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Determination of ATP related compounds in fresh and canned tuna fish by HPLC

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Reliability of two HPLC methods to determine ATP, ADP, AMP, IMP, inosine and hypoxanthine in fish was studied after modifying mobile phase composition to improve resolution. Both methods included the same extraction procedure, and used a C18 column and UV detection. Mobile phases were neutral phosphate buffer in both methods but one of them used tetrabutylammonium hydrogen sulphate as ionic supressor. Both procedures gave acceptable results, although the addition of the ionic suppressor led to various advantages, such as improved recovery, precision, and sensitivity. Furthermore, chromatographic procedure without ionic suppressor showed a lack of specificity in canned tuna samples, since an unknown peak appeared at ATP retention time. In addition, IMP, Ino and Hx stability in fish samples and in fish samples extracts stored at −18°C were studied and no changes on their contents were observed throughout 26 weeks. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Although the main changes during fish spoilage result from bacterial growth, the first changes in fish tissues result from autolytic reactions controlled by native enzymes, such as in the adenosine triphosphate (ATP) break-down process (Kennish & Kramer, 1986). The pathway of ATP catabolism in fish muscle has been extensively documented as a degradative sequence to adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx). Several differences between fish species have been reported but despite those differences a single ATP derivative or a combination of various catabolites of ATP have been used to monitor freshness in a wide variety of fish (Handumrongkul & Silva, 1994). IMP and Hx are reported to be relatively stable at sterilisation temperature and have been suggested for the quality assessment of canned tuna as well as other related species (Tokunaga *et al.*, 1982). Recoveries of 75% and 92% were measured for IMP and Hx respectively when they had been spiked in tuna before canning (Gill *et al.*, 1987).

Some ATP derivatives are related with the taste of fish and fish products. High content of Hx is related with the bitter off-taste of spoiled fish and IMP evokes a meaty taste sensation. For this reason, amongst others, freshly caught fish, in which the levels of IMP are very low, has poor organoleptic acceptance. Moreover, IMP like glutamine monophosphate (GMP) and monosodium glutamate (MSG), acts as an enhancer in improving the palatability for flavour of home-broth and commercial foods. These compounds can be used as flavour potentiators (Matoba *et al.*, 1988).

Several analytical methods have been described to determine ATP and its related compounds in fish samples. Initially, anion–cation exchange columns to separate ATP and related compounds were described (Saito *et al.*, 1959; Jones *et al.*, 1962; Kennish & Kramer, 1986). These procedures are tedious and often do not allow a complete resolution of all analytes. More recently, high-pressure liquid chromatographic (HPLC) methods have been applied to determine ATP and related compounds. Some of these HPLC methods are based on the classic anion cation-exchange techniques (Iwamoto *et al.*, 1987), but nowadays, C_{18} reverse phase columns are the most commonly used (Gómez *et al.*, 1992). In the last few years, the use of mobile phases with ionic supressors to improve HPLC resolution has been extended (Murray & Thomson, 1983; Persson & Karger, 1986).

The aim of this work was to study an LC procedure for determination of ATP related compounds in fresh fish and canned fish products. For this purpose, we checked the related compound mobile phases reported by Murray and Thomson (1983), which employ

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tetrabutylammonium hydrogen sulphate, and the report by Ryder (1985), which does not, using a Tracer Spherisorb ODS 2 C_{18} column. Modifications of original procedures were required to improve resolution. Reliability of both modified methods was studied in terms of linearity, precision, recovery and sensitivity.

Changes in ATP related compounds in biological samples stored at frozen temperatures have been reported, but these changes were mainly observed in phosphorylated compounds (Hiltz *et al.*, 1971; Watanabe *et al.*, 1987). ATP, ADP and AMP can only be found at relatively high level after killing of fish with minimal struggle (Izquierdo-Pulido *et al.*, 1992). Fish for consumption usually shows IMP as the main ATP related compound. Thus, Perez-Villareal & Howgate (1987) proposed the use of a K_1 value to evaluate fish freshness. The K_1 value is obtained only from IMP, Ino and Hx contents. Freezing samples before analysis is a routine practice in many laboratories. Despite several authors reporting that samples and standards should be stored at −70°C for ATP and related compound determination (Ryder, 1985), the use of this low temperature is not always possible in food control laboratories. In this work we check the suitability of the storage at 18°C of fish samples and in fish neutralised extract stored at −18°C.

METHOD

Apparatus

Liquid chromatograph: a KONTRON model 600 E System Controller, a detector UV/VIS UVIKON 625 LC and an integrator Hewlett-Packard 3396A series II were employed. The LC column was a Tracer Spherisorb ODS 2 C_{18}, 0.4 cm×25 cm, particle diameter 5 μm (Teknokroma).

Centrifuge: P-SELECTA model Centronic S-577.

pH meter: CRISON model Digit 501.

Domestic mincer: MOULINEX.

Magnet plate: A06 SBS.

Reagents

Solvents: Methanol LC grade (Scharlau).

Water: Ultrapure water obtained from MilliQ-System (Millipore).

Mobile phases. **Method I:** mobile phase A: 0.04 M potassium dihydrogen orthophosphate (Teknokroma) and 0.06 M dipotassium hydrogen orthophosphate (Teknokroma) adjusted to pH 7 with 0.1 N potassium hydroxide (Panreac). Mobile phase B: methanol. **Method II:** mobile phase A: 0.1 M potassium dihydrogen orthophosphate (Teknokroma) adjusted with 0.1 N potassium hydroxide (Panreac) to pH 7, containing 1.95 mM of tetrabutylammonium hydrogen sulphate (Teknokroma). Mobile phase B: methanol.

Mobile phases were always filtered through HVLP 4700 membrane 0.45 μm (Millipore).

Standards: Inosine 5′monophosphate disodium salt (IMP), Adenosine 5′-monophosphate sodium salt (AMP), Adenosine 5′-diphosphate di(monocyclohexilamonium) salt (ADP), Adenosine 5′-triphosphate trisodium salt (ATP), Hypoxanthine (Hx) and Inosine (Ino) free bases were obtained from SIGMA. Stock solution: 1000 mg/l as free base or acid of each analyte in phosphate buffer pH 7. Working solution: Dilute stock solutions to 5, 10, 50, 100, 150, 200, and 250 mg/l with phosphate buffer. Filter through HVLP 1300 membrane 0.45 μm (Millipore) and store at 4°C in refrigerator and protected from light. Equilibrate to operating temperature before use.

Samples

Samples of canned tuna and samples of their corresponding raw material, fresh *Thunnus albacares*, were obtained directly from a canned tuna factory (Spain). Sample preparation was as follows: for fresh fish remove head, bones, and guts; for canned fish open can just before analytical determination, remove covering liquid and dry sample with absorbent paper. Then grind and homogenise sample mechanically with a domestic mincer for about 1 min until obtaining a fine paste.

The extraction procedure has to be performed at 4°C. Accurately weigh 10 g homogenised sample in a 50 ml centrifuge tube (30 mm diameter). Add 15 ml 0.6 N $HClO_4$ and a magnetic stirring bar. Mix thoroughly for 10 min on a magnetic stirring plate. Centrifuge 10 min at 3000 rpm and separate the two phases. Add 10 ml 0.6 N $HClO_4$ to the solid residue obtained, mix thoroughly for 10 min and repeat centrifugation. Discard solid phase. Combine the 2 perchloric extracts in 25 ml volumetric flask and fill up to volume with 0.6 N $HClO_4$. Take quickly an aliquot of 10 ml and adjust the pH to 6.5–6.8 with 0.1 N potassium hydroxide and led stand for 30 min at 4°C. Filter to remove $KClO_4$ and fill to 25 ml the neutralised extract with phosphate buffer (pH 7). Filter the neutral extracts through a 0.45 μm before LC analysis using HVLP 1300 membrane 0.45 μm (Millipore).

LC conditions

Method I: Flow rate of mobile phase was 1 ml/min. The elution program was as follows: 0 min, 100% A, 0% B; 8 min 100% A, 0% B; 9 min 70% A, 30% B; 13 min 70% A, 30% B. Finally, program took a further 10 min to return to the initial conditions and stabilise.

Method II: Flow rate of mobile phase was 1.5 ml/min. The elution program was as follows: 0 min, 100% A, 0% B; 5 min, 100% A, 0% B; 6 min, 90% A, 10% B; 15 min, 90% A and 10% B. Finally, program took a further 10 min to return to the initial conditions and stabilise.

Statistical analysis

Statistical analysis was performed by means of the SPSS Statistical Software Package. Analysis of variance of regression, Cochran's test, and Student's *t*-test were used in the reliability study. Non-parametric Kruskall–Wallis test was used to check stability of IMP, Ino and Hx in fish samples and fish extracts kept at −18°C.

RESULTS AND DISCUSSION

Using the mobile phase proposed by Ryder (1985), ATP, ADP, AMP, IMP, Ino and Hx were resolved, but Ino showed a wide, unsymmetrical peak since it was strongly retained in the C18 column. To improve Ino resolution, isocratic conditions were changed to gradient with 30% of methanol after 8 min. Methanol yielded a reduction in the elution time of Ino from 22 to 12 min. Figure 1 (a) shows the chromatogram obtained using the elution program described in LC conditions section for Method I.

Using the mobile phase proposed by Murray & Thomson (1983), which includes an ionic suppressor and 10% of methanol, Ino and IMP eluted very early and unresolved. The elimination of methanol resolved Hx and IMP, but ATP showed a wide, unsymmetrical peak. The addition of 10% of methanol to the mobile phase after 5 min of no methanol isocratic conditions described for Method II improved the resolution Fig. 1 (b)).

In both methods, ATP, ADP, AMP, IMP, Ino, and Hx were identified on the basis of retention time by comparison with standard solutions. The relative standard deviations (RSDs) of analyte retention time were very similar for both methods, ranging from 1.1 to 1.9 in Method I and from 1.3 to 2.0 in Method II.

Results of reliability study of both methods, which were made using the same standard solutions and fish samples (fresh tuna), are shown in Table 1. Detector response was linear from 2 to 200 μg/g. Linearity was verified by analysis of variance of regression (r values from 0.9963 to 0.9999, $p < 0.001$). Within-day precision studies were made after eight determinations, of ATP and related compounds using the same sample, reagents and apparatus. Relative standard deviations obtained (RSD) were always acceptable (Kolthoff *et al.*, 1975). Recovery was tested by the standard addition procedure using two addition levels for each analyte. By statistical analysis (Cochran's test), we verified that accuracy of both methods did not depend of analyte content in

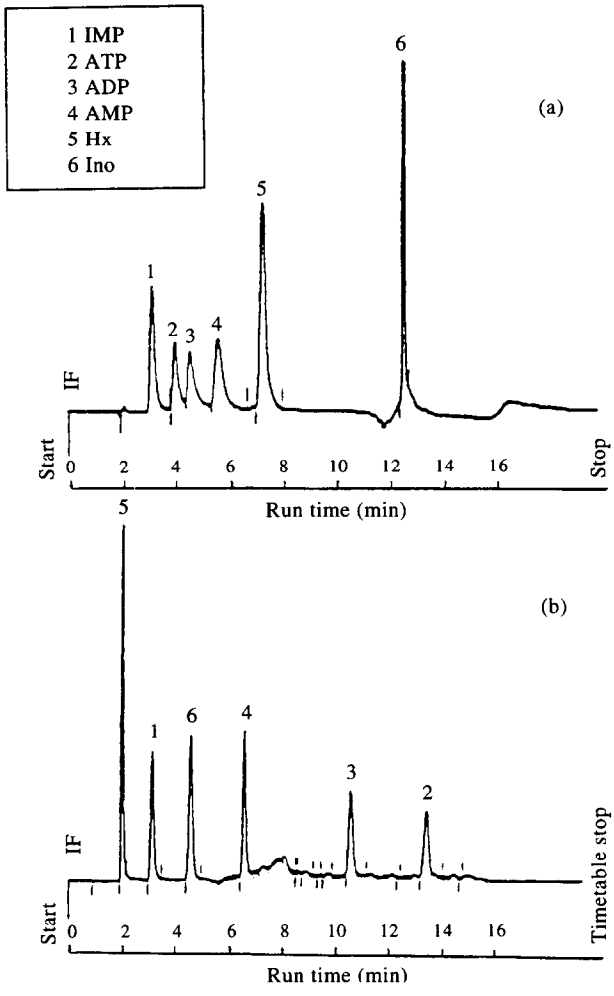

Fig. 1. Liquid chromatogram of a standard solution (20 μg/g). (a) Method I: flow 1 ml/min; mobile phase A was 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4 and mobile phase B was methanol. (b) Method II: flow 1.5 ml/min; mobile phase A was 0.1 M KH_2PO_4 adjusted with 0.1 N KOH to pH 7 and containing 1.95 mM tetrabutylammonium hydrogen sulphate and mobile phase B was methanol.

Table 1. Results of the reliability study of Method I and Method II

	Linearity (r)[a]		Precision % (RSD)[b]		Recovery % (mean ± SD)[c]		Sensitivity (Dtl, µg/g)[d]	
	Method I	Methods II	Method I	Method II	Method I	Method II	Method I	Method II
ATP	0.9985	0.9987	8.28[e]	2.66[f]	97.12 ± 8.7[g]	95.58 ± 5.5[g]	0.41	0.42
ADP	0.9999	0.9993	7.29[e]	8.46[e]	98.47 ± 6.5[g]	99.47 ± 6.9[g]	1.94	0.52
AMP	0.9999	0.9998	5.94[e]	4.62[e]	99.20 ± 9.5[g]	99.73 ± 6.6[g]	1.78	1.17
IMP	0.9968	0.9989	4.41[e]	2.82[f]	105.2 ± 9.9[g]	95.54 ± 5.5[g]	1.63	0.68
Ino,	0.9963	0.9999	8.15[e]	4.51[f]	96.32 ± 5.0[g]	95.61 ± 8.4[g]	2.10	1.91
Hx	0.9999	0.9997	3.22	5.96[e]	90.52 ± 2.5[g]	100.60 ± 6.9[h]	1.27	1.08

[a]Regression coefficient; [b]Relative Standard Deviation ($n = 8$); [c]Mean recovery ± Standard deviation ($n = 16$); [d]Detection limit; [e]Values for each analyte in precision and recovery bearing a common superscript letter are not different ($p \geq 0.05$).

samples. Then, by considering the mean recovery found for each one analyte, we also verified by Student's t-test that no significant statistical differences existed between the mean recovery found and the theoretical value of 100%, except for Hx (90.52%) when Method I was used. Since it was not possible to get a fish sample without ATP or a related compound, 0.6 N $HClO_4$ neutralised with 0.1 N KOH was the blank used to study the methods sensitivity. Detection limits were calculated according to the Long and Winefordner criterion (Long & Winefordner, 1983). Results obtained ranged from 0.41 μmol/g to 2.10 μmol/g for Method I and from 0.42 to 1.91 for Method II.

Range of linearity was similar for both methods. On the contrary, differences between methods were found in precision and recovery (Table 1). Thus, Cochran's test showed that RDS for ATP, IMP and Ino was higher in Method I than in Method II. Likewise, by means of the Student's t-test it was verified that Hx average recovery was higher in Method II than in Method I. In addition, sensitivity was, in general, better in Method II than in Method I.

Furthermore, another difference between the methods was the lack of specificity of Method I when canned tuna was analysed. A lack of specificity for ATP was observed after the comparison of chromatograms obtained from canned tuna and from its corresponding raw material (fresh tuna). Figure 2 showed, as in canned tuna, an unknown peak appears at ATP retention time. All canned tuna samples studied showed a peak response at ATP retention time which was always higher than the ATP response detected in the raw material. This was not observed using Method II (Fig. 3), since an unknown peak also appears but it

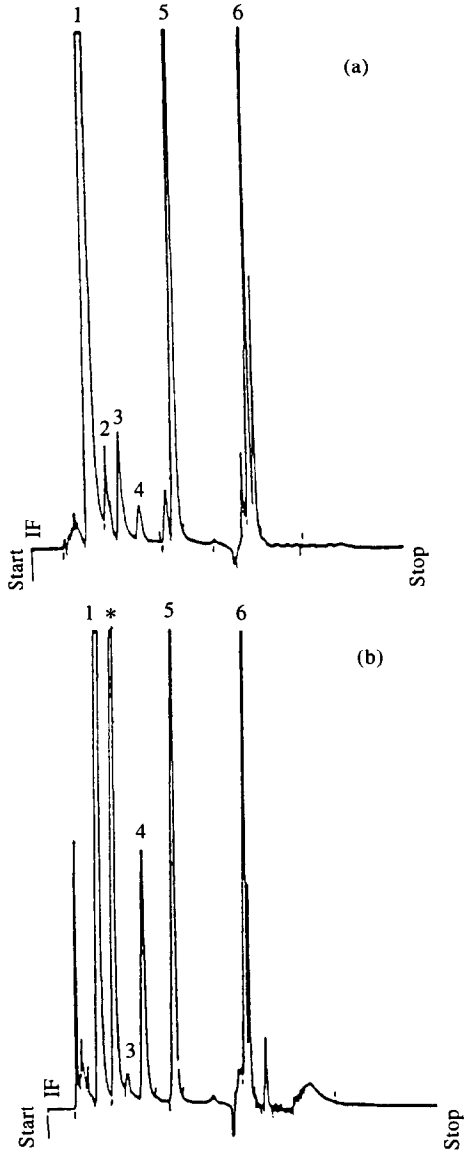

Fig. 2. Liquid chromatogram of ATP and related compounds in fresh (a) and canned tuna (b) by Method I. 1 = IMP, 2 = ATP, 3 = ADP, 4 = AMP, 5 = Hx, 6 = Ino and *, unknown peak.

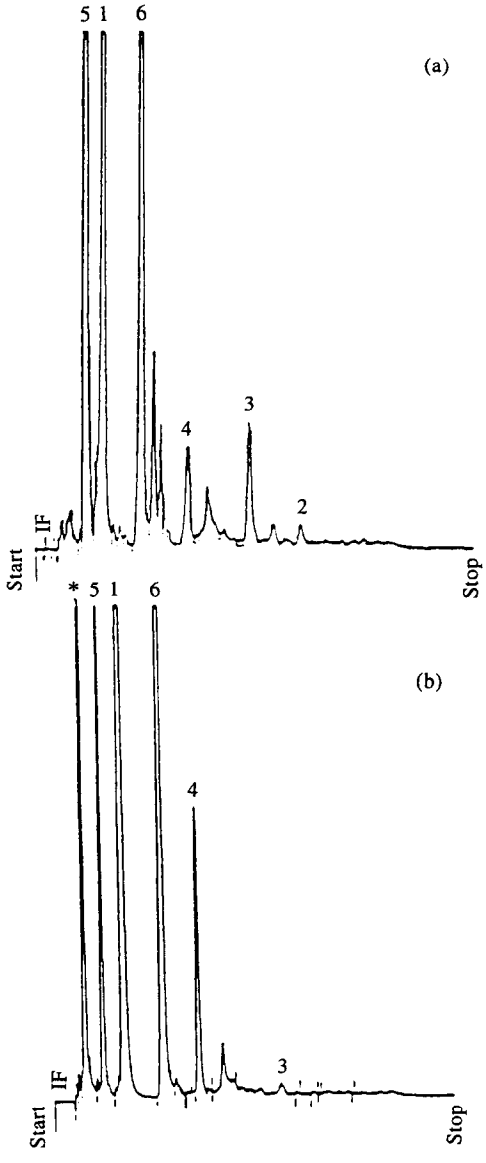

Fig. 3. Liquid chromatogram of ATP and related compounds in fresh (a) and canned tuna (b) by Method II. 1 = IMP, 2 = ATP, 3 = ADP, 4 = AMP, 5 = Hx, 6 = Ino and *, unknown peak.

Table 2. Changes of percentages of initial response (IR%) of IMP, Ino and Hx in fish samples and in fish neutralised extracts kept at 18°C

Time (weeks)	IMP		Inosine		Hypoxanthine	
	Fish sample	Fish extract	Fish sample	Fish extract	Fish sample	Fish extract
0	100	100	100	100	100	100
2	105±3	98±1	93±4	96±7	96±7	91±7
9	110±2	94+3	90±2	95±8	112+3	100±2
17	92+6	109±4	110±5	100±2	91±2	103±12
19	97±2	108±7	89±3	94+7	104±11	98±4
22	101±12	106±4	98±4	94±8	94±3	92+8
26	108±7	108±4	98±5	107±4	111±8	107+9

elutes at the very beginning of the chromatogram and it does not interfere with any ATP related compound. We verified that adenine and adenosine, which are possible heat break-down products of ATP (Murata & Sakaguchi, 1988), did not correspond to the unknown peak. Results showed that for canned tuna ATP should be quantified using Method II. The main drawback of Method II is that the use of ionic suppressor increases the economical cost of the mobile phases and the time required to condition the column before sample analysis.

Water and phosphate buffer are two solvents generally used to prepare ATP and related compounds standard solutions. We checked out the stability of ATP and related compounds in three different solvents: 1) water, 2) phosphate buffer solution (pH 7), and 3) phosphate buffer solution (pH 7.6). The standard solutions (100 mg/l) were protected from light and stored under refrigeration at 4°C for 5 weeks. Chromatographic responses were followed at zero time and periodically during storage. Results showed that both buffer phosphate solutions were suitable to keep stable standard solutions, since all analytes responses remained constant (changes lower than 1.0%) during the whole period of study. When water was used as solvent ATP, ADP and AMP responses only remained constant during 3 weeks of storage, whereas IMP, Ino and Hx responses were constant for the 5 weeks.

The stability at −18°C of IMP, Ino and Hx in fresh fish tuna samples ($n=4$) and in their corresponding neutralised fish extracts is showed in Table 2. Contents were determined at zero time (without storage) and after 2, 9, 17, 22 and 26 weeks of frozen storage. To evaluate possible changes during frozen storage, we compared for each sample the percentage of the corresponding initial chromatographic responses (IR%) throughout the 26 weeks of storage, since the four studied samples showed different concentrations of the analytes. Using a Kruskall–Wallis test no differences ($p \geq 0.05$) in the IR% were found between fish samples or fish extracts stored at −18°C throughout storage. On the basis of our results, storage at −18°C seems to be enough to keep fish samples and fish extracts before IMP, Ino and Hx determination.

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