

Development and application of a method for determination of nucleosides and nucleobases in *Macra veneriformis*

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ABSTRACT

Background: *Macra veneriformis*, a typical marine bivalve mollusk, delicious sea food while low cost, is ubiquitous and abundant in Chinese coastal areas, especially in the coastal shoals of Jiangsu province. To our knowledge, previously reported analytical methods can not meet a set of quality control. **Objective:** For the simultaneous determination of eight components (uridine, inosine, guanosine, thymidine, adenosine, xanthine, thymine and hypoxanthine) in *M. veneriformis*, a high performance liquid chromatography with UV detector method was established. **Materials and Methods:** To develop the method, a reverse phase column, BioBasic-C₁₈ (5 μm, 4.6 mm × 250 mm) was used. The mobile phase consisted of methanol and water using a gradient elution. The UV wavelength was set at 245 nm. The analysis conditions including extraction methods, extraction solvents, and HPLC parameters were optimized systematically for achieving good separation. Linearity, accuracy, repeatability and detection limit was revealed and showed good performance. **Results:** The optimized HPLC method was successfully applied for the qualification of 5 nucleosides namely, uridine, inosine, guanosine, thymidine, adenosine and 3 nucleobases namely, xanthine, thymine, hypoxanthine in *M. veneriformis*. **Conclusion:** A method with less time-consuming, more sensitive, and more precise was developed for the quantitative determination of nucleosides and nucleobases in *M. veneriformis* extractions. The established method might apply as an alternative approach for the quality assessment of *M. veneriformis*.

Key words: High-performance liquid chromatography, *Macra veneriformis*, nucleobase, nucleoside, quantitative analysis

INTRODUCTION

Macra veneriformis (family: Mactridae) grows wildly in the coastal wetland of China especially in Jiangsu province. In China, the mollusks of *M. veneriformis* is not only utilized as a delicious food, but also a traditional Chinese medicine with the functions of antihyperglycemic, anticancer, anticoagulant, protecting vascular system and so on.^[1-4] *M. veneriformis* as a mollush have a pronounced umami taste and belong to the food group of exceptional nutritive value, rich in proteins,^[5-6] nucleosides, saccharides,^[7-9] vitamins^[10,11] and minerals,^[12] while low in calories and fat.^[13] Specific organoleptic characteristics and a well-balanced diet have led to a worldwide increase in *M. veneriformis* consumption.

It has been reported that nucleosides and their bases exhibited various bioactivities. For instance, all purines can inhibit the activity of mononamine oxidase and show antioxidant effect^[14]; nucleoside analogs have been used as anti-HIV drugs.^[15] Further, additional benefits of nucleotides such as their contribution to iron absorption in the gut,^[16] enhanced repairing of gastrointestinal tract damage,^[17] impact on fatty acids metabolism^[18] and an improvement in immune response^[19] have been revealed. Nucleosides and nucleobases, involved in the regulation and modulation of various physiological processes in body through purinergic and/or pyrimidine receptors,^[20,21] are recognized to be the main bioactive components.

In present study, a method for determination of nucleosides and nucleobases was developed in order to quantify the nucleosides and nucleobases in *M. veneriformis*. Three extraction methods, including boiling water extraction (BWE), stirred tank extraction (STE), and ultrasonic

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extraction (UE) were compared, and UE was used as the best extraction method. HPLC was used for the qualitative analysis of nucleosides and nucleobases in the samples of different harvest times. Furthermore, with the developed HPLC-UV method, 8 nucleosides and nucleobases in *M. veneriformis* samples could be simply and accurately quantified. This is a report about the simultaneous determination of nucleosides and nucleobases in *M. veneriformis* by HPLC-UV, which provide an alternative, feasible approach for the quality assessment of *M. veneriformis* in addition to the methods using polysaccharides and amino acids as the markers. The chemical structures of these reference compounds are shown in Figure 1.

MATERIALS AND METHODS

Reagents and materials

Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Deionized water (18.2 MΩ) was produced by Milli-Q system (Millipore, Bedford, MA, USA). All the mobile phases were filtered through membrane (0.45 μm) and degassed with a Waters in-line degasser apparatus. Uridine, xanthine, thymine, hypoxanthine, inosine, guanosine, thymidine and adenosine were purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Other chemicals were of analytical grade and commercially available. The materials of *M. veneriformis* were obtained from Maojia port, Jiangsu. These *M. veneriformis* samples were authenticated by Dr. Xi-He Wan, Institute of Oceanology and Marine Fisheries, Jiangsu, China. The collected specimens were starved in an aquarium for 24 h to evacuate their gut contents, and then flesh was excavated from the shell and stored at -10°C condition for further using.

Preparation of standard nucleosides and nucleobases

The mobile phase was used as the solvent for stock solution preparation, and the concentrations for each standard were different within 0.4 to 100 μg·mL⁻¹ except uridine 156.13 μg·mL⁻¹ and xanthine 120.25 μg·mL⁻¹. A certain volume of stock solution was transferred to 10mL volumetric flask

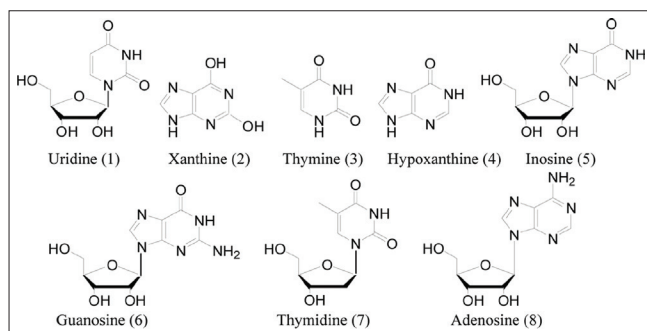


Figure 1: Chemical structures of the identified nucleosides and nucleobases

and diluted with mobile phase to the desired concentration. All the standard solutions were stored at 4°C in the dark.

Chromatographic system

Chromatographic analyses were carried out using an Acquity HPLC system (Waters, Milford, MA, USA) fitted with a binary pump, a plate autosampler, a thermostated column compartment, sample organizer and a Waters 2487 ultraviolet detector. A BioBasic-C₁₈ column (5-μm particles, 4.6 mm × 250 mm) with an in-line vanguard column was used for chromatographic separation. Solvents that constituted the mobile phase were methanol (A) and water (B). Because of a high risk of microbial contamination, each mobile phase was passed through a 0.45-μm filter. The separation was achieved using gradient elution of 0-6 min, 1% A; 6-15 min, 1-4% A; 15-45 min, 4-30% A; then keeping 50% A for 10 min to clean the column, and finally, reconditioning steps of the column was 1%A isocratic for 5 min. CH₃OH/H₂O (10:90, v/v) were used as solutions for cleaning the injection needle. The flow rate was set at 0.50 mL·min⁻¹ and the injection volume was 10 μL. The peak of the solvent front was considered to be equal to the dead time (t₀). The column eluate was monitored at 254 nm for all compounds.

Validation of the method

Calibration curves

Stock solutions containing 8 reference compounds were freshly prepared daily dilution of stock standard solutions with mobile phase. At least four concentrations of the solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the relative peak area versus the concentration of each analyte detected by UV.

Limits of detection (LOD) and quantification (LOQ)

Stock solution containing 8 reference compounds was diluted to a series of appropriate concentrations with, and an aliquot of the diluted solutions were injected into HPLC for analysis. The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Precision, repeatability, accuracy and stability

Intra- and inter-day variations were chosen to determine the precision of the developed assay. The intraday precision was examined on the mixed standards for six times within 1 day, while for interday variability test, the solution was determined in duplicates for consecutive three days. Variations were expressed by the RSD. The repeatability of the developed method was evaluated at appropriate level (4.0 g) of lyophilized powder which were extracted and analyzed by HPLC-UV as mentioned above triplicates. The RSD was used as the measurement of repeatability. A recovery test was used to evaluate the accuracy of the developed method. Known amount of standards were added to *M. veneriformis* powder, and then extracted and analyzed as described above. Three replicates were performed for the test. The average

percentage recoveries were calculated as follow formula:

$$\text{Recovery (\%)} = \frac{(\text{amount found} - \text{original})}{\text{amount spiked}} \times 100\%$$

Stability of sample solution was tested, which was analyzed in every 4 h within 24 h. Variation was expressed as RSD. To test the repeatability of extractive, three levels (4.0 g, 6.0 g and 8.0 g) of the sample were extracted and analyzed under the optimum conditions triplicates and analyzed by HPLC as mentioned above. Variations were expressed by RSD.

Sample preparations

The sample pretreatment procedure is often the most important step, which can greatly influence the repeatability and accuracy of the entire analysis. The adaptation of an appropriate selective pretreatment method for analytes usually protects the matrix purification process from interferences. In this study, 8 nucleosides, including uridine, xanthine, thymine, hypoxanthine, inosine, guanosine, thymidine and adenosine, in *M. veneriformis* were determined using several extraction solvents i.e., methanol (100%, 50%, 20%), ethanol (100%, 50%, 20%) as well as water and butanol. Each (4.0 g) was mixed with 100-mL different solvents, and then immediate ultrasonic extraction of nucleosides and nucleobases was performed at room temperature for 60 min, 2 times. After extraction, the extract was cooled down to the room temperature, and made up the lost weight with different solvents, then centrifuged at 1.5×10^4 rpm for 10 min. The supernatant was filtered through a 0.45- μm Econofilter. To get the optimization extraction method several sample preparation methods with different solvents have been used for quantitative determination of nucleosides in *M. veneriformis*, but their data are greatly various.

Boiling water extraction

Four grams of powder of *M. veneriformis* were mixed with 100 mL boiling (95-100°C) solvent in a glass tube with stopper, accurately weighted and kept at boiling water bath (95°C) for 60 min, 2 times. Extract was cooled down to the room temperature, made up the lost weight with solvent, and then centrifuged at 1.5×10^4 rpm (Centrifuge TGL-16G, ShangHai Anting Scientific Instrument Factory, China) for 10 min. The supernatant was filtered through a 0.45- μm Econofilter (Agilent Technologies, Palo Alto, CA, USA) before HPLC analysis.

Stirred tank extraction

Four grams of powder of *M. veneriformis* were mixed with 100 mL solvent in a glass tube with stir bar, accurately weighted and kept at room temperature (25°C) for 60 min, 2 times. The extract was cooled down to the room temperature, and made up the lost weight with solvent, then centrifuged at 1.5×10^4 rpm for 10 min. The supernatant was filtered through a 0.45- μm Econofilter.

Ultrasonic extraction

Four grams of powder of *M. veneriformis* were mixed with 100 mL solvent placed into an ultrasound machines, accurately weighted and kept on for 60 min, 2 times. The extract was made up the lost weight with solvent and centrifuged at 1.5×10^4 rpm for 10 min. The supernatant was filtered through a 0.45- μm Econofilter before HPLC analysis.

RESULTS AND DISCUSSION

Optimization of HPLC parameters

The selection of mobile phase should consider both separation and effect on HPLC. The main objective of this study was to obtain an efficient, reliable, and rapid method for the quantification of nucleosides on HPLC. We present a method that is able to separate many compounds with high resolution. The nucleosides and nucleobases are the compounds with high polarity, which are easily separated in high ratio of aqueous mobile phase. Therefore, BioBasic-C₁₈ column with high ratio of aqueous mobile phase was selected. The optimum chromatographic conditions are summarized in Section 2.3.

Column temperature variations did not exert much influence on the overall analysis time. However, retention with respect to separation and the peak shape were considerably affected. Different temperatures of 20, 30 and 40°C were tested and the results demonstrated that resolution increased and retention times decreased with increased temperature, which show in Figure 2. In addition, the temperature increase (40°C) resulted in

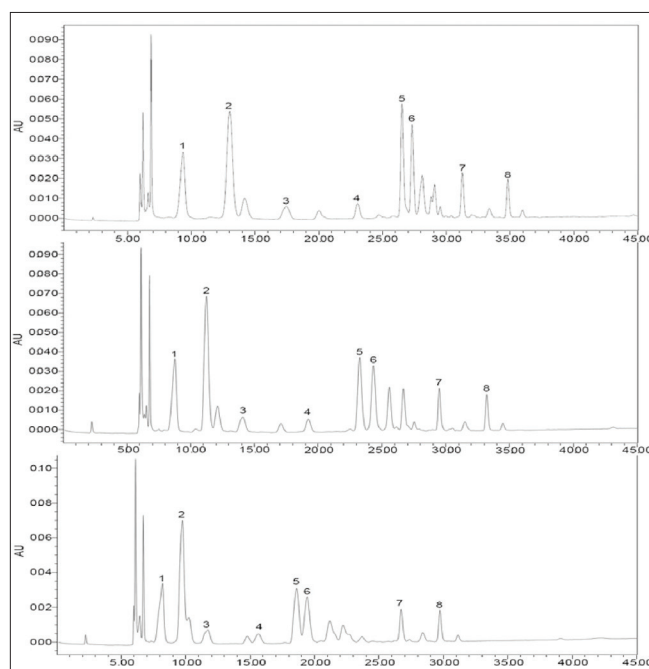


Figure 2: Effect of different column temperatures, I: column temperature 20°C; II: column temperature 30°C; III: column temperature 40°C

partial superimposed peaks. Ultimately, a temperature of 30°C was deemed optimal. HPLC chromatogram of a mixed working standard solution detected with a UV set at 254 nm is shown in Figure 3-a and the chromatogram of sample are shown in Figure 3-b.

Validation of the method

The linearity, regression and linear ranges of 8 analytes were determined using the developed HPLC method. The overall LODs and LOQs were less than 0.32 µg·mL⁻¹

and 1.01µg·mL⁻¹, respectively [Table 1] and the overall recoveries were between 95.15 and 101.07% with RSD less than 3.03%. The overall intra- and interday variations (RSDs) of the 8 analytes were less than 1.21% and 1.32% [Table 2], respectively. The developed method also had good repeatability (RSD < 0.4%).

Optimization of extraction procedure

Optimization of extraction solvents

During the preliminary investigation on the resolution

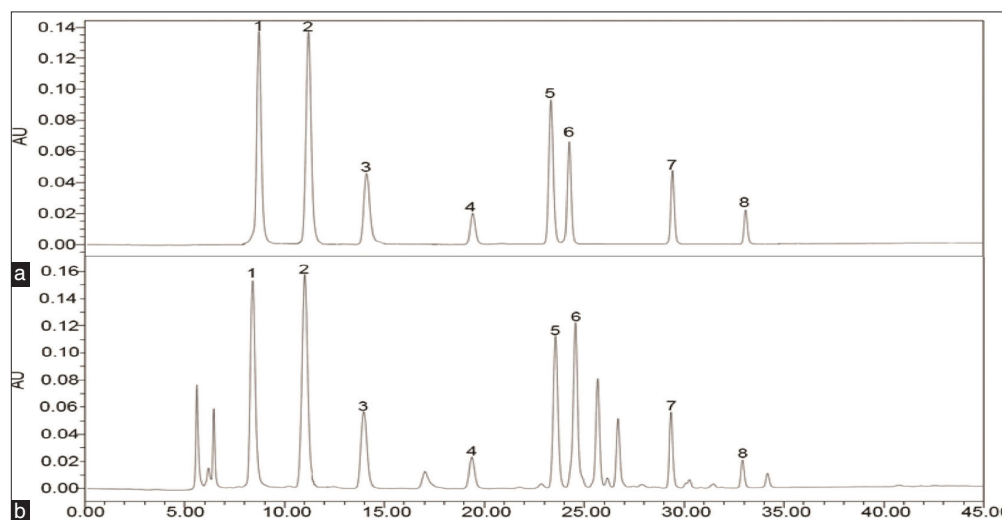


Figure 3: HPLC chromatograms of solution of standards (a) and samples (b). Peaks: 1, uridine (102.26 µg·mL⁻¹); 2, xanthine (113.48 µg·mL⁻¹); 3, thymine (46.10 µg·mL⁻¹); 4, hypoxanthine (9.80µg·mL⁻¹); 5, inosine (59.07 µg·mL⁻¹); 6, guanine (40.98 µg·mL⁻¹); 7, thymidine (45.62 µg·mL⁻¹); 8, adenosine (5.55 µg·mL⁻¹)

Table 1: Linear regression data, LOD and LOQ of the investigated compounds

Analytes	Linear regression data			LOQ ^b (µg·mL ⁻¹)	LOD ^b (µg·mL ⁻¹)
	Regressive equation ^a	Test range (µg·mL ⁻¹)	r ²		
Uridine	y = 268520x-38064	15.61-156.13	0.9999	0.348	0.101
Xanthine	y = 33272x-4270.6	6.01-120.25	0.9998	0.073	0.024
Thymine	y = 100508x-12702	0.51-50.14	0.9997	0.399	0.121
Hypoxanthine	y = 79302x-11290	0.63-63.57	0.9996	0.115	0.032
Inosine	y = 46443x-3268.7	8.02-80.21	0.9997	0.265	0.079
Guanosine	y = 115869x-17223	1.04-20.83	0.9998	0.179	0.053
Thymidine	y = 24972x-1595.6	6.92-69.24	0.9998	0.171	0.049
Adenosine	y=57693x-7251.8	0.47-4.73	0.9998	0.132	0.035

^a y is the value of peak area, and x is the value of the reference compound's concentration (µg·mL⁻¹). ^b LOD and LOQ were determined at 5/N of about 3 and 10, respectively.

Table 2: Precision, recovery, stability and repeatability of eight analytes

Analytes	Precision (RSD, %, n = 6)		Recovery (% , n = 3)		Stability (RSD, %, n = 6)	Repeatability (RSD, %, n = 3)
	Intra-day	Inter-day	Mean	RSD (%)		
Uridine	0.23	1.22	99.98	2.08	0.09	0.22
Xanthine	1.21	1.01	98.94	2.18	0.99	0.36
Thymine	0.03	1.09	99.98	2.92	1.64	0.07
Hypoxanthine	0.83	0.93	100.51	3.03	1.75	0.09
Inosine	0.39	0.32	101.07	2.68	0.34	0.23
Guanosine	0.66	0.24	99.41	1.48	3.41	0.17
Thymidine	0.11	1.32	100.95	1.12	2.76	0.21
Adenosine	0.63	0.99	100.62	1.82	4.26	0.16

of separation, the effect of eight solvent, i.e., methanol: water (1:4, v/v); methanol: water (1:1, v/v); methanol: water; ethanol: water (1:4, v/v); ethanol: water (1:1, v/v); ethanol and butanol was compared. Four grams of powder of *M. veneriformis* was used for extraction as mentioned above. After HPLC analysis, the peak areas of the eight investigated compounds were used for evaluation of the extraction efficiency. The results showed that their contents were very different though the extraction solvent. It was observed that more favorable separation resolutions of most compounds could be achieved in methanol water solution, especially in methanol: water (1:1, v/v). [Figure 4]

Optimization of extraction method

The effects of extraction methods on the quantification of nucleosides in the same sample are various, so extraction variables such as extraction method (Stirred tank extraction (STE), Boiling water extraction (BWE), Ultrasonic extraction (UE)), extraction time (30 min, 1 time; 30 min, 2 times; 60 min, 1 time; 60 min, 2 times; 90 min, 1 time; 90 min, 2 times), extraction temperature (room temperature, 50°C) and solvent volume (50, 100 and 150 mL) were investigated to obtain optimal extraction conditions. The

results of extraction methods are shown in Figure 5, which indicates that BWE was most ineffective on total content, while adenosine can not be extracted by STE. Generally, the best extraction method was found to be UE at room temperature with 100mL 50% methanol for 60 min, 2 times, which obtain the highest extraction efficiency for 8 constituents analyzed among those extraction methods.

Quantification of nucleosides and nucleobases in different month

The developed HPLC method was subsequently applied to simultaneous determination of 8 nucleosides and nucleobases. The typical chromatograms of ultrasonic extraction extracts for *M. veneriformis* samples were shown in Figure 2. The identification of the investigated compounds was carried out by comparison of their retention time and their UV spectra with those obtained by injecting standards in the same conditions. Due to research blank in *Mactra* genus, it was necessary to qualitatively and quantitatively compare the formulation of nucleosides and nucleobases among different samples.^[22] The harvest month of samples and its contents of the investigated compounds are summarized in Figure 6.

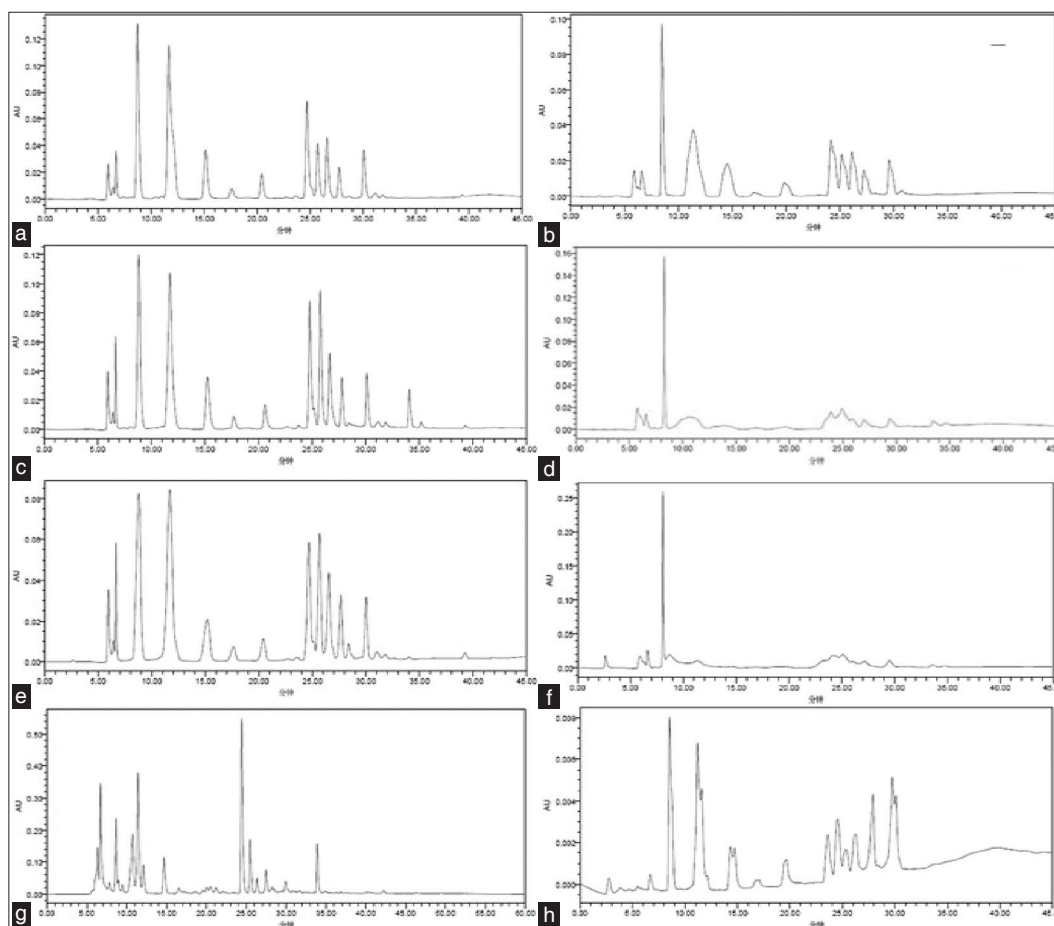


Figure 4: Effect of different extraction solvents, a: Methanol: Water (1:4,v/v); b: methanol:water (1:1,v/v); c: methanol; d: water; e: Ethanol:water (1:4,v/v); f: Ethanol:water (1:1,v/v); g: Ethanol; h: butanol

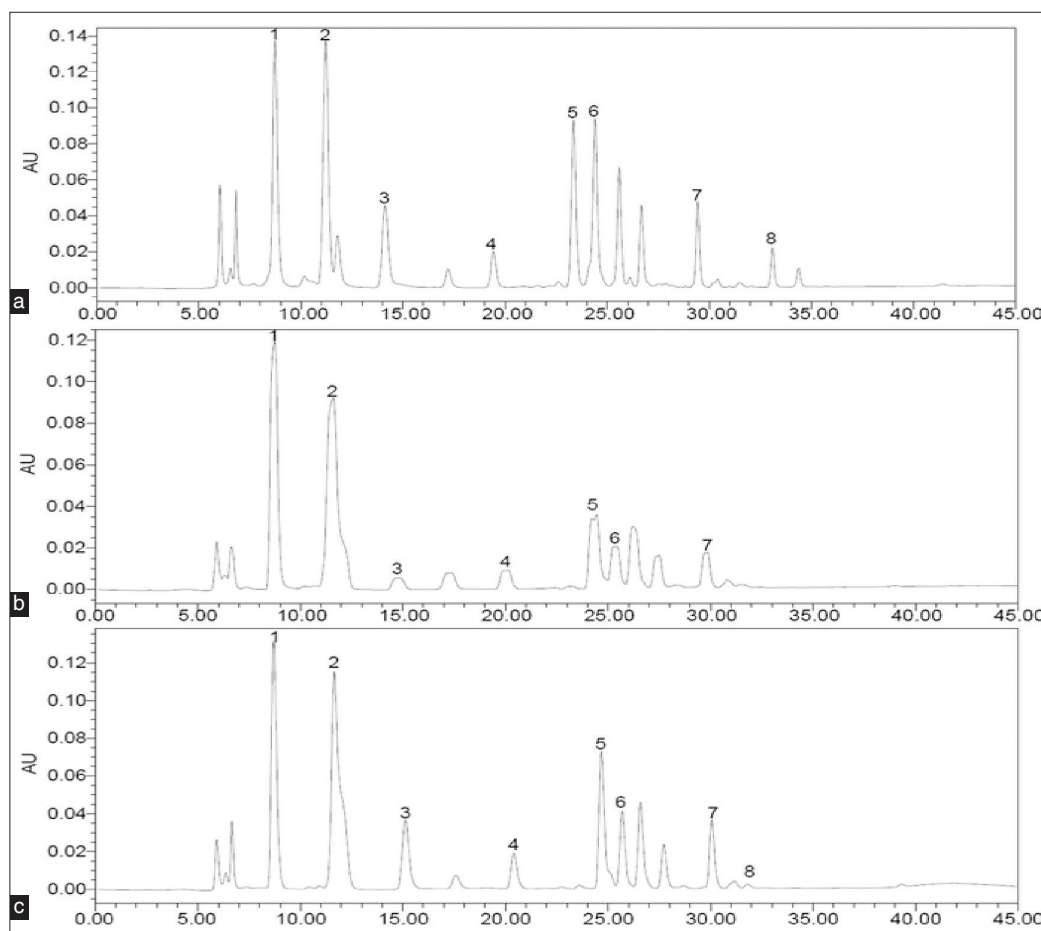


Figure 5: HPLC chromatograms of different extraction methods, i: Ultrasonic extraction (UE); ii: Stirred tank extraction (STE); iii: Boiling water extraction (BWE)

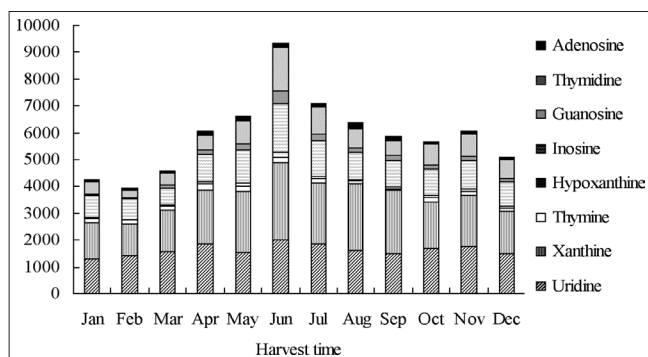


Figure 6: Content of nucleoside and nucleobases of different harvest times

The results [Figure 6] showed that almost all of those *M. veneriformis* samples were rich in nucleosides and nucleobases, and with various contents. The total nucleoside compounds in *M. veneriformis* of summer are significantly higher than those of others, which may relate with growth period of *M. veneriformis*.^[23] The most significant increase of total nucleoside compounds was found in the *M. veneriformis* of June, which increased nearly 50% on the average of others in this area.

As for the individual compounds determined in the experiments, remarkable differences were also observed. The compound uridine was found to be a predominant constituent in many samples, which contain the highest content in June and lowest in January. Likewise, xanthine, another compound found as a major constituent in many samples, varied from 1189.84 to 2877.48 $\mu\text{g}\cdot\text{g}^{-1}$. Furthermore, the content of hypoxanthine was observed to be the least in all nine markers. These results revealed that in the different harvest time, the contents of these nucleosides and nucleobases were different and the variation may attribute to many factors, including genetic variation, *Mactra* origin, and climate or geography (Ocean circulation).

CONCLUSIONS

Extraction methods greatly influence the quantitation of nucleosides in *M. veneriformis*. Therefore, sample preparation is very important, which should be carefully optimized. An HPLC method was established for the simultaneous determination of nucleosides and nucleobases in *M. veneriformis*. The method was found to be simple, precise and accurate which should be useful for other researchers

in the field. In addition, it is helpful to develop a rational method for quality control of *M. veneriformis*.

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