Analyst

RSC Publishing

PAPER

Highly efficient sample preparation and quantification of constituents from traditional Chinese herbal medicines using matrix solid-phase dispersion extraction and UPLC-MS/MS†

Cite this: *Analyst*, 2013, **138**, 2279

Xiao-Lan Cheng,‡ Lian-Wen Qi,‡ Qi Wang,‡ Xin-Guang Liu, Besma Boubertakh, Jin-Yi Wan, E-Hu Liu* and Ping Li*

In this work, a rapid and simple method based on matrix solid-phase dispersion (MSPD) and ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was developed. Guge Fengtong preparation (GGFT), a traditional Chinese herbal medicine, was investigated for validation, and eight major constituents were determined including four saponins (protodioscin, protogracillin, pseudoprotodioscin and dioscin) and four gingerols (6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol). Response surface methodology and desirability function were employed to optimize the extraction conditions, such as dispersant, dispersant/sample ratio, solvent concentration, and elution volume, of MSPD. Results showed that MSPD using C_{18} (1.75 g) as the dispersant material and methanol (89%, v/v) as the eluting solvent (12.00 mL) resulted in a high extraction efficiency. MSPD extraction had the advantages of combining extraction and clean-up in a single step, was less time consuming and required lower solvent volumes compared with conventional methods. Quantification of chemical compounds from GGFT preparations were performed using UPLC-MS/MS in multiple-reaction monitoring mode. The proposed method afforded a low limit of detection ranging from 0.02 to 0.40 ng for saponins and gingerols. For all the analytes, recoveries ranged from 80.9% to 103% and repeatabilities were acceptable with relative standard deviations of less than 6.81%. The proposed MSPD-UPLC-MS/MS method was successfully utilized to analyze five batches of GGFTs, and the results demonstrated that this method is simple, efficient and has potential to be applied for the quality control of herbal preparations.

Received 22nd November 2012
Accepted 28th January 2013

DOI: 10.1039/c3an36732k

www.rsc.org/analyst

1 Introduction

Herbal medicines (HMs) and their preparations, with mild healing effect and lower side effects, have been widely used for thousands of years in many oriental countries, such as in China, Korea, and Japan. In the past decades, with a rapid increase in developing multicomponent therapeutics for complex diseases, HMs have been receiving ever-increasing interest and attention in both academic and industrial fields.[1–3] Many pharmaceutical companies have renewed their strategies in favor of herbal drugs. Therefore, the time seems to be ripe for botanicals of better quality. Nevertheless, it is well known that most HMs and their derivative products are prepared from one or several plant extracts, and are complicated multi-component systems with hundreds or even thousands of chemical components.[4] Moreover, HMs often contain a large amount of proteins, pigments, sugars, and tannins, which in some cases, do not contribute to the pharmaceutical effects. Herein, owing to the complicated matrix and low levels of active compounds, the quality control of HMs has long been regarded as a challenging task for scientists.

Sample preparation is a crucial step to ensure the efficiency of analytical procedures, especially in the analysis of trace compounds in HMs. Efficient sample preparation depends on the matrix, as well as the properties and concentration of the analyte.[5] The sample preparation steps typically include homogenization, extraction, clean-up and concentration, followed by the final analysis. Conventional extraction methods, such as ultrasonic extraction,[6] soxhlet extraction,[7] heat-reflux extraction[8] are commonly employed. These methods, however, are usually time- and solvent-consuming and require additional clean-up or filtration steps.[9] In recent years, research has been focused on advanced sample preparation methods which allow for the elimination of additional sample clean-up and

State Key Laboratory of Natural Medicines (China Pharmaceutical University), No. 24 Tongjia Lane, Nanjing 210009, China. E-mail: liping2004@126.com; liuehu2011@163.com; Fax: +86 25 8327 1379; Tel: +86 25 8327 1382

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3an36732k

‡ These authors contributed equally to this work.

pre-concentration steps, reduced use of organic solvents, exclusion of sample component degradation, improvement of extraction efficiency, selectivity, and/or kinetics. Matrix solid-phase dispersion (MSPD), which enables the simultaneous accomplishment of both extraction and clean-up steps, has been demonstrated to be an attractive alternative to sample preparation of complex matrices.[10] MSPD is achieved mainly through the dissolution and dispersion of the organic phase bound to the sorbent, instead of solvent extraction, and thus it consumes far less organic solvent and requires a shorter extraction time compared with conventional extraction methods. The application of MSPD has shown satisfactory results in the extraction of pesticides and veterinary drug residues in food,[11,12] fatty vegetable matrices,[13] olives and olive oil,[14] and active compounds from plant materials.[15,16] The use of MSPD for the extraction of constituents in HMs has not been well explored.

Liquid chromatography-mass spectrometry (LC-MS) has now been widely accepted to be the predominant tool for the qualitative and quantitative analysis of HMs, because of its advantages in sensitivity and selectivity.[17–19] Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using a triple quadrupole instrument operated in the multiple reaction monitoring (MRM) mode is increasingly utilized for the determination of targeted constituents in HMs and food by official and routine laboratories.[20] The aim of this work was to develop and validate a simple and effective analytical procedure that combines MSPD and UPLC-MS/MS for the determination of major constituents in traditional Chinese medicines. Guge Fengtong preparations (GGFTs), composed of Dioscorea Nipponica Rhizoma, Spatholobi Caulis and Zingiberis Rhizoma, was used as a case study to validate the developed method. A multivariate chemometric approach, response surface methodology (RSM), was employed to optimize the crucial parameters of the MSPD extraction conditions. The results obtained in this work indicated that the MSPD-UPLC-MS/MS method enables rapid, simple, and selective assay and is useful for high-throughput analysis of multiple constituents in commercial HMs and their preparations.

2 Experiment

2.1 Chemicals and materials

Analytical grade methanol used for sample preparation was purchased from Nanjing Chemical Reagent Factory (Nanjing, China). Water was purified with a Milli-Q system (Milford, MA, USA). Acetonitrile and formic acid, both of MS grades, were provided by Merck (Darmstadt, Germany). Dispersants tested for MSPD including C_{18}, silica gel, multi-walled carbon nanotubes (MWCNT, 3–5 nm i.d., >233 m^2 g^{-1}), carboxyl modified multi-walled carbon nanotubes (COOH-MWCNT, 3–5 nm i.d., >233 m^2 g^{-1}, COOH-content (weight): 2.56%) and primary/secondary amine (PSA) were all obtained from Shanghai Welch Materials Co. Ltd. (Shanghai, China).

The reference standards of protodioscin, protogracillin, pseudoprotodioscin, dioscin were obtained from Beijing Emilion Science & Technology Co., Ltd (Beijing, China). Purified compounds 6-gingerol, 8-gingerol, 6-shogaol and 10-gingerol were purchased from Chengdu Master Biotechnology Co., Ltd (Sichuan, China). The purities of the standards (Fig. 1) were all higher than 98%. The GGFTs were collected from two pharmaceutical companies in China: Xiuzheng Pharmaceutical Co., Ltd. (GGFT tablets, samples 1–3) and Shandong Xinqi Pharmaceutical Co., Ltd. (GGFT capsules, samples 4–5).

2.2 Preparation of standard solutions

Each standard compound was accurately weighed and dissolved in methanol to prepare the standard stock solutions. The concentrations of protodioscin, protogracillin, pseudoprotodioscin, dioscin, 8-gingerol, 6-gingerol, 6-shogaol and 10-gingerol were 2.17 mg mL^{-1}, 1.64 mg mL^{-1}, 1.68 mg mL^{-1}, 2.50 mg mL^{-1}, 2.87 mg mL^{-1}, 1.40 mg mL^{-1}, 2.50 mg mL^{-1} and 2.62 mg mL^{-1} respectively. Working standard solutions were prepared by diluting the standard solution with methanol to provide a series of standard solutions in order to make the calibration curve. The mixture of the reference compounds stock solution was also prepared, and all the solutions were stored at 4 °C in a refrigerator.

2.3 Sample preparation and extraction

2.3.1 MSPD extraction. The GGFTs were triturated to a fine powder by use of a pestle and mortar. About 0.5 g of sample, 0.5 mL of water and 1.5 g of dispersant were placed in the agate mortar. The sample and the dispersant were blended with the pestle to obtain an homogenous mixture. Once completely dispersed, the mixture was transferred into a column with a layer of absorbent cotton at the bottom. A thin layer of absorbent cotton was then added at the top of the sample mixture. The column was eluted with 10 mL of methanol, and the target analytes were eluted out and collected in a 10 mL volumetric flask. A 1 mL aliquot of eluate was centrifuged at 13 000 × *g* for 10 min before their injection into the LC system.

2.3.2 Heat-reflux extraction (HRE). GGFTs powder (0.5 g) and 80 mL of methanol were put into a 250 mL glass flask. The mixture was heated and refluxed for 2 h. The extract was cooled to room temperature and centrifuged (5000 × *g*, 10 min). The extraction procedure was performed for 2 cycles. Finally, the extracts were combined, dried and transferred into a 10 mL volumetric flask and diluted to the mark with methanol. The resulting sample solution was centrifuged (13 000 × *g*, 10 min) before LC analysis.

2.3.3 Ultrasonic extraction (UE). The sample powder (0.50 g) was accurately weighed and put into a 100 mL conical flask, into which 50 mL of methanol was added. The flask was sonicated for 30 min (two times) using an ultrasonic cleaner (KQ-100DE Kunshan Ultrasonic Instrument Co. Ltd., Kunshan, China). The ultrasonication process was conducted at room temperature and the output power was set at 250 W. Then, the extract was concentrated and diluted to 10 mL with methanol. The resulting extract was sequentially centrifuged at 13 000 × *g* for 10 min and constituted the sample solution for LC analysis.

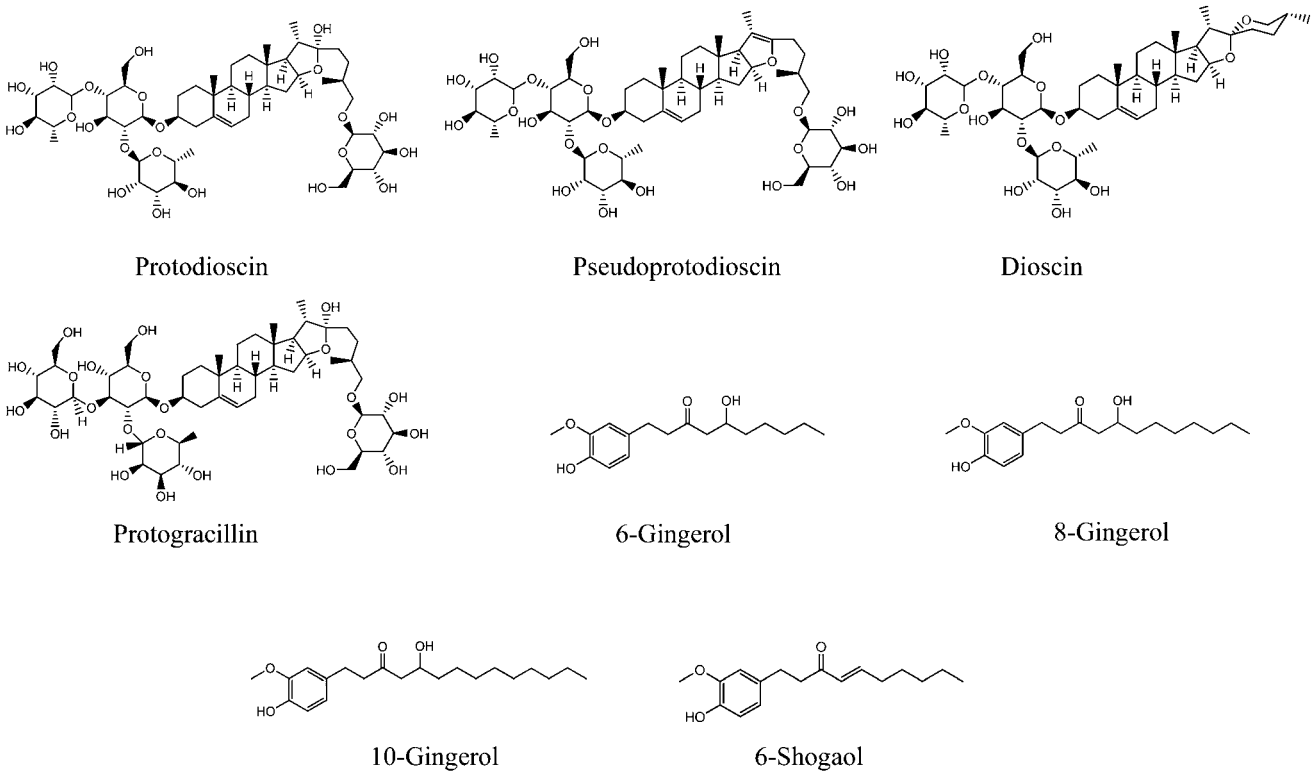

Fig. 1 Chemical structures of the tested compounds in the GGFT preparations determined by UPLC-MS/MS.

Table 1 Analytical factors and levels for RSM, and results of response surface analysis

	Levels		
Independent variables	−1	0	1
Dispersant/sample ratio (g/g, X_1)	1	2.5	4
Methanol concentration (%, v/v, X_2)	50	75	100
Elution volume (mL, X_3)	5	10	15

Results of RSM

Test numbers	X_1	X_2	X_3	Extraction yield/mAu
1	1	−1	0	202
2	0	0	0	535
3	−1	1	0	434
4	−1	−1	0	334
5	−1	0	−1	215
6	1	0	1	547
7	0	1	1	560
8	0	−1	1	127
9	0	0	0	623
10	0	−1	−1	141
11	1	0	−1	284
12	0	0	0	604
13	0	0	0	655
14	0	1	−1	435
15	−1	0	1	513
16	0	0	0	598
17	1	1	0	614

2.4 Analytical methods

The MSPD conditions were optimized by HPLC experiments. The chromatographic analyses were performed with an Agilent 1100 series liquid chromatography (Agilent Technologies, Santa Clara, CA, USA). The analytes were separated on an Extent-C_{18} column (150 mm × 4.6 mm i.d.,5 μm, Agilent Technologies, USA) at 30 °C, using water containing 0.1% formic acid (A) and acetonitrile (B) as mobile phase. The gradient profile was optimized as follows: 0–20 min, 15–30% B; 20–40 min, 30–50% B; 40–55 min, 50–55% B; 55–85 min, 55–100% B. The flow rate of the mobile phase was maintained at 1 mL min^{-1}. The injection volume of the sample solution was 20 μL. The detection wavelength was set at 203 nm and 280 nm. The data acquisition and analysis were performed by Agilent ChemStation software.

Quantitative analysis was carried out on a Waters Acquity UPLC system (Waters, Corp., Milford, MA, USA) coupled to a Xevo Triple Quadrupole MS (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization source. LC separations were achieved on an ACQUITY UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) at 30 °C. The gradient elution program was as follows: starting at 15% acetonitrile–85% water (containing 0.1% formic acid), increasing acetonitrile to 50% in 6 min and 100% in 12 min, keeping constant for 1 min, then decreasing acetonitrile to 15% in 1 min. The flow rate was 0.40 mL min^{-1}. The sample injection volume was 1 μL. MS detection was performed in the MRM mode using the $[M + H]^+$ ion as the precursor. The conditions of MS analysis were as follows: capillary voltage, 3 kV; desolvation gas flow rate, 1000 L h^{-1}; temperature, 550 °C; cone gas flow rate, 50 L h^{-1};

Table 2 Analysis of mean square deviation of regression equation

Source	Sum of squares	d*f*	Mean square	*F* value	*p*-value prob > *F*	Significance
Model	4.929×10^5	9	54 766.39	9.77	0.0033	Significant
X_1	2850.13	1	2850.13	0.51	0.4988	
X_2	1.919×10^5	1	1.919×10^5	34.25	0.0006	Significant
X_3	56 448.00	1	56 448.00	10.07	0.0156	Significant
X_1X_2	24 336.00	1	24 336.00	4.34	0.0756	
X_1X_3	306.25	1	306.25	0.055	0.8218	
X_2X_3	4830.25	1	4830.25	0.86	0.3841	
X_1^2	18 620.00	1	18 620.00	3.32	0.1111	
X_2^2	83 116.84	1	83 116.84	14.83	0.0063	Significant
X_3^2	90 676.05	1	90 676.05	16.18	0.0050	Significant
Residual	39 222.75	7	5603.25			
Lack of Fit	31 468.75	3	10 489.58	5.41	0.0683	Not significant
Pure error	7754.00	4	1938.50			
Cor total	5.321×10^5	16				

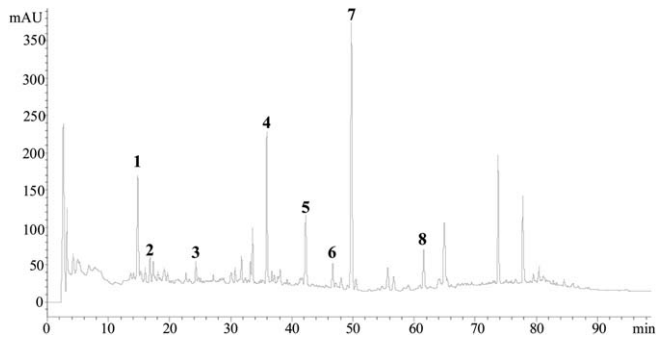

Fig. 2 Representative HPLC chromatogram of GGFT preparations extracted using MSPD (peak 1, protodioscin; peak 2, protogracillin; peak 3, pseudoprotodioscin; peak 4, 6-gingerol; peak 5, dioscin; peak 6, 8-gingerol; peak 7, 6-shogaol; peak 8, 10-gingerol).

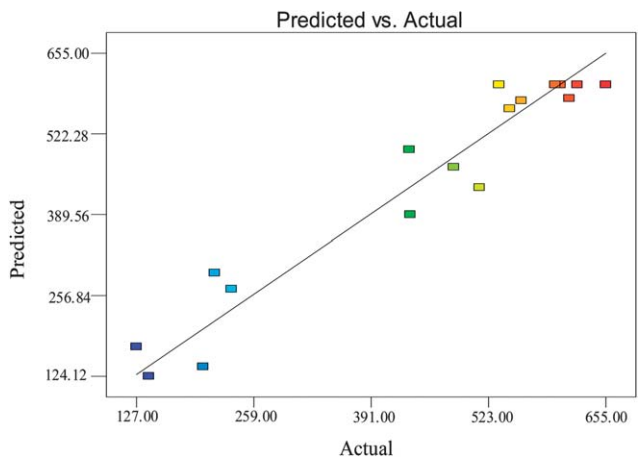

Fig. 3 Graph of actual values *versus* predicted values for extraction yield by MSPD.

source temperature, 150 °C. Both cone voltage (CV) and collision energy (CE) were set to match the MRM of each marker, and the dwell time was automatically set by the Mass Lynx software. The optimized parameters of MRM for quantification are listed in Table 3.

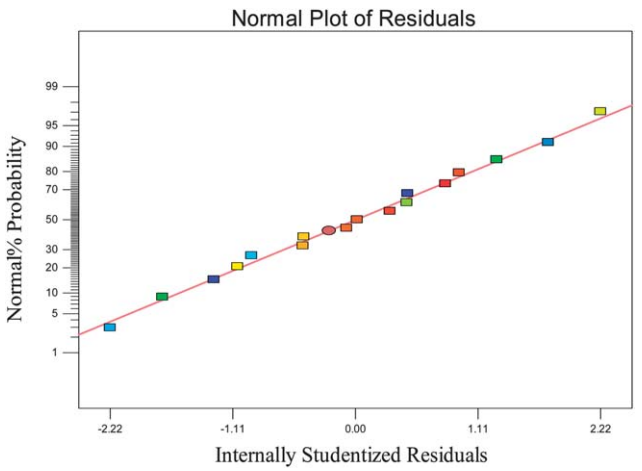

Fig. 4 Normal% probability plot of internally studentized residuals.

2.5 Experiment design to optimize the extraction conditions

The dispersant was firstly optimised by a mono-factor test, later a series of experiments by RSM were designed to optimize the MSPD process. The software Design Expert (Trial Version 7.1.6, Stat-Ease Inc., Minneapolis, MN, USA) was employed for experimental design, data analysis and model building, and a three variable, three-level Box–Behnken Design (BBD) was used. Three variables used in this study were dispersant/sample ratio (1 : 1–4 : 1, X_1), methanol concentration (%, X_2) and elution solvent volume (5–15.0 mL, X_3), with three levels of each variable, while the dependent variable was the extraction yield of GGPTs (expressed as the geometric mean of peak area of all analytes). The coded values of independent variables and their levels for the BBD are summarized in Table 1. The complete design consisted of 17 combinations and five replicates at the central point and was carried out to allow for estimation of a pure error sum of squares (Table 2). The experimental data from BBD were analyzed using response surface regression to fit the following quadratic polynomial model:

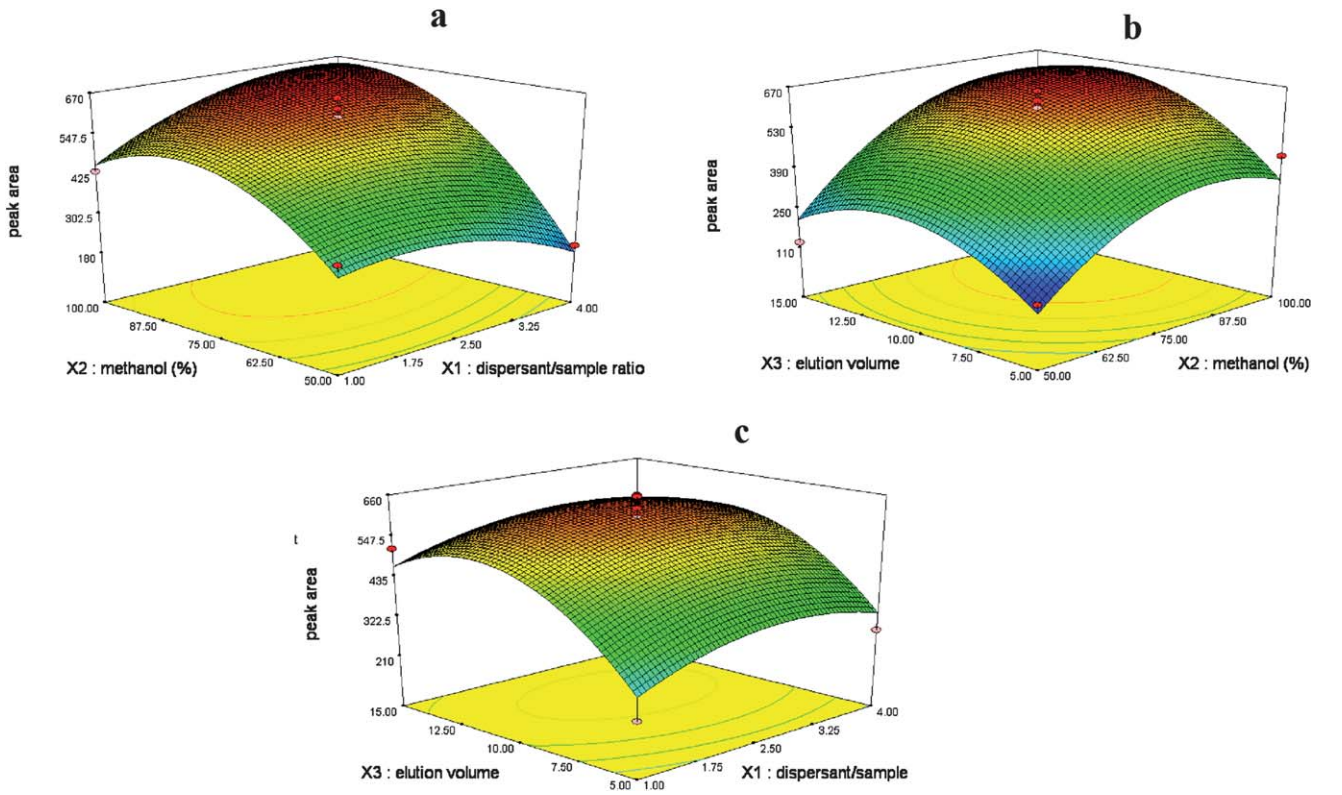

Fig. 5 Response surface and contour plots for the effect of independent variables on: the extraction of target compounds: (a) dispersant/sample ratio and methanol, (b) methanol and elution volume, (c) dispersant/sample ratio and elution volume.

$$Y = \gamma_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_i^2 + \sum_{i \neq j=1}^{3} a_{ij} X_i X_j$$

where Y is the predicted response, γ_0 is a constant and a_i, a_{ii} and a_{ij} are the linear, quadratic and interactive coefficients of the model, respectively. Accordingly, X_i and X_j represent the levels of the independent variables, respectively. The regression analyses, statistical significance and response surfaces were analyzed using Design-Expert 7.1.6 software (Trial Version, State-Ease Inc., Minneapolis, MN, USA). p values less than 0.05 were considered to be statistically significant.

3 Results and discussion

3.1 Optimization of MSPD procedure by RSM

The MSPD process was optimized by HPLC analysis of eight major constituents in GGFTs, *i.e.*, protodioscin, protogracillin, pseudoprotodioscin, dioscin, 6-gingerol, 8-gingerol, 6-shogaol and 10-gingerol. The dispersant plays an important role in the MSPD procedure. Five types of dispersants including C_{18}, silica gel, MWCNT, COOH-MWCNT and PSA were evaluated. The data (see ESI Table 1†) indicated that C_{18} provided the best extraction efficiency. Thus, C_{18} was selected as the MSPD dispersant.

The mono-factor approach is effective for optimization of extraction conditions. However, possible interaction effects between variables cannot be evaluated and misleading conclusions may be drawn. RSM, a collection of mathematical and statistical techniques, can overcome these difficulties and allows for the possible interaction effects of the variables to be accounted for. Therefore, RSM was applied to obtain other optimal parameters of MSPD. The effects of the process variables, dispersant/sample ratio (1 : 1–4 : 1, X_1), methanol concentration (%, X_2) and elution solvent volume (5–15.0 mL, X_3), were further investigated. The response was the geometric mean of the peak area for the analytes evaluated by HPLC. The HPLC profile in Fig. 2 shows good separation of the target compounds in the GGFT extracts under the system. Multiple regression analysis was performed to predict coefficients of the model, and three-dimensional (3D) surface response plots were generated according to the predicted model to visualize the relationship between the process variables and responses.

3.1.1 Fitting the response surface models. The BBD matrices in coded and un-coded forms and experimental values for the geometric mean of peak area for analytes are presented in Table 1. Employing multiple regression analysis on the experimental data of BBD, the predicted model was established by the following modified quadratic polynomial function:

$$Y = 603.00 + 18.88 \times X_1 + 154.88 \times X_2 + 84.00 \times X_3 + 78.00 \times X_1 \times X_2 - 8.75 \times X_1 \times X_3 + 34.75 \times X_2 \times X_3 - 66.50 \times X_1^2 - 140.50 \times X_2^2 - 146.75 \times X_3^2$$

where Y was the response, and X_1, X_2 and X_3 respectively corresponded to the coded values of the three independent variables dispersant/sample ratio, methanol concentration and elution volume.

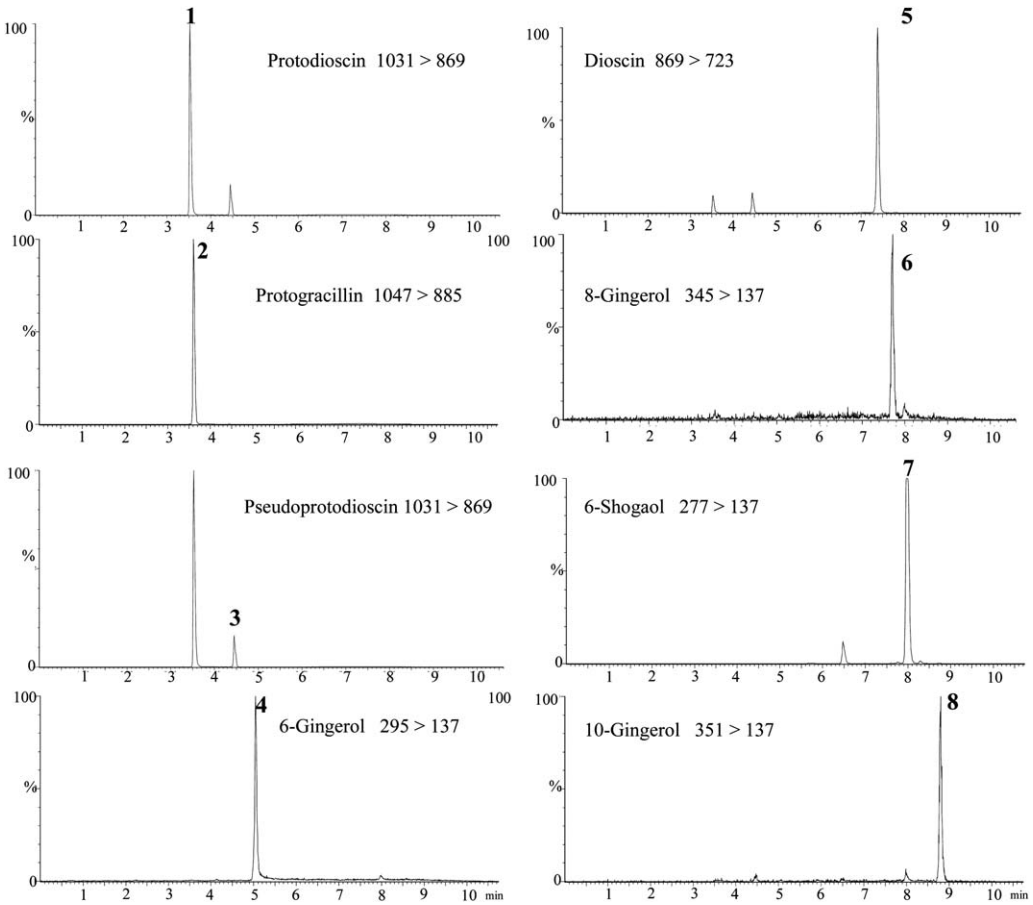

Fig. 6 The UPLC-MS/MS analysis MRM chromatogram of target compounds: 1, protodioscin; 2, protogracillin; 3, pseudoprotodioscin; 4, 6-gingerol; 5, dioscin; 6, 8-gingerol; 7, 6-shogaol; 8, 10-gingerol.

To test the significance and adequacy of the model, analysis of variance (ANOVA) is required by using the *F*-test. The ANOVA results suggest that the model had a high model *F* value and a very low *p* value for responses ($p = 0.0033$, less than 0.05), indicating the significant fitness of the model (Table 2). The coefficient of determination (R^2), defined as the ratio of the explained variation to the total variation, is also a measurement of the degree of fitness. In this work, the R^2 value ($R^2 = 0.9263$) of the regression model was satisfactory to validate the significance of the model. Besides, the lack of fit, which evaluates the failure of the model to represent the data in the experimental domain points, was insignificant for the response with *p*-value of 0.0683 (>0.05). This implied that the model equation was adequate. Based on the error analysis, it was observed that the predicted values were very close to the practical values (Fig. 3), and errors were normally distributed and insignificant (Fig. 4). All these results confirmed that the model was well fitted, and was appropriate to make precise estimations in the studied experimental area.

Table 3 MRM conditions used for UPLC-MS-MS determination of active compounds in GGFTs[a]

Compounds	Retention time/min	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Cone/V	Collision energy/eV
Protodioscin	3.51	1031 $[M - H_2O + H]^+$	869	65.0	34.0
Protogracillin	3.59	1047 $[M - H_2O + H]^+$	885	68.0	35.0
Pseudoprotodioscin	4.43	1031 $[M + H]^+$	869	68.0	38.0
6-Gingerol	5.03	295 $[M + H]^+$	137	28.0	20.0
Dioscin	7.39	869 $[M + H]^+$	723	66.0	32.0
8-Gingerol	7.69	323 $[M + H]^+$	137	30.0	22.0
6-Shogaol	7.96	277 $[M + H]^+$	137	32.0	25.0
10-Gingerol	8.79	351 $[M + H]^+$	137	40.0	36.0

[a] Bold face transitions were used to quantify.

Table 4 Linear regression data, LOD, LOQ, precision and recoveries of eight active components in GGFTs by MSPD extraction

Analyst	Regression equations	R^2	Linear range/μg	Inter-day precision ($n = 5$, %)	Intra-day precision ($n = 6$, %)	LOD/ng	LOQ/ng	Repeatability (RSD%, $n = 5$)	Recovery			
									Original/μg	Spiked/μg	Found/μg	Recovery (%)
Protodioscin	$y = 3 \times 10^6x + 11\,869$	0.9982	0.0217–2.17	0.64	1.14	0.08	0.43	5.45	235	147.5	248.9	93.9
										260	481	94.6
										542.5	740	93.1
Protogracillin	$y = 3 \times 10^6x - 56\,299$	0.9987	0.0082–0.82	1.59	0.32	0.03	0.54	6.81	32.5	19.7	50	88.9
										41	69.3	89.8
										65.6	91.7	90.3
Pseudoprotodioscin	$y = 968\,100x + 627.41$	0.9969	0.00168–0.336	5.67	6.29	0.40	1.60	5.52	5	3.4	7.8	83.9
										7.5	12	92.7
										25.2	28.7	94.3
6-Gingerol	$y = 342\,984x + 13\,541$	0.9906	0.00287–0.574	1.30	0.76	0.20	0.57	6.63	20.2	7.2	26.4	85.3
										23	39.5	83.6
										57.4	74.3	94.2
Dioscin	$y = 2 \times 10^6x + 324\,681$	0.9912	0.025–2.50	0.56	0.92	0.18	0.50	4.04	207.5	125	324	93.4
										200	414	103
										500	722	102.8
8-Gingerol	$y = 2 \times 10^6x + 4202.5$	0.9994	0.007–0.70	3.79	1.38	0.07	0.28	6.55	2.3	2.8	4.5	80.9
										7	8.5	88.9
										19.6	19.3	87
6-Shogaol	$y = 2 \times 10^7x + 1 \times 10^6$	0.9945	0.0125–1.25	0.33	1.20	0.02	0.05	4.03	66.5	37.5	102.6	96.3
										57.5	122	96.4
										150	209.2	95
10-Gingerol	$y = 586\,904x + 16\,138$	0.9923	0.00262–0.262	1.17	0.82	0.026	0.08	1.13	6.3	3.9	9.7	88.8
										9.4	14.4	86.5
										23.6	25.9	83.3

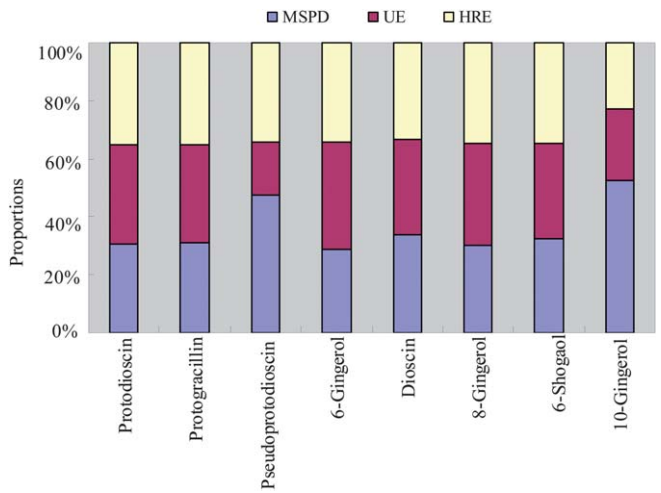

Fig. 7 100% stacked column chart for the comparison of extraction efficiency of MSPD, UE and HRE.

From the above model and Table 2, it was also seen that the factor with the largest effect on the MSPD extraction efficiency was the linear term of methanol concentration ($p < 0.05$), followed by quadratic term of elution volume ($p < 0.05$), and the quadratic term of methanol concentration ($p < 0.05$). The linear term of elution volume also had high significant effect on the yield. The data suggested that methanol concentration and elution volume were the critical variables for extraction of target constituents from GGFTs by MSPD.

3.1.2 Analysis of the response surface. In order to provide a better visualization of the effect of the independent variables on the extraction yield, 3D response surface curves of the model are given in Fig. 5. The effects of two factors on the response are shown at one time, while the third factor was kept at zero in all cases. Fig. 5a showed the 3D plot of the response surface for the extraction yield of GGPTs as related to dispersant/sample ratio and methanol concentration. It was observed that the response rose with increasing methanol concentration. When the methanol concentration reached higher levels, the response slightly decreased. The hydrophobicity of the tested saponins is relatively lower compared with other analytes, resulting in a slight decrease in extraction efficiency under higher methanol concentration. With respect to dispersant/sample ratio, the influence of this independent variable was not as significant as that of methanol concentration. No obvious effect of dispersant/sample ratio on response change was observed, as was in agreement with the results of Zhang *et al.*[21] Fig. 5b presents the interaction of elution volume and methanol concentration. With increasing elution volume, the extraction yield rose at first, but once the volume amount reached high levels, the response did not change significantly. It was found that the maximum extraction yield was achieved when the methanol concentration was 88.89% (v/v) and the elution volume was 12.22 mL. A similar interaction between elution volume and dispersant/sample ratio (Fig. 5c) on the extraction yield could be easily obtained.

3.1.3 Verification of predictive model. Based on the tested results and model equation, the optimal extraction conditions

Table 5 Comparison of content of analytes detected in commercial GGFT preparations extracted by MSPD ($n = 3$)

	Content (%) (mean ± SD)				
Analyst	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Protodioscin	0.0937 ± 0.0016	0.1017 ± 0.0015	0.04674 ± 0.0003	0.0518 ± 0.0004	0.0321 ± 0.0010
Protogracillin	0.0139 ± 0.0001	0.0153 ± 0.0002	$0.0066 \pm 4.49 \times 10^{-5}$	$0.0094 \pm 6.03 \times 10^{-5}$	$0.0054 \pm 1.51 \times 10^{-5}$
Pseudoprotodioscin	$0.0024 \pm 3.45 \times 10^{-5}$	0.0462 ± 0.0005	0.0428 ± 0.0009	$0.0017 \pm 2.36 \times 10^{-5}$	0.0272 ± 0.0001
6-Gingerol	0.0082 ± 0.0002	0.0085 ± 0.0004	0.0113 ± 0.0004	0.0086 ± 0.0002	0.0068 ± 0.0001
Dioscin	0.0832 ± 0.0008	0.0808 ± 0.0018	0.0413 ± 0.0016	0.0775 ± 0.0015	0.0027 ± 0.0011
8-Gingerol	0.0009 ± 0.0001	$0.0009 \pm 5.53 \times 10^{-5}$	0.0011 ± 0.0001	$0.0001 \pm 2.8 \times 10^{-5}$	$0.0009 \pm 1.03 \times 10^{-5}$
6-Shogaol	$0.0266 \pm 8.56 \times 10^{-5}$	0.0264 ± 0.0001	0.0386 ± 0.0003	0.0274 ± 0.0002	0.0196 ± 0.0001
10-Gingerol	$0.0026 \pm 1.95 \times 10^{-5}$	$0.0022 \pm 8.42 \times 10^{-5}$	0.0025 ± 0.0001	$0.0017 \pm 4.54 \times 10^{-5}$	$0.0015 \pm 1.63 \times 10^{-5}$

predicted by RSM were: dispersant C_{18}, dispersant/sample ratio 3.43, methanol concentration 88.89% and elution volume 12.22 mL. To validate the suitability of the quadratic equation for predicting the optimal response values, a verification experiment was carried out under the adjusted conditions: dispersant C_{18}, dispersant/sample ratio 3.5, methanol concentration 89% and elution volume 12.00 mL. It was shown that the real laboratory values were not significantly different to the predicted values, and were also better than any single factor experiments. Therefore, the extraction conditions obtained by the RSM were not only accurate and reliable, but also had practical value to reflect the expected optimization.

3.2 Optimization of UPLC-MS/MS conditions for quantitative analysis

With respect to the UPLC separation, the gradient was optimized to provide separation among the saponins and gingerols. Two brands of analytical columns, ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm) and ACQUITY HSS T3 (100 mm × 2.1 mm, 1.8 μm) were tested for their suitability. The results showed that the former one resulted in chromatograms with better resolution of adjacent peaks within a shorter time, and thus was selected for quantitative analysis. The MRM chromatograms obtained from the target compounds are depicted in Fig. 6. Efficient separation of protodioscin (3.51 min), protogracillin (3.59 min), pseudoprotodioscin (4.43 min), dioscin (7.39 min), 6-gingerol (5.03 min), 8-gingerol (7.69 min), 6-shogaol (7.96 min) and 10-gingerol (8.79 min) was achieved within 10 min. All the analytes were well separated using a mobile phase consisting of acetonitrile and water with 0.1% formic acid.

To quantify the eight major constituents in GGFT preparations, MS analysis was studied in both positive and negative ion modes. The chemical structures of eight analytes were characterized based on their retention behavior, quasi-molecular ions $[M + H]^+$ and fragment ions. It was found that the tested compounds had higher sensitivity in the positive ion mode compared to the negative ion mode. Therefore, the positive ion mode was selected. Each standard compound was investigated individually to achieve optimal CV and CE. Under the optimized UPLC-MS/MS conditions, all eight compounds in the GGFTs were identified and quantified. Retention time (t_R) and MS information for each analyte including $[M + H]^+$, quantitative ions, CV and CE are shown in Table 3.

3.3 Method validation

With the aim of verifying the suitability and performance of the MSPD-UPLC-MS/MS method for the determination of saponins and gingerols in GGFTs, method quality parameters, including linearity, intra-day precision, inter-day precision, limits of detection (LODs) and quantification (LOQs), repeatability and recovery were estimated (see Table 4).

The linearity, regression and linear ranges of the eight target compounds were obtained using the external standard method. As shown in Table 4, each calibration curve was linear over the studied concentration ranges with satisfactory correlation coefficients ($R^2 > 0.99$). The results indicated good linearity between concentrations of investigated compounds and their peak areas within the test ranges. The LOD and LOQ were considered as the analyte lowest concentrations that yield a signal-to-noise ratio (S/N) of 3 and 10, respectively. The determined LODs were in the range from 0.02 to 0.40 ng, and LOQs were in the range from 0.05 to 1.60 ng.

Precision was evaluated as intra-day and inter-day precision by measuring % RSD at the concentration used. Intra-day precision was evaluated for each analyte by injecting the same standard solution six times in a single day, and the inter-day precision was assessed by analyzing the same standard solution over three consecutive days. The RSD values across the various concentrations were less than 6.29% for intra-day precision analysis whereas the inter-day precision ranged from 0.56% to 5.67%. Six independent sample solutions of GGFT sample (sample 1) in parallel were determined for evaluation of repeatability. The repeatability RSD values of the eight compounds were less than 6.81%, indicating that the method was reliable and repeatable.

A recovery test was carried out to evaluate the accuracy of the method. Before extraction, accurately known amounts of analytes (high, middle and low) were spiked to approximately 0.5 g of the GGFT sample, and then extracted and analyzed with the described method. The recovery values were calculated by comparison of analyte responses obtained from the samples spiked before and after the extraction at the same concentration level. Each recovery test was carried out in triplicate. The overall

recoveries fell the range of 80.9–103%, with RSD less than 3.52%, indicating that the established method was accurate for the determination of major compounds in GGFTs.

3.4 Comparison of MSPD with traditional extraction methods

Conventional UE and HRE were also investigated for the extraction of the eight components in GGFT samples. The extraction efficiency of MSPD was compared with that of the UE and HRE methods (Fig. 7) and a student's *t*-test was used to statistically compare the contents. It was clear that pseudo-protodioscin failed to be extracted completely by UE, and HRE offered unsatisfactory extraction yield of 10-gingerol. The efficiency provided by MSPD was similar or even better than that obtained by the UE and HRE methods. Beside its efficiency, MSPD also requires the expenditure of smaller amounts of reagents and less extraction time. The total solvent volume utilized by the MSPD method for each assay was less than 15 mL, and the whole extraction procedure took approximately 20 min, which was much shorter than that of UE (60 min) or HRE (240 min). Furthermore, MSPD does not require sophisticated instruments or materials. Considering the extraction efficiency, the expenditure of extraction time and the consumption of solvent, MSPD is an attractive analytical alternative for the desired quick and economic determination of major components from HMs.

3.5 Application

The proposed MSPD-UPLC-MS/MS method was applied to the analysis of major saponins and gingerols in five GGFT samples from different manufacturers or different batches from the same manufacturer. It can be seen that all the eight compounds were eluted within 10 min and clearly detected with baseline separation in MRM mode. The contents were calculated with external standard methods based on the respective calibration curves, and the results are summarized in Table 5.

Among the target analytes, the most abundant saponin and gingerol are protodioscin and 6-shogaol, respectively. The higher amount of 6-shogaol can be explained by the transformation from 6-gingerol during the storage and manufacturing procedure.[22] We also observed that there were significant differences among GGFT samples in terms of individual contents of the investigated bioactive components. It is believed that the factors including plant origins, sources, cultivated year, harvest time, geographical climate and manufacturing procedure may be responsible for content variations of the bioactive markers. Standardization of planting, harvesting, processing and manufacturing of medicinal herbs is strongly recommended to assure the quality consistency of this commonly used herbal preparation.

4 Conclusions

In this study, for the first time, a rapid, simple and effective MSPD extraction method coupled with UPLC-MS/MS was developed and validated for the simultaneous determination of chemical constituents from the herbal preparation GGFTs. Compared with traditional extraction techniques, MSPD possessed the advantages of lower consumption of sample and organic solvent, a simpler extraction procedure, and a shorter extraction time. The applicability of this MSPD-UPLC-MS/MS method to the simultaneous determination of the major constituents in GGFT samples was demonstrated. The proposed method has potential to be fully exploited in the chemical analysis field to rapidly assess other traditional Chinese herbs and their preparations.

Acknowledgements

This work was supported by a Project of the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2012BAI29B07), the National Natural Science Foundation of China (81202898), the Program for the Top Doctor Innovative Talents of China Pharmaceutical University (2011BPY02) and the Research and Innovation Project for College Graduates of Jiangsu Province (CXZZ11-0809).

References

1 D. Normile, *Science*, 2003, **299**, 188.
2 B. M. Schmidt, D. M. Ribnicky, P. E. Lipsky and I. Raskin, *Nat. Chem. Biol.*, 2007, **3**, 360.
3 R. Verpoorte, D. Crommelin, M. Danhof, L. J. W. J. Gilissen, H. Schuitmaker, J. van der Greef and R. F. Witkamp, *J. Ethnopharmacol.*, 2009, **121**, 479.
4 V. M. Shinde, K. Dhalwal, M. Potdar and K. R. Mahadik, *International Journal of Phytomedicine*, 2009, **1**, 4.
5 S. A. Rodrigues, S. S. Caldas and E. G. Primel, *Anal. Chim. Acta*, 2010, **678**, 82.
6 X. J. Cao, X. M. Ye, Y. B. Lu, Y. Yu and W. M. Mo, *Anal. Chim. Acta*, 2009, **640**, 47.
7 C. W. Halstead, S. Lee, C. S. Khoo, J. R. Hennell and A. Bensoussan, *J. Pharm. Biomed. Anal.*, 2007, **45**, 30.
8 T. M. Hung, M. K. Na, B. S. Min, T. M. Ngoc, I. Lee, X. F. Zhang and K. H. Bae, *Arch. Pharmacal Res.*, 2007, **30**, 685.
9 H. J. Lee and C. Y. Kim, *Food Chem.*, 2010, **120**, 1224.
10 L. Enríquez-Gabeiras, A. Gallego, R. M. Garcinuño, P. Fernández-Hernando and J. S. Durand, *Food Chem.*, 2012, **135**, 193.
11 A. Hercegová, M. Dömötörová and E. Matisová, *J. Chromatogr., A*, 2007, **1153**, 54.
12 M. D. Marazuela and S. Bogialli, *Anal. Chim. Acta*, 2009, **645**, 5.
13 B. Gilbert-López, J. F. García-Reyes and A. Molina-Díaz, *Talanta*, 2009, **79**, 109.
14 J. F. García-Reyes, C. Ferrer, M. J. Gómez-Ramos, A. Molina-Díaz and A. R. Fernández-Alba, *TrAC, Trends Anal. Chem.*, 2007, **26**, 239.
15 T. Visnevschi-Necrasov, S. C. Cunha, E. Nunes, M. Beatriz and P. P. Oliveira, *J. Chromatogr., A*, 2009, **1216**, 3720.
16 H. J. Xu, X. W. Shi, X. Ji, Y. F. Du, H. Zhu and L. T. Zhang, *Food Chem.*, 2012, **135**, 251.

17 Q. X. Liu, S. Y. Qiu, H. Yu, Y. X. Ke, Y. Jin and X. M. Liang, *Analyst*, 2011, **136**, 4357.
18 C. C. Leandro, P. Hancock, R. J. Fussell and B. J. Keely, *J. Chromatogr., A*, 2006, **1103**, 94.
19 Q. Q. Xing, T. Liang, G. B. Shen, X. L. Wang, Y. Jin and X. M. Liang, *Analyst*, 2012, **137**, 2239.
20 É. Alechaga, E. Moyano and M. Teresa Galceran, *Analyst*, 2012, **137**, 2486.
21 Q. Zhang, D. Cai and J. Liu, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 2809.
22 X. Zhang, W. T. Iwaoka, A. S. Huang, S. T. Nakamoto and R. Wong, *J. Food Sci.*, 1994, **59**, 1338.