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Optimization and validation of a chromatographic method for the simultaneous quantification of six bioactive compounds in Rhizoma et Radix Polygoni Cuspidati

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Abstract

A reverse-phase HPLC method was developed for simultaneous quantification of six bioactive compounds in Rhizoma et Radix Polygoni Cuspidati. These compounds – polydatin (**1**), resveratrol (**2**), rhein (**3**), emodin (**4**), chrysophanol (**5**) and physcion (**6**) – were analysed from 24 authentic samples of the herb using UV HPLC. Based on the UV absorption characteristics of the six compounds, absorption wavelengths of 306 nm were chosen to quantify compounds **1** and **2**, and 290 nm for compounds **3**–**6**. A reliable and reproducible quantitative HPLC method for analysing authentic samples of Rhizoma et Radix Polygoni Cuspidati from different cultivation regions was developed. The results showed that the concentration of compound **1** in samples from Sichuan was almost 2-fold higher than that of samples acquired in Guangxi. Furthermore, compounds **3** and **5** were not found in all the samples tested. Thus, instead of using polydatin (**1**) and emodin (**4**) as markers for quality assessment, as in conventional practice, these findings show that compounds **2** and **6** are more suited to act as marker compounds for a more specific assessment of the quality of this herb.

Introduction

Rhizoma et Radix Polygoni Cuspidati (Huzhang, HZ), comes from the stem and root of *Polygonum cuspidatum* Sieb. Et Zucc., which belongs to the family Polygonaceae. This herb is one of the most commonly used traditional Chinese medicinal plants in China and is listed in the Chinese Pharmacopoeia (2005).

HZ contains a variety of chemical groups such as stilbenes, anthraquinones and flavones (Kimura et al 1983; Huang 1994; Xiao et al 2003). Previous pharmacological and clinical studies have indicated that several chemical components in HZ are bioactive. Polydatin, the most abundant chemical component in HZ, can inhibit platelet aggregation, induce apoptosis (Kimura et al 1983; Lee et al 2002; Wang et al 2002; Xiao et al 2003), lower blood cholesterol (Huang 1994; Xiao et al 2003) and enhance blood flow in capillaries (Kimura et al 1983; Xiao et al 2003). Resveratrol, which is also found in grapes but at a much lower concentration (Feng et al 2004; Yao et al 2005), has been demonstrated to inhibit the growth of several bacteria and fungi (Wang et al 2002), to lower blood cholesterol level, and exhibits anti-inflammatory properties and chemoprotective activity against cancer proliferation (Jayasuriya et al 1992; Spainhour 1997). Rhein is used in the prevention and treatment of diabetic nephropathy (Yokozawa et al 1997). Chrysophanol improves the impairment of memory acquisition associated with senility (Zhang et al 2005). Physcion can reduce damage to swelling tissues during cerebral haemorrhage (Mueller et al 1999). All these aforementioned bioactive compounds in HZ also possess anticancer, antiviral, antifungal and anti-inflammatory properties (Kong et al 2000; Wang et al 2002; Chang et al 2005). Although these compounds have been widely use in medicinal applications, limited work relating to the quantification of these bioactive components has been reported. The literature shows that most studies focus mainly on one or two chemical components (Feng et al 2004; Zhou et al 2005; Lei et al 2006). Among those

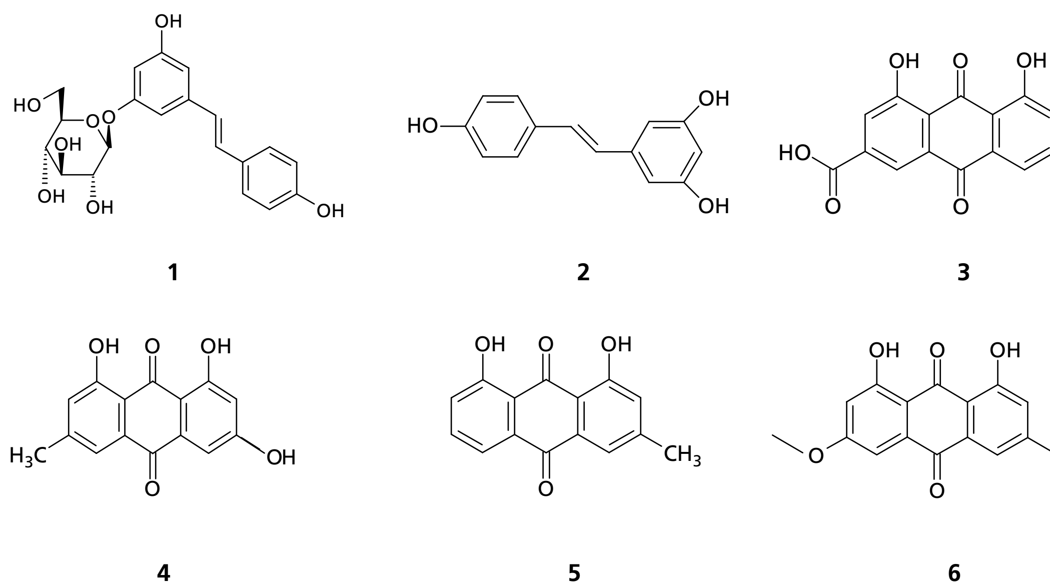

Figure 1 Chemical structures of the six bioactive compounds: (**1**) polydatin, (**2**) resveratrol, (**3**) rhein, (**4**) emodin, (**5**) chrysophanol, (**6**) physcion.

quantitation methods, thin-layer chromatography and HPLC were the major tools of analysis.

In this work, an accurate HPLC method has been used to simultaneously quantify six bioactive compounds in authentic samples of HZ from different cultivation regions. The compounds were polydatin (**1**), resveratrol (**2**), rhein (**3**), emodin (**4**), chrysophanol (**5**) and physcion (**6**) (Figure 1). They were first identified using liquid chromatography–mass spectrometry and compared with data in our previous studies (Qian et al 2006). Based on the UV absorption characteristics, optimal detection wavelengths were chosen for quantitative analysis of bioactive compounds in HZ. It is important to note that the anthraquinones in HZ are mainly in the glycoside form. Thus, in order to quantify the total anthraquinone, acidic hydrolysis was performed to cleave and release the glycosidic units.

Materials and Methods

Instrumentation

An Agilent 1100 series HPLC diode array detector (DAD) system consisting of a vacuum degasser, binary pump, autosampler, thermostatic column compartment and DAD (Agilent, Palo Alto, CA, USA) was used for acquiring chromatograms, UV spectra and 3D plots. A Branson 5210E-MTH ultrasonic bath (Branco Ultrasonics Corporation, CT, USA) was used for sample extraction.

Chemicals and reagents

Analytical-grade methanol (Labscan, Bangkok, Thailand) was used for preparation of sample and standard solutions. HPLC-grade acetonitrile (Labscan), deionized water generated using a Milli-Q water system (Millipore, Bedford, MA, USA) and formic acid (Unichem, Warsaw, Poland) were used for preparation of mobile phase. Sulphuric acid (95–97% from Tedia, CA, USA) and analytical-grade dichloromethane (Labscan) were used for acid hydrolysis to determine the total contents of emodin, physcion and rhein.

Polydatin, resveratrol, rhein, emodin, chrysophanol and physcion were purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). The purity of these chemical standards was determined to be more than 98% by normalization of the peak areas detected by HPLC–DAD.

Materials and sample preparation

Samples of HZ were collected from a number of retailers in Hong Kong and several cultivation bases in mainland China. The sources of the plant materials are listed in Table 1. These samples were authenticated by Professor ZhongZhen Zhao (School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China) by means of geographical origin identification, microscopic and macroscopic authentication and physiochemical identification. Voucher specimens were deposited in the Herbarium Centre, Hong Kong Baptist University (20060926).

The samples were cut into small pieces, ground into powder and passed through a 20-mesh (0.9 mm) sieve. The ground powders were stored in cool dry conditions. For assay of polydatin, resveratrol, rhein, emodin, chrysophanol and physcion, stock standard solutions were prepared in the concentration range 1–400 μg mL^{-1} in methanol. For the calibration curves, 10 μL aliquots of each stock solution were injected for HPLC analysis.

An accurately weighed sample of powder (0.2 g) was introduced into a 60 mL centrifuge tube and 15 mL methanol was added. The mixture was extracted by sonication at room temperature for 0.5 h, then centrifuged at 3500 rpm for

Table 1 Concentrations of the chemical components in 24 authentic samples of Rhizoma et Radix Polygoni Cuspidati (Huzhang, HZ) from different sources in P. R. China. Chrysophanol was not found in any of the 24 samples

Source and year of harvest	I.D.	Polydatin	Resveratrol	Rhein	Emodin	Physcion
Guangxi (2005)	HZ-1	15.05 ± 0.25	2.75 ± 0.06	N.D.	20.92 ± 0.34	7.47 ± 0.07
Guangxi (2005)	HZ-2	13.97 ± 0.08	1.73 ± 0.02	N.D.	17.11 ± 0.07	6.80 ± 0.02
Guangxi (2005)	HZ-3	11.10 ± 0.04	2.69 ± 0.06	N.D.	19.09 ± 0.06	8.43 ± 0.01
Guangxi (2005)	HZ-4	19.76 ± 0.07	1.13 ± 0.01	N.D.	24.75 ± 0.27	10.27 ± 0.01
Guangxi (2005)	HZ-5	13.48 ± 0.01	1.74 ± 0.02	N.D.	19.45 ± 0.31	8.47 ± 0.08
Guangxi (2005)	HZ-6	13.48 ± 0.19	2.50 ± 0.03	N.D.	20.40 ± 0.57	7.69 ± 0.12
Guangxi (2005)	HZ-7	16.52 ± 0.09	2.15 ± 0.16	N.D.	19.87 ± 0.05	6.49 ± 0.14
Abazhou, Sichuan (2006)	HZ-8	31.25 ± 0.25	3.15 ± 0.05	2.53 ± 0.02	17.06 ± 0.08	6.22 ± 0.05
Huaxi, Sichuan (2006)	HZ-9	27.14 ± 0.33	0.71 ± 0.01	N.D.	17.39 ± 0.08	6.59 ± 0.03
Changdu, Sichuan (2006)	HZ-10	27.43 ± 1.17	1.30 ± 0.01	N.D.	19.74 ± 0.04	8.71 ± 0.03
Aoping, Sichuan (2006)	HZ-11	26.71 ± 0.01	2.18 ± 0.02	1.61 ± 0.001	19.35 ± 0.18	9.23 ± 0.01
Changdu, Sichuan (2006)	HZ-12	25.10 ± 0.38	3.65 ± 0.10	1.89 ± 0.004	18.14 ± 0.20	5.97 ± 0.10
Changdu, Sichuan (2006)	HZ-13	19.12 ± 0.13	3.96 ± 0.04	N.D.	20.35 ± 0.46	9.97 ± 0.01
Changdu, Sichuan (2006)	HZ-14	26.04 ± 0.13	1.71 ± 0.01	N.D.	19.28 ± 0.71	9.95 ± 0.06
Changdu, Sichuan (2006)	HZ-15	26.07 ± 0.08	1.06 ± 0.01	N.D.	17.83 ± 0.10	7.95 ± 0.01
Changdu, Sichuan (2006)	HZ-16	25.93 ± 0.35	2.43 ± 0.01	1.29 ± 0.01	21.44 ± 0.87	8.14 ± 0.03
Emei, Sichuan (2006)	HZ-17	30.05 ± 0.01	6.46 ± 0.04	1.58 ± 0.01	20.99 ± 0.12	7.92 ± 0.08
Guangxi (2006)	HZ-18	18.04 ± 0.05	1.19 ± 0.03	N.D.	15.79 ± 0.06	6.59 ± 0.02
Guangxi (2006)	HZ-19	16.95 ± 0.06	3.31 ± 0.06	N.D.	17.52 ± 0.07	5.48 ± 0.05
Guangxi (2006)	HZ-20	18.70 ± 0.48	1.28 ± 0.03	N.D.	15.57 ± 0.26	6.02 ± 0.04
Guangxi (2006)	HZ-21	9.75 ± 0.24	2.48 ± 0.03	N.D.	17.05 ± 0.65	7.48 ± 0.16
Guangxi (2006)	HZ-22	20.93 ± 0.27	4.71 ± 0.08	N.D.	31.69 ± 0.09	14.48 ± 0.11
Guangxi (2006)	HZ-23	20.64 ± 0.45	1.95 ± 0.05	N.D.	16.92 ± 0.09	5.99 ± 0.01
Guangxi (2006)	HZ-24	16.83 ± 0.13	3.41 ± 0.04	N.D.	17.37 ± 0.32	8.75 ± 0.04

Concentrations are given in mg g^{-1}, mean ± s.d. (n = 3).
N.D., not detected.

10 min. The supernatant was transferred into a 50 mL volumetric flask. The procedure was repeated twice. The extracts were combined and transferred into the volumetric flask and made up to volume with methanol, then filtered through a Nylon membrane filter (0.45 μm; Alltech; Deerfield, IL, USA). A 10 μL aliquot of the solution was injected for HPLC analysis. Sample duplicates were prepared as described above for analysis. To compare extraction methods, 0.2 g of sample in 15 mL methanol was extracted under various conditions.

For acid hydrolysis, 10 mL of the methanolic extract described above was pipetted and introduced accurately into a 100 mL round-bottomed flask and mixed with 10 mL Milli-Q water; 3.5 mL 2.5 M sulphuric acid and 20 mL water-saturated dichloromethane were then added to the mixture, which was kept under reflux for 2 h. The organic layer was then concentrated to near dryness under vacuum using a rota-evaporator. The residue was dissolved in methanol. The solution was transferred into a 10 mL volumetric flask and made up to volume. The extract was filtered through a membrane filter. An aliquot of 10 μL was injected for HPLC analysis. Sample duplicates were prepared as above for analysis.

HPLC-DAD analysis

For chromatographic analysis, we used an Alltima C_{18} column (5 μm, 4.6 mm × 250 mm, Alltech) with a C_{18}, 4.6 mm × 7.5 mm guard column. The mobile phase consisted of 0.4% formic acid in deionized water (A) and acetonitrile (B) using a gradient programme of 15–20% B over 0–20 min, 20–40% B over 20–40 min, 40–100% B over 40–60 min followed by 100% B for 60–65 min. The solvent flow rate was 1 mL min^{-1} and the column temperature was maintained at 30°C. The DAD detector was set at 290 and 306 nm for acquiring chromatograms.

Results and discussion

Our recent work on chromatographic fingerprints of HZ has established the optimal chromatographic conditions for effective resolution of the six compounds within the chromatographic window (Qian et al 2006). The current studies focused on optimization of various chromatographic and experimental parameters for detection, crude extraction, method validation, stability evaluation and quantitative analysis.

The choice of detection wavelength is a crucial step for developing accurate quantitative analysis. Thus, in previous related work on HZ, various detection wavelengths were used for polydatin, resveratrol and emodin (Feng et al 2004; Chinese Pharmacopoeia 2005; Yao et al 2005; Zhou et al 2005; Lei et al 2006). To determine the compromise wavelength of detection for the three compounds, the wavelengths 254, 290 and 306 nm were investigated. The results indicated that 306 nm was the optimal wavelength for compounds **1** and **2**, whilst compounds **3–6** showed high molar absorptivity at 290 nm. Therefore, wavelengths of 290 nm and 306 nm were

chosen as the compromise wavelengths for detection in our study. Typical chromatograms at the two detection wavelengths are shown in Figure 2.

To achieve optimal extraction efficiency for the six compounds, we investigated different extraction solvents and methods and duration of extraction. Regarding solvent

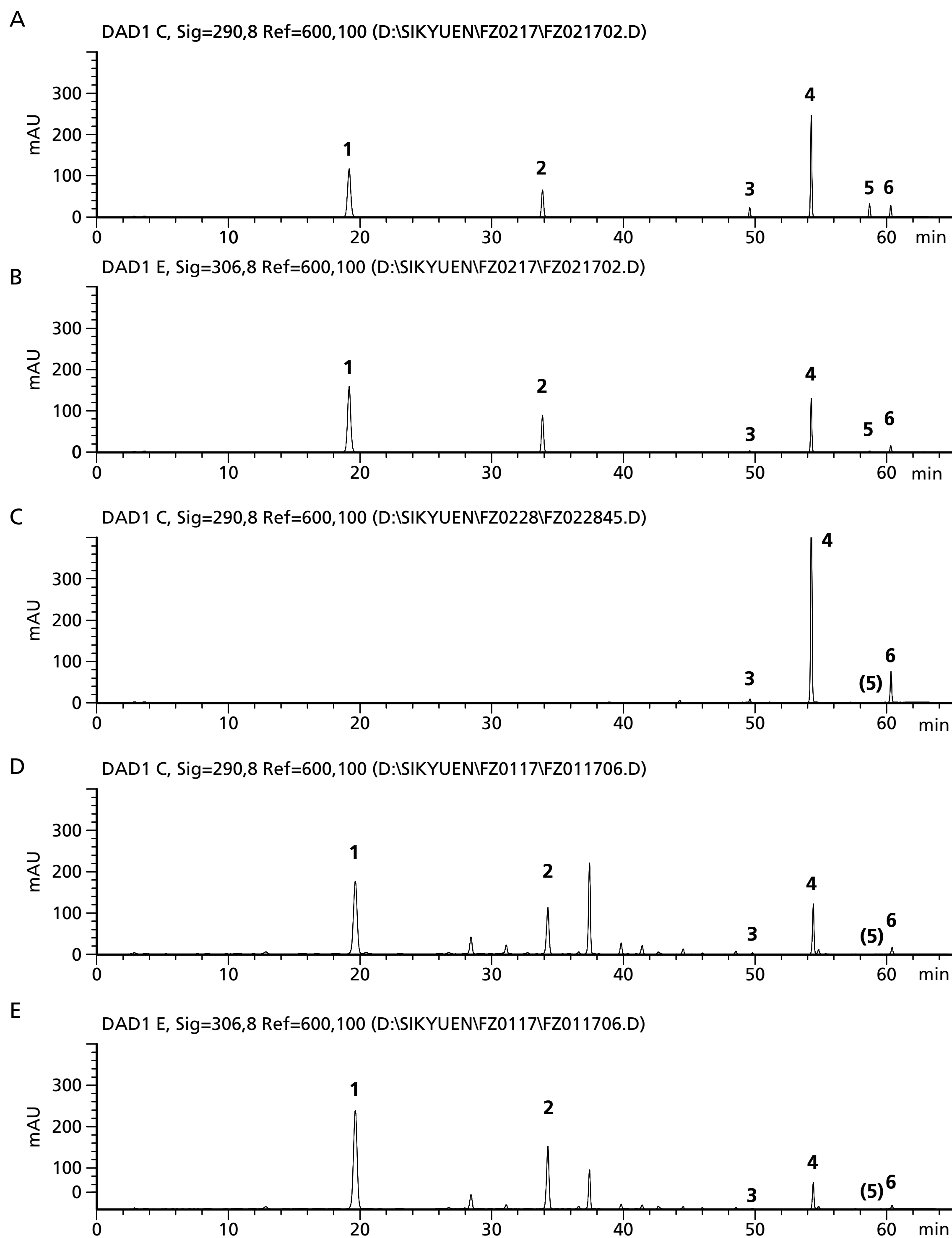

Figure 2 Typical chromatograms showing mixed standards at detection wavelengths of 290 nm (A) and 306 nm (B), and upon hydrolysis and detected at 290 nm (C), upon methanolic extraction and detected at 290 nm (D) or 306 nm (E). Compound 5 (chrysophanol) is marked in parentheses as it was not present in any of the 24 samples.

choice, methanol and ethanol are the most common solvents used for extraction, as shown in previous studies (Lee et al 2002). In this work, the extraction efficiencies of compounds **1–6** were compared (Table 2). As these chemical components contain hydroxyl functionalities (Kimura et al 1983; Huang 1994; Xiao et al 2003), extraction efficiency is likely to be enhanced by using a higher polarity organic solvent. This is shown by results which suggest that extraction efficiencies are generally higher with methanol than with ethanol (Zhou et al 2005). Subsequent studies have shown that only free anthraquinones were extracted under these conditions.

Extraction under reflux for 0.5–8 h by sonication is the general method for isolation of compounds **1** and **2**, as reported in previous work (Feng et al 2004). However, compound **1** was reported to be thermolabile and thus the yield is likely to be reduced at high-temperature extraction because of isomerization (Feng et al 2004). Optimum conditions for extraction were therefore investigated. A variety of extraction methods were compared, including extraction under reflux, Soxhlet extraction and sonication. Of these, sonication produced comparatively better yields for all target compounds. Sonication has become a widely accepted method for extraction in phytochemistry because of its high efficiency and ease of handling (Table 2). In order to ensure quantitative extraction of target compounds in HZ, the number of extraction cycles was also determined. The results showed that three extraction cycles of 30 min each led to a complete extraction of compounds **1–4** and **6**. Although compound **5** was not present in all 24 samples in our present study, it is one of the components first identified in HZ (Tsukida & Yoneshige 1954). Variations in cultivation regions, harvest season and processing methods are likely to affect the concentration of this component.

It is well-known that the anthraquinones in HZ exist mainly in a glycosidic form (Huang 1994). It was therefore unsurprising that the content of compounds **3, 4** and **6** rose considerably after acid hydrolysis. The optimum duration of acid hydrolysis to achieve maximum yields for these three compounds was 2 h reflux.

Having considered the ease of handling and the yields of the compounds, methanolic extraction was the best method to use for extracting compounds **1, 2** and **5**, whilst acid hydrolysis was used to improve extraction of the other three analytes of interest. Table 1 summarizes the total anthraquinones in HZ following acid hydrolysis.

A full validation of the method was conducted, the results of which are listed in Table 3. Good linearity ($r^2 > 0.999$) was achieved for all the quantified chemical constituents. Instrumental precision was evaluated from five replicate injections of a sample solution; method precision was investigated through another five replicate analyses of the same sample (e.g. HZ-1). The variation in content of each compound was expressed as relative standard deviation (r.s.d.). Recovery was determined by fortifying the samples with different amounts, namely 50, 100 and 150% of the respective compounds. Duplicate analysis was conducted at each fortification and the average recovery of all six compounds was found to be within 96.0% (r.s.d. 3.0%) to 101.8% (r.s.d. 1.6%) (Table 4). Based on visual evaluation with signal-to-noise ratio of about 3:1 and 10:1, the limit of detection for the six compounds was in the range 13.5–76.5 μg g^{-1}, and the limit of quantitation was within 58.5–254.5 μg g^{-1}. These results

Table 2 Effect of extraction method and solvent variation on extraction efficiency. Chrysophanol was not found in any of the 24 samples

Extraction method[a]/ solvent	Polydatin	Resveratrol	Rhein[c]	Emodin[c]	Physcion[c]
Reflux	29.68 ± 0.01	6.04 ± 0.01	0.71 ± 0.58	5.75 ± 0.03	2.11 ± 0.02
Soxhlet	30.30 ± 0.09	6.36 ± 0.04	0.73 ± 0.11	6.07 ± 0.04	2.12 ± 0.02
Sonication	31.11 ± 0.81	6.55 ± 0.07	0.75 ± 0.01	6.09 ± 0.12	2.26 ± 0.06
Methanol[b]	28.18 ± 1.02	6.23 ± 0.15	0.71 ± 0.02	5.93 ± 0.15	2.20 ± 0.05
Ethanol[b]	27.40 ± 0.70	5.95 ± 0.12	0.63 ± 0.02	4.05 ± 0.20	2.18 ± 0.15

Concentrations are given in mg g^{-1}, mean ± s.d. (n = 3). N.D. = Not detected; [a]solvent: methanol; [b]conducted under reflux; [c]unhydrolysed (free) form.

Table 3 Validation data

	Instrumental precision[a] (%)	Method precision[a] (%)	LOD (μg g^{-1})	LOQ (μg g^{-1})	Linear regression equation	r^2
Polydatin	0.664	1.65	76.5	254.5	y = 41.053x – 52.003	0.9999
Resveratrol	0.671	2.13	17.5	58.5	y = 81.185x – 20.529	0.9994
Rhein	0.464	2.76	15.5	155.0	y = 13.693x – 8.7528	0.9998
Emodin	0.494	1.61	26.0	82.5	y = 42.347x – 17.756	0.9998
Chrysophanol	0.381	2.21	15.5	155.0	y = 22.409x – 7.7957	0.9998
Physcion	0.330	2.10	13.5	136.0	y = 16.803x – 10.46	0.9993

[a]Values are relative s.d. (n = 5).
LOD, limit of detection; LOQ, limit of quantifcation.

Table 4 Recovery (%) of the six compounds at different fortifications (50, 100 and 150%)

Fortification	Polydatin	Resveratrol	Rhein	Emodin	Chrysophanol	Physcion
50%	100.8	97.1	103.2	99.6	101.6	97.4
100%	98.7	95.8	101.6	98.8	103.2	101.2
150%	101.2	95.0	98.2	98.1	100.6	95.7
Mean ± r.s.d.	100.2 ± 1.5	96.0 ± 3.0	101.0 ± 3.0	98.8 ± 0.8	101.8 ± 1.6	98.1 ± 2.9

Values were obtained from duplicate analysis at each fortification for each compound.
r.s.d., relative s.d.

indicate that the method is satisfactory for subsequent quantitative analysis.

Before sample quantitation, compound stability remains an unsolved technical concern. It has been reported that compounds **1** and **2** are readily isomerized in light conditions (Feng et al 2004). The anthraquinone-type compounds **4** and **6** were also found to exhibit similar behaviour. The stability in storage of these compounds was therefore evaluated by dissolving the respective compounds and allowing the solutions to stand. The degree of deterioration under light and dark conditions was compared over a period of 48 h. By comparing the chromatographic peak areas, the levels of compounds **1, 2, 4** and **6** in methanol were found to be within the range 70.3–94.1% upon standing for 48 h in light, indicating that these compounds are relatively sensitive to light. However, limited changes in content were observed with standing of these methanol solutions in the dark for 48 h. These findings indicate that subsequent quantitative analysis should ideally be performed within the first hour of preparation of freshly extracted samples (< 0.5% change in concentration in our study), and compounds have to be protected from exposure to light during storage.

The levels of compounds **1–6** were determined in 24 authentic samples using the established method. The results revealed that all compounds, with the exception of rhein and chrysophanol, were found in the samples (Table 1). Rhein was found in some samples from Sichuan, whilst chrysophanol was not observed at all (Chang et al 2005). HZ from Sichuan generally contains higher levels of polydatin than samples from other provinces (Feng et al 2004). Emodin, a widely occurring natural compound in herbs, was the most abundant chemical component in HZ. According to the Chinese Pharmacopoeia, both polydatin and emodin are conventionally adopted as indices to assess the quality of HZ. Our present work suggests that resveratrol and physcion offer alternatives as suitable candidates for marker compounds for quality assessment purposes. Both of these compounds were found in reasonable quantities in all 24 samples.

Conclusion

The quality evaluation of plant material remains a challenge in natural product chemistry, not only because of severe matrix interference during analysis, but also because of batch-to-batch variability between plant species. It is generally believed that differences in cultivation conditions, harvest season and storage conditions are important factors that affect the levels of certain chemical components. Therefore, the rationale for choosing particular chemical compound(s) as markers for quality evaluation has to be considered carefully. The selected markers should represent the major/entire chemical profile and preferably should be commercially available. To facilitate routine operation in the herbal industry for quality assessment purposes, tedious experimental procedures for the isolation and chromatographic identification of these compounds have to be avoided. In this study, a quantitative method for the simultaneous determination of resveratrol, polydatin, rhein, emodin, chrysophanol and physcion in Rhizoma et Radix Polygoni Cuspidati was established using HPLC–DAD. Before this study, there was no reliable evaluation of relevant chemical components. The method we have developed here was found to be reliable and suitable for analysing large number of samples with good accuracy and reproducibility.

Resveratrol and physcion are two chemical components generally available in Rhizoma et Radix Polygoni Cuspidati. Unlike emodin, these two compounds exist more specifically in HZ and are associated with proven pharmacological activities. Thus they act as potential candidates, offering an alternative for a more specific assessment of the quality of the herb as well as for quality control purposes.

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