COOLING RATE OF SHUCKED OYSTER MEATS IN ONE-GALLON PLASTIC CONTAINERS

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INTRODUCTION

Potentially hazardous foods are those foods that are capable of supporting rapid and progressive growth of infectious or toxigenic microorganisms or the growth and toxin production of *Clostridium botulinum*. The FDA Food Code (USDHHS, 1993) requires that potentially hazardous food be held under refrigeration at 5°C (41°F) or below or at temperatures above 60°C (140°F). In general the maximum time potentially hazardous foods are permitted to be held in the hazardous temperature range is four hours.

Raw molluscan shellfish are considered as a potentially hazardous food, but the guidelines which govern their processing and storage are contained in the National Shellfish Sanitation Program, Manual of Operations, Part II (USDHHS, 1995). The time live shellfish as shellstock can remain outside refrigeration after harvest depends upon the time of the year or temperature of the harvest water and their intended use (i.e. for raw consumption or for shucking). Once shellstock are placed under temperature control, the storage area must be continuously maintained at 7.2°C (45°F) or below. After initial refrigeration, shellstock may not remain outside temperature control for more than two hours. Following shucking, the meats must be delivered to the packing room within one hour. Shucked meats must be cleaned, thoroughly drained and packed using a schedule which permits the meats to be chilled to an internal temperature of 7.2°C (45°F) within two hours of delivery to the packing room.

Appendix C, Part II of the Manual (2) provides a series of cooling curves for oyster meats packed in various size containers to assist processors in developing cooling schedules to comply with the two hour cool down time. These curves were generated more than 30 years ago with metal containers at the request of the U.S. Public Health Service by the American Can Company. The data from these curves
have been extrapolated to suggest it requires two hours for meats in one gallon metal containers to cool from 10°C (50°F) to 7.2°C (45°F).

Today, plastic containers are the industry standard for packing shucked meats. The types of plastics used in the manufacture of food containers have lower thermal conductivity than metals used to construct cans. This study was undertaken to produce new cooling curves for oyster meats packed in one-gallon plastic containers which the industry can use to set cooling schedules. These curves are compared with those published in Appendix C, Part II of the Manual (2). Additionally, the cooling curves for dry pack oysters and oysters packed with water are contrasted because some processors contend that dry pack oysters cool slower resulting in a poor quality product.

MATERIALS AND METHODS

Container

The one-gallon plastic container most frequently used by the Gulf oyster industry is the "tall gallon bucket." This container and snap-on lid are constructed of high density polyethylene with a wall thickness of approximately 0.041 in. (1.04 mm). Approximate dimensions are top diameter 7 in. (177.8 mm), bottom diameter of 5.5 in. (139.7 mm) and overall container height of 7.94 in. (201.6 mm). The containers used in this study were manufactured by Venture Packaging Incorporated, Moroville, OH 44847-0246, item number LT700128.

Temperature measurement

Temperatures were measured with mercury-in-glass thermometers with the bulbs positioned at the approximate geometric center of each container. Each thermometer was calibrated against a certified thermometer over the range of 4.4 to 26.7°C (40 to 80°F). The temperature in each container during cooling was also continually recorded with a thermistor linked to an M160 data logger (Omega Engineering, Stamford, CT).

Oysters

Oyster meats were purchased from a local shop on the same day they were shucked. The meats were adjusted to the desired temperature before initiating each cooling experiment as follows. The contents of each gallon container of shucked meats was distributed into four one-gallon zip-seal bags. The bags were suspended in approximately 20 liters of water at the desired temperature for one hour. The
meats were removed from the bag and drained for two minutes on a standard skimmer before placing in the test container.

**Ice**

Commercially-produced flake ice was obtained the day prior to the study and held overnight in a cool room at 4°C (39°F).

**Experimental procedure**

Oyster meats were adjusted to the desired experimental temperature, as described above, and placed in containers, two for each study. Lids were placed on each container and thermometers and thermistors were inserted through holes in each lid to the approximate geometric center of the container. The initial temperature was recorded and the containers were placed onto a two-inch thick bed of ice in an insulated box and then surrounded with wet ice. Lids were covered to a depth of about one inch with ice. The insulated box was equipped with a drain to remove water resulting from the melting of the ice. Temperature readings on the thermometers were recorded at 30-minute intervals. As needed, fresh ice was added to the chest.

After each experiment was completed, the meats were removed from the test container and adjusted to the new starting temperature.

**Comparison of metal and plastic containers**

Data was obtained by using plastic containers. It was compared with the data taken from the curves provided on page APC-2, Appendix C, Part II of the Manual (2). Those curves were developed by using oysters packed in (610 x 708) metal cans.

**Solid vs. wet pack study**

In recent years, some Gulf processors have begun packing shucked oysters by the weight of drained meats. A common packaging weight is 6 pounds, 7 pounds or 8 pounds of drained meats in a one-gallon container with water added to fill the container. An 8-pound gallon would represent a "solid" or "dry" pack. For the solid vs. wet pack study, the cooling rates of an 8-pound gallon (no water added) and a 6-pound gallon (6 pounds of oyster meats and water to fill the container) were compared.
RESULTS

Figure 1 presents the results of five separate cooling studies in which the initial temperature of the oyster meats ranged from 9.4 to 25.6°C (49 to 78°F). Data for each study represents the average of two replicates. In all trials there was an initial lag of 0.5 to 1 hour after icing before the temperature at the center of the container began to decrease. Subsequently, the temperature decreased at a rate of 2.8°C (5°F) or less per hour. As the temperature decreased in the container, the rate of temperature decrease declined. Only packs of oyster meats with an initial temperature below 10°C (50°F) were cooled to 7.2°C (45°F) at the geometric center within two hours of icing.

Figure 1. Cooling curves for oyster meats in the geometric center of one-gallon containers packed in ice
A comparison of cooling curves for meats in one-gallon plastic and metal containers is shown in Figure 2. At the two starting temperatures shown, the curves developed for both types of containers have approximately the same slope.

Figure 2. A comparison of the cooling of oyster meats in the geometric center of one-gallon plastic (solid line) and metal (broken line) containers packed in ice. Data for the metal containers (610x708 can) were taken from National shellfish Sanitation Program, Manual of Operations, Part II, Appendix C, page 2.
Cooling curves for solid and wet packs with initial temperatures of approximately 15.6°C (60°F) are compared in Figure 3. The curves are similar which indicates that the addition of up to 25% water to a pack does not change the cooling rate of the oyster meats in the center of container.

![Graph showing temperature changes over time](image)

**Figure 3.** A comparison of the cooling of oyster meats in the geometric center of one-gallon plastic containers with and without added water. Solid pack oysters (solid line) were drained for two minutes before packing. Wet pack oysters (broken line) contained 75% oyster meat and 25% added water by weight.

**SUMMARY**

The cooling rate of shucked oyster meats in one-gallon plastic containers was approximately the same as that reported for one-gallon metal containers. Temperature at the geometric center of the one-gallon container did not begin to decrease until 0.5 to 1 hour after the container was packed in ice; the rate of decrease thereafter was 2.8°C (5°F) or less per hour. We were unable to achieve an internal temperature of 7.2°C (45°F) at the center of the container within the required two hours with the exception of meats that had been precooled to less than 10°C (50°F) before packing into gallon containers and icing. Therefore, shellfish processors should consider precooling shellfish before packing. Strategies for precooling meats include adding ice to the shucking bucket, using chilled water to wash meats on the skimmer and adding cold water or crushed ice to the tank during blowing.
REFERENCES


SURVIVAL OF FLORIDA AQUACULTURED CLAMS
IN REFRIGERATED STORAGE

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¹ Florida Dept. of Agriculture, Bureau of Seafood and Aquaculture
² Food Science and Human Nutrition Dept. and
³ Cooperative Extension Program
at the University of Florida

The northern hard clam, Mercenaria mercenaria, is thriving as an aquacultured product of Florida. About 200 shellfish growers currently farm over 700 acres of state-owned submerged lands off two counties on Florida’s west coast. Production of hard clams has fast become established in areas where neither aquaculture nor a traditional clam fishery existed. During 1995, sales of clams produced by Florida growers totaled $5.41 million. This was a 48% increase from 1993. This is equivalent to 43 million live clams sold in 1995. Seeding in 1996 also increased lending to predictions of continued growth for this industry.

The marketability of these clams has been hampered by a perceived short survival in refrigerated temperatures (Menzel, 1972). Commercial hard clams harvested wild in Florida have been both Mercenaria mercenaria and Mercenaria campechiensis and previous work showed that the survival of these clams in common refrigeration decreased significantly as the water temperatures increased throughout the summer months (Otwell et al., 1986; Menzel, 1971).

The objective of this study was to investigate the survival of the Florida aquacultured clam in variable conditions and refrigerated temperatures. Practical methods to increase the survival of the clams and thus improve their marketability were evaluated. These included several tempering techniques which allowed the clams to acclimate to the temperature changes from harvest to storage.

METHODS

This project was initiated by the clam industry in Florida. Farms in four different areas about Florida sent 500 clams from each location (totaling 2000 clams) to the University of Florida, Food Science and Human Nutrition Department the first week of every month for eight months. All the clams were harvested within the same few days and the water temperature, salinity and shipping conditions noted. The target size was 7/8 to 1 inch hinge
width. Immediately post harvest the clams were packaged and sent to Gainesville. Upon arrival at UF, the samples were separated into twelve subsamples. Six of these subsamples were stored in three different temperatures (35, 45 and 55 °F) in two different storage arrangements. Samples were stored in harvest bags to simulate shipment and in trays to simulate retail conditions. Six other subsamples were saved for miscellaneous testing such as freezing, and tempered dry and wet storage with various temperature cooling profiles. In dry storage, refrigerated incubators were programmed to slowly go from harvest water temperatures to storage temperatures. A continuous program (85°F—(20hrs)—45 °F) and a stepwise program (85°F—(6hrs)—65°F—(14hrs)—45 °F) were both investigated. For wet tempering, a recirculating tank was set at 68°F. Clams were placed in the tank immediately post harvest and samples removed after six hours and after 24 hours and placed in the three storage temperatures. All samples were checked daily. The condition of the clams (odor, drip loss and appearance) and the number of dead clams were recorded. If the clams were resting open, they were tapped gently. If they closed they were considered alive. If they remained shut briefly but reopened they were considered commercially dead. If the clams did not shut they were dead. The clams were stored in refrigeration until 50% had died.

RESULTS AND DISCUSSION

The data are presented to show the number of days 80% or more of the clams survived in refrigerated storage from May through October 1996. At the required storage temperature of 45°F the days with 80% clam survival decreased from an average of 17 days in May and June to an average of 8, 7 and 8 days in July, August and September respectively (Figure 1). In October, the average number of days with 80% survival increased up to 16. The survival of the clams from Charlotte Harbor was low in May. That area had been closed for several months due to red tide and this may have weakened and or stressed the clams prior to harvest and shipment. The survival of the clams from Indian River North was low in July following heavy rains which drastically decreased the salinity of the water in that area.
The average values for clam survival from all four locations at the three storage temperatures are presented in Figure 2. At 35°F the average days with 80% clam survival was lower than 45°F for all months except May. The greater change from water to storage temperatures stressed the clams further and decreased their survival. As expected the clams stored at 55°F had a longer survival rate than those in the other storage temperatures except in May. Although the clams stored at the higher temperatures lived longer, there were concerns related to the microbiological consequences of storage at the higher temperatures.

![Figure 2. Survival of Florida cultured clams in refrigerated storage.](image)

The water temperatures increased from an average of 76.3 °F in May to an average high of 84.3 in July (Table 1). This approximate 10 degree increase in July and August did have a negative influence on the survival of the clams in all three refrigerated storage temperatures. The salinity of the water at the time of harvest did not appear to influence the survival of the clams but did influence their growth (Table 2). The clams from the areas with the lower salinity were smaller in size throughout the study (Table 3).

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Table 2. Water salinity at harvest for all four locations.

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Table 3. Average size (mm) of the clams harvested from all locations.

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<tr>
<th>Location</th>
<th>Cedar Key</th>
<th>Charlotte Harbor</th>
<th>Indian River (N)</th>
<th>Indian River (S)</th>
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<td></td>
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<td>50</td>
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<tr>
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<tr>
<td>October</td>
<td>27</td>
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Tempering the clams from harvest temperatures to storage temperatures in both dry and wet conditions increased the survival significantly. The dry tempering in the refrigerated incubators doubled the number of days with 80% of the clams alive in both July and August, Figure 3. There was no significant difference in clam survival between the continuous and stepwise dry temperers. The number of days with 80% survival of the clams was more than doubled when they were tempered in recirculating seawater, Figure 4. The best tempering results were obtained when the clams were tempered at 68°F for 24 hours post harvest.

![Figure 3. Survival of Florida cultured clams tempered from 85 to 45°F](image-url)
CONCLUSIONS

The data showed that the primary influences on the survival of clams were the temperatures of the waters at the time of harvest and storage temperatures. When the clams were exposed to rapid and large changes in temperature (>20 degree changes) they became stressed and died. As the water temperatures increased during the summer months the change in temperature between harvest and storage became greater and the clams had shorter survival times. Tempering in both dry storage and recirculating seawater acclimated the clams to the lower temperatures, reduced stress and significantly increased their survival. More research is needed in this area.

REFERENCES


THE CHARACTERIZATION OF THE PROTEOLYTIC ENZYME(S) RESPONSIBLE FOR THE POST MORTEM SOFTENING OF SILVER HAKE (Merluccius bilinearis)

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INTRODUCTION

Silver hake is a small gadoid fish of great abundance in the North Atlantic. It has not previously been considered a useful species because of its relatively small size (< 30 cm), softening during iced storage and tissue toughening in frozen storage due to the cross linking induced by the enzymatic formation of formaldehyde. Due to the declining traditional fish stocks, the utilization of this species has increased but the problems associated with processing these fish still exist. A potential end-product for silver hake is surimi. To produce a high quality surimi, only the best quality fish muscle can be used. It has been known for some time that when silver hake is stored on ice for any length of time, the tissue begins to soften and is unacceptable for consumption (Hiltz et al., 1976; Leim and Scott, 1966). Therefore the fish must be processed quickly upon arrival at the plant or on board ship, immediately upon landing. As fresh fish must be used to get firm muscle tissue, the fishermen can only remain at sea for a limited amount of time. The fish must be processed quickly in order to ensure quality of the end product.

It is hypothesized that there may be a slow release of membrane-bound proteolytic enzymes causing subsequent softening of the muscle tissue. If this were true, one may be able to correlate the enzyme proteolytic activity in the sarcoplasmic fluid with textural softening. Because there are many proteolytic enzymes associated with fish, only those that are considered active in the physiological pH range, and intimately associated with the myofibrils were examined.

Cathepsin B is a lysosomal cysteine protease that has a major function in intracellular breakdown (Polgar, 1989). An et al. (1994a) showed that one of the most active enzymes in Pacific whiting fish fillets was cathepsin B. Like silver hake, Pacific whiting softens quickly on iced storage. Calpains are neutral thiol proteases, that require calcium as an activator. In comparison to cathepsins, calpains are high in molecular weight (27 KDal for cathepsin, 210
KDal for calpain). Calpain is an intracellular, non-lysosomal cysteine protease (Polgar, 1989). Calpain's main site of proteolysis in postmortem mammalian muscle is the Z-disc and the proteins associated with it, and is thought to be involved with the turnover of contractile proteins (Koohmaraie, 1992). However, fish calpains have been noted to also have an effect on myosin and other contractile proteins (Muramoto et al., 1989).

Using phase contrast microscopy and scanning electron microscopy (SEM), the ultrastructure of the myofibrils were examined to help verify the conclusions acquired from the enzyme/texture relationship.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical Company (St.Louis MO), unless otherwise specified. Double distilled deionized water was used and all glassware was cleaned in Decon 9, rinsed three times in warm water, and rinsed three times in double distilled, deionized water.

Collection of Samples

Fish were caught, sacrificed and stored on ice in a 3oC for not more than three days prior to examination. Fish were randomly picked for each experiment.

Preparation of Crude Sarcoplasm

For all extractions, the sample weights, the volumes of sarcoplasm and the sarcoplasmic protein concentration were measured. Total proteolytic enzymes, calpains and cathepsins were extracted in a one step extraction, described by Nilsson and Ekstrand (1994) with some minor changes. Muscle tissue was excised from the dorsal area of the fish and ultracentrifuged using a Beckman SW-27 rotor, at 100,000 x g for 60 minutes at 3°C. The supernatant was then collected and assayed for protein and enzyme activities.

Enzyme Assays

Optimal pH's and temperatures were determined for all enzyme assays. Temperatures ranging from 0°C - 50°C were tested along with pH levels ranging from 3-8. Calculation of total enzyme present was calculated for each substrate. In order to determine which enzymes were membrane-bound, some samples of muscle tissue were homogenized using a mortar and pestle and centrifuged at 100,000 x g (in order to disrupt lysosomes). The activities were compared to the more gentle treatment of centrifugation which was intended to leave lysosomes intact (Lehninger, Nelson and Cox, 1993). This procedure helped determine which enzymes were present in the sarcoplasm (Nilsson and Ekstand, 1994). All enzyme extracts were kept on ice until their addition to the reaction mixtures.
Casein Assay

The protocol for apparent calpain activity was described in Wang et al. (1993). Each reaction mixture (in a final volume of 1.0 mL) contained 4 mg of casein, 50 mM imidazole-HCl buffer (pH 7.5) containing 10 mM b-mercaptoethanol, 0.5 mL sodium azide, 0.05 M calcium chloride. To this solution, 1.0 mL of diluted sarcoplasm was added. The samples were incubated for a total of 60 minutes at pH's ranging from 3.5-8 and temperatures ranging from 0°C - 50°C. Once the optimal pH and temperature was established, all other assays were conducted at those values. The reactions were terminated by the addition of 0.5 mL of 10% trichloroacetic acid (TCA). The tubes were then placed in 3-5°C cold room overnight and filtered through Whatman #1 filter paper. Using the method of Lowry et al. (1951), the concentration of TCA-soluble peptides in the filtrate was determined using bovine serum albumin (BSA) as the standard on a Philips PU 8800 UV/VIS spectrophotometer. One unit of calpain activity was defined as the amount of enzyme that caused an increase of one absorbance unit at 280 nm after 60 minutes incubation at 25°C, and corrected by subtracting the activity of a blank that was measured in the presence of EDTA.

Z-Arg-Arg-NMec Assay

The apparent activity of cathepsin B was measured using the procedures described in Yamashita and Konagaya (1990) and Barrett and Kirschke (1981). The muscle was excised as before. The artificial substrate that was used for cathepsin B was Z-Arg-Arg-MCA (benzyloxy carbonyl-Arg-Arg-7-(4methyl) coumarlamide). The samples were assayed at pH's ranging from 3.5-8 and temperatures ranging from 0°C - 50°C. Once the optimal pH and temperature was established, all other assays were conducted at those values. For this assay, 10 mM of Z-Arg-Arg-MCA was used as the substrate. Stock substrate was prepared by dissolving 1mM Z-Arg-Arg-MCA in sulphonate, and kept at 3°C until needed and made 20 mM with 1% Brij solution daily.

The activities were measured by mixing 1 ml of the appropriately diluted amount of muscle extract with 1% Brij solution, 1 mL of 0.2 M citric acid-phosphate buffer (0.1% b-mercaptoethanol, prepared daily), waiting one minute for activation and then beginning the reaction with the addition of 1 mL of 2 mM substrate (Z-Arg-Arg-MCA). Samples were incubated at the appropriate temperature and pH, using a temperature controlled cell in a luminescence spectrophotometer (Perkin Elmer C550 Luminescence Spectrophotometer), set at excitation 370 nm, and emission 460 nm. A standard of 7-(4-methyl) coumarlamide was made to quantitatively measure the amount of product being liberated. This reaction was performed in the dark because 7-(4-methyl) coumarlamide is photo-reactive. One unit of activity was expressed as that releasing 1 mmol of aminomethylcoumarin/min at 25°C.

Inhibition/Activation Assays

Inhibitory assays were performed to confirm the identity of the enzyme(s) suspected to be the cause of the softening. In each case, the same enzymatic assay was performed both
with and without the inhibitor or activator. Refer to Table 1 for the effect of inhibitors and activators used.

Table 1. Common protease inhibitors/activators and their effects

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Effect</th>
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<tr>
<td>E-64*</td>
<td>Cysteine protease inhibitor, blocking thiol groups.</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Essential for the activity of calcium activated proteases.</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>Serine protease inhibitor.</td>
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<tr>
<td>EDTA</td>
<td>Chelates divalent cations such as Ca⁺².</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Cysteine protease inhibitor, blocking thiol groups.</td>
</tr>
<tr>
<td>Antipain</td>
<td>Cysteine protease inhibitor.</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>Carboxyl protease inhibitor.</td>
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<tr>
<td>Iodoacetic acid</td>
<td>Cysteine protease inhibitor</td>
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* E-64 (L-trans-epoxysuccinylleucylamido(4-guanidino)butane.

Inhibitor and activator concentrations were described in Yamashita and Konagaya (1992) and Kominami et al. (1984). The concentrations of each inhibitor used in each assay were 2 mM E-64, 50 mM CaCl₂, 0.05% soy trypsin inhibitor, 2 mM EDTA, 0.1 M leupeptin, 10 mg/ml antipain, 0.1 M pepstatin and 2 mM iodoacetic acid. Calcium chloride is an activator for calpain like enzymes, and was omitted from casein assay to show its affect.

Texture Analysis

Texture analysis was performed using an Instron Model 4502 (Instron, Canton, MA) equipped with a Kramer shear-compression cell (Gill et al., 1979). Fresh uncooked fish were used for texture measurements. The Instron Series IX Automated Materials Testing System (Version 5.02, Instron Corporation) was used to calculate the shear force and peak force at various user defined points on the texture profile (Figure 1).

Phase Contrast Microscopy

Aseptically dissected muscle tissue (10g) and 90g of distilled water was homogenized in a stomacher (Lab Blender 400) for 30 seconds in order to prepare a fine suspension of individual myofibrils. A drop of this homogenate was placed on a clean microscope slide with a cover slip and viewed at 400x under oil immersion on a phase contrast microscope (Nikon Optiphot) equipped with a Nikon (FX-35A) camera.

Electron Microscopy

Samples of fish fillets (three) were wrapped in plastic, iced and transferred immediately to the electron microscopy laboratory of the "Institute for Marine Biosciences" (National Research Council of Canada, Halifax, NS). The fillets were further dissected into 1 mm
samples, ensuring that for each sample, there was a longitudinal section, an oblique section, and a transverse section. These samples were plunged into liquid propane, cooled near liquid nitrogen temperatures. These samples were fractured by striking with a sharp blade, freeze dried in a Meitech Model 750 freeze drier and gold-coated in an Edwards 306A coater. The samples were examined using a scanning electron microscope JEOL model JXA35 at magnifications of 2000X and 4000X.

Statistical Analysis

Systat version 5.05 for Windows was used to analyze data using ANOVA stepwise regression, calculating the t-value and F-value for a 5% level of significance. Error bars on figures represent 2 standard deviations.

RESULTS AND DISCUSSION

Texture Analysis

Ando et al. (1991) used a puncture test to show that the firmness of muscle tissue during iced storage had a good correlation with sensory results. Although this study was used to show a relationship between the softness of sashimi (a raw fish meat dish) and the perceived mouth feel, the study also showed that the break strength of muscle tissue correlated with tissue softening.

After several trials, it became qualitatively apparent that the modified Kramer cell force deformation curves gave reasonable measurements of softening of silver hake muscle tissue. Several portions of the force deformation curves were examined. Of the 5 points measured, the maximum load and firmness (Figure 1) best measured the softening of the silver hake. Because of the size of the fish, a pooled sample was used for analysis. Therefore any variation within each test period was test variation, not sample variation. Figure 2 shows that during iced storage, the muscle tissue became softer and after 6 days of iced storage, developed a "pudding-like" texture.
Figure 1. Typical force deformation curve, illustrating possible points of significance (Voisey and Larmond, 1977).

Figure 2. Texture profile analysis of silver hake stored on ice for 4 days ($n=4$, Max. Load, $T=7.084$, $F=50.84$, $p=0.000$; Firmness, $T=10.388$, $F=17.906$, $p=0.000$). Refer to Figure 1 for definition of points.
Casein Assay

Apparent millimolar calpain activity was monitored using the method of Wang et al. (1993) using casein as a substrate. Casein hydrolyzing activity was inhibited by E-64 (99.8%), EDTA (85%), leupeptin (91%), antipain (92%) and IAA (88%). Caseinolytic activity was enhanced by the addition of Ca+2. The optimum pH was higher than the pH of the fish muscle extract, but there was activity between pH 6 and 6.5. The optimal pH was measured to be 7.1, with an optimal temperature of 30°C. There was an increase in the specific calpain-like activity in the sarcoplasm over time of iced storage (refer to Figure 3). It is speculated that mechanism for the softening of the muscle tissue may not be one agent but rather a combination of several enzymes. It would appear that calpain was one of the enzymes that had an affect on the overall texture of silver hake muscle tissue.

![Graph showing specific activity vs days.](image)

*Figure 3. Casein-hydrolysing activity from silver hake tissue extract, pH 7.1, Temp 30°C (n=6, T=11.512, F=132.532, p=0.000).*

Z-Arg-Arg-NMec Assay

It was expected that the cathepsins may have had a greater influence over the softening of the muscle tissue than the calpains although the latter are very powerful proteolytic enzymes and thought to be the causative agent in the softening of mammalian muscle tissue (Koomaraie, 1992). The fluorescence of aminomethylcoumarin liberated from the substrate was measured by excitation at 370 nm and emission at 440 nm with a fluorescence spectrophotometer. The cathepsin B specific activity increased nearly 5-fold over time of iced storage (Figure 4). The total cathepsin B (bound plus unbound activity) was also calculated and showed that 96% of the cathepsin B was solubilized during iced storage. All assays were completed at the optimal pH determined to be 6.5, with an optimal temperature of 27°C. Cathepsin B has been regarded as the most active cysteine protease in Pacific whiting fish fillets (An et al., 1994b). Because silver hake is a close relative of Pacific
whiting, it would be reasonable to assume that cathepsin B would be a very important factor in the proteolysis of silver hake muscle tissue.

![Graph showing specific activity over days](image)

**Figure 4.** Z-Arg-Arg-NMec hydrolysing activity from silver hake tissue extract, pH 6.5, temperature 25°C (n=6, T=6.744, F=45.481, p=0.000).

Inhibitors and Activators

Inhibition tests were conducted to help with the identification of each enzyme and to eliminate the possibility of others (Table 2). The inhibitors used were E-64, pepstatin, EDTA, trypsin inhibitor, leupeptin, antipain, calcium chloride and iodoacetic acid. The caseinolytic activity results suggest the enzyme was a calcium-activated cysteine protease, as illustrated by its decrease in activity with E-64, leupeptin, antipain and iodoacetic acid EDTA. CaCl2 was obviously an activator for the caseinolytic activity. The minimum decrease in activity with trypsin inhibitor, and pepstatin would also help confirm that most of proteolytic activity was not a trypsin like enzyme or a carboxyl protease. Cathepsin B which was assayed in the presence of a rather specific artificial substrate (Z-Arg-Arg-NMec) was not affected by the presence of Ca+2, pepstatin or EDTA, but was inhibited in the presence of E-64, leupeptin, antipain and iodoacetic acid.
Table 2. Results of the effects of different inhibitors and activators on the activity of silver hake sarcoplasm (percentage of activity with inhibitors present in assay)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cathepsin B</th>
<th>Calpain</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-64</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>93</td>
<td>86</td>
</tr>
<tr>
<td>EDTA</td>
<td>96</td>
<td>15</td>
</tr>
<tr>
<td>ST1</td>
<td>87</td>
<td>89</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Antipain</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>CaCl2</td>
<td>96</td>
<td>130</td>
</tr>
<tr>
<td>IAA</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

Texture and Enzyme Activity

For each of the enzymes assayed, the muscle texture was analyzed along with the specific activities of apparent calpain and cathepsin B. It is difficult to establish a "cause and effect" relationship for such a limited set of data. However, Figure 5 shows the results for casein hydrolyzing activity versus the texture (firmness) and it can be seen that there was negative relationship. That is, softening generally corresponded to higher specific activities. This would indicate that as the casein hydrolyzing activity increased, the texture of the muscle tissue became less firm. The same can be seen in Figure 6 with regard to the Z-Arg-Arg-NMec hydrolyzing activity. It should be noted that no error bars are present and ANOVA was not preformed because two dependent variables are being compared.

![Graph](image)

**Figure 5.** Relationship between texture (firmness) of silver hake muscle tissue and casein hydrolysing activity of sarcoplasmic fluid (pH 6.3).
Figure 6. Relationship between texture (firmness) of silver hake muscle tissue and Z-Arg-Arg-NMec hydrolysing activity of sarcoplasmic fluid (pH 6.3)

Phase Contract Microscopy

There was a reduction in the length of myofibrils over time on iced storage. Similar observations were made by Tokiwa and Matsumiya (1969) who were studying cod, pollack and carp at the time. The fresh silver hake myofibril strands were long and thread-like. As time on iced storage increased, there was an increase in the fragmentation and a decrease in length of the myofibrils (Figures 7 and 8). This agrees with observations seen on the myofibrillar fragmentation of stored cod muscle tissue (Tokiwa and Matsumiya, 1969).

Figure 7. Fresh silver hake myofibrils viewed using a phase contrast microscope under oil immersion (bar = 10μm)
Figure 8. Silver hake myofibrils after 6 days of iced storage viewed using a phase contrast microscope under oil immersion (bar = 10μm).

The typical striated appearance of the myofibrils changed with iced storage time. The structural definition of the Z-line decreased with time on ice, suggesting degradation of the contractile proteins. Tokiwa and Matsumiya (1969) also observed that the Z-line deteriorates during iced storage, which would make the muscle tissue more susceptible to mechanical breakage. They speculated that because of the optimal pH of the protease in question and of the hydrolyzed proteins, that the enzymes most likely responsible for this fragmentation were cathepsins. The activities of both cathepsin B and calpain had the Z-line as a potential substrate.

Scanning Electron Microscopy

Scanning electron microscopy was used to see the ultrastructural changes. Figure 9a shows a cross section of a myofibril. The myofibrillar protein network that was broken down in the iced silver hake (Figure 9b). Figure 10a shows a longitudinal view of a myofibril. Again the inter-connection present in fresh muscle tissue deteriorates during iced storage (Figure 10b). Not only was there a reduction in the proteinaceous network seen in myofibrils but also in the collagen sheath surrounding the fibre. Bremner and Hallet (1985) also reported the degradation of collagen in fish muscle during storage. The reduction in the collagen sheath surrounding the fibre could explain the mechanical breakage occurring during fragmentation of tissue samples.
Figure 9. Silver hake myofibrils (transverse section) viewed using a scanning electron microscope a) Fresh myofibrils b) Myofibrils stored on ice for 6 days (Bar = 10μm).

Figure 10. Silver hake myofibrils (longitudinal section) viewed using a scanning electron microscope a) Fresh myofibrils b) Myofibrils stored on ice for 6 days (Bar = 10μm).
CONCLUSIONS

Both cathepsin B and calpain are capable of hydrolysis under physiological conditions. Both of the enzymes tentatively identified in silver hake had specific activities which increased as time on iced storage increased. The inhibition studies showed that one enzyme was most likely a cysteine protease, and not a trypsin-like protease or a carboxyl protease. The second enzyme was more typically like cathepsin B, cleaving the artificial substrate, Z-Arg-Arg-NMec and inhibited by E64, leupeptin, antipain and iodoacetic acid. The texture of the muscle tissue became very soft after only 4 days of iced storage. The microstructure and ultrastructure of the muscle tissue showed degradation of the contractile proteins of the myofibril. The relationship between the texture softening and the activities of the two substrates indicate that the two most likely enzymes causing the softening of the silver hake muscle tissue were calpain and cathepsin B.

REFERENCES


Koomaraie, M. 1992. The role of Ca++-dependent proteases (calpains) in post mortem proteolysis and meat tenderness, Biochimie, 74, 239-245.


THE VOLUNTARY NUTRITION LABELING OF RAW PRODUCE
AND FISH: FDA'S POLICY ON DATA BASE REVIEW

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Center for Food Safety and Applied Nutrition
Office of Food Labeling (HFS-165)
200 C St., SW, Washington, DC 20204

On August 16, 1996, the Food and Drug Administration (FDA) published a final rule that revised the guidelines and the nutrition labeling values for the voluntary nutrition labeling of raw fruits, vegetables, and fish. The action, in response to the requirements of the Nutrition Labeling and Education Act of 1990 (NLEA), makes the voluntary nutrition labeling program more consistent with mandatory nutrition labeling of other foods regulated by FDA. Within the same document, the agency also explained its policy on its review of databases in both the voluntary and mandatory nutrition labeling programs.

In November, 1991, FDA published a final regulation in the Federal Register (corrected March, 1992) that:
1) identified the 20 most frequently consumed raw fruits, vegetables, and fish in the United States;
2) established guidelines for their voluntary nutrition labeling; and
3) set criteria for substantial compliance with the guidelines by food retailers.

The agency also stated that at least every two years it would publish updates on the values and provide an opportunity for comment. Otherwise, FDA would publish a notice that nutrition labeling values had not changed. [The 1996 regulation increased that time interval to every four years.] FDA also advised that once final regulations governing nutrition labeling of FDA-regulated processed, packaged foods were finalized (in 1993), it would revise the guidelines for the voluntary nutrition labeling program to make them as consistent as possible with those final rules. Consistency is defined in terms of what information is required (content), and how that information is to be presented (format).

FDA published a proposal in the Federal Register in July, 1994, to update the nutrition labeling values for the 20 most frequently consumed raw fruits, vegetables, and fish and to revise the guidelines for the voluntary nutrition labeling of these foods to reflect the final rules for mandatory nutrition labeling. The agency considered all comments to the proposal in finalizing the regulation.
Definition of "Raw Fish"

FDA considers "raw fish" to be freshwater or marine finfish, crustaceans, and mollusks in the natural state that have received minimal or no processing. Raw fish may include whole or filleted fish that are fresh or fresh frozen (unpackaged or packaged by the retailer); alive in the retail store; shrimp that have been shelled or deveined; and lobster, crab, and shrimp that have been thermally processed or shelled, but not processed or prepared in any other way. Nutrition labeling is mandatory for fish that are canned or smoked; have undergone processing such as breading, flaking, or pressing; or were packaged before reaching the retail level. FDA doesn't recommend that consumers eat raw fish, however, so the agency reports the nutrient values on a 3 oz. skinless, cooked portion, without added, salt or sauces.

The 20 Most Frequently Consumed Raw Fish

<table>
<thead>
<tr>
<th>Blue Crab</th>
<th>Orange Roughy</th>
<th>Swordfish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catfish</td>
<td>Oysters</td>
<td>Whiting</td>
</tr>
<tr>
<td>Clams</td>
<td>Pollock</td>
<td></td>
</tr>
<tr>
<td>Cod</td>
<td>Rainbow Trout</td>
<td></td>
</tr>
<tr>
<td>Flounder/Sole</td>
<td>Rockfish</td>
<td></td>
</tr>
<tr>
<td>Haddock</td>
<td>Salmon (Atlantic/Coho)</td>
<td></td>
</tr>
<tr>
<td>Halibut</td>
<td>Salmon (Chum/Pink)</td>
<td></td>
</tr>
<tr>
<td>Lobster</td>
<td>Salmon (Sockeye)</td>
<td></td>
</tr>
<tr>
<td>Mackerel</td>
<td>Scallops</td>
<td></td>
</tr>
<tr>
<td>Ocean Perch</td>
<td>Shrimp</td>
<td></td>
</tr>
</tbody>
</table>

The Guidelines

The final regulations for the voluntary nutrition labeling program continue to grant retailers flexibility in disseminating the nutrition labeling information to consumers through various means and materials, such as shelf labels, signs, posters, brochures, notebooks, or leaflets. Much of the regulation does relate to retailers, but it also relates to members of the industry who wish to label individual products and to those who want accurate nutrient levels and label values for fish.

Retailers may use a chart format or an individual label format, as long as the labeling materials are viewable from within the fish department. In addition, if raw fish are frozen within the retail establishment and presented in a different section of the store, that section should also provide the labeling information. Many of the trade associations are continuing to use a chart format in their marketing materials, but FDA encourages industry to consider using an individual label format, the Nutrition Facts panel that you see on processed, packaged foods. A poster or brochure for fish could easily contain 20 individual nutrition
labels and may be easier to read than a chart format with 20 lines and columns that contain the nutrition information.

The new regulation requires that retailers, as well as members of the fish industry who wish to provide individual nutrition labels on packaging materials for fish included in the voluntary program, should use the nutrition labeling values provided by FDA for the most frequently consumed raw fruits, vegetables, and fish. After August 16, 1997, the effective date of the regulation, retailers must use the data provided by FDA in order to be in compliance with the guidelines for the voluntary nutrition labeling program. That date is also the effective date for members of the fish industry who label individual packages of fish.

FDA recommends that labeling values be used as soon as possible, however, especially at the retail level. Because there is a relatively short amount of time before the 1996 FDA compliance survey, FDA will consider either the old (1991) or new (1996) labeling values for retail stores to be in compliance.

Individually labeled fish products will not be assessed for compliance as a part of the 1996 FDA compliance survey for the voluntary nutrition labeling program, but they will need to be in compliance with the regulations for mandatory labeling in § 101.9. Those regulations explain the requirements for the Nutrition Facts label in regard to format, type size, bold print, etc. FDA does plan to assess the prevalence of individually packaged raw foods that bear nutrient content claims, such as "low fat" and whether or not they have the nutrition facts panel.

Nutrient Levels

FDA calculated the labeling values for raw fish based upon raw data obtained from the United States Department of Agriculture (USDA). The agency based values for catfish on data in the Journal of Food Science in a 1990 article by Joyce Nettleton, William Allen, and several other authors. Those data were provided to FDA in a comment to the proposed rule.

Again, FDA analyzes raw nutrient data. FDA believes that mean values from software data bases or the scientific literature may be inappropriate for nutrition labeling and does not recommend their use except for calculating nutrients for restaurant menus. The agency derives labeling values by completing compliance calculations with the data, using 95% prediction intervals. The final labeling values will be adjusted from the mean that would be directly calculated from the data. Sometimes the levels of "bad" nutrients will be inflated; other times the levels of "good" nutrients will be deflated. Rather than using a mean value alone, FDA looks at all data and considers the variability among the data points in calculating a label value for a nutrient.

FDA strongly encourages the fish industry, trade associations, and academia to continue to test fish to determine nutrient levels and to provide those data (especially raw data) to the agency for consideration in the next update of labeling values.
The regulation states that in four years, after reevaluating the most frequently consumed fish in the United States, the agency will provide an update to the regulation. For example, there is already a question whether mackerel should be included on the list. The agency plans to continue to work with the National Fisheries Institute and other interested parties to continue to refine the list of fish. FDA strongly encourages you to send not only data for nutrient values for fish but also estimates to determine current consumption of various types of fish. Please remember that consumption is not the same as sales or catch.

FDA requires certain nutrients for food labeling (i.e., calories, calories from fat, total fat, saturated fat, cholesterol, sodium, total carbohydrate, dietary fiber, sugars, protein, vitamin A, vitamin C, calcium and iron) but also allows for optional nutrients for foods in the voluntary program. Even though potassium is optional, FDA notes that the potassium provided by fish is important—12 out of the 20 types of fish are considered a “source” of potassium—so the agency included potassium in its data. The individual label format provides an easier vehicle to list optional nutrients.

The regulations state that on charts the columns for sugars and fiber may be omitted for fish. Instead, a footnote may be included, stating "Fish provide negligible amounts of dietary fiber and sugars." With fewer columns, the charts would be more readable by consumers, but the amount of information provided would not be reduced.

Nutrient Content Claims

Nutrient content claims are label statements such as "low fat", "good source of potassium". FDA addressed the question of the need for nutrition labeling for packaged raw fruits and vegetables that bear a claim in a booklet on frequently asked questions that it issued in August of 1993. At that time, it stated: "Claims subject the food to nutrition labeling in accordance with § 101.45, which means that nutrition information will have to be available at point of purchase although not necessarily on the package." The agency is now reevaluating that policy for not only raw produce, but for raw fish.

FDA encourages members of the fish industry who put nutrient content or health claims on their packaging to also include nutrition label information because it is not possible to predict whether the products will be sold in stores where retailers make the nutrition information available to consumers. Even if it is possible to control the flow of the products into specific retail stores, it is not possible to have control over retailers' decisions to display (or to continue to display) nutrition labeling information for these products. Depending upon retailers to provide nutrition labeling values to justify nutrient content or health claims would be a gamble for those who assume liability for their products with claims. For raw fish that are not among the 20 most frequently consumed, it is even less likely that nutrition information will be available in retail stores. Therefore, FDA recommends that nutrition information be included on those products bearing claims.

Submitting Data to FDA
The nutrition labeling values provided by FDA are for generic commodities. Several groups have called to ask if a commodity group could use nutrition labeling values developed for a specific type of fish. FDA does encourage the use of names and label values for specific products, as long as the commodity group has the data to support the label values used for the product. If a commodity group wishes to amend the nutrient values for a generic item, FDA encourages the group to submit the values to the agency for consideration in the agency's next revision of the voluntary nutrition labeling program, which would be in 2000. Of course, you’d want to submit the data prior to that time. In order to log a request into the system, please send all submissions to:

Food and Drug Administration  
Center for Food Safety and Applied Nutrition  
Office of Food Labeling, (HFS-165)  
200 C St., SW  
Washington, D.C. 20204

If you have questions, please call Mary Bender 202-205-5592; send a fax to 202-205-5532; or best yet, send an E-mail to m0b@fdacf.ssw.dhhs.gov. [That’s m-zero-b.]

If FDA decides to use the labeling values you submit for the generic item, those values will be made available for public comment. Again, any nutrition labeling value for a generic item that the agency decides to incorporate will have to be used by retailers for them to be in compliance.

Issues on Raw Fish

Catfish. There were three comments to the proposed rule that dealt with the fat content of farmed catfish. I understand that the fat content is affected by species, size of fish, diet of fish, season of year, stocking rate of pond, pond size, and gender of fish. The comments also provided data from Nettleton et al. on farmed catfish composition and requested that FDA consider this information in developing revised labeling values.

FDA concluded that the data from Nettleton et al. provided more accurate label values for catfish. So the agency derived values and adopted them. The values for total fat are higher than the industry wants, however, and the industry is concerned. Again, we encourage all to continue to analyze data for raw fish and to submit those data to FDA.

Orange Roughy. FDA is still concerned about the fat value for orange roughy, because the current value for orange roughy doesn’t include wax esters and is not consistent with the definition of total fat in the regulations (i.e., the amount of total lipid fatty acids present expressed as triglycerides).

A comment to the proposed rule did question why FDA wanted a value for total fat in orange roughy that includes the presence of wax esters because wax esters are not a
metabolizable source of energy in humans and have no dietary significance. It also stated that consumers need information with which to make dietary choices, and that it is misleading to add nonmetabolizable fat to the value for fat in orange roughy. The elevated levels of fat that would result from the addition of wax esters would falsely suggest to consumers that orange roughy was contributing a substantial amount of metabolizable fat to daily intake.

FDA continues to request information that would provide a basis for revising the declaration of total fat to reflect the presence of wax esters in orange roughy but that would not be misleading to the consumer. The agency will also address the issue of declaration of available fat in a separate rulemaking.

Atlantic/Pacific Mackerel, Ocean Perch, and Lobster. In response to comments to the proposed rule, FDA reviewed the data provided to the agency for mackerel and ocean perch and discovered that there were errors in the data file. FDA obtained the correct information and then derived the nutrition labeling values correctly for the final rule. Thanks to the commenters, FDA was able to trace the data errors to their source and make the corrections. In addition, the agency did make an error and correct it in the final rule in regard to calories from fat for lobster.

FDA Review of Submitted Data Bases

FDA needed to create a more efficient, flexible and responsive data base review system that wouldn't overwhelm the resources that the agency has available, and yet provide industry with the assurance that it seeks through data base review and approval. FDA solicited comments regarding the agency's approach to data bases in the proposal on the voluntary labeling of raw produce and fish. Based on its review of the comments, FDA decided to modify its approach to data bases that are submitted to the agency for review. The new policy relates to products falling under the mandatory label program, but also includes foods in the voluntary program, such as raw fish.

All data in the form of nutrition label values that are submitted to the agency should be accompanied by raw data. If there are data that the submitter has determined as unsuitable, they should also be provided with explanation.

FDA will continue to the evaluate data submitted for the 20 most frequently consumed raw fish. In addition, FDA will evaluate data for fish NOT included in the top 20 that are submitted for review if those data are accompanied by a plan to collect additional data for the purpose of updating label values. [In other words, if you want to send data for the top 20, please do. If you want to send data for other types of fish, please do, as long as you also submit a proposal to collect additional data.]

In order to facilitate the use of the developing nutrient data base for fish and to limit the uncertainty that could result from a delay in agency review of the data base, upon submission firms may begin use of the nutrient label values and to initiate the planned studies to collect
data to update the values. During this interim period, FDA won't take action against a product bearing label values included in a database submitted to the agency for review. If any product is identified through FDA compliance activities as including label values that are out of compliance, contingent on the company's willingness to come into compliance, the agency will work with both the manufacturer and the database developer to understand and correct the problematic label values.

When FDA receives the interim data and planned studies, it will first evaluate the label values relative to the raw data. FDA will recalculate label values based solely on the raw data that have been submitted. As explained earlier, the agency will derive label values using compliance calculations based upon 95 percent prediction intervals and, when appropriate, will use weighting procedures, as recommended in the FDA Nutrition Labeling Manual.

FDA will evaluate the data for completeness and reasonableness, e.g., it will consider whether or not there are enough samples, and whether all nutrients are included. FDA requests that supporting documentation, such as analytical methodology and a sampling plan, accompany interim data. The agency acknowledges, however, that a large amount of the interim data available from manufacturers and trade associations are based upon historical data, where the analytical methodology and sampling plan are not available. Therefore, FDA will accept data even if it is not accompanied by comprehensive documentation, as long as the reason that the documentation is not provided is fully explained and is acceptable to the agency.

After FDA reviews the data, it will consider using those values in updating the top 20. For fish not included in the top 20, FDA will review the planned studies to collect additional data. The agency will concentrate on analytical methodology and on the reasonableness of the factors that could account for nutrient variability (e.g., region), rather than on the rigor of sampling design or statistical treatment of the data. FDA cautions, however, that data base submitters should follow the FDA recommendations regarding sampling strategies, weighting procedures, and statistical treatment of data that are described in the nutrition labeling manual.

FDA will respond in writing after review of the data and the planned studies. The agency will address the nutrient label values that were submitted and will indicate whether it has any objection to continuing the planned studies or to continued use of the label values for two years from the date of the agency response. After those two years, manufacturers will be expected to provide the agency with a summary update that reassesses the interim label values based upon completion of the planned laboratory analyses. The agency will evaluate how the study findings bear on the interim label values and will consider whether it would have any objection to continued use of the updated interim values for up to an additional five years. At the same time, however, the agency may suggest modifications to the ongoing plan of study. If after review of data and planned studies, FDA determines that the label values or studies are not appropriate, as indicated above, the agency will notify the manufacturer of that decision.
Please note that an initial primary focus of FDA's compliance review of product labels is on nutrient content claims (e.g., "high protein", "low fat") that are used. FDA will continue to closely monitor products making such claims and expects that the manufacturer, packer, or distributor will have sufficient data to support the validity of such claims.

Again, FDA strongly encourages industry to analyze data for raw fish and to submit those data to the agency for consideration for the next revision of nutrition labeling information for raw commodities. If an updated rule is to publish in 2000, we'll need data in the next two years in order to consider it in a proposal.