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DEVELOPMENT OF HPLC SEPARATION METHOD OF GINSENOSIDES ON A POLYVINYL ALCOHOL-BONDED STATIONARY PHASE

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ABSTRACT

A simple high performance liquid chromatographic assay for the simultaneous quantitative analysis of seven ginsenosides, Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 in commercial ginseng products is described. Chromatographic separation of the analytes was achieved in less than 20 min using a polyvinyl alcohol-bonded column with UV detection at 203 nm. Optimization of chromatographic conditions was determined by a three-factor central composite design, the variables being the percentage of acetonitrile in the mobile phase, column temperature and flow rate. A full quadratic model was found to be adequate in describing the separation of ginsenosides on the polyvinyl alcohol-bonded stationary phase. Complete separation of seven ginsenosides was achieved using acetonitrile–water (82.5/17.5) as the mobile phase run isocratically at a flow rate of 298 μL/min and with the column temperature at 9°C.The developed method was validated over the range of 10 – 120 μg/mL using a 5 μL sample injection volume. Intra- and inter-day variation for three ginsenoside standards (Rf, Rd and Rb1) at three concentration levels ranged from 0.07 to 0.83% expressed as the relative standard deviation. The accuracy based on the nominal concentration values at three concentration levels was in the range 98.7–100.8%. The limit of detection was between 0.43 and 1.03 μg/mL while the limit of quantification was from 1.42 to 3.13 μg/mL. The method is found to be applicable for the determination of ginsenosides in commercial ginseng products.

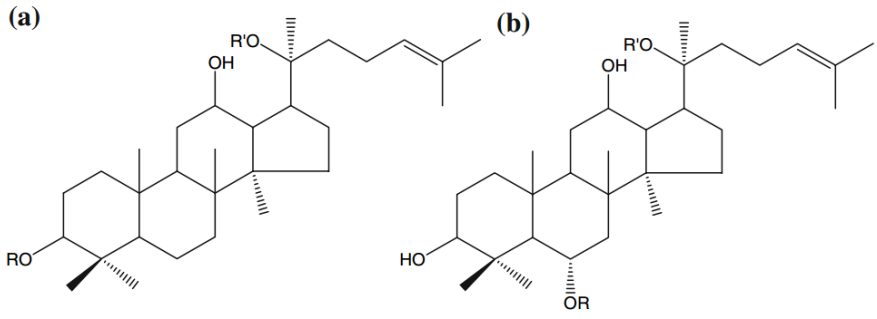
Key words: chromatography, acetonitrile, ginsenosides, acetonitrile–water, ginseng, plant, Panax ginseng, Panax quinquefolius, mass spectroscopy.

Introduction: Ginseng is one of the most widely used herbal drugs valued for its therapeutic and pharmacological activities [1, 2]. The ginseng plant belongs to the Araliaceae family and includes 13 species, the most commonly used of which are *Panax ginseng C.A*, (Asian ginseng) and *Panax quinquefolius L*. (North American ginseng). All species of ginseng contain ginsenosides, a group of dammarane saponins, which are the active components mainly responsible for the

effects attributed to ginseng [3]. Over 30 ginsenosides have been identified from *Panax spp*. [4] but the most abundant ginsenosides present are Rb1, Rb2, Rc and Rd, which possess 20(S)-protopanaxadiol as an aglycon (Fig.1**a**) and Rg1, Rf and Re, which possess 20(S)-protopanaxatriol as an aglycon (Fig.1**b**).

The standardization of ginseng and ginseng products is usually based on their ginsenoside content. Several methods have been developed for the analysis of ginsenosides, which focused on the identification and quantitative analyses of these active components in raw ginseng materials, processed ginseng, medicinal formulations and biological samples. Among these methods are thin layer chromatography (TLC) [5], gas chromatography (GC) [6] and high performance liquid chromatography (HPLC) [7-9]. HPLC methods have been the most successful and are now the most widely accepted analytical procedures for the analysis of ginsenosides. HPLC with ultraviolet (UV) detection [7-9] had been extensively used for the routine analysis of ginsenosides due to its simplicity and practicality compared to other detection techniques such as mass spectroscopy (MS) [10] or evaporative light scattering detection (ELSD) [11].

Analysis of ginsenosides by HPLC is usually done in reversed-phase wherein the stationary phase commonly used is C18 and a gradient elution is employed. A previous study reported a novel solid phase extraction (SPE) – HPLC method for the simultaneous determination of seven ginsenosides (Rg1, Re, Rf, Rb1, Rc, Rb2 and Rd) in ginseng products [12]. The separation was performed on a C18 column using an improved step gradient elution program. The method was able to solve the coelution problem of Rg1 and Re and complete separation of the seven ginsenosides was achieved within 70 min. The SPE clean-up procedure was also effective in eliminating the interference of other components present in the real samples. Although the developed method was applicable for the analysis of ginseng products, the long analysis time using the step gradient elution program is a disadvantage for routine analysis.



20(S)-Protopanaxadiol (if R = R′ = H

Rb1: R = D-Glc(β1-2) D-Glc
R′ = D-Glc(β1-6) D-Glc

Rb2: R = D-Glc(β1-2) D-Glc
R′ = L-Ara(pyr) (β1-6) D-Glc

Rc: R = D-Glc(β1-2) D-Glc
R′ = L-Ara(fur) (β1-6) D-Glc

Rd: R = D-Glc(β 1-2) D-Glc
R′ = D-Glc

20(S)-Protopanaxatriol (if R = R′ = H

Re: R = L-Rha(α1-2) D-Glc
R′ = D-Glc

Rf: R = D-Glc(β1-2) D-Glc
R′ = H

Rg1: R = D-Glc
R′ = D-Glc

insenosides investigated in this work.

The group of Bonfill et al. [13] reported the use of diol column for the separation of ginsenosides in *Panax ginseng*-based pharmaceuticals. A mobile phase consisting of aqueous orthophosphoric acid pH 2.5 and acetonitrile at a ratio of 18:82 was used isocratically to separate the seven compounds. Separation was achieved in less than 20 min but incomplete separation of Rf and Rg1 and Rd and Re was observed. Also, the percent recoveries for Rd and Rb2 were relatively low (ranging from 70 to 74%) when this method was used. Recently, our group reported the isocratic separation of ginsenosides using a diol column at subambient temperatures [14]. Complete separation of seven ginsenosides in less than 20 min was achieved using water – acetonitrile (17.5/82.5) as the mobile phase at 14°C. This report also showed the influence of temperature on the retention and separation of ginsenosides on the diol column.

This study was conducted to develop a simple chromatographic technique using a polyvinyl alcohol (PVA)-bonded stationary phase for the separation and quantitative determination of seven ginsenosides. PVA-bonded phase was chosen over the diol column due to its stability against high and low pH buffers, strong solvents, and the different selectivity that the vinyl group may offer.

Experimental

Chemicals and Reagents

Seven ginsenoside standards (Rb1, Rb2, Rc, Rd, Re, Rf and Rg1) were purchased from Extrasynthese (Lyon, France). HPLC grade acetonitrile used as the mobile phase and methanol used for the sample preparation were obtained from Wako Pure Chemical Industries (Osaka, Japan). Water was purified by a Milli-Q Water Purification System (Millipore, Tokyo, Japan).

Standard Solution Preparation

Stock solutions (500 μg/ml) of the standards were prepared by dissolving in methanol accurately weighed amounts of the standards. The stock solutions were covered with aluminum foil and stored at 20°C for a maximum of 2 months to ensure stability of the analytes according to Ji et al. [15]. Standard solutions for constructing the calibration curves were prepared daily from the stock solutions by dilution with methanol to achieve solutions with concentrations ranging from 10 to 120 μg/mL.

HPLC Measurement

The chromatograph used in this study is a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan) consisting of a two-flow channel degasser, an inert pump, an autosampler, a column oven and a UV-Vis detector. S-MicroChrom (S-MC) system controller (Shiseido, Tokyo, Japan) was used to control the operation of the HPLC system. The separation of the ginsenosides was achieved on YMC-Pack PVA-Sil (Polyvinyl alcohol-bonded column; 5 μm, 250 mm·× 2 mm i.d.) purchased from YMC Co., Ltd. (Kyoto, Japan). Optimization of the chromatographic conditions was carried out using the experimental design which is described in detail in ‘‘Results and Discussion’’. Detection of the analytes was performed at 203 nm. For data collection and processing, Borwin Chromatography Data Processing Software (Jasco, Tokyo, Japan) running on a personal computer

was used.

Method Validation

Linearity, Detection and Quantification Limits

The calibration curves for all seven standards were constructed with seven concentrations from the range 10 – 120 μg/mL (Table 3). Seven replicate injections of standards at each concentration level were performed. The peak areas of the standards were plotted against the concentration and the linearity was evaluated by linear regression analysis. Limits of detection (LOD) and quantification (LOQ) were estimated from the signal-to noise ratio. The limit of detection was calculated by LOD = 3.3 σ/S, where σ is the standard deviation of the response of the blank and S is the slope of the calibration curve. The limit of quantification was calculated by LOQ = 10 σ/S under the ICH guidelines, Q2B (Methodology for Validation of Analytical Procedures) [16].

Accuracy and Precision

Accuracy of the assay method was determined for both intra-day (repeatability) and inter-day (intermediate precision) variations using the three standards Rf, Rd and Rb1 at three concentration levels. The precision of the assay was calculated by analyzing three known samples three times in 1 day to determine the intra-day variability and on three consecutive days to determine the inter-day variation. Accuracy was calculated as the percentage of the nominal concentrations (Table 4).

Results and Discussion

Optimization of Chromatographic Conditions

The main objective of optimization in chromatography is to find a condition that will give adequate separation of all the components of a mixture in reasonable time. In this case, an experimental design (ED) was used to find the optimum chromatographic condition for the separation of seven ginsenoside standards using the polyvinyl alcohol (PVA)-bonded stationary phase (Table 1). An experimental design is a planned series of experiments with changing variables describing the experiment in the most efficient way in order to find optimal variable setting for further evaluation [17]. The aim of ED is to get the best description of the response surface which is a 3D plot showing the influence of one or more variables on an output response (fig. 3).

Table 1. Variable level obtained from central composite design and experimental *Rf* for each experiment during optimization.

Experiment number	Variables			
	ACN, %	Temperature, °C	Flow rate, μL/min	R_f
1	78.3	17.5	250	0.1072
2	86.7	17.5	250	0.0598
3	82.5	5.0	250	0.1789
4	82.5	30.0	250	0.0220

5	82.5	17.5	166	0.1127
6	82.5	17.5	334	0.1760
7	80.0	10.0	200	0.1265
8	85.0	10.0	200	0.1405
9	80.0	25.0	200	0.0547
10	85.0	25.0	200	0.0210
11	80.0	10.0	300	0.1588
12	85.0	10.0	300	0.1536
13	80.0	25.0	300	0.0646
14	85.0	25.0	300	0.0145
15	82.5	17.5	250	0.1568
16	82.5	17.5	250	0.1736

Preliminary studies were first conducted to determine the chromatographic parameters or variables that greatly affect the separation of ginsenosides on the PVA-bonded stationary phase. Among the variables, the percentage of acetonitrile (%ACN) in the mobile phase and the column temperature are the ones that have great influence on the retention and separation of the analytes. In addition to these two variables, the flow rate was also included and a three-factor central composite design (CCD) was constructed using The Unscrambler 9.6 software (CAMO Inc., Oslo, Norway). Sixteen experiments were carried out: eight cube experiments, 6 star experiments and a center experiment which was run twice. Table 1 shows the 16 experiments as well as the values of the 3 variables for each run.

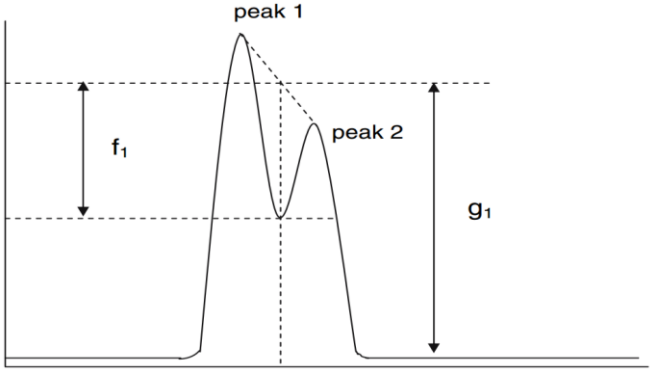


Fig. 2. Definition of f_i and g_i for the computation of the response factor *Rf*.

A response function defined by Kaiser [18] was used as the experimental response for each chromatographic run. The response function, Rf, which takes into account the separation efficiency and the analysis time, is defined by the equation:

$$R_f = \frac{1}{\sqrt{t}} \prod_{i=1}^{m-1} \frac{f_i}{g_i}$$

where the meanings of f_i and g_i are shown in Fig. 2, t is the retention time of the last peak, m is the number of peaks, i =1, 2,..., m-1. The *Rf* values for all the

runs are also listed in Table 1. The CCD data listed in Table 1 were analyzed to determine the effect of the three variables on the separation of the analytes. An analysis of variance (ANOVA) was performed on the design to assess the significance of the model. Table 2 summarizes the ANOVA for a full quadratic model showing the coefficients of the variables, *F*-ratio and *P*-values.

Table 2. Response model coefficients, *F* ratios, and *P*-values obtained in the ANOVA.

Source	Coefficient	*F*-value	*P*-value
Model		22.3926	0.0006
Constant	-36.57320		
A: ACN	0.86553	6.8416	0.0398
B: Temperature	0.06402	135.4734	<0.0001
C: Flow rate	0.00555	6.8895	0.0393
AB	-0.00062	4.1837	0.0868
AC	-0.00004	0.6184	0.4616
BC	-0.00001	0.8607	0.3893
A^2	-0.00515	37.5177	0.0009
B^2	-0.00047	24.8561	0.0025
C^2	-0.00004	4.1377	0.0882
Lack-of-fit		1.9785	0.4911

As can be seen from Table 2, the model is significant with a P-value less than 0.05. The lack-of-fit test is designed to determine if the selected model is adequate to describe the observed data. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factor. The *P*-value for the lack-of-fit is 0.4911 (>0.05) signifying that the lack of fit is not significant relative to the pure error and that the pure quadratic model can accurately describe the observed data. The significance of each term in the quadratic model can be assessed by its *P*-value. A *P*-value less than 0.05 indicates that the term is significant while a *P*-value greater than 0.05 shows that the term is not significant. In this case, the terms A, B, C, A^2 and B^2 are significant model terms. The terms A, B, and C correspond to %ACN, temperature and flow rate, respectively, indicating that the three variables significantly affect the retention and separation of the ginsenosides on the PVA-bonded stationary phase. Figure 3 shows three response surface plots obtained from the quadratic model. These plots show the variation in *Rf* when one of the three variables was held constant while the other two were changed.

It can be seen in Fig. 3a and c that high Rf values can be obtained at a lower temperature. Using the derived quadratic model and the response surface plots, the conditions that resulted to the highest *Rf* value were found to be: %ACN 82.5%, temperature 9°C, and flow rate 298 µL/min. These were chosen as the optimum chromatographic conditions. Figure 4 shows the chromatogram of the seven

ginsenoside standards separated on the PVA-bonded column using the optimized separation conditions. Complete resolution of all the 7 analytes was achieved in less than 16 min. This is significantly shorter than the previously reported 70-min analysis time using a C18 column and gradient elution [12].

The PVA-bonded stationary phase showed different selectivity for the analytes as compared to C18 phases. Using the C18 columns, the order of elution for the seven ginsenosides from the least to the most retained analyte was Rg1, Re, Rf, Rb1, Rc, Rb2 and Rd [12]. This order was completely different from the results obtained using the PVA-bonded phase wherein the order of elution was Rf, Rg1, Rd, Re, Rc, Rb2 and Rb1. This order of elution was similar to that observed using he diol column [14] signifying a similarity in the mechanism of the ginsenosides separation on both columns.

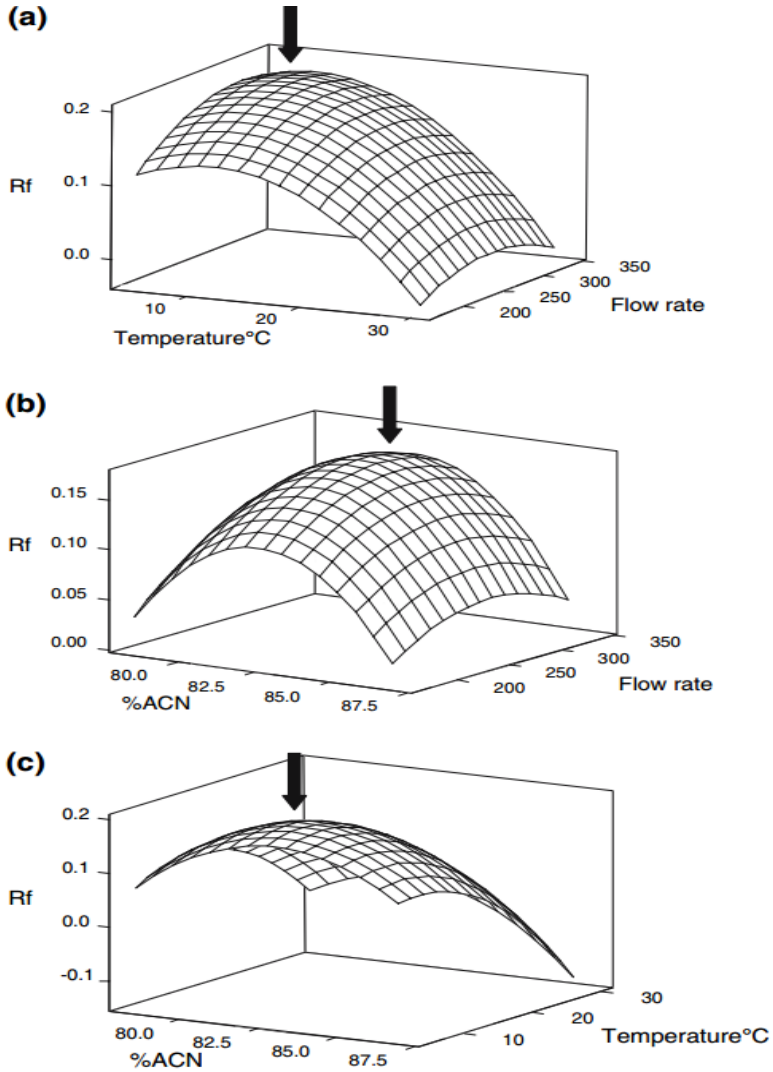


Fig. 3. Response surface plots estimated from the CCD for **a** Temperature versus Flow rate at constant %ACN of 82.5%, **b** %ACN versus Flow rate at constant Temperature of 17.5°C, and **c** %ACN versus Temperature at constant Flow rate of 250 µL/min. Arrows indicate maximum points of the graphs.

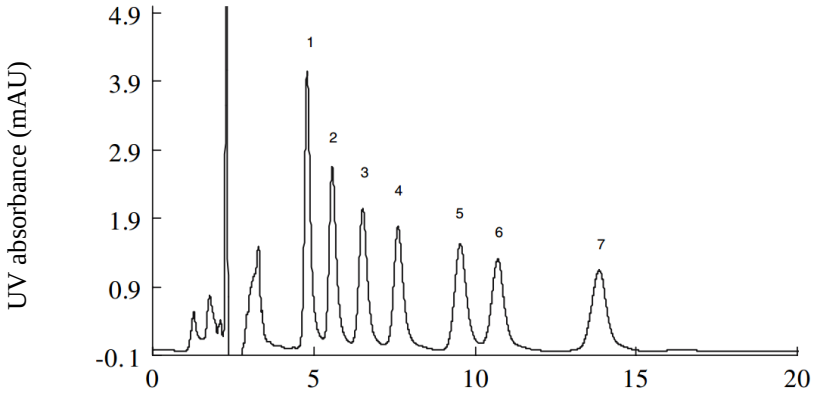


Fig. 4.Typical chromatration of seven ginsenoside standards. Chromatographic conditions: Column-YMC-Pack PVA-Sil (polyvinyl alcohol bonded silica, 5 µm, 250 mm·2.1 mm i.d.); Mobile phase, isocratic elution with acetonitrile/water (82.5:17.5); Flow rate, 298 µL/min. Column temperature 9°C; Sample concentration 200 µg/mL; injection volume 5.0 µL. Peaks 1 = Rf; 2 = Rg1; 3 = Rd; 4 = Re; 5 = Rc; 6 = Rb2; and 7 = Rb1.

Method Validation

Linearity, LOD and LOQ Calibration curves for the seven ginsenosides were constructed by plotting the peak area of the analytes versus the corresponding concentrations. The linearity of the plots was assessed in terms of the correlation coefficients (r^2). All calibration curves were linear over the concentration range of 10–120 µg/mL with r^2 as high as 0.9984. LOD and LOQ ranged from 0.426–1.033 to 1.291–3.130 µg/mL, respectively. Table 3 summarizes these results for the seven ginsenoside standards.

Table 3. Calibration curves and sensitivity for determination of ginsenosides.

Ginsenoside	Calibration curve[a]	Correlation coefficient, R^2	LOD[b], µg/mL	LOQ[c], µg/mL
Rf	y = 1391.47x-5168.46	0.9984	0.996	3.017
Rg1	y = 1370.28x-7483.43	0.9985	1.033	3.130
Rd	y = 869.68x-2457.42	0.9998	0.813	2.463
Re	y = 1264.45x-2036.59	0.9985	0.426	1.291
Rc	y = 926.54x-4562.91	0.9988	0.469	1.422
Rb2	y = 826.91x-2485.93	0.9993	0.824	2.497
Rb1	y = 844.82x-1535.14	0.9998	0.950	2.880

Seven measurements at seven concentration levels over the range 10–120 µg/mL

[a] y peak area; x concentration (µg/mL).

[b] LOD = 3.3 σ/m, where σ is the standard deviation of the response of the blank, m is the slope of calibration curve.

[c] LOQ = 10 σ/m, where σ is the standard deviation of the response of the blank, m is the slope of calibration curve.

Accuracy and Precision

Intra- and inter-day variation (repeatability) and accuracy of the method were determined from the three typical ginsenoside standard (Rf, Rd and Rb1) solutions by replicate analyses of three concentration levels for each standard. The intra-day variations were assessed by analyzing the standards at each concentration level in triplicate. Inter-day variations were determined by measuring the analytes for three consecutive days with three replicates for each concentration level. Table 4 shows the precision and accuracy data for the three standards Rf, Rd and Rb1. As can be seen from Table 4, RSD values for the intra- and the inter-day measurements were not more than 0.42 and 0.83%, respectively, indicating good intra- and inter-day precision. High accuracy for both the intraday (99.0–100.8%) and the inter-day (98.7–100.8%) measurements were also obtained for the validated method.

Table 4. Accuracy and precision data for the determination of three typical ginsenoside control samples.

Ginsenoside	Control sample concentration, μg/mL	Intra-day variations (n=3)		
		Measured[a], μg/mL	RSD[b], %	Accuracy[c], %
Rf	90.00	90.07±0.06	0.07	100.1
	50.00	50.27±0.07	0.13	100.5
	30.00	30.18±0.11	0.37	100.6
Rd	90.00	90.08±0.09	0.10	100.1
	50.00	49.48±0.21	0.42	99.0
	30.00	29.90±0.05	0.17	99.7
Rb1	90.00	90.15±0.12	0.14	100.2
	50.00	50.03±0.08	0.16	100.1
	30.00	30.23±0.11	0.36	100.8
Ginsenoside	Control sample concentration, μg/mL	Inter-day variations (n=3)		
		Measured[a], μg/mL	RSD[b], %	Accuracy[c], %
Rf	90.00	90.14±0.07	0.07	100.2
	50.00	50.42±0.20	0.39	100.8
	30.00	30.21±0.05	0.16	100.7
Rd	90.00	89.96±0.26	0.28	99.9
	50.00	49.56±0.17	0.34	99.1
	30.00	29.62±0.24	0.83	98.7
Rb1	90.00	90.10±0.07	0.07	100.1
	50.00	50.09±0.06	0.11	100.2
	30.00	30.13±0.11	0.35	100.4

[a] Values are the mean values with ± standard deviation.

[b] Relative standard deviation.

[c] Accuracy (%) = (Mean measured value/Nominal value)·100.

Conclusion

The optimized chromatographic condition derived from CCD was effective in completely separating the seven ginsenosides. The use of the PVA-bonded stationary phase showed different selectivity for the separation of the analytes as compared to C18columns. The developed method was found to be less time consuming as compared to the previously reported method using C18 column and gradient elution method. All statistical parameters (RSD, LOD, LOQ and linearity) were acceptable. Since the retention behavior of ginsenosides on polar stationary phases such as the diol and the PVA-bonded phases is not yet well understood, studies on the retention mechanisms of these analytes on these stationary phases are now being conducted in our laboratory.

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