Journal of Chromatographic Science, 2021, 1–9
doi: 10.1093/chromsci/bmab063
Article

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Ginsenoside Contents in Ginseng: Quality by Design-Coupled Two-Dimensional Liquid Chromatography Technique

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Received 2 June 2020; Editorial Decision 2 May 2021

Abstract

Red ginseng and white ginseng, with different chemical constituents, exhibit different antioxidative, anticancer, antiasthmatic and immunomodulatory properties. The aim of this study was to determine the amount of ginsenoside contents (Rg_1, Re, Rb_1, Rb_2, Rc, Rd and Ro) in red and white ginseng. A rapid and comprehensive method was developed using the quality-by-design (QbD) and heart-cutting two-dimensional liquid chromatography (2D-LC) techniques. The temperature (25°C), mobile phase constituent (0.1%H_3PO_4), flow rate (0.35 mL/min) and concentrations of the final (45%) and initial (19.5%) organic solvents were optimized to efficient chromatography-based isolation method. The gradient program was optimized by QbD Fusion AE system. A selective column (Thermo Acclaim RSLC Polar Advantage II 2.2 µm, 100 × 2.1 mm) was used for the studies. The ginsenoside Rb_1, Rc and Ro exhibiting poor separation resolution were separated using the heart-cutting 2D-LC technique. The average Rb_1, Rb_2 and Rc contents in red ginseng were significantly higher than the average Rb_1, Rb_2 and Rc contents in white ginseng. Ginsenoside Ro can be potentially used as a marker to evaluate the qualities of white and red ginseng. This comprehensive and rapid method can be potentially used to screen the quality of the markers in the future.

Introduction

Ginseng, the roots and rhizomes of *Panax ginseng* Meyer belonging to the Araliaceae family, has been used for thousands of years for preventing and treating diseases in Asian countries (1). Ginsenosides are the primary bioactive compounds present in ginseng. The ginsenosides can be classified into three groups: protopanaxadiols, protopanaxatriols and oleanolic acids. These have been used to treat cardiovascular, kidney and reproductive diseases in human beings (2–4). Ginsenosides Rg_1, Re, Rb_1, Rb_2 and Rd (isolated from ginseng) accounted for >70% of the total ginsenosides (5). These have been identified as the quality markers of ginseng products (6). In addition, ginsenoside Ro (oleanolic acid-type ginsenoside) has been isolated from ginseng (7). The bioactive properties of ginsenosides are influenced by the types of their ginsenoside monomers present and the ginsenoside monomer contents (8). Ginsenoside Rg_1 exhibits anti-inflammatory properties (9), while ginsenoside Re has been to treat type II diabetes (10). Ginsenoside Ro can potentially be used as a novel anti-thrombotic agent that can inhibit glycoprotein alpha IIb/beta3-mediated fibrinogen (11).

Numerous processed ginseng products, prepared from white, red and black ginseng, are commercially available. White ginseng is prepared by dehydrating fresh ginseng, while red ginseng is produced

by steaming fresh ginseng for 2 h at a temperature of 100°C (12). The demalonylation reaction can be carried out with malonyl ginsenosides Rb_1, Rb_2, Rc and Rd. The products are processed for the production of the corresponding neutral ginsenosides. The ginsenosides Rb_1, Rb_2 and Rb_3 could be converted to the rare ginsenosides Rg_3, Rg_5 and Rk_1 (13). The antioxidative (14), anticancer (15), antiasthmatic (16) and immunomodulatory activities (17) exhibited by white ginseng are different from those exhibited by red ginseng. The Chinese Pharmacopoeia (ChP edition 2015) lists white and red ginsengs as biomarkers for quality evaluation (18). The ChP states that the Rg_1 and Re content are ≥0.30% in white ginseng and 0.25% in red ginseng, while the Rb_1 content is >0.20% in both. It has been observed that the ginsenoside contents cannot be used to distinguish white ginseng from red ginseng. Thus, they are mis-sold and clinically misused.

The amounts of ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc and Rd in *P. ginseng* and their ratios have been determined using the high-performance liquid chromatography (HPLC) technique (7). However, the developed method was time-consuming and required the use of a large volume of the solution during the analysis process. Different researchers employed different analysis methods for product isolation, and the compounds exhibited low stability under the experimental conditions. A rapid method has been developed for the analysis of the bioactive constituents (19). An ultra-high performance liquid chromatography system coupled with a mass spectrometer (MS) was used for the studies. However, the developed method was not cost-efficient and few laboratories could afford it. Therefore, it is necessary to develop a rapid and convenient analytical method that can be used to identify the quality markers present in white and red ginsengs.

The concept of quality by design (QbD) was proposed by Joseph M. Juran in 1992 (20), and this concept was used for the quality control of drugs by U.S. Food and Drug Administration in 2004 (21). The concept of QbD has recently gained much attention. It is used to search and manufacture drugs. The QbD strategy was used to develop an analytical HPLC method based on sound statistical experimentation principles (22) that accounted for analytical target profiles (analytical performance), critical method attributes (precision, accuracy and sensitivity) and critical method parameters (pH, temperature and selective column). The QbD strategy based on an HPLC method was used to rapidly isolate amiodarone hydrochloride and its impurities present in drugs (23). Rapid and efficient separation of the peaks corresponding to the compounds was achieved. Zacharis *et al.* (24) established a simple and robust LC method for the isolation of alkyl *p*-toluenesulfonates using a QbD-based analytical method. However, reports on the use of the QbD strategy for the quantitative analysis of natural products present in white and red ginsengs are rare. However, reports on the use of the QbD strategy for the quantitative analysis of natural products present in white and red ginsengs are rare. The process of analyzing ginsenosides Rg_1, Re and Rb_1 (in white and red ginsengs) is time-consuming due to bad chromatography performance in ChP (18). The resolving power exhibited by the two-dimensional liquid chromatography (2D-LC) technique was better than the resolving power exhibited by the conventional 1D-LC technique. The tropane alkaloids present in herbal medicines could be separated well using the 2D-LC technique (25).

The objective of this work was to develop a rapid and robust method for measuring ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, Ro and Rd present in white and red ginsengs. A simple and rapid QbD-based analytical method was used to optimize the various parameters (such as the choice of the mobile phase, temperature, flow rate and gradient for markers in LC that influence separation performance). The type of column that can be used for efficient compound separation was also identified. Following this, a 2D-LC method was developed to isolate these compounds represented by closely placed peaks. Following this, a 2D-LC method was developed to separate. The samples differed in their ages and were collected from various regions. This comprehensive, simple and rapid method can be potentially used to control the quality of herbal medicines consisting of complex chemical substances.

Experimental

Reagents and materials

Ginsenosides Rg_1 (batch number: PRF10031209), Re (batch number: PRF9030201), Rb_1 (batch number: PRF15032406), Rc (batch number: PRF8022343), Rb_2 (batch number: PRF10062842) Ro (batch number: PRF9121001) and Rd (batch number: PRF8062623) were purchased from Biopurfy Phytochemicals (Chengdu, China). The ginsenosides references were >98% pure. The structural formulas of ginsenosides are shown in Supplementary Figure S1. HPLC-grade acetonitrile, methanol and phosphoric acid were purchased from Fisher Scientific (Santa Clara, CA, USA). Distilled water available in the laboratory was filtered using a Milli-Q water filtration system.

Fresh ginseng collected from different geographical origins was processed at Jilin Kangmei Pharmaceutical Co. Ltd. White ginseng was prepared by drying fresh ginseng. Fresh ginseng was steamed for 2 h and dried to produce red ginseng. Seventeen batches of white (11 batches) and red (6 batches) ginseng, collected from different regions and differing in their ages, were collected. The weight of each batch was set to 500 g. The detailed information of samples is shown in Supplementary Table SI. The dried white and red ginseng were smashed using a pulverizer (Shandong Dade Medical Machine) and passed through 65-mesh screen. The medicinal powder was stored at Ziplock bag at −20°C until further use.

Sample preparation and preparation of standard solution

The sample powder (1 g) for LC development analysis was extracted using an ultrasonic water bath (100 Hz). The ultrasonication process was conducted for 50 min, and methanol (25 mL; 70%) was used as the solvent during the process. The sample solution was cooled to 25°C, after which methanol (70%) was added to the extracted sample and initial weight of the sample was recorded. Each solution was filtered through a microporous membrane (0.22 μm) conducting the HPLC experiments. Each reference was dissolved in methanol to prepare the ginsenoside samples (1 mg/mL, Rg_1, Re, Rb_1, Rc, Ro, Rb_2 and Rd). The samples were diluted with methanol to prepare the appropriate standard samples to generate the calibration curves.

QbD design

The method development experiments were designed using Fusion AE method development software (S-Matrix, Eureka, CA, USA). It was used to develop an analytical method for detecting the markers of white and red ginseng. An experimental setup was required to conduct the Fusion QbD-based LC method. Initially, the design was generated, which was exported in the next step. Following this, the experiment was conducted. The subsequent step involved the import/entry of the response data, which were then analyzed. The details of the operational steps are as follows: (i) experiment

setup: in this step, a new data file was developed for rapid column and solvent screening is developed, and the target LC instrument was chosen based on the operational conditions. (ii) Generation of design: the desired variable ranges or levels are entered, and the setup data are updated to develop design operations. (iii) Design export to LC Lab: in this step, the operation is executed using an LC instrument. (iv) Execution of the experiment: the sequence is run on the LC instrument. (v) Import/enter response data: the imported data are analyzed. (vi) Optimization of conditions: the "best" condition for the best overall search is identified. (vii) Visualization of the acceptable performance region: the acceptable performance region is identified.

During the experiments, columns of varying selectivity were tested to achieve the best sample separation. Mobile phases containing aqueous components (water or 0.1% phosphoric acid) were used for the efficient separation of the ginsenosides. The column temperature and flow rate were also optimized. The solvent gradients were optimized to achieve the best separation possible in the shortest amount of time. The columns used were procured from Thermo Scientific (Acclaim RSLC Polar Advantage II; 2.2 µm, 100 × 2.1 mm; PA2). The composition of the optimal mobile phase (aqueous) was identified as 0.1% phosphoric acid water (A). Acetonitrile (B) was used as the organic mobile phase. The column temperatures and flow rates were set at 25°C and 0.35 mL/min, respectively. The HPLC system was equipped with an ultraviolet (UV) detector operating at 203 nm (gradient elution conditions: 0–0.5 min, 81.5%A; 0.5–30.5 min, 81.5–55%A; 30.5–32.5 min, 55%A; 32.5–33.5 min, 55–5%).

Heart cutting 2D-LC

The multiple heart-cutting 2D-LC technique was used to separate the peaks with low-resolution peaks. The experiments were conducted using a Thermo Scientific Dionex UltiMate 3000 dual gradient system equipped with SRD-3600 dual ternary pump, WPS 3000 autosampler, DAD 3000RS detector and TCC-3000RS column oven. Two position, six port switching valves connected with three loops (200 µL) were used to collect the targeted fractions during the first dimension separation processes. The same mobile phase was used to conduct the 1D-LC and 2D-LC experiments under conditions of the same UV wavelength. The column temperatures and flow rates were set at 30°C and 1 mL/min, respectively. The 2D separation process was conducted using the Thermo Scientific Accucore C30 (2.2 µm, 100 × 2.1 mm) system. The mobile phase consisted of 0.1% phosphoric acid (A), acetonitrile (B) and acetonitrile/methanol (60/40) (C). The elution programs for 2D separation process were as follows: 0–10 min, 90%B; 12–24 min, 5%B; 33 min, 50%B; 36 min, 80%B; 36.5–41 min, 5%B; 50 min, 60%C; 53–55 min, 70%C; 55.5–60 min, 5%B; 70 min, 48%B; 72 min, 90%B.

Validation method

The linearity of each calibration curve was determined by plotting the analytes peak area (Y) against the concentrations of analytes (X). Limit of detections (LOD) and limit of quantifications (LOQ) were determined from the concentrations of the analytes when the signal-to-noise ratios were 3 and 10, respectively. The intra- and inter-day precision of the methods were determined using reference standard stock solution. Experiments to determine the intra-day precision were conducted five times each day and experiments to determine the inter-day precision were conducted on three consecutive days. The repeatability of the experiments was determined using six sample solution prepared following the method used before. The stabilities of the analytes were assessed by analyzing the same sample solution stored at 4°C (analyzed within 48 h of preparing the samples). The recovery rates were determined all reference standards by adding the samples (100% concentration) to the sample extracts. The recovery rate was calculated using following formula: recovery rate (%) = (detected amount − original amount)/spiked amount × 100%.

Results

QbD-based analysis of seven ginsenosides

The QbD analysis technique was used to ginsenosides. The protocols reported by Mallik *et al.* (22) were followed for the experiments. The technique used to optimize the HPLC method was used to isolate the ginsenosides in white and red ginseng. The type of column used and column temperature dictates the separation efficiency of the developed HPLC method. Six columns exhibiting varying packing modes and selectivity were screened to identify the optimal conditions to achieve the best possible separation. The Acclaim RSLC C18 (2.2 µm, 100 × 2.1 mm), Hypersil GOLD (1.9 µm, 100 × 2.1 mm) and Accucore phenyl (2.2 µm, 100 × 2.1 mm) column could enhance the peak capacity. The resolution could also be enhanced. The HSS T3 column (1.8 µm, 100 × 2.1 mm) and BEH column (1.8 µm, 100 × 2.1 mm) could be used to isolate the polar compound. The PA2 column could be used to efficiently polar or non-polar compounds even under acidic and alkaline condition. Good peak separation and enhanced peak resolution could be achieved when the column temperatures were low, and the column pressure was high.

The peak resolution could be increased using a mobile phase containing phosphoric acid buffer. However, it limited the applicability of the column under the acidic conditions. The Fusion AE method was used to optimize the temperature, column conditions and buffer types (used in the mobile phase) to achieve good compound (ginsenoside) separation. Acetonitrile was used as the organic phase during the experiments. The detailed QbD-based experimental conditions are presented in Table I. The flow rate was maintained at 0.4 mL/min. The column was conditioned for 5 min and the wavelength under which the experiments were conducted was 203 nm. After the QbD analysis method was developed, the experiments were conducted 45 times under the optimized conditions (Supplementary Table SII). Each set of HPLC analysis was repeated three times. The number of peaks indicated the resolution for the target compounds. The guidelines laid down by the United States Pharmacopeia state that compounds with peak resolution >1.5 can be quantitatively analyzed. The optimal conditions to achieve the best separation of the target compounds were determined (PA2; 0.1% H_3PO_4; percentage of final organic solvent: 45%; oven temperature: 35°C). The number of peaks obtained under optimal conditions was 7.1, and 5.8 peaks exhibited resolution >1.5 (Supplementary Table SIII).

The flow rate and initial organic solvent content significantly affected the analysis process. The optimal oven temperature was determined again. The chromatography-specific parameters were designed by Fusion AE (Table II). Eighteen experimental conditions were analyzed and each experimental run was repeated thrice. The best parameter was determined from the acceptable performance region. An analysis of the unshaded region (with peak resolution >1.5) revealed that the criteria for the limit of acceptability for critical responses were adhered to. The center point (T) of the black-outlined box represents the best condition used to determine

Table I. Conditions for QbD Analysis for Screening Column Types, Mobile Phase and Final Organic Solvent Percentage

Column valve position	Column level	Constant name	Constant value	Units	Constant name	Constant value	Units
P1	Acclaim RSLC C18 (2.2 µm, 100 × 2.1 mm)	Initial aqueous Solvent	84	%	Ramp Up to Wash Time	2	min
P2	HSS T3 column (1.8 µm, 100 × 2.1 mm)	Initial organic Solvent	16	%	Column Wash Time	5	min
P3	Acclaim RSLC Polar Advantage II (2.2 µm, 100 × 2.1 mm)	Equilibration Time	1	min	Column Wash Organic	95	%
P4	BEH column (1.8 µm, 100 × 2.1 mm)	Initial Hold Time	0.5	min	Ramp Down from Wash Time	2	min
P5	Hypersil GOLD (1.9 µ, 100 × 2.1 mm)	Gradient Time	20	min	Re-equilibration Time	5	min
P6	Accucore phenyl (2.2 µm, 100 × 2.1 mm)	Final Hold Time	1	min	Re-equilibration Organic	16	%

Acclaim C18 = Thermo Scientific Acclaim RSLC C18 (2.2 µm, 100 × 2.1 mm); T3 = ACQUITY UPLC HSS T3 column (1.8 µm, 100 × 2.1 mm); PA2 = Thermo Scientific Acclaim RSLC Polar Advantage II (2.2 µm, 100 × 2.1 mm); BEH C18 = ACQUITY UPLC BEH column (1.8 µm, 100 × 2.1 mm); Gold = Thermo Scientific Hypersil GOLD (1.9 µm, 100 × 2.1 mm); Phenyl = Thermo Scientific Accucore Phenyl (2.2 µm, 100 × 2.1 mm)

the optimal robustness of the method. Points A, B, C and D indicate the robustness of the method. The acceptable performance regions (initial organic solvent percentage and pump flow rate) are shown in Figure 1A. The data presented in the figure indicate that the acceptable initial organic solvent percentages were in the range of 14–25% and the acceptable pump flow rate was in the range of 0.3–0.4 mL/min. The square region consists of four points: A (0.33 mL/min, 17.3%), B (0.33 mL/min, 21.7%), C (0.37 mL/min, 17.3%) and D (0.37 mL/min, 21.7%). The points indicate the robustness of the established method. The best organic solvent and pump flow rate (mL/min) conditions for the developed method (center point of square region) were 19.5 and 0.35, respectively. The number of peaks exhibiting a resolution >1.5, when the oven temperature was 25°C, was greater than the number of peaks exhibiting a resolution of >1.5 when the oven temperature was 30°C (response surface; Figure 1B and C). The gradient program was the key parameter that helped the development of the chromatographic method. The other important parameters were pump flow rate (0.35 mL/min), oven temperature (25°C), injection volume (2 µL), detector wavelength (203 nm), percentage of initial organic solvent (19.5%) and percentage of final organic solvent (47%). The data obtained from five experiments were analyzed. Each set of experiments was repeated thrice. The best separation of the mixed standards could be achieved using the optimized gradient conditions (Supplementary Figure S2).

The optimized method was used to determine the constituents of white and red ginsengs. Analysis of the typical chromatograms of red ginseng (Figure 2) revealed that the third, fourth and sixth peaks indicated a mixture of target compounds and impurities. The chromatograms recorded with white ginseng are presented in Supplementary Figure S3. Analysis of the chromatograms revealed that the 1D-LC method could not be used to analyze a large volume of a complex mixture. A rapid method (heart-cutting 2D chromatography method) was used to analyze all the target compounds present in a mixture. The optimized QbD method was considered as the first dimension in the heart-cutting 2D chromatography technique. The column type, mobile phase constituents, flow rate and program gradient were optimized to conduct the 2D chromatography experiments.

Heart cutting 2D chromatography technique

Long *et al.* (25) developed the rapid heart-cutting 2D-LC technique for the isolation of tropane alkaloids. Orthogonal selectivity is one of the important parameters that dictate the efficiency of the heart-cutting 2D LC. The column PA2 can be used to bind the polar sulfonamide moieties with the ether chains on the surface of silica gel. The Accucore C30 (2.2 µm, 2.1 × 100 mm) column, the chemical phase of which was different from the chemical phase of the column 1D study, as to have the best separation. Better orthogonal properties could be thus achieved during the 2D-LC experiments (26). The optimized flow rates were 0.6, 0.8 and 1 mL/min. The separation performance could be enhanced by increasing the flow rate. The separation performance was tested at three temperatures (column temperature 25, 30 and 35°C) and the best separation performance was achieved at the temperature of 30°C. The acetonitrile to methanol ratio in the third mobile phase was to 60:40 to efficiently separate Ro. The fractions 1–3 obtained following the first dimension chromatography technique were cut and separated using the second-dimension chromatography, technique (exhibiting better separation performance for the ginsenosides compared with the 1D chromatography technique; Figure 2).

Various parameters (linearity, LOD, LOQ, precision, repeatability, stability and recovery) were studied to determine the efficiency of the developed heart-cutting 2D-LC method. A correlation coefficient (R^2) value >0.9980 indicated that the calibration curves of all the compounds exhibited good linearity (Table III). The LOD values were in the range of 0.17–0.98; the LOQ values ranged from 0.58 to 8.30. The intra-day precision was calculated from the relative standard deviation (RSD) of the peak areas (reference standards). The precision was found to be <2.18%. The repeatability of the six samples calculated from the RSDs was <4.91%. The samples were stable for 48 h when RSD value was <2.33%. The recovery rates of all the compounds were between 90.5 and 101.5% when the RSD value was <4.65%.

Determination of ginsenosides in white and red ginseng

The Re, Rg_1, Rb_1, Rc, Rb_2, Ro and Rd contents in white and red ginseng have been presented in Table IV. The Rg_1 content ranged

Table II. Best Overall Answer Search for Obtaining the Results using the Fusion QbD Analysis Method

Column type	Weak solvent type	Final organic solvent	Oven temperature	No. peaks	No. peaks ≥1.50—Resolution (USP)
PA2	**0.1%H_3PO_4**	**45**	**35**	**7.1**	**5.8**
PA2	Water	45	25	6.3	5
Phenyl	0.1%H_3PO_4	45	25	6.8	5.4
Phenyl	Water	55	25	7	5.3
BEH C18	0.1%H_3PO_4	45	35	7.3	5.1
BEH C18	Water	45	25	6	4.3
Acclaim C18	0.1%H_3PO_4	45	35	7.3	4.5
Acclaim C18	Water	55	25	7	4.1
Gold	0.1%H_3PO_4	45	25	6.2	4.5
Gold	Water	55	25	7	4.4
T3	0.1%H_3PO_4	45	25	6.9	4.5
T3	Water	55	25	5.9	4.3

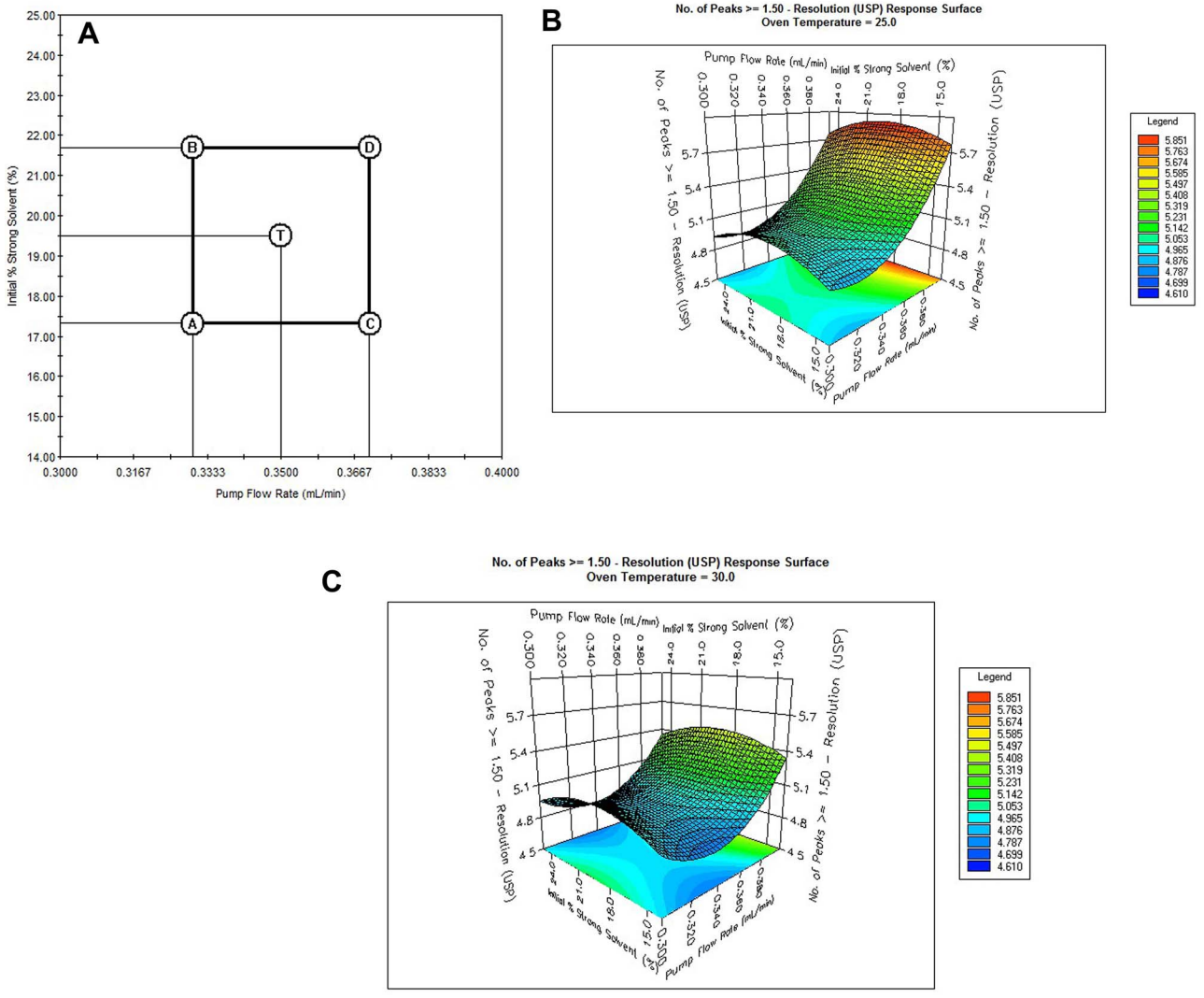

Figure 1. The acceptable performance region using % strong solvent and pump flow rate (A); compared with oven temperature of 25°C (B) and 30°C (C) based on the initial % strong solvent and pump flow rate.

from 2.21 to 3.75 mg/g in red ginseng. The Re content was in the range of 0.88–1.84 mg/g, the Rb_2 content was in the range of 0.72–2.81 mg/g, the Rd content was in the range of 0.81–1.31 mg/g, the Rb_1 content was in the range of 4.38–6.58 mg/g, the Rc content was in the range of 0.80–3.27 mg/g and the Ro content was in the range of 2.14–3.43 mg/g. In white ginseng, the Rg_1 content was in the range of

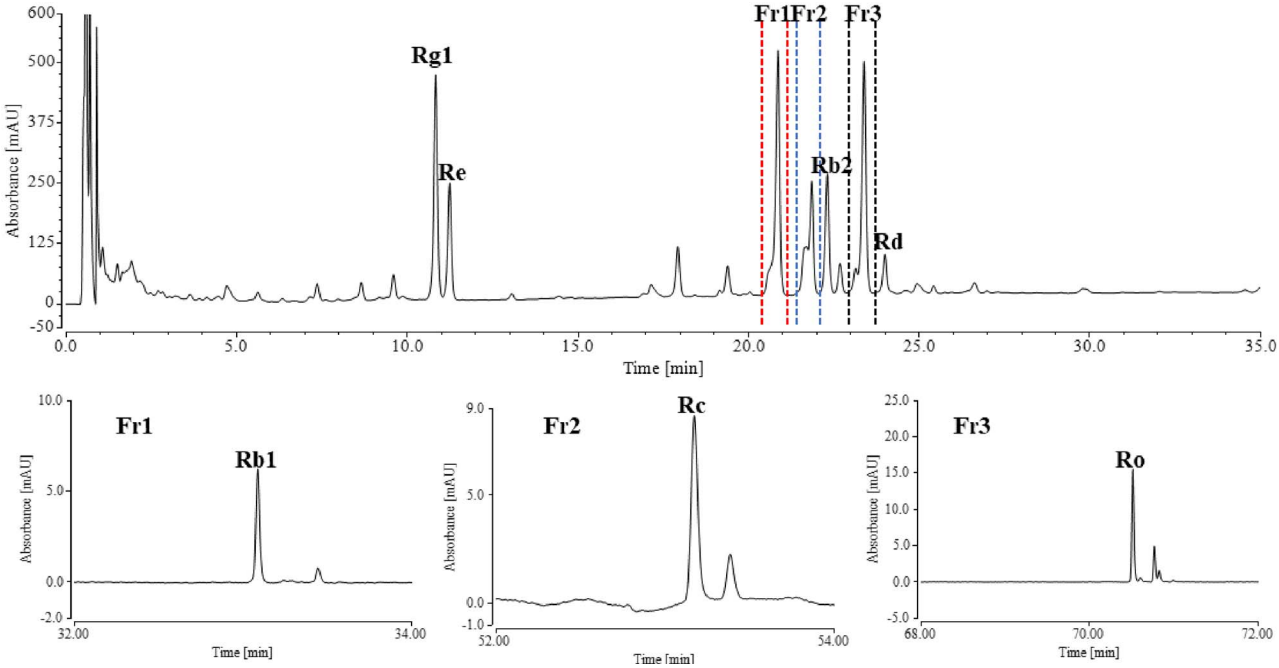

Figure 2. Optimized chromatography of red ginseng by Fusion QbD, ginsenoside Rb_1, Rc and Ro could not separate well from untargeted compounds; the fraction of ginsenoside Rb_1, Rc and Ro separated by two-dimensional chromatography.

Table III. The Calibration Curve, Linear Range, Regression, LOD, LOQ, Intra- and Inter-Day Precision, Repeatability and Recovery Rates of Analytes

Analyte	Calibration curve	Linear range (μg/mL)	R^2	LLOD (μg/mL)	LOQ (μg/mL)	Intra-day precision (%)	Inter-day precision (%)	Repeatability (%)	Recovery rate (%)	Recovery rate (RSD %)
Ginsenoside Rg_1	$y = 0.1752x + 0.8335$	4.06–812	0.9984	0.17	0.58	0.85	0.89	3.46	98.5	3.78
Ginsenoside Rb_1	$y = 0.0057x + 0.003$	3.26–651	0.9993	0.98	3.26	0.92	1.23	3.86	97.3	4.65
Ginsenoside Re	$y = 0.2977x + 2.1115$	4.06–405.5	0.9980	0.19	0.65	0.83	1.68	3.87	100.3	3.65
Ginsenoside Rd	$y = 0.1457x - 0.2367$	2.82–563	0.9992	0.27	0.89	1.09	1.79	3.94	101.5	3.97
Ginsenoside Ro	$y = 0.0066x + 0.0282$	4.17–833	0.9989	0.91	3.02	0.83	1.03	4.91	94.8	4.01
Ginsenoside Rc	$y = 0.0053x + 0.0253$	5.89–1178	0.9992	2.49	8.30	2.18	1.34	3.39	92.4	4.63
Ginsenoside Rb_2	$y = 0.1286x + 0.6042$	3.89–777.5	0.9986	0.21	0.71	1.51	1.64	3.68	90.5	4.25

1.91–3.31 mg/g, the Re content was in the range of 0.70–1.76 mg/g, the Rb_2 content was in the range of 0.72–1.92 mg/g, the Rd content was in the range of 0.34–0.83 mg/g, the Rb_1 content was in the range of 1.95–2.63 mg/g, the Rc content was in the range of 0.41–1.45 mg/g and the Ro content was in the range of 2.03–4.88 mg/g. The amount of ginsenoside Rb_1 was >3.86 mg/g in red ginseng, <2.63 mg/g in white ginseng. Ginsenosides Ro and Rg_1 (>2.03 and 1.91 mg/g, respectively) were found to be the primary constituents in red and white ginsengs. The amount of Re was <1.84 mg/g in red and white ginsengs.

Discussion

The extraction conditions for white and red ginsengs have been outlined in the ChP (2015 edition). The samples were extracted using chloroform a Soxhlet extractor was operated for 3 h during the extraction process. The solution was allowed to condition overnight and then ultrasonicated for 30 min using n-butyl alcohol (50 mL). The extracting solution was dried and the residue dissolved in methanol. Following this, the solution was filtered through a membrane filter (0.22 μm). The sample solution was injected into the HPLC system for analysis (18). The method was time-consuming

Table IV. The Contents of Ginsenosides Rg_1, Re, Rb_2, Rb_1, Rc, Ro and Rd in White and Red Ginseng ($n = 3$, mg/g)

Site	Name	Year	Rg_1	Re	Rb_1	Rc	Rb_2	Ro	Rd
Ji'an Jilin	red ginseng	four	3.75 ± 1.21	1.84 ± 1.02	6.58 ± 2.03	0.80 ± 0.97	0.72 ± 0.56	3.43 ± 1.03	1.31 ± 1.89
Tonghua Jilin	red ginseng	four	2.73 ± 1.64	1.72 ± 1.13	4.93 ± 2.31	3.27 ± 1.54	2.81 ± 1.03	2.84 ± 0.87	1.31 ± 0.98
Hailun Heilongjiang	red ginseng	four	2.83 ± 1.06	1.72 ± 1.15	4.79 ± 1.06	2.60 ± 1.05	2.37 ± 0.96	2.88 ± 1.64	0.98 ± 0.78
Fusong Jilin	red ginseng	five	2.61 ± 1.78	0.89 ± 0.46	4.38 ± 1.05	3.01 ± 1.06	2.47 ± 1.53	2.85 ± 1.30	1.05 ± 1.03
Tieli Heilongjiang	red ginseng	five	3.46 ± 2.01	0.88 ± 2.09	5.09 ± 2.03	2.58 ± 1.06	2.36 ± 1.32	2.14 ± 1.05	0.81 ± 1.06
Changbai Jilin	red ginseng	six	3.15 ± 0.97	1.69 ± 1.56	5.72 ± 1.25	2.61 ± 1.03	2.72 ± 0.94	4.31 ± 1.97	0.99 ± 0.32
Changbai Jilin	white ginseng	five	2.21 ± 1.16	1.40 ± 1.31	3.86 ± 1.14	1.51 ± 0.86	1.51 ± 0.87	2.27 ± 0.64	0.72 ± 2.05
Ji'an Jilin	white ginseng	four	3.16 ± 1.34	1.12 ± 0.32	1.95 ± 1.32	1.12 ± 1.30	1.57 ± 0.78	2.15 ± 1.35	0.44 ± 2.04
Changbai Jilin	white ginseng	four	1.91 ± 0.98	1.64 ± 0.74	1.96 ± 0.65	0.41 ± 1.21	0.73 ± 0.84	2.49 ± 1.02	0.44 ± 0.06
Tonghua Jilin	white ginseng	four	3.04 ± 1.32	1.44 ± 1.21	2.31 ± 0.54	1.45 ± 1.31	1.92 ± 1.20	2.03 ± 0.46	0.83 ± 1.15
Dunhua Jilin	white ginseng	four	2.34 ± 2.01	1.06 ± 1.30	2.33 ± 0.97	0.59 ± 2.09	1.25 ± 1.02	2.62 ± 1.03	0.58 ± 2.17
Jingyu Jilin	white ginseng	four	2.42 ± 1.69	1.76 ± 0.65	2.20 ± 0.85	0.55 ± 0.97	1.15 ± 2.09	2.05 ± 0.65	0.51 ± 0.28
Tieli Heilongjiang	white ginseng	four	2.87 ± 1.36	1.64 ± 1.34	2.36 ± 1.06	0.61 ± 0.45	1.08 ± 1.18	2.85 ± 0.97	0.48 ± 1.07
Tieli Heilongjiang	white ginseng	five	2.88 ± 1.67	0.84 ± 0.54	2.43 ± 0.96	0.73 ± 0.64	1.13 ± 1.01	3.20 ± 1.64	0.53 ± 1.18
Fusong Jilin	white ginseng	five	2.18 ± 1.02	1.52 ± 0.96	2.19 ± 1.02	0.49 ± 0.45	0.72 ± 1.52	2.48 ± 0.98	0.34 ± 0.15
Changbai Jilin	white ginseng	five	2.44 ± 0.65	0.83 ± 0.08	2.63 ± 0.15	0.86 ± 0.65	1.23 ± 1.03	2.52 ± 0.98	0.46 ± 0.97
Changbai Jilin	white ginseng	six	3.31 ± 2.49	0.70 ± 1.24	2.00 ± 1.03	0.72 ± 0.64	1.09 ± 0.95	4.88 ± 1.65	0.37 ± 1.09

and the workflow was complex. A large volume of the solvent was consumed during the process. A limited amount of chloroform could be used as it was toxic. The use of n-butyl alcohol also limits the application of the process as it emits a pungent smell.

Another method was tested for sample extraction. The sample (1 g) was dissolved in 25-mL methanol and n-butyl alcohol. The sample solutions were extracted by ultrasonicating them for 30 min. The maximum amounts of the ginsenosides could be extracted when the extraction solvent was methanol (Supplementary Figure S4). A solvent system consisting of methanol and water was used for extraction. The polarity of the solvent system varied. The percentages of methanol in water were varied as 10, 30, 70 and 90%. The results indicated that the maximum extraction ratio could be achieved with a solvent system consisting of 70% methanol in water. The extraction time was found to be an important parameter that influenced the extraction efficiency. The extraction times were varied as 20, 30, 40, 50 and 60 min. It was observed that the maximum amount of ginsenoside could be extracted when the extraction time was 50 min. Thus, the optimum amount of ginseng could be extracted from ginsenoside (1 g) using 70% methanol (25 mL) under conditions of ultrasonication (time: 50 min).

The Acclaim RSLC Polar Advantage II columns were used to embed the polar groups of the amide units. Multi-point adhesion between the ligand and the silica gel surface was detected. The best separation of the alkaline compounds was achieved using the Acclaim RSLC C18 column that contained less amounts of the silicyl group. The Hypersil GOLD columns are based on highly pure silica and novel proprietary derivatization. The high-strength silica present in the T3 columns is compatible with a 100% aqueous mobile phase. The BEH columns containing ethylene-bridged hybrid particles exhibit good chemical stability. The Accucore phenyl columns are packed with ultrapure silica and contain a solid core. They can be used to separate aromatic and moderately polar analytes. In this study, the Acclaim RSLC Polar Advantage II columns were used to isolate the ginsenosides present in red and white ginsengs, as the neutral and acidic ginsenosides interact with the polar groups of the amide units.

A 2D score scatter plot with 95% confidence ellipses (principal component analysis plots) based on the seven ginsenoside contents was analyzed to distinguish white ginseng from red ginseng. The first two principal components accounted for 70.12% of the total variances. Figure 3A revealed that the white and red ginsengs were clustered, indicating that specific ginsenosides could be used to distinguish the two ginsengs under study from each other. The average ginsenoside (Rb_2, Rb_1 and Rc) contents in red ginseng were higher than the average ginsenoside contents in white ginseng (Figure 3B). The average Rb_1 content in red ginseng (5.05 mg/g) was 2.26 times higher than the average Rb_1 content (2.24 mg/g) in white ginseng. The average Rc and Rb_2 contents in red ginseng were 2.34 and 2.14 mg/g, respectively, while the average Rc and Rb_2 contents in white ginseng were 0.75 and 1.19 mg/g, respectively. These results agreed well with the previously reported results where it was revealed that during sample processing, the ginsenoside contents varied significantly (13). The variations can be attributed to the fact that malonyl ginsenosides get converted to the corresponding neutral ginsenosides during the sample processing steps. Therefore, the criterion of $Rb_1 > 2.0$ mg/g was not applicable under these conditions (for white and red ginsengs). The Ro contents in white and red ginseng were >2.5 mg/g. The Ro content was lower than the Rb_1 content and higher than the contents of the other ginsenosides. The oleanolic acid-type ginsenoside Ro exhibited antitumor, anti-inflammatory and anti-hepatitis properties. Reports on the determination of the amount of Ro are scarce (27). The use of the mobile phase containing 0.1% phosphoric acid helped increase the resolution of the Ro peak observed in the chromatograms. The better resolution achieved by adding phosphoric acid can be attributed to the interactions between Ro and the solid sorbents present in the analytical column (28). Therefore, we believe that ginsenoside Ro can be used as a marker to identify white and red ginsengs under the given conditions.

Conclusion

Various parameters (such as column conditions, temperature, flow rate, percentages of the final and initial organic solvent used, and

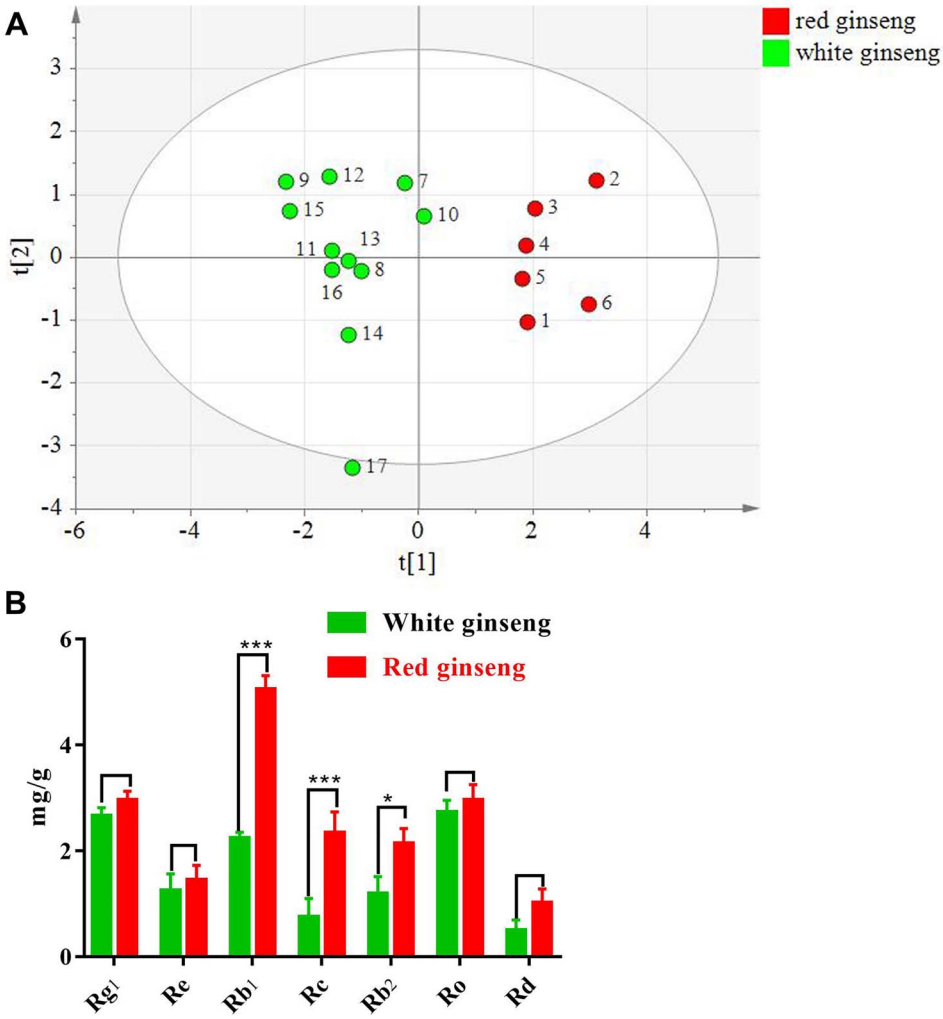

Figure 3. Average contents of ginsenosides Rg_1, Re, Rb_2, Rb_1, Rc, Ro and Rd in white and red ginseng.

gradient program) that influence the isolation of ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc, Rd and Ro were optimized using the QbD technique. A rapid heart-cutting 2D-LC technique was developed for the isolation of ginsenosides Rb_1, Rc and Ro that exhibited poor separation resolution. The average content of ginsenosides Rb_1, Rb_2 and Rc in red ginseng was much higher than the average of the same ginsenosides in white ginseng. The amount of Rb_1 was >3.86 mg/g in red ginseng and <2.63 mg/g in white ginseng. The average Rb_1 content (5.05 mg/g) was 2.26 times higher than the average Rb_1 content (2.24 mg/g) in white ginseng. The average Rc and Rb_2 contents in red ginseng were 2.34 and 2.14 mg/g, respectively, while the average Rc and Rb_2 contents in white ginseng were 0.75 and 1.19 mg/g, respectively. The Ro content (>2.5 mg/g) can potentially be used as a new marker for the identification of white and red ginsengs under the current settings. A rapid and comprehensive method for screening the quality markers should be developed in the future for the quality control of herbal medicines.

Supplementary data

Supplementary data are available at *Journal of Chromatographic Science* online.

Funding

This work was financially supported by the National Natural Science Foundation of China (81920108033, 82074011, 81703682, 81530096, 81573581).

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