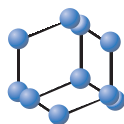
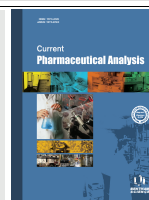


## RESEARCH ARTICLE

BENTHAM  
SCIENCE

# Compositional Monosaccharide Analysis of *Morus nigra* Linn by HPLC and HPCE Quantitative Determination and Comparison of Polysaccharide from *Morus nigra* Linn by HPCE and HPLC



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**Abstract: Background:** *Morus nigra* Linn is not only treated as health food but also medicine in Chinese history.

**Objective:** Here, we have extracted and separated the heteroglycan to monosaccharides of the dry fruits.

**Method:** After heteroglycan being hydrolysed, then were we derivatized the monosaccharides with 1-phenyl-3-methyl-5-pyrazolone (PMP), and then subsequently HPCE and HPLC were used to separate. HPCE, which held an uncoated capillary (d=75µm) and detected by PDA at 245 nm with borate buffer, the voltage was set at 15 kV and capillary temperature was 25°C. There is Yilite column (Hypersil BDS C18 5µm, 4.6mm×250mm) with HPLC and detected by UV at 245 nm and capillary temperature 25°C. The High Performance capillary electrophoresis method and High Performance Liquid Chromatography method were compared on monosaccharide composition and quantitative determination of polysaccharide from the black mulberry.

**Results:** Through several indicators including standard curve, precision, reproducibility, stability and recovery rate, the result of the two methods is basically consistent while they have complementary advantages.

**Conclusion:** Both methods are suitable for the determination of the black mulberry polysaccharide, but each has its own merits.

**Keywords:** *Morus nigra* Linn, polysaccharide, HPCE, HPLC, boric acid, linearity.

## 1. INTRODUCTION

Xinjiang mulberry is a semi-cultivated and semi-wild kind which is a rare precious resource from nature, and it's the only one *Morus nigra* Linn with 22ploid flower chromosome in China [1]. Owing to the special ecological environment in Xinjiang, *Morus nigra* Linns contain more polysaccharide, flavone, vitamins, amino acid and so on [2]. Many researches have shown that polysaccharide has antitumor [3], antioxidant [4], effect on hypoglycemic and blood fat [5], and modulate the immune [6] etc. *Morus nigra* Linn is not only treated as health food but also is used as medicine. The polysaccharide in this study is extracted and separated from the dry mulberry fruits.

At present, spectrophotometry and HPCE are used to determine monosaccharides contents of mulberry leaves, no one has gone about to analyze the heteroglycan to monosaccharides of the mulberry fruits by HPCE and HPLC at the

same time. In this study, High Performance capillary electrophoresis takes capillary as separate channel and high voltage DC current as driving force, and is famous for its high sensitivity, speed and consuming insignificant amount of sample. While High Performance Liquid Chromatography has advantages of high speed, efficiency and sample can be used repeatedly. To a certain extent, both methods communicate but complement with each other, however, kinds of monosaccharides of the mulberry fruits are varied. It's necessary and interesting to compare the two methods to analyze each one accurately. It is significant and helpful to explore monosaccharides of Xinjiang *Morus nigra* Linn.

## 2. EXPERIMENTAL

### 2.1. Reagents

D-Glucose anhydrous, Mannose, alactose, Rhamnose, GalUA, D-xylose, Arabinose, are acquired from National Institutes For Food and Drug Control (China), Mulberris (*Morus nigra* Linn) were purchased from Xinjiang kashgar which were identified by professor Palida (Department of Medicinal Plants & Pharmacognosy, xinjiang medical

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University), the dry fruits were kept at room temperature. HPCE-grade acetonitrile and methanol during our experience were purchased from Sigma (St. Louis, MO, USA): The next, ammonium acetate, acetic acid, absolute ethyl alcohol, chloroform, chlorhydric acid (HCl, assay F99%), sodium hydroxide in pellets (NaOH, assay F99%), trifluoroacetic acid (TFA), were purchased from Fu Yu chemical company (tianjin, China); And the 1-phenyl-3-methyl-5-pyrazolone (PMP) was purchased from the second Chemical Reagent Company (shanghai, China); What's more, the distilled water was obtained from Millipore (Bedford, MA, USA).

## 2.2. Mulberry Extract Preparation for HPCE and HPLC Analysis

The mulberry polysaccharides were detached by water-extraction and ethanol-precipitation method. Briefly, 100g of mulberry were ground in a blender then the powder were added through 200-mesh screen. We took 10g into ultrasonic extraction (KQ-500PE) two times with 100mL of HPCE-grade water at 60°C, 30 min every time. The combined extracts were dealt with vacuum filtration and concentrated under a reduced pressure, then ethyl alcohol was added and adjusted to 80% alcohol, and then kept at room temperature for 24 h. Precipitation is solid, then the supernatant were poured out and the precipitation is dried in the vacuum drying oven at 60°C for 12h. Finally, we got yield violet mulberry polysaccharide. After this Extraction process, 10 mg of the analyte of mulberry polysaccharide was placed in an ampoule (5mL) with 2mL of 2M TFA. The ampoule should be sealed under an alcohol blast burner and then put into an oil bath at 105°C for 4h to hydrolyze the polysaccharide into component monosaccharides. The resultant mixture was cooled to room temperature, next, we dried the mixed solution under a reduced pressure repeatedly to remove the excess TFA. Finally, some of HPCE-grade water was added and we took the solution into a volumetric flask (5ml) after filtered through a 0.45µm membrane filter for the further experiments.

The next step is derivatization procedure by PMP, which was carried out as described previously [7]. Briefly, take 500µl of monosaccharide [mixed standard solution] or the monosaccharide hydrolysate of the polysaccharides in artillery pipe, then 500µl of 0.3 M aqueous NaOH and 500µl 0.5 M PMP-methanol solution were added in turn. After 30s shaking, the mixture was kept at 70°C for 30 min, and then when it is cool and 500µl of 0.3M HCl should be neutralized with. Next, the resulting solution was extracted with chloroform 3 times, and the organic phase was discarded. Finally, the water layer was filtered through a 0.45µm membrane filtration for analysis. The reagent solution was freshly prepared before derivatization.

The last Preparation is for buffer and standard solution. The running buffers were prepared by dissolving the boric acid powder to the appropriate concentrations (180mM) and its pH values could be adjusted to 10.05 with sodium hydroxide (NaOH). The standard monosaccharides (2mM) stock solution were prepared by powder and diluted with distilled water. Both of the buffer and sample solution were filtered through a 0.45µm membrane filtration. All the solutions prepared were stored in the dark at 4°C.

## 2.3. HPCE and HPLC Equipment and Conditions

### 2.3.1. HPCE Determination of Polysaccharide

HPCE analyses in this study were played with a CE instrument P/ACE 5500 from Beckman equipped with a single wave-length UV-Vis detector and a diode array UV-Vis detector (DAD). The system was controlled by 32Karat Software from Beckman (Beckman P/ACE Station-CE Software, version 1.21). The capillary cartridge contained an un-coated fused-silica tube (I.D.75 µm) supplied by Hebei Yongnian Ruifeng Chromatography Devices Company (China). Then the 180mM boric acid (pH 10.05) was selected as our final optimised running buffer. Dissolving 1.1129g of the powder in 100mL of HPLC-grade water, titrating to pH 10.05 with sodium hydroxide in pellets, then the solution was finished. Finally, the buffer should go through 0.20 mm nylon filtration. In this study, capillary conditioning and all of the washing solutions (such as NaOH 1 and 0.1 M; HCl 1 M; H<sub>3</sub>BO<sub>4</sub> 0.1 M) were prepared in HPCE-grade water. While new capillaries must be conditioned at 25°C, by freshing with CH<sub>3</sub>OH (5min), HCl 0.1 M (5min), HPCE-grade water (5 min), NaOH 0.1 M (5 min), HPCE-grade water (5 min) and running buffer (5 min) in turn. At the beginning of everyday work, the capillary should be rinsed with running HCl 0.1 M for 2 min, HPCE-grade water for 3 min, NaOH 0.1 M (3 min), HPCE-grade water (3 min) and running buffer for 3 min, and before each run with buffer for 10 min. The choice of acidic washing was due to the necessity of avoiding hysteresis of the wall charge generated by the use of acidic run buffers. Each running buffer was a new preparation. The sample was loaded onto the capillary for 10 s at low-pressure mode (0.5 psi, 1 psi = 6894.76 Pa), whereas all the conditioning and washing steps were performed at high-pressure mode (20 psi). The electrophoretic runs were carried out at 15 kV under 25°C and the detector wavelength was set at 245 nm. There is 50 min enough for overall running [8-11].

### 2.3.2. HPLC Determination of Polysaccharide

HPLC analysis was performed using a waters e2695 separations Module (Palo Alto, CA, USA) equipped with a binary pump delivery system, degasser, autosampler and waters 2489 UV/visible detector. An analytical HPLC column was used (Hypersil BDS C18; 5µm, 4.6mm×250mm id; Phenomenex). The mobile phase consisted of ammonium formic acid pH=5.5 (A) and acetonitrile (B). The solutions were filtered on 0.22 µm cellulose acetate filter discs (Albet, Barcelona, Spain) and then sonicated for 8 min. The HPLC system was conditioned with the mobile phase at least for an hour or until a stable baseline was obtained. The following linear elution gradient was employed as follows (Table 1):

## 3. RESULTS AND DISCUSSION

### 3.1. Preliminary Studies

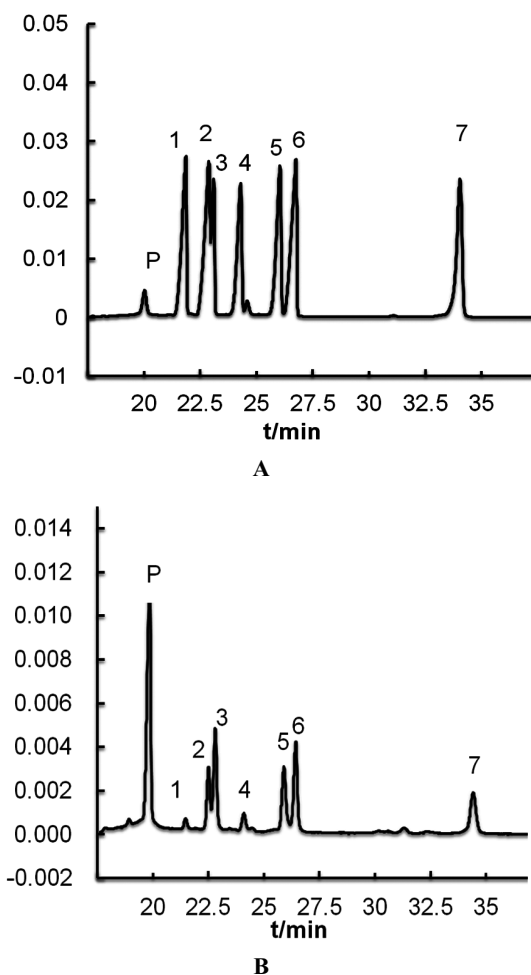
Preliminary experiments, prepared as described in Section 2, were processed differently to determine the best condition for the separation of anthocyanins. We have compared circumfluence and ultrasonic extraction on the fungus polysaccharides, and we found that ultrasonic extraction was much more comfortable and faster. Then, HPCE, which held an un-coated capillary (d=75µm) and detected by PDA at

**Table 1.** HPLC system gradient mobile phase.

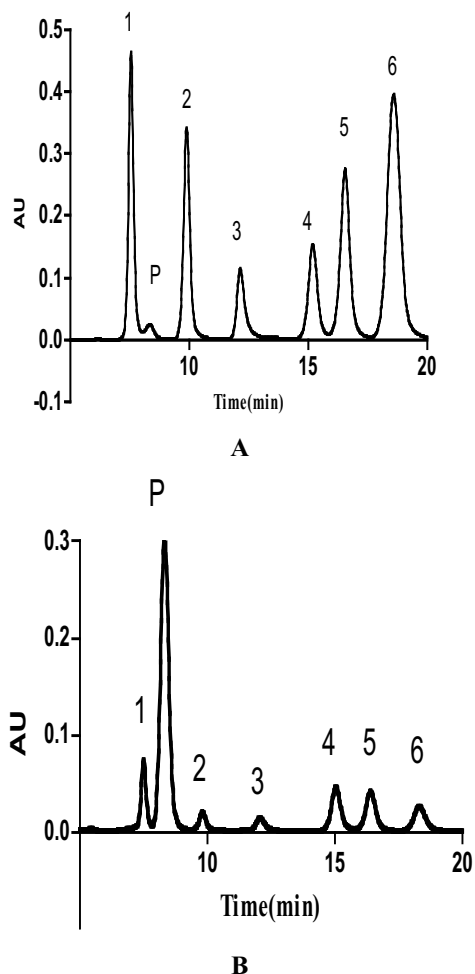
	Ammonium Formic Acid pH=5.5 (A)	Acetonitrile (B)
0min	80%	20%
5min	60%	40%
15min	40%	60%
25min	22%	78%
60min	80%	20%

A 20 min post-run equilibration was performed. The total run time was 80 min. An injection volume of 10  $\mu$ L and a flow rate of 1 mL/min were used, while the detector wavelength was set at 245nm.

245 nm with boric acid buffer at voltage 15 kV and the capillary temperature was 25°C. We have tried to change different buffer, such as, monobasic sodium phosphate dihydrate ( $\text{NaH}_2\text{PO}_4$ ) and phosphoric acid ( $\text{H}_3\text{PO}_4$ , p.a.), but the electropherogram is not very good. So, we selected 180mM boric acid pH (10.05) as buffer at last. The results you can see in Fig. (1). And there is Yilite column (Hypersil BDS C18 5 $\mu$ m, 4.6mm $\times$ 250mm) with HPLC and the ratio of the linear elution gradient was set at 22:78 after several selections. The results are shown in Fig. (2).



**Fig. (1).** Electropherogram of *Morus nigra* Linn polysaccharides from Xinjiang recorded with the optimised method. (A) standard monosaccharides ; (B) monosaccharide of the sample. P-PMP-1- Man -2- Rah -3-GalUA - 4- Glc- -5-Gal -6-Arab.



**Fig. (2).** Chromatogram of *Morus nigra* Linn polysaccharides from Xinjiang recorded with the optimised method. (A) standard monosaccharides ; (B) monosaccharide of the sample. P-PMP-1- Man -2- Rah -3-GalUA - 4- Glc- -5-Gal -6-Arab.

### 3.2. Validation Parameters

#### 3.2.1. Specification Curve

We diluted the stock solution of standard monosaccharides (2mM) with ultrapure water to 1mM, 0.75mM, 0.5mM, 0.25mM, 0.125 mM, 0.0625mM, 0.0312mM and then determined by HPLC and HPCE. The results are shown in Table 2. As shown in it, their linear dependence was not bad, and the linearity range of HPCE is wider than HPLC.

**Table 2. Standard curves of the seven standard monosaccharides.**

Monosa-Ccharides	r	Standard Curve <sup>(a)</sup>	Linearity Range mM <sup>(b)</sup>
	HPCE HPLC	HPCE HPLC	HPCE HPLC
xyl	0.9958	y=199296x-9643	0.0312 ~ 2
Arab	0.9974 0.9974	y=220944x-10493 Y=7000000x-4093	0.0312 ~ 2 0.0625 ~ 1
Glc	0.9998 0.9998	y=109568x-656.43 y=3000000x+15871	0.0312 ~ 2 0.0625 ~ 1
Rah	0.9991 0.9999	y=152886x+58.852 y=3000000x+10498	0.0312 ~ 2 0.0625 ~ 1
Man	0.9958 0.9999	y=182952x-1047.7 y=4000000-3466	0.0312 ~ 2 0.0625 ~ 1
Gal	0.9978 0.9999	y=224268x-2422.7 y=4000000+10278	0.0312 ~ 2 0.0625 ~ 1
GalUA	0.9986 0.9961	y=204605x-5703.9 y=3000000+144675	0.0312 ~ 2 0.0625 ~ 1

(a) x is the concentration of monosaccharides and y is the peak area integral value.

(b) linearity range of monosaccharides is mmol/L.

**Table 3. Precision, stability, reproducibility of the seven standard monosaccharides.**

Monosaccharides	Precision %RSD	Reproducibility %RSD	Stability %RSD
	HPCE HPLC	HPCE HPLC	HPCE HPLC
xyl	1.72	2.82	2.76
Arab	1.78 0.35	0.96 0.91	1.20 1.06
Glc	2.36 0.42	1.14 0.87	1.24 0.60
Rah	1.80 0.40	2.17 1.19	2.34 0.63
Man	2.16 0.49	1.39 0.98	3.07 0.45
Gal	1.57 0.26	2.48 0.69	1.83 0.70
GalUA	1.60 0.92	2.71 2.34	1.62 2.26

### 3.2.2. Precision' Stability and Reproducibility

The standard comparison solution was carried out with derivatization and it was determined according to the above condition. The specimen is handled six times. The seven standard monosaccharides' peak area integral values are determined. RSD% was below 3% and 1% respectively. The results obtained confirm a good precision of these developed methods, and HPLC is better than HPCE.

Take the identical sample to supply the test solution for 0, 2, 4, 8, 24 h respectively, and determine the peak area integral values. RSD % was below 4% and 3% by HPCE and HPLC respectively. The experimental results indicate that samples are stable during 24 h.

Take 0.01g *Morus nigra* Linn powder to extract, according to 2.3session by the same method. The peak area integral values are determined according to the above conditions, RSD % were both below 3%. The experiment results indicate that the accuracy is good. Above results are shown in Table 3.

### 3.2.3. Recovery

Take appropriate *Morus nigra* Linn powder of the known content for each group and add the standard monosaccha-

rides solution on the basis of 80%, 100%, 120%, according to the 2.3 session by the same method and computation content using standard curve method, and the spitting recovery rate was calculated. The average recovery (n = 6) range of by HPCE and HPLC were among 98.0% ~102.0%, RSD % were both below 3%.

### 3.2.4. Quantification of Monosaccharides in Sample

The standard addition and recovery experiments are conducted to determine the accuracy of the present method for the quantification of monosaccharides in *Morus nigra* Linn samples. According to the migration time from Fig. (1) and adding standard monosaccharides solution into sample, the peak area integral values heightened so that we can know that polysaccharides of *Morus nigra* Linn is made of D-xylose, Arabinose, D-Glucose anhydrous, Rhamnose, Mannose, Galactose, GalUA.

Calculating the results with calibration factor and the ratio of amount of substance were 1:7.4:15.8:3.4:10.8:14.3:4.5. According to the migration time from Fig. (2) by the same method, we found that HPLC determined only six monosaccharides, they were Man, Rah, GalUA, Glc, Gal, Arab, and D-xylose cannot be identified with Arabinose although its in

the linearity range. The ratios of the amount of substances were 2.96:1:1.57:3.92:2.83:1.08.

### CONCLUDING REMARKS

In this investigation, we developed a simple extraction procedure and a sensitive electrophoretic separation technique which allows the separation of the main monosaccharides from dry *Morus nigra* Linn fruits. This easy method shows excellent sensitivity, accuracy and precision and tests the traditional benefits of HPCE analysis, such as its high separation efficiency, the low consumption of solvents and samples; it leads to considerable reduction in analysis time of monosaccharides when compared with HPLC. However, the major volumes injected in HPLC enabled to reach lower repeatability RSD than HPCE.

The results we obtained indicate the large advantage of electrophoretic applications, although its quantification repeatability is really troubling. These developed methods can both therefore be applied to extract and determine component and quantitative of polysaccharide from Xinjiang *Morus nigra* Linn and other samples.

### CONFLICT OF INTEREST

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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