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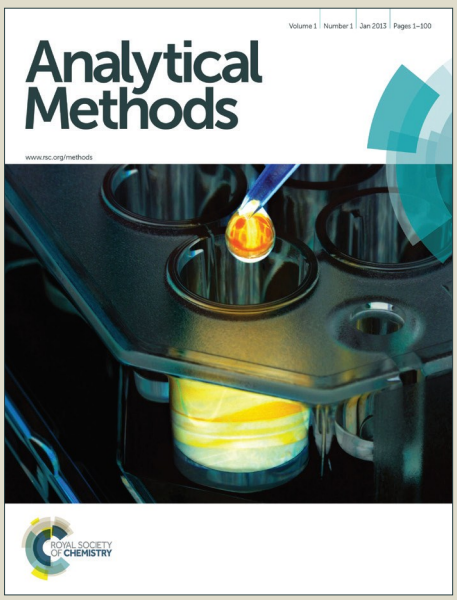

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Simultaneous Quantification of seventeen Bioactive Components in Rhizome and Aerial Parts of *Alpinia officinarum* Hance Sampled at Different Growing Periods Using Liquid Chromatography/Quadrupole Tandem Mass Spectrometry

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ABSTRACT

The rhizomes of *Alpinia officinarum* Hance (Zingiberaceae family) have been used as antiemetics, stomachics and analgesics in Asia for centuries. Unfortunately, the aerial parts were thrown away as wastes whilst harvesting the rhizomes of *A. officinarum*. Recently, scientists reported that the ethanol extract of aerial parts displayed anti-proliferation activity through mitochondrial pathway-induced cell apoptosis. However, the chemical composition information of this extract remained largely unknown. We have identified sixteen chemicals including twelve flavonoids and four diarylheptanoids from the methanol extraction of *A. officinarum* leaves using liquid chromatography/tandem mass spectrometry (LC-MS/MS). In order to better explore the potential value of the aerial parts, we need to know what the main constituents occurring in the aerial parts and how the contents of these chemicals are influenced by the growing periods. In the present study, a LC-MS/MS method was developed and validated for determination of seventeen compounds both occurring in the aerial parts and rhizomes sampled at different growing periods. Validation indices evaluated were satisfactory and the method was successfully employed to analyze the above-mentioned plant samples. Notably, we found that the contents of these compounds except for quercetin were higher in rhizomes than those of compounds in aerial parts. The six major constituents both in aerial parts and rhizomes were galangin, kaempferide, hexahydrocurcumin, pinocembrin, chrysin and isorhamnetin. Moreover, the content changes' trends of most of the monitored phytochemicals along with sampled periods were almost similar between the aerial parts and the rhizomes.

Our study should be of value in arousing everyone's interest to make the best use of aerial parts of *A. officinarum* in the future.

Keywords: *Alpinia officinarum* Hance; rhizomes and aerial parts; harvest times; LC-MS/MS

Introduction

Plant secondary metabolites are critical for not only the function and value of the compounds within the plants themselves, but also the relationship involves biological interaction of plants with other organisms and with their environment [1]. A plant that is able to synthesize some compounds that can disturb the physiological functions of an herbivore may have a selective advantage over one that does not. The secondary metabolites may be utilized to establish interlocking relationship within the contexts of pollination, seed dispersal or protection of the plant by another organism. Generally speaking, the secondary chemicals are biosynthesized, accumulated under certain conditions and then transported within the plant to a site of storage or are deposited on the surface [2]. From an evolving point of view, it appears that these products are often concentrated in the most vulnerable tissues.

Alpinia officinarum Hance (Zingiberaceae family), known as lesser galangal, is a famous traditional herb and mainly distributed in the Southern China such as Guangdong province and Hainan Island. *A. officinarum* rhizomes that used as medicinal parts have been used as antiemetics, stomachics and analgesics in Asia for centuries [3]. Recently, a review article of our group has summarized the advances in studies on chemical constituents in *A. officinarum* rhizomes and their pharmacological activities [4]. Some bioactive components of rhizomes have been listed as essential oil, flavonoids, diarylheptanoids, phenylpropanoids, glycosides and other constituents in this article. Especially, the flavonoids such as galangin and diarylheptanoids are the main constituents and have exhibited various pharmacological activities including

antimicrobial, antiviral, antitumor, antioxidant roles, gastric ulcer protective activities and usually used as a hemostat for treating gastrointestinal hemorrhages. Therefore, flavonoids and diarylheptanoids are always used as marker compounds for quality control of *A. officinarum* rhizomes and its extracts and some Chinese traditional patent medicine.

Unlike the rhizome, the aerial parts of *A. officinarum* are not widely concerned. Zhang *et al.* identified five flavonoids including galangin, 3-O-methylgalangin, pinocembrin, pinobaksin and kaempferide from the ethanol extract of the aerial parts [5]. A Chinese patent (CN104138368A) provided a process for producing a purified extract (AO-95) from the aerial parts by ethanol extraction and subsequent purification via macro-porous adsorptive resins. This AO-95 extract displayed anti-proliferation activity through mitochondrial pathway-induced cell apoptosis. However, the chemical composition information of this extract was not provided in this patent. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) technique has been used to biological molecules structure determination[6-9]. Recently, we identified sixteen chemicals including twelve flavonoids and four diarylheptanoids from the methanol extraction of *A. officinarum* leaves using LC-MS/MS~~)~~ with selected reaction monitoring mode[10]. Twelve flavonoids included chrysin, pinocembrin, tectochrysin, apigenin, galangin, 3-O-methylgalangin, acacetin, kaempferol, kaempferide, quercetin, isorhamnetin and rutin. Four diarylheptanoids were yakuchinone A, oxyphyllacinol, hexahydrocurcumin and hannokinol. These secondary metabolites may contribute to the above-mentioned anti-cancer activity.

Therefore, the aerial part of *A. officinarum* has potential to be used as the medicinal part in the future.

In order to better explore the potential value of the aerial parts, we need to know what the main constituents occurring in the aerial parts and how the contents of these chemicals are influenced by the growing periods. Furthermore, as time goes on, the transportation of these secondary metabolites from aerial parts to rhizomes is also need to be characterized. In the present study, seventeen compounds both occurring in the aerial parts and rhizomes sampled at different growing periods were monitored and quantified using LC-MS/MS. Notably, we found that the content changes' trends of most of the target phytochemicals along with sampled periods were almost similar between aerial parts and rhizomes.

Material and Methods

Chemical and Reagents

Reference standard of nootkatone was obtained from Sigma-Aldrich (St Louis, MO, USA). Yakuchinone A was purchased from Chenfun Medical Technology (Shanghai) Co., Ltd. (Shanghai, China). Hexahydrocurcumin and Hannokinol were purchased from BioBioPha Co., Ltd (Kunming, China). Apigenin and pinocembrin were obtained from Shanghai YuanYe Bio-Technology Co., Ltd (Shanghai, China). Acacetin was bought from Nanjing Zelang Pha Co. Ltd (Nanjing, China). Galangin, rutin, quercetin, kaempferol, luteolin and isorhamnetin were purchased from National Institutes for Food and Drug Control (Beijing, China). Tectochrysin, izalpinin, chrysin and kaempferide were separated from *A. oxyphylla* fruits. Diarylheptanoid was separated and prepared from the rhizomes of *A. officinarum*. The purities of these reference standards were over 98.0%. HPLC-grade methanol and acetonitrile were products of Merck (Darmstadt, Germany). HPLC-grade formic acid was purchased from Aladdin Industrial Inc. (Shanghai, China). HPLC-grade water was prepared with a Millipore Milli-Q Integral 3 cabinet water purifying system (Bedford, MA, USA). The other chemical reagents, analytical grade or better, were obtained from Hainan YiGao Instrument Co., Ltd (Haikou, China). The chemical structures of 18 plant secondary metabolites are shown in Figure 1.

(Insert Figure 1 here)

The aerial parts and rhizomes of 3-year-old *A. officinarum* were collected from

the planting base of Hainan Lin-Feng-Yuan Industrial Co., Ltd. The sampling periods and corresponding serial numbers are shown in Figure 2.

(Insert Figure 2 here)

Sample Preparation for Analysis

In order to perform the determinations on these plant secondary metabolites, we developed an extraction protocol specific for these plant samples. All the rhizomes and aerial parts of *A. officinarum* were dried in an electric drying oven (6CH-18, Xingmin Tea Machinery Co., Ltd., Anxi, China) at 40°C until dry. The freshly dried plant samples were manually shucked into little segments, which were smashed using a smashing machine (FW100, Taisite Instrument, Tianjin, China) and then sieved manually by a 60 mesh. The resulting fine powders and residue were mixed evenly. An aliquot (0.5 g) was weighed precisely and macerated with 50-fold methanol and then ultrasonicated three times for 30 min each. For each extraction, the resulting extract solutions were centrifuged at 13000 rpm for 10 min (Kubota 5922, Kubota Corporation, Tokyo, Japan). 1 mL of supernatant was sampled and the remaining was discarded. The residue was extracted with methanol for another 2 times. The sampled methanol extracts (3 mL) were combined and centrifuged at 13000 rpm for 10 min to obtain the supernatant fractions that were frozen at –20ºC until analysis. The extract solution was appropriately diluted with methanol before analysis. Finally, a 10 μL aliquot was injected into the LC-MS/MS system for quantitative analysis.

Instrumentation and LC-MS/MS Conditions

A component LC-MS/MS system consisted of an API 4000 plus mass spectrometer (AB-SCIEX, Toronto, Canada) interfaced via a Turbo V ion source with a Shimadzu Prominence UFLC chromatographic system (Shimadzu Corporation, Kyoto, Japan) and operated with AB-SCIEX Analyst software. The UFLC system is equipped with two LC-20AD pumps, a model DGU-20A_{3R} degasser unit, a SIL-20A HT auto-sampler and a CTO-20A column oven.

Chromatographic separation was achieved using a Phenomenex Kinetex 2.6 μ XB-C18 (2.10 mm i.d. × 50 mm) [11]. The LC mobile phase at a flow-rate of 0.3 mL·min^{-1} consisted of 0.1‰ formic acid in water (A) and methanol (B) using a gradient elution as follows: from 0% B to 2% B in 0.01 min, hold for 1 min; from 2% B to 35% B in 0.01 min, hold for 3 min; from 35% B to 90% B in 11 min; back to 2% B in 0.01 min; maintain 4.99 min. The temperature of the column was controlled at 40ºC. A 0.5-μm biocompatible inline filter (Upchurch Scientific, Oak Harbor, WA, USA) was used before the chromatographic column.

The parameters of the electrospray ionization (ESI) source operating in positive ionization mode were as follows: collision gas flow (N_2): level 4; curtain gas (N2): 25 psi; nebulizer gas (N_2, Gas I): 55 psi; heated dry gas (N_2, Gas II): 55 psi; IonSpray voltage (v): +5,500 v and dry temperature (TEM): 550 ºC. MS/MS operating conditions were optimized by infusion of the standard solution (1 μg·mL^{-1}) of each analytes into the ESI source via a syringe pump (11plus, Harvard Apparatus, Holliston, MA, USA). Quantification was performed using multiple reaction-monitoring (MRM) modes for the following transitions. The MRMs of nootkatone, yakuchinone A,

tectochrysin, izalpinin, chrysin, kaempferide, apigenin-4',7-dimethylther, kaempferol, galangin, hannokinol, hexahydrocurcumin, pinocembrin, isorhamnetin, luteolin, rutin, apigenin, acacetin and quercetin were *m/z* 219.2→163.0 (the optimal collision energy, 22 V), 313.2→136.9 (13 V), 269.1→226.0 (43.5 V), 285.0→242.0 (43 V), 255.1→152.9 (42 V), 301.1→286.0 (37 V), 299.2→256.0 (45 V), 287.2→153.0 (45 V), 271.2→153.0 (43.8 V), 313.8→256.3 (31.5 V), 375.2→357.2 (9 V), 257.1→153.0 (30.5 V), 317.2→302.2 (35.2 V), 287.1→153.0 (45.1 V), 611.2→303.0 (25.4 V), 271.1→153.0 (42.8 V), 285.1→242.0 (46.5 V) and 303.1→153.0 (47 V), respectively, with a scan time of 20 ms for each ion pair. The product ion spectra of protonated molecules are shown in Figure 3.

(Insert Figure 3 here)

Validation of Analytical Method

The quantitative LC-MS/MS method was validated with respect to linearity, recovery, precision and sensitivity. Twelve stock solutions containing about 1 $mg \cdot mL^{-1}$ of nootkatone, yakuchinone A, chrysin, kaempferol, galangin, hannokinol, hexahydrocurcumin, pinocembrin, luteolin, rutin, quercetin and diarylheptanoid were prepared independently in methanol excluding hexahydrocurcumin, which was dissolved in acetonitrile. Kaempferide, tectochrysin, izalpinin, apigenin, isorhamnetin and acacetin were prepared in methanol and the concentration of standards was 1.41, 1.035, 0.5, 0.5, 0.3 and 0.332 $mg \cdot mL^{-1}$, respectively.

For calibration aims, working solutions were freshly prepared via diluting each stock solution with methanol or acetonitrile. Calibration curves were established on

six data points covering the designed concentration such as 2-2000 ng·mL^{-1}. Each calibration curve was obtained by plotting the peak area *vs* the concentration of the standard. The linearity was assessed by calculation of a regression line using the least squares method. The limits of detection (LOD) and quantification (LOQ) were evaluated by analysis of the peak height *vs* the baseline noise at a signal-to-noise ratio of 3:1 and 10:1, respectively. Reliabilities of the extraction method were evaluated by adding reference standards (25 ng for ***C-02***, ***C-12*** and 50 ng for the other compounds) into the powdered plant material of the two selected rhizomes and aerial parts samples, and the recovery of the added standards was measured. Precision was assessed by the evaluation of the repeatability (intra-day precision) and by intermediate precision (inter-day precision). The intra-day precision was determined by analyzing six replicate plant samples in a day, which was performed by the same technician. The inter-day precision was obtained through measuring the above mentioned six plant samples in three consecutive days and by two different analysts.

Results and Discussion

Selection of the Extraction Method

Plant samples were extracted using published method [11-13] by our group with a slight modification. In this study, we chose methanol as the extraction solvent and sonication procedure for the extraction of the target constituents. Sonication combined with methanol extraction provides mechanical disruption and thereby aiding in extraction. In order to control the uniformity of sonication procedure, fresh running water was added into the bath of the ultrasonic extraction device (40 KHz, 80 W, Kunshan Ultrasonic Instruments, Kunshan, China) for each extraction. After having completed the third sonication extraction, the ultrasonic device was kept on standby for 1 hour in case of overheats. Our results revealed that the recoveries of the 17 plant secondary metabolites were more than 85% by single extraction and almost 100% through triple extraction for aerial parts and rhizomes samples. Therefore, the sample was macerated with 50-fold methanol and then ultrasonicated three times for 30 min each.

Method Validation

Representative chromatograms for 17 reference standards and aerial parts samples, as well as rhizomes samples of *A. officinarum*, are shown in Figure 4. No interfering peaks were noted and good resolution was achieved among the monitored chemicals, which were mainly eluted within 7.5-14 min except for hannokinol (retention time 7.07 min, data not shown) during our 20-min gradient program. Exogenous chemical coming from plastic consumables such as inserts tubes, pipette tips interfered with

hannokinol analysis. We failed to overcome this interference because we had to apply various plastic products during our assay procedure.

(Insert Figure 4 here)

Standard curves were linear over the dynamic ranges with correlation coefficients greater than 0.99 (see Table 1). The LODs and LOQs were in the range of 0.0141–2 ng·mL^{-1} and 0.1–10 ng·mL^{-1}, respectively. Mean recovery (standard addition approach) of each target compound from aerial parts samples and rhizomes samples exceeded 85.5% (range = 85.5-112%, Table 1), indicating that various plant background matrices had little effect on the quantification analysis. For this standard addition approach, the accurate amounts of the standards are added to real samples, in which the concentration of target analyte has been determined, and then extracted and analyzed. The average percentage recoveries are evaluated by calculating the ratio of detected amount versus added amount. Actually, standard addition approach was used to analyze pharmaceutical residues in environmental samples [14], diarrheic shellfish poisoning toxins [15] and pharmaceutical residues in drinking water [16].

As shown in Table 1, the RSD values of intra- and inter-day variations of the seventeen secondary metabolites occurring in aerial parts and rhizomes were almost less than 10%. Overall, these results indicated that the established method was accurate and reliable.

(Insert Table 1 here)

Quantitative Assay of 17 Constituents occurring in the Aerial parts and

Rhizomes of *A. officinarum*

The content levels of the 17 constituents are summarized in Figure 5a and 5b. Besides quercetin (***C-12***), the content levels of the sixteen monitored compounds in rhizomes were 1.26 – 14.0 times higher than those of in aerial parts. The rank order of the best six compound for their content differences was as follows: kaempferide (***C-18***, 14 times) > izalpinin (***C-16***, 10.9 times) > diarylheptanoid (***C-03***, 7.47 times) > hexahydrocurcumin (***C-05***, 7.17 times) > nootkatone (***C-01***, 6.57 times) > galangin (***C-06***, 6.08 times). For quercetin, its concentration in aerial parts was 1.42 fold higher than that of in rhizomes. The six major constituents both in aerial parts and rhizomes were the same, *i.e.*, galangin (***C-06***) > kaempferide (***C-18***) > hexahydrocurcumin (***C-05***) > pinocembrin (***C-07***) > chrysin (***C-14***) > isorhamnetin (***C-08***). Their highest content levels were larger than 100 $\mu g \cdot g^{-1}$. Of particular, the highest content levels of galangin (***C-06***) were 17.7 $mg \cdot g^{-1}$ (1.77%) in rhizomes and 2.92 $mg \cdot g^{-1}$ (0.29%) in aerial parts, respectively. Similarly, kaempferide and hexahydrocurcumin in rhizomes were 5.11 $mg \cdot g^{-1}$ (0.51%) and 1.29 $mg \cdot g^{-1}$ (0.13%), respectively. Zhai *et al.* measured the active flavonoids including galangin, kaempferide, quercetin and isorhamnetin of *A. officinarum* rhizomes from 19 different localities of Hainan province [17]. The content of galangin and kaempferide was at 0.07-1.56% and 0.06-0.64%, respectively. Therefore, galangin was the predominant constituent both in aerial parts and rhizomes of *A. officinarum*.

(Insert Figure 5a and 5b here)

As shown in Figure 5a and 5b, the contents of the seventeen constituents

occurring in aerial parts and rhizomes changed obviously with different growing periods (from 19 February to 31 August). The differences of these phytochemicals covered 1.96-61.5 and 1.93-116 times for rhizomes and aerial parts, respectively. The content of apigenin (***C-11***) in rhizomes sampled at 19 February (***S-01***) was 61.5 times higher than that at 30 May (***S-07***). Similarly, the content of chrysin (***C-14***) in aerial parts sampled at August 16 (***S-11***) was 116 fold higher than that at 15 June (***S-08***). The content variability of the best six phytochemicals along with different growth time both in rhizomes and aerial parts samples was as follows: galangin (***C-06***, 6.84/3.74), kaempferide (***C-18***, 3.37/2.71), hexahydrocurcumin (***C-05***, 14.2/37.4), pinocembrin (***C-07***, 5.01/2.45), chrysin (***C-14***, 58.9/166) and isorhamnetin (***C-08***, 3.22/2.43). Of particular, the contents of these five major flavonoids except for chrysin in rhizomes maintained at a relatively higher level during the period of 15 June –15 July and amounted to highest levels at 31 August. As for galangin, Deng *et al.* determined its content in rhizomes of *A. officinarum* harvested in different months using a reversed-phase HPLC method [18]. The content levels ranged from 0.74% (May) to 1.39% (October). During the period of July to October, the content was relatively stable ranging from 1.06% to 1.39%. Our results revealed that the content variability of galangin in rhizomes covered 0.26-1.77%. On the other hand, the content levels of galangin in aerial parts changed from 0.08% to 0.29%. Therefore, one must fully consider the impact of harvest time on the contents of phytochemicals. The Chinese Pharmacopeia recommends that the best harvest period for rhizomes is in late summer and early autumn (*i.e.*, August and September) [19]. This recommendation looks like

reasonable based on our results.

As shown in Figure 5a and 5b, the content changes of the seventeen phytochemicals with growth time were almost similar between aerial parts and rhizomes. The content-time curves presented two types, "dumbbell" or "parallel bars". For the "dumbbell" type curves, such as yakuchinone a (***C-02***), hexahydrocurcumin (***C-05***), luteolin (***C-09***), apigenin (***C-11***), acacetin (***C-13***), chrysin (***C-14***) and kaempferol (***C-17***), the contents during the period of 14 April -15 July retained at relatively low levels. This period looked like a handle of the dumbbell and the handle thickness was different for this type phytochemicals indicating the content variability among aerial parts or rhizomes samples. On the other hand, for the "parallel bars" type chemicals, including nootkatone (***C-01***), diarylheptanoid (***C-03***), galangin (***C-06***), pinocembrin (***C-07***), isorhamnetin (***C-08***), and so on, the contents both in aerial parts and rhizomes had smaller fluctuation from 19 February (***S-01***) to 31 August (***S-12***). Currently, it is still unclear how to explain the content variations.

The rhizomes of *A. officinarum* are utilized as medical parts for traditional medicine, such as stimulant and carminative. It is especially useful in flatulence, dyspepsia, vomiting and sickness at stomach, being recommended as a remedy for sea-sickness, sometimes for fever or as a stimulant. In addition, galangal is used in cattle medicine [20]. Unfortunately, the aerial parts were thrown away as wastes whilst harvesting the rhizomes of *A. officinarum*. Like rhizome, the aerial parts contain multiple phytochemicals such as flavonoids (*e.g.* galangin), diarylheptanoids (*e.g.* hexahydrocurcumin) and sesquiterpenes (*e.g.* nootkatone) although their contents are

relatively lower than those of rhizomes based on our results. In addition, the yield of aerial parts of *A. officinarum* was comparable or less than that of rhizomes. Therefore, additional work is required to assess the bioactive properties of the aerial parts and make the best use of these valuable medicinal plant resources.

In summary, in this study we reported the development and validation of a LC-MS/MS and successfully employed this method to analyze the seventeen phytochemicals present in aerial parts and rhizomes of *A. officinarum*. Our results revealed that (1) validation indices evaluated were satisfactory; (2) the target seventeen phytochemicals were measurable in aerial parts and rhizomes sample; (3) the contents of these compounds except for quercetin were higher in rhizomes than those of compounds in aerial parts; (4) the six major constituents both in aerial parts and rhizomes were galangin, kaempferide, hexahydrocurcumin, pinocembrin, chrysin and isorhamnetin; (5) the content changes' trends of most of the monitored phytochemicals along with sampled periods were almost similar between aerial parts and rhizomes; and (6) the amplitude of variation along with growth period for each phytochemical was different. Our study should be of value in arousing everyone's interest in the future to make the best use of the aerial parts, rather than just rhizomes, of *A. officinarum*, valuable medicinal plant resources.

Acknowledgements

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Conflicts of Interest

There are no competing interests to declare.

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Figure legends

Figure 1 Chemical structures of interest occurring in *A. officinarum* aerial parts and rhizomes. These chemicals are given ID numbers in parentheses.

Figure 2 Cartoon for different sampling periods for *A. officinarum* aerial parts and rhizomes.

Figure 3 Product ion spectra of protonated phytochemicals of interest.

Figure 4 Representative chromatograms for seventeen reference standards (upper panel, the concentration level was 2000 ng/mL) and aerial parts samples (bottom panel, harvested at August 16, 2014 with sample no. *S_11*), as well as rhizomes samples (middle panel, harvested at March 29, 2014 with sample no. *S_03*) of *A. officinarum*.

Figure 5 Content-sampling period curves for seventeen phytochemicals.

Figure 1

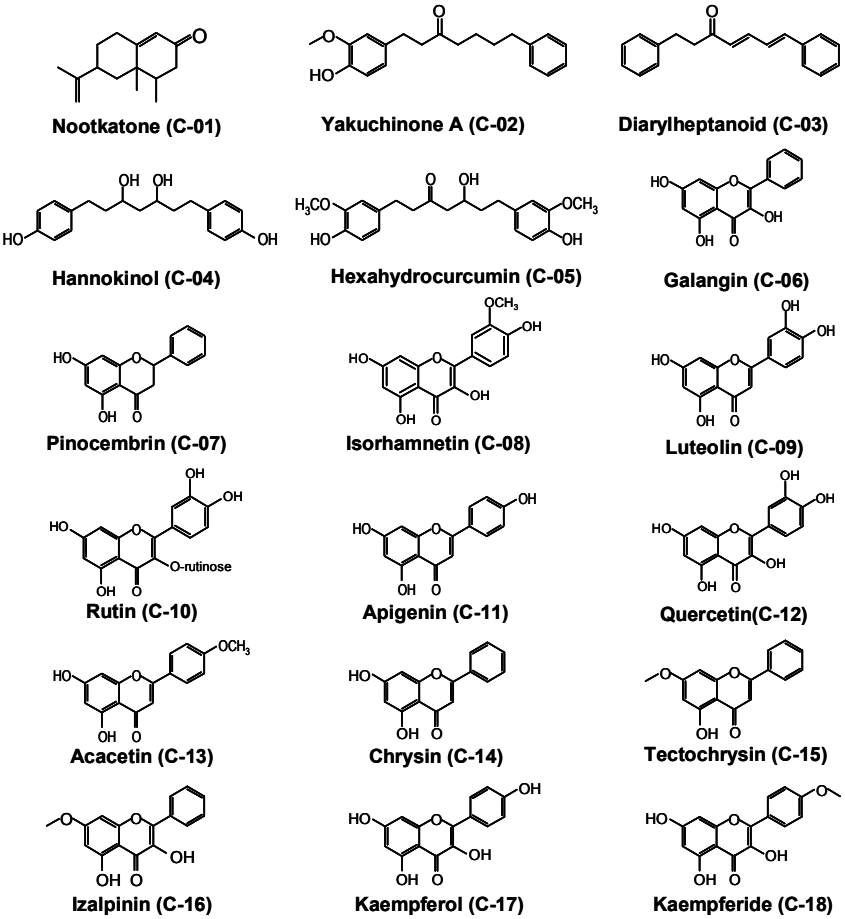

Figure 2

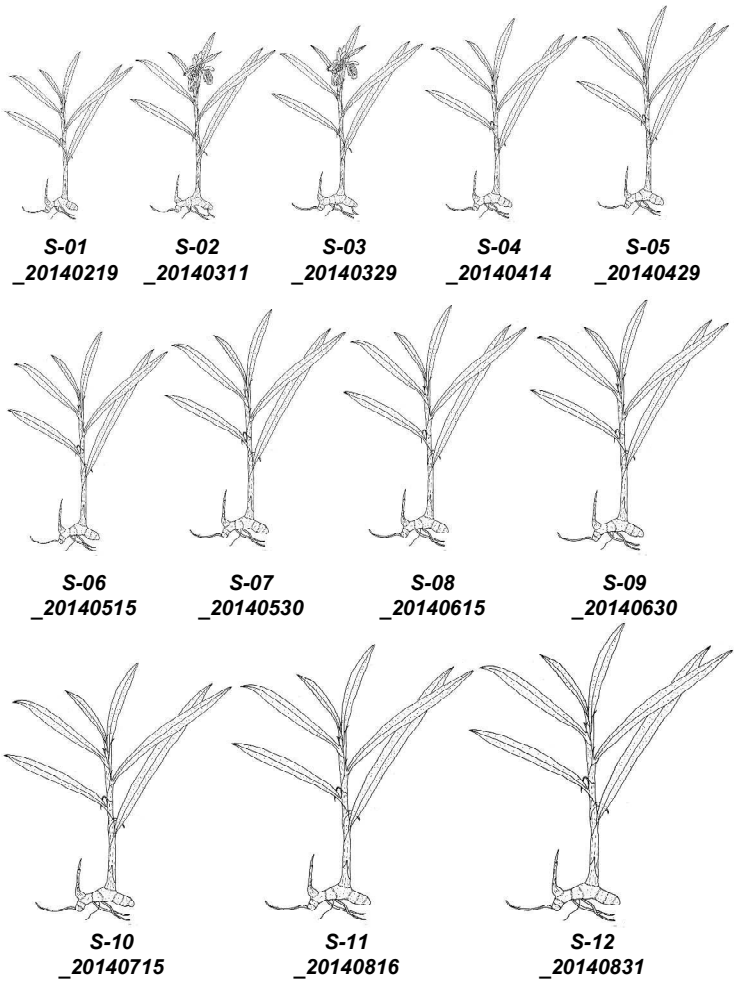

Figure 3

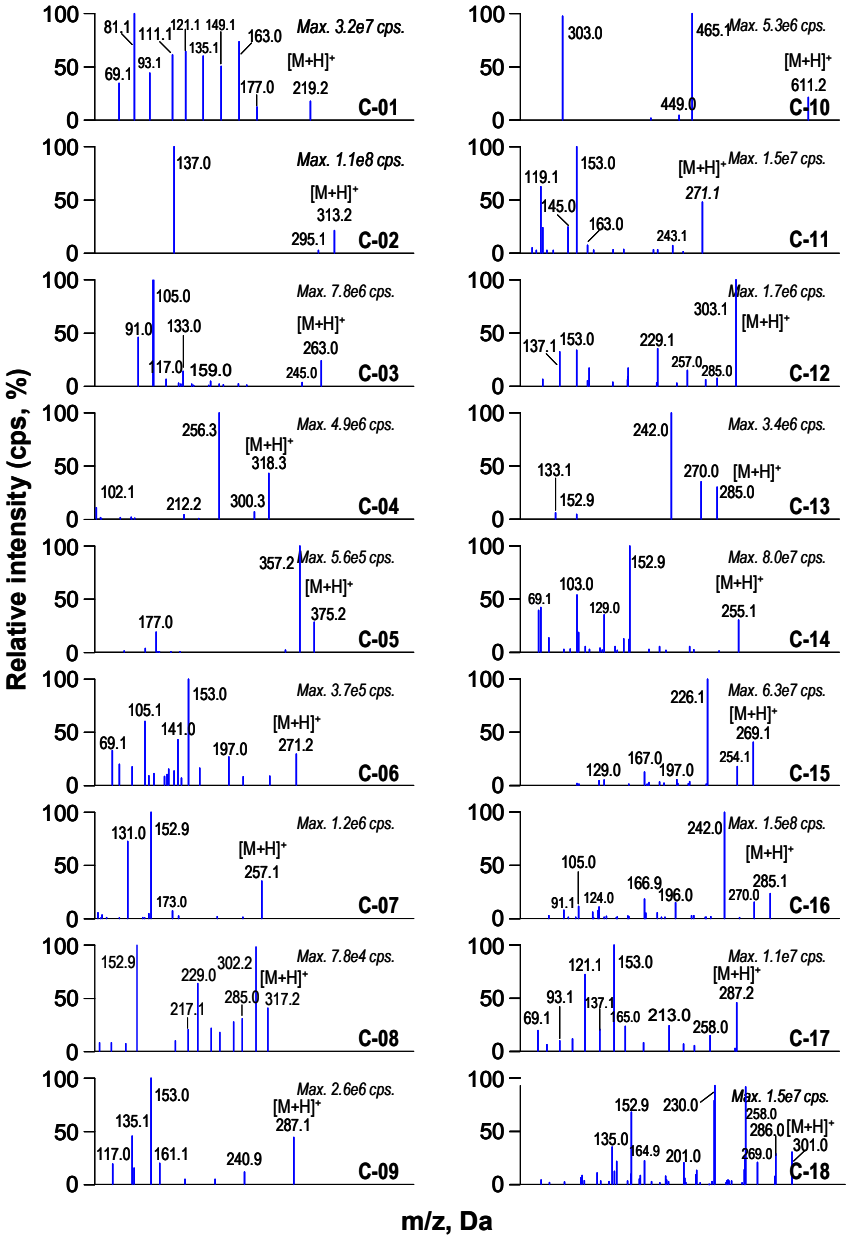

Figure 4

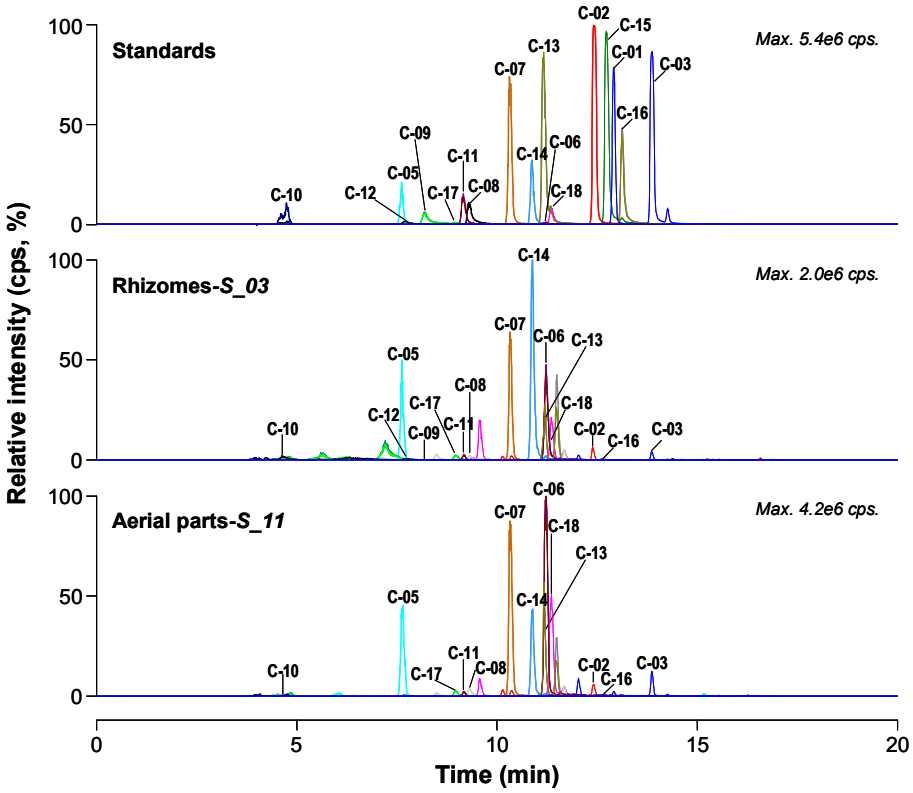

Figure 5a

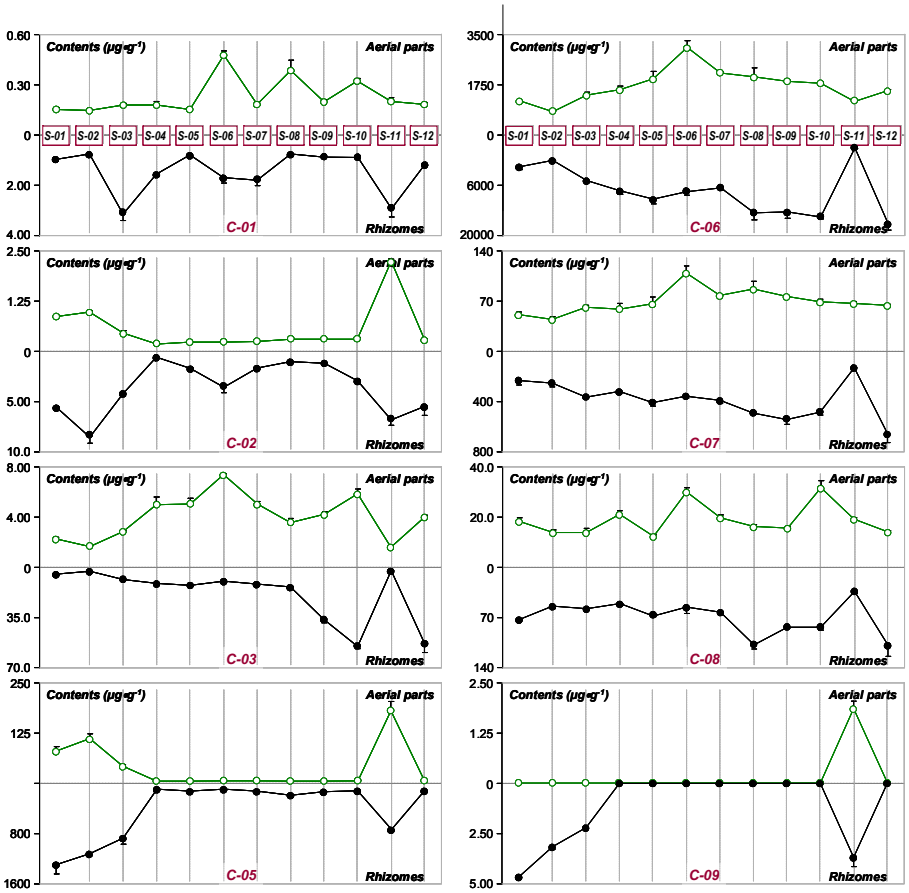

Figure 5b

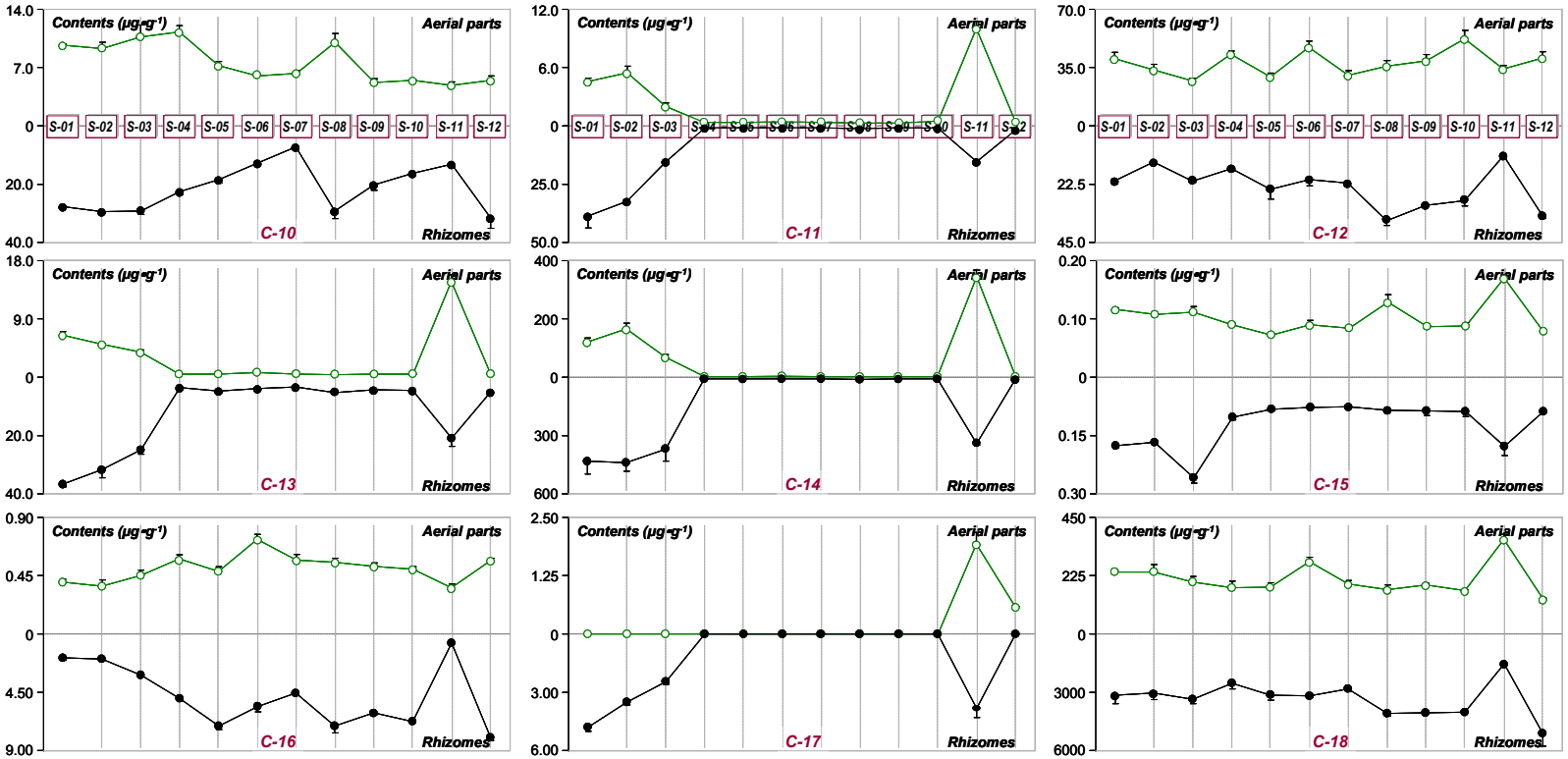

Table 1 Validation parameters

Analytes	LOD ($ng \cdot mL^{-1}$)	LOQ ($ng \cdot mL^{-1}$)	Linearity (range, *r*, weighting factor)	Precision (Intra-/inter-day; RSD, %)		Recovery (mean %; RSD %)	
				Aerial parts	Rhizomes	Aerial parts	Rhizomes
C-01	0.05	1	1-100, r=0.9963, $1/x^2$	2.37%; 9.96%	2.28%; 3.41%	105 (2.61%)	102 (2.90%)
C-02	0.05	1	1-100, r=0.9933, $1/x^2$	4.53%; 7.51%	4.81%; 5.57%	85.9 (0.46%)	91.5 (1.15%)
C-03	0.01	0.1	1-100, r=0.9943, $1/x^2$	4.83%; 4.45%	5.04%; 4.08%	86.7 (2.54%)	85.9 (4.28%)
C-05	1	2	2-2000, r=0.9975, 1/x	5.21%; 6.20%	9.28%; 8.76%	91.6 (1.98%)	88.1 (9.66%)
C-06	0.01	2	2-1000, r=0.9991, 1/x	8.38%; 8.01%	8.40%; 5.42%	98.1 (5.77%)	90.8 (9.32%)
C-07	0.05	0.5	1-100, r=0.9936, $1/x^2$	3.27%; 4.04%	3.24%; 4.38%	88.9 (1.88%)	87.6 (2.32%)
C-08	1	2	2-1000, r=0.9993, 1/x	2.44%; 3.22%	7.86%; 6.21%	102 (3.68%)	94.2 (5.03%)
C-09	1	2	2-2000, r=0.9985, 1/x	7.11%; 5.89%	5.64%; 5.80%	110 (4.31%)	109 (2.98%)
C-10	1	2	2-2000, r=0.9992, 1/x	3.85%; 4.12%	4.04%; 4.32%	106 (6.56%)	103 (10.6%)
C-11	0.5	1	1-100, r=0.9971, $1/x^2$	4.36%; 3.36%	2.07%; 2.90%	112 (1.98%)	95.2 (4.58%)
C-12	5	10	10-2000, r=0.9989, 1/x	7.23%; 8.12%	5.03%; 6.63%	97.6 (7.85%)	93.4 (5.48%)
C-13	0.05	0.1	1-100, r=0.9939, $1/x^2$	4.15%; 2.94%	7.78%; 2.27%	111 (3.19%)	95.2 (3.96%)
C-14	0.5	1	1-100, r=0.9947, $1/x^2$	2.76%; 4.44%	6.30%; 14.8%	94.5 (2.98%)	91.6 (2.78%)
C-15	0.05	0.1	1-100, r=0.9904, $1/x^2$	2.02%; 2.31%	3.98%; 3.29%	86.0 (0.84%)	85.5 (2.67%)
C-16	0.05	0.1	1-100, r=0.9943, $1/x^2$	6.98%; 6.38%	5.28%; 5.13%	89.0 (3.86%)	87.0 (1.18%)
C-17	2	5	2-2000, r=0.9980, 1/x	5.39%; 6.82%	8.41%; 6.82%	106 (7.57%)	102 (4.47%)
C-18	0.0141	1.41	1.41-1410, r=0.9987, 1/x	2.30%; 9.30%	7.27%; 7.55%	103 (5.47%)	112 (3.57%)