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Short communication

Simultaneous determination of eleven bioactive compounds in *Saururus chinensis* from different harvesting seasons by HPLC-DAD

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ABSTRACT

A high performance liquid chromatography method coupled with diode array detection (HPLC-DAD) was developed for simultaneous determination of five major active flavonoids, two aristolactams and four main lignans in *Saururus chinensis*, namely rutin (**1**), isoquercitrin (**2**), quercetin-3-O-β-D-glucopyranosyl (1 → 4)-α-L-rhamnoside (**3**), quercitrin (**4**), quercetin (**5**), aristolactam A II (**6**), sauristolactam (**7**), dihydroguaiaretic acid (**8**), sauchinone (**9**), licarin A (**10**) and licarin B (**11**). The analysis was performed on an Agilent Eclipse XDB C_{18} column (4.6 mm × 150 mm, 5 μm) with gradient elution of 0.4% aqueous phosphoric acid and acetonitrile. The detection wavelengths were 280 and 360 nm. All calibration curves showed good linearity ($r^2 > 0.9991$) within test ranges. The method was reproducible with intra- and inter-day variation less than 3.2%. The recovery of the assay was in the range of 95.1–103.9%. The validated method was successfully applied for the analysis of the eleven bioactive compounds in seven samples from different harvesting seasons and significant variations were revealed. The results indicated that the developed method can be used as a suitable quality control method for *S. chinensis* and it should be harvested in August (fruiting period) for Jiangsu cultivation regions, taking the yield into consideration, with the highest amounts of lignans, relative higher amounts of flavonoids and lower amounts of aristolactams.

1. Introduction

Saururus chinensis has been used as a diuretic and a detoxifying agent for the treatment of edema, jaundice, gonorrhea and several inflammatory diseases in China and Korea [1–3]. Previous phytochemical and pharmacological studies have demonstrated that flavonoids [4,5] and lignans [6–8] are the main bioactive compounds of *S. chinensis*, which possess a wide array of pharmacological and biochemical activities, such as antidiabetic [9], anti-carcinogenic [10,11], anti-inflammatory [12,13], antioxidant [7,9] and hepato-protective [14]. Aristolactams, the aristolochic acid derivatives, exhibited neuro-protective activity [15,16], nephrotoxic [17], carcinogenic [18], mutagenic [19] and have more or less cytotoxic effect [20]. A series of methods have been developed for the determination of *S. chinensis*, including coulometric titration [21], UV–vis spectrophotometry [22], high performance liquid chromatography (HPLC) [23–26] and capillary electrophoresis (CE) [27,28]. However, these methods suffered from low resolution, low sensitivity or few analytes (less than five analytes).

It is well known that the therapeutic effects of traditional Chinese medicines (TCMs) are usually attributed to multiple bioactive compounds [29,30]. Therefore, simultaneous determination of flavonoids, aristolactams and lignans in *S. chinensis* is required for the evaluation of its quality and the control of dosage during clinical studies. Moreover, it was demonstrated that herbs collected from different harvesting seasons are discrepant in the types and quantities of chemical constituents, which influence their therapeutic effects significantly [31,32]. However, Pharmacopoeia of the People's Republic of China (vol. I, 2005) specified *S. chinensis* can be harvested in every season [1]. *S. chinensis* is a perennial plant and the aerial parts will be withered in a vegetative cycle. Thus, it is essential to study the harvesting seasons of *S. chinensis*.

In this study, a reliable HPLC-DAD method was developed for simultaneous determination of five major active flavonoids, two aristolactams and four main lignans in seven *S. chinensis* samples collected from different harvesting seasons. The validated method can be used as a valid analytical method for intrinsic quality control of *S. chinensis*. The quantitative analysis results may be helpful for choosing the best harvesting seasons of *S. chinensis*.

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2. Experimental

2.1. Chemicals, reagents and materials

Seven samples of *S. chinensis* (S1–S7) from different harvesting seasons were collected on the 10th of every month from May to November in 2008 from the same field of Nanjing, Jiangsu province. The botanical origin of materials was identified by Jian-wei Chen, Professor of Pharmacognosy, Nanjing University of Chinese Medicine. Voucher specimens were deposited at Herbarium of Nanjing University of Chinese Medicine. Standard compounds were isolated from the aerial parts of *S. chinensis*. Their structures were confirmed based on spectroscopic analysis (^{1}H NMR, ^{13}C NMR and ESI-MS). The purity of each compound was more than 98% detected by HPLC-DAD. Their structures are shown in Fig. 1. Acetonitrile was of HPLC grade from Tedia Company (United Stated of America) and deionized water was purified by EPED superpurification system (Eped, Nanjing, China). Other reagent solutions were of analytical grade from Beijing Reagent Company (Beijing, PR China).

2.2. HPLC-DAD analysis

The analyses were performed on an Agilent 1200 liquid chromatograph system (Agilent Technologies, Palo Alto, CA, USA), equipped with a double pump and a DAD detector. The separations were carried out on an Agilent Eclipse XDB C_{18} column (4.6 mm × 150 mm, 5 μm). The column temperature was set at 25 °C. The mobile phases consisted of 0.4% aqueous phosphoric acid (A) and acetonitrile (B). The gradient condition was: 15% B (v/v) at 0–16 min, 15–25% B at 16–30 min, 25% B at 30–32 min, 25–30% B at 32–35 min, 30% B at 35–37 min, 30–33% B at 37–40 min, 33% B at 40–45 min, 33–48% B at 45–55 min, 48–55% B at 55–75 min, 55–80% B at 75–82 min, 80% B at 82–88 min and the re-equilibration time of gradient elution was 15 min. The flow rate was 0.8 ml/min and the injection volume was 10 μl. Detection wavelength was set at 360 nm for analytes **1**, **2**, **3**, **4** and **5**, and 280 nm for analytes **6**, **7**, **8**, **9**, **10** and **11**. The absorption spectra of compounds were recorded between 200 and 400 nm. The compounds were identified by comparing their retention times and UV spectra with those of the markers. Purity angles of all analytes did not exceed the purity

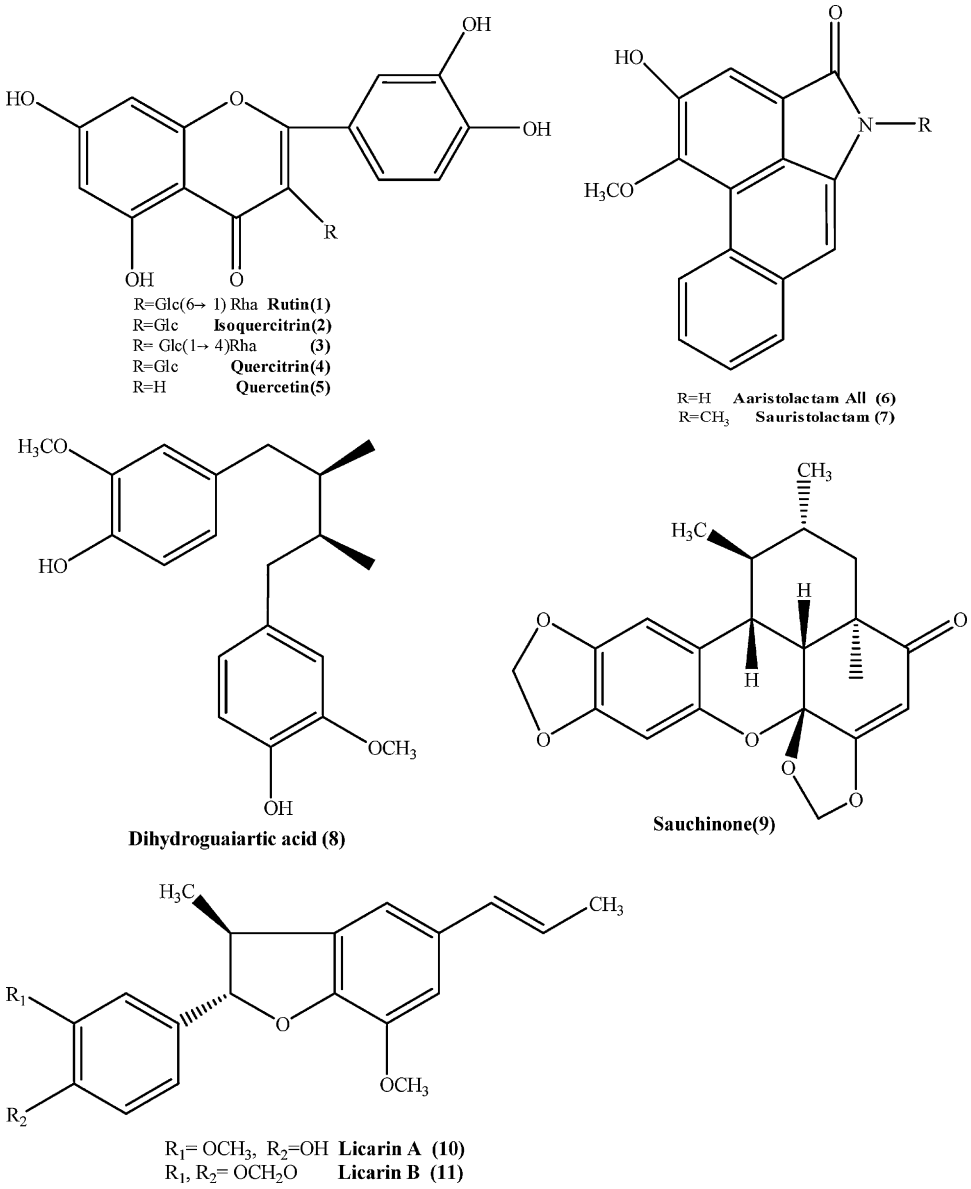

Fig. 1. Structures of eleven reference compounds.

thresholds, in addition, spiking samples with the reference compounds showed no additional peaks. Thus, the purity of each peak in chromatography was well.

2.3. Preparation of standard solutions

Mixed standard stock solution containing rutin (**1**), isoquercitrin (**2**), quercetin-3-O-β-D-glucopyranosyl-(1 → 4)-α-L-rhamnoside (**3**), quercitrin (**4**), quercetin (**5**), aristolactam AII (**6**), sauristolactam (**7**), dihydroguaiaretic acid (**8**), sauchinone (**9**), licarin A (**10**) and licarin B (**11**) was prepared in 80% methanol. Working standard solutions were prepared by diluting the mixed standard solution with 80% methanol to give different concentrations for calibration curves. The solutions were filtered through a 0.45-μm membrane prior to injection.

2.4. Preparation of sample solutions

The accurately weighed powder (0.5 g, 40-mesh) was extracted with 25 ml of 80% methanol in an ultrasonic bath for 30 min (250 W, 40 kHz). The solution was filtered through filter paper and residue was washed with 20 ml of 80% methanol for twice. All the solutions were combined and evaporated to dryness in a rotary evaporator. The extract was dissolved in 5 ml of 80% methanol in a volumetric flask. The resulting solution was filtered through a 0.45-μm membrane before injected into HPLC system for analysis.

3. Results and discussion

3.1. Optimization of extraction procedure

In order to obtain satisfactory extraction efficiency, extraction method, extraction solvent and extraction time were investigated. Ultrasonication for 30 min was proven to give a similar efficient extraction as refluxed for 2 h. Different solvents including, methanol–water (40:60, v/v; 60:40, v/v; 80:20, v/v) and methanol were screened. Methanol–water (80:20, v/v) exhibited complete extraction of all the major constituents.

3.2. Optimization of chromatographic conditions

Various compositions of mobile phase were tried: methanol–0.4% phosphoric acid, methanol–0.1% formic acid and acetonitrile–0.4% phosphoric acid. As a result, 0.4% phosphoric acid–acetonitrile in the gradient mode was chosen to give the desired separation and acceptable tailing factor. Furthermore, other chromatographic variables were also optimized, including analytical columns (Agilent Eclipse XDB C_{18} and Agilent TC-C_{18}), column temperatures (25 and 30 °C) and flow rates (0.8 and 1.0 ml/min). Eventually, the optimal separation was achieved on an Agilent Eclipse XDB C_{18} column (150 mm × 4.6 mm, 5 μm) at a column temperature of 25 °C with a flow rate of 0.8 ml/min. As the three types of compounds showed different UV absorption properties, different detection wavelengths were simultaneously set to monitor these compounds in a single run, viz. 360 nm for analytes **1**, **2**, **3**, **4** and **5**, and 280 nm for **6**, **7**, **8**, **9**, **10** and **11**.

3.3. Validation of the method

3.3.1. Calibration curves, limits of detection and quantification

Standard stock solutions containing the eleven analytes were prepared and diluted to appropriate concentrations for plotting the calibration curves. At least six concentrations of the 11 analytes solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The limit of detection (LOD) and the limit of

Table 1
Linear regression data, LOD and LOQ of 11 analytes.

Analyte[a]	Regression equation[b]	Linear range (μg/ml)	r^2	LOD (μg/ml)	LOQ (μg/ml)
1	$y = 21.1620x + 14.9820$	1.93–192.8	0.9998	0.12	0.39
2	$y = 17.7320x + 1.7941$	1.26–252.5	0.9991	0.09	0.31
3	$y = 14.7500x + 10.0830$	2.70–270.0	0.9998	0.06	0.19
4	$y = 21.3220x - 5.5197$	0.86–216.0	0.9997	0.04	0.13
5	$y = 17.7676x - 13.4560$	1.11–22.10	0.9994	0.05	0.17
6	$y = 66.6416x - 14.5762$	1.51–30.20	0.9999	0.02	0.07
7	$y = 56.0873x - 12.6819$	1.64–32.80	0.9998	0.02	0.06
8	$y = 10.1164x - 19.1045$	5.52–114.0	0.9997	0.12	0.41
9	$y = 4.8744x - 6.1388$	13.70–548.0	0.9999	0.15	0.49
10	$y = 25.1933x - 33.1534$	5.03–100.6	0.9993	0.05	0.18
11	$y = 25.6630x - 4.6347$	1.52–62.64	0.9999	0.03	0.08

[a] The notation for analyte refers to Fig. 1.
[b] y is the peak area, x is the concentration injected.

Table 2
Precision, repeatability, stability and recovery of 11 analytes.

Analyte[a]	Precision (R.S.D., %)		Repeatability (R.S.D., %, $n = 6$)	Stability (R.S.D., %, $n = 7$)	Recovery (%, $n = 3$)	
	Intra-day ($n = 6$)	Inter-day ($n = 3$)			Mean	R.S.D.,%
1	0.42	1.78	2.49	1.93	100.1	2.78
2	0.21	2.07	2.13	2.41	99.6	2.14
3	0.17	1.93	1.78	2.03	99.3	2.49
4	0.28	1.21	2.54	2.52	100.5	3.17
5	0.36	1.74	2.97	2.64	102.1	4.42
6	0.44	2.14	3.06	2.17	103.9	2.86
7	0.37	2.06	2.81	2.56	99.7	3.21
8	0.29	2.63	1.93	3.01	96.1	2.05
9	0.23	2.41	1.89	1.69	102.3	1.92
10	1.18	3.18	2.81	4.11	95.1	3.62
11	0.71	2.31	2.72	4.27	96.2	2.91

[a] The notation for analyte refers to Fig. 1.

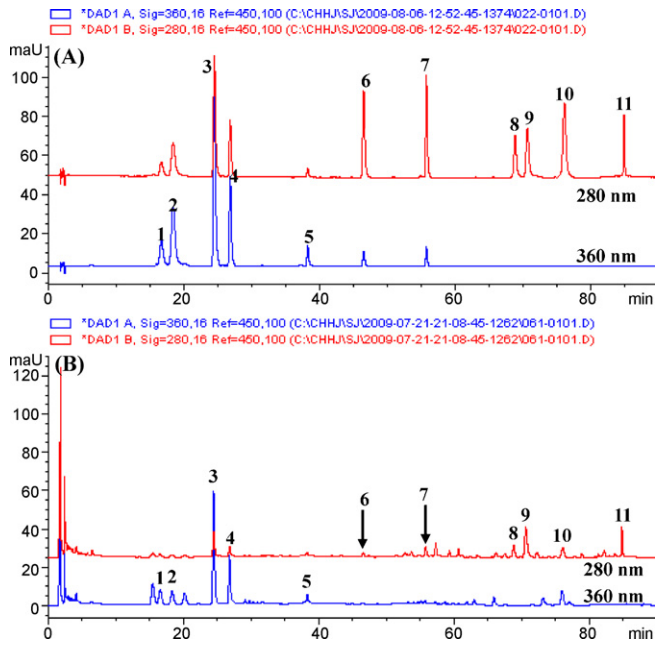

Fig. 2. Typical HPLC chromatograms of (A) mixed standards and (B) *Saururus chinensis*. Rutin (**1**), isoquercitrin (**2**), quercetin-3-O-β-D-glucopyranosyl(1 → 4)-α-L-rhamnoside (**3**), quercitrin (**4**), quercetin (**5**), aristolactam A II (**6**), sauristolactam (**7**), dihydroguaiaretic acid (**8**), sauchinone (**9**), licarin A (**10**) and licarin B (**11**).

quantification (LOQ) were determined at a signal-to-noise ratio of about 3 and 10, respectively. The calculated results are summarized in Table 1. All the analytes showed good linearity ($r^2 > 0.9991$) in a wide concentration range. The LOD and LOQ of the eleven analytes were 0.02–0.15 and 0.06–0.49 μg/ml, respectively.

3.3.2. Precision, repeatability and stability

The intra- and inter-day precisions were investigated by determining a mixed standard solution in six replicates during a single day and by duplicating the experiments on 3 consecutive days. To further evaluate the repeatability of the developed assay, *S. chinensis* was analyzed in six replicates with the above established method. Stability of sample solution was analyzed at 0, 2, 4, 8, 12, 24 and 48 h within 2 days at room temperature, respectively. Variations were expressed by relative standard deviations (R.S.D.). All the results were shown in Table 2, indicating that the intra and inter-day, repeatability and stability R.S.D. values of the eleven compounds were all less than 4.11%.

3.3.3. Accuracy

A recovery test was used to evaluate the accuracy of the method. The recoveries were determined by spiking accurately known amounts of the eleven analytes solution to approximately 0.25 g of the *S. chinensis* and then extracted and analyzed with the described method. The results were shown in Table 2. The recovery of the method was in the range of 95.1–103.9%, with R.S.D. less than 4.42%, indicating that the established method was accurate for the determination of eleven bioactive compounds in *S. chinensis*.

3.4. Sample analysis

The established method was subsequently applied to the simultaneous determination of the investigated compounds in *S. chinensis* from different harvesting seasons. Each sample was analyzed in triplicate to determine the mean content (mg/g) and the results were tabulated in Table 3. Representative HPLC-DAD chromatograms of standards and samples were shown in Fig. 2.

Table 3
Contents (mg/g) of 11 analytes in *Saururus chinensis* from different harvesting seasons.

Samples	Harvesting month	Analyte[a]											Total
		1	2	3	4	5	6	7	8	9	10	11	
S1	May	2.31 ± 0.01	2.03 ± 0.02	2.44 ± 0.02	1.92 ± 0.05	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.01	0.67 ± 0.01	3.18 ± 0.04	0.25 ± 0.00	0.28 ± 0.00	13.25
S2	June	0.44 ± 0.01	0.25 ± 0.01	1.51 ± 0.02	0.41 ± 0.01	0.07 ± 0.00	0.04 ± 0.00	0.10 ± 0.01	0.96 ± 0.01	3.28 ± 0.07	0.25 ± 0.01	0.32 ± 0.01	7.63
S3	July	0.55 ± 0.03	0.30 ± 0.01	1.17 ± 0.04	0.26 ± 0.02	0.07 ± 0.01	0.12 ± 0.00	0.11 ± 0.01	1.06 ± 0.05	4.05 ± 0.18	0.25 ± 0.01	0.37 ± 0.04	8.31
S4	August	0.90 ± 0.04	0.33 ± 0.01	1.43 ± 0.05	0.28 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.09 ± 0.00	1.08 ± 0.04	3.70 ± 0.16	0.29 ± 0.02	0.43 ± 0.02	8.65
S5	September	0.35 ± 0.01	0.11 ± 0.00	0.62 ± 0.02	0.12 ± 0.00	0.06 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.55 ± 0.03	1.99 ± 0.01	0.18 ± 0.01	0.23 ± 0.02	4.47
S6	October	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	tr	0.04 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.13 ± 0.01	0.75 ± 0.03	0.04 ± 0.00	0.04 ± 0.01	1.20
S7	November	–	–	–	–	–	0.04 ± 0.00	0.06 ± 0.00	0.09 ± 0.01	0.67 ± 0.03	0.04 ± 0.00	0.02 ± 0.00	0.92

"–": below the LOD. "tr": below the linear range of calibration.
[a] The notation for analyte refers to Fig. 1.

Table 3 shows that the amount of the analyzed compounds varies with the harvesting seasons remarkably, especially the four flavonoid glycosides (FGs), namely analytes **1**–**4**. Total flavonoids were at the highest content in May (seedling stage) and decreased significantly from May to June (from 8.76 to 2.68 mg/g). In August (fruiting period), total flavonoids reached another peak (3.00 mg/g). Aristolactams, namely analytes **6** and **7**, varied with harvesting seasons not as significantly as FGs and the contents were in the range of 0.04–0.13 mg/g. Among the analyzed compounds, the content of sauchinone was the highest (0.67–4.05 mg/g). It accumulated at the highest amount between July and August. At the same time, another three lignans, namely analytes **8**, **10** and **11**, all accumulated at their highest amounts in August, with relative higher amounts of flavonoids and lower amounts of aristolactams. After September, the contents of all analytes decreased significantly, even cannot be detected. The total amounts of the eleven analyzed compounds accumulated at the highest amounts in May and relative higher amounts in August. These results indicated that *S. chinensis* should be harvested in August (fruiting period) for Jiangsu cultivation regions, taking the yield into consideration.

4. Conclusion

A simple and accurate method was developed for simultaneous determination of eleven bioactive constituents in *S. chinensis* from seven different harvesting seasons. This was the first report on the simultaneous quantification of eleven bioactive constituents in *S. chinensis*. The developed method can be used as a reliable method for intrinsic quality control of *S. chinensis* and *S. chinensis* should be harvested in August (fruiting period) for Jiangsu cultivation regions, taking the yield into consideration.

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