High-Pressure Liquid Chromatographic Determination of Hypoxanthine in Refrigerated Fish

A procedure based on high-pressure liquid chromatography was developed to measure the hypoxanthine content of fish tissue. The extraction, separation, and quantification procedures gave a rapid and direct measure of hypoxanthine in tissue. Coefficent of variation for the method was 4.0%, and recovery of hypoxanthine added to fish tissue averaged 92.5%. Whitefish samples held at 1 °C under various gaseous environments showed an accumulation of hypoxanthine with storage time that was readily measured with this method. This procedure has potential for use as an index of fish freshness.

Various chemical analyses have been suggested for use as indices of the deterioration of fish quality during storage. Martin et al. (1978) reviewed some chemical methods used to assess fresh fish quality and suggested that monitoring the accumulation of hypoxanthine (Hx) has several advantages, including reflecting both autolytic and bacterial activity in fish tissue. Hx formation has been shown to correlate to storage time and eating quality for a variety of species and storage conditions (Martin et al., 1978). Increases in the Hx content in the order of 5–10-fold have been demonstrated for perch and flounder after 9 days of storage at 0 °C (Spinelli et al., 1964; Jahns et al., 1976). Decreased sensory ratings were correlated to increases in Hx during storage of channel catfish in a study by Beuchat (1973). Taste panel evaluations also correlated well to Hx accumulation in chill-stored cod and haddock (Jones et al., 1964).

Procedures for measuring Hx in fish tissue have relied primarily on the principle of an enzymic reaction that converts Hx to xanthine and eventually to uric acid. After extraction of Hx from the tissue, xanthine oxidase is added to the extract and the increase in uric acid can be measured spectrophotometrically. Various modifications of the procedure have been proposed so that it can be automated (Jones et al., 1965), used with paper test strips (Jahns et al., 1976), or used with a redox indicator dye (Burt et al., 1969). While the use of the xanthine oxidase makes the method specific, the more rapid methods involving an indicator dye may suffer from interferences due to other positive-reacting materials (Beuchat, 1973).

Alternative methods for the determination of nucleotides in biological samples have been proposed based on recent developments in the area of high-pressure liquid chromatography (LC). In a relatively short period of time, nucleotides such as Hx can be extracted from tissue, separated from other nucleotides by reverse-phase chromatography, and quantified (Anderson and Murphy, 1976).

The use of a CO_2-enriched atmosphere has been investigated as a way to extend the shelf life of fresh fish (Brown et al., 1980). While organoleptic and microbiological evaluations are important criteria for determining fish freshness, the use of an index of freshness can be a guide in studying various gaseous atmospheres or a more rapid indicator of freshness when gathering preliminary data on quality changes during storage. The direct measurement of Hx in fish tissue by LC could be a useful approach to monitoring the freshness of refrigerated fish. The objective of this study was to evaluate the use of LC in measuring the Hx content of fish tissue for potential use as a quality index.

EXPERIMENTAL SECTION

Extraction. Fifteen grams of fish muscle was cut into small strips and blended with 30 mL of 7.5% trichloroacetic acid (Cl_3AcOH) at high speed for 1 min in a Waring blender. The blended material was filtered through Whatman No. 1 filter paper into a 100-mL volumetric flask. The blender jar and filter paper were washed 3 times with 10-mL volumes of Cl_3AcOH. The extract was adjusted to pH 4–5 with 1.0 N NaOH and brought to volume with 4% potassium dihydrogen phosphate buffer at pH 4.4. Samples were stored under refrigeration and analyzed within 1 week for Hx content.

Chromatography. A Waters Associates Model 204 liquid chromatograph was used for the Hx analysis. The column was a μBondapak C_{18} (3.9 mm i.d. × 30 cm, Waters Associates) used with a mobile phase of 4% potassium dihydrogen phosphate buffer (pH 4.4) pumped at a flow rate of 1.5 mL/min. The absorbance detector was set at a wavelength of 254 nm, and the output was recorded on a Model 3380A Hewlett Packard recorder-integrator. The fish extracts were filtered through a 0.2-μm membrane filter prior to injection of 10 μL. The amount of Hx was quantified by comparing the peak height from the fish samples to injections of standard Hx (Sigma Chemical Co.) prepared in 4% phosphate buffer.

Use of Xanthine Oxidase. Identification of Hx in a fish extract was made on the basis of elution time and cochromatography with Hx standard. For further verification of the Hx identification on the chromatogram, an extracted sample was treated with xanthine oxidase (EC 1.2.3.2; Grade III, Sigma Chemical Co.) to convert the Hx to xanthine and uric acid (Kalckar, 1947). A fish extract containing Hx was adjusted to pH 7 with 1.0 N NaOH, and 1.0 mL of the extract was treated with 10 μL of the enzyme preparation (0.1 unit). The mixture was held at room temperature and analyzed by LC after 25 and 80 min to determine changes in the Hx peak. Chromatograms of the enzyme-treated sample were compared to the extract without added enzyme to provide further evidence as to the identity of the Hx peak.

Analysis of Fish Tissue. Fish species investigated in this study, whitefish and herring, were obtained at a commercial outlet in Minneapolis, MN. Reproducibility of the Hx method was determined by analyzing six 15-g aliquots of herring muscle that had been blended for homogeneity. Recovery was estimated by spiking the tissue with 0.5 μmol of Hx/g and analyzing six replicate samples. The calculation was based on recovery of the added Hx.

The storage study was conducted by packaging whitefish fillets in Nylon/SURLYN pouches (26 × 38 cm) that were flushed with various gas mixtures and heat sealed. The pouches were held at 1 °C, and the contents were sampled periodically for Hx. The gases used for packaging were 100% CO_2, a mixture of 57% N_2, 42% CO_2, and 1% O_2, and a mixture of 69% N_2, 30% CO_2, and 1% O_2.

RESULTS AND DISCUSSION

The measurement of Hx in fish tissue by LC is a promising approach for assessment of fish quality. In developing

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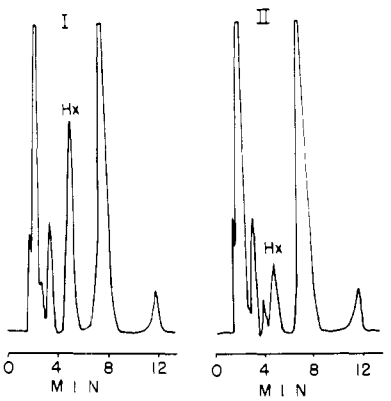

Figure 1. Chromatogram I is from a fish extract showing the elution of hypoxanthine as Hx. Chromatogram II is from the same extract after treatment with xanthine oxidase for 25 min.

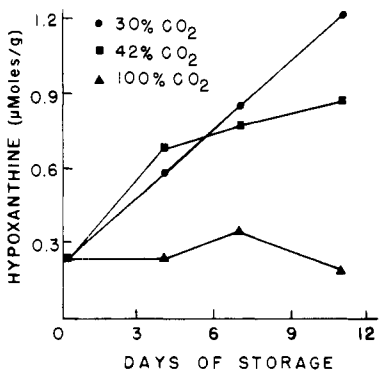

Figure 2. Accumulation of hypoxanthine in whitefish stored at 1 °C in various CO_2 environments.

LC methodology for fish tissue, it was necessary to consider factors such as chromatographic identification of Hx, reproducibility, and recovery. When fish muscle was extracted under the conditions described, numerous peaks appeared on the chromatogram, one of which cochromatographed with the Hx standard at approximately 5 min. Additional verification of the identity of Hx was obtained with the xanthine oxidase treatment. The chromatograms in Figure 1 represent untreated and enzyme-treated extracts. The HX peak decreased by 67% after 25 min of incubation with the enzyme. Additional incubation time resulted in a 90% decrease in the Hx peak after 80 min. Since xanthine oxidase action is relatively specific for the Hx degradation, the results provide further evidence for Hx identification and also suggest that there is little chance of an interfering compound in the extract that would coelute and complicate the Hx measurement.

When varied concentrations of Hx standard were injected, the response in peak height was linear over the range of concentrations tested up to 1 μg injected. The minimum detectable amount of Hx was 2 ng/injection. Replicate analysis of homogeneous tissue gave Hx levels ranging from 0.89 to 1.0 μmol/g with a mean of 0.96 μmol/g and a 4.0% coefficient of variation. When the muscle was spiked with Hx, recovery ranged from 89.8 to 96.9% with a mean of 92.5%. Extracts could be held at refrigerator temperature (5 °C) for up to 2 weeks without changing the measurement of Hx.

The change in the Hx content with storage time for the chilled-stored whitefish in several gas environments is shown in Figure 2. Increases in Hx were generally noted with increasing storage time, and increasing the level of CO_2 in the package seemed to decrease the accumulation of Hx. The results indicate that Hx measurement by LC can be a useful tool for investigations of storage stability and that the Hx level is responsive to varied levels of CO_2 enrichment. Correlations of Hx content to other freshness criteria such as organoleptic evaluation, microbiological populations, or oxidative changes must be considered for various species and conditions before conclusions can be drawn about the value of a CO_2-controlled atmosphere or the CO_2 level giving highest quality. Nevertheless, the Hx procedure studied in this investigation is objective, rapid, and accurate and it represents an alternative to the xanthine oxidase procedure. This study shows that the LC method for Hx is a potentially valuable technique and can be useful in collecting data that can serve as an index of fish quality.

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