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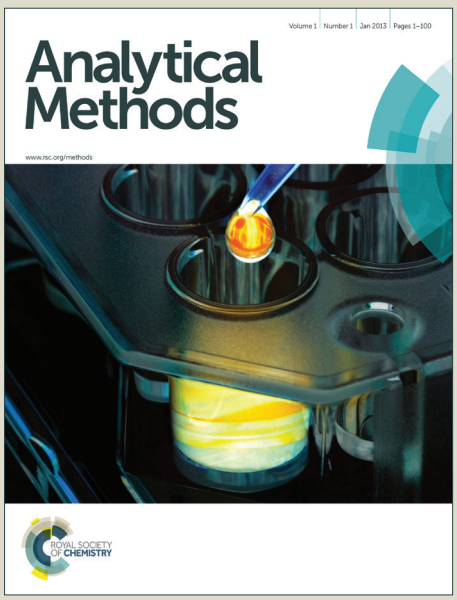

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Identification of dried shark fins by seven sugars analysis using pre-column derivatization high performance liquid chromatography and stable carbon isotope ratio analysis

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ABSTRACT

A simple and sensitive method of pre-column high performance liquid chromatography (HPLC) was developed for the simultaneous determination of seven sugars, which was also applied for the identification of dried shark fins preliminarily. In the meantime, $\delta^{13}C$ values of dried shark fins samples also were determined by element analyser-isotope ratio mass spectrometry (EA-IRMS). The dried shark fins were decomposed by enzymolysis of papaya protease and amylase enzyme, then samples were analysed by HPLC after the derivatization of enzymatic hydrolysate, using 1-phenyl-3-methyl-5-pyrazolone (PMP) as derivating agent. The results indicated that seven sugars were well separated by this method. When the derivatization time of 75 minutes and the phosphate buffer-acetonitrile (80 : 20, *v/v*) mobile phase system (pH = 6.8) were applied, the detection results and separation effects of seven standard substances can be the best. Moreover, the peak areas of two kinds of uronic acid derivative products were decreased significantly ($P < 0.01$) along with the increase of time, so it is necessary to perform the HPLC analysis within 24 h after the derivatization reaction. The developed method is suitable for the identification analysis of artificial dried shark fins with good accuracy, reproducibility and sensitivity. In addition, $\delta^{13}C$ values were highly significant ($P < 0.01$) within real dried shark fins and fake dried shark fins. Thus, the combination of these methods could potentially be useful for dried shark fins identification, no matter fake dried shark fins or artificial dried shark fins.

Keywords: Dried shark fin; Pre-column derivatization; High performance liquid chromatography (HPLC); 1-phenyl-3-methyl-5-pyrazolone; Sugar; Identification; Stable carbon isotope ratio

Introduction

Dried shark fins, made by dried fins of sharks or rays, which especially consist of filament cartilage with fibrous protein collagens, are traditionally used in soups served at important occasions.[1-3] Generally, the main ingredient in dried shark fins is protein. In addition, they also contain rich essential amino acid and a small amount of fat, sugar and necessary minerals for human body. Hence, dried shark fins have very high medical and medicinal value, which makes them a highly prized commodity. The biggest market for dried shark fins is China, especially Hong Kong and Guangdong province. For decades, Hong Kong has been the center of the world trade in dried shark fins handling between 50% and 85% of global dried shark fins imports from at least 85 countries.[4-7] In general, the larger the fin and higher fin needle content (collagen fibers), the more expensive the dried shark fins. Unfortunately, whenever price differences exist, there is also a potential for those dishonest traders to attempt to make money by passing off the cheaper product as the more expensive one, resulting in extremely widespread of counterfeit dried shark fins. Consequently, although the adulteration and counterfeit of dried shark fins are not injurious to health, problems of dried shark fins fraud negatively influence market growth by damaging consumer confidence.[8-9]

Under this kind of circumstance, appropriate methods in identifying fake from real dried shark fins to ensure a fair competition among producers and to protect consumers against fraud are extremely required. Traditional identification method mainly depends on the accumulation of experience, which has a great deal of subjectivity and uncertainty. In recent years, lots of experts and scholars have tried to apply some new methods to identify dried shark fins and great achievements were obtained. Polymerase chain reaction (PCR) and SYBR Green PCR techniques were applied in authenticity identification of composition of sharks in food, these methods, in addition, can also be used in species identification of sharks.[10-14] Interestingly, the method of attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was established, which could perform the undamaged and rapid

identification of dried shark fins, and can be used to evaluate the quality of dried shark fins, moreover, for those fake dried shark fins made by leftover material of sharks, which cannot be judged by PCR technique, this method can be applied to clearly differentiate real dried shark fins with them, thus can make up the defect of PCR method.[15] Generally speaking, these methods mentioned above could play a certain role in identification of dried shark fins, however, as the dried shark fins are multifarious and disorderly, and the specificity differences between the samples are obvious, which can hardly form routine and daily distinguishing standard.

Generally, carbohydrate compounds are lack of characteristics of ultraviolet absorption. In order to improve the sensitivity of carbohydrate compounds in high performance liquid chromatography (HPLC) examination, the derivatization method, which makes them into derivatives that have the ultraviolet absorption or fluorescence, was adopted. As it can react with reducing sugar quantitatively under mild conditions and without stereoisomerism products, 1-benzene-3-methyl-5-pyrazolone (PMP) has been successfully applied to the analysis of monosaccharide composition of polysaccharide compounds.[16] The reaction scheme of PMP with reducing sugars (Glu as an example) was illustrated in Fig.1. Shark fins not only contain acid mucopolysaccharide and small amounts of glucan, which can be enzymolysised, under certain conditions, into glucose, in addition, but also have amino sugars and uronic acids.[17] Generally, the carbon isotopic composition of plant materials strongly depends on the carbon fixation process such as the C-3, C-4 or crassulacean acid metabolism (CAM) cycle,[18] therefore, characteristics of the isotopic compositions have been widely used to investigate the authenticity of food materials or the animals feed on these plants.

In this work, pre-column derivatization high performance liquid chromatography (PD-HPLC) method was developed to simultaneously determine five saccharides and two uronic acids in dried shark fins, and the method was also applied for the identification of dried shark fins by detecting the differences of derivative products of real and fake dried shark fins. In the meantime, $\delta^{13}C$ values of dried shark fins samples were determined by EA-IRMS, and the carbon isotope ratio analysis was also

used in the identification of dried shark fins. The combined methods are suitable for the identification analysis of artificial dried shark fins and fake dried shark fins with good accuracy, reproducibility and sensitivity.

Materials and methods

Instruments, Reagents, and Materials

The Agilent 1200 HPLC coupled with an ultraviolet detector was obtained from the Agilent Company, USA. The centrifuge, vortex mixer, and Milli-Q Gradient system were obtained from LD5-2A (Jingli, Beijing, China), MS3 (IKA, Germany), and Millipore (Bedford, USA), respectively.

For stable isotopic ratio analysis, all samples were determined by EA-IRMS using a DELTA V PLUS IRMS (Thermo Electron Corporation) interfaced with a Flash 2000 EA (Thermo Electron Corporation) to determine carbon isotope ratios. Both samples and standard materials were measured after the balance of reference gases, the standard deviations (SD) of reference gas, CO_2, were less than 0.06‰ (n = 10).

Ultrapure water (18.2 MΩ) was obtained from a Milli-Q system (Millipore, Bedford, USA). Glucose (Glc, ≥95.0%), lactose (Lac, ≥95.0%), galactose (Gal, ≥95.0%), trehalose (Tre, ≥95.0%), mannose (Man, ≥95.0%), glucuronic acid (GlcUA, ≥96.0%), galacturonic acid (GalUA, ≥95.0%) were purchased from Dr. Ehrenstorfer, Germany. Derivatization reagent of PMP was obtained from Aladdin and papain and amylase were purchased from Sigma, USA. Methanol and acetonitrile (HPLC-grade) were obtained from the Fisher Company, USA.

Standard substances and Samples

13 dried shark fins standard substances were obtained from Guangzhou Dried Seafood & Nut Industry Association, China. The other 51 dried shark fins samples were purchased from dry cargo market of Guangzhou, China. All standard substances and samples were dried in an oven for 8 h (45℃) and then were ground to a fine powder in the mortar before analysis.

Enzymolysis of dried shark fins

Powdered sample of 0.50 g was accurately weighed into a 10 mL glass centrifuge tube,

then 5 mL of the 0.2 mol/L Tris-HCl buffer solution (pH = 6.9) were added into the tube and the powder were uniformly dissolved into which by vortexing. The 10 g/L amylase and papain prepared by the 0.05 mol/L Tris-HCl buffer solution (pH = 6.9), were also added into the tube and mixed by vortex shaking. The sample solution was enzymatic hydrolysised more than 12 hours under 45℃ to ensure the enzymolysis of samples completely. After vortexing for 2 minutes and centrifuging at 12846 *g* for 5 minutes, the liquid supernatant was passed through a 0.22 μm nylon filter and was finally transferred into a sample tube for the next step.

Preparation of derivates of standard substances and samples

200 μL of 20 mmol/L standard substances and sample solution obtained above were transfered into 10 mL glass centrifuge tubes, respectively. Then 200 μL PMP-methanol solution (0.5 mol/L) and 200 μL sodium hydroxide solution (0.3 mol/L) were added and homogenized for an additional minute. The solution were put into the water bath of 70℃ for 30-105 minutes and neutralized by 200 μL of 0.3 mol/L hydrochloric acid solution. The solution was extracted subsequently using trichloromethane, after vortexing and centrifuging, trichloromethane under layer was discarded. This step was repeated five times and the solution upper layer was finally transferred into sample bottles for the HPLC analysis.

HPLC instrumentation and chromatographic conditions

HPLC analysis was performed on an Agilent 1200 System equipped with a PDA detector and a Diamonsil C18 column (250 × 4.6 mm, 5 μm). All derivatized monosaccharide, disaccharide and uronic acid were quantified on a PDA detector at 250 nm. Ultrapure water with 0.1% formic acid-acetonitrile (80 : 20, *v/v*), 20 m mol/L ammonium acetate-acetonitrile (80 : 20, *v/v*), phosphate buffer (pH = 6.8)-acetonitrile (80 : 20, *v/v*) were used as the mobile phase, respectively, with flow rate of 1.0 mL/min and oven temperature was maintained at room temperature. The effects of these three mobile phase systems on the separation of all target substances were investigated and the optimal mobile phase system was chosen.

Standards of IRMS and $\delta^{13}C$ analysis

The δ notation was used to describe the isotopic difference between the sample and an

international standard, which was defined as the following formula (1),

$$\delta\ ^{13}C(‰) = (R_{sa} / R_{st} - 1) \times 1000 \qquad (1)$$

Where R_{sa} represented the isotope ratio ($^{13}C/^{12}C$) of the sample, and R_{st} was that of the reference standard substance. Variations in stable isotope ratios were reported as parts per thousand (‰) deviation from internationally accepted standards: Vienna Pee Dee Belemnite (V-PDB) for carbon isotope ratio. Each sample was analysed at least three times and the values were averaged and adopted for the results. In addition, the analysis was repeated if the difference between the two values was higher than 0.20‰ for $\delta^{13}C$ analysis. Moreover, for each run at least one in-house standard (casein) was analysed to check the accuracy of the analysis.

Each powdered dried shark fins was weighed 1 mg into a small tin capsule (3 mm × 2 mm × 5 mm). Then, the capsule was folded and compressed to contain the sample and minimise any air present. The prepared samples were introduced into the elemental analyser (EA) by an auto-sampler. The stable carbon isotopic composition was recorded in the delta (δ) notation relative to the VPDB standard. The CO_2 reference gas was calibrated against a casein reference material and was found to have a value of $\delta^{13}C = -26.98‰ \pm 0.15‰$ ($n = 10$).[19] The linearity region for the isotope amount ratio n ($^{45}CO_2$) / n ($^{44}CO_2$) as a function of the intensity of m / z = 44 was 4 to 10 v. Only analyses within this range were used in the final values.[20] Each sample was analysed a minimum of three times and the mean value was adopted.

Statistical analysis

Data were analyzed using SPSS (SPSS Inc., Chicago, IL, USA) and presented as mean ± SD with triplicates. Significance was determined at $P < 0.01$ by analysis of variance (ANOVA) followed by Duncan's least significant test.

Results and discussion

Optimization of chromatographic conditions

We first tested the mobile phase of ultrapure water with 0.1% formic acid-acetonitrile (80: 20, *v/v*). Since complete separation of the peaks was not possible, we orderly tested the mobile phase of 20 mmol/L ammonium acetate-acetonitrile (80: 20, *v/v*),

and phosphate buffer (pH = 6.8)-acetonitrile (80: 20, *v/v*), and fortunately encountered the best resolution and effective separation of the chromatographic peaks with the mobile phase of phosphate buffer (pH = 6.8) and acetonitrile (80: 20, *v/v*). Fig. 2 shows the typical chromatograms of saccharides and uronic acids standards in different mobile phase systems mentioned above.

Optimization of derivatization time

When saccharides and uronic acids were determined by pre-column derivatization method, the derivatization time is of importance to the yield of derivatization products, researchers have proved that the best derivatization time was generally from 30 minutes to 120 minutes. In this work, under the other conditions are the same, the effects of 30, 45, 60, 75, 90 and 105 minutes of derivatization time on the peak areas of standard substances were studied, and the results were shown in Fig. 3. As can be seen from Fig. 3, in the initial stage of derivatization reaction, the peak areas of derivatization products significantly ($P < 0.01$) increased along with the increase of derivatization time. When the derivatization time was 75 minutes, the peak areas of 7 derivatization products all reached maximum values. However, when the derivatization time was more than 75 minutes, the peak areas of derivatization products, on the contrary, decreased remarkably ($P < 0.01$). So, in the following experiment, the derivatization time was selected as 75 minutes.

Derivatizing agent PMP

In order to verify whether the derivatization reaction was complete or not, effects of the proportions (*v/v*) of standards solution (0.02 mol/L) to PMP (0.5 mol/L) on the peak areas of the derivatization products were investigated. The results were presented in Fig. 4 with the proportions of 2 : 1, 2 : 2, 2 : 3 and 2 : 4 (the proportions (*v/v*) of standards solution (0.02 mol/L) to PMP (0.5 mol/L) were 200 μL : 100 μL, 200 μL : 200 μL, 200 μL : 300 μL and 200 μL : 400 μL, respectively), respectively. As shown in Fig. 4, when the proportion (*v/v*) of standards solution (0.02 mol/L) to PMP (0.5 mol/L) decreased from 2 : 1 to 2 : 2, the peak areas of the derivatization products increased sharply, and the *P*-value analysis was highly significant ($P < 0.01$), which indicated that the PMP addition was highly significant based on the peak areas

of the derivatization products. When the proportion (*v/v*) of those decreased from 2 : 2 to 2 : 3 and 2 : 4, the peak areas of which also decreased, indicating that the peak areas presented significant differences ($P < 0.01$) as the proportions of standards solution and PMP changed.

Stability of the derivatization products

In order to investigate the stability of the derivatization products (The derivatization products were stored in a refrigerator with the constant temperature of 4□), the peak areas of HPLC analysis after the derivatization reaction of 1, 3, 5, 10, 20 and 60 days were presented in Fig. 5. As can be seen in Fig. 5, along with the increase of storage time, the peak areas of Gal, Man, Lac, Glc and Tre remained unchanged ($P < 0.01$), while those of GlcUA and GalUA decreased significantly ($P < 0.01$) and with the linear relations of $Y_1 = -1032 \ln(X_1)+11473$ ($R^2 = 0.945$) and $Y_2 = -1898 \ln(X_2)+11984$ ($R^2 = 0.941$), respectively. Hence, it is of importance to determine the derivatization products within 1 day to obtain precise results of GlcUA and GalUA.

Linearity and LOD

A series of mixed saccharides and uronic acids standard solutions were prepared. The series concentrations of which were set at 0.1, 0.5, 1.0, 5.0, 20.0 mmol/L. Under the HPLC conditions optimized above, the standard chromatogram was obtained and the linear equations were obtained by setting the peak areas of each target compound as ordinate (Y) but the corresponding molar concentrations as abscissa (X). The LODs were calculated by analyzing the spiked aqueous sample that underwent pretreatment and yielded a signal-to-noise ratio of 3 ($S/N = 3$). The equations of linear correlation, correlation coefficient, and the LODs of the target analytes are shown in Table 1, which indicates that the seven sugars presented favorable linearity with the correlation coefficient larger than 0.998 within the corresponding concentration range and LODs of 0.006-0.010 mmol/L and limit of quantitation (LOQ) ($S/N = 10$) of 0.020-0.033 mmol/L.

Recoveries and reproducibility

Negative samples at three spiked levels of analyte with 1, 5, 10 mmol/L mixed

standard substances were used to test the recoveries of analytes according to the proposed method with 6 identical samples tested at each concentration. The results indicated that the recoveries of seven sugars were satisfactory with values in the range of 98.0%-118.6% (Table 2). Moreover, relative standard diversities (RSDs, $n = 6$) of 3.21-6.32% were observed, which means the accuracy and precision can meet the requirements of analysis method.

Effects of enzyme concentrations on the peak areas of HPLC analysis

Effects of the enzyme concentrations on the peak areas of HPLC analysis were shown in Table 3. As can be seen in Table 3, the peak areas of Gala, Lac and Glu increased significantly ($P < 0.01$) along with the increase of enzyme concentrations, however, when the enzyme concentration increased to 2.0 mL of papain (10 g/L) and 2.0 mL of amylase (10 g/L), the peak areas of which reached to maximums, then with the increase of enzyme concentrations, the peak areas of which decreased markedly ($P < 0.01$). The results indicated that the enzymolysis reaction was complete with the enzyme concentrations of 2.0 mL papain and 2.0 mL amylase. Therefore, 2.0 mL papain and 2.0 mL amylase were chosen to perform the enzymolysis reaction.

Application to identification of practical samples

The HPLC method established in this work was adopted to determine the derivatization products of saccharides and uronic acids in dried shark fins, the results indicated that GalUA, Lac and Glc of 13 dried shark fins standard substances can be detected and with the concentration range of 0.104-1.481 mmol/L, 0.111-1.819 mmol/L and 0.101-1.016 mmol/L, respectively (Table 4). Then the analytical method described here was utilized to determine saccharides and uronic acids in 51 dried shark fins samples, and the results showed that 20 artificial shark fins can be easily distinguished by this method as GalUA, Lac and Glc cannot be detected in these shark fins (the chromatograms of a real shark fin sample and an artificial dried shark fin sample were presented in Fig.6). Unfortunately, GalUA, Lac and Glc can be detected in the other 31 dried shark fins and their concentrations were in the range of the dried shark fins standard substances. So, it is inapplicable to use this method to identify those fake dried shark fins made by leftover material of shark.

δ^{13}C analysis of 64 dried shark fins samples

In order to differentiate the dried shark fins samples, the method of EA-IRMS was established to analyse the carbon isotopic composition of dried shark fins. In fact, the δ^{13}C values of all the samples were detected by EA-IRMS, using casein as the standard of carbon isotope (δ^{13}C value =—26.98‰). The determination results were presented in Table 5. As shown in Table 5, the δ^{13}C values of real dried shark fins, dried shark fins identified real, dried shark fins identified fake and artificial dried shark fins were —14.231 ± 1.204‰, - 14.456 ± 0.981‰, —17.247 ± 0.621‰ and —14.561 ± 1.016‰, respectively. The *P*-value analysis was highly significant ($P < 0.01$) between two sets of real dried shark fins and fake dried shark fins, indicating that the fake dried shark fins made by leftover material of sharks can be easily differentiated from real dried shark fins, making the stable carbon isotope ratio an additional way in the identification of fake dried shark fins from real dried shark fin.

Conclusion

A pre-column high performance liquid chromatography (HPLC) method was developed for the determination of seven sugars. The developed method was applied in the identification of dried shark fins. The results indicated that this method is of importance in distinguishing the artificial dried shark fins from the real dried shark fins. Combined this HPLC method with the stable carbon isotope ratio analysis, both the artificial dried shark fins and the fake dried shark fins made by leftover material of shark can be identified.

Author contributions

[§]The first two authors contributed equally to this work.

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Fig. 1 Reaction scheme of PMP with reducing sugars (Glu as an example)

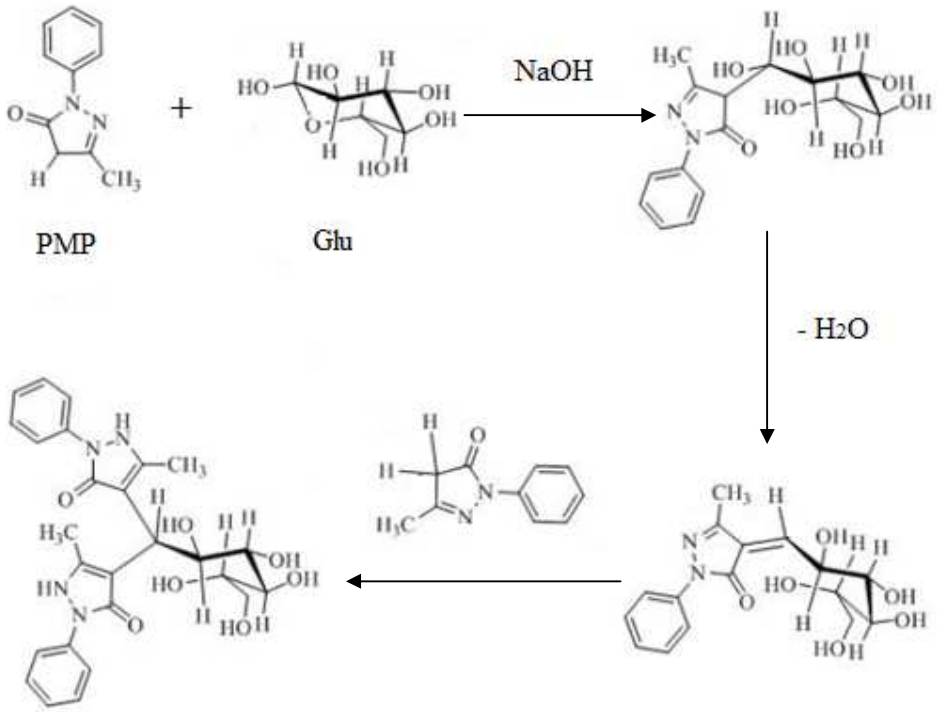

Fig. 2 Typical chromatograms of standard substances by HPLC method in different mobile phase systems. (1-7 stand for Man, GluUA, GalUA, Lac, Glu, Gal and Tre, respectively)

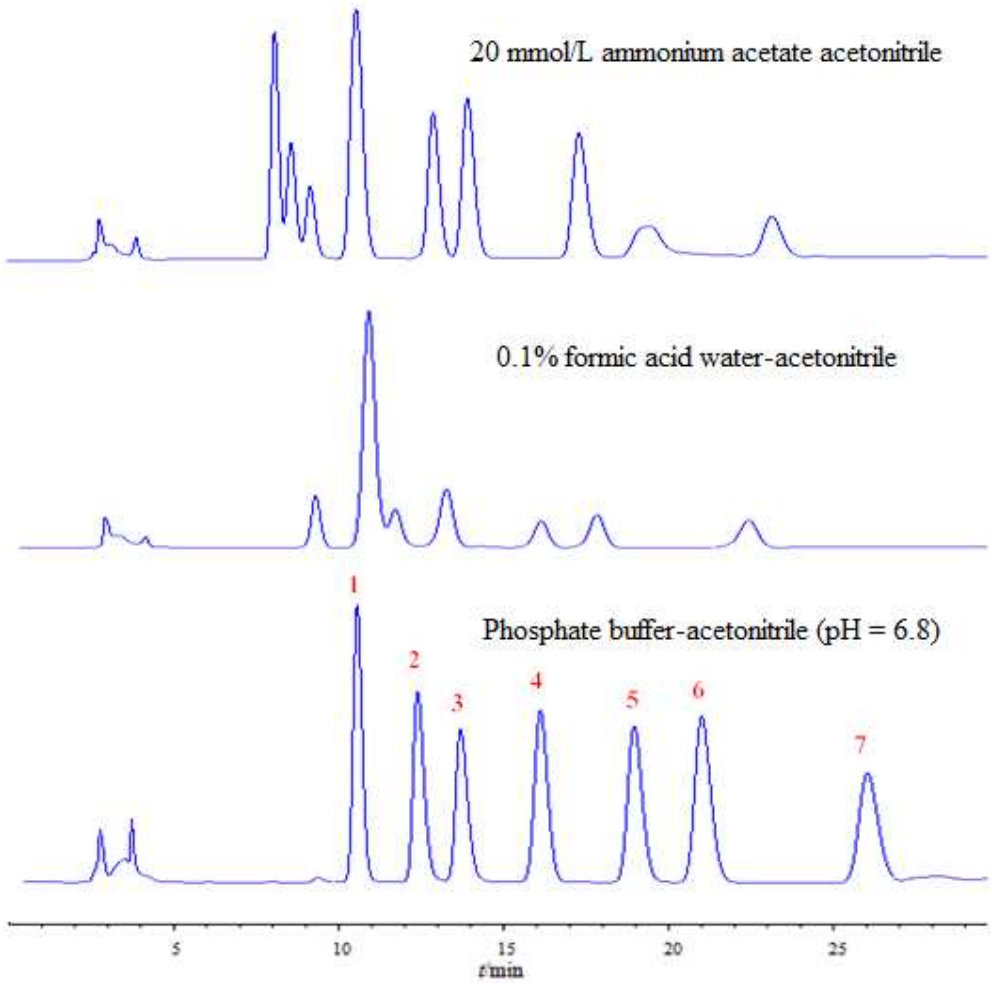

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Fig. 3 Effects of derivatization time on the peak areas of the derivatives. The derivatives from left to right are Man, GluUA, GalUA, Lac, Glu, Gal and Tre. The data are expressed as means ± SD. Values within the same mullion with different letters above are significantly different at $P < 0.01$

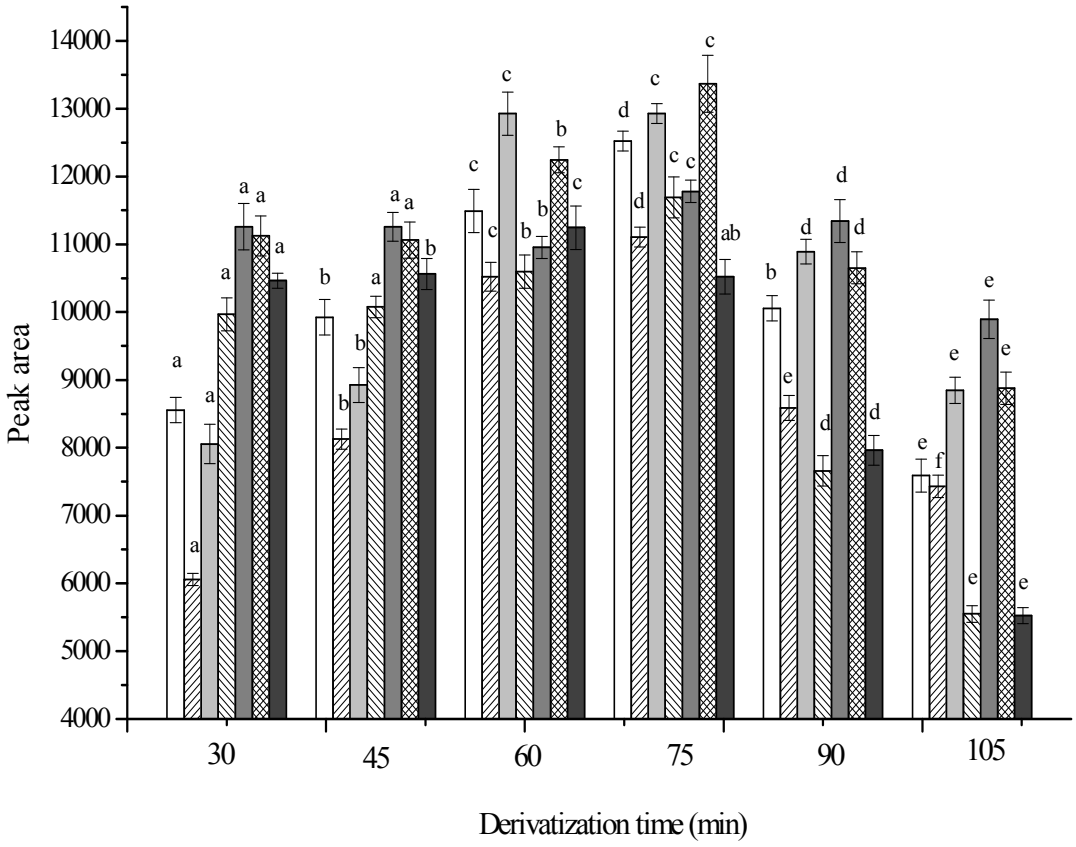

Fig. 4 Effects of derivative agent of PMP on the peak areas of the derivatives. The derivatives from left to right are Man, GluUA, GalUA, Lac, Glu, Gal and Tre. The proportions (*v/v*) of standards solution (0.02 mol/L) to PMP (0.5 mol/L) were 2: 1, 2: 2, 2: 3 and 2: 4, respectively. The data are expressed as means ± SD. Values within the same mullion with different letters above are significantly different at $P < 0.01$

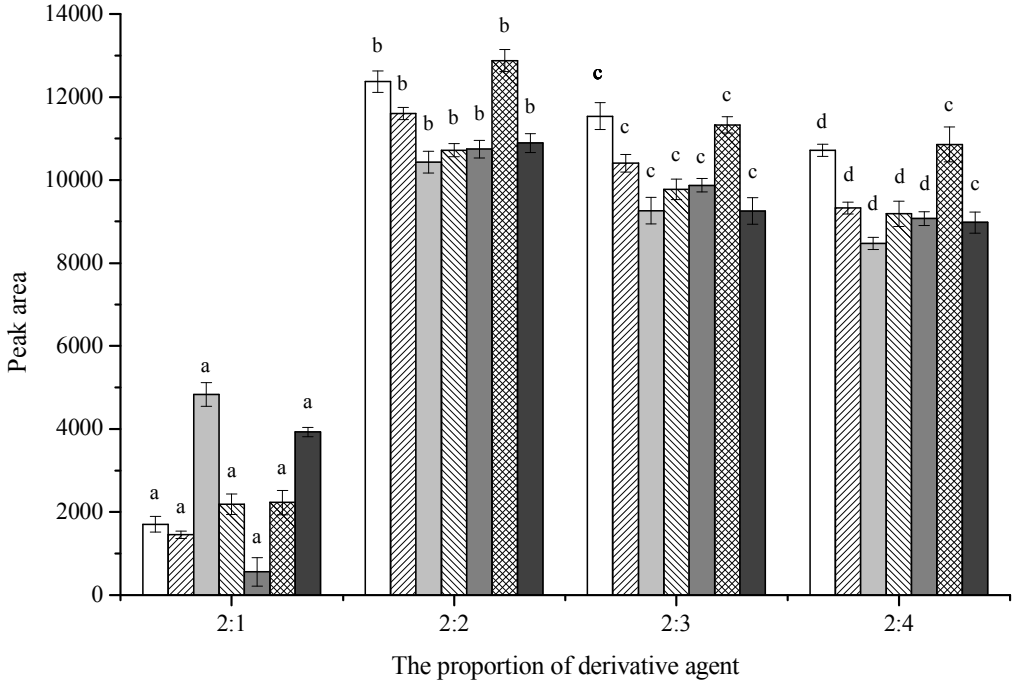

Fig. 5 Effects of storage time after derivative reaction on the peak areas of the derivatization products (a: Gal, b: Man, c: Lac, d: Glc, e: Tre, f: GlcUA, g: GalUA)

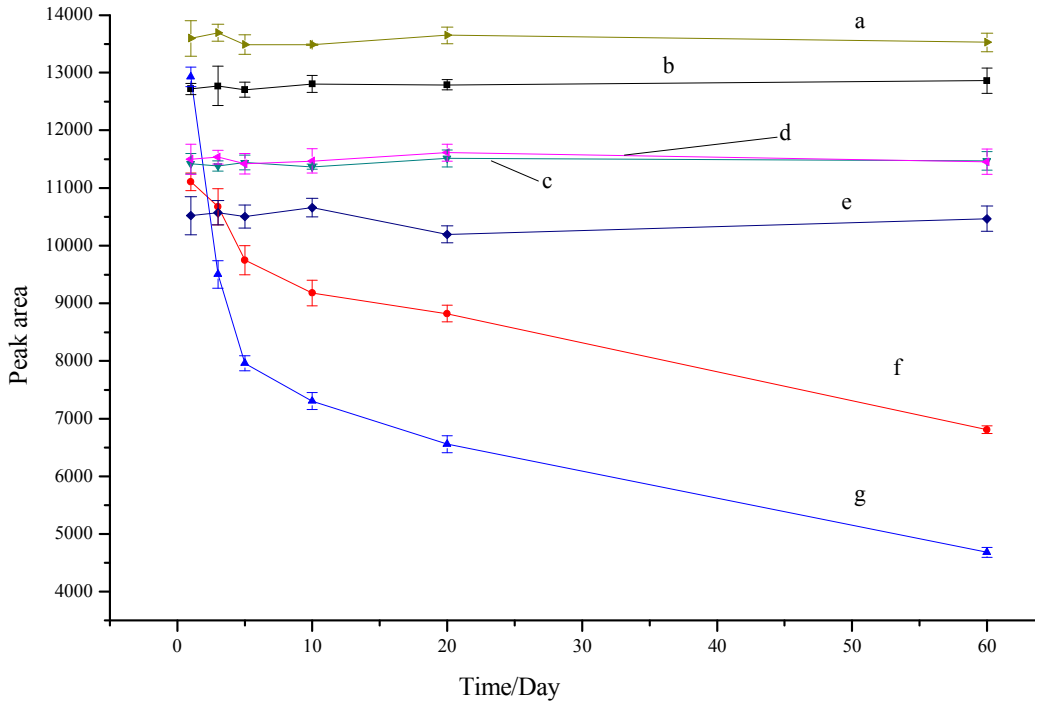

Fig. 6 Chromatogram of a real shark fin sample and an artificial dried shark fin sample

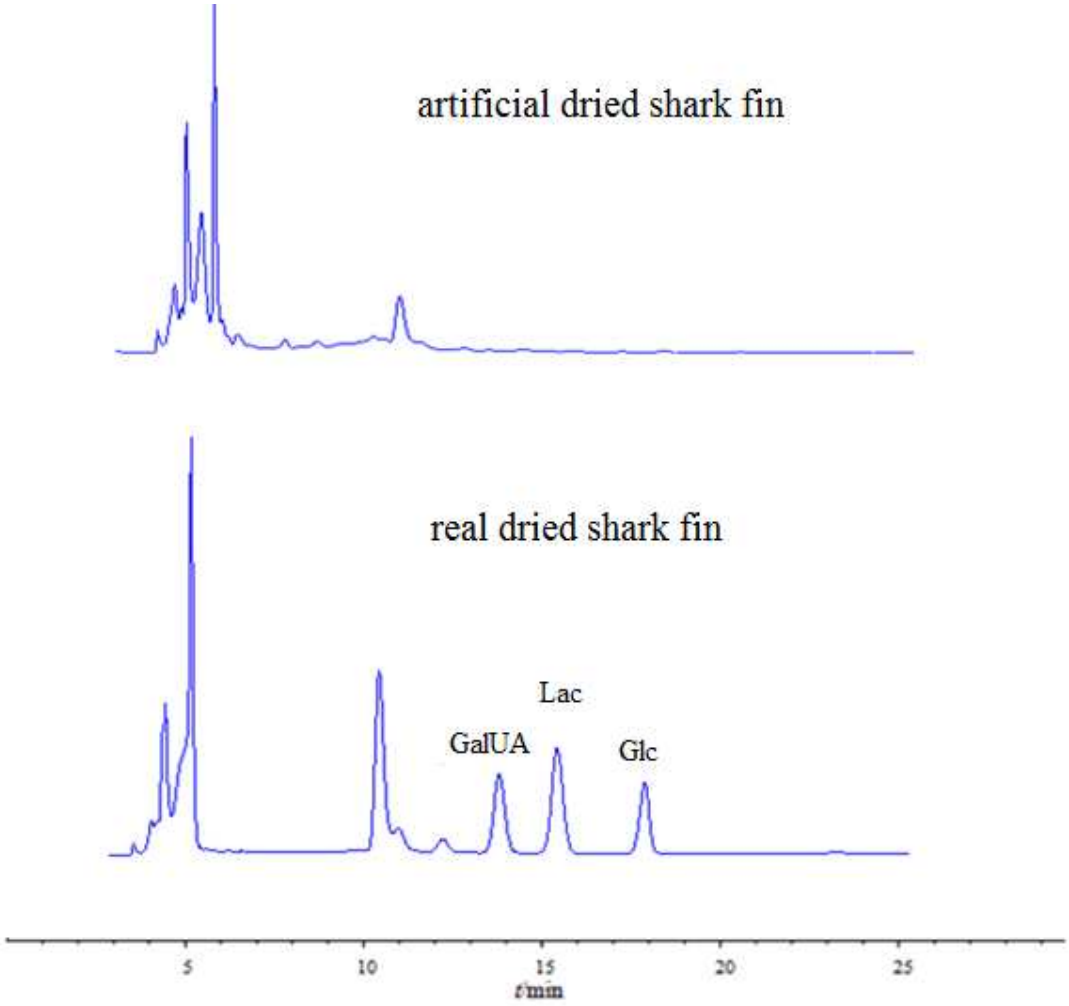

Table 1 Linear equations and R^2, LODs and LOQs of seven saccharides and uronic acids

Standard substance	Retention time (min)	Linear equation	R^2	Linear range (mmol/L)	LODs (mmol/L)	LOQs (mmol/L)
Man	10.861	Y = 2337.8X+85.7	0.998	0.1-20.0	0.006	0.020
GlcUA	12.056	Y = 2199.4X+39.6	0.999	0.1-20.0	0.008	0.025
GalUA	13.222	Y = 2554.9X+35.1	0.999	0.1-20.0	0.010	0.033
Lac	15.921	Y = 2099.4X+121.3	0.998	0.1-20.0	0.010	0.033
Glc	18.946	Y = 2134.5X+133.2	0.999	0.1-20.0	0.010	0.033
Gal	20.921	Y = 2681.5X-31.3	0.999	0.1-20.0	0.006	0.020
Tre	25.869	Y = 2197.3X-82.6	0.999	0.1-20.0	0.008	0.025

Table 2 The recovery of the different sugars in the negative samples at three spiked levels of analyte with 1, 5, 10 mmol/L mixed standard substances

Compound	Added/(mmol/L)	Recovery (n = 6)	
		Mean/%	RSD/%
Gal	1	112.5	4.21
	5	109.7	4.07
	10	111.8	4.05
Man	1	118.6	4.02
	5	116.2	4.57
	10	116.9	5.21
Lac	1	98.0	6.12
	5	106.2	6.25
	10	104.3	6.32
Glc	1	110.9	5.11
	5	107.6	5.26
	10	113.5	5.02
Tre	1	98.3	4.23
	5	99.7	5.92
	10	104.2	6.06
GlcUA	1	102.5	3.21
	5	106.4	4.34
	10	105.9	4.66
GalUA	1	103.9	4.62
	5	111.3	5.12
	10	107.8	5.25

Table 3 Effects of enzyme concentrations on the peak areas of HPLC analysis*

Contents of enzyme (mL)		Peak area		
Papain	Amylase	GalUA	Lac	Glc
0.5	0.5	1536 ± 189[a]	1662 ± 78[a]	1153 ± 46[a]
1.0	1.0	2580 ± 202[b]	3666 ± 143[b]	3818 ± 125[b]
1.5	1.5	3120 ± 168[c]	4719 ± 252[c]	4829 ± 222[c]
2.0	2.0	3416 ± 192[d]	5505 ± 165[d]	5642 ± 309[d]
2.5	2.5	2116 ± 96[e]	3250 ± 176[e]	3119 ± 80[e]

*The data are expressed as means ± SD with triplicates. Values within the same column with different lower-case letters are significantly different at $P < 0.01$.

Table 4 A summary of the results of the dried shark fins*

Samples	GalUA (mmol/L)			Lac			Glc		
	Min	Max	mean	Min	Max	mean	Min	Max	mean
13 standard samples	0.104	1.481	0.785	0.111	1.819	1.112	0.101	1.016	0.645
20 artificial samples	ND	ND	ND	ND	ND	ND	ND	ND	ND
The other 31 samples	0.479	1.225	0.715	0.366	1.560	0.850	0.255	1.448	0.725

*ND means “not detected”.

Table 5 δ^{13}C values of different kinds of samples*

Samples	δ^{13}C values (‰)
Real dried shark fins (n = 13)	- 14.231 ± 1.204[a]
Dried shark fins identified as real (n = 24)	- 14.456 ± 0.981[ab]
Dried shark fins identified as fake (n = 7)	- 17.247 ± 0.621[c]
Artificial dried shark fins (n = 20)	- 14.561 ± 1.016[b]

*The data were expressed as means ± SD (each sample was determined a minimum of three times and the average was adopted). Values within the same column with different letters were significantly different at $P < 0.01$.