Industrial Crops and Products

Supercritical CO_2 extraction and pre-column derivatization of polysaccharides from *Artemisia sphaerocephala* Krasch. seeds via gas chromatography

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A B S T R A C T

Optimal process conditions for supercritical CO_2 extraction of polysaccharides from *Artemisia sphaerocephala* Krasch. seeds and their monosaccharides were analyzed to provide a theoretical basis for identification and development. Single-factor experiments and orthogonal designs were performed to determine the effects of extraction conditions on polysaccharide yields. The polysaccharides were fractionated through ion-exchange chromatography, and were shown to be homogeneous via high-performance size-exclusion chromatography (HPSEC). The resultant polysaccharide samples were hydrolyzed with trifluoroacetic acid, and then acetylated and analyzed for monosaccharide composition by gas chromatography. The optimum extraction parameters were established with extraction pressure of 45 MPa, extraction temperature of 45 °C, flow rate of CO_2 at 20 L/h, extraction time of 2 h, separation pressure of 10 MPa, and separation temperature of 56 °C. The corresponding *A. sphaerocephala* Krasch. seeds polysaccharides (ASKR) yield was 18.59% (w/w). The polysaccharide contained mannose, rhamnose, galactose, glucose, arabinose, xylose, and fucose. The predominant monosaccharides in ASKR were glucose (38.48 mg/g) and xylose (16.2 mg/g). The average molecular weight of ASKR was 551.3 ± 11.2 kDa, and its radius of gyration was 22.6 ± 0.5 nm based on HPSEC–MALLS–RI system.

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1. Introduction

Artemisia sphaerocephala Krasch. (Compositae) is a perennial psammophyte subshrub species grown in desert regions in China (Guo et al., 2011, 2013). The plant is widely distributed throughout the region, and is rich in resources. Polysaccharides comprise a class of complex and large molecular structures of polymer carbohydrates that are compounded by monosaccharide polymerization. The molecular weight of polysaccharides ranges from 10 kDa to 1000 kDa (Guo et al., 2012). *A. sphaerocephala* Krasch. seed polysaccharide (ASKR) is a reducing polysaccharide extracted from its seed oil residues. ASKR possesses a medium molecular weight (Hao et al., 2010; Wang et al., 2010). Studies have confirmed that ASKR exhibits medicinal effects, including anticancer properties, antioxidant properties (Xing et al., 2009), and immunity enhancement (Wang et al., 2009; Zhang et al., 2006).

Water extraction, microwave, and ultrasonic methods have been used to extract ASKR (Xing et al., 2009; Zhang et al., 2012). However, the traditional methods are time consuming, cause undesirable decomposition of ASKR contents, and possess low extraction efficiency. Supercritical extraction determines the special properties of a substance near the critical point (Chen et al., 2010; Jin and Tong, 2014). Appropriate entrainers or modifying agents (e.g., methanol and ethanol) are used in supercritical CO_2 systems to improve the extraction efficiency of strong-polarity and large-molecule materials, as well as to increase the probability of polysaccharide extraction (del Valle et al., 2008; Diaz-Reinoso et al., 2006). Previous studies have used supercritical CO_2 extraction for specific plant polysaccharides (Diaz-Reinoso et al., 2006; Pradhan et al., 2010; Raeissi et al., 2008). However, data on ASKR extraction remain limited (Guo et al., 2013; Wang et al., 2010).

In the current research, we used supercritical CO_2 to extract ASKR through initial hydrolysis with trifluoroacetic acid. The substance was derivatized with acetic anhydride, gas chromatograph with capillary column, and external standard method to detect the physical properties of hydrolyzed ASKR. We also provided details for efficient ASKR extraction.

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2. Materials and methods

2.1. Materials and reagents

A. sphaerocephala Krasch. seeds were collected in Ningxia, China. The seeds were air dried, sealed in plastic bags, and stored in the refrigerator at 4 °C. CO_2 (food grade, purity >99%), methanol, ethanol, methylene chloride, dimethyl sulfoxide, sulfuric acid, glucose, 1-methyl imidazole, anthrone, acetic anhydride, diethylaminoethanol (DEAE)–Sepharose fast flow ion exchange resin, and trifluoroacetic acid were purchased from Beijing Chemical Reagent Co. (Beijing, China). Standard monosaccharides (L-rhamnose, D-galactose, D-arabinose, D-mannose, D-fucose, xylose, and glucose) were purchased from Sigma–Aldrich Co. (USA). All other reagents were of analytical grade. Deionized water was used throughout the experiment.

2.2. Extraction and purification of ASKR

Two hundred grams of ASKR was placed into an extraction kettle. The process parameters of supercritical CO_2 extraction were set; cooling and kettle-heating devices were opened. The main pressure pump was opened after the temperature reached the desired value. CO_2 flow and extraction time were 20 L/h and 2 h, respectively. The pressure and temperature of kettle II were 4 MPa and 36 °C, respectively. Influential factors of ASKR yield included extraction pressure, extraction temperature, as well as pressure and temperature of kettle I. The extracted ASKR was collected from a recycled bottle. Chloroform and n-butanol (ratio, 4:1) were added to the polysaccharide. The ASKR-containing bottle was fully shaken for 30 min and centrifuged at 5000 rpm for 20 min. The protein was replaced 10 times from the upper water phase. The dialysis supernatant was placed into a fluid bag; dialysis was performed using tap and distilled water for 48 and 24 h, respectively. The solution was concentrated and freeze-dried under vacuum to obtain crude ASKR (Guthalugu et al., 2006; Nascimento da Fonseca Contado et al., 2010).

Crude ASKR was dissolved and added to the DEAE–Sepharose fast flow ion exchange resin. The polysaccharide solution was eluted with distilled water and different concentrations of NaCl solution at a flow rate of 1.5 mL/min. The eluent was collected (30 min/pipe), and detection was performed using sulfuric acid phenol. Approximately 0.2 mL of the sample solution was drawn and distilled up to 2.0 mL. Then, 1.0 mL of 6% phenol, as well as 1.0 and 5.0 mL of concentrated sulfuric acid, was added. The resulting mixture was shaken and cooled for 20 min and then left to stand at room temperature. The absorbance was then measured at 490 nm, and the polysaccharide content was calculated based on the glucose standard curve. Similar flow points were merged, and the polysaccharide solution was concentrated. Dialysis was then performed on the solution. Crude ASKR solution was freeze-dried under vacuum to yield various polysaccharide fractions (Wang et al., 2010).

2.3. Determination of ASKR content

2.3.1. Glucose standard curve scheme

Seven large tubes were respectively added with 0, 0.1, 0.2, 0.3, 0.4, 0.6, and 0.8 mL glucose standard solutions (100 μg/mL) and filled with 1 mL distilled water.

Four milliliters of anthrone reagent (0.2 g anthrone dissolved in 100 mL sulfuric acid) was immediately placed into each tube. The glass tubes were then soaked in ice water for rapid cooling. The tubes containing anthrone solution were immersed in boiling water for 10 min; nozzle and reflux funnel were used to prevent evaporation. The solution was cooled with flowing water and placed at room temperature for 10 min. The absorbance at 620 nm was determined, and the standard curve was obtained (Chen et al., 2012).

2.3.2. Determination of soluble polysaccharide content

Fifty milliliters of ASKR was dissolved in 5 mL of 30% ethanol solution; the polysaccharide solution was diluted 100 times. One milliliter of the diluent was taken from the polysaccharide solution, and 4.0 mL anthrone reagent was immediately added into each tube. The tubes were soaked in ice water for rapid cooling. The tubes containing anthrone solution were immersed in boiling water for 10 min; evaporation was inhibited by using a nozzle and reflux funnel. The solution was cooled with flowing water and placed at room temperature for 10 min. Subsequently, the absorbance at 620 nm was determined. The standard curve was used to calculate polysaccharide concentration (C). The polysaccharide content in ASKR extract (Liu et al., 2012; Xin et al., 2012) was calculated as follows:

$$polysaccharide \;\; content = \frac{C(\mu g/mL) \times 5\,mL \times 5 \times 100 \times 10^{-6}\,g/\mu g}{0.05\,g} \times 100$$

2.4. Homogeneity of ASKR and determination of average molecular weight

Polysaccharide fractions were filtered with soluble cellulose acetate membrane (3.0 μm). High-performance size-exclusion chromatography–multi-angle laser light scattering–differential detector coupled system (HPSEC–MALLS–RI) was used, and comprised three chromatographic column series: TSK G5000 PW (7.5 mm × 600 mm), TSK G3000 PWxl (7.8 mm × 300 mm), and TSK G2500PWxl (7.8 mm × 300 mm). Mobile phases included 0.15 mol/L $NaNO_3$ and 0.02% NaN_3; the velocity was 0.4 mL/min. The average molecular weight of the polysaccharide was based on data from MALLS and RI detectors; the molecular weight was directly calculated by ASTRA 5.3 software (Gengec and Kobya, 2013; Maina et al., 2014).

2.5. Monosaccharide characteristics in ASKR prior to column derivatization via gas chromatography (GC)

2.5.1. Preparation of ASKR hydrolysis solution

Fifty milliliters of ASKR was added in the test tube containing 2 mL of 4 mol/L trifluoroacetic acid. The solution was hydrolyzed at 120 °C for 2 h, and the test tube was cooled to room temperature. Anhydrous methanol was added for distillation. Trifluoroacetic acid was removed and yielded the ASKR hydrolysis solution. Each sample underwent the process three times.

2.5.2. Preparation of standard monosaccharide mixture

Ten milliliters of monosaccharide standards (L-rhamnose, D-galactose, D-arabinose, D-mannose, D-fucose, xylose, and glucose) were used. The solution was mixed and dissolved in 10 mL volumetric flask; the mixture was diluted with water to 5.00, 3.00, 2.00, 1.50, 1.00, 0.50, and 0.25 mg/mL (Xin et al., 2012).

2.5.3. Column derivatization

Ten microliters of monosaccharide standard mixture and polysaccharide hydrolysis solution were taken; two milliliters of sodium borohydride solution was added in the mixture and oscillated at 42 °C for 90 min. The solution was cooled to room temperature. About 0.5 mL of acetic acid, 50 μL of 1-methyl imidazole, and 1 mL of acetic anhydride were added and allowed to react in the solution at room temperature for 10 min. Eight milliliters of water was added to the system and cooled to room temperature. Four milliliters of methylene chloride methane was then added to the system and oscillated. The upper water phase was discarded, and
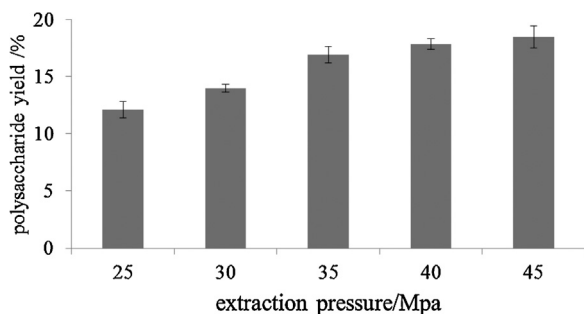
Fig. 1. Effects of extraction pressure on ASKR yield.
Note: Measurements for each treatment were performed in triplicate.

Fig. 2. Effects of extraction temperature on ASKR yield.
Note: Measurements for each treatment were performed in triplicate.

methylene chloride was incorporated in the system used for distillation. The residual solution (0.5 mL) was obtained, 1 μL of which was drawn for GC (Xin et al., 2012).

2.5.4. GC conditions

The chromatographic column included Rtx-5 fine quartz wool column (30 m × 0.25 mm, 25 μm). The temperatures of the program were set as follows: 80–180 °C at a rate of 20 °C/min and 180–192 °C at a rate of 1 °C/min for 5 min; 192–240 °C at 5 °C/min and 280 °C at 30 °C/min for 5 min. The temperatures of FID detector and injection port were 300 and 280 °C, respectively. The flow rate of H_2 and N_2 was 30 mL/min. The air velocity was 300 mL/min, the split ratio was 20:1, and the volume of the sample quantity was 1 μL (Liu et al., 2012).

2.6. Statistical analysis

Final data were expressed as mean ± standard deviation ($\bar{x} \pm s$). ANOVA was employed to assess the differences among groups. Statistical analysis was performed using SPSS 21.0. Significant differences were denoted by $p < 0.05$.

3. Results and discussion

3.1. Supercritical CO_2 for ASKR extraction

The regression curve for glucose concentration and absorbance was given as $y = 30.065 + 0.0872x$ (x, absorbance; y, glucose standard solution concentration in μg/mL). The correlation coefficient R^2 was 0.9992. Standard glucose exhibited good linearity from 8 μg to 24 μg based on the experiments.

3.2. Single-factor tests

3.2.1. Effects of pressure on the yield of extracted polysaccharide

Fig. 1 shows the effect of extraction pressure on polysaccharide yield. The following parameters were recorded: extraction temperature, 45 °C; separation pressure of kettle I, 10 MPa; and separation temperature of kettle I, 56 °C. Polysaccharide yield increased with extraction pressure and reached its maximum when the extraction pressure was 40 MPa. The extraction pressure is important to efficiently obtain the supercritical fluid. Density of the supercritical fluid increases with pressure, and its solubility improves given the same temperature. Increased pressure reduces the mass transfer resistance, which is beneficial in extracting the target components (Diaz-Reinoso et al., 2006). In this study, the compressibility of the supercritical fluid was reduced, and the polysaccharide yield slowly increased when the pressure increased to a certain degree. We selected the pressure of 40 MPa for further research based on equipment costs and safety factors (the maximum pressure of the equipment, which was set as 45 MPa).
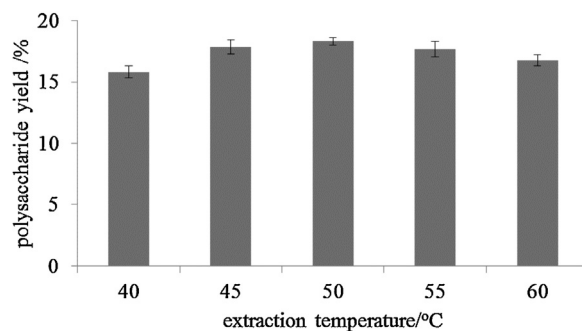
3.2.2. Effects of extraction temperature on polysaccharide yield

Fig. 2 reveals the effects of temperature on polysaccharide yield, in which the extraction pressure was 40 MPa, and the separation pressure and temperature of kettle I were 10 MPa and 56 °C, respectively. Maximum ASKR yield was obtained at 50 °C; the influence of temperature on the density of the supercritical fluid was complex. Solvent volatility and diffusion coefficient increased with temperature. Furthermore, the solubility of the supercritical fluid and extraction effects increased. Temperature reduced the fluid density and retained the dissolving capacity and extraction effects (Chen et al., 2010). Given the results, we chose 45 °C for subsequent research.

3.2.3. Influence of kettle I pressure on polysaccharide yield

The effects of separation pressure of kettle I on polysaccharide yield are depicted in Fig. 3. The following parameters were also recorded: extraction temperature, 45 °C; extraction pressure, 40 MPa; and separation temperature of kettle I, 56 °C. Maximum polysaccharide yield was obtained when the pressure of kettle I was 9 MPa. The appropriate separation pressure of the reactor was beneficial for polysaccharide precipitation and complete separation of impurities. Thus, we selected the separation pressure of kettle I to be 9 MPa for further research.

3.2.4. Influence of the separation temperature of kettle I on polysaccharide yield

Fig. 4 depicts the effects of the separation temperature of kettle I on polysaccharide yield given the corresponding extraction temperature, extraction pressure, and separation pressure of kettle I of 45 °C, 40 MPa, and 10 MPa. Maximum polysaccharide yield was obtained at 56 °C. CO_2 solubility decreased with temperature at 9 MPa, which promoted polysaccharide precipitation. However, the impurities in high-temperature grease affected the polysaccharide quality; thus, the optimum temperature for separation in kettle I was 56 °C.
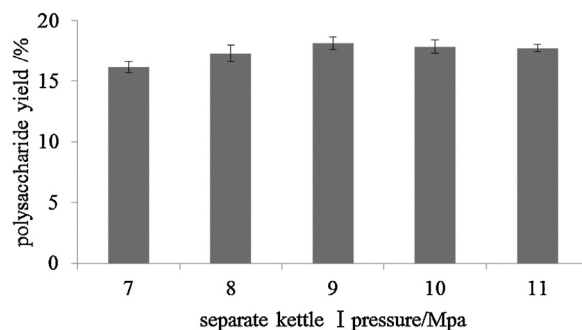

Fig. 3. Effects of the separation pressure of kettle I on ASKR yield.
Note: Measurements for each treatment were performed in triplicate.

Table 1
Orthogonal array design matrix and experimental results.

Test no.	Factors				
	A Extraction pressure (MPa)	B Extraction temperature (°C)	C Kettle I pressure (MPa)	D Kettle I temperature (°C)	Polysaccharide yield
1	35	45	8	52	17.81
2	35	50	9	56	17.64
3	35	55	10	60	17.52
4	40	45	9	60	18.51
5	40	50	10	52	18.41
6	40	55	8	56	18.35
7	45	45	10	56	18.57
8	45	50	8	60	18.37
9	45	55	9	52	18.19
K_1	17.66	18.30	18.18	18.14	
K_2	18.42	18.14	18.11	18.19	
K_3	18.38	18.02	18.17	18.13	
R	0.77	0.28	0.07	0.06	

3.3. Orthogonal experiment results

L_9 (3^4) orthogonal table was selected according to the results of single-factor experiments (Guthalugu et al., 2006). Table 1 illustrates the corresponding results and analyses. The factors that influence polysaccharide yield followed the order extraction pressure > extraction temperature > kettle I temperature > separation kettle I pressure. The polysaccharide yield was the index and the optimum combination was $A_3B_1C_3D_1$ based on the following k factors: extraction pressure, 45 MPa; extraction temperature, 45 °C; separation pressure of kettle I, 10 MPa; and separation temperature of kettle I, 56 °C. Under optimal conditions, the polysaccharide yield of ASKR was found to be 18.59%.

The F values of extraction pressure were greater than the critical value ($p < 0.05$) based on ANOVA with polysaccharide yield as the index. Thus, extraction pressure significantly affected the results. The F of the extraction temperature, separation pressure and temperature of kettle I were less than the critical F ($p < 0.05$); the parameters yielded little effects on the results. Pressure and temperature, which mainly influenced the product distribution in extraction kettles, varied between the kettles. Water, polysaccharide, impurities, and other main extraction parameters were obtained from kettle II when kettle I exhibited high pressure.

3.4. Separation and purification of ASKR

Alcohol sink and sevag methods were used to remove the protein and obtain crude polysaccharide during supercritical CO_2 extraction of *A. sphaerocephala* Krasch. seeds. In addition, DEAE–Sepharose fast flow ion exchange column was utilized for purification. The procedure possesses an ion-exchange function and exhibits a rapid elution rate and good effects on the molecular
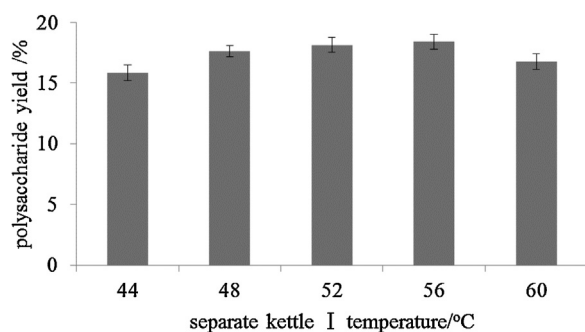
Fig. 4. Effects of the separation temperature of kettle I on ASKR yield. *Note:* Measurements for each treatment were performed in triplicate.
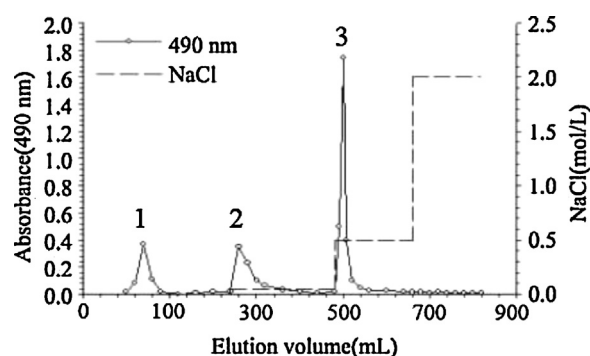
Fig. 5. Elution patterns of polysaccharides from *A. sphaerocephala* Krasch. seeds on DEAE–Sepharose fast flow chromatography.

sieve. Fig. 5 shows the elution curve of ASKR. The crude polysaccharide was separated into three components, and the eluent was condensed. Dialysis was performed on the solution, which was then freeze-dried in vacuum to obtain the flocculent polysaccharides, namely, ASKR-1, ASKR-2, and ASKR-3. ASKR-1 and ASKR-2 possessed minimal amounts, and ASKR-3 was the primary component. Thus, we selected ASKR-3 to analyze the substructure.

3.5. Determination of ASKR-3 homogeneity and molecular weight

Polysaccharide purity cannot be measured using the standards of the purity of small-molecule compounds because polysaccharides are biological macromolecules that exist in microscopic heterogeneity. The homogeneity and molecular weight of *A. sphaerocephala* Krasch. seed polysaccharide ASKR-3 were analyzed through HPSEC. ASKR-3 yielded a single peak on the symmetrical HPSEC from RI chromatograms (Fig. 6). The result suggested that ASKR-3 was a uniform polysaccharide component. The average molecular weight of ASKR was 551.3 ± 11.2 kDa, and the radius of gyration was 22.6 ± 0.5 nm based on MALLS. The molecular

Table 2
Regression equations for seven monosaccharides.

Monosaccharide	Linear equation	Correlation coefficient (R^2)
Mannose	$y = 4.41853x - 2.91526$	0.9936
Glucose	$y = 5.30675x - 3.38203$	0.9933
Galactose	$y = 7.77211x - 5.03253$	0.9942
Rhamnose	$y = 5.43322x - 2.33986$	0.9941
Arabinose	$y = 6.40882x - 1.79609$	0.9916
Xylose	$y = 2.11232x - 0.76912$	0.9957
Fucose	$y = 3.39304x - 1.30368$	0.9961

Table 3
Monosaccharide composition of ASKR.

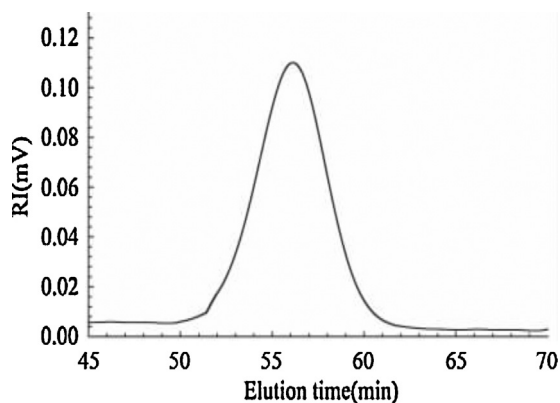| Species		Mannose	Glucose	Galactose	Rhamnose	Arabinose	Xylose	Fucose
ASKR	Monosaccharide content (mg/g)	10.8	16.2	9.86	8.78	8.48	38.48	10.09
	Relative content/%	10.490	16.090	9.55	8.518	8.243	37.301	9.802

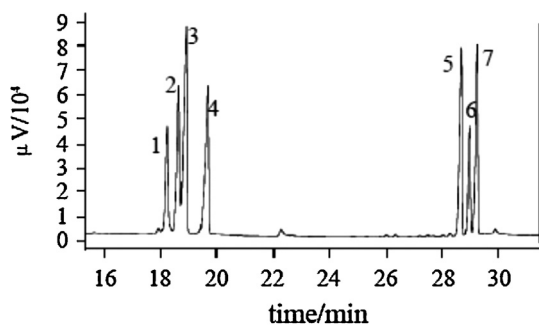**Fig. 6.** HPSEC chromatograms of ASKR-3 based on RI detector.

Fig. 7. GC chromatogram of the mixture of seven monosaccharide standards. *Note:* 1. mannose; 2. glucose; 3. galactose; 4. rhamnose; 5. arabinose; 6. xylose; 7. fucose.

weight between polysaccharides extracted from different *A. sphaerocephala* Krasch. seeds were relatively large and ranged from 30 kDa to 600 kDa. The large differences in molecular weights were caused by the different types of *A. sphaerocephala* Krasch. and their different growth cycles (Wang et al., 2009; Zhang et al., 2012).

3.6. Quantitative linear relationships of the external standard for seven monosaccharides

The reference substance concentration (abscissa) was plotted against the chromatography peak area (ordinate) of the standard curve (Fig. 7). Table 2 presents the linear equations and the corresponding correlation coefficients (R^2) of seven monosaccharide standard derivatives. Experiments revealed that the monosaccharides exhibited good linear relationships between 0.25 and 5.00 mg/mL in accordance to the quantitative requirement. Fig. 7 shows the GC chromatography results for seven monosaccharides.

Five measurements were performed for each treatment based on chromatographic conditions. Values of area RSD and retention time for mannose (3.660%, 0.016%), rhamnose (4.399%, 0.034%), galactose (4.600%, 0.050%), glucose (3.827%, 0.045%), arabinose (3.635%, 0.027%), xylose (3.991%, 0.022%), and fucose (2.041%, 0.036%) were obtained.
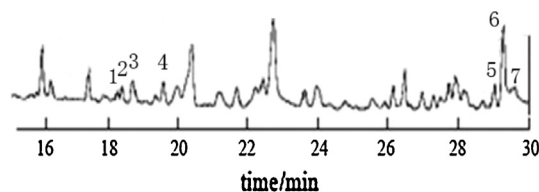

Fig. 8. GC chromatograms of monosaccharides from ASKR polysaccharide. *Note:* 1. mannose; 2. glucose; 3. galactose; 4. rhamnose; 5. arabinose; 6. xylose; 7. fucose.

3.7. Characteristics of monosaccharides in ASKR

Fig. 8 shows the derivative chromatogram of the acetylated ASKR polysaccharide based on gas chromatograph. Hydrolysis results for ASKR included the monosaccharides (rhamnose, galactose, arabinose, mannose, fucose, xylose, and glucose). Some unknown components also appeared, results revealed that these unknown components were not carbohydrates, but rather may be parts of the residual proteins that remained after purification, hydrolysis, and derivatization. These residues may have generated signals in the gas chromatography spectra, but they exhibited insignificant effects on monosaccharide content in the polysaccharide.

Table 3 shows the monosaccharide composition of ASKR, which mainly included rhamnose, galactose, arabinose, fucose, and glucose. Small amounts of xylose and mannose were also observed, and the total monosaccharide content reached 102.69 mg/g. Glucose and galactose contents were high (58%).

4. Conclusions

A. sphaerocephala Krasch. ASKR was extracted via supercritical CO_2 under optimal extraction conditions. The extraction pressure, extraction temperature, CO_2 flow rate, extraction time, separation pressure, and separation temperature were 45 MPa, 45 °C, 20 L/h, 2 h, 10 MPa, and 56 °C, respectively. The ASKR yield was 18.59% from purification via ion-exchange column chromatography. HPSEC results indicated that ASKR-3 was a homogeneous component that comprised glucose (16.20 mg/g) and xylose (38.48 mg/g). The average molecular weight and radius of gyration of ASKR were 551.3 ± 11.2 kDa and 22.6 ± 0.5 nm, respectively.

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