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Phenanthrene Derivatives, 3,5-Dimethoxyphenanthrene-2,7-diol and Batatasin-I, as Non-Polar Standard Marker Compounds for Dioscorea Rhizoma

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Abstarct – Phenathrene derivatives, such as batatasins, are well-known constituents in Dioscorea Rhizoma. Although phenanthrenes have been reported as representative compounds in this plant, standard markers for quality control have been focused on the polar constituents (saponins and purine derivatives). Herein, simple, rapid and reliable HPLC method was developed to determine 3,5-dimethoxyphenanthrene-2,7-diol (DMP) and batatasin-I (BA-I) as non-polar standard maker compounds of Dioscorea Rhizoma. DMP and BA-I were analyzed under optimized HPLC conditions [column: Columbus 5 μ C18 100A (30 × 4.6 mm i.d., 5 μm; mobile phase: H_2O with 0.025% CH_3COOH (v/v) for solvent A and CH_3CN with 0.025% CH_3COOH (v/v) for solvent B, gradient elution; flow rate: 2 mL/min; detection: 260 nm), and each experiment was finished within 13 min. Good linearity was achieved in the range from 0.5 to 10.0 μg/mL for each compound, and intra- and inter-day precision were in the acceptable levels. The recovery test were performed with three different Dioscorea Rhizoma samples (*D. opposita, D. batatas* and *D. japonica*), and showed its accuracy values in the range of 97.2 - 102.8% for three different concentrations of DMP and BA-I. The content levels of DMP and BA-I were ranged under 0.0020%. These results demonstrated that amounts of DMP and BA-I are easily determined with conventional HPLC-UV-DAD method although the content levels were lower than those of saponins and allantoin in Dioscorea Rhizoma. This HPLC method could be used for quality control of various *Dioscorea* preparations.
Keywords – Dioscorea Rhizoma, Phenanthrenes, Quality control

Introduction

Dioscorea Rhizoma has been widely used as an important medicinal plant in the worlds, and used for stimulating the stomach and spleen against poor appetite and has a tonic effect on the lungs and kidneys (Bae, 1999; Frombi, *et al*., 2000). Various pharmacological activities of Dioscorea Rhizoma have been reported for anti-oxidative (Chang, *et al*., 2004), anti-fungal (Sautour, *et al*., 2004), anti-mutagenic (Miyazawa, *et al*., 1996), hypoglycemic (McAnuff, *et al*., 2002), and immunomodulatory effect (Choi, *et al*., 2004). The major components of this plant are known as saponins (Liu, *et al*., 2007) and sapogenins (Akahori, 1965), starch (Jayakody, *et al*., 2007), purine derivatives, and mucilage (Fu, *et al*., 2006).

So far, the standard markers for quality control of Dioscorea Rhizoma have been focused on polar constituents such as saponins (dioscin derivatives) (Liu, *et al*., 2007) and purine derivatives (allantoin) (Zhang, *et al*., 2004). However, it is difficult to determine saponins in plant with conventional UV detector due to very few UV-absorbing chromophore groups. Therefore, HPLC-MS and evaporative light scattering detector (ELSD) have been used to detect saponins (He, 2000; Ha, *et al*., 2006). Allantoin, one of the purine derivatives, is regarded as another standard marker due to high-level abundance in *Dioscorea* species (Fu, *et al*., 2006). Allantion has very polar nature causing no or little retention in conventional C18 columns. To overcome above problem, complicated derivatization methods have been used to determine allantoin (Czauderna, *et al*., 2000). However, *Dioscorea* spp. possesses not only polar compounds but also non-polar constituents such as phenanthrene derivatives (Takasugi, *et al*., 1987; Coxon, *et al*., 1982; Ireland, *et al*., 1981; Hashimoto, *et al*., 1978; Hashimoto, *et al*., 1974; Letcher, 1973; Hashimoto, *et al*., 1972).

In the course of searching for standard maker substances in Dioscorea Rhizoma (*D. opposita* Thunb., *D. batatas* Decne. and *D. japonica* Thunb.), two major peaks were observed in HPLC-UV chromatograms. The compounds, corresponding two peaks, were isolated and their chemical

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structures were identified as to be 3,5-dimethoxyphenanthrene-2,7-diol (DMP) (Leong, *et al*., 1997; Takasugi, *et al*., 1987) and batatasin-I (BA-I) (Coxon, *et al*., 1982). Batatasins have been reported as typical constituents of Dioscorea Rhizoma, showing growth inhibitory properties and antifungal activities (Coxon, *et al*., 1982). Moreover, BA-I particularly appeared to be the most widespread in *Dioscorea* spp. than any other batatasins (Ireland, *et al*., 1981). Although the contents of DMP and BA-I are present at low level in Dioscorea Rhizoma, yet they have been known for the representative constituents of Dioscorea Rhizoma. In addition, they are easily detected with small amounts when compared with saponins and allantoin on conventional HPLC-UV method, which enable us to evaluate them as possible standard maker compounds for Dioscorea Rhizoma.

The present study describes the development and validation of HPLC-UV-DAD method for simultaneous determination of DMP and BA-I as non-polar standard maker compounds in Dioscorea Rhizoma.

Experimental

Instrumentations and chromatographic conditions – HPLC analysis was performed by a Gilson HPLC system (Middleton, WI, USA) equipped with two pumps (321 pump), UV/Vis detector (UV/VIS-151) and autosampler (234 autoinjector). Data acquisition and analysis was carried out with Gilson Unipoint software. UV spectra for standard maker compounds were achieved on Waters 996 photodiode array detector (Milford, MA, USA), and data were processed with the Waters Millenium^{32} version 4.00 software. The columns used in this study was Phenomenex Columbus 5 μ C18 100A (30 × 4.6 mm i.d.; 5 μm; Torrance, CA, USA). The mobile phases were H_2O with 0.025% CH_3COOH (v/v) for solvent A and CH_3CN with 0.025% CH_3COOH (v/v) for solvent B, and started a linear gradient increasing B from 20% to 50% at 10 min followed by being back to initial condition (B 20%) at 11 min and maintained to 13 min. Flow rate was 2 ml/min, and all samples were injected with the volume of 20 μL. HPLC chromatograms were obtained with UV wavelength at 260 nm.

Plant material for extraction and isolation – *D. opposita* (Dioscoreaceae) was provided by Tong Yang Moolsan Co., Ltd. (Young-In, Kyungi-DO, South Korea), and identified by Prof. Gwang Jin Chang, the Korea National Agricultural College.

Extraction of *D. opposita* – The fresh roots of *D. opposita* (4 kg) were cut into small peace and extracted with 100% MeOH (24 L) with ultrasonication to obtain methanol extract (55 g). The MeOH extract was suspended with distilled water and partitioned with CH_2Cl_2 to give CH_2Cl_2-soluble extract (6.5 g).

Isolation of standard marker compounds from CH_2Cl_2-soluble extract – The CH_2Cl_2-soluble extract (6.5 g) was subjected to column chromatography on Sephadex LH-20 (70 g) with MeOH (each 18 mL) as eluent, and afforded forty fractions (fractions 1 - 40). Each fraction was analyzed by HPLC, and HPLC chromatograms of all fractions were compared with that of *D. opposita* extract to determine peak identity for standard marker compounds. From the analyses results, fractions 18 - 19 were combined and evaporated in dryness to give compound **1** (18 mg), and fraction 22 was concentrated to dryness under vacuum yielding compound **2** (9.4 mg). The purities of isolated compounds were evaluated as over 98% by above HPLC method, and they were used as standard marker compounds in this study.

3,5-Dimethoxyphenanthrene-2,7-diol (**1**). Colorless solid; ^{1}H-NMR (CD_3OD, 300 MHz) δ 9.03 (s, H-4), 7.14 (1H, s, H-1), 7.79 (1H, d, J = 8.8 Hz, H-9), 7.76 (1H, d, J = 8.8 Hz, H-10), 6.78 (1H, d, J = 2.4 Hz, H-8), 6.70 (1H, d, J = 2.4 Hz, H-6), 4.07 (3H, s, 5-OC$\underline{H}_3$), 4.01 (3H, s, 3-OC$\underline{H}_3$); ^{13}C-NMR (CD_3OD, 100 MHz) δ 161.4 (C-5), 157.0 (C-7), 149.7 (C-3), 146.7 (C-2), 137.0 (C-4a), 129.4 (C-8a), 128.8 (C-10), 126.8 (C-1a), 126.5 (C-9), 116.6 (C-5a), 113.5 (C-1), 110.7 (C-4), 106.4 (C-8), 100.8 (C-6), 57.0 (3-O$\underline{C}H_3$), 56.9 (5-O$\underline{C}H_3$); EI/MS *m/z* 270 $[M]^+$ (100), 255 (19), 227 (18), 184 (10), 149 (25), 77 (47)

Batatasin-I (**2**). Yellowish solid; ^{1}H-NMR (CD_3OD, 300 MHz) δ 8.98 (s, H-4), 7.58 (1H, d, J = 8.6 Hz, H-9), 7.45 (1H, d, J = 8.6 Hz, H-10), 7.27 (1H, s, H-1), 6.90 (1H, d, J = 2.5 Hz, H-8), 6.70 (1H, d, J = 2.5 Hz, H-6), 4.06 (3H, s, 5-OC$\underline{H}_3$), 3.97 (3H, s, 3-OC$\underline{H}_3$), 3.90 (3H, s, 7-OC$\underline{H}_3$); ^{13}C-NMR (CD_3OD, 100 MHz) δ 161.5 (C-5), 159.8 (C-7), 147.9 (C-2), 137.0 (C-4a), 129.4 (C-9), 129.0 (C-8a), 127.5 (C-1a), 126.2 (C-10), 116.9 (C-5a), 114.5 (C-1), 110.1 (C-4), 103.1 (C-8), 100.6, (C-6), 56.8 (3-, 5-O$\underline{C}H_3$), 56.6 (7-O$\underline{C}H_3$); EI/MS *m/z* 284 $[M]^+$ (100), 269 (25), 241 (15), 226 (10), 183 (10), 142 (9), 77 (5)

Plant materials and sample preparation for HPLC analysis – *D. opposita, D. batatas* and *D. japonica* were collected from different regions (Ham-An and Jin-Ju) in Korea, and identified by Prof. Gwang Jin Chang, the Korea National Agricultural College. All samples were freeze-dried and pulverized to give fine powder. Fine powder (5 g) of each Dioscorea Rhizoma sample was added to methanol (200 mL) and extracted by ultrasonication for 60 min at room temperature and filtered. The

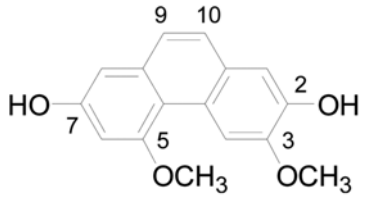
filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in methanol (10 mL), and the solution was filtered through 0.22 μm Millipore membrane filter and stored at 4 °C prior to HPLC analysis.

Calibration – Sequential diluted solutions of standard marker mixture (0.5, 1.0, 5.0 and 10.0 μg/mL) were tested to obtain the linearity of calibration curve. Each standard solution was injected five times consecutively. The calibration curves were achieved by plotting the ratio of peak area versus concentration (μg/mL). The linearity was obtained by linear regression analysis calculated by the least square regression. The limit of detection (LOD) was calculated as signal-to-noise (S/N) ratio of 3, while the limit of quantification (LOQ) was defined as S/N ratio of 10 for each column.

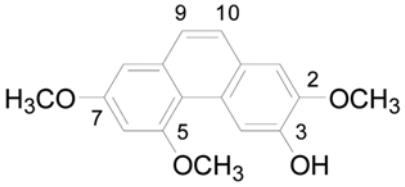
Validation of HPLC method – The precision of this HPLC method was obtained by intra- and inter-day variations. The intra-day precision was evaluated by analyzing the results of five consecutive injections of sequential diluted solutions of standard marker mixture (0.5 - 10.0 μg/mL) as described above, while inter-day variation was performed by five consecutive injections of sequential diluted solutions of standard marker mixture on five different days. The precisions were expressed by the calculation of relative standard deviations [RSD (%) = (SD/mean) × 100%]. The accuracy of the HPLC method was achieved by performing recovery test. Methanol (blank) and three concentrations (1.0, 5.0 and 10.0 μg/mL) of standard marker mixtures were spiked (each 500 μL) directly to the methanol extract of each Dioscorea Rhizoma sample (500 μL) and analyzed according to described method. The recoveries were calculated as: Recovery (%) = EC/TC × 100 (%) where TC and EC are theoretical concentration (μg/mL) and experimental concentration (μg/mL) of standard markers, respectively.

Results and Discussions

Isolation and identification of standard marker compounds - In the course of searching for standard marker compounds in Dioscorea Rhizoma with HPLC-UV-DAD, two major peaks with retention times at t_R 4.40 and 7.81 min were observed from three different Dioscorea Rhizoma samples (*D. opposita*, *D. batatas* and *D. japonica*). Therefore, compounds corresponding two peaks were isolated from *D. opposita*, and their chemical structures were identified as 3,5-dimethoxyphenanthrene-2,7-diol (DMP, **1**) and batatasin-I (BA-I, **2**) by comparison of their spectroscopic data (^{1}H- ,^{13}C-NMR and MS) with those of published literature values (Fig. 1) (Coxon, *et al*., 1982; Leong, *et al*., 1997; Takasugi, *et al*., 1987). Isolated DMP and BA-I from *D. opposita* displayed maximum UV absorption at 260 nm, and two peaks from *D. batatas* and *D. japonica* showed its λ_{max} value at 260 nm on UV spectra (Fig. 2). Various analytical conditions (columns, solvent conditions, flow rates) were evaluated to obtain rapid and reliable HPLC method for determining DMP and BA-I. Among the tested methods, the best HPLC condition was selected. HPLC chromatograms were recorded with UV wavelength at 260 nm in order to achieve the optimum sensitivity and precision for DMP and BA-I.



3,5-Dimethoxyphenanthrene-2,7-diol (**1**)



Batatasin-I (**2**)

Fig. 1. Chemical structures of standard maker compounds for Dioscorea Rhizoma.

Calibration, LOD and LOQ – Good linearity was achieved in the range from 0.5 to 10.0 μg/mL for DMP and BA-I mixture. The regression equation were $y = 94.196x + 61633$ for DMP and $y = 156.33x + 283.74$ for BA-I, where y is the peak areas, and x (μg/mL) is the concentration of standard marker compounds. The correlation coefficients were $R^2 = 0.9999$ for DMP and $R^2 = 1$ for BA-I (Fig. 3). The LOD and LOQ values were evaluated as 0.15 and 0.45 μg/mL for DMP, and 0.1 and 0.35 μg/mL for BA-I. The retention time were t_R 4.40 ± 0.04 min for DMP and 7.81 ± 0.09 for BA-I.

Validation of HPLC method – The intra-day precisions (expressed as RSD) were ranged from 0.80 to 6.00% for DMP, and 0.10 to 4.17% for BAT, while inter-day precisions were 0.10 - 13.04% for DMP and 0.20 - 4.17% for BA-I (Table 1). The accuracy (expressed as recovery) of DMP and BA-I were in the range of 100.2 - 101.6% (RSD 1.38 - 3.18%) and 97.2-100.0% (RSD 0.53 - 1.51%) for *D. opposita*, 99.0 - 101.2% (RSD 2.60 - 7.82%) and

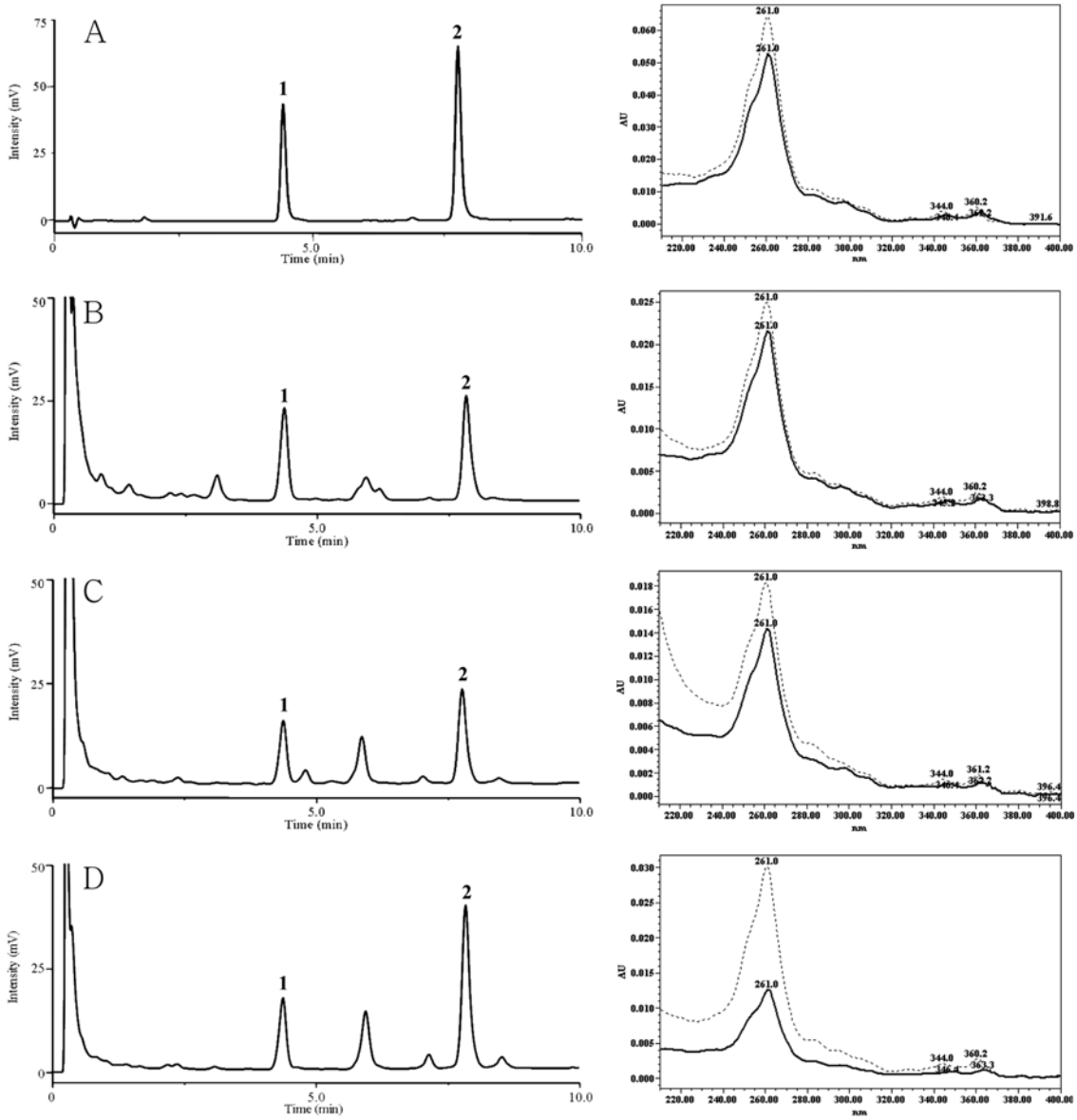


Fig. 2. Chromatograms of (A) mixture solution of DMP (**1**) and BA-I (**2**), (B) *D. oppositа*, (C) *D. batatas* and (D) *D. japonica* extracts monitored at 260 nm by HPLC-UV-DAD and UV spectra of peaks **1** and **2** [solid line, DMP (**1**); dotted line, BA-I (**2**)].

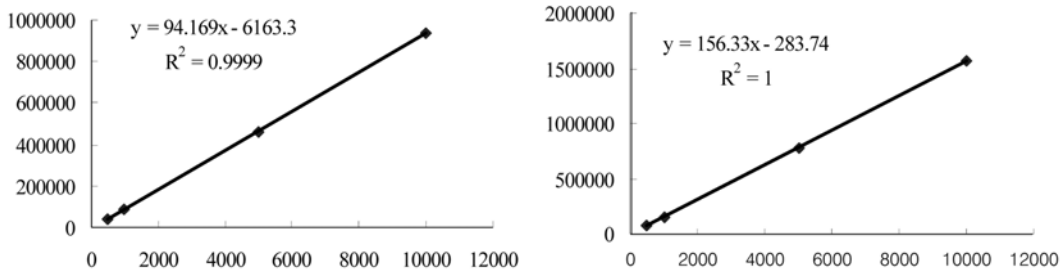


Fig. 3. Calibration curves for DMP and BA-I.

99.5 - 102.8 (RSD 1.79 - 2.03%) for *D. batatas* and 99.1 - 100.6% (RSD 1.32 - 2.91%) and 100.4 - 101.0 (RSD 1.33 - 2.24%) for *D. japonica* (Table 2). These results demonstrated that present analysis method is reliable method.

Quantitative analysis of DMP and BA-I in Dioscorea Rhizoma – The contents of DMP and BA-I in three kinds of Dioscorea Rhizoma were determined by above HPLC method. The contents were in the range at 10.46 - 20.60 μg/g (0.0010 - 0.0021%) for DMP while BA-I levels were at 12.52 - 18.96 μg/g (0.0012 - 0.0019%) (Table 3). These results are similar to those of previously published literature (Ireland, *et al*., 1981) for BA-I, but DMP contents were about fivefold higher in present study. It have been reported that batatasins were native dormancy-inducing substances in yam bulbils and they were induced by a plant hormone such as gibberellin (Hasegawa, *et al*., 1974). The environmental stimuli such as light and temperature, harvesting time and storage conditions might affect endogenous hormonal change (Girardin, *et al*., 1998, Phillips., 1998), which resulted in the possible differences of contents for batatasins.

Table 1. Intra-day and Inter-day variations of DMP[a] and BA-I[b]

Sample	TC[c]	EC[d]	RSD[e]	Ac[f]
DMP[a]	Intra-day (n =5)			
	0.5	0.50 ± 0.03	6.00	100.0
	1.0	0.98 ± 0.04	4.08	98.0
	5.0	5.03 ± 0.10	1.99	100.6
	10.0	9.99 ± 0.08	0.80	99.9
	Inter-day (n = 5)			
	0.5	0.51 ± 0.02	3.92	102.0
	1.0	0.99 ± 0.02	2.02	99.0
	5.0	4.98 ± 0.03	0.60	99.6
	10.0	10.01 ± 0.02	0.20	100.1
BA-I[b]	Intra-day (n = 5)			
	0.5	0.46 ± 0.06	13.04	92.0
	1.0	0.97 ± 0.06	6.19	97.0
	5.0	5.14 ± 0.02	0.39	102.8
	10.0	9.93 ± 0.01	0.10	99.3
	Inter-day (n = 5)			
	0.5	0.48 ± 0.02	4.17	96.0
	1.0	0.99 ± 0.02	2.02	99.0
	5.0	5.04 ± 0.05	0.99	100.8
	10.0	9.98 ± 0.02	0.20	99.8

[a]3,5-Dimethoxyphenanthrene-2,7-diol; [b]Batatasin-I; [c]Theoletical concentration (μg/mL); [d]Experimental concentration (μg/mL); [e]Relative standard deviation = SD/mean × 100 (%); [f]Accuracy = EC/TC × 100 (%)

Summary

Quantitative analysis of two phenanthrene derivatives, 3,5-dimehtoxyphenanthrene-2,7-diol (**1**) and batatasin-I

Table 3. Contents of DMP and BA-I in Dioscorea Rhizoma samples (n = 5)

Samples	Amounts of standard makers (μg/g dry weight)	
	DMP[a] (%)	BA-I[b] (%)
D. opposita	20.60 ± 0.40 (0.0020%)	12.76 ± 0.04 (0.0013%)
D. batatas	10.36 ± 0.68 (0.0010%)	12.52 ± 0.44 (0.0013%)
D. japonica	16.04 ± 0.20 (0.0016%)	18.96 ± 0.12 (0.0019%)

[a]3,5-Dimethoxyphenanthrene-2,7-diol; [b]Batatasin-I

Table 2. Recovery of DMP and BA-I added to Dioscorea Rhizoma samples (n=5)

Sample	DMP[a]					BA-I[b]			
	ASC[c]	TC[d]	EC[e]	R[f]	RSD	TC	EC	R	RSD
D.opposita	Blank (MeOH)	(5.15)[g]	5.15 ± 0.10	–	1.96	(3.19)[g]	3.19 ± 0.01	–	0.31
	0.5	5.65	5.66 ± 0.18	100.2	3.18	3.69	3.69 ± 0.04	100	1.08
	2.5	7.65	7.68 ± 0.14	100.4	1.82	5.69	5.61 ± 0.03	98.6	0.53
	5.0	10.15	10.31 ± 0.12	101.6	1.38	8.19	7.96 ± 0.12	97.2	1.51
D.batatas	Blank (MeOH)	(2.59)[g]	2.59 ± 0.17	–	6.56	(3.13)[g]	3.13 ± 0.11	–	3.51
	0.5	3.09	3.07 ± 0.24	99.4	7.82	3.63	3.66 ± 0.12	100.8	3.28
	2.5	5.09	5.04 ± 0.17	99.0	3.37	5.63	5.60 ± 0.10	99.5	1.79
	5.0	7.59	7.68 ± 0.20	101.2	2.60	8.13	8.36 ± 0.17	102.8	2.03
D.japonica	Blank (MeOH)	(4.01)[g]	4.01 ± 0.05	–	1.25	(4.74)[g]	4.74 ± 0.03	–	0.63
	0.5	4.51	4.47 ± 0.11	99.1	2.46	5.24	5.26 ± 0.07	100.4	1.33
	2.5	6.51	6.53 ± 0.19	100.3	2.91	7.24	7.32 ± 0.11	101.1	1.50
	5.0	9.01	9.06 ± 0.12	100.6	1.32	9.74	9.84 ± 0.22	101.0	2.24

[a]3,5-Dimethoxyphenanthrene-2,7-diol; [b]Batatasin-I; [c]Amount of added standard compound (μg/mL); [d]Theoletical concentration (μg/mL); [e]Experimental concentration (μg/mL); [e]Recovery = EC/TC × 100 (%); [f]Relative standard deviation = SD/mean × 100 (%); [g]Mean value of EC of blank was used as value of TC of blank

(**2**), in Dioscorea Rhizoma was accomplished by HPLC-UV-DAD. To our knowledge, this is the first paper to report HPLC method of non-polar marker compounds in Dioscorea Rhizoma although a number of papers have been reported that batatasins are representative constituents of Dioscorea Rhizoma. The present results demonstrated that DMP and BA-I could be standard marker compounds in Dioscorea Rhizoma. The introduced HPLC method is simple, rapid and reliable, and it could be used for quality control of various *Dioscorea* preparations together with HPLC methods to determine polar substances such as saponins and purine derivatives.

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