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Ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS) for time-dependent profiling of raw and steamed *Panax notoginseng*

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ABSTRACT

The metabolic profiles of *Panax notoginseng* and its associated therapeutic values are critically affected by the duration of steaming. The time-dependent steaming effect of *P. notoginseng* is not well-characterized and there is also no official guideline on its duration of steaming. In this paper, a UHPLC/TOFMS-based metabolomic platform was developed for the qualitative profiling of multiparametric metabolic changes of raw *P. notoginseng* during the steaming process. Our method was successful in discriminating the differentially processed herbs. Both the unsupervised principal component analysis (PCA) score plot ($R^2X = 0.664$, Q^2 (cum) = 0.622, and PCs = 2) and the supervised partial least square-data analysis (PLS-DA) model ($R^2X = 0.708$, $R^2Y = 0.461$, and $Q^2Y = 0.271$) demonstrated strong classification and clear trajectory patterns with regard to the duration of steaming. The PLS-DA model was validated for its robustness via a prediction set, confirming that the UHPLC/TOFMS metabolic profiles of the raw and differentially steamed *P. notoginseng* samples were highly reproducible. Based on our method, the minimum durations of steaming for the maximum production of bioactive ginsenosides such as Rg3 and Rh2 were also predicted. Our novel time-dependent metabolic profiling approach represents the paradigm shift in the quality control of *P. notoginseng* products.

1. Introduction

Panax notoginseng (Burk.) F.H. Chen (commonly known as Tianqi or Sanqi) is a highly valuable and important Chinese medicinal herb cultivated mainly in Yunnan Province, China. Extensive phytochemical and pharmacological studies on *P. notoginseng* have been reported. The constituents of *P. notoginseng* consist of various saponins, amino acids, polysaccharides and flavonoids [1]. The dammarane-type saponins, which include ginsenosides and notoginsenosides, account for 12% of the total root contents [1]. These are the main bioactive components of the herb, contributing to its pharmacological activities [2]. To date, a total of 56 dammarane-type saponins have been isolated and characterized [3] most of which have been proven to be bioactive for the prevention and treatment of cardiovascular and cerebrovascular diseases, immunoregulation, hepatoprotection and anti-carcinogenesis [2].

Two different forms of *P. notoginseng* are available, namely, the raw and steamed forms [1]. These two forms have different clinical indications due to their different pharmacological activities. The raw form of *P. notoginseng* is known for its hemostatic and cardiovascular properties to arrest various internal or external hemorrhage, eliminate blood stasis, improve blood circulation, disperse bruises, reduce swelling and pain [4]. However, the steamed form has been claimed to be a tonic used to nourish blood and increase production of various blood cells in anaemic conditions [4]. As the raw and steamed forms of *P. notoginseng* have different pharmacological actions, it is pertinent to administer the correct form of herb to avoid any undesirable consequences. Therefore, it is of paramount importance to characterize the raw and steamed forms of *P. notoginseng*.

Metabolites are the end products of cellular regulatory processes, and their fluxes and levels can be regarded as the ultimate response of biological systems to respond to genetic or environmental changes. Therefore, metabolomics has emerged as a valuable technology for the comprehensive profiling and comparison of metabolites [5]. The curative effects of Traditional Chinese Medicine (TCM) are principally based on the synergic effect of their multi-targeting, multi-ingredient preparations, in contrast to modern pharmacology and drug development that often focus on a single chemical entity [6]. Therefore, the method employing a few markers or pharmacologically active constituents to assess the quality and authenticity of the complex preparations

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has a number of severe challenges. Currently, quality consistency evaluation of TCM products or herbal medicines based on similarities among chromatograms is extensively used [7]. But, very little efforts have been paid in interpreting or understanding of the differences in the chromatograms for the purpose of quality control [7]. Metabolomics enables comprehensive assessment of complex TCMs or herbal remedies and sample classification of diverse biological status, origin or quality in samples, by means of chemometrics, such as principal component analysis (PCA). Some examples of the applications of metabolomics in the quality control of herbal remedies or TCM include differentiation between different parts of herb such as *P. notoginseng* [3], quality assessment and authentication of herbs such as *Atractylis chinensis* DC [8], and monitoring of batch-to-batch or manufacturer-to-manufacturer differences for product such as Liuwei Dihuang Pills [9].

In our previous work [10], a UHPLC/TOFMS-based metabolomic platform was developed for the profiling of both raw and steamed *P. notoginseng*. Our method measures qualitatively multiparametric metabolic responses of raw *P. notoginseng* to the steaming process and discriminates the differentially processed herbs. Although *P. notoginseng* is a well-known Chinese herbal medicine, its associated time-dependent steaming effect is not well-characterized. As the duration of steaming affects the metabolic profiles of *P. notoginseng* and its associated therapeutic effects, it becomes important to characterize the metabolic changes related to the different hours of steaming. To the best of our knowledge, there is also no official guideline on the duration of steaming of *P. notoginseng*. In the present study, the time-dependent metabolic changes of *P. notoginseng* when it is subjected to different hours of steaming were investigated. With the developed UHPLC/TOFMS method, the metabolic profiles of the raw and the differentially steamed samples are acquired and compared using multivariate data analysis. This novel study is important to address the current gap in correlating the herbal contents with the duration of steaming of *P. notoginseng*.

2. Experimental

2.1. Materials and reagents

Leucine enkephalin and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from Merck (Rahway, NJ, USA). Methanol (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Distilled water was purified 'in-house' using a MilliQ system (Millipore, Bedford, MA, USA). Ginsenosides Rg1, Rb1, Rc, Rd and Re were purchased from Indofine Chemical Company (Somerville, NJ, USA). Notoginsenoside R1 was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rg3 was purchased from Chroma Dex Inc. (Irvine, CA, USA). Rh1, Rh2 and Rg2 were purchased from Delta Information Centre for Natural Organic Compounds (Anhui, China). The raw *P. notoginseng* root was obtained as a single batch from one Chinese medical shop in Singapore. All other chemicals used were of analytical grade.

2.2. Sample preparation

Samples of the powdered raw *P. notoginseng* root were steamed at 120 °C using an autoclave (Hirayama, Japan) for different time periods. The first batch (batch 1) of the *P. notoginseng* powder was steamed for 1, 2, 4, 6, 9, 12, 15 and 24 h while the second batch (batch 2) was steamed for 2, 6, 9, 12 and 15 h. The steamed powder was then dried in a vacuum oven at about 80 °C until constant weight and extracted using the method as described [10]. 10 mL of 70% methanol was added to 1 g of the powdered sample of raw *P. notoginseng* root. The suspension was mixed for 20 min using an ultrasonicator (230 V, Branson model 5510, Danburry, CT, USA) and filtered. This extraction was repeated two additional times. The combined filtrate was evaporated to dryness *in vacuo*. The residue was then dissolved in 5 mL of 70% methanol. All extracted samples were further diluted 100-fold with 50% methanol (v/v). The diluted samples were centrifuged at 14,000 rpm for 10 min and 2 μL of the supernatant was injected for UHPLC/TOFMS analysis. For each batch, six individual extractions were performed on the raw and steamed samples to generate six replicates each of the raw and steamed extracts.

2.3. Ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS)

All samples were analyzed using an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) interfaced with a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (QTOFMS) equipped with ESI source (Q-Tof Premier™, Waters, Manchester, UK). The UHPLC/TOFMS system was controlled by MassLynx 4.1 software (Waters, UK). The chromatographic separation was performed on an ACQUITY BEH C_{18} 1.7 μm 100 mm × 2.1 mm i.d. column (Waters, UK). The mobile phases consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile, v/v). The optimized UHPLC elution conditions were: 0–0.2 min, 2% B; 0.2–2.0 min, 2–30% B; 2.0–4.7 min, 30–60% B; 4.7–6.5 min, 60–80% B; 6.5–8.3 min, 80–90% B; 8.3–9.5 min, 98% B and 9.5–10.0 min, 2% B. The flow rate was 0.5 mL/min. The column and autosampler were maintained at 45 and 15 °C, respectively. Each wash cycle consisted of 200 μL of strong solvent (95% methanol, v/v) and 600 μL of weak wash solvent (10% methanol, v/v).

The TOFMS system was tuned for optimal sensitivity and resolution in electrospray negative ionization mode (ESI^-) using leucine enkephalin (50 pg/μL infused at 5 μL/min). The TOFMS was operated in the 'W' mode and tuned using the standard compound ginsenoside Rg1. The optimized MS conditions were as followed: capillary voltage 3.1 kV, sampling cone voltage 45 V, extraction cone voltage 4.5 V, ion guide 3.0 V, desolvation temperature 350 °C, source temperature 120 °C, cone gas flow 0 L/h, desolvation gas flow 700 L/h, collision energy 5 eV, MCP detector voltage 1800 V, pusher voltage 938 V, pusher voltage offset −2.00 V and puller voltage 670 V. The analyzed mass range was 400–1500 Da and centroid data were acquired with a 0.1 s scan time and a 0.01 s interscan delay. Prior to analysis, the system was calibrated routinely using sodium formate solution infused at a flow rate of 5 μL/min. All analyses were acquired using an independent LockSpray™ interface to ensure high mass accuracy and precision. The $[M-H]^+$ ion of leucine-enkephalin (2 ng/μL infused at 3 μL/min) was used as a reference lock mass (*m/z* 554.2615). The LockSpray™ was operated at a reference scan frequency, a reference cone voltage and collision energy of 10 s, 35 V and 5 eV, respectively. Dynamic range enhancement (DRE™) was applied throughout the MS experiment to ensure accurate mass measurement over a wider dynamic range.

2.4. Method validation

A standard mixture containing ginsenosides Rb1, Rb2, Rc, Rd, Re, Rg1, Rg2, Rg3, Rh1, Rh2 and notoginsenoside R1 was prepared in 50% (v/v) methanol. This standard mixture was used during UHPLC/TOFMS method development/validation and in the actual analysis where it is injected once after every ten extracted samples (2 μL each time) to monitor the retention time variation and mass accuracy. Likewise, blank sample consisting of 50% methanol

(2 μL) was injected routinely using the same sequence to monitor inter-sample cross-over effect.

2.5. Multivariate data analyses

The ESI$^-$ raw data were analyzed by the MarkerLynx applications manager version 4.1 (Waters, UK). The parameters used were RT range 2–8 min, mass range 400–1400 Da, mass tolerance 0.01 Da, internal standard detection parameters were deselected for peak retention time alignment, noise elimination level was set at 7.00, intensity threshold 10 counts, mass window 0.05, RT window 0.05. MarkerLynx uses ApexTrack^{TM} peak integration to detect chromatographic peaks. No specific mass or adduct was excluded. A list of the intensities of the peaks detected was generated using retention time (RT) and *m/z* data pairs. An arbitrary ID was assigned to each of these RT–*m/z* pairs in the order of their UHPLC elution (i.e. 1, 2, 3, 4, etc.) for data alignment. The process was repeated for each run until the final sample. For each sample, the correct peak intensity data for each RT–*m/z* pair was aligned in the final data table. Ions of different samples were considered to be the same ion when they demonstrated the same RT (tolerance of 0.05 min) and *m/z* value (tolerance of 0.01 Da). If a peak was not detected in the sample, the ion intensity was documented as zero in the final data table. The ion intensities for each detected peak were normalized against the sum of the peak intensities within that sample using MarkerLynx. The resulting three-dimensional data comprising of peak number (RT–*m/z* pair), sample name (observations), and ion intensity were analyzed by unsupervised principal component analysis (PCA) and supervised partial least square-discriminant analysis (PLS-DA) SIMCA-P+ software (Umetrics AB, Umea, Sweden). All variables were pareto-scaled prior to analyses. In our study, the batch 1 data set was visualized by unsupervised PCA to check for outliers and classification trends among the raw and differentially steamed (1, 2, 4, 6, 9, 12, 15 and 24 h) *P. notoginseng* samples. The batch 1 raw and steamed samples (1, 2, 4, 6, 9, 12, 15 and 24 h) were utilized subsequently to build the PLS-DA model while batch 2 raw and steamed samples (2, 6, 9, 12 and 15 h) were used as the external validation or prediction set to cross-validate the PLS-DA model.

2.6. Univariate data analyses

Therapeutically important marker ginsenosides that contributed significantly to the clustering of observations were further subjected to univariate statistical analyses. Statistical differences between time points for the steamed samples (batch 1) were compared against the raw samples (control) using repeated measure for one-way analysis of variance (ANOVA) and Bonferroni post-tests (GraphPad Prism 4, San Diego, CA, USA). Statistical significance was established when $p < 0.05$.

3. Results and discussion

3.1. Ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOFMS)

It was reported in earlier studies that 14 saponins were identified in raw and steamed *P. notoginseng* using a 80 min analysis method [1] while 9 saponins were detected within a 50 min analysis [11]. In our previous study, a 12 min UHPLC/TOFMS metabolic profiling assay was reported for the profiling of raw and steamed *P. notoginseng*, shortening the sample analysis time significantly [10]. The significant reduction in analysis time was a combination of enhanced selectivity of TOFMS detection and enhanced chromatographic resolution with the use of sub-2 μm particle column chemistry. In the present study, the UHPLC method was further optimized to 10 min without compromising the chromatographic peak resolution. This was achieved mainly through the use of step-gradients and optimizing the gradient time especially during the earlier phase of the chromatography (0.2–4.7 min). The representative chromatograms of the raw and steamed *P. notoginseng* extracted samples are presented in Fig. 1. The UHPLC/TOFMS system proved to be robust as the retention time shift and the mass accuracy based on the analysis of a standard mixture containing ginsenosides Rb1, Rb2, Rc, Rd, Re, Rg1, Rg2, Rg3, Rh1, Rh2 and notoginsenoside R1 were <0.02 min and 10 ppm, respectively. There was also minimal carry-over with <2.6% peak area percentage in the blank samples. These validations were of utmost importance as any significant shifts in retention time and *m/z* values and any carry-over peaks can potentially complicate the chemometric data analysis by adding false positive results.

ESI$^-$ was selected as the ionization mode for the TOFMS experiments since our previous paper [10] demonstrated that the ginsenosides were detected with greater ion sensitivities using this ionization mode and hence provided richer information on the saponins. Each saponin was identified by comparing its retention time and accurate *m/z* value of the extracted ion peak to that of its standard. Three adduct ions namely, $[M-H]^-$, $[M+HCOO]^-$ and $[M-2H]^{2-}$ of the ginsenosides were observed and documented. However, the $[M+HCOO]^-$ ions were eventually used as the target ions for data analyses since they generally showed good abundance and ion count consistency, as previously reported [10]. The chromatographic peaks at 2.34 and 2.62 min (Fig. 1) were associated with exogenous substances derived from sample preparation. As these unknown compounds were present at the same levels in both raw and steamed samples, they did not affect the subsequent chemometric data analysis.

3.2. Principal component analysis (PCA)

PCA is an unsupervised technique that assumes no prior knowledge of class structure and transforms the variables in a data set into a smaller number of latent variables known as principal components (PCs), which are uncorrelated with each other and account for decreasing proportions of the total variance of the original variables. Each new PC is a linear combination of the original variables such that a compact description of the variation within a data set is generated. Observations are assigned scores according to the variation measured by the PCs with those having similar scores clustering together. In our study, the data set was visualized by unsupervised PCA to check for outliers and classification trends among the batch 1 raw and differentially steamed (1, 2, 4, 6, 9, 12, 15 and 24 h) *P. notoginseng* samples. Preliminary PCA was performed on all observations using 787 pareto-scaled variables. Our results indicated out of 54 samples, there were four severe outliers belonging to the 2, 12, 15 and 24 h steamed samples. These samples were found to lie outside the Hotelling T2 (0.95) ellipse of the PCA score plot and removed from the subsequent PCA analysis. The final PCA score plot obtained using the batch 1 samples demonstrated clear classification trends among the raw and differentially steamed *P. notoginseng* samples with all the observations falling within the Hotelling T2 (0.95) ellipse (Fig. 2; $R^2X = 0.664$, Q^2 (cum) = 0.622, and PCs = 2). While there were some overlaps among the observations (raw and 1 h; 12 and 15 h), most of the raw and differentially steamed samples were clearly clustered in the score plot. This strong classification pattern underscored the feasibility of using time-dependent metabolic profiling of *P. notoginseng* in elucidating the optimal duration of steaming to achieve its desired herbal contents. Based on these results, we proceeded to use supervised PLS-DA to build a prediction model using the batch 1 samples.

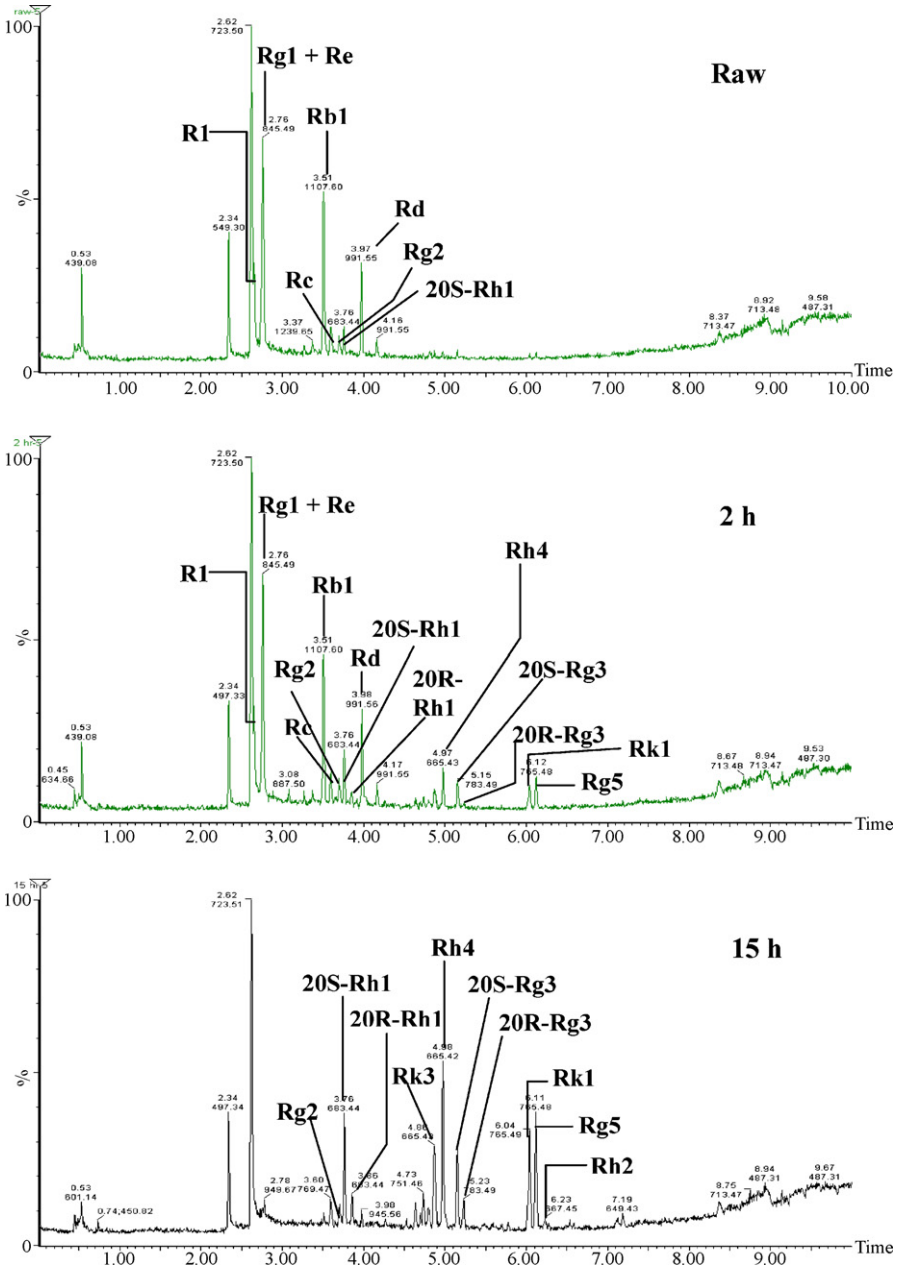


Fig. 1. Representative UHPLC/TOFMS chromatograms of raw and steamed *P. notoginseng* extracted samples (2 and 15 h).

3.3. Partial least square-discriminant analysis (PLS-DA)

PLS-DA is a supervised technique of multivariate data analysis; used to distinguish two or more classes by searching for variables (*X* matrix) that are correlated to class membership (*Y* matrix). In this approach, the axes are calculated to maximize the separation between groups and can be used to examine separation that would otherwise be across three or more PCs. PLS is used to maximize the correlation between the design matrix *X* (the normalized peak areas) to matrix *Y* (e.g. duration of steaming) so that the response variable *Y* can be predicted from *X*. In this study, batch 1 raw and steamed samples were utilized to build a PLS-DA model where the model performance was evaluated using the R^2 and Q^2 parameters, both of which vary between 0 and 1, where 1 indicates a perfect fit. R^2X is the cumulative modeled variation in *X*, indicating how much of the variation within a data set can be explained by the various components of the model; R^2Y is the cumulative modeled variation in *Y*; and Q^2Y is the cumulative predicted variation in *Y*, indicating how accurately the data can be predicted according to cross-validation. Batch 2 raw and steamed samples (2, 6, 9, 12 and 15 h) were used as the external validation or prediction set to cross-validate the PLS-DA model.

A preliminary PLS-DA model was constructed where 3 severe outliers (two raw samples and one 12 h sample) were found to lie outside the Hotelling T2 (0.95) ellipse of the PLS-DA score plot. These three observations were removed before constructing the final PLS-DA model. A four latent variable (LV = 4) PLS-DA model was constructed ($R^2X = 0.708$, $R^2Y = 0.461$, and $Q^2Y = 0.271$) where clear classification trends of the raw and differentially steamed samples were observed as shown in the three-dimensional score plot (Fig. 3A). The raw and steamed samples demonstrated a clear trajectory pattern with regard to the duration of steaming (Fig. 3A). Our results confirmed that the observed changes in the levels and occurrence of ginsenosides evolved in a specific and time-

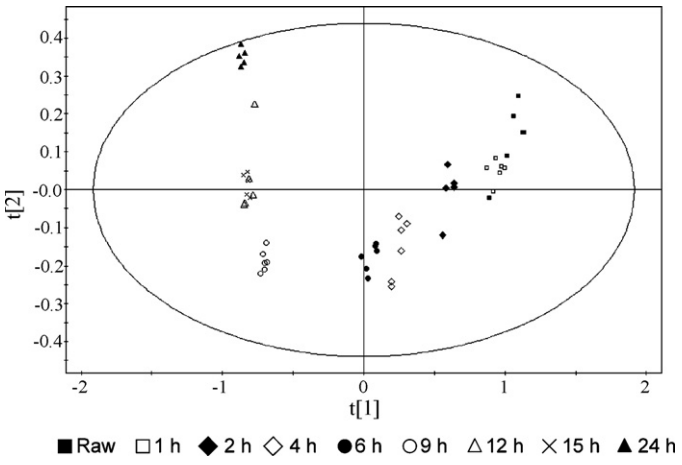


Fig. 2. PCA score plot of raw and steamed *P. notoginseng* samples (1, 2, 4, 6, 9, 12, 15 and 24 h).

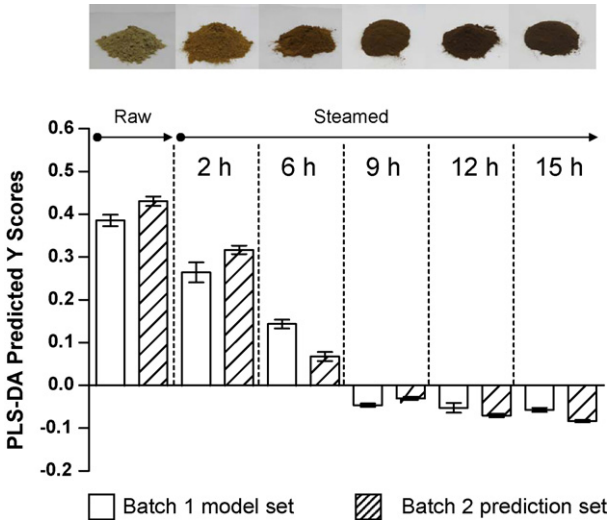


Fig. 4. Plot of PLS-DA cross-validated predicted *Y* scores of raw and steamed *P. notoginseng* samples (2, 6, 9, 12 and 15 h) belonging to the batch 1 model and batch 2 prediction set, respectively. Photographs of the corresponding raw and steamed dried powder samples are also presented.

dependent manner with regard to the steaming process. However, we were not certain if such a time-dependent trajectory of the observations could be repeated when another batch of samples was analyzed. As it was important to validate the robustness of the PLS-DA model before it can be used to correlate the desired herbal constituents with the duration of steaming process, an external validation or prediction set (batch 2) comprising both raw and differentially steamed samples was used to test the PLS-DA model. To aid visualization of the prediction, observations belonging to the model (batch 1) and prediction (batch 2) sets were plotted in the same three-dimensional score plot (Fig. 3B). It was evident that the observations belonging to the different groups of the prediction set, namely the raw, 2, 6, 12 and 15 h steamed *P. notoginseng* samples, were accurately clustered near their corresponding groups in the PLS-DA model (Fig. 3B). The cross-validated predicted *Y* scores (latent variable 1) of the observations belonging to the model and prediction sets were extracted and plotted to further ascertain the robustness of the PLS-DA model (Fig. 4). Our results indicated that these predicted *Y* scores were comparable between the model and prediction observations across all differentially steaming time points (2, 6, 9, 12 and 15 h) including the raw samples. Accordingly, we also observed a gradual darkening in the colors of the *P. notoginseng* samples with increasing duration of steaming (Fig. 4). Collectively, our external cross-validation results confirmed that the UHPLC/TOFMS metabolic profiles of the raw and differentially steamed *P. notoginseng* samples were highly repeatable. This pertinent finding suggested that UHPLC/TOFMS metabolic profiling could be applied to predict the status of an unknown *P. notoginseng* sample whereby the duration of steaming could be estimated. Our novel approach represents a paradigm shift in the quality control of *P. notoginseng* products, which will lead to the correct identification of herb form and ensure that the correct form is consumed by our patients.

3.4. Univariate data analyses of therapeutically important ginsenosides

The steamed form of *P. notoginseng* has different therapeutic uses as compared to the raw form. During the steaming process, the ginsenosides in the raw powder undergo hydrolysis to produce different ginsenosides. In our study, we found that the levels of some ginsenosides (Rg1, Rb1, Rc, Rd, Re and notoginsenoside R1) were reduced with steaming while the levels of other ginsenosides (Rg3, Rh1, Rh2, Rk3, Rh4, Rk1 and Rg5) were increased according to the duration of steaming. The structures of these ginsenosides are shown in Fig. 5. Ginsenosides 20S-Rg3 and Rh2 which were reported to have anticancer activity were produced upon steaming. As shown in Fig. 6, the levels of ginsenosides 20S-Rg3 and Rh2,

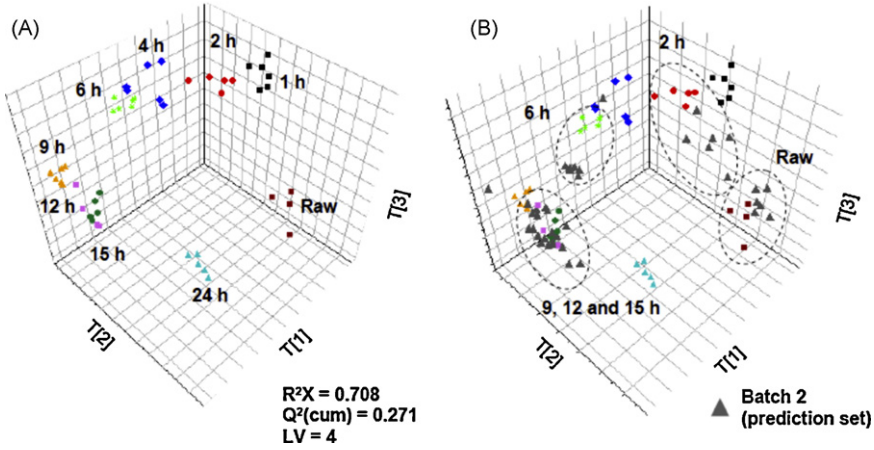
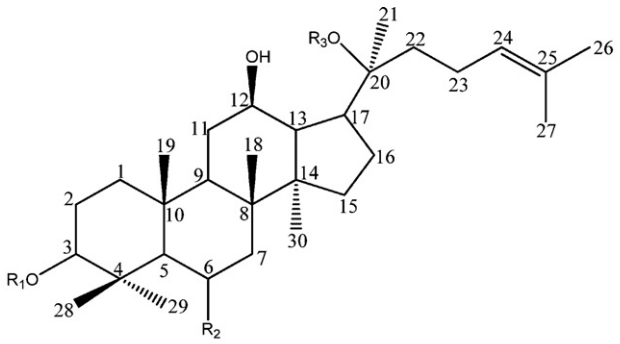


Fig. 3. (A) Three-dimensional PLS-DA score plot of batch 1 raw and steamed *P. notoginseng* samples (1, 2, 4, 6, 9, 12, 15 and 24 h) and (B) three-dimensional PLS-DA score plot of batch 1 model set and batch 2 prediction set samples comprising raw, 2, 6, 9, 12 and 15 h steamed samples.



Saponins	R_1	R_2	R_3
Protopanaxatriol			
Rg1	-H	-O-Glc	-Glc
Re	-H	-O-Glc^{2}-^{1}Rha	-Glc
Rg2	**-H**	**-O-Glc^{2}-^{1}Rha**	**-H**
Rh1	**-H**	**-O-Glc**	**-H**
R1	-H	-O-Glc^{2}-^{1}Xyl	-Glc
Protopanaxadiol			
Rb1	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Glc
Rc	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Ara(f)
Rd	-Glc^{2}-^{1}Glc	-H	-Glc
Rg3	**-Glc^{2}-^{1}Glc**	**-H**	**-H**
Rh2	**-Glc**	**-H**	**-H**
Rb2	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Ara(p)

Fig. 5. Chemical structures of some saponins present in raw and steamed *P. notoginseng*. The saponins in bold are characteristic for steamed samples. Abbreviations: Glc, glucose; Ara(f), arabinose in furanose form; Ara(p), arabinose in pyranose form; Rha, rhamnose; Xyl, xylose.

as estimated using integrated peak area of the $[M+HCOO]^-$ ions, were found to increase steadily upon steaming. Using repeated measures one-way ANOVA, it was confirmed that for both 20S-Rg3 and Rh2, their levels in the steamed samples were statistically significant compared to the raw samples after 6 and 9 h of steaming process respectively ($p < 0.05$). For 20S-Rg3, it was found that its levels among 6, 9, 12, 15 and 24 h samples were not statistically significant. This implied that for 20S-Rg3, steaming up to 6 h is sufficient. On the other hand, for Rh2, it was determined that its level at 24 h was statistically different from the 15 h sample. Hence, for Rh2, steaming up to 24 h is necessary. Official information on the duration of steaming used traditionally is not available [4]. According to Ye and Zhang, processed *P. notoginseng* can be prepared using two methods [12]. For the first method, raw *P. notoginseng* was first cut into pieces and they were fried using edible oil until the surface color becomes dark yellow. The oil was then removed and the sample was cooled and powdered. In the second method, raw *P. notoginseng* sample was washed and "steamed thoroughly". It was cut into small pieces and dried [12]. However, the duration was not specified. In this study, various steaming durations up to 24 h were selected for comparisons of the chemical compositions. The maximum duration of 24 h was chosen for convenience.

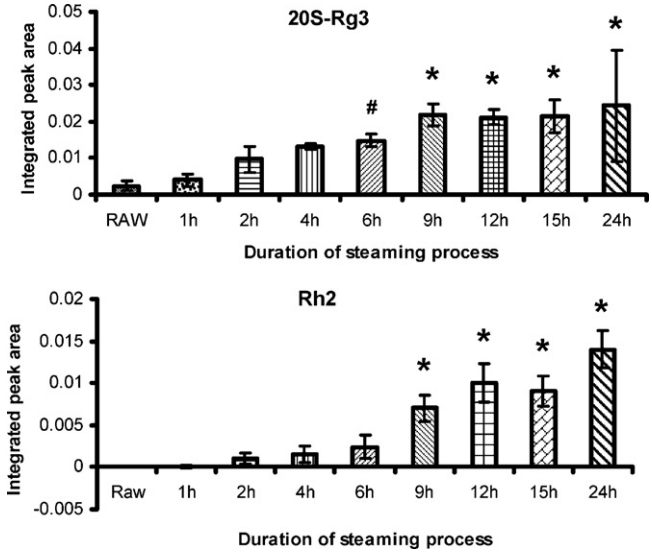


Fig. 6. Plots of integrated peak area of the $[M+HCOO]^-$ ions of (A) 20S-Rg3 and (B) Rh2 against the duration of steaming process (hours) for the batch 1 samples (# is $p < 0.05$ while * is $p < 0.001$ when compared to the raw samples).

For the raw form of *P. notoginseng*, its traditional use as a hemostatic was supported and proven by several studies [2]. Dencichine, a hemostatic agent is found present in the raw *P. notoginseng*, giving hemostatic activity of *P. notoginseng* [13]. The raw form is also claimed to remove blood stasis and improve circulation. Such pharmacological activities were also proven by studies that showed it has blood thinning effect such as antiplatelet and anticoagulant effects [2]. In addition, *P. notoginseng* was reported to be able to regulate lipid levels to prevent atherosclerosis [2,14]. These therapeutic effects will aid in the prevention of thrombosis and cardiovascular events and improve microcirculation. Overall, the traditional uses of raw *P. notoginseng* are well supported by the modern scientific findings of its pharmacological actions and clinical studies.

It has been reported that the hemostatic effect was abolished by autoclaving [15]. Study have shown that steamed *P. notoginseng* was found to contain less dencichine than the raw *P. notoginseng* and its level diminished with longer duration of steaming such as 6 h [13]. Decline in the amount of dencichine upon steaming

corresponds to the less or no hemostatic effect in the steamed *P. notoginseng*. And, the steamed form is used traditionally as a hematinic to increase blood production. A study has shown that *P. notoginseng* saponins can increase proliferation and upregulation of hematopoiesis [16]. *In vitro* studies have shown that panaxadiol saponin can stimulate proliferation of bone marrow and panaxatriol saponin will inhibit the proliferation [17]. Some examples of panaxadiol saponin are ginsenosides Rb1, Rb2, Rc, Rd, Rg3, Rh2, Rk1 and Rg5. Panaxatriol saponin will include Re, Rg1, Rh4, Rg2, Rh1, notoginsenoside R1, Rg6, and Rk3. Ginsenoside Rg3 which is a panaxadiol saponin and a deglycosylated ginsenoside, formed during steaming, was proven to possess hematopoietic effects whereby it enhanced the proliferation of the total spleen and bone marrow (BM) cells [18]. Based on these findings, hematopoietic activity of ginsenoside Rg3 is supported. In terms of modern scientific findings, there are limited amount of information on the effects of steamed *P. notoginseng* on the blood. Further research is required to address the gap between the traditional use and modern scientific findings.

In terms of the modern scientific findings on the pharmacological activity of raw and steamed *P. notoginseng*, our group has recently reported that the raw and steamed form demonstrated differences in antiplatelet and anticoagulant activities, with the steamed form having greater activity and the activities were enhanced with increasing steaming durations [19]. In addition, the *in vitro* antiplatelet and anticoagulant effects are positively translated into a prolongation of *in vivo* rat bleeding time after oral administration of the raw and steamed extracts [19]. This finding agrees with the antithrombotic activity found in the Korean red ginseng, which is a steamed form of *P. ginseng* [20]. It was demonstrated in a study that the red ginseng which is a steamed form of *P. ginseng* has a significantly stronger activity in promoting blood circulation, antithrombotic and fibrinolytic activity, as compared to the white ginseng (untreated *P. ginseng*) [21]. Ginsenosides Rg3 and Rg2 were shown to have strong inhibitory activity on platelet aggregation and inhibitory effect on the conversion of fibrinogen to fibrin [22]. In addition, ginsenoside Rk1, Rg5 and Rg3 were found to inhibit platelet aggregation induced by various agonists [23]. These ginsenosides which were formed upon steaming could be the ones contributing to the antithrombotic effect in the steamed *P. notoginseng*.

Differences in pharmacological activities of the raw and steamed/heated form of herb have also been reported in other *Panax* species. It is reported that steaming or heating of *P. ginseng* causes changes in the saponin content/composition. This, in turn affects their properties such as bioactivity with significant implications for product quality and product development. Enhanced biological activity (antioxidant and anticancer activities) of heat-treated ginseng (such as red ginseng produced by steaming and drying) has put the research focus on the identification of trace compounds formed during heating (Rh2, Rg3, Rg5, and Rh1). Steaming of raw ginseng at temperatures more than 100 °C enhanced its biological properties such as its vasodilation, radical scavenging activity and anticarcinogenicity [24]. The enhanced activity was in turn attributed to the changes in the composition of the ginsenoside mixture induced by the heat treatment. These findings have led to the use of processing as a means to enhance the biological activity of ginsenosides [24]. A ginseng product (sun ginseng, with a ratio of ginsenoside [Rg3 + Rg5] to [Rc + Rd + Rb1 + Rb2] above 1) produced by heat treatment at 120–180 °C for 0.5–20 h with enhanced pharmacological effects such as antioxidant, anti-tumorigenic and vasodilation has also been patented [25]. In another study, it has been demonstrated that heat treatment of *P. ginseng* at a temperature and under pressure higher than those applied to the conventional preparation of red ginseng, gave rise to increased production of saponins such as Rg3, Rg5, Rg6, Rh2, Rh3, Rh4 and Rs3, which are absent or present, if any, in trace amounts in conventional white or red ginseng [26]. For *Panax quinquefolius*, it had also been shown that heat-processing of the roots and berries increased the antiproliferative [27] and radical scavenging activity [28] of American ginseng significantly. The antiproliferative activities of red American ginseng are augmented when ginsenoside Rg3 is increased [27].

This finding supported the fact that steaming causes changes to the metabolic profile of *Panax* species and lead to differences in pharmacological activity and therapeutic effects of the raw and steamed herb. Studies on the steamed *P. notoginseng* are still lacking and inconclusive. Hence, more research on its activities is necessary. It is critical to study the differences between raw and steamed *P. notoginseng* using metabolomics as a first step to investigate their profiles and relating them to their different therapeutic uses. It is pertinent to administer the correct form of herb to avoid any undesirable clinical consequences. Therefore, it is paramount to characterize the raw and steamed forms of *P. notoginseng* for the investigation of their pharmacological use and safety.

4. Conclusion

An optimized UHPLC/TOFMS metabolic profiling method was developed for the analysis and evaluation of raw and steamed *P. notoginseng*. The UHPLC/TOFMS metabolic profiles combined with multivariate statistical analysis represents a powerful platform for the time-dependent profiling of raw and differentially steamed *P. notoginseng*. The methodology holds potential to be applied to the quality control of the steaming process to achieve desired herbal contents of the steamed products.

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References

[1] A.J. Lau, S.O. Woo, H.L. Koh, Analysis of saponins in raw and steamed *Panax notoginseng* using high-performance liquid chromatography with diode array detection, J. Chromatogr. A 1011 (2003) 77–87.
[2] T.B. Ng, Pharmacological activity of sanchi ginseng (*Panax notoginseng*), J. Pharm. Pharmacol. 58 (2006) 1007–1019.
[3] M. Dan, M. Su, X. Gao, T. Zhao, A. Zhao, G. Xie, Y. Qiu, M. Zhou, Z. Liu, W. Jia, Metabolite profiling of *Panax notoginseng* using UPLC–ESI-MS, Phytochemistry 69 (2008) 2237–2244.
[4] The State Pharmacopoeia Commission of People's Republic of China, Pharmacopoeia of the People's Republic of China, vol. 1, Chemical Industry Press, Beijing, 2005.
[5] R. Bhalla, K. Narasimhan, S. Swarup, Metabolomics and its role in understanding cellular responses in plants, Plant Cell Rep. 24 (2005) 562–571.
[6] P. Xie, S. Chen, Y.Z. Liang, X. Wang, R. Tian, R. Upton, Chromatographic fingerprint analysis – a rational approach for quality assessment of traditional Chinese herbal medicine, J. Chromatogr. A 1112 (2006) 171–180.
[7] D.K.W.C. Mok, Foo-Tim Chau, Chemical information of Chinese medicines: a challenge to chemist, Selected Papers from the International Conference on Chemometrics and Bioinformatics in Asia – CCBA 2004 82 (2006) 210–217.
[8] Y. Ni, Y. Peng, S. Kokot, Fingerprinting of complex mixtures with the use of high performance liquid chromatography, inductively coupled plasma atomic emission spectroscopy and chemometrics, Anal. Chim. Acta 616 (2008) 19–27.
[9] B. Xie, T. Gong, M. Tang, D. Mi, X. Zhang, J. Liu, Z. Zhang, An approach based on HPLC-fingerprint and chemometrics to quality consistency evaluation of Liuwei Dihuang Pills produced by different manufacturers, J. Pharm. Biomed. Anal. 48 (2008) 1261–1266.
[10] E.C.Y. Chan, S.L. Yap, A.J. Lau, P.C. Leow, D.F. Toh, H.L. Koh, Ultra-performance liquid chromatography/time-of-flight mass spectrometry based metabolomics of raw and steamed *Panax notoginseng*, Rapid Commun. Mass Spectrom. 21 (2007) 519–528.
[11] J.B. Wan, P. Li, S. Li, Y. Wang, T.T. Dong, K.W. Tsim, Simultaneous determination of 11 saponins in *Panax notoginseng* using HPLC–ELSD and pressurized liquid extraction, J. Sep. Sci. 29 (2006) 2190–2196.

[12] D. Ye, S. Zhang, Traditional Chinese Medicine Processing Science, People's Medical Publishing House, Beijing, 1999.
[13] H.L. Koh, A.J. Lau, E.C.Y. Chan, Hydrophilic interaction liquid chromatography with tandem mass spectrometry for the determination of underivatized dencichine (beta-N-oxalyl-L-alpha,beta-diaminopropionic acid) in *Panax* medicinal plant species, Rapid Commun. Mass Spectrom. 19 (2005) 1237–1244.
[14] W. Ji, B.Q. Gong, Hypolipidemic effects and mechanisms of *Panax notoginseng* on lipid profile in hyperlipidemic rats, J. Ethnopharmacol. 113 (2007) 318–324.
[15] H.M. Chang, P.P.H. But, Pharmacology & Applications of Chinese Materia Medica, World Scientific Singapore, Philadelphia, PA, USA, 1986.
[16] R.L. Gao, X.H. Chen, X.J. Lin, X.D. Qian, W.H. Xu, B.H. Chong, Effects of notoginosides on proliferation and upregulation of GR nuclear transcription factor in hematopoietic cells, Acta Pharmacol. Sin. 28 (2007) 703–711.
[17] Y.P. Niu, X.D. Qian, W.X. Wang, Effect of panaxadiol saponin and panaxtrol saponin on proliferation of human bone marrow hemopoietic progenitor cells, Zhongguo Zhong Xi Yi Jie He Za Zhi 24 (2004) 127–129, 132.
[18] S.S. Joo, T.J. Won, M.S. Kim, D.I. Lee, Hematopoietic effect of ginsenoside Rg3 in ICR mouse primary cultures and its application to a biological response modifier, Fitoterapia 75 (2004) 337–341.
[19] A.J. Lau, D.F. Toh, T.K. Chua, Y.K. Pang, S.O. Woo, H.L. Koh, Antiplatelet and anticoagulant effects of *Panax notoginseng*: comparison of raw and steamed *Panax notoginseng* with *Panax ginseng* and *Panax quinquefolium*, J. Ethnopharmacol. 125 (2009) 380–386.
[20] Y.R. Jin, J.Y. Yu, J.J. Lee, S.H. You, J.H. Chung, J.Y. Noh, J.H. Im, X.H. Han, T.J. Kim, K.S. Shin, J.J. Wee, Y.P. Yun, Antithrombotic and antiplatelet activities of Korean red ginseng extract, Basic Clin. Pharmacol. Toxicol. 100 (2007) 170–175.
[21] X. Li, R. Guo, L. Li, Pharmacological variations of *Panax ginseng* C.A. Meyer during processing, Zhongguo Zhong Yao Za Zhi 16 (1991) 3–7, 62.
[22] L.P. Christensen, Ginsenosides chemistry, biosynthesis, analysis, and potential health effects, Adv. Food Nutr. Res. 55 (2009) 1–99.
[23] J.G. Lee, Y.Y. Lee, S.Y. Kim, J.S. Pyo, H.S. Yun-Choi, J.H. Park, Platelet antiaggregating activity of ginsenosides isolated from processed ginseng, Pharmazie 64 (2009) 602–604.
[24] O. Guclu-Ustundag, G. Mazza, Saponins: properties, applications and processing, Crit. Rev. Food Sci. Nutr. 47 (2007) 231–258.
[25] N.D. Kim, M.K. Park, S.K. Lee, J.H. Park, J.M. Kim, in: Park, Man Ki (Eds.), Processed Ginseng Product with Enhanced Pharmacological Effects, KR, Seoul, 1998.
[26] J.H. Park, J.M. Kim, S.B. Han, N.Y. Kim, A new processed ginseng with fortified activity, in: H. Huh, K.J. Choi, Y.C. Kim (Eds.), 7th International Symposium on Ginseng, Seoul, South Korea, 1998, pp. 146–159.
[27] C.Z. Wang, H.H. Aung, B. Zhang, S. Sun, X.L. Li, H. He, J.T. Xie, T.C. He, W. Du, C.S. Yuan, Chemopreventive effects of heat-processed *Panax quinquefolius* root on human breast cancer cells, Anticancer Res. 28 (2008) 2545–2551.
[28] K.S. Kang, N. Yamabe, H.Y. Kim, T. Okamoto, Y. Sei, T. Yokozawa, Increase in the free radical scavenging activities of American ginseng by heat processing and its safety evaluation, J. Ethnopharmacol. 113 (2007) 225–232.