# Determination of Adenosine Triphosphate and Its Breakdown Products in Fish Muscle by High-Performance Liquid Chromatography

John M. Ryder

Adenosine mono-, di-, and triphosphates, inosine monophosphate, inosine, and hypoxanthine in neutralized perchloric acid extracts of fish muscle were resolved in a single run by high-performance liquid chromatography. Recoveries of reference compounds added to fish muscle/perchloric acid homogenates were 94–100% and responses to repeated injections of a mixed standard were highly reproducible. Analysis time was reduced to 20% of that for previous methods which use ion exchange chromatography. $K$ values were computed directly from the results.

## INTRODUCTION

Many methods have been used for the objective assessment of fish muscle quality during storage. Most common among these are the determination of trimethylamine (TMA), dimethylamine (DMA), hypoxanthine (Hx), inosine 5′-monophosphate (IMP), and $K$ value (see below). Martin et al. (1978) reviewed these methods (except $K$ value) and suggested that changes due to autolysis and bacterial activity during chill storage were better revealed by the presence of adenosine 5′-triphosphate (ATP) catabolites rather than by the presence of TMA. However, reservations were expressed regarding the variability of both the nucleotide pools between individuals within a species and the rate of ATP catabolism between species. On the other hand Saito et al. (1959) had estimated the freshness of fish muscle from the ratio of the sum of the final breakdown products of ATP catabolism, namely inosine (HxR) and Hx, to the sum of ATP, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), IMP, HxR, and Hx. This ratio expressed as a percentage was called the $K$ value. Thus, the $K$ value would seem to obviate the reservations expressed by Martin et al. (1978) and may be one of the most appropriate chemical indicators of freshness devised (Ehira, 1976). In spite of this, $K$ values are infrequently reported presumably because of the difficulties encountered in the analysis.

Many different methods have been used for quantitative determination of ATP and its breakdown products. These can be divided into two major categories: those methods that measure the total nucleotides, nucleosides, and bases and provide information on ATP and its related compounds and those that measure one particular compound, for example, hypoxanthine.

Methods of the first category have traditionally used time consuming ion exchange chromatography (Jones and Murray, 1964; Spinelli and Kemp, 1966; Saito et al., 1959), whereas methods of the second category have used enzyme techniques predominantly to measure the relevant compound. These have been less time consuming but yield information on one of the compounds alone (Jahns et al., 1976; Burt et al., 1968).

More recently, high-performance liquid chromatography (HPLC) has been used to determine hypoxanthine (Warthesen et al., 1980) and $K$ value (Lee et al., 1982) in fish muscle. An enzyme sensor method (Karube et al., 1984) has also been used to determine $K$ value. None of these methods however, can be used to quantitate the levels of each of the ATP catabolites in fish muscle.

HPLC has also been used to determine ATP and its breakdown products in fish muscle (Murray and Thomson, 1983). However, this method employs a complex mobile phase employing ion pairing techniques and a noncommercial column.

The system reported here presents an HPLC method which utilizes simple reverse-phase separation with a commercially available column, which provides rapid, quantitative analyses of ATP and its breakdown products from which $K$ values may be computed.

## EXPERIMENTAL SECTION

**Materials.** Purine standards were obtained from Sigma Chemical Company (St. Louis, MO) and were checked for purity by the method described below.

**Extraction Procedure.** Five grams of muscle from the anterior dorsal region of the fish were homogenized with 25 mL of 0.6 M perchloric acid at 0 °C for 1 min with an Ultra Turrex T18/10 homogenizer (Janke & Kunkel GMBH and Co., Stanton, West Germany). The homogenate was centrifuged at 3000$g$ for 10 min, and 10 mL of the supernatent immediately neutralized to pH 6.5–6.8 with 1 M potassium hydroxide. After standing at 0 °C for 30 min, potassium perchlorate was removed by filtration through sintered glass and the filtrate diluted to 20 mL prior to storage at −70 °C for subsequent analysis.

**HPLC Analysis.** A Waters Associates Model 204A liquid chromatograph (Milford, MA) equipped with two Model 6000A pumps, a Model 730 Data Module, a Model 720 Systems Controller, a Model 440 Absorbance Detector, and a WISP Model 710B Autosampler was used for all analyses. Separations were achieved on a reverse-phase μBondapak C18 stainless steel column (3.9 mm I.D. × 30 cm Waters Associates) equilibrated at 30 °C. The mobile phase of 0.04 M potassium dihydrogen orthophosphate and 0.06 M dipotassium hydrogen orthophosphate dissolved in Milli Q purified distilled water (Millipore, Bedford, MA) was used at a flow rate of 2 mL/min. Buffer solutions were prepared daily and were filtered through a 0.45-μm aqueous filter (Type HAWP, Millipore, Bedford, MA) before use. The eluant was monitored at 254 nm with full scale deflection set at 0.2 absorbance units. The detector response for each of the six nucleotides, nucleosides, and bases found in fish muscle (Figure 1) was calibrated daily by injecting varying amounts of a solution containing 0.166 mM of each reference compound. Quantification was by use of external standards, and $K$ values were computed with the Model 730 Data Module. All solutions were passed through a 0.45-μm aqueous filter prior to injecting onto the column.

**Peak Identification and Percent Recovery.** Peaks obtained from fish muscle extracts were identified by co-

Division of Horticulture and Processing, Department of Scientific and Industrial Research, Auckland, New Zealand.

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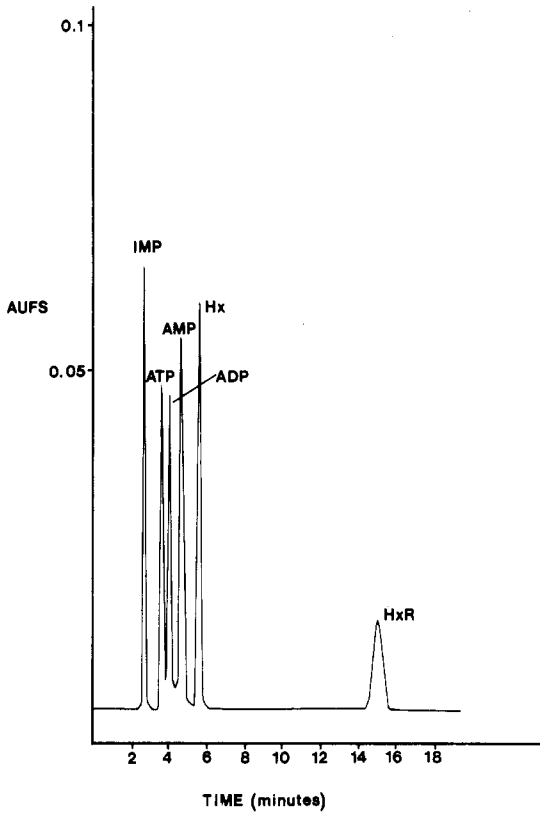


**Figure 1.** Separation of approximately 3.5 nmol of six standards relevant to ATP catabolism in fish muscle.

**Table I. Coefficient of Variation of Quantitation of 10 Replicate Standard Injections**

| compd | coeff of variation, % |
|---|---|
| IMP | 1.48 |
| ATP | 0.93 |
| ADP | 1.83 |
| AMP | 1.67 |
| Hx | 1.57 |
| HxR | 1.89 |

chromatography with standard solutions. The recovery of each compound was determined by spiking aliquots of fish muscle/perchloric acid homogenates with varying amounts of each compound. Analyses were carried out in duplicate. The homogenates were then analyzed as above. Endogenous concentrations were obtained from unspiked aliquots of the homogenates.

## RESULTS AND DISCUSSION

When this method was used, the six purines relevant to ATP catabolism in fish muscle (Jones and Murray, 1964) were sufficiently well resolved to permit accurate quantitation within 16 min (Figure 1). The coefficient of variation for each compound after 10 replicate injections of the standard solutions (Table I) demonstrates the reproducibility attainable by use of this method even when the adenine nucleotides are not completely resolved (Figure 1). Whereas this lack of resolution was not a problem in fin fish muscle extracts (Figures 2 and 3), it may become of more significance in analysis of crustacea muscle, where these compounds represent a major proportion of the combined nucleotide, nucleoside, and base pool (Dingle et al., 1968). However, quantitation of these compounds in crustacea muscle would still seem feasible using this method (Table I).

The ATP catabolites of hoki (*Macruronus novaezelandiae*) after storage in ice for 2 days and 21 days are shown in Figure 2 and 3, respectively. These analyses are typcial

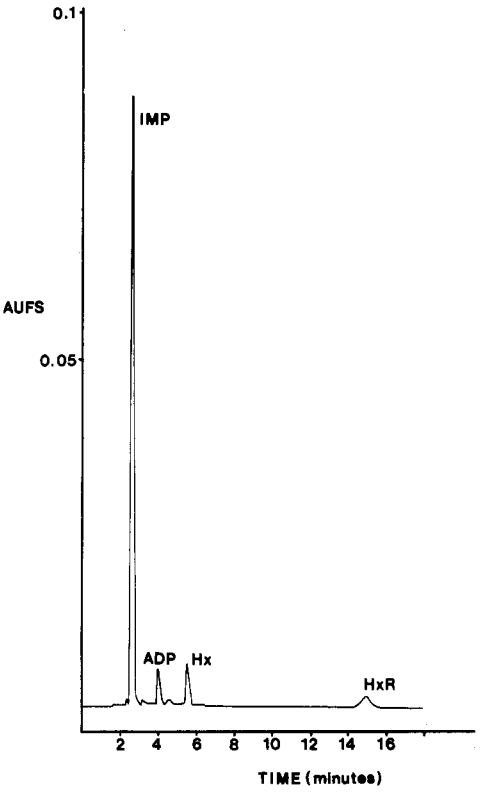


**Figure 2.** Analysis of ATP and its breakdown products in hoki. The fish had been in ice for 2 days.

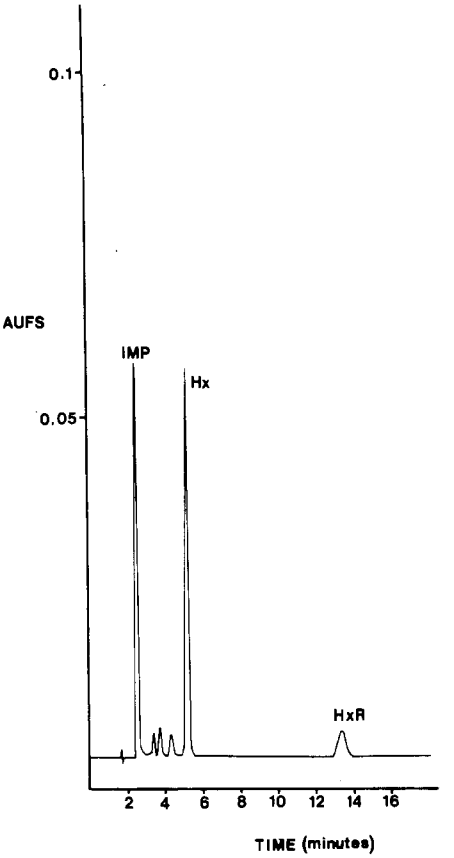


**Figure 3.** Analysis of ATP and its breakdown products in hoki. The fish had been in ice for 21 days.

of those obtained from other fin fish species (jack mackerel, *Trachurus novaezelandiae*; orange roughy, *Hoplostethus atlanticus*; unpublished data). Figure 4 depicts the changes in IMP, Hx, and HxR concentrations in hoki during storage in ice. ATP, ADP, and AMP were not

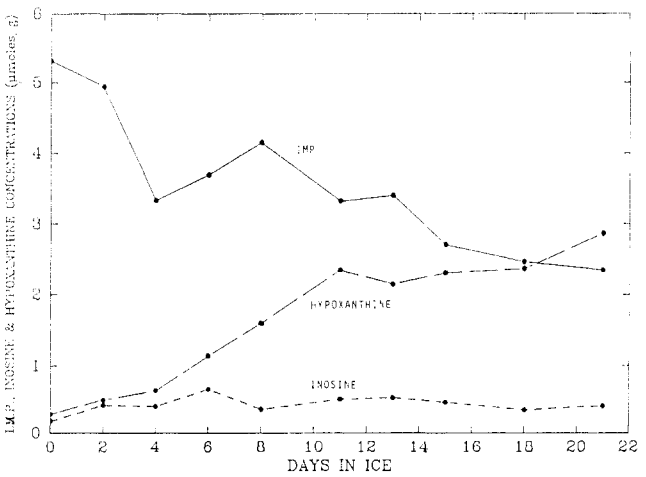


**Figure 4.** Changes in concentrations of IMP, inosine, and hypoxanthine in hoki during storage in ice.

**Table II. Percentage Recovery of ATP and Its Breakdown Products from Standard Added to a Perchloric Acid/Fish Homogenate of Known Initial Concentration of the Relevant Compounds**

| | recovery, % | | | | |
|---|---|---|---|---|---|
| | amount added, μmol/g[a] | | | | |
| compd | 0.60 | 0.30 | 0.15 | 0.08 | mean |
| IMP | 89.7 | 86.5 | 91.7 | 110.1 | 94.5 |
| ATP | 100.9 | 96.9 | 104.0 | 89.7 | 97.9 |
| ADP | 92.6 | 94.6 | 95.9 | 117.3 | 100.1 |
| AMP | 96.9 | 92.3 | 101.5 | 92.3 | 95.8 |
| Hx | 96.4 | 92.1 | 96.5 | 105.1 | 97.5 |
| HxR | 91.2 | 88.9 | 93.6 | 102.9 | 94.2 |

[a] μmol/g of fish/perchloric acid homogenate.

plotted due to their consistently low levels throughout the storage period. Hypoxanthine increased while IMP decreased and HxR remained constant. The *K* value also increased during this period. These chromatograms demonstrate that ATP and its breakdown products in fish muscle can be monitored by using this technique.

The mean recoveries for each compound, over the range of amounts of added standard, ranged from 86.5 to 117.3% with a mean recovery of each compound varying from 94.2 to 100.1% (Table II). Peak areas increased linearly with increasing concentrations of reference compounds up to 2 μg injected, and the minimum detectable amount was 5 ng/injection. The IMP and hypoxanthine peaks cochromatographed with guanosine 5′-monophosphate and guanine, respectively, but the latter compounds have seldom been reported in fish muscle extracts.

A radially compressed column (Waters Associates Z Module) with μBondapak C18 packing was evaluated but gave no advantages over the stainless steel column for routine analyses. In spite of the lower pressures and high flow rates associated with radially compressed columns enhancing the ease and speed of solvent changeover, the resolution between the adenosine nucleotide peaks was inferior to that achieved on the stainless steel column.

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**Registry No.** AMP, 61-19-8; ADP, 58-64-0; ATP, 56-65-5; IMP, 131-99-7; inosine, 58-63-9; hypoxanthine, 68-94-0.

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# Lipid Oxidation in Fish Tissue. Enzymatic Initiation via Lipoxygenase

J. Bruce German and John E. Kinsella*

Mechanisms underlying the initiation of lipid peroxidation in fish were investigated with trout skin tissue. Conversion of docosahexaenoic and arachidonic acid into polar products by skin tissue was consistent with enzymatic activity sensitive to lipoxygenase inhibitors stannous chloride and esculetin. GC/MS analyses of the monohydroxy derivatives of arachidonic acid identified 12-hydroxyeicosatetraenoic acid as the major monohydroxy product suggesting a 12 lipoxygenase activity in the skin. Thus, enodgenous skin lipoxygenase released post-mortem may constitute a significant source of initiating radicals leading to subsequent lipid peroxidation in fish tissue.

INTRODUCTION

Lipid oxidation is a very important event leading to the deterioration of foods containing highly unsaturated fats. Fish, in particular contain high levels of cellular unsaturated lipids (Kinsella et al., 1977) which readily deteriorate via peroxidation resulting in the development of objectionable odors and flavors. Although the process of lipid oxidation is highly favored thermodynamically, the direct reaction between oxygen and even highly unsaturated lipid is kinetically hindered. Hence, an activating reaction is

Institute of Food Science, Cornell University, Ithaca, New York 14853.

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