.11	3.90	97.60	6.91
Tectoridin	y=194.144121x+627.090215	0.9999	0.01048–1048	0.51	1.55	3.76	6.46	6.95	104.05	19.81
Iristectorin B	y=260.258806x+509.853615	0.9999	0.0101–1010	0.23	0.90	0.17	7.06	8.68	107.00	10.38
Iridin	y=308.733764x+1291.773626	0.9997	0.01080–1080	0.08	0.41	7.55	5.71	6.15	103.55	7.96
Tectorigenin	y=713.315543x+2692.443012	0.9995	0.01040–1040	0.07	0.52	10.42	5.67	5.75	90.67	6.92
Iristectorigenin A	y=268.772846x+401.828886	0.9999	0.0109–1090	0.14	0.55	5.02	6.59	6.82	95.64	13.44
Irigenin	y=272.609184x+323.266003	1.0000	0.01013–1013	0.25	1.01	9.11	2.12	2.22	133.42	10.99
Irisflorentin	y=726.140118x+351.116169	0.9944	0.00969–969	0.10	0.48	10.91	5.93	6.33	104.58	7.52

Table 5
Contents of flavonoids in the laser dissected tissues.

Sample no.	Dissected part	Tissue	Amount in unit area (ng/10^5 μm^2)							
			Mangiferin	Tectoridin	Iristectorin B	Iridin	Tectorigenin	Iristectorigenin A	Irigenin	Irisflorentin
1	The top of the rhizome aged 1	Cork	29.29	21.06	2.79	31.85	25.72	18.38	182.67	138.53
		Cortex	2.32	100.49	10.64	98.41	6.21	1.50	9.73	0.40
		Center	0.82	130.70	11.80	87.09	5.40	0.48	4.93	0.07
	The bottom of the rhizome aged 1	Cork	11.91	8.38	0.90	19.18	5.38	4.74	64.75	70.27
		Cortex	0.96	27.42	3.89	53.60	1.00	0.47	7.69	0.13
		Center	1.05	34.012	4.81	70.92	1.07	0.31	7.17	0.96
2	Primordium of sprout	Cork	67.93	16.70	14.82	37.18	53.84	46.11	253.08	273.24
		Cortex	14.34	313.03	56.92	214.71	5.97	3.07	9.59	1.96
		Center	1.63	117.34	21.57	63.15	3.78	0.95	3.06	0.10
	Lateral part	Cork	74.98	44.06	16.12	76.46	39.62	59.52	293.48	106.79
		Cortex	3.80	554.08	66.14	514.62	6.87	3.94	12.85	1.39
		Center	1.46	304.94	28.57	128.58	5.75	1.19	4.03	0.11
	Vertical part	Cork	58.78	54.69	16.95	115.36	24.02	41.50	397.71	442.13
		Cortex	0.17	397.56	56.23	299.81	2.54	0.43	5.41	0.29
		Center	3.46	513.70	50.92	276.80	0.99	3.75	19.74	14.58
3	Primordium of sprout	Cork	18.28	202.02	22.01	45.13	17.49	25.02	190.62	35.90
		Cortex	2.31	264.08	35.24	86.68	4.14	1.47	4.40	–*
		Center	1.27	20.39	11.83	29.32	7.90	3.04	3.82	0.005
	Bulb part	Cork	1.90	67.00	8.21	22.98	52.10	74.78	225.79	157.37
		Cortex	0.25	353.09	13.98	64.86	20.49	6.80	32.53	1.29
		Center	0.40	247.78	8.74	36.20	10.70	2.94	13.66	0.28
	Lateral part	Cork	20.92	94.33	30.91	118.46	53.81	81.56	637.07	267.86
		Cortex	0.13	282.22	254.43	218.36	16.66	6.44	26.03	0.53
		Center	0.12	645.90	216.56	536.86	10.24	4.02	14.51	0.26
	Vertical part	Cork	2.94	66.07	10.38	66.60	17.42	26.01	339.08	296.69
		Cortex	0.44	321.62	109.41	210.95	4.45	1.59	7.15	0.33
		Center	0.63	402.04	230.17	220.96	13.13	4.41	11.31	0.86
4	Lateral part	Cork	9.66	168.55	16.84	79.35	98.23	123.78	291.29	246.43
		Cortex	0.13	429.24	38.82	218.30	0.92	0.35	5.52	1.56
		Center	0.11	353.06	27.80	114.77	–*	–	1.46	0.17
5	Lateral part	Cork	64.08	11.24	1.89	33.95	15.90	30.47	1290.44	414.70
		Cortex	2.03	112.21	19.35	154.64	0.87	0.92	13.01	2.32
		Center	0.16	78.20	10.93	78.00	5.21	–	13.48	0.05
6	Lateral part	Cork	125.64	176.25	32.49	1.91	125.74	166.83	223.27	44.38
		Cortex	2.08	506.83	100.62	21.32	11.57	5.22	45.94	5.77
		Center	0.54	306.61	47.40	12.85	–	1.47	12.45	0.30
7	Lateral part	Cork	75.06	42.76	4.29	64.44	29.85	36.82	522.28	160.03
		Cortex	2.04	336.57	34.03	231.22	1.84	–	6.71	0.64
		Center	0.44	180.03	14.83	75.83	–	0.05	1.46	–
8	Lateral part	Cork	59.69	49.05	8.02	55.87	12.93	37.21	231.99	111.84
		Cortex	2.27	270.35	40.44	339.82	3.15	1.84	18.83	5.65
		Center	0.64	435.83	61.96	324.38	3.82	1.27	5.71	0.08
9	Lateral part	Cork	12.45	47.37	8.13	60.51	33.56	102.19	119.38	43.94
		Cortex	0.73	115.86	21.50	107.97	0.21	0.26	2.43	0.06
		Center	0.55	156.60	21.18	93.13	1.82	0.56	5.43	0.52
10	Lateral part	Cork	82.21	191.91	39.45	1.68	232.28	288.17	337.48	52.91
		Cortex	8.92	397.03	52.09	399.57	4.91	2.24	12.20	0.81
		Center	0.17	224.89	12.28	42.60	1.40	–	1.50	–
11	Lateral part	Cork	63.17	167.887	42.07	69.00	105.51	263.15	1007.41	273.43
		Cortex	4.17	413.27	95.78	174.45	8.33	3.70	29.11	7.45
		Center	2.09	518.01	94.91	149.88	31.72	8.86	28.39	0.81
12	Bulb part	Cork	4.02	17.03	0.80	5.66	19.98	7.33	31.03	11.93
		Cortex	0.28	128.79	17.24	131.0746	8.00	2.27	6.95	0.35
		Center	0.03	71.54	37.02	111.68	2.60	0.40	2.77	0.02
Root	The root of *B. chinensis* (L.) DC. aged 2/3	Cork	114.01	16.86	4.02	245.42	21.88	81.56	358.79	162.13
		Cortex	6.44	16.75	4.48	236.43	–	0.45	20.84	3.46
		Center	13.47	178.82	33.36	493.91	0.28	0.95	57.15	3.86

* Undetected.

to contain more flavonoids than the bottom. The total flavonoid content of one-year-old rhizome was generally lower than other samples. The lateral part of sample 2 showed more contents of mangiferin, tectoridin, iristectorin B, iridin, tectorigenin, iristectorigenin A, irigenin, and irisflorentin in the cross section; while the primordium section had 4.61, 88.67, 7.98, 28.36, 2.73, 1.63, 5.78 and 6.49 ng per unit area of those analytes, and the vertical section contained 1.60, 63.54, 6.52, 45.23, 0.75, 0.58, 10.52, and 9.19 ng per unit area of those analytes. Thus, for the two-year-old rhizome, the lateral part had more flavonoids than the vertical part or primordium.

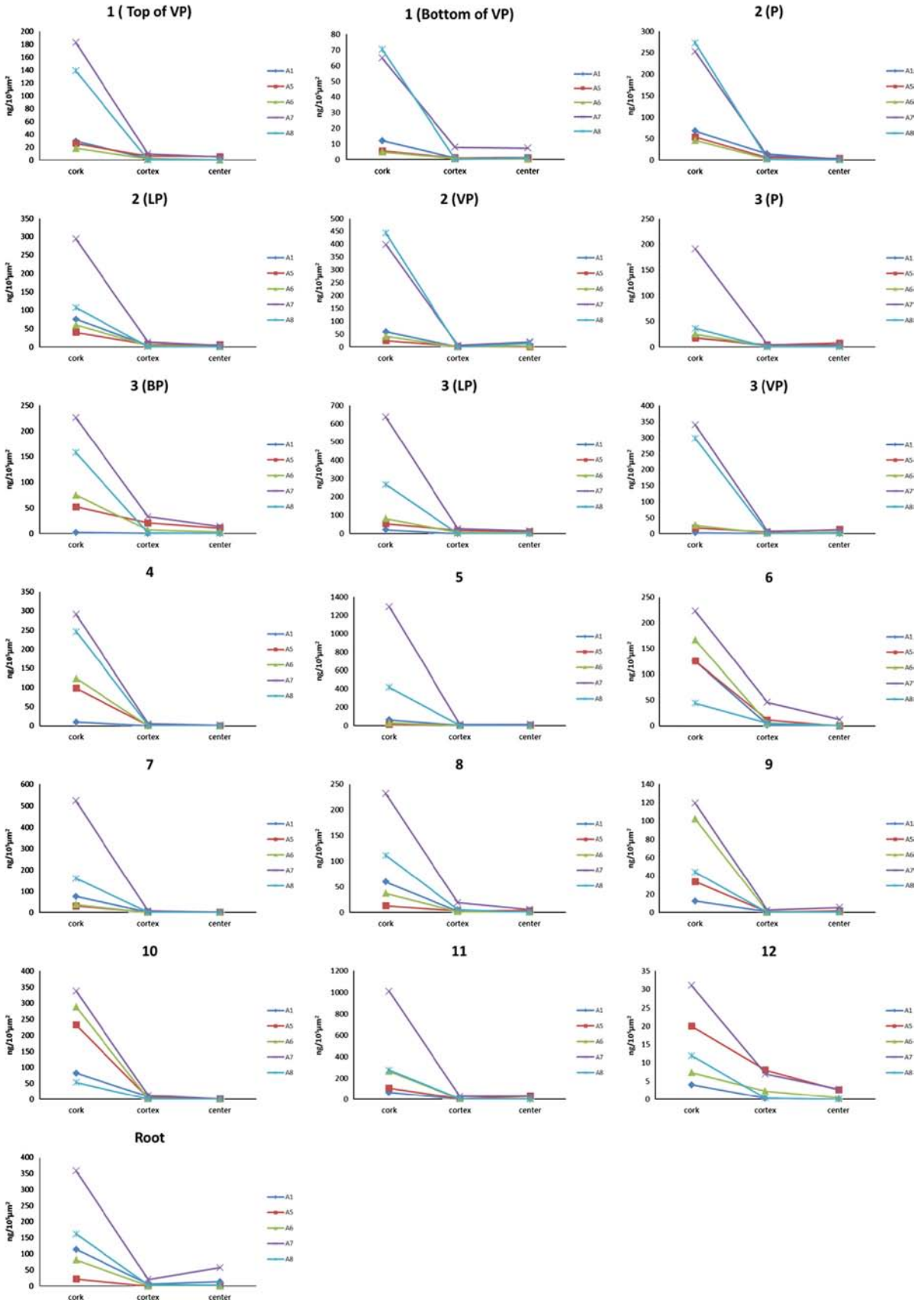


Fig. 5. The contents of mangiferin, tectorigenin, iristectorigenin A, irigenin, and irisflorentin in the samples. A1, A5, A6, A7, A8 represents mangiferin, tectorigenin, iristectorigenin A, irigenin, and irisflorentin respectively. P, BP, LP and VP is on behalf of the primordium, bulb part, lateral part and vertical part respectively; 1(T) and (B) means the top and bottom of the vertical part of sample 1 aged 1.

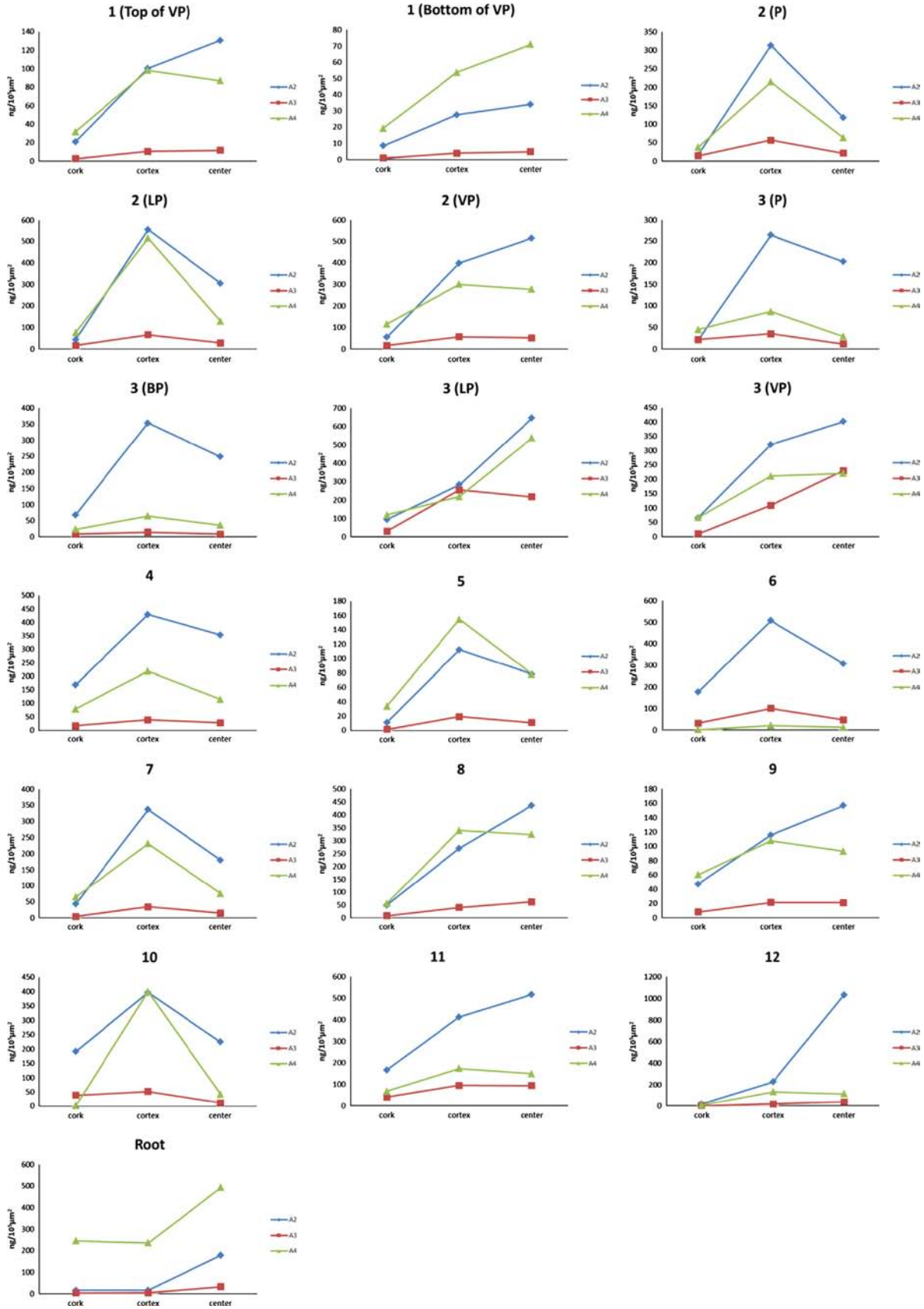


Fig. 6. The contents of tectoridin, iristectorin B, iridin in the samples. A2, A3, A4 represents tectoridin, iristectorin B, iridin respectively. P, BP, LP and VP is on behalf of the primordium, bulb part, lateral part and vertical part respectively; 1(T) and (B) means the top and bottom of the vertical part of sample 3 aged 1.

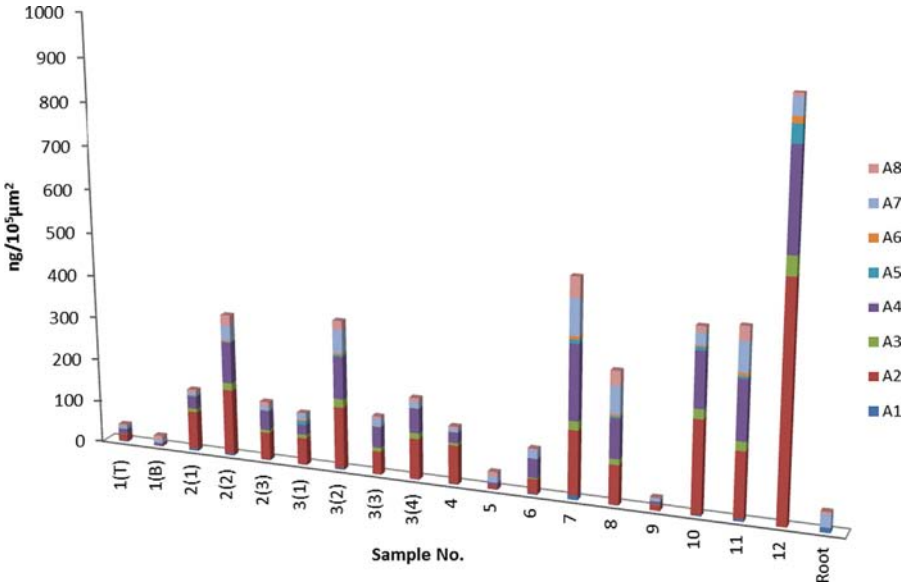


Fig. 7. The flavonoid contents of the cross section extractions. A1–8 represents mangiferin, tectoridin, iristectorin B, iridin, tectorigenin, iristectorigenin A, irigenin, and irisflorentin respectively. 1(T) and (B) meant the top and bottom of the vertical part of sample 3 aged 1; 2(1), 2(2), 2(3) signified the primordium, lateral part and vertical part of the rhizome respectively; 3(1), 3(2), 3(3) and 3(4) were on behalf of the primordium, bulb part, lateral part and vertical part separately.

Table 6
Contents of flavonoids in the cross sections of the samples.

Sample no.	The section of the dissected part of the rhizome	Amount in whole cross sections ($ng/10^5\ \mu m^2$)							
		Mangiferin	Tectoridin	Iristectorin B	Iridin	Tectorigenin	Iristectorigenin A	Irigenin	Irisflorentin
1	The top of the rhizome aged 1	2.20	15.61	0.77	11.67	1.02	0.40	6.19	3.87
	The bottom of the rhizome aged 2	1.14	1.93	0.03	3.80	0.54	0.01	7.26	9.94
2	Primordium of sprout	4.61	88.67	7.98	28.36	2.73	1.63	5.78	6.49
	Lateral part	3.53	151.71	16.99	95.93	1.47	2.94	34.19	25.25
	Vertical part	1.60	63.54	6.52	45.23	0.75	0.58	10.52	9.19
3	Primordium of sprout	0.79	62.12	8.61	22.94	8.46	3.74	14.53	1.50
	Bulb part	2.97	142.98	19.50	97.74	4.18	3.77	54.26	20.69
	Lateral part	0.31	53.55	8.89	48.04	0.83	0.92	17.17	5.87
	Vertical part	0.61	93.70	13.48	56.23	0.99	0.98	14.34	8.31
4	Lateral part	1.40	88.54	6.07	24.77	0.03	0.11	7.79	5.32
5	Lateral part	0.90	8.22	0.07	6.91	0.09	0.04	14.04	10.07
6	Lateral part	2.17	33.62	2.66	43.84	0.45	0.04	19.89	2.43
7	Lateral part	9.54	148.73	20.07	172.17	9.31	7.86	83.53	47.31
8	Lateral part	1.38	89.91	13.31	91.00	4.03	4.36	64.00	33.35
9	Lateral part	0.26	12.64	0.91	6.67	0.08	0.01	6.54	1.73
10	Lateral part	2.35	212.73	22.33	126.92	7.57	3.48	27.65	14.28
11	Lateral part	5.57	150.69	20.69	136.34	5.70	7.13	68.08	32.61
12	Lateral part	0.08	539.66	43.75	227.84	40.97	14.77	40.33	6.58
Root	The root aged 2 or 3	9.72	–*	–	1.74	0.06	0.09	29.45	5.02

*Undetected.

For the three-year old rhizome, the bulb part showed the most flavonoids, while the primordium section bore the fewest flavonoids. Such variation in flavonoid concentration might be the reason why different sections of rhizomes of different growth ages show different fluorescence. In order to facilitate comparison, other rhizome samples were sectioned at the portions aged 1 (lateral part for the rhizome aged 2, and bulb part for the rhizome aged 3) for analysis.

Several hypotheses had been proposed, asserting that secondary metabolism was modified from nutrients, and thus was limited by substrate availability, such as carbon supply and energy [49]. This could explain why the flavonoid contents were low in the sprout primordium or the one-year-old rhizome, since the primordium needed more nutrients to develop and allocated less substrate to support secondary metabolism synthesis. Also, vulnerable tissues were defended more than old, and thus some young tissues often sequestered larger amounts of secondary metabolites than the old [49]. This might explain the higher flavonoid quantity in the bulb or lateral parts than in the vertical parts.

As shown in Fig. 7 and Table 6, sample 9 contained lower levels of mangiferin, tectoridin, iristectorin B, iridin, tectorigenin, iristectorigenin A, irigenin, and irisflorentin than those of the lateral samples of two-year old rhizomes. The microscopic studies described above had found that the fluorescence characteristics of sections of sample 9 were different. So the fluorescence characteristics of *B. chinensis* sections could be correlated with the distribution and accumulation of secondary metabolites, which was identical with the previous published reports [3,4,46].

Moreover, the distribution of the three hydrophilic flavonoid compounds, namely tectoridin, iristectorin B and iridin, detected within sample 2 and 3 revealed an interesting pattern: those compounds tended to accumulate in the cortex of the young rhizome but were stored in the center rather than the cortex of

the old rhizome. For example, tectoridin in the cortex and center portion of primordium of sample 2 was 313.03 and 117.34 ng per unit area respectively, then became 554.08 and 304.94 ng per unit area respectively in the lateral part, and finally transformed as 397.56 and 513.70 respectively in the vertical part. In other words, tectoridin tended to store more in the center of the vertical part than in the lateral part or primordium. The same pattern appeared with iristectorin B and iridin. Thus, it was reasonable to speculate that such flavonoid components might participate in growth or growth regulation.

Considering the flavonoid accumulation patterns and the size of developed rhizome, for maximum harvest, *B. chinensis* should be harvested at 3 years after seeding. This was identical with the traditional cultivation method. In terms of flavonoid content, sample 12, which was collected in late October, possessed the best quality as compared with other samples harvested during flowering. This further confirmed the wisdom of the traditional collected periods of late October to early November.

Multiple roots typically grow out from *B. chinensis* rhizome, and are conventionally excluded from medicinal use. To ascertain if these roots could be used medicinally, a root sample was prepared for determination. The results indicated that the root had less total content of flavoniods but with higher proportions of irigenin and iridin compared with the rhizome. This result confirmed the traditional practice of excluding roots, but suggested that they might possess some potential medicinal use different from the rhizome.

4. Conclusion

In this study, LMD, fluorescence microscopy, and UHPLC-Q/TOF-MS were applied to profile tissue-specific chemicals of the rhizomes of *B. chinensis* at different growth ages. UHPLC–QqQ-MS was further used to determine the contents of 8 flavonoid analytes. The methods established were precise, convenient and effective for assay. As a result, 43 signals were detected, and 26 flavonoid compounds combined with seven triterpene compounds were identified or tentatively identified through qualitative analyses. Meanwhile, flavonoid distribution patterns were revealed: the hydrophobic compounds, including flavonoid and isoflavonoid aglycones and xanthone were mainly distributed in the cork, while the hydrophilic compounds, namely the flavonoid and isoflavonoid glycosides usually accumulated in the cortex or center part. *B. chinensis* samples of different growth ages from different origins were compared. The bulb or lateral part of rhizome were found to generally possess more flavonoids than the vertical part or the primordium. In addition, medicinal organ of *B. chinensis* was explored by analyzing rhizome and root samples. The results indicated that the root might possess certain potential medicinal use different from the rhizome.

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