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Optimization of extraction methods with alkali and determination of stachyose, sucrose, and raffinose in fresh rehmannia (*Rehmannia glutinosa* Libosch) using high-performance liquid chromatography with evaporative light scattering detection

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A high-performance liquid chromatography (HPLC) method with evaporative light scattering detection (ELSD) was used to determine the stachyose, sucrose, and raffinose in *Rehmannia glutinosa* Libosch simultaneously. Carbohydrates were separated on an XBridge™ Amide column using a mixture solvent of acetonitrile: water (70:30, v/v) as mobile phase. Four different methods for the extraction and purification of stachyose in fresh rehmannia (FR) tubers were detailed and evaluated. According to the analysis of the three factors in orthogonal array design (OAD), the optimal combination parameters of the processing technology were as follows: pH value of 12.0, extraction time of 2 h and ethanol concentration of 5%. The extraction method with alkali can be used to extract the carbohydrates completely from the FR. The stachyose content (652.01 mg/g) obtained using the extraction method with alkali was obviously higher than that using other methods. Interestingly, the results show that the stachyose content decreased by approximately 30 to 40% when precipitated with the addition of ethanol. Therefore, the current method is suitable for the determination of sugars in FR and provides the basis for industrial production and purification in future studies.

Key words: Fresh rehmannia, alkali, oligosaccharide, evaporative light scattering detection, orthogonal array design.

INTRODUCTION

Rehmannia glutinosa Libosch. (RL), which is a comprehensive traditional Chinese medicinal herb, belongs to the Scrophulariaceae family. Three types of *R. glutinosa*, namely, fresh rehmannia (FR) root (Xian Dihuang), rehmannia dried rhizome (Sheng Dihuang), and prepared rehmannia root (Shu Dihuang), are used as medicinal materials for the processing method (Capek and Hribalova, 2004). About 70 monomeric compounds have been separated from *R. glutinosa*, of which saccharides, including polysaccharides and oligosaccharides, particularly stachyose and monosaccharides, have the largest contents (Zhang et al., 2008). An initial

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report indicated that FR root contains the highest content of 64.9% stachyose of the total sugar (Kubo et al., 1996), whereas only 30% was found in Rehmannia dried rhizome.

Recently, oligosaccharides, such as raffinose and stachyose, have been identified as potential development prospects that mainly exist in RL, legume seeds (Martínez-Villaluenga et al., 2005), such as soybeans (Leske et al., 1993; Kim et al., 2003), lupins (Martínez-Villaluenga et al., 2005), lentils (Hoch et al., 1999), peas (Frias et al., 1996), and chickpeas (Xiaoli et al., 2008), among others, and other plants, such as Chinese artichoke (Yin et al., 2006) and *Lycopus lucidus* Turcz (Yang et al., 2010). Oligosaccharides may influence animals and humans in various ways: they can improve intestinal health, modify lipid metabolism, modulate the immune response, and decrease the risk of intestinal and systemic diseases. Recently, studies have shown that oligosaccharides, particularly stachyose, have good hypoglycemic effects (Zdunczyk, 2004; Zhang et al., 2004; Zhang et al., 2004). Although RL has not been popularly consumed as an agricultural vegetable product and botanical food supplement among peasants in China, the oligosaccharides it contains can be used as sources for industrial production.

Previous studies have provided various RL extraction and detection methods, such as the boiling water method (Ekvall et al., 2007) and 50 and 80% ethanol extraction methods (Ni et al., 1992). The current study presents an extraction method with alkali using a high-performance liquid chromatography (HPLC) method with evaporative light scattering detection (ELSD) for the simultaneous determination of stachyose, sucrose, and raffinose in RF. In the current study, the orthogonal array design (OAD), which is a type of factorial design, was used to optimize the extraction method with fewer experiments, compare the content of oligosaccharides with other extraction methods, establish a stable chromatographic condition, and determine the stachyose loss in precipitation with the addition of ethanol.

METHODS AND MATERIALS

Plant material and chemicals

The *R. glutinosa* Libosch. tubers were provided by the Institute of Medicinal Plant Development in Beijing. The samples were thoroughly washed with water and cut into small slices or strips. The stachyose, sucrose, and raffinose standards (all > 98%) were purchased from Yifang Technology Inc. (Tianjin, China). AR-grade acetonitrile was purchased from J. T. Baker (USA). All other chemicals and reagents were of analytical grade.

Sample preparation

Method 1

20.0 g FR was cut into small slices or strips, which were then placed into 240 ml water with 5% ethanol and 0.01 M NaOH and heated for 2 h to reflux. The solvent was removed via filtration, and the RL residue was extracted using 200 ml of the same solvent for 2 h. The extracts were collected and combined, and their pH values were adjusted to 7.0. The combined extracts were concentrated to 32 ml via reduced pressure distillation and then precipitated with the addition of ethanol (80%, v/v) to remove the polysaccharide. The extract was kept refrigerated at 4°C overnight to allow the oligosaccharides and polysaccharides to precipitate completely. The combined extract was centrifuged for 15 min at 3,000 rpm, and the supernatant and filtrate were collected via filtration. The supernatant was concentrated under reduced pressure of below 80°C, and the polysaccharides were eluted thrice with ethanol and acetone. The two samples were freeze-dried to obtain the supernatant and precipitate.

Method 2

20.0 g FR was cut into small slices or strips, which were then placed into 240 ml water with 50% ethanol. Then, the succeeding extraction and freeze-drying steps were similar to those in method 1. The supernatant and precipitate were obtained.

Method 3

20.0 g FR was cut into small slices or strips, which were then placed into 240 ml water with 80% ethanol (Ni et al., 1992; Yang et al., 2010). Then, the remaining extraction and freeze-drying steps were similar to those in method 1. The supernatant and precipitate were obtained.

Method 4

20.0 g FR was cut into small slices or strips, which were then were placed in 240 ml of water (Ekvall et al., 2007). Then, the remaining extraction and freeze-drying steps were similar to those in method 1. The supernatant and precipitate were obtained.

Optimization of method 1

The OAD design used in the current study focused on the effects of the following three most important variables: A) ethanol concentration (5, 10 and 50%); B) pH value (10.0, 11.0, and 12.0); and C) extraction time (1, 1.5 and 2 h). The OAD $L_9(3^4)$ matrix used for the optimization and the level settings of the individual factors are shown in Table 1. The individual factors at different levels were detected and used to evaluate the efficiency and optimize the extraction method.

HPLC analysis and condition

The experiment was performed using a Waters 600 Multisolvent Delivery System with an online degasser and a Waters 2424 ELSD Detector System, both from Waters, USA. Empower® Software was used for the processing and integration of data. The oligosaccharides were separated on Waters XBridge™ Amide 3.5 µm (4.6 × 150 mm, Waters, USA), which was maintained at a temperature of 25°C. The mobile phase was acetonitrile: water (70:30, v/v) with a flow rate of 0.5 ml min^{-1}. The ELSD used nitrogen as the nebulizing gas at 30 psi pressure, and the temperature of the drift tube was fixed at 40°C. The value of gain was 300, and the spray mode was cooling.

Table 1. Factors and levels of orthogonal test.

Variable	Level		
	1	2	3
Ethanol concentration (%)	5	10	50
B PH value	10	11	12
C extraction time (h)	1	1.5	2

Table 2. L9 $(3)^3$ orthogonal test result.

No.	Ethanol concentration	B pH value	C pH value	Blank	Extraction yield (mg)
1	1	1	1	1	257.49
2	1	2	2	2	254.32
3	1	3	3	3	280.41
4	2	2	3	1	253.50
5	2	3	1	2	267.55
6	2	1	2	3	275.21
7	3	3	2	1	279.19
8	3	1	3	2	264.02
9	3	2	1	3	239.74
I	792.21	772.44	804.22		
II	796.26	747.55	808.71		
III	782.95	827.14	797.92		
k	k1 = 3	k2 = 3	k3 = 3		
I/k1	264.07	257.48	268.07		
II/k2	265.42	249.18	269.57		
III/k3	260.98	275.71	265.97		
R	4.43	26.52	3.59		

RESULTS AND DISCUSSION

Optimization of extraction method conditions

FR is a traditional Chinese medicinal plant that has been studied for many years (Kubo et al., 1996; Zhang et al., 2004; Zhang et al., 2008). In the current study, after the analysis of OAD and variance (Table 2), the pH value was found to have the most significant effect on the extraction yield among the three factors, and the order of importance that influenced extraction yield was found to be as follows: pH value (B) > extraction time (C) > ethanol concentration (A). The optimal combination parameters of the processing technology were $B_3A_1C_3$, namely, pH value of 12.0, extraction time of 2 h, and ethanol concentration of 5%.

HPLC analysis and separation

Previous studies have reported that HPLC-ELSD can determine sucrose, raffinose, and stachyose using as mobile phase methanol: acetonitrile: water (55:25:20, v/v/v) (Yin et al., 2006). In the current study, the HPLC-ELSD method had significant effects on oligosaccharide retention using the mobile phase of acetonitrile: water (70:30, v/v).

Figure 1 shows a chromatogram of the mixed standard solution of sugars (sucrose, raffinose and stachyose), each at a concentration of 0.2 mg/ml, the retention times being 10.43, 16.32, and 26.89 min for sucrose, raffinose, and stachyose, respectively. Compared with the other solvent systems, the HPLC-ELSD method had a flow rate of 0.5 ml/min. Moreover, no other interfering peaks are present near the analyte peaks, showing the simple, stable, and good resolution of the method.

Ten-microliter samples obtained using methods 1, 2, 3, and 4 were individually injected into the HPLC system at 25°C. Figure 2 shows the oligosaccharides in the supernatant and polysaccharides. The XBridge™ Amide had a good separation for sucrose, raffinose and stachyose within 30 min.

Sucrose, raffinose and stachyose were found in the supernatant, but only stachyose was found in the polysaccharides. The results indicate that the precipitation with the addition of ethanol to remove the polysaccharide has a certain influence on stachyose during precipitate production.

Table 3. Performance characteristics of the proposed method.

Analytes	Linearity range (µg)	Correlation coefficient	LOD (µg)	LOQ (µg)	Precision (R.S.D.% n = 5)	
					Intra-day	Inter-day
Sucrose	0.2 - 2.0	0.9972	0.05	0.10	2.17	2.26
raffinose	0.41 - 3.09	0.9996	0.07	0.14	1.56	2.38
Stachyose	0.41 - 4.16	0.9992	0.10	0.19	1.88	2.07

Table 4. Recovery of stachyose by spiking method.

Background (µg)	Added (µg)	Found (µg)	Average recovery (%)	R.S.D. (%)
181.18	144.94	327.15 ± 2.61	100.8	4.71
181.18	181.18	368.13 ± 2.49	103.2	4.89
181.18	217.41	394.99 ± 0.88	98.3	1.62

Method and validation

Standard calibration curves were obtained by injecting the standard solutions (2, 4, 6, 8, 10, 15, 20, and 30 µl) of sucrose (0.103 mg/ml), raffinose (0.109 mg/ml), and stachyose (0.110 mg/ml) in triplicates. The logarithm of the peak areas and those of the sucrose, raffinose, and stachyose quality were linearly correlated, as shown in Table 3. The precision of injection was demonstrated through the repetitive injection of each (0.2 mg/ml) standard solution (sucrose, raffinose and stachyose). The mean values for precision were 2.23 and 1.68% for sucrose, 1.71% for raffinose, and 1.68% for stachyose. Intra- and inter-day precision values were checked by analyzing the average amount of sugar in the supernatant of method 1. R.S.D values are shown in Table 3. The limits of quantification (LOQ, S/N = 10) and detection (LOD, S/N = 3) for sucrose, raffinose, and stachyose were experimentally calculated using serial dilution. The LODs of 0.05, 0.07, and 0.10 µg and the LOQs of 0.10, 0.14, and 0.19 µg were calculated for sucrose, raffinose, and stachyose, respectively, indicating that the employed ELSD was sensitive enough to the aforementioned sugars.

The accuracy of the method was determined by investigating the recovery of stachyose at three levels, that is, 80, 100 and 120% of the known amount (181.18 µg), by spiking it with the standard stachyose. The result of recovery shown in Table 4 was between 98.3 and 103.2%, and the average R.S.D. was below 4.89%, indicating that the method was precise, accurate, and sensitive for the quantitative determination of sucrose, raffinose and stachyose in FR.

Evaluation of the extraction method

Three sugar contents (sucrose, raffinose and stachyose) were determined in the current experiment using the HPLC-ELSD method. As shown in Figure 2, the contents of sucrose (282.11 mg/g), raffinose (167.57 mg/g), and stachyose (652.01 mg/g) were obviously highest in method 1, indicating that the extraction with a certain amount of alkali could increase the sucrose, raffinose, and stachyose contents in method 1 compared with those of the other methods (Table 5). These results can be attributed to the fact that sugar is mainly composed of polyhydroxy aldehydes and polyhydroxy ketone. Moreover, the addition of alkali in the extraction method could inhibit the enzyme activity in rehmannia extracts. Thus, the oligosaccharides, particularly stachyose, did not experience loss in extraction method 1, which has been previously studied (Ekvall et al., 2007).

The sugar contents in the polysaccharide and supernatant were compared. Sucrose and raffinose were surprisingly found to mainly exist in the supernatant, and they almost did not exist in the polysaccharide. However, as shown in Figure 2, stachyose existed in both polysaccharide and supernatant, corresponding to its ability to precipitate with the addition of ethanol, which was determined in previous studies. Thus, stachyose was lost during ethanol precipitation. In the four methods, the stachyose losses in polysaccharide and those of the supernatant were compared, obtaining 36.08, 35.29, 37.74 and 30.05% in methods 1 to 4, respectively.

In 1993, the Japanese Ministry of Health approved food with stachyose and raffinose oligosaccharides as a “specific health food” (Zdunczyk, 2004). Stachyose and raffinose are the only oligosaccharides that the U.S. Food and Drug Administration recognized as generally regarded as safe (GRAS).

Oligosaccharides, which are defined as prebiotics (Zdunczyk, 2004), have the highest content in FR, but rehmannia could not be widely used as food. The current experiment provides a stable, simple, and practical extraction method with the addition of alkali to increase the contents of oligosaccharides, particularly stachyose, in *R. glutinosa* Libosch. extracts. The stachyose content

Table 5. Contents of the sugars in the supernatant and precipitate determined using different methods.

RF (g)	Method	Sucrose (mg/g)		Raffinose (mg/g)		Stachyose (mg/g)	
		Supernatant	Precipitated	Supernatant	Precipitated	Supernatant	Precipitated
20.0	Method 1	652.01	282.11	167.57	0	8.29	0
20.0	Method 2	479.32	165.77	106.95	0	5.72	0
20.0	Method 3	437.11	155.21	80.79	0	4.09	0
20.0	Method 4	506.89	197.64	118.04	0	5.89	0

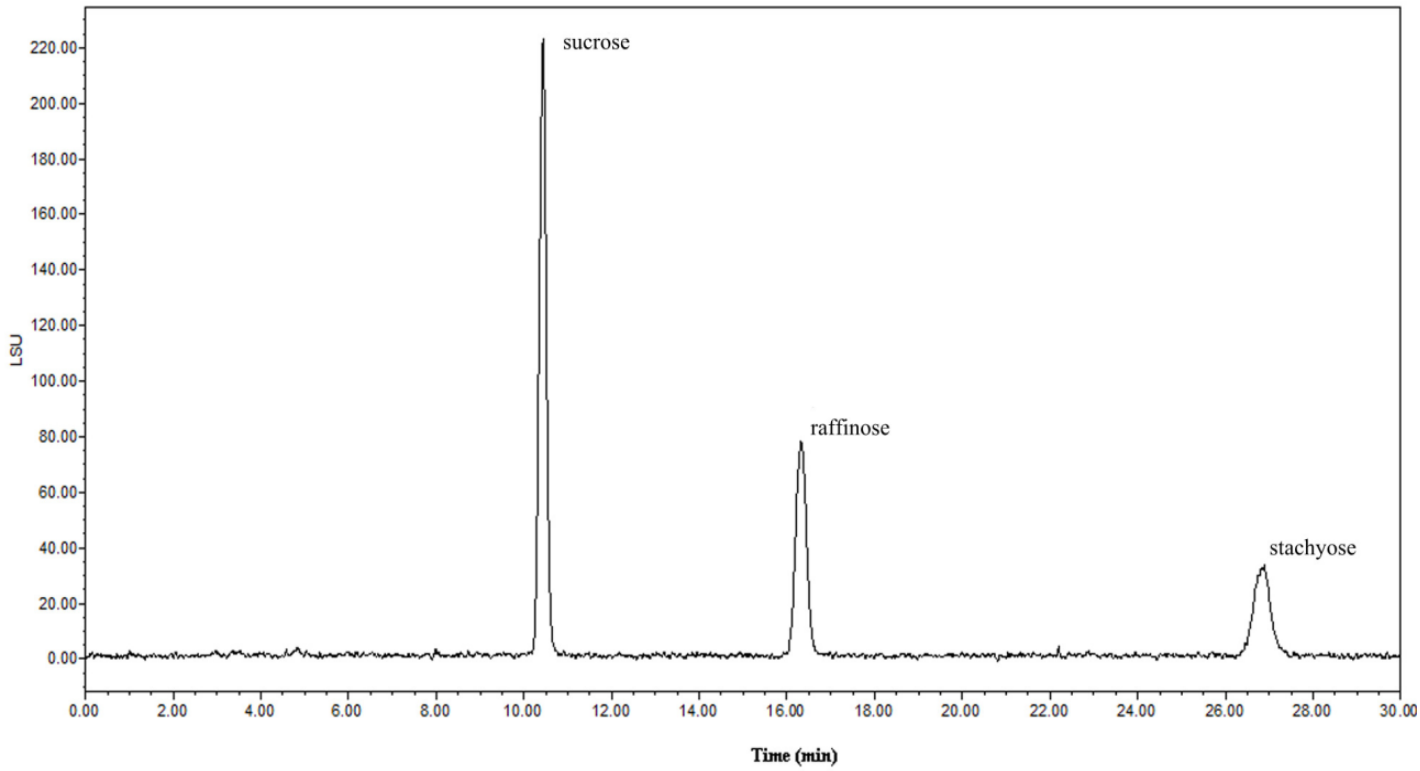

Figure 1. Chromatogram of a standard mixture of sucrose, raffinose and stachyose (0.2 mg/ml for each sugar).

in method 1 was higher than that in the other extraction methods in previous studies. Thus, method 1 provides a new basis for industrial production and purification processes in future research.

Conclusion

The proposed HPLC-ELSD method is simple, reliable, and appropriate for the simultaneous determination of sucrose, raffinose, and stachyose in FR. The extraction method with alkali completely extracts the oligosaccharides in FR and provides a basis for industrial production and purification. The stachyose content was interestingly found to decrease (approximately 30 to 40%) when precipitated with the addition of ethanol.

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ABBREVIATIONS

HPLC, High-performance liquid chromatography; **ELSD,** evaporative light scattering detection; **RL,** *Rehmannia glutinosa* Libosch; **FR,** fresh rehmannia; **OAD,** orthogonal array design.

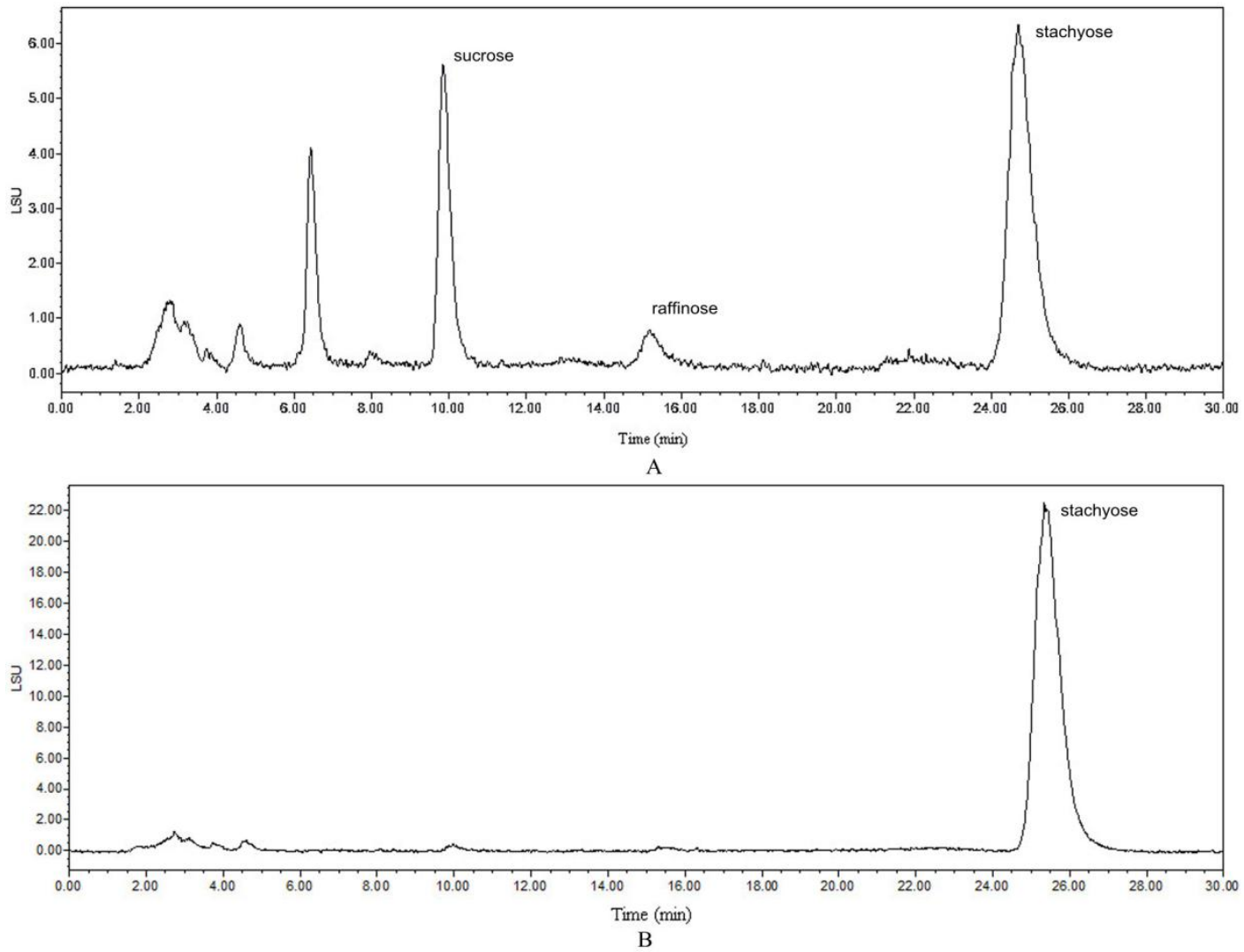

Figure 2. Chromatogram of (A) the supernatant components in the sample and (B) the precipitated components in the same sample using method 1.

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