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Analysis of the monosaccharide composition of fucoidan by precolumn derivation HPLC*

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Abstract We developed an HPLC method for analysis of the monosaccharide composition of fucoidans. The fucoidan was hydrolyzed into monosaccharides with 2 mol/L trifluoroacetic acid. Using ribose as the internal standard, the monosaccharide derivatives, obtained with 1-Phenyl-3-methyl-5-pyrazolone (PMP), were separated by reverse-phase HPLC using a gradient elution process, and monitored by ultraviolet detection at 245 nm. In the concentration range of 0.1–2.0 mmol/L, the peak area of each monosaccharide had a good linear relationship with its concentration (r^2>0.998). The average recoveries of mannose, rhamnose, glucuronic acid, glucose, galactose, xylose, and fucose were 86.2%, 95.1%, 62.5%, 102.0%, 94.8%, 66.6%, and 105.1%, respectively. This method was accurate and had good reproducibility and could be used to determine the monosaccharide contents of fucoidans.

Keyword: fucoidan; monosaccharide; derivation; HPLC

1 INTRODUCTION

Fucoidans are sulfated polysaccharides found within the cells of brown algae. They are composed primarily of highly branched (1→2) or (1→3) linked α-L-fucose-4-sulfate units. However, the chemical composition of fucoidans varies among algal species (Zhang et al., 1996). Some species contain simple sulfated fucans, while others have complex sulfated heterogeneous polysaccharides that contain several monosaccharides, such as mannose, rhamnose, glucuronide, glucose, galactose, xylose, and fucose (Percival, 1979). Fucoidans have traditionally been quantified colorimetrically using hydrochloric acid homocysteine-sulfuric acid (Gibbons, 1955). Gas chromatography (GC) has also been used for determination of L-fucose in fucoidans (Zhao et al., 2004). Monosaccharides cannot be detected directly by absorption due to the lack of chromophores in their molecular structure. Honda et al., (1989) developed 1-phenyl-3-methyl-5-PyraZolone (PMP), a pre-column derivatization reagent, which provided a high yield of single highly absorbent derivatives. Based on this, we developed a new method for the analysis of the monosaccharide compositions of fucoidans by precolumn derivation HPLC. This method has the advantage of speed, sensitivity, and reproducibility.

2 MATERIALS AND METHODS

2.1 Materials

We used a SHIMADZU-20A high performance liquid chromatography system, consisting of two LC-20AT pumps, a SPD-M20A photodiode array UV-VIS detector, a CTO-20A chromatography oven (25°C), an injection valve with a 20 μl injection loop. The monosaccharide derivatives were separated on a CBM-20A YMC-Pack ODS-AQ (250×4.6 mm, 5 μm) column. The data was recorded and processed using Shimadzu LC-Solution.

We purchased PMP (AR) from Tianjin Guangcheng Chemical Reagents Ltd. (China). The reagent was recrystallized twice using methanol before use. We obtained trifluoroacetic acid (AR) from the Tianjin Guangfu Fine Chemical Research Institute (Nankai, Tianjin, China). The standard reference materials (mannose, rhamnose, glucuronic acid, glucose, galactose, xylose, and fucoidan) were purchased from Sigma (St. Louis, MO, USA). All other

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chemicals were guaranteed reagent-grade. We extracted fucoidan from *Laminaria japonica* as described previously (Zhang et al., 2003).

2.2 Hydrolysis of fucoidan

A sample of fucoidan (15.0 –20.0 mg) was dissolved in 2 mol/L trifluoroacetic acid (2.0 ml) in a 10 ml ampoule. The ampoule was sealed in a nitrogen atmosphere and incubated for 4 h at 110°C. Following incubation, the ampoule was cooled to room temperature. The reaction mixture was then neutralized to pH 7 with 2 mol/L sodium hydroxide, and we added 2.0 ml of the internal standard solution. The mixture was shaken well, diluted to 10 ml and filtered. The filtrate was retained for PMP determination.

2.3 Preparation of the monosaccharide reference solutions

An accurate amount of ribose (~1 mmol) was dissolved in water, diluted to 50 ml, and used as the internal standard solution. A mixture of mannose, rhamnose, glucuronic acid, glucose, galactose, xylose, and fucose (~0.1 mmol of each monosaccharide) was dissolved in water. We then added 5.0 ml internal standard solution into the solution. The mixture solution was then diluted to 50 ml, and retained for PMP derivation.

2.4 Derivatization with PMP

We added 0.5 mol/L methanolic solution of PMP (100 μL) and 0.3 mol/L aqueous sodium hydroxide (100 μL) to the monosaccharide reference solution or a reducing fucoidan solution (100 μL each). The mixture was incubated at 70°C for 30 min. The reaction mixture was then cooled at 8°C, and neutralized with 0.3 mol/L hydrochloric acid. We then added 1 ml of chloroform to the solution. The mixture was shaken well and centrifuged at 5 000 r/min for 10 min at 6–8°C. The chloroform layer was discarded and the aqueous layer was extracted twice with chloroform. The final aqueous layer was analyzed directly by HPLC.

2.5 Chromatography

Chromatographic conditions were generally as follows: column, YMC-Pack ODS-AQ (250×4.6 mm, 5 μm); temperature, 25°C; solvent A, 0.4% triethylamine in 20 mmol/L ammonium acetate buffer solution (pH 6.30 with acetic acid)-acetonitrile (9:1); solvent B, 0.4% triethylamine in 20 mmol/L ammonium acetate buffer solution (pH 6.30 with acetic acid)-acetonitrile (4:6); gradient, 10%–14% in 9 min, 14%–64% from 9 min to 30 min, 64% during the next 5 min at 1 ml/min. The eluate was monitored at 245 nm.

2.6 Calculation of monosaccharide contents

Using ribose as an internal standard, the correction factor for each monosaccharide was calculated using Equation (1):

$$f_{i/s}=(W_i/W_s)/(A_i/A_s) \quad (1)$$

where A_s and A_i are the peak areas of the internal ribose standard and the standard monosaccharide in the reference solution, respectively; W_s and W_i are the content of the internal ribose standard and the standard monosaccharide in the reference solution, respectively.

The content of every monosaccharide in the polysaccharide hydrolysis solution (W) was calculated with Equation (2):

$$W=f_{i/s}(A_i/A_s)W_s \quad (2)$$

where A_s and A_i are the peak areas of the internal standard ribose and the monosaccharide in the polysaccharide reducing solution, respectively; W_s is the content of the internal standard ribose in the polysaccharide hydrolysis solution.

3 RESULTS AND DISCUSSION

3.1 Optimization of the conditions for pre-column labeling

The monosaccharides were derivatized following the methods of Honda et al. (1989). However, care was taken to ensure that the aqueous phase had been fully neutralized prior to extraction with chloroform, and the neutralization and extraction process were conducted at the same temperature. At high pH the extractions were inefficient and significant amounts (>50%) of the PMP-sugars were also extracted (Strydom, 1994). At different temperatures, PMP-sugars have different distribution coefficients (Ma et al., 2002), which leads to instability in the correction factors. Based on our results, larger amounts of PMP-sugars are lost at higher temperatures. To ensure stability of the correction factor and successful removal of PMP, the neutralization and extraction processes were conducted at 8°C, and the centrifugation was conducted at 6–8°C.

3.2 Separation of standard PMP-sugars and PMP-hydrolyzed fucoidan sugars

Analysis of standard PMP-sugars by HPLC is shown in Fig.1a. The broad reagent peak was well

separated from all the PMP-sugars. Furthermore, we achieved good separation of all the PMP-sugars.

The separation of PMP-degraded monosaccharides from fucoidan is illustrated in Fig.1b. We also detected two additional peaks, Peak a and Peak b, located between Peak 1 and Peak 2. We hypothesize that these represent mannuronic acid and guluronic acid formed by degradation of alginate, an impurity.

3.3 Optimization of separation of monosaccharide derivatives

Initially, we separated the PMP-sugars under the conditions described by Rao et al., (2007). Accordingly, solvent A consisted of 15% acetonitrile and 20 mmol/L ammonium acetate buffer; solvent B consisted of 40% acetonitrile and 20 mmol/L ammonium acetate buffer; and the gradient was 0%–50% solvent B over 25 min. However, under these conditions we were not able to separate PMP-galactose, PMP-xylose, and PMP-fucose. Furthermore, the tailing peaks of the PMP-sugars resulted in the inaccurate calculation of some peaks, particularly for glucuronic acid. Therefore, based on the methods of Strydom (1994), we employed a very flat gradient in the initial 9 min and a steep gradient in the following 21 min. We also added 0.4% triethylamine into the mobile phase to alleviate the tailing peaks.

We evaluated the effect of the mobile phase pH on retention time (Fig.2). Retention time decreased as pH increased. However, we did not observe any change in the order of elution. At pHs below 6.8, PMP-galactose, PMP-xylose, and PMP-fucose were completely separated. However, when the pH was <6.3, PMP-glucose and PMP-galactose were not well separated. Thus, a pH range from 6.3 to 6.8 provided good separation of all the PMP-sugars. We observed

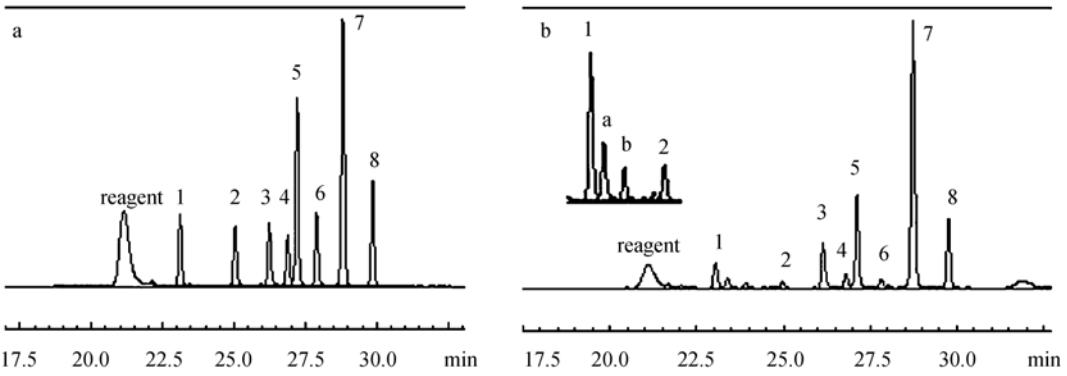

Fig.1 a: High performance liquid chromatographic (HPLC) separation of PMP-labeled monosaccharide standards; b: High performance liquid chromatographic (HPLC) separation of PMP derivatives of acid hydrolysates of polysaccharide

1. Mannose (Man); 2. Rhamnose (Rham); 3. Glucuronic Acid (GlcUA); 4. Glucose (Glc); 5. Galactose (Gal); 6. Xyiose (Xyl); 7. Fucose (Fuc); 8. Ribose (Rib)

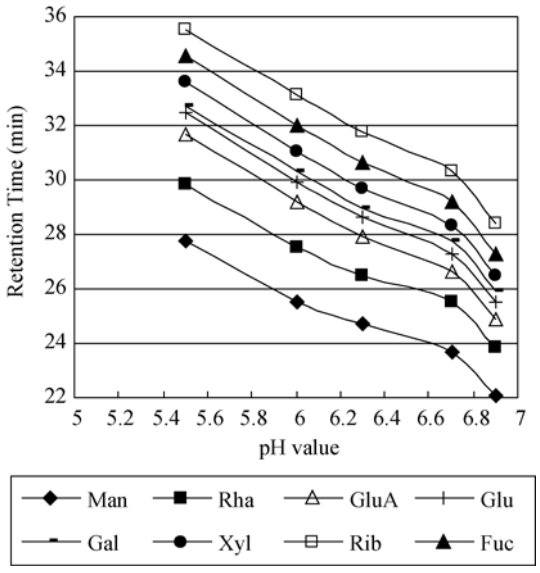

Fig.2 Chromatographic elution times of PMP-sugarsat different pH values of the eluent

an impurity peak immediately after the PMP-ribose peak in the magnified chromatogram obtained at pH 6.8. (Honda et al., 1991) concluded that this represented a mesityl oxide peak. This peak affected the calculation of the PMP-ribose peak area. Lower pH values further delayed elution of the mesityl oxide peak after the PMP-ribose peak. At pH 6.3, the mesityl oxide peak and PMP-ribose peak were well separated.

3.4 Selection and treatment of the internal standard

We obtained good separation between ribose-PMP and the other PMP-sugars (Fig.1a). Therefore, ribose-PMP may be used as an internal standard. However, care must be taken to ensure that the internal standard solution is added to the polysaccharide hydrolysis solution after it is neutralized with

2 mol/L sodium hydroxide. We hypothesized that the structure of ribose was changed during the process of polysaccharide reduction.

3.5 Verification of methodology

3.5.1 Standard curves

The standard curves for each sugar-PMP showed excellent linearity in the concentration range of 0.1–2.0 mmol/L (Table 1).

Table 1 Calibration curves for every PMP-monosaccharide

PMP-sugar	Standard curve	Correlation coefficient r^2 (n=5)
Mannose	y=9E+06x–127767	0.9997
Rhamnose	y=6E+06x–135965	0.9994
Glucuronic acid	y=7E+06x–347262	0.9997
Glucose	y=6E+06x–11798	0.9996
Galactose	y=8E+06x–103571	0.9997
Xylose	y=8E+06x–162561	0.9993
Fucose	y=7E+06x–121529	0.9995
Ribose	y=7E+06x–317346	0.9984

3.5.2 Reproducibility

Repeated determinations (n=5) of the same PMP-sugar sample yielded relative standard deviations of 1.150%, 1.357%, 2.34%, 1.251%, 1.216%, 1.159%, 1.122%, and 1.181% for PMP-mannose, PMP-rhamnose, PMP-glucuronic acid, PMP-glucose, PMP-galactose, PMP-xylose, PMP-fucose, and PMP-ribose, respectively. Thus, reproducibility was good for all PMP-sugars, with the exception of PMP-glucuronic acid.

3.5.3 Stability

We measured the same PMP-sugar sample 2, 4, 8, 12, and 24 h after it was neutralized with hydrochloric acid. The relative standard deviations of PMP-mannose, PMP-rhamnose, PMP-glucuronic acid, PMP-glucose, PMP-galactose, PMP-xylose, PMP-fucose and PMP-ribose were 2.29%, 2.59%, 6.16%, 2.45%, 1.99%, 1.70%, 2.03, and 1.12%, respectively. Thus, the PMP-sugars were stable, with the exception of PMP-glucuronic acid.

3.5.4 Recovery

A sample of fucoidan (~5.0 mg) was weighed into a 10 ml ampoule. We then added 1.0 ml of the monosaccharide standard solution and 4 mol/L trifluoroacetic acid. The mixture was then processed as described previously. We observed good recovery for rhamnose, glucose, galactose, and fucose (Table 2). Recovery of mannose was slightly lower, possibly because the calculation of its peak area was impacted by Peak a. Recovery of glucuronic acid was quite low, likely due to the poor reproducibility and stability of PMP-glucuronic acid. The low recovery of xylose resulted from the small adjacent impurity peak. Because the xylose content was very low, the impurity had a greater effect on the calculation of its peak area.

Table 2 Recovery experiment results of every monosaccharide in fucoidan (n=5)

Monosaccharide	Scope of recovery	Average recovery	RSD(%)
Mannose	0.82–0.88	0.86	2.64%
Rhamnose	0.94–0.96	0.95	1.00%
Glucuronic acid	0.57–0.66	0.63	5.93%
Glucose	1.01–1.04	1.02	1.23%
Galactose	0.92–0.97	0.95	2.34%
Xylose	0.65–0.70	0.67	5.03%
Fucose	1.02–1.08	1.05	1.85%

3.6 Analytical results of fucoidan

Using the method outlined above, we determined that the contributions of mannose, rhamnose, glucuronic acid, glucose, galactose, xylose, and fucose were: 2.32%, 0.61%, 5.25%, 1.40%, 8.29%, 0.16%, and 29.94%, respectively. The hydrochloric acid homocysteine-sulfuric acid method (Gibboms, 1955) yielded 28.70% fucose from the same sample, which was lower than the value obtained using HPLC.

4 CONCLUSION

We developed a simple, effective, and sensitive method to analyze the monosaccharide composition of fucoidan using precolumn derivation HPLC. We used the method to identify the monosaccharide composition and determine the monosaccharide contents in fucoidan. Determination of xylose and mannose may be impacted by impurities in fucoidan. However, their content is generally very low and they are not the primary active components. Thus, this method can be used for determination of monosaccharide contents in fucoidan. In addition, this method can be used for determination of monosaccharide contents in other algal polysaccharides by changing the pH value of the mobile phase.

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