Multicomponent quantification of *Astragalus* residue fermentation liquor using ion chromatography-integrated pulsed amperometric detection

YING ZHANG[1], JIARONG WU[1], QUANHUI NI[1] and HONG DONG[2]

[1]School of Chinese Herbal Medicine, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong 510006; [2]Beijing Key Laboratory of Traditional Chinese Veterinary Medicine, Beijing University of Agriculture, Beijing 102206, P.R. China

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Abstract. Chinese medicine residues contain abundant cellulose and hemicellulose, which are potential renewable carbon sources for ethanol production. The aim of the present study was to develop a rapid and reliable method to evaluate the cellulose and hemicellulose utilization in Chinese medicine residues. In the present study, key hydrolysates (arabinose, galactose, glucose, xylose, and cellobiose) of the cellulose and hemicellulose in fermentation liquor of *Astragalus* residues were simultaneously quantified by ion chromatography using an integrated pulsed amperometric detector (IPAD). HPLC analysis was performed on a Dionex ICS-2500 equipped with GP50 gradient pump and ED50 IPAD. The working and reference electrodes were gold electrode and Ag/AgCl electrode, respectively. Separation was achieved on serial no. 002627 Dionex Analytical column (2x250 mm). Sodium hydroxide of 250 mM and water were used as the mobile phase with a flow rate of 0.2 ml/min. The temperature of column was kept at 30˚C. This method was validated for accuracy and precision. The regression equation revealed a good linear relationship (R^2=0.9959-0.9984) within the test ranges. The limits of detection and quantification for five standard analytes (arabinose, galactose, glucose, xylose and cellobiose) were in the range of 0.067-0.091 and 0.08-0.23 mg/l, respectively. The method showed good reproducibility for the quantification of five analytes in fermentation liquor of *Astragalus* residue with intra-and inter-day variations less than 3.843%.

Correspondence to: Dr Ying Zhang, School of Chinese Herbal Medicine, Guangzhou University of Chinese Medicine, 232 Waihuan Road, Guangzhou, Guangdong 510006, P.R. China
E-mail: tjxyzyzy@hotmail.com

Dr Hong Dong, Beijing Key Laboratory of Traditional Chinese Veterinary Medicine, Beijing University of Agriculture, Beijing 102206, P.R. China
E-mail: donghong523@163.com

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Introduction

Cellulosic ethanol generated from lignocellulosic biomass such as agricultural residues has been recognized as one of the most sustainable biofuels for transportation (1,2). Chinese medicine residues, which have been treated at high temperature, contain cellulose, hemicellulose, lignin, fat, protein, and polysaccharide (3,4). It is a very promising biomass material for its soft texture and large production.

Cellulose and hemicelluloses are the substrates for ethanol production (5-8). In fact, cellulose and hemicelluloses are first hydrolyzed to yield arabinose, galactose, glucose, xylose, and cellobiose. Then, glucose and xylose are fermented to produce ethanol. The traditional strain used to produce ethanol is *Saccharomyces cerevisiae* (*S. cerevisiae*). Due to *S. cerevisiae* not having a specialized xylose transport system, it utilizes xylose only after glucose is depleted in the fermentation broth and then xylose is transferred by the hexose transport system (9,10). For the sake of simultaneously converting glucose and xylose to ethanol, we used a fusant, obtained by protoplast fusion, combining *Pichia stipitis* with *S. cerevisiae*. Thus, in the process of simultaneous saccharification and co-fermentation (SSCF), a variety of sugars and monosaccharides can be produced. With the purpose of valuation of cellulose and hemicellulose utilization, it is necessary to develop an accurate analysis method for the simultaneous determination of these compounds.

Several detectors coupled with chromatographic systems have been used to quantify sugars. Common detection methods include mass spectrometry (MS) (11,12), refractive index detection (RID) (13,14), evaporative light scattering detection (ELSD) (15-17), charged aerosol detection (CAD) (18,19), and integrated pulsed amperometric detector (IPAD) (20). Ligand-exchange and cation-exchange chromatography with RID (21), and high-performance anion-exchange chromatography with IPAD (HPAEC-IPAD) (20,22) have been successfully applied to monitor carbohydrates.

HPAEC-IPAD enables the rapid monitoring of monomeric and dimeric sugars in the aqueous extracts and hydrolysates of biomass. The pKa of carbohydrates ranges from 12 to 14 (23). Carbohydrates exist in the forms of negative ion in strong

alkaline neurogen. Therefore, they can be separated by the IEC (24). Since carbohydrate is separated in strong alkaline neurogen, its detection methods should be applicable to the alkaline conditions. IPAD is technologically suitable. More importantly, derivatization reaction and sample purification are not needed. Moreover, IPAD can precisely detect the quantity of carbohydrate from pmol to fmol. Pulsed amperometric detection is suitable for trace component analysis and it is a universal and non-specific method with high sensitivity (24-26). The method can provide a stable baseline even with steep gradients. Pulse amperometric detection with the larger response signal is suitable for the quantification of sugars.

In our study, the strain was a fusant. SSCF was used. Chinese medicine residue was used as the liquid fermentation substrate. Products and intermediate products are more complex because the technological line is long, the strain is unique and the substrate composition is complicated. We tried to use ELSD, RID to determine monosaccharides and disaccharides but the results were not ideal. Finally, a simple, rapid, and reliable IEC method combined with IPAD was developed for the simultaneous determination of arabinose, galactose, glucose, xylose, and cellobiose. The method facilitated the rapid analysis of sugars and degradation products in biomass degradation products.

Materials and methods

Materials and reagents. Sodium hydroxide stock solution (50%) was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA) (B0921993 334 UM824). Ultrapure water prepared with Milli-Q was used in the experiment. *Astragalus* residues were collected from Chinese medicine factory. Xylanase and cellulase were purchased from Xiangbo Biological Technology Co., Ltd. (Guangzhou, China). HPLC-grade arabinose, galactose, glucose, xylose, and cellobiose (purity >99%) were purchased from Sigma-Aldrich (Shanghai, China). The test strain was a fusant prepared by protoplast fusion, combining *S. cerevisiae* with *Pichia stipitis*.

Preparation of standard solution. Stock solutions of arabinose (2.5 mg/l), galactose (2.5 mg/l), glucose (7.5 mg/l), xylose (2.5 mg/l), and cellobiose (2.0 mg/l) were prepared in ultrapure water. The working standard solutions were prepared as required by appropriate dilution of stock solutions with ultrapure water. Standard solutions sugar (arabinose, galactose, and xylose) were prepared daily according to various concentrations (0.1, 0.25, 0.5, 1.0 and 1.5 mg/l), and standard glucose solutions of different concentrations (0.3, 0.75, 1.5, 3.0 and 4.5 mg/l) were prepared. The concentrations of standard cellobiose solutions were, respectively, 0.08, 0.75, 1.5, 3 and 4.5 mg/l.

Sample preparation. *Astragalus* residue was first pretreated with 1% sulphuric acid for 2 h at 120˚C. Then 0.3% yeast extracts, 0.25 g/l $(NH_4)_2HPO_4$, and 0.025 g/l $MgSO_4 \cdot 7H_2O$ was added into the fermentation medium and pH was adjusted to 4.8 with sodium hydroxide solution. Two-step SSCF technology was employed (27,28). Xylanase was added to perform 8 h prehydrolysis. Strain and a small percentage of cellulose were then added into the hydrolyzate to carry out SSCF (step one) for 48 h. After 48 h, the remaining cellulose was fed to release glucose from glucan and subsequently fermented to generate ethanol with the fusant (Fig. 1).

Table I. HPLC gradient elution program.

Time (min)	250 mM NaOH (%)
0-15	4
15-20	4-30
20-35	30

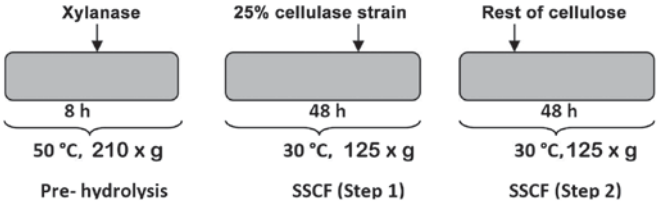

Figure 1. Schematic diagram of two-step simultaneous saccharification and co-fermentation (SSCF).

The fermentation liquor was centrifuged at 10,000 x g for 10 min. Then the supernatant was diluted 5,000-fold and filtered through 0.45 μm Millipore filter. The filtrate was collected as the sample solution.

HPAEC-IPAD instrumentation and chromatographic conditions. HPLC analysis was performed on a Dionex ICS-2500 equipped with GP50 gradient pump and ED50 IPAD. Working and reference electrodes were, respectively, gold and silver electrodes. Separation was achieved on serial no. 002627 Dionex Analytical column (2x250 mm) (Dionex, Sunnyvale, CA, USA). The mobile phase consisted of 250 mM sodium hydroxide and water. The elution program is shown in Table I. Each elution run was completed within 35 min. The rate of the flow was 0.2 ml/min and an aliquot of 25 μl of sample solution was injected into the HPAEC-IPAD system. The column temperature was set to 30˚C for separation and determination.

Calibration curves and limits of detection and quantification. First, 25 μl of each solution was injected in duplicates to construct calibration curves. The limits of detection (LOD) and quantification (LOQ) were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. The LOD and LOQ values were experimentally verified by injecting standard solutions of the compounds at the LOD and LOQ concentrations.

Accuracy. The recovery test was used to evaluate the detection accuracy. In the recovery experiments, the selected samples were also spiked with known amounts of arabinose, galactose, glucose, xylose, and cellobiose (0.1 mg/l), and then analyzed according to the chromatographic conditions described above. The average recoveries were calculated as: Recovery (%) = (the observed amount - original amount)/the spiked amount x 100%.

Table II. Regression equation and LOD and LOQ for arabinose, galactose, glucose, xylose, and cellobiose.

Compounds	Retention time (min)	Regression equation	Linearity range (mg/l)	R^2	LOD (mg/l), S/N=3	LOQ (mg/l), S/N=10
Arabinose	7.269	Y=10.998X-0.0866	0.10-1.50	0.9959	0.067	0.10
Galactose	9.085	Y=10.701X-0.0297	0.10-1.50	0.9984	0.082	0.10
Glucose	9.852	Y=12.929X-0.2877	0.30-4.50	0.9979	0.074	0.23
Xylose	11.009	Y=10.669X-0.0978	0.10-1.50	0.9977	0.091	0.10
Cellobiose	29.469	Y=7.3485X-0.0588	0.08-1.20	0.9971	0.080	0.08

LOD, limits of detection; LOQ, limits of quanitification; S/N, signal-to-noise ratio.

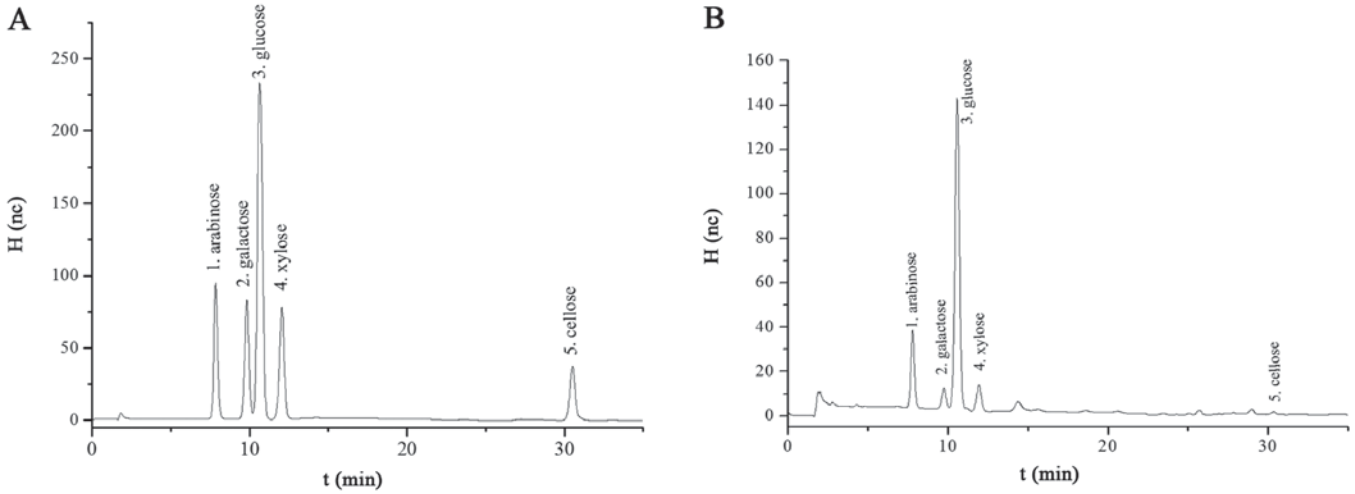

Figure 2. IEC-PAD chromatogram of arabinose, galactose, glucose, xylose, and cellobiose. (A) Standard solution and (B) sample. Calibration curves were plotted with LC Chameleon software. The regression equations, R^2, RSD, retention time, limits of detection (LOD) and quantification (LOQ) of the five compounds are listed in Table II. All calibration curves showed good linear regressions in the range (R^2=0.9959-0.9984). The LODs (S/N=3) and LOQs (S/N=10) for sugars were within the ranges of 0.067-0.091 and 0.19-0.25 mg/l, respectively).

Precision. In 3 consecutive days, the intra- and inter-day precisions were determined through five injections of the sample solution. Additionally, the reproducibility and repeatability of the advanced method were determined by measuring the retention time and peak areas.

Results and Discussion

The chromatogram by HPAEC-IPAD for the standard compounds and sample is shown in Fig. 2, and Table II. The results demonstrated that the five compounds were well separated by the serial no. 002627 Dionex analytical column. From this point of view, the quantitative determinations of arabinose, galactose, glucose, xylose, and cellobiose in the fermentation liquor of *Astragalus* residue were feasible.

Table III gives the recovery and precision data for five compounds. Recovery experiments were performed to determine the detection accuracy of the method. The samples were analyzed before and after the addition of known amounts of arabinose, galactose, glucose, xylose, and cellobiose. The recoveries ranged from 97.83 to 102.05%. The intra- and inter-day %RSD of retention time and peak areas have low values (<3.843%). Therefore, the developed IEC-PAD showed a high precision, accuracy, and sensitivity for the simultaneous quantitative evaluation of arabinose, galactose, glucose, xylose, and cellobiose in fermentation liquor of *Astragalus* residues.

The HPAEC-IPAD method was applied to analyze arabinose, galactose, glucose, xylose, and cellobiose (Table IV). Glucose concentration (17.57 g/l) in fermentation liquor was the highest, indicating that glucose utilization rate was not high. It was then necessary to study the way to improve the utilization of glucose. The following step was based on the process optimization to improve the utilization of glucose and increase the ethanol yield.

The ion chromatography (IC) pulse ampere detection method has a high detection sensitivity to sugar and reaches the level in μg/l. The method is suitable for the detection of compounds with a low concentration. If the sample concentration was higher, REDOX reaction may not have been fully completed; thus leading to a lower measurement value than the actual value. As a result, it is necessary to dilute the sample to the suitable concentration. In our experiments, the supernatant was diluted 5,000-fold and the concentration was in the range of the standard curve.

When the mobile phase was 250 mm NaOH: Water = 4:96%, monosaccharide components can be completely removed.

Table III. Precision data of retention time and peak areas.

	Intra-day precision (n=3, mean), %RSD									Inter-day precision (n=9, mean), %RSD			
	Day 1			Day 2			Day 3						
Compounds	t_R[a]	P_A[b]	H_C[c]	t_R	P_A	H_C	t_R	P_A	H_C	t_R	P_A	H_C	Recovery (%)
Arabinose	0.780	0.967	2.386	1.697	2.135	3.242	0.865	0.122	1.975	1.895	1.427	3.090	97.83
Galactose	0.886	0.555	1.492	1.505	2.961	2.712	0.809	0.633	2.258	2.043	2.351	2.713	99.06
Glucose	0.801	0.301	0.270	1.424	0.373	0.982	0.877	0.699	1.082	1.906	0.643	1.766	98.47
Xylose	0.785	1.105	1.229	1.718	0.691	0.671	0.840	1.609	0.483	1.359	1.265	1.401	99.49
Cellobiose	0.362	0.806	1.215	0.383	2.600	2.313	0.165	2.630	3.233	0.895	2.639	3.843	102.05

[a]%RSD of retention time; [b]%RSD of peak area; [c]%RSD of hydrolysate concentration.

Table IV. Results of HPAEC-IPAD.

Compounds	Arabinose	Galactose	Glucose	Xylose	Cellobiose
Concentrations (g/l)	4.19	1.33	17.57	0.81	0.035

However, cellobiose was retained in the chromatographic column. To strengthen the elution effect, the gradient elution program was used. After increasing the proportion of NaOH to 20%, cellobiose which has strong adsorption ability can be eluted. The final eluent condition was provided as follows: 0-15 min, 250 mm NaOH with 4%; 15-20 min, from 4% to 30%; 20-35 min, maintaining 30%.

The content determination method was suitable for *Astragalus* residues fermentation medium, as well as for other fermentation liquids of Chinese medicinal drugs. We have applied the method to other media, such as *Kudzu*, *Patchouli* and it showed good adaptability. The developed experimental means is very useful for evaluating resource utilization of traditional Chinese medicine.

In conclusion, the analysis was carried out on a Dionex Analytical column (2x250 mm). Water and 250 mM sodium hydroxide were used as the mobile phase. The rate of the flow was 0.2 ml/min. The column temperature was kept at 30˚C. The accuracy and precision of the detection method were validated. The obtained regression equation revealed a good linear relationship (R^2=0.9959-0.9984) within the test ranges. The LOD and LOQ for five analytes (arabinose, galactose, glucose, xylose, and cellobiose) were in the range of 0.067-0.091 and 0.08-0.23 mg/l, respectively. The detection method showed good reproducibility for the quantification of five analytes in fermentation liquor of *Astragalus* residues and the intra- and inter-day variations were <3.843%.

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