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Determination of the composition of Chinese ligustrum lucidum polysaccharide by capillary zone electrophoresis with amperometric detection

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Abstract

In this paper, capillary zone electrophoresis with amperometric detection was firstly applied to indirectly determine the composition of Chinese ligustrum lucidum polysaccharide (LLPS) by analyzing its hydrolyzates: fucose, glucose, arabinose and rhamnose. Under the selected optimum conditions, the four monosaccharides could be perfectly separated within 30 min and showed significant current responses at the copper electrode. The linear ranges of fucose, glucose and arabinose were all from 5.0×10^{-6} to 1.0×10^{-4} mol l^{-1} and that of rhamnose was from 1.0×10^{-5} to 1.0×10^{-4} mol l^{-1}, and their detection limits were lower or near 1.0×10^{-6} mol l^{-1} ($S/N = 3$). Experiments showed that the mole ratio of fucose, glucose, arabinose and rhamnose in Chinese LLPS was 1.80:4.58:2.55:1.91, and the purity of this polysaccharide leached by the introduced leaching method was 93.3%. Analyzing polysaccharide by this method has some merits of quickness, low-volume sampling, simple instrument, high sensitivity and high reproducibility.

Keywords: Ligustrum lucidum polysaccharide; Capillary zone electrophoresis; Amperometric detection

1. Introduction

Ligustrum lucidum polysaccharide (LLPS), which exists in a common Chinese traditional herb of ligustrum lucidum fruit, is a kind of plant heteropolysaccharide and composed of fucose, glucose, arabinose and rhamnose with β-bond. Some researches have proved that LLPS can markedly enhance the production of interlenkin 2 (IL2) from the helper T cells (TH) of mice, which can further promote the hyperplasia of thymus dependent lymphocyte cells (T cells), the activation of bone marrow dependent lymphocyte cells (B cells), and the maturity and differentiation of both natural killer cells (NK cells) and lymphokine activated killer cells (LAK cells). So, LLPS is very potential to be used as an effective medicine in immunological enhancement and cancer inhibition [1–4].

Since LLPS and other polysaccharides are very large molecules without very active chemical characters and usually combined with proteins,

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pigments and other disturbing substances, their analysis is relatively difficult. Many analytical methods, such as NMR [5,6], TLC [7], GCC [8], UV [9], GC [10] and HPLC [11], have been studied and applied in the structural and quantitative analysis of polysaccharides. However, most above methods are suffered from complicated instruments and expensive agents, while the others can only be used in qualitative or approximately quantitative analysis.

Capillary electrophoresis (CE), which performs separations depending on the different electrophonetic velocities of studied analytes in a very thin fused silica capillary under a high voltage electric field, has been developed as an attractive analytical method owing to its high separation efficiency, low sample consumption, short analysis time and relatively simple instrumentation. Recently, CE has also been introduced into the analysis of monosaccharides and simple polysaccharides. As polysaccharides can be hydrolyzed into one or some kinds of monosaccharides with definite ratios in the first step of their metabolisms in animal bodies, their compositions, metabolic kinetics in bodies and purity in medicines could be determined by analyzing the types and contents of their hydrolyzed monosaccharides after the effective CE separations [12–14].

Currently, some detection techniques, such as UV-visible absorption, laser-induced fluorescence and electrochemical detection (ED), are used in combination with CE. Since there is no chromophoric group in both monosaccharide molecules and polysaccharide molecules, extra procedures of pre- or post-column derivations are required for the detection of monosaccharides after CE separations when UV-visible absorption or fluorescence detection method is used [15]. But detecting monosaccharides by using electrochemical method can overcome above disadvantage, because the electroactive hydroxyl groups in carbohydrates can be catalytically oxidized on the surface of copper or other metallic electrodes and show significant current responses. Furthermore, the ED possesses higher sensitivity and lower detection limit than UV-visible absorption. Hence, the CE–ED method is successfully used in the analysis of carbohydrates, especially monosaccharides [16–19].

In this paper, CE–ED method was firstly applied to study the composition of Chinese LLPS by determining its hydrolyzed monosaccharides. Experiments showed that the mole ratio of fucose, glucose, arabinose and rhamnose, which are the constituents of LLPS, was 1.80:4.58:2.55:1.91, and the purity of the LLPS leached by the introduced leaching method was 93.3%. The results also showed that analyzing a heteropolysaccharide by this method was of quickness, low-volume sampling, simple instrument and operation, high sensitivity and high reproducibility.

2. Experimental

2.1. Apparatus

CE with amperometric detection system was laboratory-built [20,21]. Electrophoresis was driven by a high-voltage supplier (±30 KV, Shanghai Institute of Nuclear Research, China). Separations were performed in a fused silica capillary (Hebei Yongnian Laser-fiber factory, China) with 25 μm i.d., 360 μm o.d. and 75 cm long. Potential control and current output were employed by a BAS LC-3D amperometric detector (Bioanalytical System, West Lafayette, IN, USA). Electropherograms were recorded by a chart recorder (Model XWT-204, Shanghai Dahua Instrument Factory, China). Electrochemical experiments were carried out by a CHI 630 electrochemical analyzer (CHI Instruments, USA). A three-electrode system was used in both electrochemistry and detection experiments, which consisted of a disk-shaped copper working electrode (Φ120 μm), a saturated calomel reference electrode (SCE) and a platinum wire counter electrode.

2.2. Preparation of copper working electrode

One side of the used copper wire(Φ120 μm) was firstly polished by emery paper and jointed with one thin copper rod (Φ1 mm), then the copper wire was inserted through one prepared glass tube

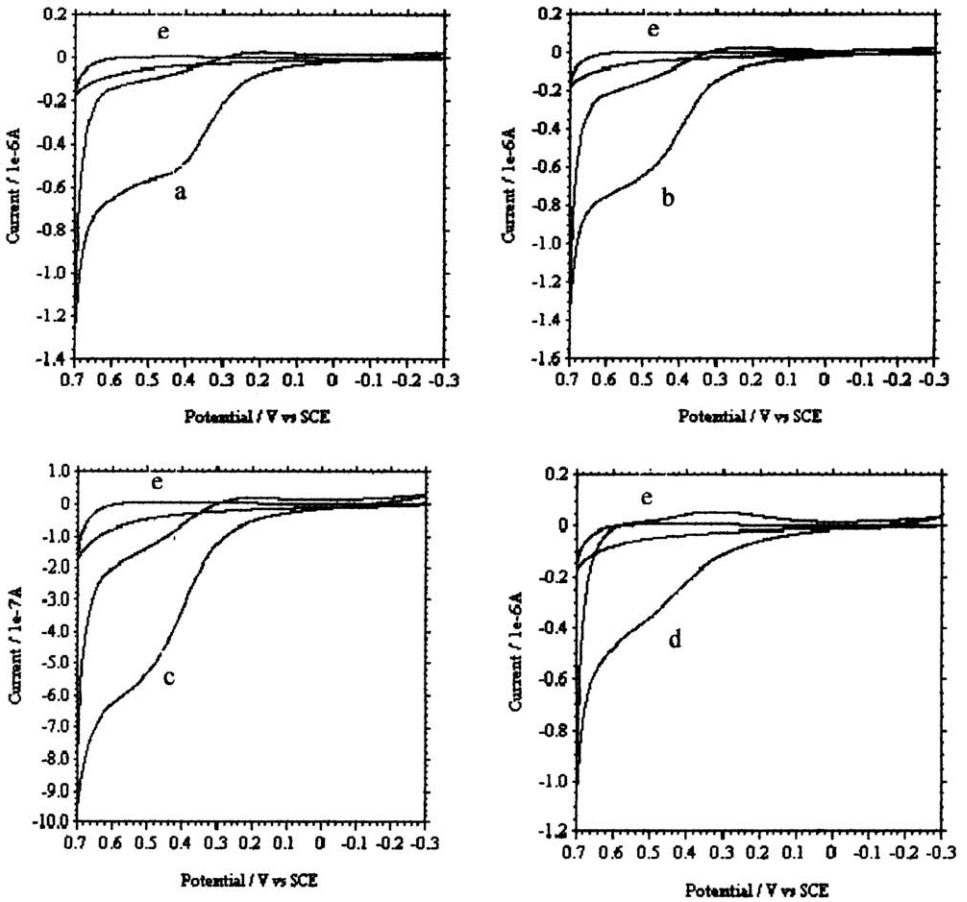

Fig. 1. Cyclic voltammograms of four monosaccharides with a concentration of 5.0×10^{-4} mol l^{-1} at disk-shaped copper electrode in 0.045 mol l^{-1} NaOH at a scan rate of 100 mV s^{-1}. (a) Fucose, (b) glucose, (c) arabinose, (d) rhamnose. (e) 0.045 mol l^{-1} NaOH.

with a normal side (Φ5 mm) and a capillary side (Φ1 mm). Finally, the two sides of the glass tube were sealed by nonconducting glue to make the copper wire and copper rod stable. This copper electrode could be used after at least 24 h air-dryness.

Prior to use, the surface of the copper electrode was polished with emery paper and alumina powder, respectively, then it was sonicated in doubly distilled water for 3 min to get enough cleanness.

2.3. Reagents

All reagents were of analytical-reagent grade. Fucose, glucose, arabinose and rhamnose were purchased from Shanghai Yuanjiu Reagent Company and their stock solutions with a concentration of 1.0×10^{-2} mol l^{-1} were prepared with doubly distilled water and diluted to needed concentrations in CE experiments.

Chinese ligustrum lucidum was purchased from Chinese Traditional Medicine Department of Shanghai Medicine Company.

Before CE separations, all used solutions were filtered through 0.45 μm polypropylene acrodisc syringe filter and sonicated for 5 min to remove bubbles.

2.4. Sampling

Accurate weight of Chinese ligustrum lucidum powder was firstly refluxed with acetone and 1:1

ethanol–ether, respectively, under a boiled-water bath for 2 h to remove pigments. Then, the residue was leached by stirring in water at 90–100 °C three times each for 6 h. Next, the leaching solution from above operation was concentrated and precipitated by adding nonaqueous ethanol. The precipitate was separated from above solution by centrifugation, dialyzed and purged with 4:1 chloroform–isopentanol to remove hetero-proteins. After frozen and dried, the LLPS was obtained.

One gram of LLPS was refluxed with 2 mol l^{-1} sulfuric acid for 10 h under a boiled-water bath and the protection of nitrogen. The filtered liquid from above operation was a mixture of monosaccharides from the hydrolyzation of LLPS, which was diluted into 250 ml and further diluted 200 times for CE analysis.

2.5. CE operations

Before experiments, the three-electrode system was fixed in corresponding positions of the electrochemical cell and the disk-shaped copper working electrode was carefully adjusted to make an effective injection to the off-side of the capillary by the three-dimension positioner.

Before each run in CE experiments, the capillary was sequentially rinsed with 1.0 mol l^{-1} hydrochloric acid, doubly distilled water, 1.0 mol l^{-1} sodium hydroxide 3 min for each and running buffer till the current inside of the capillary reached stable state. This was important to get a reproducible EOF.

The optimal conditions of this experiment were 12 kV as separation voltage, 0.045 mol l^{-1} NaOH as buffer solution, 8 s as sampling time and 0.60 V as detection potential.

3. Results and discussion

3.1. Selection of working electrode

The usually used working electrodes in CE–ED system are carbon electrodes, but they are not suitable to determine carbohydrates because carbohydrates have bigger overpotentials at the carbon electrodes. However, carbohydrates can express stronger current responses at copper electrodes under a basic environment. So the copper electrode was selected as working electrode in this experiment.

Fig. 1 are the cyclic voltammograms of fucose, glucose, arabinose and rhamnose with same concentration of 5.0×10^{-4} mol l^{-1} in 0.045 mol l^{-1} NaOH solution at a scan rate of 100 mV s^{-1}, in which all monosaccharides exhibited obvious anodic peaks in the voltage range of +0.4 to +0.7 V, while the blank solution had not anodic peak in this voltage range.

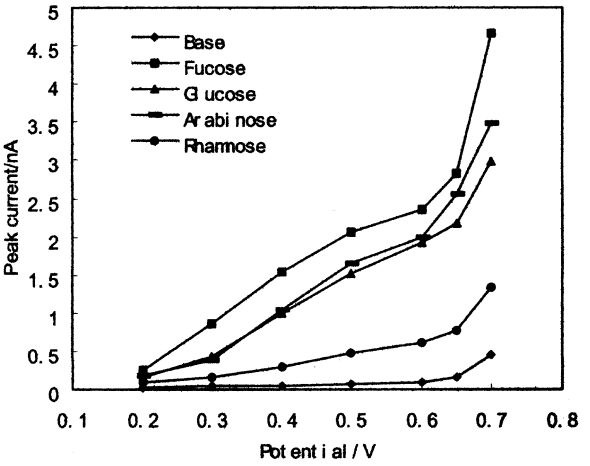

Fig. 2. HDVs of four standard monosaccharides in CE under different detection voltages from +0.2 to +0.7 V. Other conditions as the optimum.

3.2. Selection of detection potential

Fig. 2 are the hydrodynamic voltammograms (HDVs) of fucose, glucose, arabinose and rhamnose, which were obtained by monitoring their current responses after CE separations at different applied potentials. It was found that the current responses of both the monosaccharides and the blank solution increased with the increase of the applied potential. In order to get higher sensitivity and the best signal-to-noise ratio, 0.60 V was selected as the detection potential in this experiment.

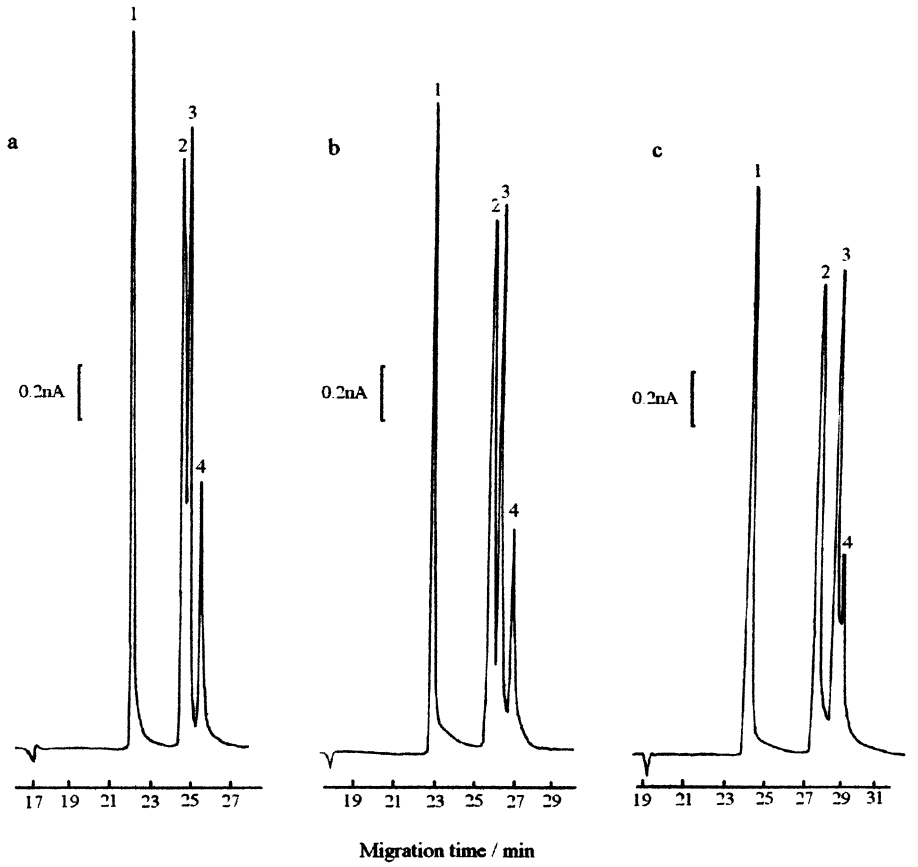

Fig. 3. Electropherograms of four monosaccharides under different NaOH concentrations. (a) 0.035 mol l^{-1} NaOH, (b) 0.045 mol l^{-1} NaOH, (c) 0.055 mol l^{-1} NaOH; 1, fucose; 2, glucose; 3, arabinose; 4, rhamnose. Other conditions as the optimum.

3.3. Separation conditions

Under strong basic conditions, carbohydrates are negatively charged by ionizing their hydrogen ions in hydroxyl groups and different carbohydrates have different charge–mass ratios, so they can be separated by CE by their different electrophoretic mobility under a high electrical field. In this experiment, sodium hydroxide was chosen as buffer solution. Fig. 3 are the electropherograms of fucose, glucose, arabinose and rhamnose at different concentrations of NaOH buffers when other conditions were same as the optimum conditions. The results showed that the separation efficiency of the four monosaccharides was sensitive to NaOH concentration, because they have similar dissociation constants and molecular weights. When the concentration of NaOH was less than 0.030 mol l^{-1}, the current peaks of glucose and arabinose were overlapped while other current peaks were perfectly separated; When the concentration of NaOH was in the range of 0.030–0.040 mol l^{-1}, the current peaks of glucose and arabinose were partly overlapped; When the concentration of NaOH was in the range of 0.040–0.050 mol l^{-1}, all the current peaks of the four monosaccharides are perfectly separated; And when the concentration of NaOH was more than 0.050 mol l^{-1}, the current peaks of arabinose and rhamnose were partly overlapped. So, 0.045 mol l^{-1} NaOH was chosen for achieving the best separation rhamnose.

It was also found that the migration time of the analytes would be prolonged with the increase of

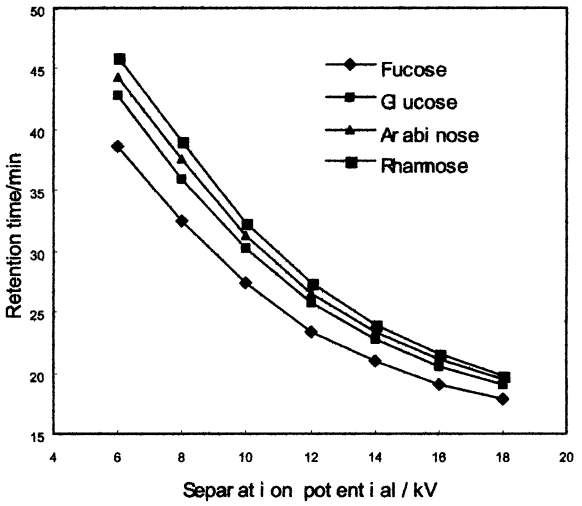

Fig. 4. Effects of separation voltage changed from 6 to 18 kV on retention time. Other conditions as the optimum.

the NaOH concentration, since when the concentration of NaOH increases, the ionic strength, which influences the mobility of the analytes is increased correspondingly.

The separation efficiency of the four monosaccharides was investigated in the separation voltage range of 6–18 kV and the results are showed in Fig. 4. When the separation voltage was less than 10 kV, the four current peaks were distant enough, meaning a very perfect separation, but the peaks were somewhat wide and the migration time was too long. The migration time of the analytes was significant shortened and their corresponding current peaks were sharpened when the separation voltage was increased. However, if the separation voltage was more than 16 kV, despite the migration time was less than 20 min, the current peaks of glucose, arabinose and rhamnose were partly overlapped owing to the more Joule heat produced by the higher current inside of the capillary. For the comprehensive consideration of the migration time and separation efficiency, 12 kV was selected as separation voltage and all the four monosaccharides could be perfectly separated within 30 min under this condition.

The electrokinetic sampling time changed from 4 to 16 s was tested with the other conditions as the optimum. It was found that when the sampling time was changed from 5 to 12 s, the peak currents were increased correspondingly. However, the current peaks of the analytes were obviously broadened if the sampling time was more than 12 s. Eight seconds were selected as sampling time in this experiment and satisfactory results were obtained under this condition.

3.4. Linearity, reproducibility and detection limits

A series of standard solutions of the four monosaccharides with a concentration range from 1.0×10^{-6} to 5.0×10^{-4} mol l^{-1} were analyzed under the optimum conditions and the results are showed in Table 1. The linear ranges of fucose, glucose and arabinose were from 5.0×10^{-6} to 1.0×10^{-4} mol l^{-1} and that of rhamnose was from 1.0×10^{-5} to 1.0×10^{-4} mol l^{-1}, and the detection limits of the four monosaccharides were lower or near 1.0×10^{-6} mol l^{-1} ($S/N = 3$), which showed that this method was very sensitive. Table 2 is the relatively standard deviations (R.S.D.) of both the migration time and peak currents of the analytes with a concentration of 5.0×10^{-5} mol l^{-1} when the analysis was repeated for six times under the same conditions. All the R.S.D.'s were less than 4%, which demonstrated that this method was of good reproducibility.

Table 1
Regression equation and detection limit[a]

Analyte	Regression equation I(nA); C(mol l^{-1})	R	Linear range (mol l^{-1})	Detection limit (mol l^{-1})
Fucose	$I = 0.133 + 4.53 \times 10^4 C$	0.9993	5.0×10^{-6}–5.0×10^{-4}	6.5×10^{-7}
Glucose	$I = 0.133 + 3.42 \times 10^4 C$	0.9971	5.0×10^{-6}–5.0×10^{-4}	8.5×10^{-7}
Arabinose	$I = 0.160 + 3.53 \times 10^4 C$	0.9974	5.0×10^{-6}–5.0×10^{-4}	8.5×10^{-7}
Rhamnose	$I = 0.023 + 1.60 \times 10^4 C$	0.9995	1.0×10^{-5}–5.0×10^{-4}	1.8×10^{-6}

[a], Detection limit was estimated according to three times of signal–noise ratio.

Table 2
Precision of the present method ($n = 6$)[a]

Analyte	Migration time (min)		Peak height (nA)	
	Average	R.S.D. (%)	Average	R.S.D. (%)
Fucose	23.03	0.86	2.40	1.91
Glucose	25.71	0.59	1.84	2.12
Arabinose	26.30	1.02	1.92	2.62
Rhamnose	27.00	1.33	0.82	3.07

[a], The concentrations of all the four monosaccharides were 5.0×10^{-5} mol l^{-1}.

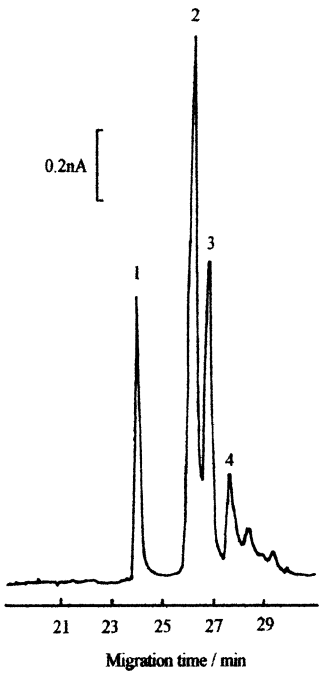

Fig. 5. Electropherograms of the monosaccharides hydrolyzed from Chinese LLPS under the optimum conditions of CE–ED. 1, Fucose; 2, glucose; 3, arabinose; 4, rhamnose.

3.5. Sample analysis

This method was used to analyze the hydrolyzates of LLPS under the selected optimum conditions and the electropherograms were obtained as Fig. 5. After four times of repetition, the mole ratio of fucose, glucose, arabinose and rhamnose, which are the constituents of Chinese LLPS, was calculated as 1.80:4.58:2.55:1.91. The purity of the LLPS leached according to the procedure in Section 2.4 was calculated as 93.3%. All above results are shown in Table 3.

Recovery experiments were performed four times by adding the four monosaccharides into the LLPS sample before hydrolysis and making their analytical concentrations as 5.0×10^{-5} mol l^{-1}. The results are listed in Table 4, showing that the recoveries of all the four monosaccharides were ranged from 96 to 102%, which meant this method was precise and practical for the analysis of LLPS.

4. Conclusion

The experimental results showed that determining the composition of Chinese LLPS by CZE-AD

Table 3
Contents and mole ratio of LLPS hydrolyzates ($n = 4$)

Component	Measured concentration (mol l^{-1})	Mass ratio (%)	Mole ratio
Fucose	1.60×10^{-5}	14.6	1.80
Glucose	4.85×10^{-5}	41.6	4.58
Arabinose	2.55×10^{-5}	19.1	2.55
Rhamnose	1.91×10^{-5}	18.0	1.91

Table 4
Recoveries of four monosaccharides in sample analysis ($n = 4$)

Component	Added amount(mol l^{-1})	Found amount (mol l^{-1})	Recovery (%)	R.S.D. (%)
Fucose	5.0×10^{-5}	5.1×10^{-5}	102.0	2.4
Glucose	5.0×10^{-5}	5.0×10^{-5}	100.0	3.1
Arabinose	5.0×10^{-5}	4.9×10^{-5}	98.0	2.2
Rhamnose	5.0×10^{-5}	4.8×10^{-5}	96.0	2.8

was of quickness, high sensitivity and high reproducibility especially when compared with the usual UV detection. This method also embodied the advantages of low-volume sampling, simple instrument and operation and could be used to study the compositions of other plant polysaccharides.

References

[1] L. Li, A. Ding, L. Meng, Zhongyao Yaoli Yu Linchuang 17 (2001) 11.
[2] M. Li, X. Tian, H. Feng, Zhongguo Zhongyao Xinxi Zazhi 3 (1996) 72.
[3] H. Yuan, Z. Lu, Zhongguo Zhongyao Zazhi 24 (1999) 691.
[4] H. Yuan, Zhongguo Yaoke Daxue Xuebao 18 (1987) 301.
[5] Z. Wang, J. Fang, Fenxi Huaxue 28 (2000) 240.
[6] D.W. Hopkins, J.A. Chudek, D.E. Bignell, J. Frouz, E.A. Webster, T. Lawson, Biodegradation 9 (1998) 423.
[7] J. Xu, Y. Xie, H. Ji, Zhejiang Daxue Xuebao (Yixue) 28 (1999) 206.
[8] Y. Zhang, Shengwu Huaxue Yu Shengwu Wuli Jinzhang 5 (1983) 18.
[9] H. Liu, Y. Huang, Y. Wu, G. Wang, Shenyang Yaoke Daxue Xuebao 16 (suppl) (1999) 60.
[10] D. Ingrid, L. Erika, J. Bo, Proc. Biochem. (Oxford) 35 (1999) 119.
[11] P. Shang, Q. Mei, Z. Cao, D. Zhao, Zhongguo Yaoxue Zazhi 35 (2000) 332.
[12] E. Grill, C. Huber, P. Oefner, A. Vorndran, G. Bonn, Electrophoresis 14 (1993) 1004.
[13] W. Zhou, R.P. Baldwin, Electrophoresis 17 (1996) 319.
[14] A. Guttman, F.A. Chen, R.A. Evangelista, Electrophoresis 17 (1996) 412.
[15] K. Ding, J. Fang, Sepu 17 (1999) 346.
[16] L.A. Colon, R. Dadoo, R.N. Zare, Anal. Chem. 65 (1993) 476.
[17] J. Ye, R.P. Baldwin, Anal. Chem. 65 (1993) 3525.
[18] C. Fu, L. Song, Y. Fang, Anal. Chim. Acta 371 (1998) 81.
[19] Q. Hu, T. Zhou, L. Zhang, Y. Fang, Analyst 126 (2001) 298.
[20] Y. Fang, X. Fang, J. Ye, Gaodeng Xuexiao Huaxue Xuebao 10 (1995) 1514.
[21] C. Fu, L. Song, Y. Fang, Anal. Chim. Acta 399 (1999) 259.