Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

Journal Name

RSCPublishing

ARTICLE

An enzymatic protocol for absolute quantification of analogues: application to specific protopanoxadiol-type ginsenosides

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Chang-Jiang-Sheng Lai, [‡] Ting Tan, [‡] Su-Ling Zeng, Lin-Ru Xu, Lian-Wen Qi*, E-Hu Liu* and Ping Li*

Comprehensive quantification of specific class of natural products using liquid chromatography tandem mass spectrometry with greener approaches is of significant importance. In this study, a green protocol was proposed for determining analogues with a small number of reference standards. The protocol was conducted by combining the chromatography-based fraction collection, the calibration curves matrix materials and the identical enzymatic hydrolysate. This strategy could accurately calibrate the concentration ratios and successfully calculate the relative response factors (*F*) of the specific protopanoxadiol-type ginsenosides. The recoveries of compounds in fractions were monitored by the modified non-standard recovery evaluation strategy instead of the commonly used method with total reference standards. Coupled with the calculated *F*, the quantitative analysis of multi-components with a single marker was successfully applied to simultaneously determine the analogues of interest in Sanqi (*Panax notoginseng*) extract. The use of snailase for quantification of ginsenosides especially in application to the commercially unavailable ginsenoside Rd isomer was reported for the first time. Consuming less solvent and fewer reference standards, the developed green protocol facilitates its application to other analogues in herbal and food samples by utilizing suitable enzymes or derivative techniques.

Introduction

Natural products have been used as an abundant, productive and low-cost source for food and pharmaceutical industries.[1-4] The same class of natural products always contain similar chemical skeletons, such as glycerides[5, 6] and glycosides (flavonoids[1, 7] or saponins[8-10]). With the development of sophisticated methods, hundreds of analogues are discovered in one herbal or food sample.[9] A large number of them exert nutritional and pharmacological activities or toxic effects.[2, 11] Currently, the traditional quality control mode with single marker is gradually being replaced with the multi-components.[5, 12-14] However, lack of a complete profile of reference standards hampers the determination of each compound.[15] Therefore, development of a greener protocol with a small number of reference standards for accurate quantification of the specific class of compounds is of significant importance.

Compared with conventional acid or base approach,[16] the enzymatic hydrolysis[5, 15] was greener for transforming specific class of compounds into the identical product. The produced compound could be used as the quantitative skeleton for simultaneous absolute quantification of analogues. The protopanoxadiol (PPD)-type ginsenosides have been recognized as one representative class of bioactive glycosides.[8-10] In this study, the specific PPD-type ginsenosides with their identical enzymatic product, compound K (C-K) or PPD, were used for illustrating the standard enzymatic protocol for glycoside analysis (Fig. S1). In order to seek the effective enzyme for selective and complete hydrolysis of the model compounds into PPD or C-K, we screened some glycosidase preparations, including xylanase, accellerase, glucanase, naringinase, snailase[17, 18] and hesperidinase[19]. The snailase was discovered as the first quantitative enzyme preparation for specific PPD-type ginsenosides. Subsequently, we developed a set of green and economical methods to make the proposed protocol be popularized in quantification of analogues.

In direct enzymatic quantification of numerous practical samples, the tedious individual fractions collection and the complete substrates transformation are unavoidable.[5] Therefore, the enzymatic hydrolysis[5, 15, 20] was adopted to calibrate the concentration ratios and to calculate the relative response factors (*F*)[21] for convenient multi-components quantification (Fig. 1). In the quantitative analysis of multi-components with a single marker (QAMS) analyses, calculation of the *F* is important for accurate quantification.[21, 22] However, the

conventional method for calculation of *F* using the slope ratios for the calibration curves of authentic compounds (CCAC)[21] was unsuitable for the analytes with reference standards commercially unavailable. Therefore, we proposed a universal strategy based on the calibration curves matrix materials (CCMM)[23] and the identical enzymatic product[5] (C-K in this work) to calculate the *F* of specific PPD-type ginsenosides. After calibration of the concentration ratios, the *F* for the analytes to the ginsenoside Rd (GRd) using the CCMM were identical to the values determined by the CCAC. The newly proposed strategy not only simplified the QAMS analysis but also achieved the determination of minor compounds and even specific unknown analogues in Sanqi (*Panax notoginseng*) extract.

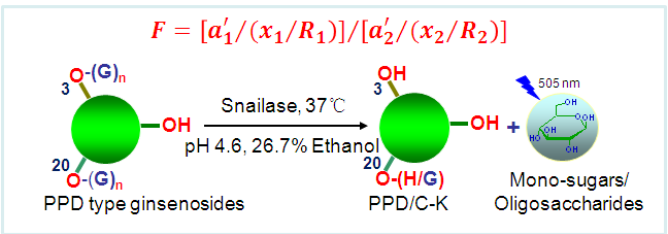


Fig. 1 The scheme for calculation of the relative response factors (*F*) of analogues using the complete enzymatic hydrolysate (compound K, C-K) and the calibration curves matrix materials. The protopanoxadiol (PPD)-type ginsenosides were selected as the model compounds.

A significant advantage of this protocol was that the total reference standards were replaced with collected chromatography-based extract fractions for simultaneous determination. This protocol avoided the time- and organic solvent-consuming isolation and purification of total reference standards. The collected fractions were collected by only single injection of the standard extract. Their losses were comprehensively monitored by the modified non-standard recovery evaluation (NSRE) strategy.[5] For economical quantification of the same class of natural products and minor analogues, this universal protocol was more solvent and standards friendly, and thus more sustainable. The proposed protocol based on the similar chemical skeletons could be widely applied for other types of analogues or *in vivo* conjugated compounds.[1, 15, 24]

Results and discussion

The hydrolysis conditions for PPD-type ginsenosides

The complete enzymatic hydrolysis depended on several factors, such as hydrolysis time, temperature, enzyme-substrate ratio, organic solvents and their volumes. In the initial hydrolysis experiments, 10 μL of 0.1 mg mL^{-1} substrates dissolved in ethanol was incubated with 100 μL of 10 mg mL^{-1} enzyme solutions at 37 °C. After centrifuging at 900 rpm, we observed that these PPD-type ginsenosides linked with saccharide chains at C-20 position, such as GRb_1, GRd and GF_2, could be completely hydrolysed into C-K but not PPD within 3 h. This might be caused by the intra hydrogen bond[25] in the group of C-12 position and the corresponding adjacent sugar moiety (Fig. S2). However, using the above conditions, the ginsenosides Rg_3 and Rh_2 without saccharide chains at C-20 position could not be completely transformed into the predicted product PPD. The low polarity of PPD and the inter hydrogen bond[25] occurred between the group of C-20 and/or C-12 position with the active regions of snailase might be responsible for the low hydrolysis ratios. Subsequently, 10 μL, 20 μL, 30 μL, 40 μL, 50 μL and 60 μL of acetone, methanol, ethanol, isopropanol and *n*-butanol was tested to break the influences of product polarity and substrate inter hydrogen bond, respectively. Ethanol was verified as the favourable solvent to increase their biotransformation ratios. With the increase of ethanol concentration, the hydrolysis equilibrium was broken and the GRg_3 and GRh_2 were completely hydrolysed into PPD within 5 h, but the enzyme hydrolysis activity for other substrates decreased simultaneously. Finally, 40 μL of ethanol was added into the incubation mixtures. To further increase the hydrolysis efficiency, 0.05 mg mL^{-1} substrates was incubated with 10 mg mL^{-1} and 15 mg mL^{-1} of snailase at 25 °C, 37 °C, 50 °C and 60 °C, respectively. The results indicated that 15 mg mL^{-1} of enzyme exhibited satisfactory activities for all substrates in 5 h at 37 °C. The optimized hydrolysis time was shorter than that of the reported β-glucosidase generated by *Aspergillus niger*[26, 27] for completely transforming the GRb_1 into C-K.

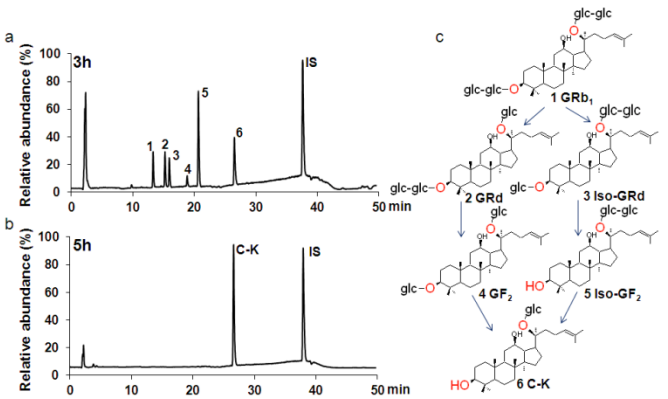


Fig. 2 Enzymatic hydrolysis of ginsenoside Rb_1 (GRb_1) at 3h (a) and 5h (b) and the hydrolysis pathway with successive loss of sugar moiety (c). All marked numbers were consistent.

Incubated for 3 h under the optimized conditions, GRb_1 was hydrolysed into series of intermediate products (Fig. 2a), including some rare products, such as GRd isomer (Iso-GRd, 3) and GF_2 isomer (Iso-GF_2, 5)[9]. At 5 h, the GRb_1 was transformed into C-K completely (Fig. 2b). Therefore, we proposed a successive hydrolysis pathway for GRb_1 (Fig. 2c), which was verified by other similar substrates (GRd and GF_2). In order to confirm the detailed hydrolysis mechanism of snailase, the assays[28] presented in Fig. S3 were used to determine the glucose in the pooled hydrolysed samples including 0.1353 mmol L^{-1} of GRb_1 or GRd enzymatic products. However, no signals were produced, which prompted us to speculate that the random degradation of saccharide chains also occurred. The predicted way with successive loss of

the terminal units[29] from the sugar chains attached at C-3 and C-20 positions was not the only mechanism. The random hydrolysis mechanism of snailase for GRb_1 and GRd was reported for the first time and it was similar to the biotransformation behaviour of *Microbacterium sp. GS514*.[27, 30] Since the snailase and other enzymes[20, 25] were difficult to hydrolyse the produced oligosaccharides into mono-sugars, we ultimately selected the C-K or PPD as the marker for quantification of individual ginsenosides. Notably, some enzyme preparations under other conditions might make the ginsenosides produce the side products, such as hydrolytic [31] or derivative compounds (i.e. ginsenoside Re-β-xylosides)[32].

Table 1 The calibration curve, linear range, LOD, LOQ and precision for the determined hydrolysates (C-K and PPD)

Analyte	Regression equation	R^2	Linear range μg mL^{-1}	LOD μg mL^{-1}	LOQ μg mL^{-1}	Precision (RSD, %)					
						Intra-day			Inter-day		
						High	Med.	Low	High	Med.	Low
C-K	$y = 0.0379x - 0.0743$	0.9950	2.5000 – 50.0000	0.2500	0.7500	3.0	1.2	3.1	2.0	3.7	1.6
PPD	$y = 0.0041x - 0.1046$	0.9918	10.0000 – 500.0000	4.0000	12.5000	2.7	0.2	1.8	3.2	1.6	1.8

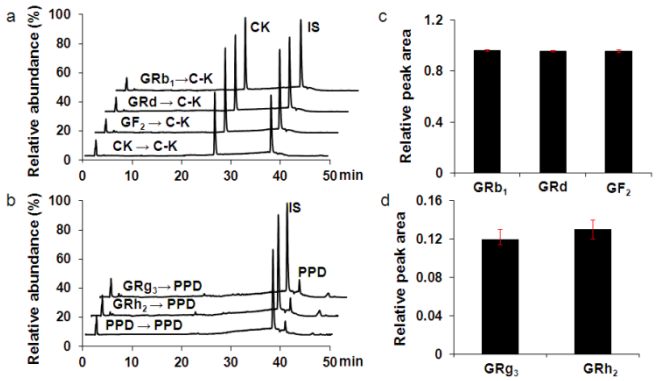


Fig. 3 The quantification potential of the enzymatic hydrolysis. The protopanoxadiol (PPD)-type ginsenosides were completely transformed into compound K (C-K) (a) and PPD (b) within 5 h. Equimolar quantities of substrates could produce the same content of C-K (c) and PPD (d), respectively.

Selection of the C-K or PPD as functional moiety for the absolute quantification

To verify the quantification potential of enzymatic hydrolysis, 10 μL of 0.0451 mmol L^{-1} GRb_1, GRd, GF_2 and C-K (Fig. 3a) as well as 0.0902 mmol L^{-1} GRg_3, GRh_2 and PPD (Fig. 3b) were incubated with the snailase. The tested ginsenosides could produce the same contents of C-K (Fig. 3c) and PPD (Fig. 3d), indicating that this method could completely transform the analytes into the targeted hydrolysates. It also effectively avoided the inevitable C-20 epimers, C-25, 26 hydrated derivatives and C-25, 26 hydroxylated side products occurred in mild acid hydrolysis,[16] alkaline cleavage, or microbial transformation.[33] Since the mass spectrometric response of PPD was weaker than the C-K, the molar concentrations of substrates for producing PPD were set as two folds than the other tested ginsenosides. The $[M-H]^-$ and $[M+HCOO]^-$ ions of C-25, 26 hydroxylated side products[16] were not detected (Fig. S4). PPD (Fig. 4a) and C-K (Fig. 4b) were showed stable under the optimized conditions, further demonstrating that no side reactions occurred.

The calibration curves were prepared by C-K or PPD (Table 1), which corresponded to the class of the PPD ginsenosides with or without sugar chain at C-20 position, respectively. The curves were linear from 2.5000 to 50.0000 μg mL^{-1} for C-K and 10.0000 to 500.0000 μg mL^{-1} for PPD. The correlation coefficients (R^2) were above 0.9918. The limit of detection (LOD) and limit of quantification (LOQ) was determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD of C-K and PPD were 0.2500 μg mL^{-1} and 4.0000 μg mL^{-1}. Their LOQ were 0.7500 μg mL^{-1} and 12.5000 μg mL^{-1}. Their overall intra- and inter-day variations (RSD %) were less than 3.7%. These results indicated that the C-K and PPD could be utilized as the functional moieties for quantifications and their *F* calculations.

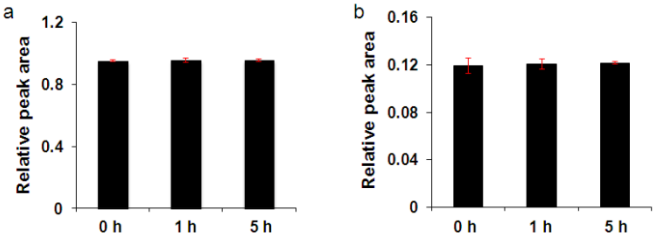


Fig. 4 The stability of compound K (a) and protopanoxadiol (b) incubated under the optimized condition.

Recoveries calculated by non-standard recovery evaluation strategy

In this study, the GRb_1, GRd, Iso-GRd and GF_2 (Fig. 5) were selected to illustrate the protocol for absolute quantification. The protopanaxatriol (PPT)-type and other subgroup of PPD-type ginsenosides were not selected owing to the activity and specificity of snailase for production of C-K or PPD. The snailase could be utilized to quantify all PPD-type ginsenosides containing outer glucose moiety linked with the C-20 position and containing linear β-D-glucosyl units attached at C-3 position. Our observations were consistent with Bgp3.[34] For those trace PPD-type ginsenosides containing the outer α-L-arabinopyranosyl, α-L-arabinofuranosyl and β-D-xylopyranosyl linked with C-20 position, the compound Y, compound Mc and compound Mx might be the preferred markers for absolute quantification, respectively.[34, 35]

Table 2 The calibration curves of the four saponins using the enzymatic hydrolysis and the typical LC-ESI/MS method

Analyte	Regression equation		Correlation coefficient (R^2)		Linear range	
	I[a]	II	I	II	I (mg mL^{-1})	II (μg mL^{-1})
GRb_1	$y = 2.5327x + 0.0283$	$y = 165.8100x + 0.0422$	0.9998	0.9998	0.0200-1.0000	0.0260-65.0000
GRd	$y = 1.3221x + 0.0130$	$y = 216.6700x + 0.0309$	0.9994	0.9992	0.0200-1.0000	0.0285-71.2500
Iso-GRd	$y = 0.2549x + 0.0401$	–	0.9987	–	0.1000-100.0000	–
GF_2	$y = 0.1076x - 0.0579$	$y = 394.5300x + 0.0476$	0.9993	0.9994	0.2000-100.0000	0.0290-72.5000

I: enzymatic hydrolysis; II: typical LC-ESI/MS method; [a], y represents response ratio and x represents concentration of crude medicine; –, not detected

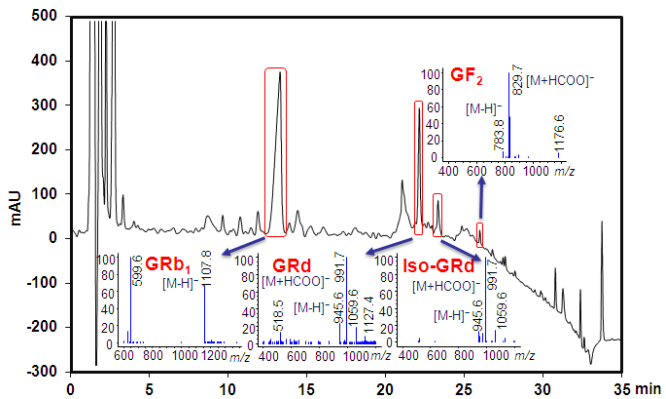


Fig. 5 The scheme for collection of the model compounds using single-injection of standard extract. All collected compounds were identified as ginsenosides Rb_1 (GRb_1), F_2 (GF_2), Rd (GRd) and its isomer (Iso-GRd) by LC-ESI/MS.

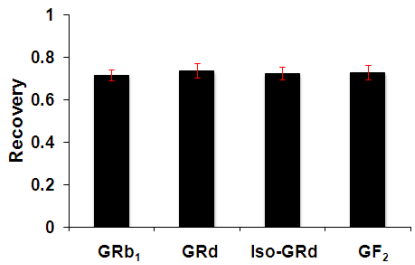


Fig. 6 The collected recoveries calculated by modified non-standard recovery evaluation (NSRE) strategy.

The collection recoveries were calculated by dividing the collected concentrations by the original ones. However, the reported incorrect method[36] using the determined peak area ratios is still applied in LC-ESI/MS analyses, especially for these compounds without the authentic standards. In this study, LC-ESI/MS rather than the LC coupled with evaporative light-scattering detection was employed. The modified NSRE[5] (Eq. 5) was developed to conveniently and universally calculate the collection recoveries for all compounds (Fig. 6). Theoretically, this method could also be applied to LC with ultraviolet detection analysis. In brief, without using the reference standards, the CCMM (Table 2) was established to calculate the concentration ratios for corresponding compounds in Sanqi extract before and after collection. The crude medicine was diluted 1-5000 folds and the MS responses of GRb_1 and GRd (Fig. S5) showed the saturation in 5–100 mg mL^{-1}. Therefore, all the collected samples were diluted 100 folds for accurate analysis. The correlation coefficients of their CCMM were above 0.9987 and their lower limits of quantification were above 0.02 mg mL^{-1}. All the collection recoveries were stable with RSD less than 3.89%, which ensured the following analyses.

Small amounts of collected extract fractions with snailase and C-K for calibration of the concentration ratios

Enzymatic quantification of the targeted compounds in Sanqi extract and calibration of the concentration ratios of the series of analogues in extract were important for F calculation and QAMS analysis, not depending on the availability of chemical standards. The mass concentrations in Sanqi extract (Table 3) were calculated by equimolar conversion[5] of the determined quantities of C-K. For the unknown ginsenosides completely hydrolysed into C-K or PPD, they could be determined by the developed enzymatic approach coupled with the high resolution MS. If some compounds were transformed partially, they could also be quantified by the enzymatic method coupled with NSRE. In practice, one calibration curve could not directly determine all the interest compounds due to the significant content deviation. To overcome this defect, the direct enzymatic incubation, combination of several targeted collected samples or increase of the injection amount could be used. Moreover, the samplings and analyses at multiple time points could be used for simultaneous quantification of numbers of analogues in one collected fraction.

To evaluate the accuracy of enzymatic hydrolysis, the typical LC-ESI/MS was adopted to determine the above selected ginsenosides (Fig. S6). The calibration curves were linear over the set concentration ranges for GRb_1, GRd and GF_2

(Table 2). The R^2 of CACC was almost identical to that of CCMM. Since the pure Iso-GRd was unavailable, its calibration curve, concentration and other parameters were not determined. At the lowest concentration, their S/N ratios were all above 10. Consequently, the concentrations determined by the two methods were closely matched (Table 3), which verified that the snailase was the first reported enzyme preparation for quantitative determination of the specific PPD-type ginsenosides. After calibration of their concentration ratios, the developed enzymatic hydrolysis coupled with the Eq. 4 offered potential for the *F* calculation and the QAMS analysis.

Table 3 The comparison for the concentration determined by the enzymatic hydrolysis and the typical LC-ESI/MS

Method	GRb_1	GRd	Iso-GRd	GF_2
Enzymatic hydrolysis (mg mL^{-1})	2.0840 ±0.0136	0.7901 ±0.0402	0.2060 ±0.0009	0.0293 ±0.0070
LC-ESI/MS (mg mL^{-1})	2.0006 ±0.0300	0.7518 ±0.0074	–	0.0227 ±0.0008

⁻, not detected.

Calculation of the relative response factors (*F*) for absolute quantification of specific PPD-type ginsenosides in Sanqi extract using QAMS

According to Eq. 3, the *F* for pure compounds could be calculated by the slope ratios of CCAC.[21] GRd was selected as the internal standard. The calculated data were shown in Table 4. The largest *F* was 1.8209 for GF_2, indicating its MS response was better than other determined PPD-type ginsenosides under this condition. Herein, we also used the new strategy to calculate their *F* based on Eq. 4 and observed that the calculated results were closely matched with those of the CCAC. Moreover, the *F* of Iso-GRd was reported for the first time and it was nearly equal to the value of GRb_1. The developed strategy only used the identical hydrolysate and small amounts of extract to calculate the *F* for some analogues or even for the compounds without authentic standards (like Iso-GRd).

Table 4 The relative correction factors of the four saponins

Relative correction factor [a]	GRd [b]	GRb_1	Iso-GRd	GF_2
F	1.0000	0.7653	–	1.8209
F'	1.0000	0.7268	0.7395	2.1 946

[a] *F* : calculated by the typical CCAC; *F'*: calculated by enzymatic hydrolysis; [b] GRd was selected as the internal standard; ⁻, not detected.

Subsequently, the concentrations of GRb_1, Iso-GRd and GF_2 were calculated according to the equation $x = F \times x_{GRd} \times y/y_{GRd}$,[22] where y_{GRd} and x_{GRd} is the peak area and the concentration of GRd,[21] respectively. Their calculated content was 21.1360 ± 0.3200 mg g^{-1}, 7.9010 ± 0.4020 mg g^{-1}, 2.4120 ± 0.0330 mg g^{-1}, and 0.2240 ± 0.0070 mg g^{-1} for GRb_1, GRd, Iso-GRd and GF_2, respectively. These results were closely matched with those of previous study.[37] The content of Iso-GRd was reported for the first time, which was supplementary to quality control of Sanqi with multi-markers.[38] Since the ruggedness of LC-ESI/MS was not as good as the LC with ultraviolet detection analysis,[39] the internal standard or the reference extract[38] could be used for correcting the fluctuation of the mass spectrometric response and simultaneously increasing the accuracy and general applicability of the developed QAMS approach based on enzymatic hydrolysis. Besides, the mobile phase compensation could further stabilize MS response in QAMS analysis.[37]

The new approach using CCMM for calculation of *F* was universal, which could be coupled with some enzymes or derivative strategies for global analyses. As for other types of ginsenosides in Sanqi extract, metabolic engineering,[40] complex glycosidase preparations,[41-43] recombinant enzyme[34] and physical/chemical methods[6, 44] might be the alternatives because of the strong hydrolysis abilities, high production ratios and the good organic solvent tolerant potential for effective degrading different glycosyl linkages and specific transforming into secondary products or mono-sugars. The derivative methods[20] and the quantified derivative tags[45] specialized for the hydrolysed mono-sugars could make the proposed protocol have wider applications and better sensitivity for analyses of the glycosides in complex samples.

Experimental

Chemicals and reagents

Betulinic acid (BA; internal standard, IS), PPD, C-K and ginsenosides Rb_1 (GRb_1), F_2 (GF_2), Rg_3 (GRg_3), Rh_2 (GRh_2) and GRd were purchased from Chengdu Must Bio-technology Co., Ltd (Chengdu, China), and all their purities were above 98%. Glucose assay kits were purchased from Shanghai Rongsheng Bio-technology Co., Ltd (Shanghai, China). Anhydrous alcohol, trisodium cirate dihydrate and citric acid monohydrate of analytical grade were purchased from Nanjing Chemical Reagent Co., Ltd (Nanjing, China). Snailase was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Deionized water (18.0 MΩ cm^{-1}) was prepared using the Milli-Q system from Millipore (Billerica,

MA, USA). Acetonitrile and formic acid of HPLC grade were obtained from Merck (Darmstadt, Germany).

Crude medicine extract preparation

The dried root of Sanqi was purchased from Dishen-sanqi Science and Technology Innovation Centre Co., Ltd (Yunnan, China) in October, 2011. The identities of *Panax notoginseng* were confirmed by Professor Ping Li, and the voucher specimens of samples are deposited at the Department of Pharmacognosy, China Pharmaceutical University.

The extraction is according to the published method.[37] In brief, 100 mg Sanqi was used for preparation of 100 mg mL^{-1} of crude medicine extracted by ultrasonic extraction at 100 Hz. The extract was centrifuged at 13000 rpm for 10 min prior to injection into the LC systems (Palo Alto, CA, USA).

Fraction collection and enzymatic digestion

According to the economical protocol for enzymatic quantification of analogues in complex extract, fractions were obtained using Agilent 1260 LC systems at 203 nm coupled with the published fraction collection strategy.[5, 46] 20 μL of 100 mg mL^{-1} of crude extract was separated by an Agilent Eclipse XDB-C_{18} column (250 mm × 4.6 mm, 5 μm) at 25 °C. The LC conditions: 0.1% (v/v) formic acid water, solvent A; 0.1% (v/v) formic acid acetonitrile, solvent B; timetable: 0 min, 28% B; 6.0 min, 28% B; 6.5 min, 31% B; 18.0 min, 31.5% B; 18.5 min, 36% B; 22.0 min, 42% B; 27.0 min, 80% B; 30 min, 100% B; 35.0 min, 100% B. The flow rate was 3.0 mL min^{-1}. According to the proposed protocol, the collected fractions were evaporated to dryness under a stream of nitrogen and re-dissolved for the following enzymatic digestion studies.

PPD-type ginsenosides were used to optimize the digestion conditions. The snailase was dissolved in 10 mmol L^{-1} sodium citrate buffers (pH 4.6) to the concentration of 15.0 mg mL^{-1}. The optimized digestion condition was that 10 μL of a certain concentration of substrates (including chemical standards or collected fractions), 100 μL of enzyme solutions (15.0 mg mL^{-1}) and 40 μL of anhydrous alcohol were incubated 5 h at 37 °C and 900 rpm. All reactions were performed in 1.5 mL Eppendorf tubes, which were conducted in a thermomixer compact (Eppendorf, Germany) and performed in triplicate. After being added 5 μL of 0.05 mg mL^{-1} BA (IS), the mixtures were precipitated with 200 μL of anhydrous alcohol and centrifuged at 13000 rpm for 10 min prior to LC-ESI/MS analyses. Since the concentrations of the determined analytes have large deviations, 2000 μL of anhydrous alcohol was added for re-dissolving the GRb_1 and GRd as well as 200 μL for Iso-GRd. As for GF_2, 10 μL of redissolved anhydrous alcohol was directly added into drying tubes to ensure parallel incubation with the optimized enzyme reaction system.

LC-ESI/MS conditions and PPD-type ginsenoside hydrolysates determinations

The purities and enzymatic hydrolysate analyses were performed on an Agilent 1100 LC with an Agilent 1100 single quadrupole mass spectrometer equipped with an ESI source (LC-ESI/MS). 10 μL of samples were separated at 25 °C on an Agilent Zorbax Extend C_{18} column (5 μm, 250 mm × 4.6 mm) preceded by an Agilent guard column (5 μm, 12.5 mm × 4.6 mm). The mobile phase was not changed. The gradient elution program was 0 min, 15%; 40 min, 100% B. The composition of 100% B was maintained for 10 min to clean the column. The flow rate was 1.0 mL min^{-1} with splitting mode.

The MS conditions: drying gas (N_2) flow rate, 10.0 L min^{-1}; nebulizer pressure, 35 psig; drying gas temperature, 300 °C; capillary voltage, 3500 V; fragmentor voltage, 120 V. In negative mode, the ions of *m/z* 455.4 (BA, IS), *m/z* 505.4 (PPD), *m/z* 667.4 (C-K and isomer), *m/z* 829.4 (GF_2 and isomer), *m/z* 991.5 (GRd and isomer) and *m/z* 1107.6 (GRb_1) were determined using the single ion monitoring mode. To test the purities of collected fractions and analyse the enzymatic mixtures, the scan mode with a range of *m/z* 500–1500 was applied. The 10 folds diluted Sanqi extract was used to calculate the collected recoveries (*R*) of the interested analytes by the modified NSRE.[5] The proposed protocol coupled with the QAMS and the calculated *F* was used for economical quantification of Sanqi extract. The novel relative response factors and collection recovery calculations could be seen in the electronic supplementary information.

Conclusions

The snailase was verified as the first enzyme preparation for quantitative determination of specific PPD-type ginsenosides. The enzymatic hydrolysis coupled with CCMM and the identical hydrolysate is a novel strategy to calculate the relative response factors for the analytes of interest, providing a new means for QAMS analyses and effectively saving the reference standards in analogues quantification. The proposed green protocol for absolute quantitation of specific PPD-type ginsenosides and calculation of the *F* only required a single run of analytical LC for collecting small amounts of individual fractions from matrix material extract, avoiding time- and organic solvent-consuming isolation and purification of reference standards. The QAMS with the calculated *F* was a practical and economical method for absolute quantification. The content of Iso-GRd and its *F* were reported for the first time. The results of other determined ginsenosides were closely matched with those of the typical LC-MS. Further work will focus on the determination of more types of ginsenosides with suitable enzyme preparations or derivative techniques.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81130068), the Program for New Century Excellent Talents in University (NECT-13-1034) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. The authors thank Si-Hui Nian, Hui-Peng Song and Meng-Ying Lv for helpful discussions.

Notes and references

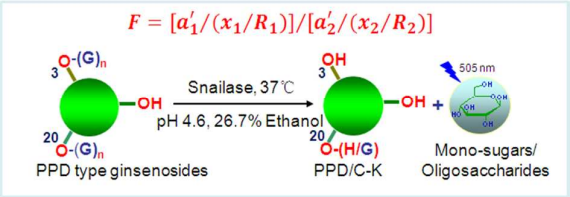
State Key Laboratory of Natural Medicines (China Pharmaceutical University), No.24 Tongjia lane, Nanjing 210009, China.
E-mail: liping2004@126.com (P. Li), liuehu2011@163.com (E.-H. Liu).

‡ These authors contributed equally to the paper.

† Electronic Supplementary Information (ESI) available: the novel calculations for the relative response factors and collection recovery. See DOI: 10.1039/b000000x/

1. S. Lindahl, A. Ekman, S. Khan, C. Wennerberg, P. Börjesson, P. J. R. Sjöberg, E. N. Karlsson and C. Turner, *Green Chem.*, 2010, **12**, 159.
2. J. M. Augustin, V. Kuzina, S. B. Andersen and S. Bak, *Phytochemistry*, 2011, **72**, 435.
3. A. L. Harvey, *Drug Discov. Today*, 2008, **13**, 894.
4. W. Wang, X. Zhang, J.-J. Qin, S. Voruganti, S. A. Nag, M.-H. Wang, H. Wang and R. Zhang, *PLoS ONE*, 2012, **7**, e41586.
5. T. Tan, C. J. S. Lai, S. L. Zeng, E. H. Liu and P. Li, *Anal. Bioanal. Chem.*, 2014, **406**, 4921.
6. D. Yu, L. Tian, D. Ma, H. Wu, Z. Wang, L. Wang and X. Fang, *Green Chem.*, 2010, **12**, 844.
7. J. Y. Zhang, Q. Zhang, H. X. Zhang, Q. Ma, J. Q. Lu and Y. J. Qiao, *J. Agric. Food Chem.*, 2012, **60**, 9023.
8. Q. Xing, T. Liang, G. Shen, X. Wang, Y. Jin and X. Liang, *Analyst*, 2012, **137**, 2239.
9. W. Z. Yang, M. Ye, X. Qiao, C. F. Liu, W. J. Miao, T. Bo, H. Y. Tao and D. A. Guo, *Anal. Chim. Acta*, 2012, **739**, 56.
10. L. W. Qi, C. Z. Wang and C. S. Yuan, *Nat. Prod. Rep.*, 2011, **28**, 467.
11. X. Wang, H. Wang, A. Zhang, X. Lu, H. Sun, H. Dong and P. Wang, *J. Proteome Res.*, 2012, **11**, 1284.
12. X. Zhang, Y. Liu, Z. Guo, J. Feng, J. Dong, Q. Fu, C. Wang, X. Xue, Y. Xiao and X. Liang, *Anal. Bioanal. Chem.*, 2012, **402**, 573.
13. X. Wang, H. Sun, A. Zhang, G. Jiao, W. Sun and Y. Yuan, *Analyst*, 2011, **136**, 5068.
14. H. W. Park, G. In, S. T. Han, M. W. Lee, S. Y. Kim, K. T. Kim, B. G. Cho, G. H. Han and I. M. Chang, *J. Ginseng Res.*, 2013, **37**, 457.
15. B. Álvarez-Sánchez, F. Priego-Capote and M. D. Luque de Castro, *Analyst*, 2009, **134**, 1416.
16. B. H. Han, M. H. Park, Y. N. Han, L. K. Woo, U. Sankawa, S. Yahara and O. Tanaka, *Planta Med.*, 1982, **44**, 146.
17. W. Li, M. Zhang, Y. N. Zheng, J. Li, Y. P. Wang, Y. J. Wang, J. Gu, Y. Jin, H. Wang and L. Chen, *Molecules*, 2011, **16**, 10093.
18. H. Luan, X. Liu, X. Qi, Y. Hu, D. Hao, Y. Cui and L. Yang, *Process Biochem.*, 2006, **41**, 1974.
19. S. R. Ko, K. J. Choi, K. Suzuki and Y. Suzuki, *Chem. Pharm. Bull.*, 2003, **51**, 404.
20. I. Boldizsár, Z. Füzfai and I. Molnár-Perl, *J. Chromatogr. A*, 2013, **1293**, 100.
21. X. Y. Gao, Y. Jiang, J. Q. Lu and P. F. Tu, *J. Chromatogr. A*, 2009, **1216**, 2118.
22. E. Cífková, M. Holčapek, M. Lísa, M. Ovčačíková, A. Lyčka, F. Lynen and P. Sandra, *Anal. Chem.*, 2012, **84**, 10064.
23. Y. Liang, H. Hao, A. Kang, L. Xie, T. Xie, X. Zheng, C. Dai, L. Wan, L. Sheng and G. Wang, *J. Chromatogr. A*, 2010, **1217**, 4971.
24. M. W. Nam, J. Zhao, M. S. Lee, J. H. Jeong and J. Lee, *Green Chem.*, 2015, DOI: 10.1039/c4gc01556h.
25. J. Fan, M. De Bruyn, V. L. Budarin, M. J. Gronnow, P. S. Shuttleworth, S. Breeden, D. J. Macquarrie and J. H. Clark, *J. Am. Chem. Soc.*, 2013, **135**, 11728.
26. J. N. Hu, X. M. Zhu, K. T. Lee, Y. N. Zheng, W. Li, L. K. Han, Z. M. Fang, L. J. Gu, B. S. Sun, C. Y. Wang and C. K. Sung, *Biol. Pharm. Bull.*, 2008, **31**, 1870.
27. L. Liu, X. M. Zhu, Q. J. Wang, D. L. Zhang, Z. M. Fang, C. Y. Wang, Z. Wang, B. S. Sun, H. Wu and C. K. Sung, *Phytochemistry*, 2010, **71**, 1514.
28. M. H. Zhang, L. Feng, M. M. Zhu, J. F. Gu, J. Jiang, X. D. Cheng, S. M. Ding, C. Wu and X. B. Jia, *J. Ethnopharmacol.*, 2014, **151**, 591.
29. X. Zhao, J. Gao, C. Song, Q. Fang, N. Wang, T. Zhao, D. Liu and Y. Zhou, *Phytochemistry*, 2012, **78**, 65.
30. L. Q. Cheng, J. R. Na, M. H. Bang, M. K. Kim and D. C. Yang, *Phytochemistry*, 2008, **69**, 218.
31. Y. Han, B. Sun, X. Hu, H. Zhang, B. Jiang, M. I. Spranger and Y. Zhao, *J. Agric. Food Chem.*, 2007, **55**, 9373.
32. S. R. Ko, Y. Suzuki, Y. H. Kim and K. J. Choi, *Biosci. Biotechnol. Biochem.*, 2001, **65**, 1223.
33. G. Chen, M. Yang, Z. Lu, J. Zhang, H. Huang, Y. Liang, S. Guan, Y. Song, L. Wu and D. A. Guo, *J. Nat. Prod.*, 2007, **70**, 1203.
34. L. H. Quan, J. W. Min, Y. Jin, C. Wang, Y. J. Kim and D. C. Yang, *J. Agric. Food Chem.*, 2012, **60**, 3776.
35. K. He, Y. Liu, Y. Yang, P. Li and L. Yang, *Chem. Pharm. Bull.*, 2005, **53**, 177.
36. A. Liu, H. Lou, L. Zhao and P. Fan, *J. Pharm. Biomed. Anal.*, 2006, **40**, 720.
37. C. J. S. Lai, T. Tan, S. L. Zeng, X. Dong, E. H. Liu and P. Li, *J. Pharm. Biomed. Anal.*, 2015, **102**, 150.
38. S. P. Li, C. F. Qiao, Y. W. Chen, J. Zhao, X. M. Cui, Q. W. Zhang, X. M. Liu and D. J. Hu, *J. Chromatogr. A*, 2013, **1313**, 302.
39. J. J. Hou, W. Y. Wu, J. Da, S. Yao, H. L. Long, Z. Yang, L. Y. Cai, M. Yang, X. Liu, B. H. Jiang and D. A. Guo, *J. Chromatogr. A*, 2011, **1218**, 5618.
40. Z. Dai, B. Wang, Y. Liu, M. Shi, D. Wang, X. Zhang, T. Liu, L. Huang and X. Zhang, *Sci. Rep.*, 2014, **4**, 3698.
41. C. S. Park, M. H. Yoo, K. H. Noh and D. K. Oh, *Appl. Microbiol. Biotechnol.*, 2010, **87**, 9.
42. S. R. Ko, Y. Suzuki, K. J. Choi and Y. H. Kim, *Biosci. Biotechnol. Biochem.*, 2000, **64**, 2739.
43. J. K. Kim, C. H. Cui, Q. Liu, M. H. Yoon, S. C. Kim and W. T. Im, *Food Chem.*, 2013, **141**, 1369.
44. Y. Bai, L. Zhao, C. Qu, X. Meng and H. Zhang, *J. Agric. Food Chem.*, 2009, **57**, 10252.
45. R. Liu, X. Hou, Y. Lv, M. McCooeye, L. Yang and Z. Mester, *Anal. Chem.*, 2013, **85**, 4087.
46. W. J. Kong, J. B. Wang, Q. C. Zang, C. Jin, Z. W. Wang, X. Y. Xing, Y. Y. Wu, Y. L. Zhao, M. H. Yang and X. H. Xiao, *J. Chromatogr. B*, 2011, **879**, 3565.

Table of contents entry:



The enzymatic protocol for simultaneous quantification of analogues in complex extract was proposed.