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Determination of purine bases in sea urchin (*Paracentortus lividus*) gonads by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) method is described to determine the levels of adenine, guanine, xanthine and hypoxanthine in the gonads of sea urchin *Paracentrotus lividus*. Purine bases were separated using a C-18 column as the stationary phase, 0.3 M KH_2PO_4 buffer solution as the mobile phase, and ultraviolet detection at 255 nm. The total amount of purine bases determined in sea urchin canned and frozen storage at −18 °C during 3 and 6 months was high [1.6 g/100 g (dry wt.)] The concentrations of purine bases changed during this time; there was a steady increase in hypoxanthine and decrease of adenine. Good results were obtained with respect to linearity ($R > 0.9995$), reproducibility (RSD% < 4.5) and recovery ($> 98.5\%$).

Keywords: High-performance liquid chromatography; Purine bases; Sea urchin

1. Introduction

The sea urchin is a shellfish belonging to the *Echinoidea* family. More than 750 species of sea urchin have been identified, of which *Paracentrotus lividus* is the most appreciated for human consumption, and they are very plentiful off the coast of Spain. This shellfish is bowl-shaped, dark in colour, and 7–8 cm in diameter; the shell is covered with spines (Campbell, 1989). The edible portions are the five gonads, which are half-moon-shaped and yellow-orange in colour. The sea urchin has been favoured by consumers because of its distinctive aroma and good taste. The gonads are eaten fresh in sauces, soups, creams and omelettes.

The season for *Paracentrotus lividus* is from the beginning of January to the middle of April when the development of gonads is at its maximum (Ramonell, 1985). The sea urchin market was originally limited to the coastal region because of the highly perishable nature of the product; however, the development of a canning process has allowed for the expansion of the market, and the sea urchin industry has become increasingly important to the economy in Galicia in recent years (Miguez & Catoira, 1990).

Purine bases are of great interest for several reasons; in particular, together with certain pyrimidine bases, they are constituents of DNA and RNA and consequently of fundamental importance in life processes. Additionally, as nucleosides, and nucleotides, they act as hormones and neurotransmitters and are present in some co-enzymes (Joule, 1995).

Uric acid is the end-product of purine base metabolism in humans. Dietary purine intake influences serum uric acid levels, and this has been reported to be associated with hyperuricemia and gout. On the other hand, the quality and quantity of purine compounds in food might change during storage and processing (Lou, 1998). For fish, the level of hypoxanthine is often used as an index of quality; for example, hypoxanthine shows a progressive increase during storage, and can be used to estimate the freshness of the sea urchin.

Fish products have moderate or high concentrations of purine bases. Hypoxanthine predominates in fish, crustacea and molluscs, while adenine is the highest purine base in shellfish (Lou, Chen, & Chen, 1996). In humans, the effect of nucleic acids, in elevating serum

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uric acid levels, varies with the different purines present in the nucleic acids. Oral doses of adenine and hypoxanthine were found to be more uricogenic than were guanine and xanthine (Clifford, Riumallo, Young, & Scrimshaw, 1976). The differences in the metabolic effect of individual purines would suggest that the restriction of some foods should be based on the content of uricogenic purines rather than on the total nucleic acid content.

Chromatography has been shown to be a technique for the determination of the purine bases. Gas chromatography was used by Hamberg and Zhang (1995) and by Cambero Pereira-Lima, Ordoñez, and De Fernando, (2000). Reversed-phase (RP) HPLC is particularly suited for the separation of purine metabolites. Generally, octadecylsilica packing materials have been most widely used for the analysis of this class of compounds, with UV (Czauderna and Kowalczyk, 1997; Gamberini, Ferioli, Zeneroli, Rustichelli, & Baraldi, 1992; Lou, 1998; Lou and Chen, 1997) or voltametric detection (Lu, Liu, & Ni, 1993).

The objective of this study was to determine the purine (adenine, guanine, hypoxanthine and xanthine) contents of sea urchin gonads by reversed-phase HPLC, and to investigate changes during storage of gonads for 3 and 6 months at −18 °C.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. Standards (adenine, hipoxanthine, xanthine, guanine, adenosine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate and xanthosine 5′-monophosphate) were obtained from Sigma Chemical, Madrid, Spain. Solvents and other chemicals (potassium dihydrogen phosphate, 85% orthophosphoric acid, formic acid) were purchased from Merck, Darmstadt, Germany and, trifluoroacetic acid was obtained from Sigma Chemical, Madrid, Spain.

2.2. Sample treatment

Sea urchins, *Paracentrotus lividus*, were caught in the Atlantic coastal region around Touriñán in Galicia (north west Spain). The gonads were then separated using a knife, kept on ice, and immediately transported to a canning factory (Conservas y Ahumados Lou, Ribeira, A Coruña, Spain). Gonads (90 g) were placed in an RR-90 can, and salt and tap water were added. The cans were vacuum-sealed and sterilized at 112 °C for 50 min. Gonads were also transported to the laboratory and immediately frozen at −18 °C. The samples were stored in sealed bags. Then the analysis was carried out with recently unfrozen samples.

The purine base contents were determined according Lou and Chen (1997) with some modifications. A sample (200 mg) of gonad was digested in a flask with a mixture of trifluoroacetic acid/formic acid (1/1, v/v) at 90 °C for 15 min. The resultant hydrolysate was transferred into a 250 ml flask and dried by rotary vacuum evaporator at 75 °C. To dissolve the purine bases, 10 ml 0.3 M potassium dihydrogen phosphate buffer solution (pH 4.0) were added to the flask. This solution was then filtered through a 0.5 μm membrane filter and injected into the chromatograph.

2.3. HPLC instrumentation

The HPLC equipment consisted of a Spectra Physics (San Jose, CA) HPLC apparatus comprising an SP8800 ternary pump, a 20-μl Rheodyne (Cotati, CA) injection loop, an SP8792 column heater kept at 28 °C, a UV–visible forward optical scanning detector (adjusted to 255 nm) linked via Labnet to a Pentium computer running PC-1000 version 3.0 software (all from Thermo Separation Products, Fremont, CA) on IBM OS/2 Warp version 3.0 hardware. A column (250×4.6 mm) packed with 5-μm Spherisorb C_{18} (Sugelabor, Madrid, Spain) was used for the separation. The analyses were performed in the gradient mode, using a 0.3 M KH_2PO_4 buffer solution, pH = 4; the initial flow-rate was 1 ml/min, then 2 ml/min for 16.1–35 min.

2.4. Identification and quantification of the purine bases

Peaks were identified by comparing their retention times and UV absorbance spectra with those of purine standards of known concentration. The accuracy of the procedure was tested by adding known quantities of purine standards to the samples and calculating the percentage recoveries.

To determine the precision of the method, six aliquots of the same homogenized sea urchin sample were subjected to the complete procedure and injected in duplicate. The relative standard deviations (RSD%) are shown in Table 1.

Recovery percentages (see Table 1) were evaluated by spiking samples of sea urchin with a mixed standard and then subjecting them to the rest of procedure. Also the detection limits (calculated in accordance with the

Table 1
Relative standard deviations (RSD%) of the method, recovery percentages and detection limits

Compound	RSD%	Recovery percentage	Detection limits (μg/ml)
Guanine	3.4	98.5	0.04
Hypoxanthine	3.8	99.7	0.06
Xanthine	4.5	99.2	0.068
Adenine	2.9	99.8	0.076

American Chemical Society guidelines, 1980) are shown in the same table.

The method was calibrated using a series of purine precursors (guanosine 5′-monophosphate, inosine 5′-monophosphate, xanthosine 5′-monophosphate and adenosine 5′-monophosphate) which were subjected to the total process, so that the hydrolysis was completed (to guanine, hypoxanthine, xanthine and adenine, respectively).

3. Results and discussion

In developing the method, several conditions were assayed. Different mobile phases were tried (0.02 M KH_2PO_4; 0.3 M KH_2PO_4; 0.3 M KH_2PO_4:Methanol:Acetonitrile:tetrahydrofuran, 979:10:10:1) but only 0.3 M KH_2PO_4 allowed the adequate resolution of adenine, guanine, hypoxanthine and xanthine. The best resolution was obtained when analysis was performed at 28 °C (but was also probed at room temperature, 25 and 30 °C). Finally, we assayed different flow rates (0.5, 1 and 2 ml/min) but we chose a gradient elution because it reduced analysis time and improved the separation.

Figs. 1 and 2 show the chromatograms of the purine standards mixture and a typical sea urchin sample, respectively. Four purines were identified and quantified: adenine, guanine, hypoxanthine and xanthine.

Results of the determination of purines in canned and frozen samples (3 and 6 months) are given in Table 2. The total amount of purines determined in sea urchin was high (1.6 g/100 g dry weight in the three samples);

Table 2
Purine contents (mg purine bases/100 g dry wt.) in canned and frozen sea urchin gonads

	Guanine	Hypoxanthine	Xanthine	Adenine
Canned	699±61.9[a]	38.8±2.2	7.7±1.6	823±58.3
Frozen (3 months)	683±46.4	180±13.8	59.3±17.8	732±28.5
Frozen (6 months)	632±48.9	231±18.7	37.9±15.8	661±59.5

[a] Mean±standard deviation ($n=8$).

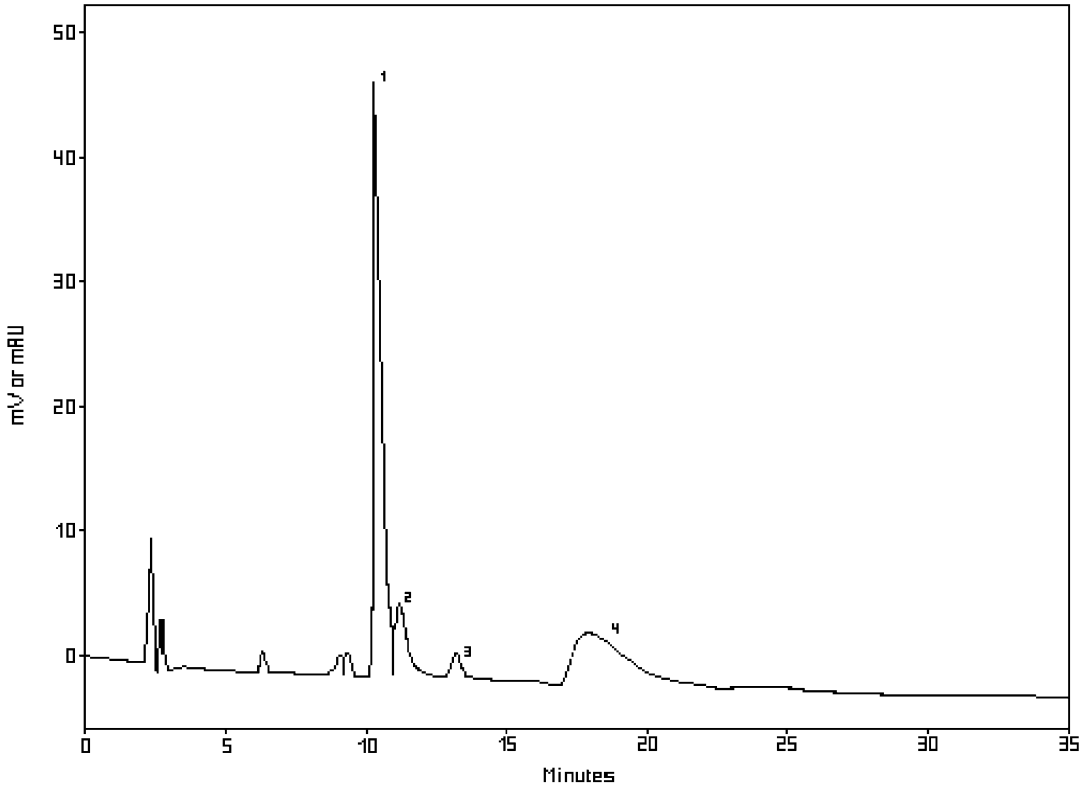

Fig. 1. Chromatogram of purine base standards. Reverse-phase HPLC; injection volume, 20 μl; column, Spherisorb ODS 2 C-18 (250×4.6 mm, 5 μm particle size); mobile phase, 0.3 M KH_2PO_4 buffer solution, pH = 4; flow-rate, 1 ml/min to 16th min and 2 ml/min for 16.1–35 min. Peaks: 1, guanine; 2, hypoxanthine; 3, xanthine; 4, adenine.

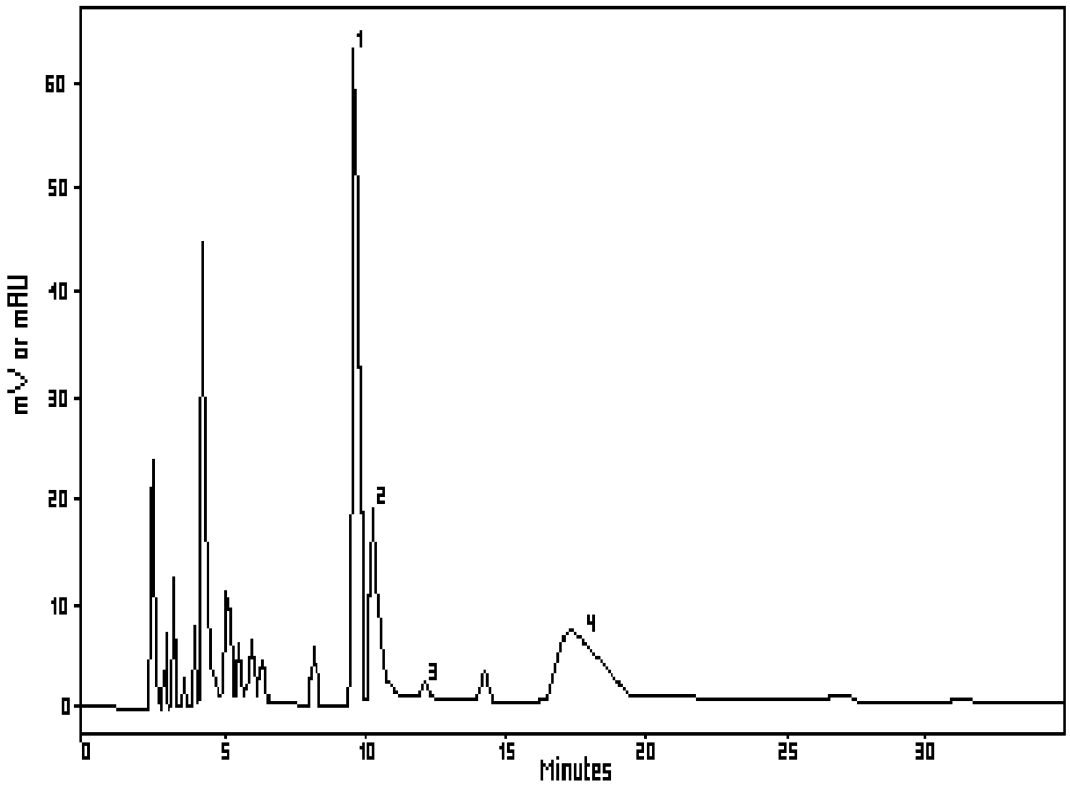

Fig. 2. Chromatogram of a sea urchin gonad sample. Reverse-phase HPLC; injection volume, 20 μl; column, Spherisorb ODS 2 C-18 (250×4.6 mm, 5 μm particle size); mobile phase, 0.3 M KH_2PO_4 buffer solution, pH = 4; flow-rate, 1 ml/min to 16th min and 2 ml/min for 16.1–35 min. Peaks: 1, guanine; 2, hypoxanthine; 3, xanthine; 4, adenine.

the Student's *t*-test showed no significant differences ($P \leqslant 0.05$) between their means. However, concentrations of purines changed during this time, although adenine and guanine were always the predominant bases.

There was a decrease in the concentration of guanine from the third month of storage at −18 °C and a steady increase of the hypoxanthine from the time of collection; xanthine increased to 59.3 mg/100 g after 3 months but then diminished again; there was a steady decrease of adenine from the time of collection.

These variations may be due to the enzymatic degradation of the bases to hypoxanthine after the death of the animal. According to Robinson (1987), significant amounts of nucleotide degradation products, such as IMP and hypoxanthine can arise as a result of post-mortem autolysis. For the canned samples the degradation did not happen because the temperature reached in the packaging inactivated the enzymes.

These results are similar to those found in other studies (Lou, 1998), where the storage of food involves a decrease in the concentrations of adenine, guanine and xanthine, but an increase in that hypoxanthine during this time, related to the freshness of food (Brulle, Ghulam, & Savoiet, 1988). During frozen storage the enzymatic activity is not completely arrested; for this reason the proportion of the purine bases is modified.

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