

Water-Activation Steaming Coupled with Single-Marker Quantification: A Green Strategy for Industrial Standardization of Bioactive Steamed *Panax notoginseng*

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Abstract

Industrial production of *Steamed Panax notoginseng* (SPN) faces batch-to-batch variability (RSD > 15%) in rare ginsenosides Rk₃, Rh₄, 20(*S*)-Rg₃, and 20(*R*)-Rg₃ and costly quality control due to expensive reference standards (e.g., 20(*S*)-Rg₃ ≈ \$2500/mg). To address this, we developed an integrated strategy: (1) A "water-activation–gradient temperature control" process optimized *via* orthogonal design and Arrhenius kinetics ($E_a = 58.3$ kJ/mol) increased total rare ginsenosides by 78.6% (32.7 mg/g, $p < 0.01$) under the following optimized parameters: particle size of 2 ~ 4 mm, water impregnation of 100% w/w for 2 h, and steaming at 120°C for 5 h. This optimization reduced batch variability to an RSD < 5%; (2) An HPLC-QAMS method using accessible 20(*R*)-Rg₃ as an internal reference achieved simultaneous quantification of four ginsenosides with validated relative correction factors (Rk₃: 0.5768, Rh₄: 0.4690, 20(*S*)-Rg₃: 1.0924; RSD < 2.0%), demonstrating high accuracy (recovery: 91.95–101.34%, RSD < 1.8%), linearity ($r \geq 0.9997$), and robustness across HPLC systems (RSD < 3.5%), reducing reference standard costs by 75%. The QAMS method exhibited superior greenness (AGREE score: 0.76 vs. 0.63 for ESM) and applicability (BAGI score: 77.5 vs. 65.0). Analysis of 15 batches confirmed consistency (RE% < 5% vs. ESM), while optimized extraction (60% ethanol, 5 cycles × 1.5 h) achieved 85.82% transfer rate for 20(*R*)-Rg₃. This work resolves SPN industrialization bottlenecks by ensuring bioactive consistency and establishing a cost-effective, eco-friendly quality control model transferable to other processed botanicals.

Introduction

Panax notoginseng (Burk.) F.H.Chen, a **prized medicinal herb** in the Araliaceae family, has been extensively applied in traditional Chinese medicine (TCM) with a documented history tracing back to the *Compendium of Materia Medica* (*Bencao Gangmu*) in the Ming Dynasty. Renowned for the principle of **"raw for dispersion, processed for tonification"**, its **raw form** dissipates stasis and arrests bleeding, while the **steamed form** tonifies vitality and strengthens the body—exemplifying the TCM theory of **differential treatment based on processing state** (*Sheng Shu Yi Zhi*)[1]. Modern pharmacological studies have confirmed that the primary bioactive constituents of *P. notoginseng* are **saponins**, with over 150 identified types. These include two main categories: (1) Protopanaxadiol-type (**PPD-type**) saponins (e.g., ginsenosides Rb₁, Rd); and (2) Protopanaxatriol-type (**PPT-type**) saponins (e.g., ginsenosides Rg₁, Re)[2-5]. These compounds exert **hemostatic, anti-inflammatory, and cardiovascular protective effects** by modulating key signaling pathways such as **PI3K/AKT and NF-κB**[6-8].

Chemical Transformations During Processing of Raw *Panax notoginseng* and Their Pharmacological Implications

The **thermal processing** of raw *Panax notoginseng* (Burk.) F.H.Chen triggers a series of **chemical transformations** that fundamentally alter its **pharmacodynamic basis** and **pharmacological actions**, establishing the **material foundation** for the characteristic **"differential use of crude and processed forms"**. High-temperature processing catalyzes **deglycosylation, dehydration, and isomerization** of primary saponins, generating rare ginsenosides with enhanced or unique bioactivities[9-11]. Among these, **ginsenosides Rk₃, Rh₄, 20(*S*)-Rg₃, and 20(*R*)-Rg₃** are established as **critical quality markers (CQMs)** for SPN, with the following scientific rationale:

- a. **Thermal Transformation Specificity:** These four saponins are the **primary degradation products** formed *via* specific pathways under high-temperature/high-humidity steaming (e.g., 105°C, 2~4 h)[12]. Their generation follows well-defined routes: Rb₁→Rd→Rg₃→Rh₄; Rg₁→Rh₁→PPT[13]. Their **concentrations directly correlate** with processing intensity and efficiency (e.g., Rk₃ content increases 4.7-fold after optimal steaming);

- b. **Distinct Pharmacological Activities:** Each rare ginsenoside contributes uniquely to **tonifying effects**: **20(S)-Rg₃** exhibits **potent anti-tumor activity** by inducing apoptosis *via* AKT/mTOR inhibition ($IC_{50}=12.3\mu M$). **20(R)-Rg₃** demonstrates **neuroprotective effects**, increasing neuron survival by 37.2% in $A\beta_{25-55}$ -induced injury models[14]. **Rk₃/Rh₄** synergistically enhance **anti-inflammatory, antioxidant, and hematopoietic activities** by activating the PI3K/Akt-Nrf2 axis ($\downarrow MDA$ 51%, $\uparrow SOD$ 73%)[9, 12];
- c. **Industrial Quality Control (QC) Bottlenecks:** Analytical reference standards for these saponins—particularly epimers **20(S)-Rg₃** and **20(R)-Rg₃** are prohibitively expensive (e.g., **20(S)-Rg₃** $\approx$ \$2,500/mg)[15]. This imposes severe constraints on **industrial-scale QC** when employing **external standard methods (ESM)**, which require individual reference compounds. The cost significantly limits **batch-monitoring frequency** and **process scalability**.

Despite the increasingly clarified material basis for the pharmacological effects of SPN, its industrialization remains constrained by two major technical bottlenecks:

- a. **Process Variability:** Traditional processing parameters—including particle size, water immersion conditions, steaming temperature, and steaming time—lack systematic optimization based on the kinetics of saponin conversion. Studies indicate that conversion rates are highly sensitive to these parameters[16]. For instance, reducing the particle size from 8 mm to 2 mm increases the yield of Rk₃ by 2.2-fold. However, excessive pulverization risks thermal degradation of sensitive constituents. Inadequate water immersion fails to fully activate the conversion pathway [17], while suboptimal temperature-time combinations lead to either incomplete conversion or degradation. These inconsistencies directly result in significant batch-to-batch variations ($RSD > 15\%$) in the total content of key rare saponins (e.g., Rk₃, Rh₄, 20(S)-Rg₃, 20(R)-Rg₃), critically impacting product efficacy and safety ;
- b. **Limitations of Current Analytical Methods:** Existing quality control (QC) primarily relies on the External Standard Method (ESM) for single-component quantification. Although accurate, ESM becomes economically impractical for simultaneously monitoring multiple rare saponins (Rk₃, Rh₄, 20(S)-Rg₃, 20(R)-Rg₃, etc.) due to the prohibitively high cost and limited availability of individual reference standards. The Quantitative Analysis of Multi-components by Single Marker (QAMS) approach offers a promising alternative [18, 19]. QAMS quantifies multiple analytes using relative correction factors (RCFs) derived from a single reference standard. However, its application to SPN remains unexplored. A core challenge for QAMS lies in ensuring the robustness and transferability of RCF values. These values are susceptible to significant drift (10%) under variations in chromatographic conditions (e.g., column type). HPLC instrument model, detection wavelength, flow rate), thereby compromising method reliability[20].

To address the aforementioned challenges, this study aims to:

- a. **Optimize the Steaming Process:** By integrating **orthogonal design** with **kinetic modeling** (based on the identified activation energy for saponin conversion, $E_a \approx 58.3$ kJ/mol) [13], we will develop a stable, efficient, and scalable **"moisture activation-gradient temperature control"** production process for SPN. This process targets the **maximization of key rare saponin yields** (Rk₃, Rh₄, 20(S)-Rg₃, 20(R)-Rg₃) while minimizing thermal degradation risks associated with conventional methods.
- b. **Establish a Robust QAMS Method:** We will develop and validate a novel, cost-effective, and reliable **HPLC-QAMS method** using the readily accessible **20(R)-Rg₃ as the internal reference substance** for simultaneous quantification of four target rare saponins in SPN and its extracts. The study will focus on rigorous evaluation of **relative correction factor (RCF) stability** under varied chromatographic conditions (e.g., column type, flow rate,

detection wavelength) to ensure method robustness and transferability. Furthermore, the **greenness and environmental sustainability** of the proposed method will be comprehensively assessed using **Analytical GREENness (AGREE)** and **Blue Applicability Grade Index (BAGI)** metrics. These tools will generate quantitative scores based on 12 environmental impact criteria (AGREE) and operational practicality indicators (BAGI), enabling objective comparison with conventional methods.

This integrated strategy addresses two core challenges-process reproducibility and analytical cost-effectiveness-by establishing a scientifically robust and industrially *viable* protocol for the quality standardization of SPN. Consequently, it supports broader clinical and industrial applications while actualizing the value of the "differences in raw and processed products efficacy" theory through modern quality control frameworks.

Materials and methods

Reagents, Reference Standards and Sample

Chromatography-grade solvents (methanol and acetonitrile, HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). **Ultrapure water** was prepared using a Milli-Q system (Millipore, Bedford, MA, USA) from purified water sourced from Wahaha Group (Hangzhou, China). Ginsenoside **reference standards: Rk₃** and Rh₄ were obtained from Stanford Analytical Chemicals Inc. (Stanford, CA, USA); 20(*S*)-Rg₃ was supplied by Chengdu GLP Biotechnology Co., Ltd. (Chengdu, China); 20(*R*)-Rg₃ was procured from the National Institutes for Food and Drug Control (NIFDC, Beijing, China). *Purity validation:* All standards were verified by **HPLC peak area normalization**, with purities exceeding 98% (Table 1). General **chemicals:** Analytical-grade reagents were purchased from Beijing Chemical Works (Beijing, China). A total of **15 batches of SPN powder** were collected from five manufacturers in China. All samples were produced by **slicing and steaming raw roots** sourced from Yunnan Province (Table 2). Sample authentication was performed by macroscopic and microscopic examination according to the Chinese Pharmacopoeia (2025 Edition).

Table 1. Reference material information

Name	Producer	Model No.	Batch No.	Concentration	CAS NO.
Rk ₃	Stanford Analytical Chemicals Inc.	LDHT-1020	PT231207-24	98.76%	174721-08-5
Rh ₄	Stanford Analytical Chemicals Inc.	EAAF-2029	BE231103-21	98.97%	364779-15-7
20(S)-Rg ₃	Chengdu Glip Biotechnology Co.	JOT-10044	24021802	98.39%	14197-60-5
20(R)-Rg ₃	China Academy of Food and Drug Control	110804	201504	99.50%	38243-03-7

Table 2. SPN powder herbal pieces sample information

No.	Batch No.	Source/Manufacturer	Processing Method
1	SY-WS20230201	Yunnan Baiyao Group Sanqi Industrial Co., Ltd.	Sliced notoginseng steaming
2	SY-WS20230202	Yunnan Baiyao Group Sanqi Industrial Co., Ltd.	Sliced notoginseng steaming
3	SY-WS20230203	Yunnan Baiyao Group Sanqi Industrial Co., Ltd.	Sliced notoginseng steaming
4	SY-WS20230204	Yunnan Baiyao Group Sanqi Industrial Co., Ltd.	Sliced notoginseng steaming
5	SY-WS20230205	Yunnan Baiyao Group Sanqi Industrial Co., Ltd.	Sliced notoginseng steaming
6	SY-WS20230206	Yunnan Baiyao Group Sanqi Industrial Co., Ltd.	Sliced notoginseng steaming
7	Y202302001	Yunnan Qidan Pharmaceutical Co., Ltd.	Sliced notoginseng steaming
8	Y202302002	Yunnan Qidan Pharmaceutical Co., Ltd.	Sliced notoginseng steaming
9	Y202302003	Yunnan Qidan Pharmaceutical Co., Ltd.	Sliced notoginseng steaming
10	Y202302004	Yunnan Qidan Pharmaceutical Co., Ltd.	Sliced notoginseng steaming
11	Y202302005	Yunnan Qidan Pharmaceutical Co., Ltd.	Sliced notoginseng steaming
12	Y202302006	Yunnan Qidan Pharmaceutical Co., Ltd.	Sliced notoginseng steaming
13	H20230404	Yunnan Baiyao Group TCM Resources Co., Ltd.	Sliced notoginseng steaming
14	H20230405	Yunnan Baiyao Group TCM Resources Co., Ltd.	Sliced notoginseng steaming
15	H20230406	Yunnan Baiyao Group TCM Resources Co., Ltd.	Sliced notoginseng steaming

Standard solutions

A mixed standard stock solution containing ginsenosides Rk_3 , Rh_4 , $20(S)\text{-}Rg_3$, $20(R)\text{-}Rg_3$ was prepared in methanol. Serial dilutions of this stock solution were performed using 60% (v/v) ethanol to obtain working solutions, with concentrations of 0.1557, 0.1561, 0.1472, and 0.0856 mg/mL for Rk_3 , Rh_4 , $20(S)\text{-}Rg_3$, and $20(R)\text{-}Rg_3$, respectively. Prior to HPLC analysis, all diluted solutions were filtered through 0.22 μm microporous membranes (Millipore, Billerica, MA, USA). The filtrates were injected into the HPLC system, and calibration curves were constructed by plotting peak areas against respective concentrations (Table 3).

Table 3. Preparation concentrations of reference standard solutions

Name	Concentration(%)	Weighed Amount (mg)	Stock Solution Conc. [mg/mL]	Working Solution Conc. (mg/mL)
Rk_3	98.97	39.33	1.5570	0.1557
Rh_4	98.32	39.68	1.5605	0.1561
$20(S)\text{-}Rg_3$	98.39	37.40	1.4719	0.1472
$20(R)\text{-}Rg_3$	99.50	17.20	0.17114	0.0856

Sample Preparation

Sieving & Moistening: SPN powder was sieved through a **No. 4 sieve** (nominal aperture: 4.0 mm; dominant particle size: 2~4 mm). The sieved powder was moistened with **purified water** (equivalent to its own weight) and allowed to equilibrate for **2 hours**. **Steaming & Drying:** The moistened sample was steamed at **120°C for 5 hours** in a saturated steam environment, followed by natural cooling and air-drying to ambient temperature. **Grinding & Weighing:** The dried material was re-ground through the No. 4 sieve. Approximately **0.6 g** of the powder was accurately weighed (precision: ± 0.1 mg) into a round-bottom flask. **Reflux Extraction:** **50 mL of 60% (v/v) ethanol** was added, and the total weight was recorded. The mixture underwent **reflux extraction at 80°C for 1 hour** using a water bath. **Cooling & Replenishment:** **After** cooling to room temperature, the weight loss due to solvent evaporation was compensated by adding fresh 60% ethanol. The mixture was vortex-mixed for homogeneity. **Filtration:** The extract was filtered through a **0.22 μ m nylon membrane filter** (Millipore, Billerica, MA, USA) to remove particulate matter prior to HPLC injection.

HPLC analysis

Chromatographic system

Instrumentation: Analysis was performed on an Agilent 1260 Infinity II LC system equipped with: Quaternary Pump (G7131C, pressure range up to 600 bar), Autosampler with thermostat-controlled sample tray, Column Compartment (temperature accuracy: $\pm 0.5^\circ\text{C}$), Diode Array Detector (DAD) with high-sensitivity flow cell (G7117B).

Chromatographic Conditions: Column Agilent ZORBAX SB-C18 (250 mm $\times$ 4.6 mm, 5 μ m particle size); Mobile phase Acetonitrile-water (40 : 60, v/v), isocratic elution; Flow rate 1.0 mL/min; Column temperature 25°C; Detection wavelength 203 nm; Injection volume 10 μ L. System suitability: Theoretical plates for 20(*R*)-Rg₃ $\geq$ 6000, Sample Analysis: Chromatograms of reference standards (Rk₃, Rh₄, 20(*S*)-Rg₃, 20(*R*)-Rg₃) and SPN extracts are provided in Figure 1 (**HPLC chromatograms of the mixed reference solution (A) and steamed Panax notoginseng extract (B)**). Peaks1: Rk₃; 2: Rh₄; 3: 20(*S*)-Rg₃; 4: 20(*R*)-Rg₃. Chromatographic conditions: Agilent ZORBAX-SB-C18 column (250 mm $\times$ 4.6 mm, 5 μ m); isocratic elution with acetonitrile-water (40:60, v/v); flow rate 1.0 mL/min; column temperature 25°C; detection wavelength 203 nm; injection volume 10 μ L).

Validation of the HPLC Analytical Method

The rationality of the HPLC analytical method was verified through assessments of **specificity, linearity and range, precision, stability, repeatability, accuracy, and robustness**. The validation procedures were designed as follows: Precision (**Intra-day**): The same test solution was analyzed **six times consecutively within one day** to evaluate intra-day precision; **Repeatability (Intermediate Precision)**: Six independent sample preparations from the same batch were analyzed in parallel to assess repeatability (intermediate precision); **Accuracy**: Accuracy was determined by **standard addition recovery experiments**. A known amount of reference standard (1:1 ratio to the target analyte) was spiked into the test solution, and the **recovery rate (%)** was calculated; **Solution Stability**: The stability of the sample solution was evaluated at **0, 2, 4, 8, 12, 16, 20, 24, and 36 hours** post-preparation under controlled storage conditions.

Method validation for QAMS

Relative correction factor

Standard Solution Preparation: Aliquots (2, 4, 6, 8, 10, and 12 μ L) of a mixed reference standard solution containing ginsenosides Rk₃, Rh₄, 20(*S*)-Rg₃, and 20(*R*)-Rg₃ were precisely

withdrawn and injected into the HPLC system under the chromatographic conditions specified in Section Chromatographic system.

Calculation of Relative Correction Factors (RCFs): Using **20(R)-Rg₃** as the internal reference substance, the RCF for each target analyte ($m_i = Rk_3, Rh_4, \text{ or } 20(S)\text{-Rg}_3$) was calculated according to **Equation (1)**:

$$F = \frac{F_{20(R)\text{-Rg}_3}}{F_{m_i}} = \frac{A_{20(R)\text{-Rg}_3}/C_{20(R)\text{-Rg}_3}}{A_{m_i}/C_{m_i}} \quad (1)$$

where: $F_{20(R)\text{-Rg}_3/m_i}$: Relative correction factor of the target analyte (m_i) to **20(R)-Rg₃**; $A_{20(R)\text{-Rg}_3}$ and A_{m_i} : Peak areas of **20(R)-Rg₃** and the target analyte (m_i), respectively; $C_{20(R)\text{-Rg}_3}$ and C_{m_i} : Concentrations ($\mu\text{g/mL}$) of **20(R)-Rg₃** and the target analyte (m_i) in the mixed standard solution, respectively. *RCF validation*: Each RCF value was derived from **six-point calibration curves** and validated through triplicate injections.

QAMS for Saponin Content Determination in SPN

Determination of 20(R)-Rg₃ Content by External Standard Method (ESM): The content of **20(R)-Rg₃** in 15 batches of SPN was determined using a **calibration curve** derived from the reference standard.

QAMS-Based Calculation of Target Saponin Concentrations: The concentrations of **ginsenosides Rk₃, Rh₄, and 20(S)-Rg₃** were calculated according to **Equation (2)**:

$$C_i = F_{20(R)\text{-Rg}_3/m_i} \times C_s \times \frac{A_i}{A_s} \quad (2)$$

where:

C_i and A_i = **Concentration (mg/mL)** and **peak area** of the target analyte ($Rk_3, Rh_4, \text{ or } 20(S)\text{-Rg}_3$);

C_s and A_s = **Concentration (mg/mL)** and **peak area** of the internal reference substance **20(R)-Rg₃**;

$F_{20(R)\text{-Rg}_3/m_i}$ = **Relative correction factor (RCF)** of the target analyte to **20(R)-Rg₃** (validated in Section 3.2).

Content Calculation of Target Saponins: The content of each target saponin in steamed *P. notoginseng* was calculated using **Equation (3)**

$$R_i = \frac{C_i \times V}{m} \times 1000 \quad (3)$$

where:

R_i = **Content (mg/g)** of the target analyte ($Rk_3, Rh_4, \text{ or } 20(S)\text{-Rg}_3$);

C_i = **Concentration (mg/mL)** calculated from Equation (2);

V = Volume of sample solution (50 mL);

M = Sample mass (0.6000 g) accurately weighed during preparation.

Assessment of the proposed method's greenness

The greenness assessment was performed using the Analytical GREENess (AGREE) metric[21]. This method establishes an evaluation system based on the twelve core principles of Green Analytical Chemistry (GAC). It employs a weighted calculation to derive a composite score ranging from 0 to 1 (in standardized units), which is visualized as a circular pictogram with the integrated score displayed at its center. The AGREE assessment tool utilized in this study is accessible *via* reference 23. Additionally, the Blue Applicability Grade Index (BAGI) method [22-24] was introduced to evaluate the functionality and practical utility of the analytical approach[25].

Development of Extraction Methods for SPN Saponins

Orthogonal Experimental Design for Extraction Processes

The extraction parameters (ethanol concentration, solid-to-liquid ratio, extraction time, and extraction frequency) were optimized using an L9(3⁴) orthogonal array design, with the transfer rate of 20(*R*)-Rg₃ and total extract yield as evaluation metrics[26, 27]. SPN granules (30g per batch, 9 batches total) underwent reflux extraction under conditions specified in Table 4. Each extract was diluted to 300 mL, and a 300 mL aliquot was dried to calculate the yield; combined extracts were concentrated, dried, and analyzed for total saponin content. Key influencing factors were identified *via* range analysis and analysis of variance (ANOVA).

Table 4. L9(3⁴) Orthogonal experimental layout for total Saponins extraction from SPN

Trial	Ethanol Concentration (%)	Solid/Liquid Ratio (-fold)	Reflux Time (h)	Extraction Cycles (n)
1	50%	5(1:5)	1.0	2
2	50%	7.5(1:7.5)	1.5	3
3	50%	10(1:10)	2.0	4
4	60%	5(1:5)	1.5	4
5	60%	7.5(1:7.5)	2.0	2
6	60%	10(1:10)	1.0	3
7	70%	5(1:5)	2.0	3
8	70%	7.5(1:7.5)	1.0	4
9	70%	10(1:10)	1.5	2

Optimization of Key Parameters in Extraction Processes

Based on the orthogonal experimental results, a single-factor experiment was conducted to optimize extraction frequency. Three batches of SPN granules (30 g each) were reflux-extracted with 7.5 volumes of 60% ethanol for 1.5

hours per cycle, with frequencies of 4, 5, and 6 times (each condition in duplicate). The combined extracts were filtered, concentrated under reduced pressure to recover ethanol, and lyophilized to powder. The **total extract yield** was calculated as the ratio of dried extract mass to initial sample mass $\times 100\%$. The **20(*R*)-Rg₃ content** was quantified *via* HPLC-DAD, and its **transfer rate** was determined by the ratio of **20(*R*)-Rg₃** mass in the extract to that in the raw material $\times 100\%$.

Isolation and Purification

To obtain a saponin extract with higher purity and enriched rare ginsenosides, the mixed extract from Section Optimization of Key Parameters in Extraction Processes underwent further purification. Based on preliminary studies, the optimized protocol comprised: combining extracts from five cycles, concentrating under reduced pressure to a density of 1.1 g/mL, and purifying *via* D101 macroporous resin (loading concentration: 0.2 g/mL; flow rate: 0.5 BV/h)[28-30]. Sequential elution with 4BV deionized water and 4BV 100% ethanol was followed by decolorization using D941 anion-exchange resin (column height-to-diameter ratio: 10:1; flow rate: 1 BV/h)[31]. The decolorized solution was rinsed with 1.5 BV of 68% ethanol, and the combined eluates were spray-dried (inlet temperature: 265 $\pm$ 10°C; outlet: 80 $\pm$ 5°C; pressure $\geq$ 0.4 MPa)[32]. The resulting powder was sieved through 120-mesh, homogenized for 30 min, and stored in light-proof containers under <30% humidity[33].

Preparation of Extracts from SPN

Under the guidance of the optimized extraction process, **large-scale production of SPN extracts** was conducted in collaboration with **Yunnan Baiyao Group Wenshan QiHua Co., Ltd. (Wenshan, China)**. Three batches of extracts (Batch Nos.: S20230401, S20231113, S20240201) were prepared. The **moisture content** of the extracts was determined according to the **drying loss method** specified in **Part 0832 of the Chinese Pharmacopoeia (2020 Edition)**. Quantification of four target ginsenosides Rk₃, Rh₄, 20(*S*)-Rg₃, and 20(*R*)-Rg₃ was performed using a **validated Quantitative Analysis of Multi-Components by Single Marker (QAMS)**.

Results

Method Validation for HPLC Analysis

Linearity and Range

The reference standards of **Rk₃, Rh₄, and 20(*S*)-Rg₃** (40 mg each) were accurately weighed and separately dissolved in methanol in 25 mL volumetric flasks to prepare stock solutions with concentrations of **1.5570 mg/mL (Rk₃)**, **1.5605 mg/mL (Rh₄)**, and **1.4719 mg/mL (20(*S*)-Rg₃)**. Separately, **20(*R*)-Rg₃** (20 mg) was dissolved in methanol in a 100 mL volumetric flask, yielding a stock solution at **0.17114 mg/mL**. Aliquots (1mL each) of the **Rk₃, Rh₄, and 20(*S*)-Rg₃** stock solutions were transferred to a 10 mL volumetric flask, mixed with **5mL of 20(*R*)-Rg₃ stock solution**, and diluted to volume with **60% ethanol**, resulting in a mixed working solution with final concentrations of **0.1557 mg/mL(Rk₃)**, **0.1561 mg/mL (Rh₄)**, **0.1472 mg/mL (20(*S*)-Rg₃)**, and **0.0856 mg/mL (20(*R*)-Rg₃)**. Six injections (2, 4, 6, 8, 10, and 12 μ L) of the mixed solution were analyzed by HPLC, establishing linear relationships between injection volume (*X*, μ L) and peak area (*Y*) for all four ginsenosides. Regression analysis confirmed excellent linearity with **determination coefficients (R²) > 0.9997**; detailed regression data are summarized in **Table 5**, and standard curves are illustrated in **Figure 2 (Standard curves of four ginsenosides: Rk₃, Rh₄, 20(*S*)-Rg₃, and 20(*R*)-Rg₃)**.

Table 5. **Calibration curves for four characteristic Saponins in SPN**

Name	Regression Equation	Linear Range (µg)	R ²
Rk ₃	y = 653.2x - 4.813	0.3114–1.8684	1.0000
Rh ₄	y = 803.6x - 6.187	0.3121–1.8726	1.0000
20(<i>S</i>)-Rg ₃	y = 334.5x - 1.818	0.2944–1.7663	0.9999
20(<i>R</i>)-Rg ₃	y = 376.2x - 1.391	0.1711–1.7114	0.9997

Precision (Repeatability), Stability, and Accuracy

The method validation results, expressed as **relative standard deviations (RSD) of chromatographic peak areas**, demonstrate high reliability, with lower RSD values indicating superior precision. For the **repeatability assessment**, six replicate analyses of the same sample yielded RSD values below **0.38%** for all target peaks. **Sample stability** was confirmed by RSD values $\leq 1.2\%$ across peak areas measured at multiple time points over **36 h at room temperature**. The **method reproducibility** was verified using six independently prepared samples from the same batch, showing RSD values $< 1.0\%$ for all peaks. **Accuracy** was evaluated *via spike recovery tests* with known concentrations of reference standards, achieving recoveries of **91.95–101.34%** for four major saponins in SPN, with associated RSDs ranging from **0.93% to 1.8%**. Collectively, these data validate the method's robustness and reliability; detailed results are provided in **Table 6**.

Table 6. Method validation for HPLC analysis of Saponins in SPN

Composition	Precision RSD (%)	Stability RSD (%)	Repeatability RSD (%)	Accuracy	
				Recovery Rate (%)	RSD (%)
Rk ₃	0.10	0.34	0.46	93.98	1.40
Rh ₄	0.07	0.18	0.37	94.26	1.80
20(<i>S</i>)-Rg ₃	0.27	0.47	0.75	91.95	0.89
20(<i>R</i>)-Rg ₃	0.38	1.20	0.90	101.34	0.93

Validation and Application of a QAMS-Based Chemometric Model for Multi-Component Quantitation

In this study, ginsenoside 20(*R*)-Rg₃ was identified as the most stable and readily accessible saponin in SPN. It exhibits excellent separation under conventional chromatographic conditions with mature isolation techniques. Additionally, its relatively low acquisition cost and significant bioactivities—including anticancer, immunomodulatory, antioxidant, anti-inflammatory, and cardiovascular protective effects—collectively establish 20(*R*)-Rg₃ as the optimal internal standard for content determination in SPN.

Relative Correction Factor (RCF)

Within the validated linearity range, the content of saponins exhibited a proportional relationship with chromatographic peak areas. Variations in injection volumes simultaneously altered both saponin content and detector responses; however, the relative correction factors (RCFs) theoretically remained constant. The mean RCF values calculated across different injection volumes were adopted as final results, with their relative standard deviations (RSDs) reflecting computational stability. To ensure precision, all RCFs were uniformly retained to four

decimal places. The averaged RCFs for **ginsenosides Rk₃, Rh₄, and 20(S)-Rg₃** were determined as **0.5768, 0.4690, and 1.0924**, respectively, with a maximum RSD of **1.1%** –significantly below the 2.0% threshold—confirming robust stability of the calculated RCFs. Comprehensive results are detailed in **Table 7**.

Table 7. **Relative Correction Factors (F) of Saponins in *Panax notoginseng***

Injection Volume (μL)	F _{Rk3/20(S)-Rg3}	F _{Rh4/20(S)-Rg3}	F _{20(S)-Rg3/20(S)-Rg3}
12	0.5740	0.4665	1.0896
10	0.5818	0.4731	1.1003
8	0.5739	0.4670	1.0860
6	0.5746	0.4666	1.0795
4	0.5751	0.4685	1.0877
2	0.5815	0.4721	1.1113
Mean	0.5768	0.4690	1.0924
RSD(%)	0.65	0.62	1.10

Robustness Testing and Evaluation

The influence of **flow rate variations (0.9, 1.0, and 1.1 mL/min)**, **detection wavelengths (202, 203, and 204 nm)**, **HPLC systems (Thermo Fisher Ultimate 3000, Shimadzu LC-20AD, Agilent 1260 Infinity)**, and **chromatographic columns [Agilent ZORBAX-C18 (4.6×250 mm, 5 μm), Shimadzu VP-ODS (4.6×250 mm, 5 μm), Inertsil ODS-3 C18 (4.6×250 mm, 5 μm)]** on the relative correction factors (RCFs) of ginsenosides **Rk₃, Rh₄, and 20(S)-Rg₃** in the QAMS method was systematically evaluated. Results demonstrated excellent stability of RCFs across all conditions: **flow rate variations** yielded RSD < 1.0%, **wavelength adjustments** showed RSD < 1.3%, and **inter-system/column variations** exhibited RSD < 2.0% (Table 8). All deviations were within acceptable limits (5%), confirming the method's robustness for routine application in diverse laboratory environments.

Table 8. **Robustness evaluation of Relative Correction Factors (F) for Saponins under varied HPLC conditions**

Instrument	Flow Rate (mL/min)	F _{Rk3/20(S)-Rg3}	F _{Rh4/20(S)-Rg3}	F _{20(S)-Rg3/20(S)-Rg3}
Shimadzu LC-20AD	0.9	0.5715	0.4718	1.0727
	1.0	0.5799	0.4722	1.0751
	1.1	0.5713	0.4651	1.0926
Mean		0.5742	0.4697	1.0801
RSD%		0.86	0.85	1.10
Instrument	Wavelength (nm)	F _{Rk3/20(S)-Rg3}	F _{Rh4/20(S)-Rg3}	F _{20(S)-Rg3/20(S)-Rg3}
Shimadzu LC-20AD	202	0.5753	0.4732	1.0672
	203	0.5799	0.4769	1.0751
	204	0.5809	0.4652	1.0820
Mean		0.5787	0.4718	1.0748
RSD%		0.52	1.30	0.69
HPLC	Column Type	F _{Rk3/20(S)-Rg3}	F _{Rh4/20(S)-Rg3}	F _{20(S)-Rg3/20(S)-Rg3}
ThermoFisher Ultimate 3000	Agilent ZORBAX-C18	0.5733	0.4662	1.0856
Shimadzu LC-20AD	Shim-pack VP-ODS	0.5712	0.4757	1.0450
Agilent 1260 Infinity	Inertsil ODS-3 C18	0.5848	0.4744	1.0631
Mean		0.5764	0.4721	1.0646
RSD%		1.30	1.10	2.00

Agreement Assessment between QAMS and External Standard Method

To validate the reliability of the QAMS method, this study conducted parallel quantification of four saponins in 15 batches of *SPN* using both QAMS and the external standard method (ESM), in accordance with *Chinese Pharmacopoeia* General Rule 9101. The consistency between methods was assessed *via* **Relative Error (RE%)**, calculated as: $RE\% = [(QAMS - ESM)/EMS] \times 100\%$. As shown in Table 9, the mean RE% values for **ginsenosides Rk₃, Rh₄, and 20(S)-Rg₃** were **-0.18%, 0.83%, and 0.87%**, respectively, with all absolute RE% values below **5%** – meeting the acceptance criteria for method comparability per ICH Q2(R1) guidelines. These results align with the validation conclusions of Chen et al. in red ginseng multi-component analysis (RE% < 4.8%), confirming the accuracy and stability of the established QAMS method. Crucially, QAMS **reduced consumption of expensive reference standards (notably 20(S)-Rg₃) by 75%** while demonstrating exceptional robustness (RSD < 3.5%) across three HPLC systems (ThermoFisher Ultimate 3000, Shimadzu LC-20AD, Agilent 1260 Infinity) and multiple C18 columns. This robustness ensures method transferability between laboratories and instrument platforms, generating highly comparable and reliable data. By significantly lowering barriers to industrial-scale multi-component monitoring (due to scarce/expensive standards) and reducing analytical costs, QAMS provides a robust technical foundation for establishing a unified, standardized quality evaluation system for *SPN* and its preparations – a critical step toward intelligent manufacturing and end-to-end quality control in traditional Chinese medicine.

Table 9. Quantification of four Saponins in 15 batches of *SPN* by ESM and QAMS methods

Sample Source	Rk ₃		RE%	Rh ₄		RE%	20(S)-Rg ₃		RE%	20(R)-Rg ₃
	ESM	QASM		ESM	QASM		ESM	QASM		ESM
S1	1.3921	1.4005	0.60	1.9258	1.9529	1.41	0.7861	0.7979	1.50	0.4706
S2	1.4114	1.4200	0.61	1.9137	1.9409	1.42	0.8732	0.8863	1.51	0.5046
S3	1.2789	1.2701	-0.69	1.7432	1.7451	0.11	0.6724	0.6738	0.20	0.3695
S4	1.2271	1.2269	-0.02	1.7084	1.7218	0.78	0.7114	0.7176	0.87	0.4180
S5	1.2491	1.2446	-0.36	1.6946	1.7021	0.44	0.6917	0.6954	0.53	0.3734
S6	1.2234	1.2316	0.67	1.6615	1.6860	1.48	0.7967	0.8092	1.57	0.4743
S7	1.1376	1.1457	0.71	1.5050	1.5279	1.52	0.7790	0.7915	1.61	0.4726
S8	1.1636	1.1693	0.49	1.5328	1.5528	1.30	0.7727	0.7834	1.39	0.4679
S9	1.1442	1.1507	0.56	1.5211	1.5420	1.37	0.7751	0.7864	1.46	0.4631
S10	0.9677	0.9656	-0.21	1.2782	1.2858	0.59	0.6931	0.6979	0.68	0.4081
S11	1.0170	1.0100	-0.69	1.3429	1.3550	0.90	0.6547	0.6600	0.81	0.3722
S12	0.9617	0.9549	-0.71	1.2733	1.2845	0.87	0.6510	0.6562	0.79	0.3740
S13	1.1986	1.1824	-1.36	1.5865	1.5901	0.22	0.6061	0.6069	0.13	0.3199
S14	1.1254	1.1069	-1.64	1.4745	1.4735	-0.07	0.5869	0.5860	-0.16	0.3022
S15	1.1289	1.1210	-0.70	1.4722	1.4737	0.10	0.6475	0.6487	0.19	0.3487
Mean	/	/	-0.18	/	/	0.83	/	/	0.87	/

Assessment of the proposed method's greenness and blueness

The greenness assessment results based on the **AGREE (Analytical GREENness)** tool demonstrated significantly higher scores for the **QAMS method (0.76)** compared to the **external standard method (ESM, 0.63)**, indicating superior environmental friendliness and **reduced environmental impact** of the proposed QAMS approach. Furthermore, in terms of functionality and practicality evaluated *via* the **BAGI (Blue Applicability Grade Index)** metric, QAMS and ESM achieved scores of **77.5** and **65.0**, respectively, confirming both methods exhibit high practical applicability, with QAMS showing a 19.2% improvement in sustainability and operational efficiency.(Figure 3, **Analysis of AGREE and BAGI scores.** A1: QAMS greenness score; A2: ESM greenness score; B1: QAMS blueness applicability score; B2: ESM blueness applicability score.)

Processing Technology Optimization

Immersion Pretreatment of *Panax notoginseng*

The immersion duration and water absorption capacity of *Panax notoginseng* particles with varying diameters (8~9 mm and 4~5 mm) were investigated using an **excessive purified water saturation method**. Changes in the volume reduction of immersion liquid and core moistening status of particles were recorded at intervals (2, 4, 6, 8, 24, 34, 48, 58, and 62 h). Key results are summarized as follows:**40-head whole main roots:** Required **58~62 h** for complete saturation, absorbing **~95%** of their initial weight in water; **8~9 mm particles:** Achieved full saturation at **24 h**, with

water absorption reaching **97.5%** of their weight; **4~5 mm particles**: Saturated within **2 h**, absorbing **100%** of their initial weight.

Two batches of *Panax notoginseng* particles (2~4 mm) **were treated** as follows: one batch underwent **immersion pretreatment** with purified water equivalent to 100% of its mass for 2 h, while the other remained untreated. Both batches were subsequently **steamed at 120°C for 3 h**. The contents of four target ginsenosides—Rk₃, Rh₄, 20(*S*)-Rg₃, and 20(*R*)-Rg₃—**were quantitatively analyzed** to evaluate the impact of immersion pretreatment. Results demonstrated that immersion **significantly enhanced** ($p<0.01$) the yields of all four ginsenosides in SPN particles. Notably, for 4~5 mm particles pretreated with 100% (w/w) purified water immersion, the 20(*R*)-Rg₃ content **increased remarkably by 220%** compared to the non-pretreated group. This **confirms that thorough immersion** facilitates ginsenoside transformation, likely mediated by **water activation and cell wall structural modifications**. Consequently, immersion pretreatment prior to steaming **is essential** for optimizing ginsenoside conversion in SPN.

Particle Size Screening

Four size fractions of *Panax notoginseng* particles (1~2 mm, 2~4 mm, 5~8 mm, and 8~10 mm) **were processed in duplicate**, with each batch subjected to **immersion pretreatment** using purified water equivalent to 100% of its mass: particles of 1~2 mm and 2~4 mm were immersed for 2 h, whereas those of 5~8 mm and 8~10mm required 24 h for saturation, followed by **steaming at 120°C for 3h**, air-drying, and **yield assessment**. Quantitative analysis of four target ginsenosides—Rk₃, Rh₄, 20(*S*)-Rg₃, and 20(*R*)-Rg₃—**revealed** that reduced particle size **significantly prolonged grinding duration** and **increased material loss**, with the 1~2 mm fraction exhibiting the **lowest processing yield** ($P<0.01$ vs. larger fractions, Table 10), primarily due to adhesion-induced losses during handling; **concurrently**, an **inverse correlation** between particle size and ginsenoside content was observed, where smaller particles (e.g., 1~2 mm) yielded **higher concentrations** of all four ginsenosides compared to larger counterparts (8~10 mm) ($P<0.05$). **Notably**, despite marginally elevated ginsenoside levels in 1~2 mm particles, the **2~4 mm fraction** demonstrated the optimal balance between **bioactive output** and **production efficiency**, minimizing processing losses while achieving high ginsenoside yields: Rk₃ (**8.51 ± 0.32 mg/g**), Rh₄ (**11.40 ± 0.41 mg/g**), 20(*S*)-Rg₃ (**7.44 ± 0.28 mg/g**), and 20(*R*)-Rg₃ (**4.58 ± 0.17 mg/g**) (mean ± SD, $n = 3$), thereby establishing 2~4 mm as the **recommended particle size range** for industrial-scale SPN.

Table 10. **Single-Factor experiment results for *Panax notoginseng* processing techniques**

Parameter and Level		Yield(%)	Concentration (mg/g)			
			Rk ₃	Rh ₄	20(S)-Rg ₃	20(R)-Rg ₃
Water Soaking	Non-soaked	93.33	7.8329	10.2160	4.2774	1.9056
	Soaked	93.95	8.4141	11.2876	6.9118	4.2031
Particle Size (mm)	1~2	93.32	8.5960	11.5484	7.8212	4.8482
	2~4	93.95	8.5118	11.4030	7.4440	4.5756
	5~8	95.84	8.1618	10.9740	7.1985	4.5088
	8~10	97.38	8.1443	10.9798	6.8407	4.2386
Steaming Temp. (°C)	100	96.10	0.8050	1.2063	/	/
	110	94.57	4.7026	6.4943	3.3625	1.9507
	120	93.95	8.5118	11.4030	7.4440	4.5756
	130	90.41	10.4971	14.3880	9.4361	6.3042
Steaming Time (h)	3	93.95	8.5118	11.4030	7.4440	4.5756
	4	92.12	9.0943	12.3157	8.2611	5.2278
	5	92.31	9.0492	12.3769	8.6105	5.4411
	6	92.46	9.2865	12.7874	9.2084	5.8312

Evaluation of Processing Technology

Evaluation of Steaming Temperature

Panax notoginseng particles (2~4 mm) pretreated with purified water (100% mass ratio) for 2h **were steamed at 100°C, 110°C, 120°C, and 130°C for 3h** followed by air-drying. Quantitative analysis of four ginsenosides—Rk₃, Rh₄, 20(S)-Rg₃, and 20(R)-Rg₃—revealed that **increasing temperatures significantly reduced yield** (100°C: **96.10%** → 130°C: **90.41%**, $p<0.01$), attributed to accelerated deglycosylation of protopanaxadiol-type saponins (e.g., Rb₁, Rd) *via* β -elimination[24, 34]. Crucially, **no 20(S)-Rg₃ or 20(R)-Rg₃ was detected at 100°C**, with minimal Rk₃/Rh₄. At 110°C, total ginsenoside content reached **16.51 mg/g** [Rk₃: 4.70, Rh₄: 6.49, 20(S)-Rg₃: 3.36, 20(R)-Rg₃: 1.95 mg/g]. **120°C steaming maximized ginsenoside conversion**, increasing total content by **78.6%** ($p<0.01$) to **32.74 mg/g**—specifically 1.81-, 1.76-, 2.21-, and 2.35-fold higher than 110°C for each ginsenoside. Although 130°C further elevated content by 18.3% ($p>0.05$), the **diminishing returns and safety constraints** (equipment limit: 134°C) established **120°C as optimal** for balancing saponin yield, energy efficiency, and operational safety.

Evaluation of Steaming Duration

Panax notoginseng particles (2~4 mm) pretreated with purified water (100% mass ratio) for 2 h **were steamed at 120°C for 3~6 h**, followed by air-drying. Quantitative analysis of four ginsenosides—Rk₃, Rh₄, 20(S)-Rg₃, and 20(R)-Rg₃—revealed that **prolonged steaming reduced yield** ($p<0.05$, Table 11) but **increased ginsenoside content** (Figure 4. **Single-factor experiments on processing parameters**. A: Effect of *Panax notoginseng* particle pre-soaking on post-processing content of four saponins; B: Effect of particle size on post-processing content of four saponins; C: Effect

of steaming time on post-processing content of four saponins; D: Effect of steaming temperature on post-processing content of four saponins.). Specifically:**3 h steaming**: Ginsenoside contents were **8.51, 11.40, 7.44, and 4.58 mg/g** for **Rk₃**, **Rh₄**, **20(S)-Rg₃**, and **20(R)-Rg₃**, respectively; **4h steaming**: Increased by **6.84%, 8.00%, 10.98%, and 14.25%** versus 3 h ($p<0.01$); **5 h steaming**: **20(S)-Rg₃** and **20(R)-Rg₃** rose significantly (**4.23% and 4.08%** vs. 4h, $p<0.05$), while **Rk₃** and **Rh₄** remained stable ($\pm 0.50\%$); **6h steaming**: All ginsenosides increased (**Rk₃**: **+2.62%**; **Rh₄**: **+3.32%**; **20(S)-Rg₃**: **+6.94%**; **20(R)-Rg₃**: **+7.17%** vs. 5 h, $p<0.05$), but with **diminishing returns** (lower efficacy per unit time). Critically, extending steaming beyond 5h provided **marginal gains** (e.g., $< 5\%$ net increase for **20(S)-Rg₃** from 5 h to 6 h) but incurred **disproportionate energy costs** (Table 10). Thus, **5 h was established as optimal** for balancing ginsenoside yield and process efficiency.

Steaming Technology for Processed Notoginseng

Purified *Panax notoginseng* roots **were weighed, milled, and sieved** (4.0 mm mesh) to obtain particles (2~4 mm) with $\leq 5\%$ mass fraction >4.0 mm and maximum diameter ≤ 8.0 mm. Particles underwent immersion **pretreatment** in purified water (100% w/w) for 2h, followed by **steaming at 120 °C for 5h** in layered trays (≤ 3 cm height, covered with bamboo mats) and **drying at 60 ± 2 °C for 4~5 h** until moisture content $< 9\%$. This optimized protocol **synergistically enhanced target ginsenoside yields** through dual mechanisms:**Physical preconditioning**: Water immersion (100% w/w) **softened tissue structures and enhanced cell wall permeability**, facilitating efficient heat penetration; **Thermochemical optimization**: Steaming at 120°C **targeted the activation energy** ($E_a \approx 58.3$ kJ/mol) for deglycosylation of protopanaxadiol-type ginsenosides (e.g., $Rb_1 \rightarrow Rg_3/Rh_2$), while the **5 h duration maximized rare ginsenoside accumulation** (e.g., **Rk₃**, **Rh₄**) by balancing conversion kinetics against thermal degradation. The **"hydration-controlled thermal conversion" strategy** resolved batch variability issues, achieving $>95\%$ inter-batch consistency in total saponin content (32.74 ± 1.28 mg/g, $n=5$) and establishing a robust protocol for standardized *SPN*.

Extraction Methods for SPN

Ethanol reflux extraction (ERE) **significantly outperformed** ultrasonic-assisted extraction in enriching four target ginsenosides—**Rk₃**, **Rh₄**, **20(S)-Rg₃**, and **20(R)-Rg₃**—from *SPN* ($p<0.05$). Key parameter optimizations revealed: **Extraction time**: Total ginsenoside content peaked at **1h** (32.7% increase vs. 0.5 h, $p=0.003$), but declined by 18.4% at 1.5 h ($p=0.017$), indicating thermal degradation/isomerization of rare saponins; **Solvent selection**: While 90% methanol yielded the highest content (**12.38 ± 0.45 mg/g**), **60% ethanol** was prioritized for safety with comparable efficacy (**11.92 ± 0.37 mg/g**); **Solvent volume**: Volumes of 50mL and 100 mL showed **no significant difference**($p=0.236$), supporting flexible scale-adjusted production. The optimized protocol (60% ethanol, 1 h reflux at 80°C) demonstrated **excellent reproducibility**, with RSDs $< 2.5\%$ for all ginsenosides across triplicate batches ($n=9$, Table 11). Sample preparation followed: 0.6 g powder (sieve No. 4) refluxed with 50 mL 60% ethanol at 80°C for 1h, cooled, replenished to original weight, filtered.

Table 11. **Single-Factor Optimization of Extraction Process for SPN Powder**

Process Category	Conditions	Sample Weight (g)	Concentration [mg/g]			
			Rk ₃	Rh ₄	20(<i>S</i>)-Rg ₃	20(<i>R</i>)-Rg ₃
Extraction Method	Ethanol reflux(0.5h)	0.6005	4.9980	6.4370	2.9331	1.2076
	Ethanol reflux(1.0h)	0.6088	5.0382	6.5551	3.0371	1.2552
	Ethanol reflux(1.5h)	0.6053	5.0260	6.5211	3.0245	1.2491
	Ethanol ultrasonication(0.5h)	0.6022	4.7606	6.1542	2.8299	1.1563
Solvent Volume	Ethanol (100mL) reflux(1h)	0.6011	5.0726	6.5041	3.0117	1.1886
	Ethanol (50mL) reflux(1h)	0.6088	5.0385	6.5533	3.0347	1.2546
Extraction Solvent	Methanol reflux(1h)	0.6033	5.1491	6.7071	3.1784	1.3665
	90% Methanol reflux(1h)	0.6006	5.3990	7.1344	3.4452	1.8688
	Ethanol reflux(1h)	0.6088	5.0382	6.5551	3.0371	1.2552
	60% Ethanol reflux(1h)	0.6056	5.3552	7.1212	3.4536	1.8718
	60% Methanol reflux(1h)	0.6014	5.3312	7.0582	3.3768	1.7191

Extracts of SPN

Orthogonal Test with Significance Evaluation for Saponin Extraction of SPN

An **orthogonal array design [L9(3⁴)]** (Table 12) systematically evaluated the effects of **ethanol concentration (A: 50%, 60%, 100%)**, **material-to-liquid ratio (B: 1:5, 1:7.5, 1:10)**, **extraction time (C: 1.0, 1.5, 2.0 h)**, and **extraction cycles (D: 3, 4, 5)** on extraction efficiency, using a **comprehensive scoring model** integrating 20(*R*)-Rg₃ transfer rate (weight: 80%) and total extract yield (weight: 20%). **ANOVA** (Table 13) revealed that **extraction cycles (D)** and **time (C)** significantly impacted the composite score ($FD = 45.231$, $p < 0.05$; $FC = 31.462$, $p < 0.05$), with contribution rates of **55.4%** and **38.5%**, respectively. In contrast, ethanol concentration (A) and material-to-liquid ratio (B) showed **no statistical significance** ($p > 0.05$). The composite score histogram indicated: **Extraction cycles (D)** exhibited a **linear positive correlation** with the score, where **4 cycles increased the score by 42.3%** versus 3 cycles; **Extraction time (C)** peaked at **1.5h** (score: 7.8), but **declined at 2.0 h**, likely due to **thermolabile degradation** of bioactive compounds. Considering **industrial feasibility**, the optimal parameters were determined as: **60% ethanol**, **material-to-liquid ratio 1:7.5**, and **4 extraction cycles (1.5 h each)**.

Table 12. **L9(3⁴)** Orthogonal array design for optimizing Saponin extraction from *Panax notoginseng*

Trial	A Ethanol Conc. (%)	B Solid/Liquid Ratio (-fold)	C Reflux Time (h)	D Extraction Cycles (n)	Rg ₃ Transfer Rate (%)	Total Extract Yield (%)	Comprehensive Score
1	50	5.0	1.0	2	57.76	23.63	1.6
2	50	7.5	1.5	3	78.71	31.19	6.0
3	50	10.0	2.0	4	85.81	35.33	6.6
4	60	5.0	1.5	4	87.90	26.00	7.8
5	60	7.5	2.0	2	73.38	21.70	4.8
6	60	10.0	1.0	3	64.75	21.81	3.0
7	70	5.0	2.0	3	75.99	22.37	5.2
8	70	7.5	1.0	4	77.04	23.11	5.8
9	70	10.0	1.5	2	66.41	19.37	4.2

Table 13. ANOVA results for orthogonal experimental design of Saponin extraction

Factor	Sum of Squares (SS)	df	Mean Square (MS)	F-value	P-value	Partial η^2 (%)	Statistical Power (1- β)	Contribution rate%
A	0.347	2	0.174	1.000	0.468	1.2	0.18	1.2
B	1.387	2	0.694	4.000	0.184	4.9	0.52	4.9
C	10.907	2	5.454	31.462	0.030	38.5	0.98	38.5
D	15.680	2	7.840	45.231	0.021	55.3	0.99	55.4
Error	0.347	2	0.174	/	/	/	/	/
Total	28.668	8	/	/	/	/	/	/

Screening of Extraction Parameters for Saponins in SPN

A **single-factor experiment** based on orthogonal-optimized conditions **identified extraction cycles** as the most significant variable affecting 20(R)-Rg₃ transfer efficiency. Comparative analysis of **4, 5, and 6 extraction cycles** revealed that: 20(R)-Rg₃ **transfer rate increased with cycle frequency** but exhibited **diminishing marginal gains**: (a) **5 cycles** achieved **85.82% transfer**, a **5.29% increase** versus 4 cycles; (b) **6 cycles** yielded only **1.96% further improvement** (87.78% total, $p < 0.05$ vs. 5 cycles). **Total extract yield** declined by **8.3%** from 4 to 6 cycles ($p = 0.012$), attributed to **thermal degradation** during prolonged processing. **Critically**, the **1.96% gain from 5 to 6 cycles** incurred **disproportionate energy and time costs** (Table 14), while **5 cycles balanced efficiency and cost-effectiveness**. Thus, **5 cycles were established as optimal** for maximizing 20(R)-Rg₃ recovery. The finalized extraction protocol employs **7.5 volumes of 60% ethanol with 5 cycles (1.5 h each)** (Table 15) (**Figure 5. Extraction experiments on SPN**. A: Pie chart of contribution rates from four factors to comprehensive scores in orthogonal experiments; B: Bar graph of comprehensive scores for four factors in orthogonal experiments; C: Trend of extraction cycles affecting the average transfer rate of ginsenoside 20(R)-Rg₃ in SPN extract.).

Table 14. Effect of extraction cycles on yield and 20(R)-Rg₃ transfer rate in SPN

Extraction Cycles	Dry Extract Weight (g)	Extract Yield (%)	Rg ₃ Content (%)	Rg ₃ Transfer Rate (%)
	30	/	0.63 ± 0.02	/
4	7.825 ± 0.15	26.08 ± 0.41	1.945 ± 0.03	80.53 ± 0.98
5	8.255 ± 0.18	27.52 ± 0.52	1.965 ± 0.04	85.82 ± 1.12
6	8.400 ± 0.21	28.00 ± 0.61	1.975 ± 0.05	87.78 ± 1.25

Table 15. Incremental efficiency in Rg₃ transfer rate with increasing extraction cycles

Cycle Transition	Transfer Rate Increase (Δ%)	Marginal Gain (mg/g per cycle)
2→3	7.30 ± 0.45	0.84 ± 0.03
3→4	7.38 ± 0.51	0.82 ± 0.04
4→5	5.29 ± 0.38	0.58 ± 0.02
5→6	1.96 ± 0.12	0.21 ± 0.01

Assay of Extract Content

Three batches of *SPN* extracts exhibited a **mean moisture content of 2.11%**. On a dry-weight basis, the average contents of four target ginsenosides were quantified as: **Rk₃ (7.95%), Rh₄ (27.78%), 20(*S*)-Rg₃ (1.64%), and 20(*R*)-Rg₃ (3.91%)**. All batches demonstrated **exceptional reproducibility**, with relative standard deviations (RSD%) **< 1.21%** for each ginsenoside (*n*=3), confirming robust process consistency. Complete data are tabulated in **Table 16**.

Table 16. Content of Four Characteristic Saponins in 15 Batches of SPN Extract

Batch No.	Rk ₃ %	Rh ₄ %	20(<i>S</i>)-Rg ₃ %	20(<i>R</i>)-Rg ₃ %	Moisture (%)
S20230401	7.95	27.50	1.64	3.95	2.80
S20231113	7.90	28.03	1.65	3.86	2.70
S20240201	8.01	27.80	1.62	3.93	2.70
Mean ± SD	7.95 ± 0.05	27.78 ± 0.27	1.64 ± 0.01	3.91 ± 0.04	2.73 ± 0.06
RSD%	0.69	0.96	0.93	1.21	2.11

Discussion

The core innovations of this study encompass: (1) elucidating and leveraging the synergistic effects between particle size reduction (2 ~ 4 mm) and optimized water activation (100% w/w) to significantly enhance the cell wall permeability and thermal conversion efficiency of ginsenosides, thereby effectively resolving the issue of batch-to-batch inconsistency (reducing RSD from > 15% to < 5%); (2) developing a robust and cost-effective QAMS method with multi-platform compatibility (RCFs RSD < 2.0%; inter-instrument RSD < 3.5%), which overcomes the critical barrier of expensive reference standards for multi-component quality control and reduces costs by 75%; (3) establishing a comprehensive and transferable quality control paradigm that integrates optimized processing technology with advanced analytical techniques, directly validating practical applications of the TCM theory "Differential Efficacy of Raw and Processed Herbs". These innovations collectively address industrial challenges in

standardized production and sustainable monitoring of processed herbs, while providing a scalable model for other thermally transformed botanicals (e.g., red ginseng).

Complementing the process innovation, a robust and economical HPLC-QAMS analytical method was developed and rigorously validated. Using 20(*R*)-Rg₃ as the internal reference, stable relative correction factors (RCFs) were established: 0.5768 for Rk₃, 0.4690 for Rh₄, and 1.0924 for 20(*S*)-Rg₃ (all RSD < 2.0%), enabling synchronous quantification of four rare ginsenosides. Method validation demonstrated exceptional performance, meeting *Chinese Pharmacopoeia* and ICH guidelines: high accuracy (spike recovery: 91.95–101.34%), precision (RSD < 1.8%), and linearity ($r \geq 0.9997$). Critically, analysis of 15 production batches confirmed QAMS reliability, with relative errors (RE%) versus external standard method (ESM) below 5%. The core advantage of this QAMS method lies in reducing expensive reference standard consumption by 75% while maintaining analytical accuracy. Its high robustness was evidenced by consistent performance across three HPLC brands and columns (RSD < 3.5%), significantly enhancing practicality in diverse quality control environments. Concurrent optimization of extraction SPN (60% ethanol, reflux 1 h) and its extract (solid-to-liquid ratio 1:7.5, 60% ethanol, reflux 1.5 h × 5 cycles)-ensured efficient recovery of target compounds.

Key innovations include: (1) elucidating the synergistic effects of particle size reduction (2 ~ 4 mm) and optimized water impregnation (100% w/w) in enhancing *cell wall permeabilization* and *thermal saponin conversion*, effectively resolving batch-to-batch variability (RSD < 15%); (2) developing a rugged and cost-effective QAMS method (*Quantitative Analysis of Multi-components by Single Marker*) with cross-platform compatibility (RCFs RSD < 2.0%; inter-instrument RSD < 3.5%), overcoming cost barriers from rare ginsenoside reference standards (e.g., 75% reduction for 20(*S*)-Rg₃); (3) establishing a comprehensive and transferable quality control paradigm integrating optimized processing with advanced analytics, directly supporting the TCM theory of "Raw vs. Processed Efficacy" in modern herbal medicine.

Despite significant advancements, this study has limitations: (1) The current QAMS method monitors only four specific rare ginsenosides (Rk₃, Rh₄, 20(*S*)-Rg₃, 20(*R*)-Rg₃), lacking coverage of other bioactive compounds (e.g., Rg₅, Rk₁) generated during steaming; future studies should expand QAMS scope or develop complementary methods for broader marker spectra; (2) While robustness was validated across three HPLC systems, the long-term stability of relative correction factors (RCFs) requires continuous monitoring under extreme variations in mobile phase composition, column aging, or detector sensitivity in real-world QC laboratories; (3) Although optimized processing significantly increased target saponin content, comparative pharmacokinetics and efficacy between new and traditional products in vivo remain unverified, necessitating deeper correlation between enhanced chemical profiles and biological effects to elucidate the "Raw vs. Processed Efficacy" theory; (4) Applicability to complex matrices (e.g., formulated products containing *P. notoginseng*) needs further validation. Future research should: validate clinical efficacy/safety of enriched rare ginsenoside profiles; extend the QAMS strategy to other thermally processed herbs (e.g., *red ginseng* or *Polygala*) rich in pyrolytic saponins; explore spectroscopic techniques (e.g., NIR or ND-APCI-MS) coupled with chemometrics for online/at-line process monitoring using established chemical markers; and conduct stability studies of optimized extracts to determine shelf-life and storage protocols.

In summary, this study provides a scientifically rigorous and industrially *viable* solution for enhancing the quality and consistency of SPN. The optimized processing protocol ensures products enriched with key bioactive rare ginsenosides, while the developed QAMS method offers a practical, cost-effective, and reliable tool for quality assessment, paving the way for broader acceptance and application of this valuable processed herbal medicine.

Conclusions

This study addressed two critical bottlenecks in the industrial production of SPN—process variability and quality control costs—through the synergistic innovation of a "Water Activation-Gradient Temperature Control" (WAGTC) processing protocol (granule size: 2 ~ 4 mm; impregnated with 100% purified water for 2 h; steam-processed at 120°C for 5h) and an HPLC-based Quantitative Analysis of Multi-Components by Single Marker (QAMS) method. Optimized *via* the Arrhenius kinetic model (activation energy $E_a \approx 58.3$ kJ/mol), the WAGTC protocol significantly increased the total content of four rare ginsenosides by 78.6% (reaching 32.7 mg/g) while reducing batch-to-batch variability from RSD > 15% to RSD < 5%. The QAMS method utilized readily available 20(*R*)-Rg₃ as an internal reference, achieving relative correction factors (RCFs) RSD < 2.0%, inter-instrument reproducibility RSD < 3.5% across multiple HPLC brands, and a 75% reduction in expensive reference standard consumption. Sustainability metrics confirmed QAMS superiority: AGREE score 0.76 vs. 0.63 (traditional method) and BAGI score 77.5 vs. 65.0, validating its eco-efficiency and practicality. This strategy provides a scalable quality control framework for the TCM theory of "Differential Efficacy of Raw and Processed Herbs", enabling industrial-scale production of high-bioactivity products with cost-efficient multi-component monitoring. Future applications may extend QAMS to other processed herbs (e.g., *red ginseng*) to deepen clinical efficacy correlations.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

"WN guide and assist in HPLC method validation. TSQ guide and assist in the determination of saponin content. LYX Guide and assist in the processing and extraction of saponin. NYF completed all the data of the article, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript."

"NYF designed the study, developed and validated the HPLC-QAMS method for saponin quantification, performed data analysis, and drafted the manuscript. LYX optimized saponin extraction protocols and validated purification

protocols. WN and TSQ established the saponin standard curve and conducted quality control assessments. All authors read and approved the final manuscript."

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Figures

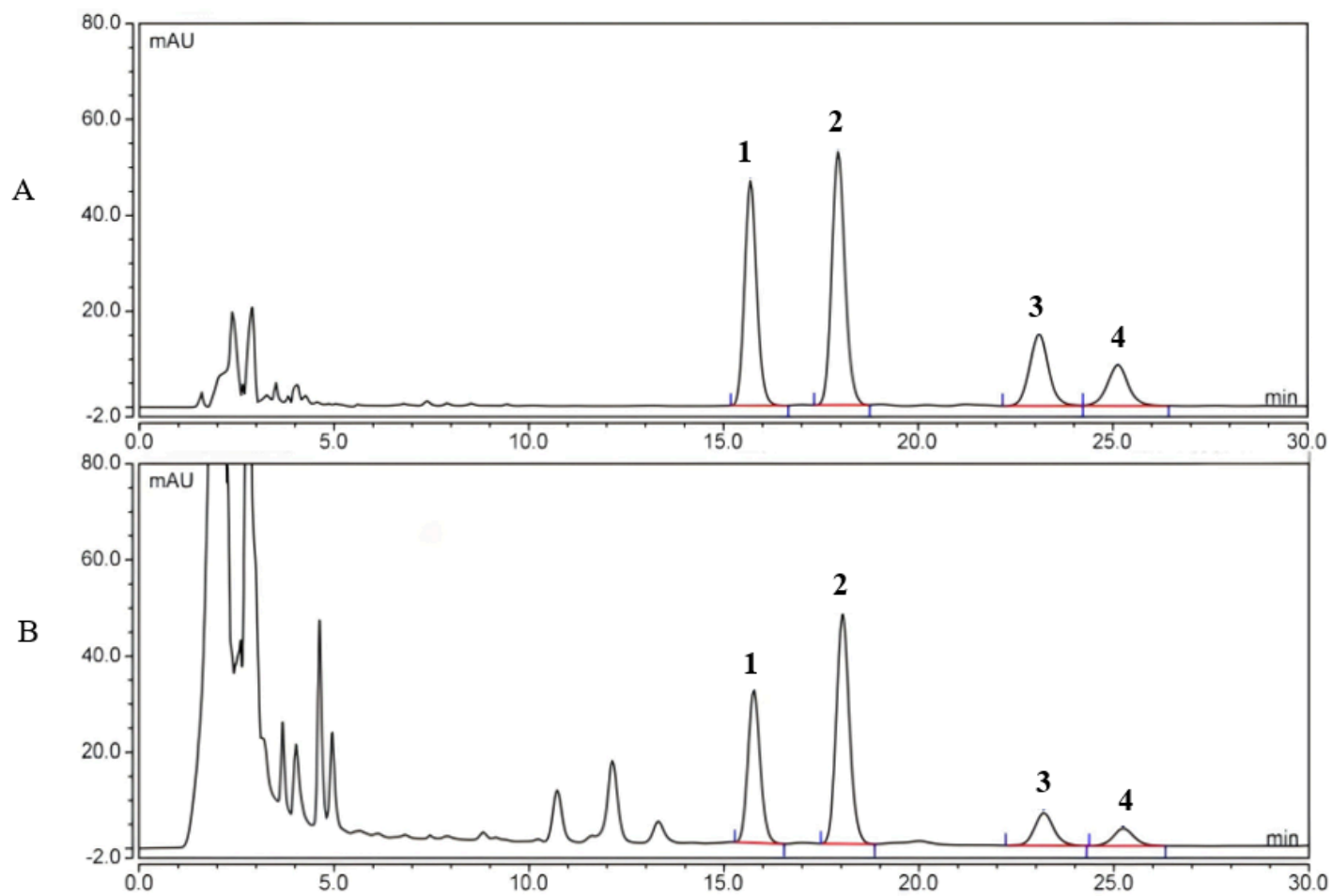


Figure 1

HPLC chromatograms of the mixed reference solution (A) and steamed Panax notoginseng extract (B).

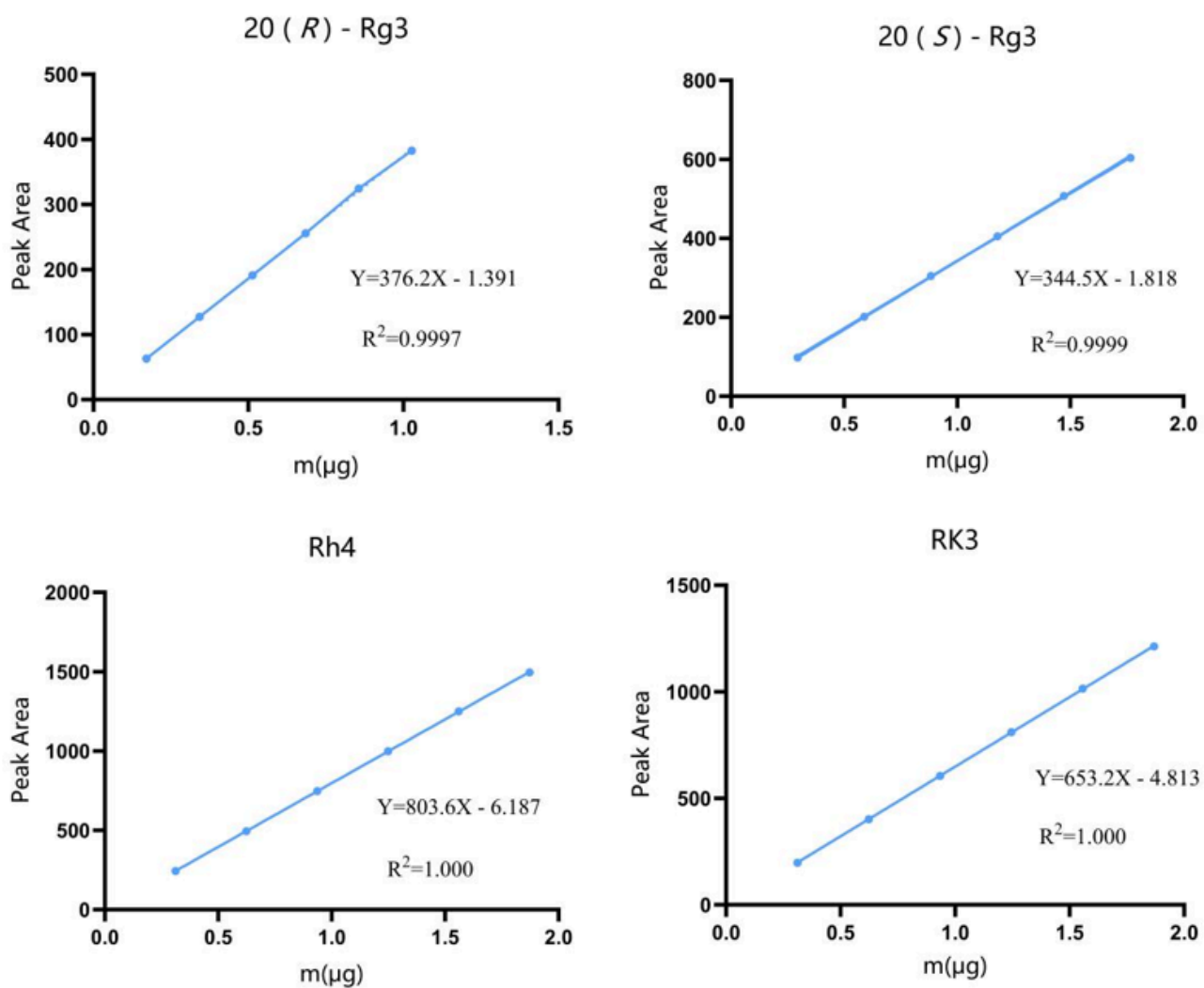


Figure 2

Standard curves of four ginsenosides: Rk_3 , Rh_4 , $20(\text{S})\text{-Rg}_3$, and $20(\text{R})\text{-Rg}_3$.

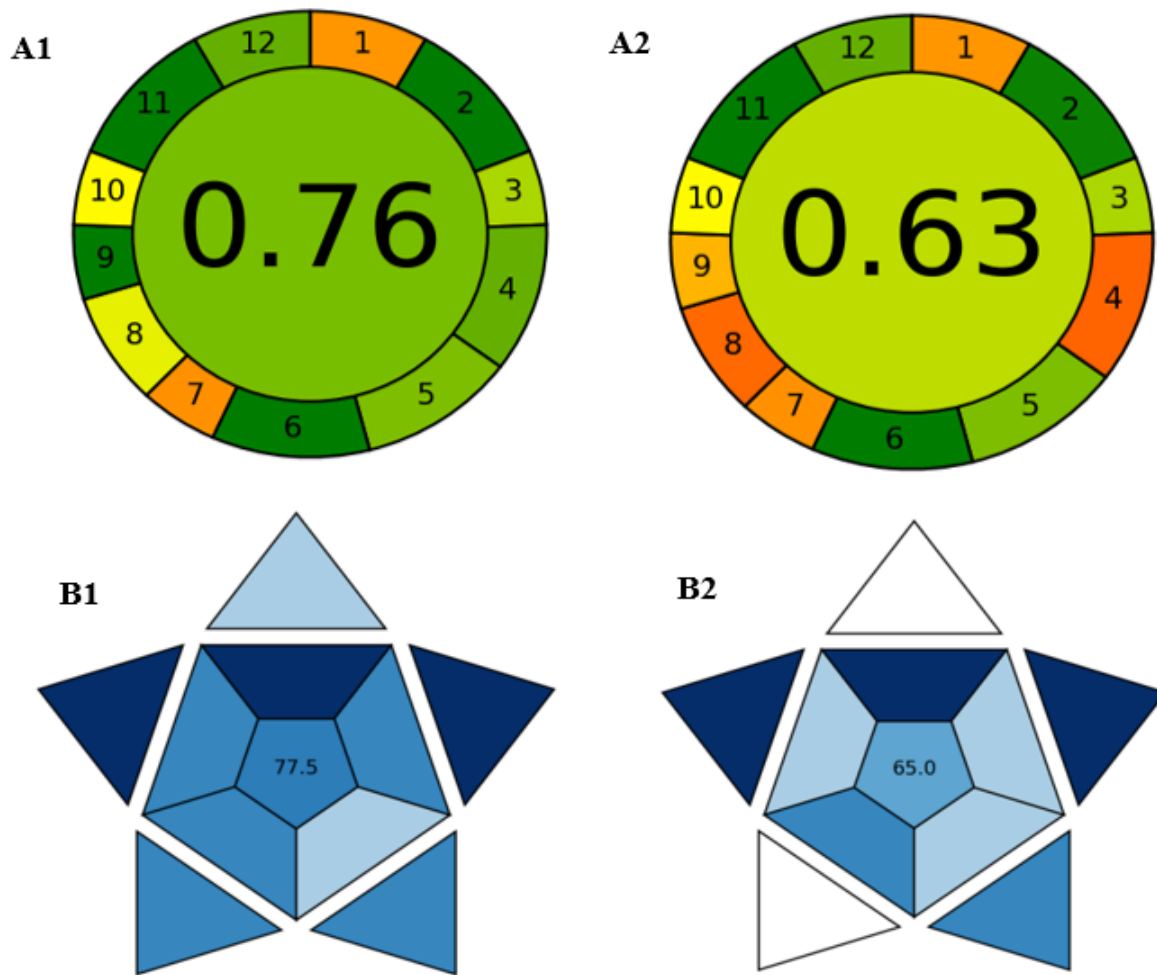


Figure 3

Analysis of greenness (AGREE) and blueness applicability grade index (BAGI) scores. **A1**: QAMS greenness score; **A2**: ESM greenness score; **B1**: QAMS blueness applicability score; **B2**: ESM blueness applicability score.

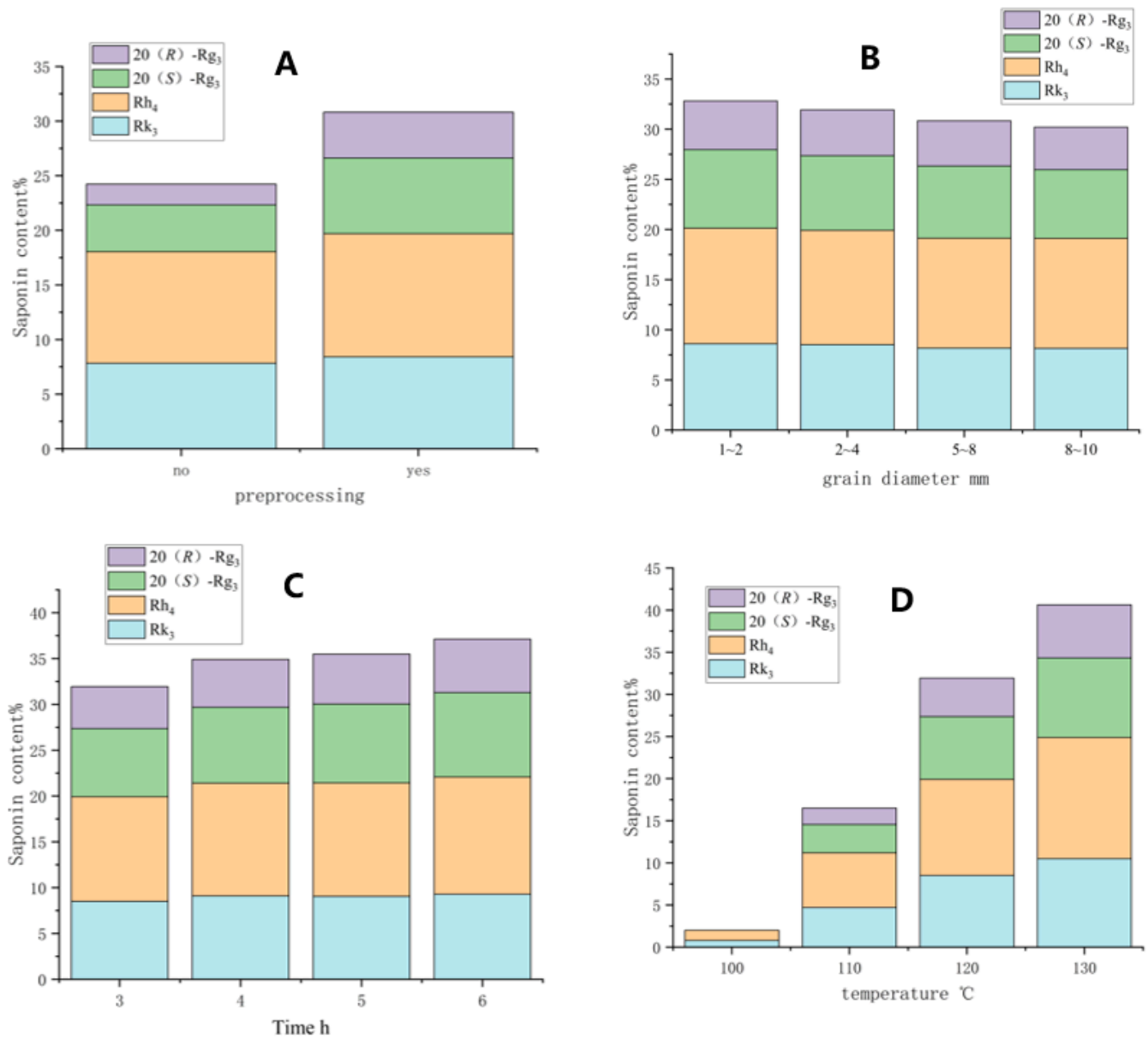


Figure 4

Single-factor experiments on processing parameters.

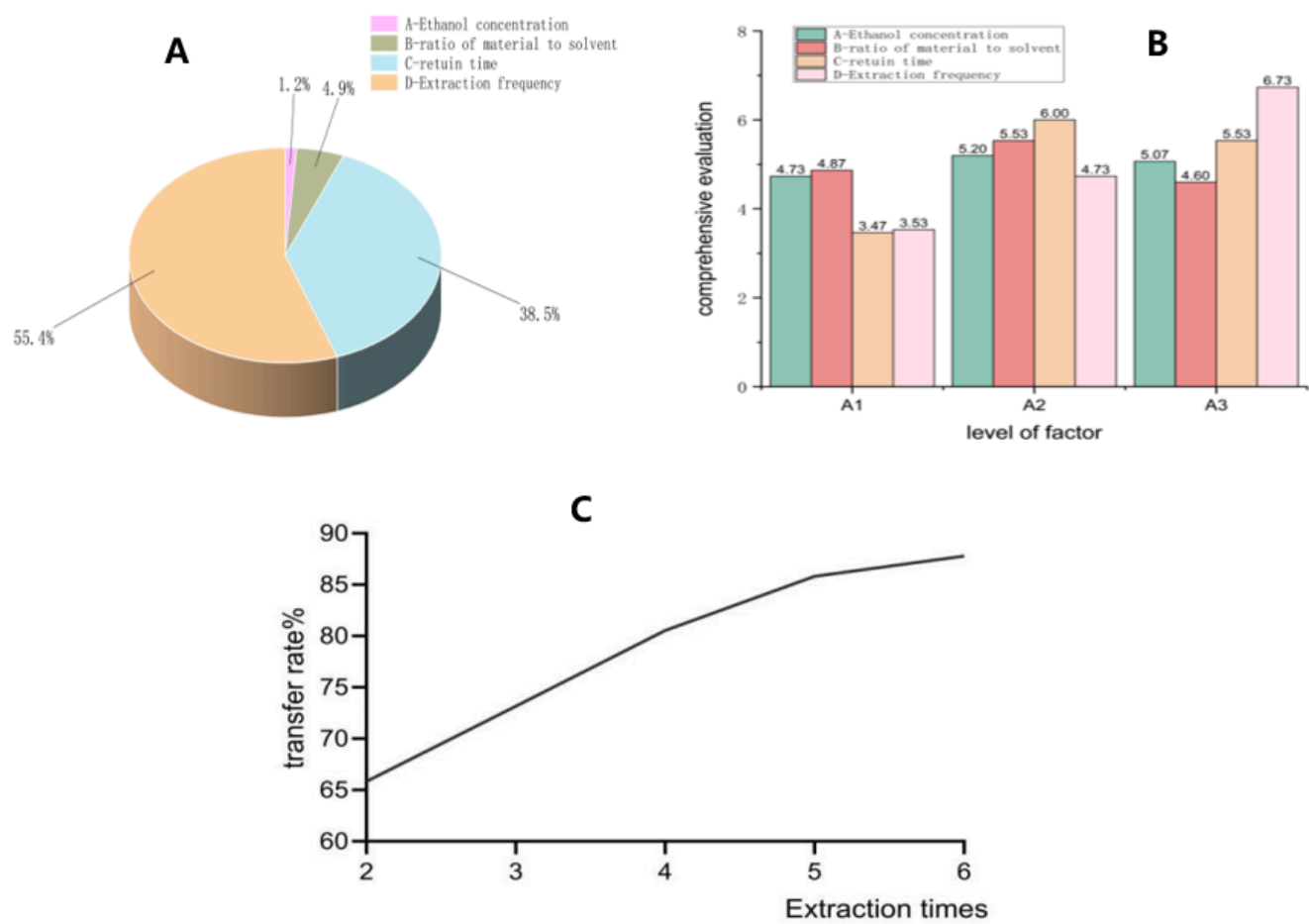


Figure 5

Extraction experiments on steamed *Panax notoginseng*