JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Determination of Triterpene Glycosides in Sea Cucumber (*Stichopus japonicus*) and Its Related Products by High-Performance Liquid Chromatography

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A creative and sensitive method has been developed for the determination of triterpene glycosides concentrations in sea cucumber (*Stichopus japonicus*) and related products by using D-quinovose (6-deoxyglucose) as the measurement standard by reverse-phase high-performance liquid chromatography (HPLC) and variable-wavelength detection. D-quinovose, which is a unique monosaccharide in holostane triterpene glycosides, was liberated by acid hydrolysis and precolumn derivatized by 1-phenyl-3-methyl-5-pyrazolone (PMP). PMP−quinovose was analyzed by HPLC with 22% acetonitrile in 0.05 M KH_2PO_4 aquatic solution (pH 5.2) as mobile phase. The calibration curves of D-quinovose were linear within the range of 6.56−164 mg/L ($r^2 > 0.995$). The contents of triterpene glycosides in various *S. japonicus* products were determined after appropriate pretreatment methods. The concentration of triterpene glycosides was calculated by the formula $C = C_{qui} \times \alpha$ ($\alpha = 8.5$). The result showed that this method was a simple, rapid, and stable method for the determination of triterpene glycosides in *S. japonicus* products.

KEYWORDS: Sea cucumber; *Stichopus japonicus*; triterpene glycoside determination; 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization; high-performance liquid chromatography (HPLC)

INTRODUCTION

Sea cucumber (*Stichopus japonicus*) belongs to the Echinodermata phylum. It has been a traditional tonic food in China and other Asian countries for thousands of years. It is common in shallow waters along eastern Asian coasts and has become the most important cultured aquatic species in recent years. It is well-known that *S. japonicus* is of high market value, and it has great global production and world trade. (*1, 2*) With the development of the food industry, various kinds of nutriments and tonic products are now made of *S. japonicus*, including dried products (beche-de-mer), pickled products, water-risen products, oral liquids, capsules, et al. Triterpene glycosides, the most important secondary metabolites and bioactive compositions of sea cucumbers, are proven to exhibit various biological activities including antifungal, cytotoxic, hemolytic, cytostatic, and immunomodulatory effects. (*3, 4*) They are a very important index for the nutrition evaluation and quality control of sea cucumber products. But so far there has not been a suitable method for the determination of these active compounds in sea cucumbers. Triterpene glycosides or saponins distributed in other organisms were always determined by spectrophotometry, (*5, 6*) chromatography (including TLC, HPLC-ELSD, LC-ESI/MS, LC-UV-NMR, LC-PDA/ESI/MS/MS, et al.), (*7–10*) and capillary electrophoresis (CE). (*11*) These traditional methods always need special saponin compounds to identify the chromatogram peaks or to be used as measurement standards. Therefore, many plant or animal saponins determination methods were complicated by the preparation of standard compounds. These shortcomings limit the applications of these traditional methods.

Triterpene glycosides from the sea cucumber *S. japonicus* collected in different areas consist of the same components, although the relative quantities of these components within a fraction will possibly vary. (*12*) In analyses of literature data, the glycosides isolated from sea cucumber (*S. japonicus*) mainly include holotoxins A, holotoxins B, holotoxins A_1, and holotoxins B_1 (**Figure 1**). (*12–14*) They are all related to holostane glycosides with an 18(20)-lactone and a sugar chain composed of six monosaccharide units. D-quinovose is a unique monosaccharide in holostane glycosides located in the second position of the sugar chain. Every molecular triterpene glycoside contains one D-quinovose unit, and it is not present in other compositions of *S. japonicus*. (*15–18*) Therefore, the concentration of D-quinovose could represent the concentration of triterpene glycosides in *S. japonicus* products.

The monosaccharide determination method with 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization by reverse-phase high-performance liquid chromatography (HPLC) (*19, 20*) or CE (*21, 22*) has more recently been proposed. The methodology of detection by HPLC-UV has been successfully employed in

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10.1021/jf800893r CCC: $40.75 © 2008 American Chemical Society
Published on Web 06/17/2008

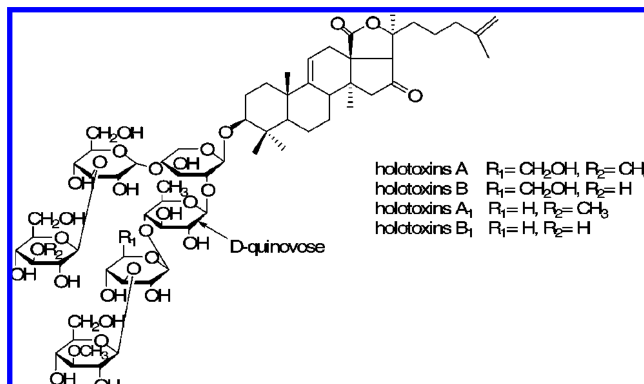

Figure 1. Chemical structures of holotoxins A, holotoxins B, holotoxins A_1, and holotoxins B_1, the four main triterpene glycosides isolated from *S. japonicus*.

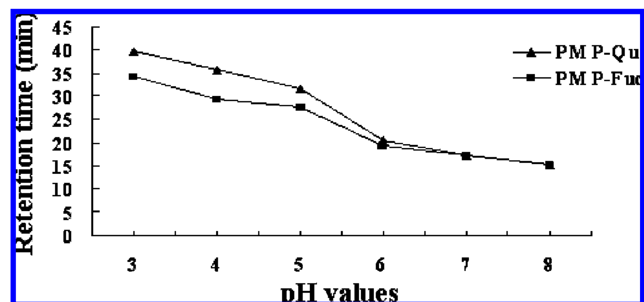

Figure 2. Elution times of PMP−fucose and PMP−quinovose at different pH values of phosphate buffer solution.

many laboratories and proven to be effective, sensitive, reproductive, and accurate. (*23, 24*) We applied this method for the determination of D-quinovose to decide the concentration of triterpene glycosides in sea cucumber (*S. japonicus*) products. An optimized chromatographic condition was established to make a good separation of PMP−quinovose from other sugars in sea cucumber samples. The pretreatments for different kinds of products are also discussed here to enhance the HPLC analysis peak of PMP−quinovose and minimize the interferences of other compositions.

MATERIALS AND METHODS

Standards and Reagents. D-quinovose was purchased from Sigma Chemical (St. Louis, MO). Acetonitrile and methanol were of HPLC grade (Burdick & Jackson, Muskegon, MI). Ultrapure water was obtained from a Milli Q-System (Millipore, Bedford, MA). 1-phenyl-3-methyl-5-pyrazolone (PMP) was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and was recrystallized from methanol before use. Ethanol, *n*-butanol, and trifluoroacetic acid (TFA) were of analytical grade.

Derivatization with PMP. A volume of 250 μL of standard D-quinovose solution was derivatized with 250 μL of PMP (0.5 M in methanol) and 250 μL of 0.3 M NaOH at 70 °C for 30 min. After being cooled to room temperature, the derivatives were neutralized with 250 μL of 0.3 M HCl solution and extracted three times with 1 mL of chloroform to remove the excess reagent. The aqueous layer was analyzed directly by HPLC.

Chromatography Conditions. An Agilent 1100 Series (Palo Alto, CA) liquid chromatograph equipped with an Agilent G1314A variable-wavelength detector (VWD) and an Agilent ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, particle size 5 μm) was used for the analysis of PMP−quinovose. The flow rate was set at 1.0 mL/min, the column temperature was 25 °C, the wavelength for VW detection was 250 nm, and the injection volume was 20 μL. The mobile phases were an isocratic condition of a mixture of acetonitrile and 0.05 M potassium dihydrogen phosphate (KH_2PO_4). The pH value of the KH_2PO_4 solution was adjusted by adding 0.3 M NaOH solution. The volume ratios of acetonitrile to phosphate buffer and the pH level of phosphate buffer, which had a great effect on the variation of elution times of PMP−sugars, (*24*) are discussed in this experiment to optimize the separation of PMP−quinovose from other PMP−sugars in *S. japonicus*, especially for fucose, which is an isomer of quinovose.;0?>

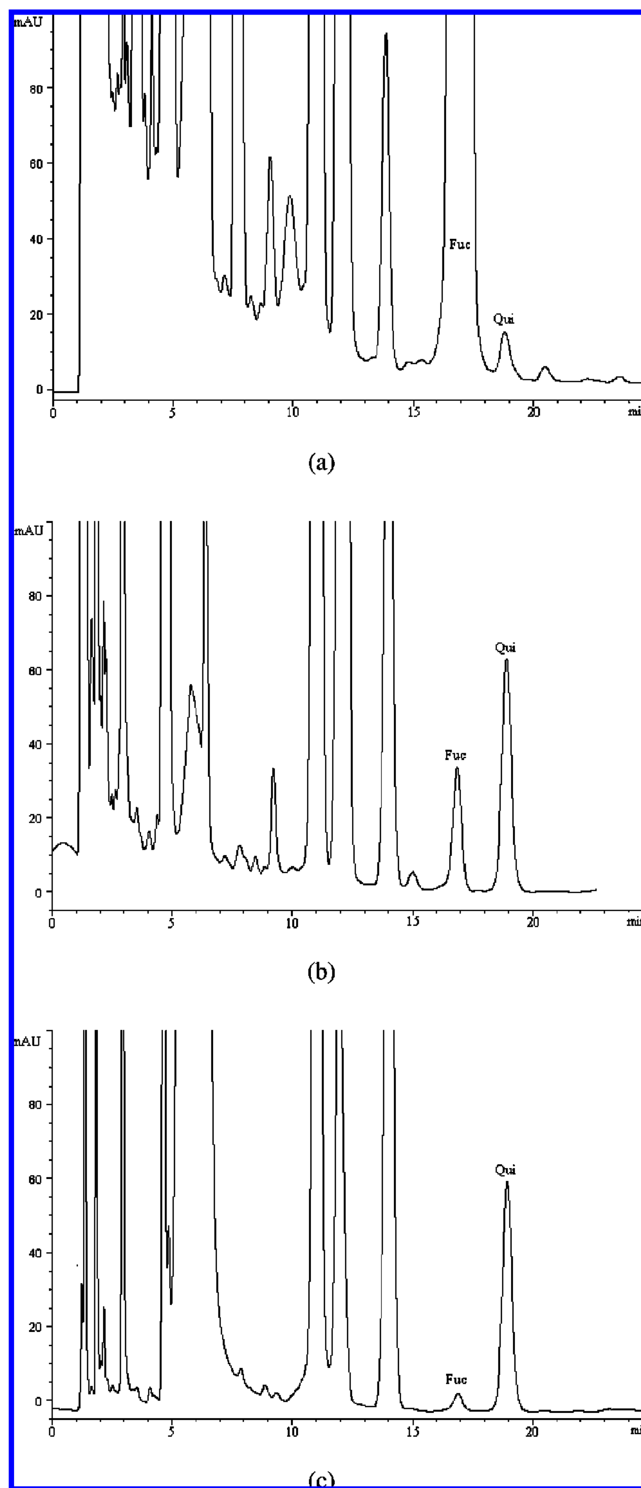

Figure 3. Chromatograms of PMP derivatives of the dried *S. japonicus* hydrolysate using different pretreatments: (**a**) without pretreatment, (**b**) extracted by 60% ethanol, and (**c**) extracted by 60% ethanol and partitioned by *n*-butanol. Experimental conditions: column, Agilent ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm,5 μm); mobile phase, 0.05 M phosphate buffer (KH_2PO_4, pH 5.2) with 22% acetonitrile; flow-rate, 1.0 mL/min; column temperature, 25 °C.

Table 1. Different Pretreatment Methods on Dried *S. japonicus*

	non-pretreatment	60% EtOH[a]	60% EtOH + *n*-butanol[b]
yields of dry solids content (%)	100	38.6	2.7
PMP−Fuc/PMP−Qui	311.44	0.473	0.0618
triterpene glycosides content	0.107 ± 0.0018%	0.0947 ± 0.0022%	0.099 ± 0.0068%

[a] Dried *S. japonicus* with pretreatment extracted by 60% EtOH. [b] Dried *S. japonicus* with pretreatment extracted by 60% EtOH and partitioned between water and *n*-butanol.

Table 2. Concentrations of Triterpene Glycosides in *S. japonicus* Products

	D-quinovose concentration (%)	triterpene glycosides concentration (%)
pickled product	0.00116	0.00984
capsule A	0.32	2.72
capsule B	0.00163	0.0139
tonic oral liquid	0.0000891	0.000757
wine	nd[a]	nd[a]

[a] Not detected.

Validation of the Quantitative Method. Standard D-quinovose solutions at nine concentration levels between 6.56 and 164 mg/L were derivatized and analyzed by HPLC, as described above. The calibration curve was obtained by peak areas of PMP−quinovose against the concentration of standard solutions (mg/L). Every concentration level was analyzed in three replications. The limit of quantification (LOQ) and limit of detection (LOD) values were calculated by determining the signal-to-noise ratios (S/N) of the lowest measured concentrations and extrapolating to the S/N values of 10 and 3, respectively. Recovery was tested by the standard addition procedure using two addition levels (16.4 and 125 mg/L).

Samples and Pretreatments. Dried sea cucumbers *S. japonicus* (beche-de-mer) collected from Japan, pickled *S. japonicus* products, *S. japonicus* active capsules A and B produced by two companies, *S. japonicus* tonic oral liquid, and *S. japonicus* wine were all purchased from the local market in Qingdao, China.

In order to enrich the glycosides compositions and reduce the interference of other components in the products, the sea cucumber products were pretreated with different methods before hydrolysis.

Dried sea cucumbers were pretreated with two simple methods together with a nontreatment sample to compare the effects of these treatments on the determination result. Six whole dried sea cucumbers (*S. japonicus*) were powdered. The first share with 500.8 mg of *S. japonicus* powder was directly hydrolyzed by 2 mL of 2 M TFA. The second share with 2.79 g of *S. japonicus* powder was extracted by 60% ethanol (20 mL/g dry powder) five times at 90 °C, the extract was concentrated, and the residue was ready for hydrolysis. The third share with 2.80 g of *S. japonicus* powder was extracted by 60% ethanol as described above, the ethanol was evaporated, and the residue was partitioned between water and *n*-butanol (20 mL/g dry powder) five times. The *n*-butanol extract was combined and evaporated to dry; the residue was then ready for hydrolysis.

Pickled products were processed by boiling fresh *S. japonicus* with a saturated salt solution. One whole pickled sea cucumber (22.3 g) was dried to 4.7936 g (water content was close to 80%) and powdered, then extracted by 60% ethanol (20 mL/g dry powder) five times at 90 °C. The extract was concentratated and hydrolyzed.

A mass of 1.2192 g of *S. japonicus* active capsule A which contained dried *S. japonicus* powder was extracted by 60% ethanol five times at 90 °C, and then the concentrated residue was ready for acid hydrolyzation. A mass of 1.1748 g of capsule B which contained *S. japonicus* powder, aloe powder, etc. was extracted by 60% ethanol and *n*-butanol five times, and then the extract was concentrated and hydrolyzed.

A volume of 100 mL of *S. japonicus* tonic oral liquid processed with *S. japonicus*, medlar, and erythritol was partitioned between water and an isocratic volume of *n*-butanol five times. The *n*-butanol extract was evaporated to dryness and was then ready for hydrolysis.

A volume of 100 mL of *S. japonicus* wine was evaporated to remove the ethanol. The residue was partitioned between water and an isocratic volume of *n*-butanol five times and was then ready for hydrolysis.

The pretreated samples above were hydrolyzed by the treatment of 2 mL of 2 M TFA at 120 °C for 2 h. (*25, 26*) D-quinovose, which was employed as the measurement standard, was liberated from the triterpene glycosides by acid hydrolysis. The hydrolyzed solutions were neutralized by 5 M NaOH solution and extracted three times with 1 mL of chloroform to remove the aglycon parts of the glycosides. The aqueous layers were complemented to a volume of 5 mL by the addition of water. Then the hydrolyzed sample solutions were derivatized with PMP and analyzed by HPLC, as described above.

Calculations. The concentrations of triterpene glycosides in *S. japonicus* products were calculated by the formula $C = C_{qui} \times \alpha$, where C_{qui} was the concentration of D-quinovose in products which could be calculated by the peak area of PMP−quinovose and the calibration curve. The value of α was defined as the ratio of the average molecular weight (M_w) of triterpene glycosides in *S. japonicus* against the molecular weight of D-quinovose. The triterpene glycosides isolated from sea cucumber (*S. japonicus*) mainly include holotoxins A (M_w = 1422), holotoxins B (M_w = 1408), holotoxins A$_1$ (M_w = 1392), and holotoxins B$_1$ (M_w = 1378). (*12–14*) Although their relative quantity would possibly vary, (*12*) the average molecular weight could be calculated as approximately 1400 Da. Therefore, the value of $\alpha = 1400/164 \approx 8.5$.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions. In previous studies, (*27–29*) a phosphate buffer consisting of acetonitrile was often used to separate the PMP−sugars. The volume ratios of acetonitrile to phosphate buffer and the pH level of the mobile phase, which had great effects on the retention and separation of the PMP derivatives of D-quinovose and other sugars in *S. japonicus*, are discussed here.

The monosaccharides of *S. japonicus* were mainly composed of galactosamine (GalN), glucosamine (GluN), glucuronic acid (GalUA), mannose (Man), galactose (Gal), and fucose (Fuc). (*30–33*) Fucose (6-deoxy-galactose), an isomer of quinovose, was the most important interference in the separation of PMP−quinovose. The variation of elution times of PMP−fucose and PMP−quinovose as a function of mobile phase pH with 20% acetonitrile are shown in **Figure 2**. PMP−Fuc and PMP−Qui could not be separated when the pH values were higher than 7 and were then found as a broad single peak in the chromatogram. When the pH = 6, PMP−Fuc and PMP−Qui were separated slightly to exhibit shoulder peaks. And a good separation would be gained by a lower pH value mobile phase. However, a lower pH value would increase the elution time of the PMP−sugars. Therefore, the highest pH value which could separate PMP−Fuc and PMP−Qui well should be applied to the mobile phase. Then, the pH values of phosphate buffer were tested between 5 and 6, and a pH value 5.2 was finally chosen. In order to shorten the HPLC analysis time, the volume ratios of acetonitrile were increased to 22% and the retention time of PMP−Qui was shorten to 18.9 min as a result. According to the results above, the mobile phase was confirmed as 0.05 M phosphate buffer (KH_2PO_4, pH 5.2) with a 22% acetonitrile composition.

Validation of the Quantitative Method. A series of calibration standard solutions were injected into the analytic column under selected conditions to test the linearity of the calibration graphs for D-quinovose after the derivatization with PMP. The corresponding calibration curve of the standard PMP−Qui peak areas against the standard D-quinovose solution concentrations (mg/L) was obtained in the range of 6.56−164 mg/L. The

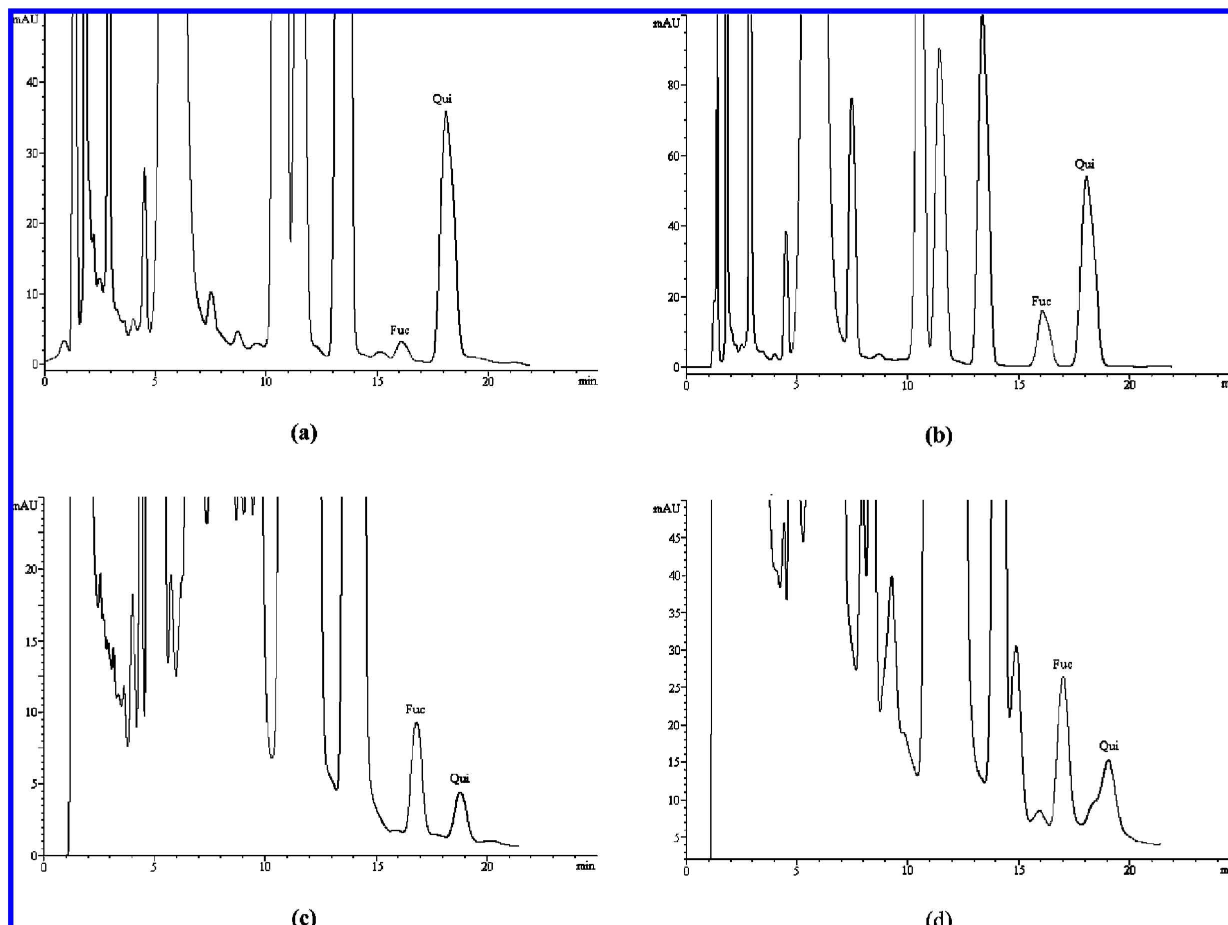

Figure 4. Chromatography of *S. japonicus* products: (**a**) pickled *S. japonicus*, (**b**) active capsule A, (**c**) active capsule B, and (**d**) tonic oral liquid.

correlation coefficient (r^2) was more than 0.995 ($y = 28.391x + 6.4485$), indicating a good linearity. The relative standard deviation (RSD) of the response factors was less than 5.0% in all cases, ranging from 0.90 to 4.76%.

Six replicate determinations of D-quinovose standard solutions at three levels of concentration (6.56, 32.8, and 164 mg/L) were performed to evaluate the method's reproducibility. The RSD values obtained from all determinations were less than 4% (3.01, 1.95, and 2.74%). The results demonstrated that the method was reproducible for the detection.

The LOD and LOQ values of D-quinovose were calculated as 0.132 and 0.52 mg/L, respectively. Taking into consideration the coefficient α which was defined in the Calculations section, the LOD and LOQ values of the triterpene glycosides in *S. japonicus* were 1.122 and 4.42 mg/L, respectively.

After derivatization, the peak areas of PMP−Qui (65.6 mg/L) were supervised every 2 h for 24 h, while the samples were stored at room temperature. The RSD of ($n = 13$) of the PMP−Qui peak area values was determined as 4.33%. The stability results indicated that the analysis method could be considered stable for the determination stay overnight at room temperature.

Recovery was tested by the standard addition procedure using two addition levels (16.4 mg/L and 125 mg/L) in dried *S. japonicus* sample. Three replicas were carried out for each addition level. The low concentration recovery was 111% ± 10.9%, and the high concentration recovery was 103.53% ± 1.93%.

Sample Pretreatments. Figure 3a−c shows the analysis chromatograms of dried *S. japonicus* with two pretreatment methods together with a nontreatment sample. Compared with the chromatogram of dried *S. japonicus* without pretreatment that was hydrolyzed directly (**Figure 3a**), the extraction procedure by 60% ethanol (**Figure 3b**) and the partitioned procedure between water and *n*-butanol (**Figure 3c**) were both able to enrich the triterpene glycosides in the sample matrix. The enrichment could also be proved by the yields of dry solids content after extraction or partition (**Table 1**). The *n*-butanol extracted solids contained triterpene glycosides equal to only 2.7% of the dried organism content, and more than 90% of the other composition was eliminated. Fucose was one of the major monosaccharides of *S. japonicus* polysaccharide, (*27–29*) which would be the primary influence for the determination of PMP−Qui; therefore, the peak area ratios of PMP−Fuc/PMP−Qui shown in **Table 1** would represent the elimination of the influence of the compositions in the sample matrix. The results (**Table 1**) indicated that the PMP−Fuc/PMP−Qui ratio values were significantly reduced after either pretreatment procedure. It was confirmed that the triterpene glycosides could be enriched after pretreatments. Although without pretreatments, PMP−Qui could also be separated from other PMP−sugars in dried *S. japonicus*, appropriate pretreatments were also recommended especially for some *S. japonicus* products with very low triterpene glycosides concentrations; these procedures could reduce the column efficiency decrease caused by other complicated compositions in the sample matrix as well. The determination results of the triterpene glycosides in dried *S. japonicus* with different pretreatment procedures shown in **Table 1** were calculated by the calibration curve and α value described above. The triterpene glycosides concentrations of pretreated samples were between 84.4 and 100.2%, compared with those of the nontreatment samples. This indicated that the pretreatments described above would not

significantly affect the determination results. After all, the pretreatments by 60% ethanol and *n*-butanol extractions would be simple and suitable methods to enrich the triterpene glycosides and advance the separation of PMP−Qui from other PMP−sugars in *S. japonicus* samples.

Applications to *S. japonicus* Products. Pickled *S. japonicus* products, two *S. japonicus* active capsules (A and B) produced by different companies, *S. japonicus* tonic oral liquid, and *S. japonicus* wine were analyzed to extend the utility of this determination method. The chromatograms are shown in **Figure 4**, and the triterpene glycosides concentrations of *S. japonicus* products are presented in **Table 2**. All the PMP−Qui peaks had good separation from the other PMP−sugars of the tested products employing some simple pretreatments except for the oral tonic, which has more complicated compositions. Possibly other pretreatments should be applied to the oral tonic to remove the shoulder peak of PMP−Qui (**Figure 4d**). The triterpene glycosides concentrations were varied in different processed products. The concentrations found in pickled product and tonic oral liquid were much lower than those of dried *S. japonicus* and capsules, and this can be attributed to the high water content in these products. Otherwise, neglecting the water composition, the concentration of triterpene glycosides in pickled *S. japonicus* (0.0458%) was still lower than that of dried products (0.107%). It may be caused by the loss of glycosides during the boiling process with exposure to a saturated salt solution several times, and the salt in the products could also diminish the triterpene glycosides concentration. Two brands of capsules are analyzed in this paper, and the concentrations were much different. The possible reason was that capsule B had many other added materials (such as the aloe powder revealed in the instructions), and the concentration of *S. japonicus* would be decreased. Triterpene glycosides were not detected in the tested *S. japonicus* wine (the chromatogram was not given). The applications to several *S. japonicus* products purchased from a market indicated that the triterpene glycosides determination method established in this paper was simple and applicable. All these results are reported for the first time. This would be an applicable method for the triterpene glycosides determination, nutrition estimation, and quality control of sea cucumber food, tonics and drugs, and so forth.

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Received for review January 20, 2008. Revised manuscript received April 28, 2008. Accepted May 5, 2008. This work was supported by the National Natural Science Foundation of China (No. 30471319), the Program for New Century Excellent Talents in University (NCET-04-0642), the Open Test Fund of the Ocean University of China (No. 05009), and the National High-tech Research and Development Project of China (2007AA091805).

JF800893R