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Original Research Article

HPLC Method Development for the Separation and Quantitative Determination of Rg1 and Re Ginsenosides from Tissue Culture

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ABSTRACT

Ginsenosides are triterpenoid saponins found in *Panax ginseng*. They are well known for their health benefits, including antioxidant, anticancer, and antidiabetic effects, along with their support for cardiovascular and neurological function. Our previous study showed that cultivating *Panax ginseng* in tissue culture successfully led to its rapid growth. Several active compounds, including Rg1 and Re ginsenosides, were identified in the powdered extract of cultivated *Panax ginseng*. As part of a continuous study, an analytical method was developed to ensure the consistency of yield composition with the required quality standards. The analytical method optimization involved selecting the mobile phase composition and adjusting the flow rate in both isocratic and gradient systems to achieve optimal separation. The gradient elution system, with a constant flow rate of 1.0 mL/min, was more effective than the isocratic system for separating ginsenosides. The optimized method was then validated, showing acceptable validation parameters. The validated method was applied to the ginsenosides assay in the powdered extract of the *Panax ginseng* sample. Eight out of fourteen ginsenosides were identified, including Rg1 and Re, which were quantified at 0.16% and 0.45%, respectively. A baseline resolution of about 5.16 between Rg1 and Re ginsenosides was achieved. The analytical method developed using HPLC in this study effectively separated ginsenoside compounds simultaneously. These findings proposed an analytical method to evaluate the quality standards of ginsenosides in *Panax ginseng*.

***Keywords*:** Tissue culture, Powdered extract, *Panax ginseng*, Rg1 ginsenoside, Re ginsenoside, High-Performance Liquid Chromatography.

Introduction

Ginseng (*Panax ginseng*) belongs to the Araliaceae family. It has been used as a functional food and medical plant in Korea, Japan, and China for over 2000 years.[1] In recent decades, ginseng has been used for antioxidant effects, anti-cancer properties, enhanced immune function, and the treatment of cardiovascular disease, diabetes mellitus, and neurological disease.[2,3] As health trends have developed, this herbal product has become increasingly popular and dominant in demand in the international market. Generally, ginseng is cultivated in fertile soil conditions and requires about 4-6 years to harvest.[4,5,6] Tissue culture techniques have advanced rapidly to produce bioactive compounds with high content and activity in recent years.[7] Various efforts to enhance the quality of ginseng are increasingly focused on conventional approaches, such as optimizing root culture techniques through bioreactors.[8]

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PT. Bintang Toedjoe (Pulomas, Jakarta, Indonesia), a subsidiary of Kalbe Company (Jakarta, Indonesia), has collaborated with Kalbe Ubaya Hanbang-Bio (KUH) (Surabaya, Indonesia), a pioneering ginseng tissue culture laboratory in Indonesia. Recent research shows that in vitro tissue culture methods produce ginsenosides successfully from *Panax ginseng*.[9] The root part of *Panax ginseng* is beneficial as a medicinal material and contains saponins and non-saponins, including phenolic compounds, flavonoids, and polysaccharides.[10,11] As a group of glycoside saponins, ginsenosides is the main triterpenoid saponin component, in addition to protopanaxadiol (PPD), protopanaxatriol (PPT), ocotillol, and oleanolic acid.[12,13,14] In particular, ginsenosides have been reported to have potential anticancer, antidiabetic, anti-inflammatory, hepatoprotective, anti-aging, and antioxidant effects.[15] The main classification of the active compounds in *Panax ginseng* species is shown in (Figure 1).

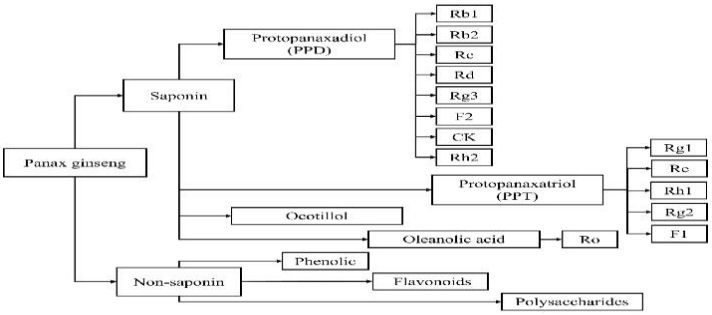

Figure 1: Classification of the active compounds in *Panax ginseng*[16]

Protopanaxatriol (PPT) includes Rg1, Re, Rh1, Rg2, and F2.[16] Rg1 ginsenoside has the chemical formula $C_{42}H_{72}O_{14}$ and a molecular weight of 801.0 g/mol. Rg1 ginsenoside has been used to reduce liver damage,[17,18] reduce oxidative stress, Alzheimer's disease, and anti-aging.[19,20] Re ginsenoside has the chemical formula $C_{48}H_{82}O_{18}$ and a molecular weight of 947 g/mol. Re ginsenoside has been used as an anti-diabetic, anti-inflammation, cardiovascular condition, anticancer treatment, and for nervous system disorders.[21] The chemical structure of Rg1 and Re ginsenosides is shown in (Figure 2).

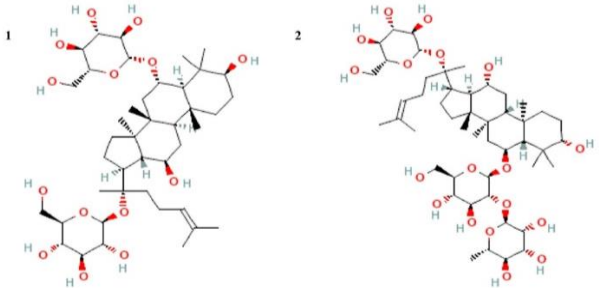

Figure 2: Structure of Rg1 ginsenoside (1),[22] Re ginsenoside (2)[23]

Rg1 and Re compounds are the main ginsenosides, dominating about 71.1% to 89.9% of the total.[24] Quality control is necessary to ensure that the products are consistent with the desired quality standards.

Analytical methods, such as chromatography and spectroscopy, are essential for analyzing ginsenoside compounds. Ginsenoside identification can be achieved through Thin Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR), and Nuclear Magnetic Resonance (NMR). At the same time, quantitative analysis is conducted using High-Performance Thin Layer Liquid Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), and Ultra High-Performance Liquid Chromatography (UHPLC).[25,26] Studies on the determination of ginsenosides in *Panax ginseng* using Thin Layer Chromatography (TLC),[27,28] Fourier Transform Infrared Spectroscopy (FTIR),[29] Proton Nuclear Magnetic Resonance (H-NMR),[30] Carbon Nuclear Magnetic Resonance (C-NMR),[31] High-Performance Thin Layer Liquid Chromatography (HPTLC),[32] Ultra High-Performance Liquid Chromatography (UHPLC) have been reported.[33,34]

Among the analytical methods mentioned above, High-Performance Liquid Chromatography (HPLC) is commonly used to analyze various ginseng species.[35,36] The High-Performance Liquid Chromatography (HPLC) method has been proven to be the most selected analytical and purification technique due to its selectivity, sensitivity, and ability to handle a wide range of polarity compounds. A reversed-phase High-Performance Liquid Chromatography (HPLC) mode is typically applied using a C18 silica column (150 or 250 × 4.6 mm) with a particle size of 5 µm and an organic solvent such as acetonitrile or methanol.[36] The gradient elution system of aqueous and acetonitrile and detection wavelength was set at 203 nm.[37]

A study by Thu Thi *et al.* (2021) reported the development and optimization of an analytical method using Solid Phase Extraction (SPE) and High-Performance Liquid Chromatography with a Diode Array Detector (HPLC-DAD) for the simultaneous determination of Notoginsenoside R1 and three ginsenosides (Rg1, Re, Rb1) in dietary supplements. The validated method provides an efficient analytical procedure for measuring ginseng and pseudo-ginseng.[38] Previous studies developed a rapid and efficient method for isolating ginsenosides Re and Rg1 from *Panax ginseng* roots. It employed a combination of High-Speed Counter Current Chromatography (HSCCC) techniques and MCI gel column chromatography, followed by High-Performance Liquid Chromatography with a Mass Spectra (HPLC-MS) analysis. The method successfully improved the speed and efficiency of ginsenoside separation and purification, and results in high purity levels were achieved, with 99.3% for Rg1 and 98.2% for Re.[39]

Due to the similarity and heterogeneity of ginsenoside structures, ginsenosides Rg1 and Re compounds have not been able to separate well. Therefore, this limitation reduces the number of detected components and the accuracy of peak integration, so inaccurate quantification may occur.[24] Therefore, this study proposed a development method and optimized separation method for Rg1, Re, and 12 other ginsenosides, which were identified simultaneously using High-Performance Liquid Chromatography (HPLC) methods and also to develop a quantitative method for Rg1 and Re ginsenosides.

Materials and Methods

Chemicals and reagents

Powdered extract of *Panax ginseng simplisia* from variation tissue culture developed by Kalbe UBAYA-Hanbang Bio Laboratory (Surabaya, East Java, Indonesia), ethanol 80%, maltodextrin DE 10 - 12, methanol HPLC grade (Merck KGaA, Darmstadt, Germany), acetonitrile HPLC grade (Merck KGaA, Darmstadt, Germany), water purification systems from Milli-Q (Darmstadt, Germany), orto - phosphoric acid 85% for analysis (Merck KGaA, Switzerland) and reference standard G-Rg1 (99.1%), G-Re (99.1%), G-Rb1 (95%), G-Rc (92.9%), G-Rh1 (100%), G-Rg2 (98.8%), G-Rb2 (100%), G-Ro (97.9%), G-F1 (99.8%), G-Rd (99.9%), G-F2 (95.1%), G-Rg3 (100%), G-CK (100%), G-Rh2 (100%) (Sigma Aldrich, USA).

HPLC instrumentation

The method was developed using HPLC Agilent, 1260 Infinity II (USA). A Symmetry Shield C-18 column 3.5 µm with a size of 4.6 × 150 mm was used. The injected sample volume was 10 µL, and the Diode Array Detector was 205 nm.

Extraction and sample preparation of culture results

Panax ginseng from tissue culture developed by Kalbe UBAYA-Hanbang Bio Laboratory was evaluated. For sample preparation, 200 g of *Panax ginseng simplisia* powder was weighed and macerated in 1 L of 80% ethanol for three cycles at 50°C, with agitation at 500 rpm. The filtrate was separated from the solid using vacuum filtration and then evaporated. In the second step, the filtrate was concentrated using a rotary evaporator (Buchi R300, Swiss). The concentrated extract was evaluated for its % Brix value (40-50%) using a refractometer (Pocket Refractometer ATAGO, Japan). In the final step, 600 mL of Milli-Q water was prepared, and the resulting thick extract was mixed with maltodextrin until fully dissolved. The mixture was then spray-dried (Buchi S-300, Swiss), yielding the powdered *Panax ginseng* extract as the final product.

Reference standards (14 ginsenosides) preparation for analysis

Reference standards of ginsenosides were prepared by dissolving them in HPLC-grade methanol. This was a 50 ppm standard stock solution. Further, each 50 ppm standard stock solution was taken about 1000 µl and transferred to 1.5 mL HPLC vials.

Preparation of sample for analysis

Approximately 1 g of powdered extract was transferred into a 50 mL volumetric flask and dissolved in HPLC-grade methanol. The flask was sonicated for 30 minutes, and the volume was then adjusted to the mark with methanol, resulting in a concentration of 20.000 ppm. The samples were filtered using 0.22 µm PTFE membrane filters and transferred into 1 mL HPLC vials.

Optimization of chromatography conditions

Based on previous studies and the conditions in our laboratory, we investigated the following parameters: elution system, mobile phase composition, flow rate, gradient composition changes, and run time, as these factors significantly affect chromatographic separation. The optimized conditions were selected based on criteria for good separation (resolution, *Rs* >1.5),[40] absence of interference from matrix peaks, and appropriate pressure. Both isocratic and gradient elution systems were applied, with the detailed isocratic system in Table 1 and the gradient system in Table 2.

Table 1: The applied isocratic system

Composition		Flow Rate (ml/min)			
Buffer	**Acetonitrile**	**0.8**	**1.0**	**1.2**	**1.5**
75	25	*n.e*	1.13	*n.e*	*n.e*
78	22	2.58	2.41	*n.e*	*n.e*
80	20	*n.e*	7.52	4.69	4.58

**n.e* (not evaluated)

Table 2: The applied gradient system

System	Time (min)	Buffer	Acetonitrile	Rt (min)		Width		Rs
				Rg1	Re	Rg1	Re	
1	0 - 8	80	20	18.312	18.889	0.118	0.120	2.12
	8 - 32	65	35					
	32 - 40	60	40					
	40 - 45	40	60					
	45 - 47	0	100					
	47 - 52	0	100					
	52 - 55	80	20					
	55 - 60	80	20					
2	0 - 35	80	20	28.208	32.344	0.473	0.631	5.16
	35 - 40	72	28					
	40 - 67	60	40					
	67 - 72	40	60					
	72 - 74	0	100					
	74 - 79	0	100					
	79 - 82	80	20					
	82 - 87	80	20					

Results and Discussion

The initial step of this study involved method development and optimization for the separation of ginsenoside Rg1 and ginsenoside Re. In previous experiments, method development was carried out under varying conditions, including changes to the mobile phase composition and column type efficiency.[41] Further modifications were made to the column type, gradient system, flow rate, and injection volume.[38] Based on the laboratory conditions in this study, the method was optimized by adjusting the elution system, mobile phase composition, flow rate, gradient composition, and run time.

The first experiment focused on modifying the elution system and flow rate, with the results presented in Table 1. The system was operated using an isocratic method, where the composition of the eluent remains constant throughout the analysis, allowing for separation based on consistent interactions with the stationary phase.[42]

In the first experiment (Figure 3A), the initial phosphate buffer (pH 2.0) and acetonitrile (75:25) were used, resulting in a resolution of 1.13 at a flow rate of 1.0 mL/min. The second experiment (Figure 3B) used a composition of phosphate buffer (pH 2.0) and acetonitrile (78:22), achieving a resolution of 2.41 at the same flow rate of 1.0 mL/min. The third experiment (Figure 3Ci) maintained the phosphate buffer (pH 2.0) and acetonitrile (78:22) composition, but the flow rate was reduced to 0.8 mL/min, yielding a resolution of 2.58. The adjustment to a lower flow rate aimed to improve resolution compared to 1.0 mL/min. However, additional peaks were observed between the ginsenoside Rg1 and Re compounds, resulting in poor separation (Figure 3Cii). Previous research suggests that a flow rate of 0.8 mL/min does not yield optimal separation.[43,44]

Therefore, further method development was conducted in this study to achieve improved separation results. Based on previous studies, a composition of 20% acetonitrile has been shown to provide good separation for ginsenoside compounds.[41] In the fourth experiment (Figure 3Di, ii), a composition of phosphate buffer (pH 2.0) and acetonitrile (80:20) was used at a flow rate of 1.0 mL/min, resulting in good separation with a resolution value of 7.52. However, the retention times (RT) for ginsenoside Rg1 and Re were relatively long. Further experiments addressed this by increasing the flow rates to 1.2 mL/min and 1.5 mL/min to shorten the retention times. In the fifth experiment (Figure 3Ei), the flow rate was increased to 1.2 mL/min, yielding a resolution value of 4.69. However, the baseline became unstable when the flow rate was further increased (Figure 3Eii), leading to less effective separation. In the final experiment (Figure 3Fi), an isocratic elution system with a flow rate of 1.5 mL/min was used, achieving good separation with a resolution value of 4.58. However, when the flow rate was further increased (Figure 3Fii), the peaks for ginsenoside compounds Re and Rg1 showed broadening.

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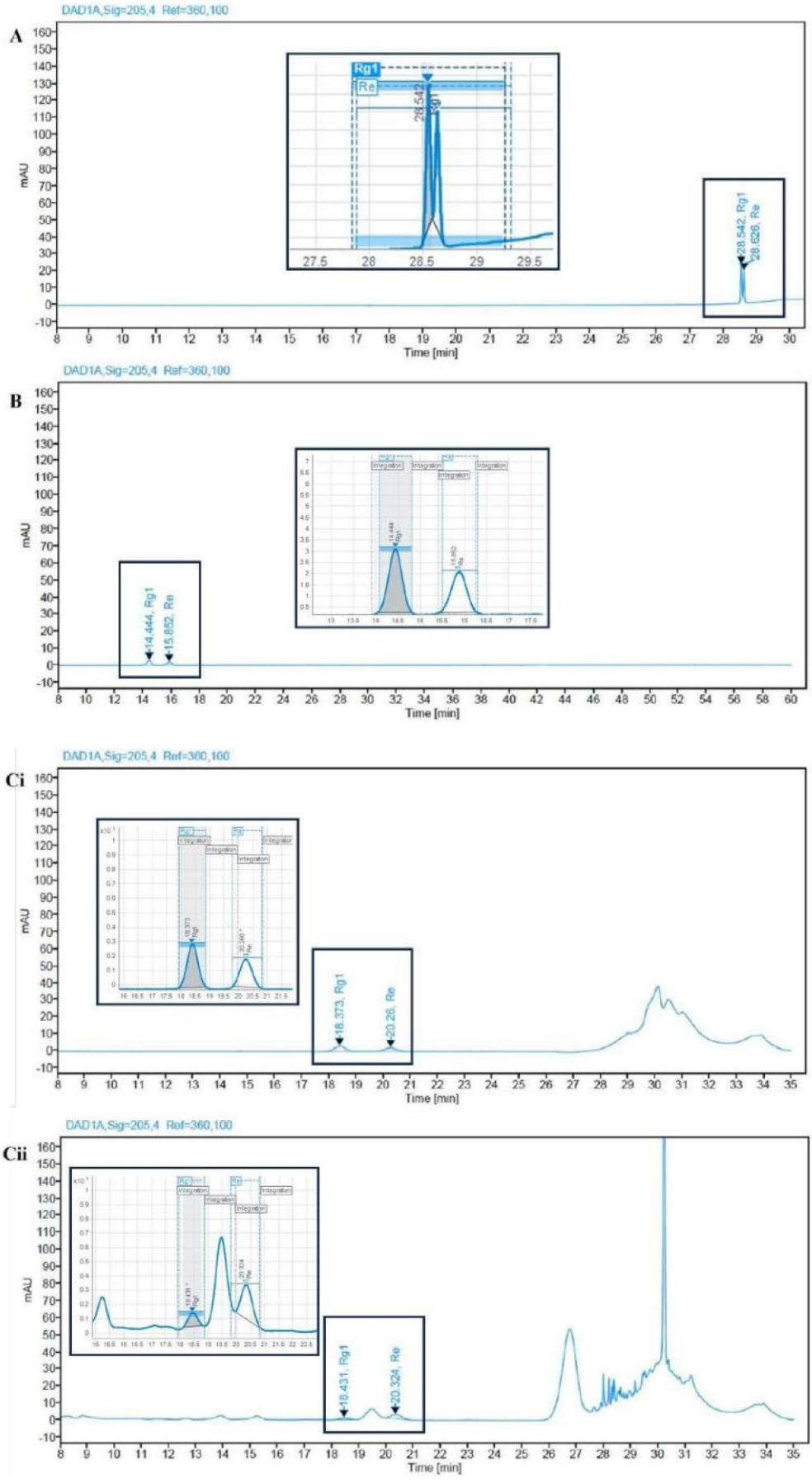

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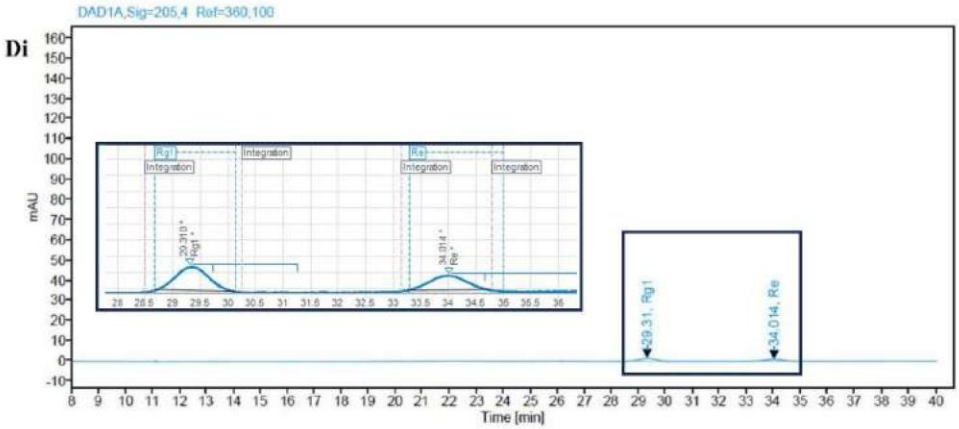

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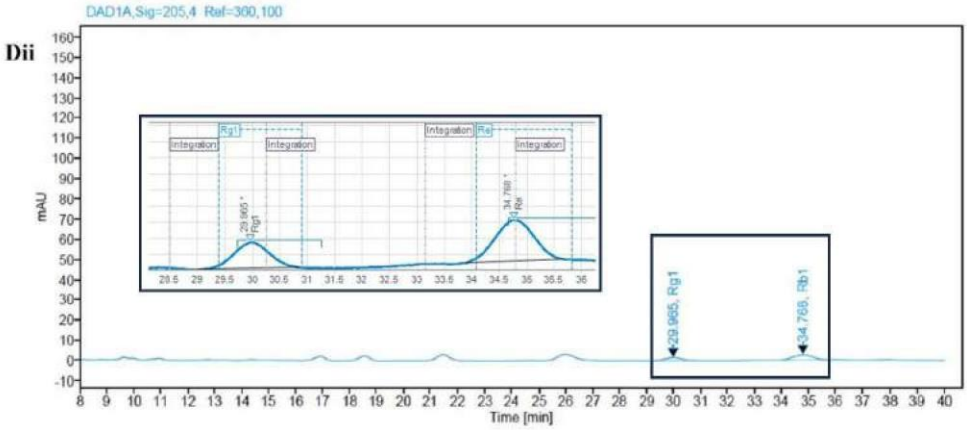

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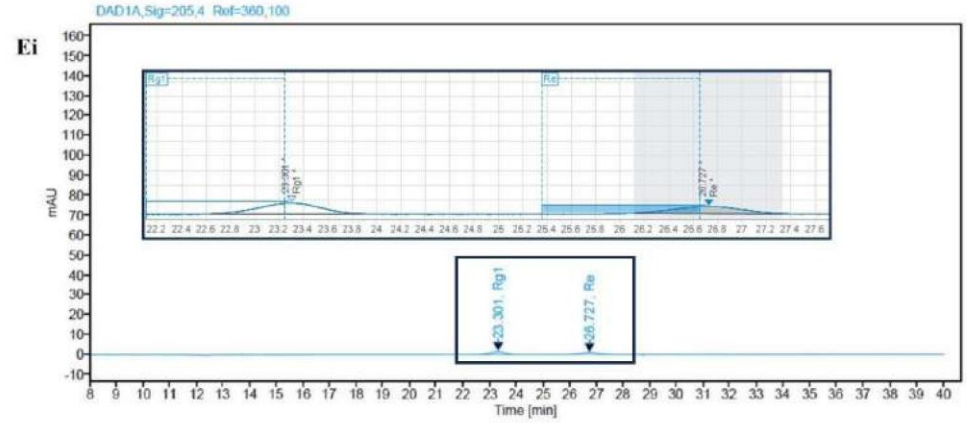

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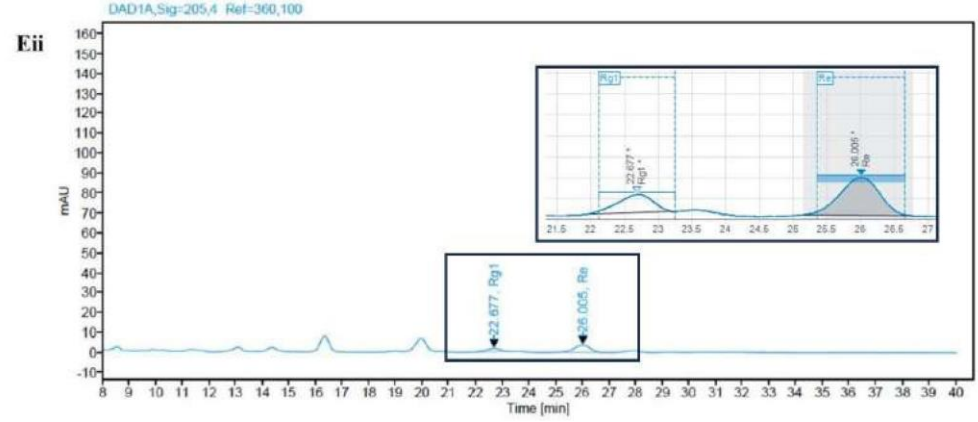

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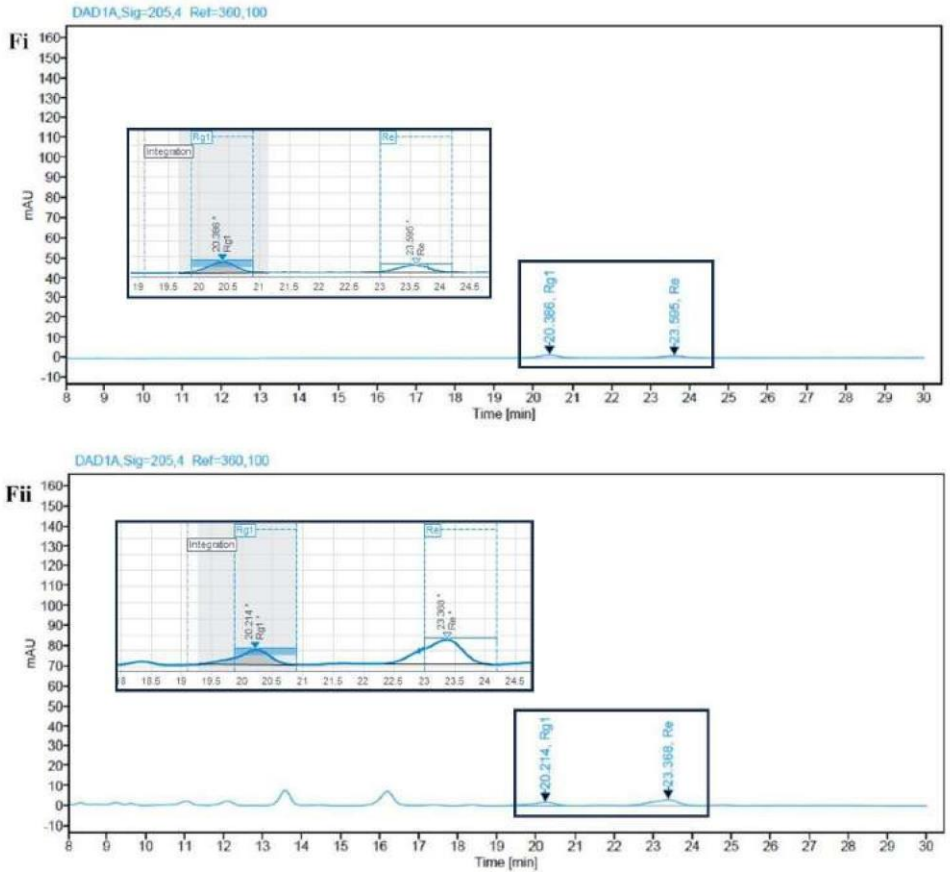

Figure 3: Representative chromatograms of Rg1 and Re ginsenosides at isocratic separation conditions of mobile phase: phosphate buffer pH 2.0 and acetonitrile (75:25); at flow rate, 1.0 mL/min (A), phosphate buffer pH 2.0 and acetonitrile (78:22); at flow rate, 1.0 mL/min (B), phosphate buffer pH 2.0 and acetonitrile (78:22); at flow rate, 0.8 mL/min (Ci, Cii), phosphate buffer pH 2.0 and acetonitrile (80:20); at flow rate, 1.0 mL/min (Di, Dii), phosphate buffer pH 2.0 and acetonitrile(80:20); at flow rate, 1.2 mL/min (Ei, Eii), and phosphate buffer pH 2.0 and acetonitrile (80:20); at flow rate, 1.5 mL/min (Fi, Fii); injection volume 10 µL, and detection wavelength, 205 nm.

Based on these results, the method selected for further optimization was used in the fourth experiment, with phosphate buffer (pH 2.0) and acetonitrile at a flow rate of 1.0 mL/min. The HPLC system employed in this study uses reverse-phase separation, where non-polar compounds interact more strongly with the stationary phase and elute more slowly. In contrast, polar compounds elute faster.[45] Since ginsenoside compounds Re and Rg1 are classified as polar compounds,[46] a composition of 80% aqueous phase was used to achieve baseline separation.

A gradient elution system was employed in this study to identify 14 ginsenosides, as it is well-suited for analyzing mixtures of complex compounds.[47] The results obtained using the gradient system are presented in Table 2. Two optimal methods with run times of 60 minutes and 87 minutes were identified. The identification of ginsenoside compounds using the gradient system is presented in Table 3. Each method successfully identified eight types of ginsenosides, followed by the calculation of the % ginsenoside content for these compounds. The highest % ginsenoside content was observed in Rg1, Re, Rb1, and Rc. Previous studies have reported that ginseng roots contain 2-3% ginsenosides, predominantly found in compounds Rg1, Rc, Rd, Re, Rb1, Rb2, and Rb0.[48] Ginsenosides can be categorized into major and minor groups. Major ginsenosides include Rb1, Rb2, Rc, Rd, Re, and Rg1, while minor ginsenosides include Rg3, Rh1, and Rh2.[49] This research focused on the separation of Re and Rg1, which belong to the major ginsenoside group. According to Table 3, ginsenoside compounds Rg1 and Re had the highest % ginsenoside content compared to other ginsenosides. Based on the method development results, the optimized method selected was the 87-minute method (system 2), as it achieved a separation resolution of 5.16 for ginsenoside compounds Re and Rg1. Previous research has also shown that the gradient system for ginsenoside compounds Rg1 and Re yields good separation results, although Rf compounds were present between the separation of Rg1 and Re. In contrast, the isocratic system produced a resolution of 3.7 for compounds Rg1 and Re.[50] Another study reported an Rg1 and Re separation with a resolution of 1.6 and a run time of 80 minutes.[38] The ginsenosides identified in this study included Rg1, Re, Rb1, Rh1, Rc, Rb2, Rd, and F2. According to previous research, ginsenosides can be classified into polar compounds (Rg1, Re, Rb1, Rc, Rb2, Rb3, F1, and Rd) and non-polar compounds (Rf, Rg2, 20(S)-Rh1, 20(R)-Rg2, F4, 20(S)-Rg3, 20(R)-Rg3, Rg5, and 20(R)-Rh2).[46] The results of this study identified ginsenosides Rg1, Re, Rb1, Rh1, Rc, Rb2, and Rd (classified as polar compounds) and F2 (classified as a non-polar compound).

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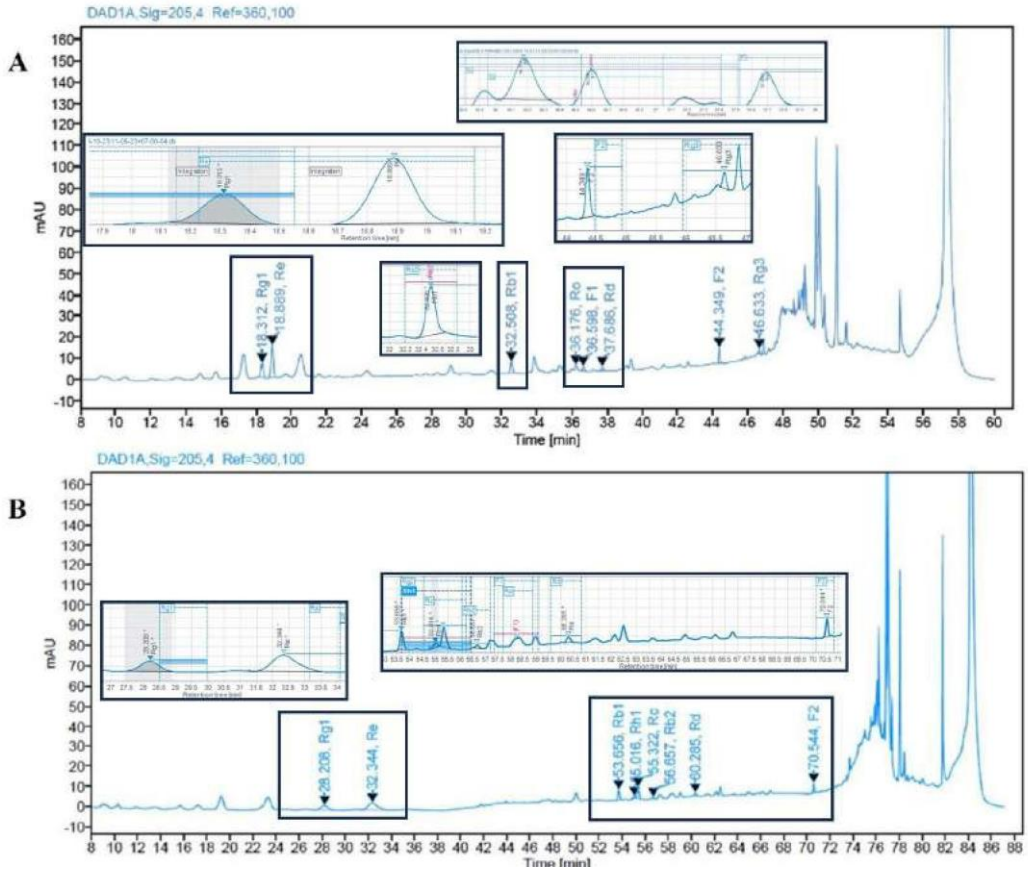

Figure 4: Representative chromatograms of samples *Panax ginseng* at gradient separation conditions of mobile phase phosphate buffer pH 2.0 (A): acetonitrile (B); at flow rate, 1.0 mL/min, injection volume 10 µL, and detection wavelength, 205 nm. Method 60 min (A), method 87 min (B).

Table 3: Identification of ginsenosides

System	% Content Compound										
	Rg1	**Re**	**Rb1**	**Rc**	**Rb2**	**Rd**	**F2**	**Rg3**	**Rh1**	**Ro**	**F1**
1	0.12	0.34	0.13	*n.i*	*n.i*	0.03	0.06	0.02	*n.i*	0.03	0.02
2	0.16	0.45	0.13	0.17	0.02	0.03	0.06	*n.i*	0.01	*n.i*	*n.i*

**n.i*(not identified)

Conclusion

An HPLC method analysis for separating ginsenosides was optimized using isocratic and gradient elution systems. Under the optimized gradient system, the method was validated and successfully applied to analyze ginsenosides in ginseng powder extract derived from tissue culture. Eight out of fourteen ginsenosides were successfully separated and quantified simultaneously. This study established an analytical method that can be applied to determine ginsenosides, especially Rg1 and Re, as part of quality control for ginseng extracts. Further development of the analytical conditions is feasible and can be explored to optimize the separation of all 14 ginsenosides.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that they will bear any liability for claims relating to the content of this article.

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