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Carbonyl Metabolism as an Index of Shrimp Decomposition.

Patrick Delano Settoon

Louisiana State University and Agricultural & Mechanical College

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Louisiana State University and Agricultural and Mechanical College, Ph.D., 1967
Food Technology

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SHRIMP DECOMPOSITION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in
Food Science and Technology

by
Patrick Delano Settoon
B.S., Southeastern Louisiana College, 1957
M.S., Louisiana State University, 1961
August, 1967
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ABSTRACT

Supplemental chemical analyses must be developed for measuring changes in shrimp quality during storage. Such tests will augment existing objective measurements, permitting a more valid determination of incipient spoilage and providing essential data required by the Food and Drug Administration for establishing reasonable standards of quality for this food product.

Carbonyl metabolism in shrimp decomposition was investigated. Methodology was developed for the extraction, isolation, and concentration of carbonyl containing compounds. An ethanolic homogenate of peeled and deveined shrimp was subjected to a batch-type, countercurrent, liquid-liquid extraction process employing n-pentane as the extraction solvent. Isolation and concentration of the extracted carbonyls was achieved through fractional distillation and column chromatography. Total carbonyl determinations were made on the eluate from the chromatographic step.

The eluate volume was concentrated to less than 0.5 ml, and the carbonyls present were separated by gas chromatographic analysis. Tentative identification of the extracted carbonyls was made by comparing their resolution times on two different column packings with those for known carbonyls.

Distinct advantages of the methodology are: small sample size (300 g), low temperature (less than 60°C), and short time
for complete analysis (less than 8 hours). These factors minimize the probability of artifact production.

Concentrations of extractable carbonyls in fresh shrimp averaged 3.1 ppm. During ice storage studies, carbonyl contents increased through 21 days' storage and then tended to fall off. Deterioration in quality was observed after the carbonyl content exceeded 5 ppm. Fresh shrimp subjected to spoilage conditions exhibited a similar pattern of carbonyl change.

Carbonyl contents of gamma irradiated shrimp (0.2 Mrad) remained essentially constant (less than 3 ppm) through 28 days' storage. The shrimp were considered to be of good quality at the end of the test period. Non-irradiated controls were spoiled at 21 days' storage and exhibited high carbonyl concentrations.

Trimethylamine nitrogen (TMA-N), ammonia nitrogen (NH₃-N), and total bacterial plate counts paralleled carbonyl changes in all studies. Total plate counts at 20°C were considered more important in quality evaluation than those at 35°C. Carbonyl formation in shrimp apparently originates primarily from sources other than those responsible for trimethylamine and ammonia production.

Shrimp quality cannot be evaluated in terms of any one objective measurement; however, consideration of the aggregate results of a number of such tests should provide a reliable index of spoilage. Results of these studies indicated that incipient spoilage was present when the following values were exceeded: total carbonyl—5 ppm; TMA-N—15 mg per 100 grams; NH₃-N—30 mg per 100 grams; and total bacterial plate counts—50 million per
gram at 20°C or 5 million per gram at 35°C.

Acetaldehyde, acetone, diacetyl, and 3-methyl-2-butanone were tentatively identified as carbonyls produced during shrimp decomposition. Plausible biochemical mechanisms were presented to account for their production.
INTRODUCTION

Mankind's survival in this physical world will not be determined by nuclear weaponry but will be predicated on the ability of the world's food scientists to provide him with a sufficient food supply. In his Food for Peace message to Congress on March 31, 1965, President Lyndon B. Johnson pointed out that millions still suffer from some form of hunger or malnutrition and that an insufficient food supply is one of the leading contributors to human misery and political instability.

With less land available for the production of food and an increased food demand of a ballooning population, the food scientist has begun to exploit other food sources. It is only natural that he has looked to the oceans and seas which cover three-fourths of the world's surface as a possible solution. The survival of the human race may well depend on the efficiency with which he is able to tap the wealth of the oceans. Organized cultivation of the seas, mariculture, now looms as an inevitable and intriguing task for future generations of food scientists.

Fish and shellfish constitute one of the great and growing food harvests of the world. Production reached over 16 million metric tons in 1965 as shown in Table I (114). However, the importance of this catch is even greater than the annual figures indicate because the most urgent food need in many countries is for
<table>
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<th>Fish</th>
<th>U.S.A.</th>
<th>World</th>
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<tr>
<td>Herring, sardine, anchovy</td>
<td>0.83</td>
<td>17.45</td>
</tr>
<tr>
<td>Molluscs</td>
<td>0.65</td>
<td>2.88</td>
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<td>Crustacean</td>
<td>0.27</td>
<td>1.18</td>
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<tr>
<td>Redfish, bass, etc.</td>
<td>0.17</td>
<td>3.05</td>
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<tr>
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<td>6.46</td>
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<td>1.19</td>
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<td>Salmon, trout, etc.</td>
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<td>0.86</td>
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<td>Flounder, halibut, etc.</td>
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<td>0.97</td>
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<tr>
<td>Jack, mullet, etc.</td>
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<td>2.12</td>
</tr>
<tr>
<td>Freshwater</td>
<td>0.06</td>
<td>6.06</td>
</tr>
<tr>
<td>Unsorted, unidentified, and others</td>
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<td>7.08</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>2.72</strong></td>
<td><strong>19.30</strong></td>
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protein, especially high-quality protein, rather than for calories. Thus, although fish probably supply less than one percent of the world's calorie needs, their contribution as a source of protein is much greater. Borgstrom (10) estimated that over 670 million of the earth's population are "fish supported". In other words, this number of people could theoretically be supplied their entire protein need from the amount of fish now produced. He calculated also that fish supply 12 percent of all animal protein on a global basis. The addition of even moderate amounts of fish or fish products to the low-protein diets that are typical of many underdeveloped countries considerably enhances nutritional value. More efficient exploitation of world fisheries could therefore make an important contribution to the relief of mounting world malnutrition.

United States fishery landings in 1965 totaled 1.7 billion pounds (4 percent higher than in 1964) with a record ex-vessel value of $451 million (16 percent more than the $390 million value in 1964). Per capita consumption of fishery products in the United States of 11.0 pounds was one-half pound more than in 1964. The United States remained fifth among the world's fishing nations. For the first time the United States imported more than half its supply of edible fishery products in 1965.

Shrimp was again the most valuable species—the 1965 catch had an ex-vessel value of $82 million. The salmon catch was valued at $67 million, and tuna at $42 million. Louisiana again led all states in the volume of catch in 1965—794 million pounds—followed by Alaska, Virginia, California, and Massachusetts (17).
A major problem which has plagued the food scientist in his efforts to utilize the resources of the sea had been the highly perishable nature of the products. Even when stored in ice, marine food products lose their delicate flavor in a short period of time. Deterioration begins immediately from the time the product is removed from its natural environment and proceeds more or less rapidly depending on the manner in which it is handled, processed, and stored.

High quality shrimp has a fine texture, bright appearance, and fresh flavor, the latter a positive quality that is found in newly-caught shrimp but gives way fairly rapidly on storage to a somewhat neutral flavor. Later, definite undesirable off-flavors develop which render the product in time stale, inedible, and putrefied. Death of the shrimp initiates a complex series of changes, the exact course of which depends on a number of factors such as species, nutritional status, location of catch, degree of struggle, and temperature of holding. Bacterial attack is delayed until the resolution of rigor, but in the meantime, post-mortem enzymatic action leads to a series of degradations.

Muscle of freshly-caught shrimp is sterile, but the external covering and intestines many times carry a large bacterial load. Bacteria increase in number very rapidly by handling and by contact with gear, deck, holding pens, etc., and multiply during storage in ice. The intestine, when full, develops intense bacterial and enzymatic activity which at elevated temperatures can cause very rapid decomposition to start from the visceral cavity. When rigor
is resolved, bacteria invade the muscle, causing a number of degra-
dative changes which commence slowly, but at a rate largely
determined by species and temperature. At a later point there is
a sharp increase in the rate of these changes as evidenced by a
rapid rise in the bacterial count and by a development of marked
off-flavors. The latter are due largely to formation of a number
of chemical groups associated with spoilage, such as trimethylamine,
indole, ammonia, volatile reducing substances, and volatile acids.

Prevention of flavor changes by chemical or physical means
would obviously be the most desirable solution to this problem.
Efforts in this direction have met with fair success, particularly
with respect to the utilization of gamma irradiation in both
pasteurization and sterilization procedures. However, all the
methods previously employed result in some flavor changes or
creation of off-flavors. While such changes are indicative of
product deterioration, it is often the case that only the esthetic
value of the product has been decreased and not necessarily the
nutritive value.

Knowledge of the chemical constituents responsible for
desirable flavors and off-flavors will provide a rational and
objective approach for selecting high quality raw material, for
controlling the processing operation, and for checking the quality
of the finished product. In addition it will permit the food
processor to follow deterioration of the product during subsequent
storage. Such knowledge would also permit the replenishment of the
product with desirable flavor constituents, or their precursors.
lost during processing or storage thus upgrading the esthetic quality. This improvement of the flavor of a given food is the ultimate practical objective of food flavor research.

Changes in seafoods during storage have been the subject of many investigations. No completely satisfactory test has been developed for evaluation of the quality of these products. There is agreement that off-odors and flavors characterizing spoilage result primarily from microbiological activity and secondarily from enzymatic and nonenzymatic changes within the tissue. Organoleptic evaluation of quality has been used almost exclusively for purchasing and grading shrimp products at various stages during their processing and storage. Such tests are based on personal judgment and consequently result in numerous subjective differences which lead to disagreement in quality assessment of individual samples. The most desirable evaluation would be an exact analysis based upon changes of certain constituents in the tissue during storage. The shrimp fisherman, shrimp handler, and processor of frozen shrimp would be especially interested in a rapid objective test for evaluating quality of their product.

If some compound originally present in the shrimp tissue could be shown to increase gradually in amount through microbial or enzymatic action, it should be possible to arrive at a numerical quality index. From such data it would be possible to state the length of time the product had been stored, its probable future storage life, and its quality at any given instant.

It would be of inestimable monetary value to the shrimp
industry, in particular, and the shellfish industry, in general, if some simple and rapid method for evaluating product quality could be developed. The shrimp catch by Louisiana shrimpers for the period June 1966 through May 1967 will amount to over 100 million pounds worth conservatively $10 million. Over 20 percent of this total poundage will never reach the consumer because of deterioration in flavor. Thousands of pounds of shrimp will be rejected on the basis of a rather dubious testing procedure for the simple reason that too little is known about the changes occurring in flavor components. The availability of such knowledge could lead to the development of more exact analytical procedures for determining quality and predicting storageability. Such knowledge could result in the reclamation of these thousands of pounds of shrimp through the interruption of the spoilage mechanism and the supplementation with desirable flavor components lost during processing and storage.

Establishment of reasonable standards for shrimp quality by the Food and Drug Administration (FDA) has been hindered by a lack of knowledge of the biochemical mechanisms underlying shrimp decomposition. The attitude of the FDA toward standards for fishery products in general has been one of reticence, apparently based on a scarcity of toxicological data, particularly with respect to irradiated products.

Investigation of those chemical components in shrimp tissue associated with flavor changes is necessary to determine their biochemical origin, eventual metabolic fate, flavor significance, toxicity, and nutritive value.
The established importance of carbonyl compounds as flavor contributors in other foods demands studies of their significance in shrimp decomposition.

This investigation was undertaken with the following objectives:

(a) develop methodology for the isolation, purification, and identification of carbonyl compounds in shrimp;
(b) evaluate changes in carbonyl content of shrimp (gamma irradiated and non-irradiated), maintained under various storage conditions, as possible indices of deterioration;
(c) elucidate the biochemical mechanisms producing these changes in carbonyl content.
REVIEW OF LITERATURE

Identification of compounds not previously studied in shrimp, and a determination of their quantitative values in fresh and decomposed states will contribute materially to procedures for measuring the degree of spoilage of this product. A thorough review of the microbiological aspects of shrimp decomposition and the suggested methods for evaluation of shrimp quality is essential to understanding the complex nature of this spoilage process. For this reason and because studies concerned with carbonyl compounds in fishery products are scarce and almost nonexistent for shrimp, the literature review of carbonyl studies is presented after a consideration of these other topics.

I. Microbiology of Shrimp:

Almost all shrimp are handled raw. Within minutes after the shrimp are landed on the deck of the trawler, they generally die. Death of the shrimp sets in train a complex series of changes. Microbial spoilage starts immediately through marine bacteria on the surface or through microorganisms which happen to contaminate the shrimp on deck, in handling and washing. Fish and other marine organisms following the shrimp may also, chiefly through slime and exuded intestinal contents, smear the shrimp (38).

Prevention of deterioration in shrimp quality involves two distinct problems: (a) maintaining low numbers of detrimental
microorganisms, and (b) controlling oxidations, chiefly of phenols, into melanins. This latter reaction is guided by specific tissue enzymes—phenolases—and results in the appearance of black zones or spots at the edge of the shell segments of the flesh (3, 33).

This dark color is produced by melanin pigments which form on the internal shell surfaces or, in advanced stages, on the underlying meat (31). These pigments are produced by an oxidative reaction of tyrosinase on tyrosine. The reaction is accelerated by copper and other metallic ions (34). Earlier assumptions that this discoloration was connected with microbial activities are definitely ruled out (1). Idyll et al. (51) confirmed the concept of black spotting as a nonmicrobial phenomenon in a comprehensive study. Kopfler (57) concluded that low dose gamma irradiation of fresh shrimp reduced melanosis and resulted in a significant extension of their iced-storage life. However, if shrimp were held beyond the onset of melanogenesis, subsequent low dose irradiation accelerated black-spot formation, which reduced consumer appeal, and therefore was definitely undesirable.

Several important comprehensive studies on the microbiology of Gulf of Mexico shrimp have appeared in the literature. During three trips aboard commercial shrimp trawlers, Green (43, 44, 45, 46) followed changes in the bacterial numbers of shrimp when caught, the effect of head removal, washing, and of ice storage in top and bottom layers of storage bins, and changes that occur during frozen storage.

Green (44) showed that whole shrimp examined immediately
after emptying of the trawler net varied in bacterial counts from 1,600 to 1,200,000 per gram. The latter count was from shrimp caught in Barataria Bay, Louisiana, a shallow inland bay which receives drainage from the settled areas adjacent to the west bank of the Mississippi River. A more representative value of bacterial counts of shrimp caught in commercial fishing nets in widely scattered areas on the open Gulf of Mexico off the Louisiana coast was found to be 42,000 bacteria per gram for 11 different samples (range 1,600 to 160,000).

When whole or headless shrimp from the same catch were washed with Gulf of Mexico water, there was an average reduction in the bacterial count (\\textsuperscript{11}). Clark and MacNaughton (13) stated that the heads should be removed from freshly caught shrimp, which should be washed and iced as quickly as possible. The dark liquid in the stomach contains partially digested plant and animal material which readily decomposes. They stressed that this liquid and the surface slime must be removed by thorough washing before icing. Bacterial counts on headless shrimp were somewhat lower than on whole shrimp from the same catch. Removal of the heads in all cases reduced the count somewhat, the heads carrying approximately 75 percent of the bacteria (32, 39). Bacterial counts of freshly-caught headless shrimp are largely determined by the bacteria and debris adhering to the surface. The average bacterial count on the shrimp as prepared for icing under commercial conditions, headed and washed by fishermen, was 7,400 per gram. It is evident from this that under commercial conditions, with expeditious handling and thorough
washing, headless shrimp may be placed in ice storage on board trawlers and carry a relatively low microbial load. This was also reported by Lantz (58). Removal of the heads extended the storage life of uncooked, iced shrimp by 2 days.

Washed, headless shrimp packed in alternate layers of ice in bins in the hold of the trawlers showed steadily rising bacterial counts measured at the bottom layer despite adequate icing (U). The highest single count, 1,200,000 per gram, recorded on the sixth day was not accompanied by change in color or appearance. When unloaded at the end of the ninth day, the shrimp were an acceptable market product. A few spoiled shrimp, selected during inspection in the processing house, contained 73,500,000 bacteria per gram.

Since the melting ice from the upper layers of shrimp washes down over the lower layers, Green (111) also studied the influence of position in the bin on bacterial counts. After 9 days' storage, headless shrimp in the layer next to the top increased from 1,800 bacterial per gram to 2,400 per gram, whereas the bacteria in the bottom layer increased a thousandfold. Whole shrimp showed a similar difference, the bottom layer containing 250 times and the upper layer 125 times as many bacteria after 4 days' storage as the freshly-caught whole samples. Water draining through a bin of one-day-iced whole shrimp showed counts of 5,900,000.

Studies by Williams (111), Williams et al. (112), and Williams and Rees (113) complement those of Green reported above in that they determined the types of bacteria initially present in
fresh Gulf of Mexico shrimp caught adjacent to the Texas coast. They showed the main groups present were *Achromobacter*, *Bacillus*, *Micrococcus*, and *Pseudomonas*. These four groups made up 78 percent of the 1,200 isolates. In biochemical characteristics they found 62 percent of the isolates were proteolytic, 35 percent lipolytic, 18 percent reduced trimethylamine oxide, and 12 percent formed indole.

Radiation pasteurization not only reduced the bacterial load but also had a marked selective action on the various groups of bacteria normally present. Shewan and Liston (98), working with cod, noted that after irradiation the percentage of *Pseudomonas* species fell from about 40 percent in the untreated controls to about 5 percent after irradiation, and that the main group was *Achromobacter*. Liuzzo et al. (62) have reported the isolation of seven radiation-resistant bacteria from fresh Gulf shrimp. These bacteria were classed into the following genera: *Brevibacterium*, *Gaffkya*, *Achromobacter*, *Sarcina*, and *Bacillus*.

Fieger et al. (32) investigated the best methods of freezing, packaging, and storing headless shrimp. Waxed cartons overwrapped with glassine or not and heat-sealed were used. This was compared with regular glazing. Samples were stored at various temperatures for 12 months. Bacteriological studies of these samples were reported by Holmes and McCleskey (49). The major problem in keeping and packaging frozen shrimp is the gradual drying of the product with ensuing chemical changes (79). Peeled shrimp had lower bacterial counts than unpeeled shrimp. The present practice of
peeling and deveining prior to freezing means the removal of potential sources of microorganisms, which otherwise, after defrosting, exert their deteriorating effect.

II. Quality Evaluation of Shrimp:

Much effort has been expended in attempts to establish objective quality tests for shrimp. Such tests would be of immeasurable value to the shrimp industry in the distribution and processing of individual catches of shrimp according to their expected storage life. Subjective sensory measurements have been used almost exclusively in the determination of shrimp quality. Studies by Fieger and Friloux (37) indicated that definite taste changes occurred during ice storage.

During the first few days of ice storage the shrimp flavor may be described as "fresh" and slightly sweet. There then follows a period in which the sweet flavor disappears and the taste becomes "flat"; however, off-flavors associated with spoilage are not present during this period. Finally, the shrimp undergo a sharp change in flavor indicative of spoilage. These three general flavor changes have been substantiated by experienced taste panel personnel through repeated organoleptic studies with ice-stored shrimp.

These investigators attempted to correlate the organoleptic stages in shrimp deterioration with chemical and bacteriological changes. Chemical determinations were made for tyrosine reaction, amino nitrogen, trimethylamine nitrogen, and volatile acids. Bacteriological examination consisted of determining total plate
counts. Tyrosine reaction values showed no definite trend and were considered of no value as an index of quality or length of storage of fresh Gulf shrimp.

Values for both trimethylamine nitrogen and volatile acids were found to be of limited value as indices in that values for both tests remained relatively constant for an initial period of about 14 days and then increased significantly; however, these increases lagged somewhat behind a similar increase in total bacterial plate count. Amino nitrogen determinations showed the most promise as a quality measurement of the ice-stored shrimp. These values decreased in magnitude as the length of storage time increased.

Several reviews have appeared in the literature concerning biochemical changes in fish during ice storage (2, 90, 99, 108), and these contain information which is generally applicable to changes in ice-stored shrimp. Most investigators believe that spoilage of such shrimp is caused mainly by bacterial enzymes, but autolytic changes are definitely important (68). A sudden increase in bacterial load is often taken to indicate onset of spoilage in fish, and comparisons with organoleptic changes in shrimp show the same relationship (35). Bacterial counts alone are unreliable in differentiation of various degradation stages in quality of shrimp.

Some investigators consider tests for ammonia unreliable as indicators of bacterial spoilage of fish (90, 99), and there is no general agreement concerning ammonia content as a quality index for these products. Studies conducted by Fieger et al. (36) showed
that Nessler nitrogen increases slightly with the onset of spoilage and then rapidly as spoilage progresses. These investigators were unable to suggest a definite spoilage index for Nessler nitrogen.

Beatty and Collins (6) found similar changes in amino nitrogen values in their studies on press juice from cod tissue. The decreases approximately equaled the increases in ammonia nitrogen leading these investigators to suggest that bacterial deamination of amino acids liberated from tissue protein would account for such results. Campbell and Williams (12) in studies with shrimp and Sigurdsson (99) in studies with herring obtained increases in amino nitrogen during refrigerated storage. It has been suggested that these differences can possibly be explained on the basis of different protein degradation products. It is also possible that the rapid association of amino groups with denatured protein molecules mask them from detection by the procedure employed (4).

Bailey et al. (4) reviewed chemical, physical, and microbiological tests useful for determining the relative quality of ice-stored shrimp. They classified the acceptable tests into three categories: (a) those indicating definite changes in prime quality; (b) those signifying the onset of spoilage; and (c) other applicable tests for determining relative quality. Table II shows the tests studied, the fraction examined, and references concerning the analytical method.

Three of the chemical tests surveyed measure definite changes during the prime quality period; these are tests for glycogen-sugar content, lactic acid content, and acid-soluble
### TABLE II

**OUTLINE OF USEFUL TESTS IN QUALITY STUDIES OF ICE-STORED SHRIMP**

<table>
<thead>
<tr>
<th>Test</th>
<th>Extraction or separation employed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Tests that indicate changes in prime quality:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycogen-sugar</td>
<td>5% Trichloroacetic acid containing 0.1% silver sulfate extract</td>
<td>(54)</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>5% Trichloroacetic acid extract</td>
<td>(108)</td>
</tr>
<tr>
<td>Acid-soluble orthophosphate</td>
<td>10% Trichloroacetic acid extract</td>
<td>(108)</td>
</tr>
<tr>
<td><strong>II. Tests that signify the onset of spoilage:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethylamine nitrogen</td>
<td>5% Trichloroacetic acid extract</td>
<td>(28)</td>
</tr>
<tr>
<td>Volatile acids</td>
<td>Steam distillation</td>
<td>(40)</td>
</tr>
<tr>
<td>Bacterial plate counts</td>
<td>Aqueous extract</td>
<td>(43)</td>
</tr>
<tr>
<td>Sulfhydryl groups</td>
<td>Aqueous extract</td>
<td>(65)</td>
</tr>
<tr>
<td><strong>III. Other applicable tests for determining relative quality:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen ion concentration</td>
<td>Tissue homogenated in water</td>
<td>(4)</td>
</tr>
<tr>
<td>Amino nitrogen</td>
<td>Aqueous extract</td>
<td>(70)</td>
</tr>
<tr>
<td>Hydration of water insoluble protein</td>
<td>Tissue homogenated in water</td>
<td>(4)</td>
</tr>
<tr>
<td>Sulfhydryl groups (ferricyanide reduction)</td>
<td>Aqueous extract</td>
<td>(43)</td>
</tr>
</tbody>
</table>
orthophosphate content. Glycogen-sugar content rapidly increased during the first three days of ice storage, then slowly decreased until the sixth day, after which time it rapidly decreased to a minimal level. Lactic acid values increased initially and then decreased. This initial increase was attributed to the rapid breakdown of carbohydrates. Collins (16) reported a similar increase in the lactic acid content of press juice of cod but could not correlate the increase with glycolytic breakdown alone. Other workers have also failed to obtain accurate balances between glycogen decreases and lactic acid increase (64, 102). After four days' storage the lactic acid in shrimp was being oxidized faster than it was produced, resulting in a rapid decrease of this constituent. This was followed by a less rapid decrease for the remainder of the storage period.

Watson (110) postulated that lactic acid in the presence of trimethylamine oxide reacted to form carbon dioxide, water, and trimethylamine, and also demonstrated the disappearance of lactic acid and appearance of carbon dioxide and trimethylamine in reducing Achromobacter suspension incubated with lactic acid and trimethylamine oxide. Collins (16) also found that both acetic acid and carbon dioxide increases concomitantly with decreases in lactic acid as further evidence for the operation of this reaction during the ice storage of fish. These changes are possibly applicable to shrimp deterioration, but other mechanisms are also operative since lactic acid does not decrease appreciably during the period of rapid rise in trimethylamine.
Acid-soluble orthophosphate decreases very rapidly during the first few days of storage and then less rapidly throughout the remainder of the storage period. This change in the progression of decrease in orthophosphate occurs shortly after the shrimp have lost their prime quality. The mechanism for this decrease in acid-soluble orthophosphate has not been elucidated.

Using commercially frozen shrimp which were thawed, then stored in beakers in refrigerators after various treatments, Farber (30) showed that volatile reducing substances offer a useful means for the chemical evaluation of the state of freshness of shrimp.

Among the numerous breakdown processes in seafood, proteolysis is prevalent. Holding shrimp at 32°F or 20°F generally results in a much slower rate of protein breakdown. Similarly, keeping shrimp at air temperature prior to ice storage involves an increased protein decomposition with the release of amino acids, including tyrosine, and the subsequent rapid conversion of the latter to melanin through the action of phenolases. Novak and Fieger (69) have shown increases in certain free amino acids during ice storage. Simidu and Hujita (100, 101) clearly established in no less than ten commercial shrimp varieties that the flavor is related to the content of amino nitrogen, particularly of the monoamino group. More than half of this was represented by glycine, which gradually declined in amount during storage. This decidedly reduces the palatability. It is notable that no cysteine or cystine has been found in shrimp (87).
Duggan and Strasburger (26) postulated two principal types of decomposition in raw shrimp. One of these, the putrefactive type, occurs when shrimp, before icing, are exposed for a time and at a temperature favorable for bacterial growth. This breakdown is characterized by the appearance of indole, presumably formed from tryptophan through bacterial action. No indole is ever found in fresh shrimp. Once started, this type of decomposition proceeds fairly rapidly even though the shrimp are subsequently well iced. The ammoniacal type of decomposition is necessarily slow and is characterized by an odor of free ammonia, in addition to other odors associated with protein decomposition.

Indole has been recommended as a reliable indicator of spoilage of the raw material prior to freezing and canning (25, 26). It has been commonly used as a criterion of inferior sanitation in processing plants or deterioration in storage prior to final processing (5). Lartigue et al. (60) evaluated the indole and trimethylamine tests for oyster quality. These investigators found that while the indole test developed by Clarke et al. (11) and the trimethylamine test of Dyer (27) were sensitive and capable of detecting microgram quantities of these compounds, they could not be used as indices of oyster spoilage since the values showed no definite pattern of change during storage of the oysters. Spinelli et al. (105) have concluded that trimethylamine is unsatisfactory as an index for flavor changes in irradiated and non-irradiated king crab meat.

The preceding literature review validates the need for
development of additional objective methods to measure deterioration in shrimp and other shellfish. While indole, trimethylamine, and ammonia production are excellent indicators of gross spoilage of shrimp, they are of limited value as gauges of marginal deterioration.

Industry-wide standards of shellfish quality have not been established for the simple reason that there are no completely satisfactory methods by which deteriorative changes can be accurately and quantitatively measured throughout their course. The oldest and still most widespread means of evaluating the acceptability and edibility of shrimp are the senses: smell and sight, supplemented by taste and touch.

Farber (30) summarized the advantages and disadvantages of such subjective testing with the following statement:

The reasons for the preferential use of sensory tests are obvious: no special laboratory equipment is needed; the fish can be examined wherever they happen to be; the tests can be carried out quickly; and many samples can be evaluated in a relatively short time. These obvious advantages, however, are to a great extent counterbalanced by a number of disadvantages inherent in the organoleptic method that significantly detract from its usefulness. The use of the senses is a subjective procedure and at best only very roughly quantitative. The impressions registered are the result of the interaction of a number of physiological, psychological, environmental, and even economic factors, including state of health, personal prejudices, preferences and interests, sensory acuity, freedom from disturbing and influencing conditions in the examining environment, and motives of possible profit or loss. The demand upon the senses becomes most critical and difficult when they are required to distinguish and assess the so-called borderline stage of freshness or the stage of incipient spoilage (that is, when a fish sample to be judged is in the last stages of freshness or in the first stages of spoilage). This task is well recognized as a difficult one, where even experienced judges often differ in their evaluation.
III. Carbonyl Studies:

Development of analytical techniques, such as gas-liquid chromatography, capable of measuring microgram or nanogram quantities of certain organic compounds, has rendered shrimp flavor research practical. It is now possible to investigate groups of chemical components which, though present in extremely small quantities in the live product, undergo significant changes following death.

Among the possible products of bacterial action and of the chemical breakdown of unsaturated fats and amines are the carbonyl-containing substances, including keto acids, ketones, and aldehydes. Diacetyl, for example, is a well-known product of lactobacilli and certain lactic streptococci. Monocarbonyl and dicarbonyl compounds have been used as indicators of rancidity.

A variety of carbonyl compounds are found in cooked and processed foods. Cooked chicken has been shown to contain diacetyl, acetone, methyl ethyl ketone, and saturated and unsaturated aldehydes (61). In any mode of detection, however, it must be kept in mind that aldehydes are very reactive compounds, and care must be taken that they do not undergo reactions such as aldol condensations during isolation or chromatography.

Extraction and subsequent isolation of carbonyl-containing compounds from plant and animal tissues has been accomplished by a number of general procedures. They are part of the neutral fraction of the extract or distillate, inasmuch as they cannot be isolated from alcohols, ethers, or sulfur-containing compounds by adjustment of pH. However, they can be separated from other neutrals by
making use of the capacity of the carbonyl group for condensing with reagents containing labile hydrogen, such as 2,4-dinitrophenylhydrazine. Pippen et al. (81) stripped the carbonyl compounds free from cooked chicken by aerating an aqueous slurry of the macerated tissue under reflux. Carbonyls were collected by passing the air stream through a trap containing 2,4-dinitrophenylhydrazine in dilute acid solution. Aeration was continued for 20 hours, after which the insoluble hydrazones were collected by filtration and divided into monocarbonyl and polycarbonyl fractions by selective solubility in hot alcohol or chloroform.

Ralls (85) isolated carbonyls from green peas by steam-distilling them at atmospheric pressure for two hours and collecting the first 250 ml of distillate. Carbonyls in the distillate were precipitated with 2,4-dinitrophenylhydrazine and recovered by filtration. These methods contain distinct disadvantages in that large samples are required (2 to 5 kg) and the samples are exposed to relatively high temperature (above 80°C) for relatively long periods of time (more than an hour). These conditions predispose the system to the possible creation of carbonyls as artifacts.

Carbonyls have been separated successfully on several types of chromatographic columns. Stephens and Teszler (107) used dinonyl phthalate at 87°C as the stationary phase for the separation of C_2 to C_5 aldehydes. Ralls (86) used carbowax 15M at 90°C and 150°C for separation of C_2 to C_6 aldehydes and ketones. Hawke et al. (47) compared several types of columns and found that Pluronic F68 and tri-m-tolylphosphate gave better separations at
110°C than paraffin wax, silicone oil or dicapryl phthalate. There was some tailing of peaks under the conditions employed by the latter investigators, and column temperatures were higher than those of the first two workers.

Yeates (115) obtained excellent separation of C$_2$ to C$_{10}$ aldehydes and ketones employing carbowax 20M and temperature programming. Corkem (18) used 1,2,3-tris-(2-cyanoethoxy) propane in partitioning over 15 carbonyl compounds under isothermal conditions.

Working with fish products other than shrimp, Farber (29) found that the content of the bisulfite-binding substances increased with spoilage and varied directly with the sensory findings. Proctor et al. (83) found carbonyl compounds in haddock flesh but reported no differences between fresh and spoiled fish.

Obata et al. (74, 75) reported the identification of crotonaldehyde, iso-butyaldehyde, formaldehyde, and iso-valeraldehyde as being partially responsible for the malodor of spoiling fish. Ota (77, 78) found volatile carbonyl concentration to be very small in fresh fish. Carbonyl contents increased with length of storage time and tended to mount in advanced spoilage. A gradual formation of acetaldehyde, n-butyaldehyde, and acetoin occurred during storage.

Studies concerned with the isolation and metabolism of carbonyls in shrimp have not been observed in the literature.
IV. Radiation Preservation of Fishery Products:

Raw fish is an unstable food. Unless it is preserved in some way, it is subject to bacterial, enzymatic, and chemical changes that soon render it unpalatable, then inedible. The primary objective of preservation processes is to inhibit or retard these changes.

Several methods for the preservation of shellfish products as adjuncts to refrigerated storage at various temperatures are described in the literature, none of which offer a panacea to the problem. Irradiation is the most effective of the newer techniques and has widespread applicability.

The amount of radiation treatment employed depends on the food and the result desired. If the main objective is prolongation of shelf life, or storage time, a "pasteurization" dose of less than 1.0 Mrad (generally between 0.2 and 0.6 Mrad) is employed. If the aim is "sterilization" of the product for long-term storage without refrigeration, the required dose is greater than 1.0 Mrad (usually between 2.0 and 4.5 Mrad).

Gamma irradiation effects in prolonging storage are due primarily to a reduction in the bacterial load and secondarily to autolytic enzyme inactivation. Bacterial growth is inhibited by killing them outright or by slowing their metabolism and reproductive rate. Actual mechanisms governing these changes are not known at the present time. Sterilization involving inactivation of bacterial spores, particularly those of Clostridium botulinum, requires a dose of about 4.8 Mrad to give the degree of inactivation
customary for "commercial" sterility. Vegetative bacteria, on the
other hand, are destroyed by low dose exposure in the neighborhood
of 0.5 Mrad (95).

Enzyme systems are apparently considerably less susceptible
to destruction by gamma irradiation. Many enzymes have been shown
to maintain their activity even after exposure to doses of 20 to
70 Mrad (23, 24, 93).

Sterilizing doses of radiation frequently cause a reduction
in food quality due to the production of objectionable odors and
flavors. Because of this limitation there is considerable interest
in the use of lower doses of radiation, which while not giving
sterile products, usually result in increased storage life of the
product under chilled conditions.

Although there is increasing emphasis on the use of small
amounts of radiation to minimize quality changes, there are several
general methods of reducing adverse effects which are effective at
all dose levels. Preventing access by oxygen during irradiation
and subsequent storage usually has a beneficial effect. In any case,
prolonged storage of many foods in the presence of oxygen would lead
to oxidative rancidity, a process which is usually hastened by
irradiation (61).

A considerable amount of work has been done on the effects
of ionizing radiations on nutrients in vitro and in situ. Reviews
by Read (89) and Raica et al. (84) provide complete and up-to-date
information on the subject and are quite replete with detailed
references. It can safely be said, in light of 18 years of w"j}
documented experimental data, that destruction of nutrients by ionizing radiations, even at high sterilizing dose levels, either parallels or is less than that caused by cooking.

Toxicological studies have been conducted on 21 representative foods which were subjected to radiation dose levels of 2.79 and 5.58 Mrad. In general, it is agreed that foods irradiated at 5.58 Mrad contain no toxic materials induced by this treatment. In another series of tests rats were fed a composite diet of nine foods supplemented with non-irradiated vitamins and minerals. No differences could be found between these and animals consuming a diet containing 100 percent of the calories as irradiated foods (84). As a part of the U. S. Army irradiated foods program, short-term (15 day) human feeding experiments were undertaken. No toxic effects were observed, and the digestibility of the macronutrients and metabolizable energy was similar in both the irradiated and non-irradiated diets.

Goldblith (12) surveyed the wholesomeness of irradiated foods in terms of past history, present status, international aspects, and future outlook. The author estimated that approximately $8 million has been expended in the past 12 years in demonstrating that foods sterilized by ionizing radiation are safe and wholesome. The process of substerilization is still being evaluated, but there is every reason to believe that this process will also prove to be safe and wholesome.

Fish and other seafoods were among the first foodstuffs to be tested by irradiation. Proctor et al. (82) pioneered the
initial work in this area. It early became apparent that doses required for the commercial sterility of fishery products created such undesirable changes in flavor and odor as to render them organoleptically unacceptable. These odors and flavors have been variously described as "metallic", "burnt-feather-like", and "rubbery". In addition, storage of the sterilized products resulted in the development of strong bitter flavors, brown and other discolorations, particularly in the white-fleshed species, and a toughening of the texture. Irradiation effects were also found to be specie-specific in many instances, i.e., the radiation dosage that could be applied without producing excessive undesirable changes varied with the type of fish. The maximum permissible dose that could be given to whiting was found to be 0.20 to 0.25 Mrad; that for shrimp and crab meat was 1.0 Mrad (7).

Coleby (15) reported that levels of radiation needed to bring about pasteurization of food products depend on the initial bacterial load and on the type of product. Pasteurization was usually effected at levels ranging from 0.05 to 1.0 Mrad, followed by refrigerated storage.

Shewan (97) summarized the status of preservation with ionizing radiation. Maximum dose levels that could be used for the preservation of 20 different species of marine products were presented, and some of the problems encountered in processing the different species were pointed out.

Miyauchi et al. (66) irradiated king crab meat at levels of 0.2 and 0.4 Mrad. Lower dosage resulted in a product with high
quality characteristics and with a significant extension in storage life at refrigerated temperatures (33°F and 42°F). At the higher level changes in odor and flavor occur, but storage life is extended greatly (1 to 6 weeks).

Novak et al. (73) found that irradiation of Gulf Coast oysters at levels above 0.2 Mrad produced adverse organoleptic changes. Oysters irradiated at 0.2 Mrad and stored in crushed ice for periods up to 21 days were superior in flavor, odor, and appearance to non-irradiated controls after 7 days' storage and were still acceptable after the 21 day period.

Sinnhuber (103) found that shrimp irradiated with 0.5 and 0.75 Mrad were not spoiled after 180 days at 45°F, and shrimp irradiated with 0.25 Mrad remained unspoiled for 60 days. A loss of irradiated flavor was detected during storage of the shrimp at 45°F.

Scholz et al. (92) reported that cooked shrimp irradiated at 0.5 and 0.75 Mrad remained in good condition throughout 18 weeks' storage. No viable organisms were found, and production of decomposition products was slight as measured by the chemical tests.

Novak and Liuzzo (71) reported that the dose of radiation which was judged to be optimum for preservation of Gulf shrimp was 0.15 to 0.2 Mrad.
EXPERIMENTAL METHODS

I. Introduction:

Subjective testing procedures are presently used by the FDA in determining shrimp quality. As pointed out in the literature review, sensory testing is important in food quality evaluation but is severely limited in its nonquantitative and roughly qualitative nature. The literature is equally implicit in noting that incipient spoilage in shrimp cannot be determined with consistency by sensory evaluation. Only in cases where objective tests are not available is it justifiable to employ subjective methods as substitutes in quality measurements.

In its zeal to protect the consumer public from adulterated foods, the FDA has imposed such stringent requirements on shrimp quality that the very life of the industry is threatened. Rigid controls are desirable and constant surveillance imperative. At the same time such controls must be realistic and based, where possible, on the experience of objective analytical procedures. While several tests have been developed to measure known products of shrimp decomposition, e.g., indole, trimethylamine, and ammonia, it is recognized that they are of insufficient reliability to be independently employed as absolute indices. A satisfactory solution to this dilemma is urgent and can be achieved by developing a sufficient number of analyses whose results, considered as a whole, will serve
as a quantitative measurement of shrimp decomposition.

With this motivation, the development of a method for the measurement of carbonyls in shrimp was undertaken. These compounds had been implicated in other types of food spoilage, but references were not available of their specific involvement in shrimp decomposition. A review of methods employed in the removal and isolation of carbonyls from these other foodstuffs indicated that, with modifications, the principles involved would be applicable in the development of methodology for their determination in shrimp.

Most of the reported methods were fraught with the potentiality of artifact generation due to the degree and time of relatively high temperature exposure. Hence, major consideration in method development was given to the selection of those analytical techniques which would minimize the production of artifacts and maximize the removal of the desired components from the tissue.

During experimentation involving established analytical tests, e.g., trimethylamine and ammonia, modifications were made which would reduce the errors associated with these methods. This emphasizes the need for continuous scrutiny of existing procedures to improve their level of reliability.

Fresh shrimp were employed in the experimental work when available. They were purchased directly from a trawler operator and were caught in butterfly nets in Redfish Bay and other inland bays located in the vicinity of Venice, Louisiana. Immediately following removal from the water, the shrimp were packed in crushed ice and transported to the laboratory where they were deheaded.
peeled, and deveined. Elapsed time from catching until receipt at the laboratory was generally less than 15 hours.

Utilization of shrimp of known past history was considered of utmost importance in attaching proper significance to the analytical results. Unfortunately, some of the referenced data on shrimp decomposition was obtained using samples of unknown past history. This is particularly true of results published by investigators far removed, both in space and time, from the original source of the sampling.

All samples of shrimp contain tissue in various states of deterioration. The nature and concentration of the metabolites present at the start of the experimentation will be dependent on many factors such as age of the sample, treatment prior to shipment, method of packing for shipment, method of shipment, handling in transit, and preparation for study. Experimental values obtained will be dictated to a great extent by these factors. By knowing the past history of the shrimp samples, analytical results can be viewed in the proper perspective and valid conclusions drawn.

Experimental methods employed in the study of carbonyl metabolism in shrimp decomposition are described below.

II. Extraction of Carbonyls from Shrimp:

A. Preparation of Shrimp for Extraction:

Shrimp samples were deheaded, peeled, and deveined immediately after receipt at the laboratory. Deveined tissue was washed three times with fresh portions of ice water. After
draining, weighed amounts of the tails—usually 336 g—were placed in Scotchpak 20A5 pouches* and heat sealed.

B. Solvent Extraction of Shrimp:

A batch-type, countercurrent, liquid-liquid extractor (Figure 1) similar to that described by Kolfenbach et al. (56) and modified by Senn (94) was employed in all extractions of shrimp tissue. A 250 ml bulb at the base of the column was adequate to prevent emulsion buildup. The small amount of oil-in water emulsion that tended to form broke readily on standing. The 25 mm ID extraction column was one meter long from the 0.75 mm jet orifice to the inlet for extractant. Diameter of the jet was found to be rather critical in controlling the size of droplet and the rate of flow of the shrimp homogenate.

A 300 g sample of the peeled and deveined shrimp was placed in a 1000 ml Waring blender jar. Three hundred milliliters of 50 percent ethanol (prepared from 95 percent unde¬natured ethanol) were added, and the tissue was homogenized at high speed for 15 minutes. Additional 50 percent ethanol was added as required to maintain a constant turnover of tissue. Temperature of the homogenate was maintained below 60°C throughout the blending operation to keep the loss of volatiles to a minimum. The homogenate was transferred to a 2000 ml beaker, and the blender jar washed with sufficient 50 percent ethanol to bring the total of ethanol employed to a volume of 900 ml

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*Film and Allied Products Division, 3M Company
Figure 1

Batch-type, Countercurrent, Liquid-liquid Extractor
(approximately three times the volume of shrimp tissue employed in the extraction).

Sonication of the homogenate for 5 minutes using a Biosonik II sonicator* at an output intensity of 9 kilocycles was employed to further reduce the particle size of the homogenate. The sonicated sample was then vacuum filtered (water aspiration) through a No. 50 (48 mesh) brass sieve screen into a 2000 ml suction flask to remove any residual coarse particles that could clog the jet.

The filtrate was placed in a 2000 ml separatory funnel employed as the extractor reservoir. An inert nitrogen atmosphere was maintained in this reservoir throughout the extraction period to minimize both carbonyl creation and destruction by air oxidation. Initial passage through the extractor was made under gravity flow. Second and succeeding passages were conducted under positive nitrogen pressure (3-5 psig) at a rate of approximately 3 liters per hour, the extracted homogenate being collected and returned to the reservoir for a total of four extractions with the same batch of solvent. Homogenate level was not permitted to rise above the extractant inlet during the extraction. Total elapsed time of the extraction period was approximately 90 minutes.

Considerable foam was produced during the homogenation and sonication phases. Consequently, during the first passage of homogenate through the column large vapor bubbles were

*Bronwill Scientific, Rochester, New York
created, the upward movement of which tended to carry small amounts of homogenate over into the extractant boiling flask. While the presence of this material in the boiling flask was not considered detrimental to the extraction, it could readily be controlled by permitting the initial passage to take place under controlled gravity flow.

Regulation of the flow rate was required. When pressure was applied to increase the flow rate, the homogenate emerged from the jet in a finely dispersed spray which tended to impede the upward movement of fresh extractant and thereby reduced the extraction efficiency. Careful control of the flow rate through the jet resulted in the aqueous phase being sufficiently dispersed without hindering the flow of extractant.

Technical grade n-pentane (from petroleum, b.p. 33 to 36°C), subjected to purification, was used as the extractant solvent. Gas chromatographic adsorption characteristics of this material were found to be identical with those for spectroquality grade n-pentane and, for economy reasons, was considered to be the more desirable grade. Approximately 700 ml of n-pentane were required to fill the extractor column and bulb completely and the 500 ml boiling flask to a level of about 200 ml. The extractant boiling flask was heated with a steam bath. Condensers were cooled to 0°C with a mixture of water and 2-propanol circulated by a water bath cooler (E. H. Sargent and Company). About 5 percent of the solvent was lost in the extraction phase through condenser inefficiency
and minor emulsion formation. A 95 percent recovery of solvent in this type of extraction is considered to be quite excellent.

III. Isolation and Concentration of Carbonyls from Shrimp:

Following the final passage of the homogenate through the extractor column, the system was allowed to cool to room temperature. Extract in the boiling flask was transferred to a 500 ml separatory funnel and washed with two 20 ml portions of distilled water to remove water solubles such as simple sugars. Ten grams of anhydrous sodium sulfate (reagent grade) were added to the funnel and the contents were shaken vigorously to remove traces of water. The dried extract was passed through fluted filter paper into a 500 ml boiling flask, and the residue was washed with 20 ml of n-pentane.

n-Pentane removal was accomplished by connecting the boiling flask to a short Vigreux fractionating column (1.70 mm, packed with 1/4 in. glass helices) and placing the flask in a 50°C circulating water bath. When the liquid volume was reduced to approximately 75 ml, the flask was removed from the bath and allowed to cool. Flask contents were transferred, with n-pentane washing, to a 2-neck 100 ml boiling flask which, after connection to a micro fractionating column (10 mm x 300 mm, packed with 1/8 in. glass helices), was then placed in the 50°C water bath. Upon reduction of the extract volume to 5 ml, the flask was removed from the bath and allowed to cool to room temperature.

The flask was then connected to the fractionating column (10 mm x 120 mm, packed with 1/8 in. glass helices) of a distil-
lation assembly (Figure 2). Column heating was accomplished by means of a heating tape covering the entire length of the column. As shown in Figure 2, the column was connected to an absorption tube (12 mm x 100 mm) immersed in liquid nitrogen which functioned as a trap for the volatiles. Evacuation of the system was accomplished by means of a vacuum pump connected to the trap. The still pot was immersed in boiling water and evacuation maintained for a period of 15 minutes. Vacuum on the system was released with nitrogen admitted through the side-neck of the boiling flask.

Distillate material was brought to room temperature and then added to a chromatographic column (10 mm x 150 mm) packed to a height of 5 inches with 60 to 100 mesh florisil (reagent grade). The trap was washed with 3 ml of \( n \)-pentane, washings being added to the column. Hydrocarbons present in the distillate were removed from the column by elution with 25 ml of \( n \)-hexane (spectroquality grade). Carbonyl compounds were eluted from the column with 30 ml of carbonyl-free methanol, the eluate being collected in a 125 ml separatory funnel. Two milliliters of this eluate were removed for total carbonyl determination.

Remaining eluate was diluted with 25 ml of distilled water and extracted with five 15 ml portions of \( n \)-pentane. Pentane layers were collected in a 125 ml separatory funnel. Five grams of anhydrous sodium sulfate were added to the funnel; contents were shaken vigorously and filtered through fluted filter paper into a 100 ml beaker. The residue was washed with a 5 ml portion of \( n \)-pentane. Reduction of the filtrate volume to approximately
Figure 2

Distillation Assembly
0.5 ml was achieved by evaporation from a glass vial (21 mm x 70 mm) in a stream of nitrogen at room temperature. Sealing of the vial with a serum type stopper facilitated sampling for the gas chromatographic analysis.

IV. Separation of Carbonyls from Shrimp:

Carbonyl compounds present in the concentrated fraction were separated by gas chromatography. Chromatographic unit employed was a Microtek Instruments Model GC-2000R equipped with both dual flame and thermal conductivity detectors and with automatic temperature programmer. The recorder was an E. H. Sargent Model TR with a 1.0 mv range and a sensitivity of 0.1 percent full scale.

Conditions established for efficient separation of the carbonyls are shown in Table III.

Sample injections were made using a Hamilton No. 701-N gastight syringe.

Programming was initiated 1 minutes from the time of injection. Analysis was completed within 10 minutes after injection.

V. Identification of Carbonyls from Shrimp:

Tentative identification of shrimp carbonyls was accomplished by subjecting the concentrate obtained from the extraction of 2000 g of tissue to chromatographic separation on two different column packings. Retention times of the sample component peaks were compared with those for known carbonyls.

Columns employed were the carbowax 20M described in Table III

*Hamilton Company, Inc., Whittier, California
### TABLE III

OPERATING PARAMETERS FOR GAS CHROMATOGRAPHIC ANALYSIS OF CARBONYLS IN SHRIMP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>1/8 in. by 8 ft stainless steel, packed with 20 percent carbowax 20M on 60 to 80 mesh Chromport XXX</td>
</tr>
<tr>
<td>Detector</td>
<td>Dual hydrogen flame</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>225°C</td>
</tr>
<tr>
<td>Inlet temperature</td>
<td>200°C</td>
</tr>
<tr>
<td>Column temperature</td>
<td>Programmed from 50°C to 150°C</td>
</tr>
<tr>
<td>Program rate</td>
<td>10°C per minute</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Helium</td>
</tr>
<tr>
<td>Carrier flow rate</td>
<td>35 cc per minute at 50 psig</td>
</tr>
<tr>
<td>Air flow rate</td>
<td>283 cc per minute at 40 psig</td>
</tr>
<tr>
<td>Hydrogen flow rate</td>
<td>60 cc per minute at 40 psig</td>
</tr>
<tr>
<td>Chart speed</td>
<td>1.0 in. per minute</td>
</tr>
<tr>
<td>Sample size</td>
<td>As noted (usually 1.5 to 2.0 ml)</td>
</tr>
<tr>
<td>Attenuation</td>
<td>As noted (usually 1 x 8)</td>
</tr>
</tbody>
</table>
and a 1/8 in. by 10 ft stainless steel column packed with 5 percent 1,2,3-tris-(2-cyanoethoxy)-propane on 60 to 70 mesh silanized Chromport XXX*. Operating conditions as noted in Table III were employed for both columns with the exception that the latter column was operated isothermally at 50°C.

VI. Determination of Total Carbonyl Content of Shrimp:

A. Procedure:

Procedure used for the determination of total carbonyl content was that of Lappin and Clark (59) with modifications.

To 2.0 ml of the unknown solution were added 2.0 ml of the 2,4-dinitrophenylhydrazine reagent, 1.0 ml of distilled water, and two drops of concentrated hydrochloric acid. Addition of distilled water prevented the formation of salt crystals when the solution was rendered basic in a later step in the procedure. The tube was loosely stoppered and heated in a water bath at 50°C (±0.1°C) for 30 minutes. After cooling, 5.0 ml of the potassium hydroxide solution were added. The almost black solution which resulted rapidly cleared to a characteristic wine-red color. Optical density of the solution was determined at 480 μm using a Beckman Model DU spectrophotometer with photomultiplier attachment. The instrument was adjusted for 100 percent transmittance for the solution from the blank determination. Carbonyl-free methanol was used in the blank.*

*Microtek Instruments Inc., Baton Rouge, Louisiana.
The spectrophotometer was standardized, and a calibration curve was constructed from the observed optical density. Figure 3 represents a typical standard curve determined by this procedure.

B. Preparation of Reagents:

Reagents used in the procedure were prepared according to the following directions.

Carbonyl-free methanol was prepared by adding 5 g of 2,4-dinitrophenylhydrazine (reagent grade) to 500 ml of methanol (reagent grade). Ten drops of concentrated hydrochloric acid were also added. After refluxing for two hours the methanol was distilled through a short Vigreux column and stored in a tightly stoppered bottle.

A saturated solution of 2,4-dinitrophenylhydrazine was prepared by adding one gram of 2,4-dinitrophenylhydrazine, which had been twice recrystallized from carbonyl-free methanol, to 100 ml of carbonyl-free methanol. This solution was prepared fresh each day.

Potassium hydroxide solution was prepared by dissolving 10 g of potassium hydroxide (reagent grade) in 20 ml of distilled water and making to 100 ml with carbonyl-free methanol.

VII. Determination of Indole, Ammonia Nitrogen, and Trimethylamine Nitrogen in Shrimp:

A. Indole:

Indole content was determined using the method
Figure 3

Typical Standard Curve for Total Carbonyl
described by Turner (109). Twenty-five grams of cut shrimp were placed in a quart-size Waring blender jar with 100 ml of 5 percent trichloroacetic acid (reagent grade) and 25 ml of distilled water. Contents were blended for 5 minutes and then quantitatively transferred to a 250 ml volumetric flask. After making to volume with distilled water and thoroughly mixing, approximately 50 ml of the blended material were centrifuged at 2500 rpm for 5 minutes. From 1 to 20 ml of the clear supernatant were placed in a 250 ml separatory funnel and made up to a volume of 20 ml with distilled water. Size of the aliquot employed was determined by the estimated indole content of the extract.

Ten milliliters of petroleum ether (reagent grade) were added to the funnel and the contents were shaken vigorously. The aqueous layer was transferred to another 250 ml separatory funnel and extracted again with a 10 ml portion of petroleum ether. After the ether fractions were combined, 10 ml of p-dimethylaminocinnamaldehyde reagent were added, the contents were shaken vigorously, and the colored aqueous layer was drained into a tube and centrifuged at 2500 rpm for 5 minutes. A blank determination was made using distilled water. After standing for 30 minutes from the time of addition of the p-dimethylaminocinnamaldehyde, optical densities were determined at 640 mu using a Bausch and Lomb "Spectronic 20" spectrophotometer. Concentrations of indole present were read from a calibration curve.
B. **Ammonia Nitrogen**:

Ammonia nitrogen determinations were made according to a modification of the procedure described by Moen (67).

A modified Conway microdiffusion cell (76) consisting of three chambers was employed in the determinations. Two milliliters of standard acid containing indicator were placed in the inner chamber. One and a half milliliters of saturated potassium carbonate (reagent grade) solution were added to the closing chamber. In the outer diffusion chamber was placed an appropriate amount (usually 1 or 2 ml) of the clear supernatant from the indole extraction. One milliliter of the saturated potassium carbonate solution was added to the outer diffusion chamber and the lid was immediately put in place. The dishes were rotated to mix the solutions in the outer diffusion chamber and then placed in an incubator at 37°C for 2 hours. Excess acid in the center well was titrated with standard barium hydroxide solution.

Calculation of the ammonia nitrogen present was made according to the following relationship:

\[
\text{NH}_3\text{-N in mg per } \frac{3100 \text{ g shrimp}}{100 \text{ g shrimp}} = (V_B \times N_B \times 11.3 \times \frac{1000}{V_S}) = \text{TMA-N}
\]

where

- \( V_B \) = volume of \( \text{Ba(OH)}_2 \) in ml
- \( N_B \) = normality of \( \text{Ba(OH)}_2 \)
- \( V_S \) = volume of sample in ml
Use of the modified Conway cell eliminated many of the problems encountered with the two-chambered cell. The modified cell avoided the use of grease by having an extra chamber (the closing chamber) which was half filled with the same solution as the outer diffusion chamber with the exception of the sample fluid to be analyzed. A liquid trap was formed by the clear plastic top which was dipped into the outer closing chamber thereby excluding the possibility of gas leakage, and, as the liquid in the trap was also that used to liberate the ammonia in the outer diffusion chamber, the risk of absorption was eliminated.

C. Trimethylamine Nitrogen:

Trimethylamine nitrogen was determined by a modification of the procedure of Moen (95). The procedure was identical to that described above for the determination of ammonia nitrogen with the following additional steps. Two milliliters of 10 percent formaldehyde solution were added to the closing chamber and also to the outer diffusion chamber prior to the addition of saturated potassium carbonate to the outer chamber.

Calculation of the trimethylamine nitrogen present was made according to the following relationship:

\[
\text{TMA-N in mg per 100 g shrimp} = (V_B \times N_B \times 14 \times \frac{1000}{V_S})
\]

where

\[V_B = \text{volume of Ba(OH)}_2 \text{ in ml}\]
\[ N_B = \text{normality of } \text{Ba(OH)}_2 \]
\[ V_S = \text{volume of sample in ml} \]

VIII. Microbiological Examination of Shrimp:

Microbiological examination of shrimp consisted of total plate count determinations at 20°C and 35°C. Eleven grams of peeled and deveined shrimp were aseptically blended with 99 ml of sterile water, and appropriate serial dilutions were made from this primary dilution. Tryptone-glucose extract agar was used as the plating medium (116). Total count plates incubated at 20°C were examined at 5 days. Plates incubated at 35°C were examined at 48 hours. The bacterial count was expressed as the number of colonies per gram of the sample.

Classification of isolates in certain of the studies were made according to Bergey's Manual of Determinative Bacteriology (8).

IX. Ultraviolet and Infrared Analyses:

Ultraviolet spectra were obtained using a Beckman Model DU spectrophotometer equipped with a hydrogen lamp source and photomultiplier attachment.

Infrared spectra were obtained using a Perkin-Elmer Model 237 infrared spectrophotometer.

X. Storage and Irradiation Studies:

Storage studies in crushed ice were conducted by placing the sample pouches in a styrofoam ice chest with a layer of crushed ice surrounding the sample. The chest was placed in a coldroom at
5.5°C (±0.2°C). Water was drained from the chest at 48 hour intervals and crushed ice replenished as required.

Irradiation was made at a dosage level of 0.2 Mrad with cobalt-60 in the 11,000 curie irradiator located at the Nuclear Science Center on the Baton Rouge campus of Louisiana State University.
RESULTS AND DISCUSSION

When analyzing the quality of foods and other biological materials, it is necessary to perform chemical, microbiological, physical, and organoleptic tests. This includes evaluation of the nutritional status and biological availability of the tissue. Shrimp quality is a manifestation of the presence of many metabolic components produced by a multitude of metabolic pathways. It should not be anticipated, in fact it is highly improbable, that a single analytical test will be established as an absolute index of shrimp quality.

Presence or absence of certain chemical components alone does not give a true picture of the food quality or its pattern and mechanism of decomposition. For example, indole may be present in measurable quantities, and yet the product may be organoleptically acceptable. Trimethylamine values may be low, and the product may be inedible. Indole and trimethylamine values are meaningful chemical indices of shrimp quality only when considered in light of other chemical, microbiological, physical, and organoleptic data.

Every analysis that can be developed for measuring metabolic components in shrimp will contribute to a solution of the overall problem of quality evaluation. The more interrelationships that can be established and mechanisms elucidated the better will be the understanding of those factors controlling flavor production.
Flavor is a subtle and complex sensation and is a combination of taste, smell, and feel. Every chemical component of a given food contributes to the flavor sensation experienced for that food. Certain components influence the flavor to a greater degree than others, and the particular group of these major contributors will vary from food to food, and within a given food depending on age, temperature, pH, etc.

Odor is an integral part of the flavor machinery and in general results from the volatility of certain groups of chemical components whose quantities may or may not be proportional to the effects induced since synergistic and additive aspects must also be taken into consideration. Prime flavor components, unfortunately, are not necessarily those present in the largest concentrations. Many of the compounds are encountered only in vanishingly minute amounts. Many are so reactive and unstable that they are easily altered during examination. Investigators in the field are impressed by the great complexity of the mixture of volatile flavoring substances that have been obtained from most of the foods studied.

A review of the literature in the field of flavor chemistry readily discloses that most investigations have centered around these volatile components, notably carbonyls, acids, amines, ethers, and alcohols. Studies by Stanley (106) and Senn (94) on fruit flavors, by Pippen (80) on poultry flavor, by Day (20) on milk flavors, and by Hornstein (50) on lean meat flavor are representative of work involving these groups of compounds. Without exception, carbonyls are implicated as major flavor contributors in all of the
foods studied.

Scarcity of references to carbonyl determinations in shellfish is reflective of: (a) presence of these materials at micro or submicro levels, and (b) lack of satisfactory methods for the quantitative removal of these compounds from the tissue under consideration. This latter condition has presented a major hindrance to studies of the chemical composition of biological materials. Most studies consequently have been concerned with qualitative relationships, and even these have left much to be desired, especially with respect to interrelationships between metabolic components.

Procedures which are satisfactory for the removal of a given component from one type of tissue may be completely unsatisfactory for the isolation of that same component from a different type of tissue. As noted in the literature review there are a number of references to methods used in the removal of carbonyls from various biologics, none of which are concerned with shrimp. Consequently, it was necessary to develop a procedure for the isolation and concentration of these components from shrimp tissue.

Countercurrent liquid-liquid extraction is a very common procedure employed in isolation and purification work. It is extremely valuable in the isolation of substances from two-phase systems which undergo extensive emulsion formation when more conventional extraction techniques are used. It is surprising that this valuable technique had not been employed by more investigators in the field, since it is ready-made for isolation of many components from biological systems.
Selection of an efficient extraction solvent was the key to the successful employment of this technique in the isolation of carboxyls from shrimp. Homogenation and sonication of the shrimp tissue in 50 percent aqueous ethanol facilitated liberation of the carboxyls from the cells through cell wall destruction. This dictated that the extraction solvent be nonpolar for maximum immiscibility with the aqueous phase. Concomitantly, the solvent system had to fulfill the criteria of: (a) exhibiting infinite solubility for the carboxyls present in the homogenate, and (b) having a low boiling point which would permit the maintenance of low temperature throughout the extraction and isolation procedures. Diethyl ether and n-pentane were solvents fulfilling these criteria. Senn (9h) found that ether extracted large amounts of undesirable nonvolatile material. Because of this and the fact that n-pentane is less expensive and less hazardous, it was employed as the extraction solvent.

Vacuum distillation of the concentrated extract enabled a separation of the volatile components from the nonvolatiles. Distillation was conducted with caution to prevent entrainment of pigment residue into the distillation column. It was found that this residue tended to decompose when contact was made with the heated column, yielding several large component peaks in the chromatographic analysis. These decomposition products exhibited an unusual tenacity for the carbowax 20M column packing, necessitating several days of helium purging and high inlet temperature to eliminate the bleed of these materials onto the column during temperature programming.
This is pointed out to emphasize the need for continually guarding against the generation of artifacts which could easily prejudice results and add to confusion which already exists in the area of flavor chemistry research. Subsequent trapping of the volatiles by liquid nitrogen cooling insured a minimal loss of the carbonyls during the volatilization step. Some aerosol formation was noticeable as the distillate was brought to room temperature, but this was practically eliminated by intermediate warming in ice water.

Presence of nonoxygenated volatiles in the distillate necessitated the employment of an additional isolation technique. Column chromatography, using florisil as a selective adsorbent, effected an excellent separation of oxygenated components from nonoxygenated volatiles. These latter compounds were not adsorbed and could be readily removed by elution with n-hexane (hl). Reclamation of the carbonyls was accomplished by elution with carbonyl-free methanol.

Gas-liquid partition chromatography is one of the most powerful separation methods available. It can be used in flavor investigations for separation and detection of volatile compounds isolated from food products. Ionization detector equipped instruments are much more sensitive and are more stable with respect to changes in operating parameters than are thermal detector equipped instruments. The former type respond to as little as $10^{-10}$ to $10^{-15}$ mole of solute in the carrier gas.

Selection of the most efficient column for separating the carbonyls was based primarily on the recommendations of chromatographic
authorities (18, 115). Other operating conditions were determined by trial and error. High n-pentane to carbonyl ratio in the final concentrate resulted in only fair resolution of the lower molecular weight carbonyls. During the identification studies, some improvement in resolution was achieved by coupling a 4 ft by 1/4 in. stainless steel column, packed with 5 percent SE-30 on 60 to 80 mesh Chromport XXX, to the sample column.

Major advantages of the methodology developed in this study are: (a) small sample size required (less than one pound), (b) low temperature required (less than 60°C), and (c) relatively short period of time required for complete analysis (8 hours). Further developmental study would be desirable to: (a) evaluate the extraction efficiency of other solvent systems, (b) reduce the time required for complete analysis, and (c) improve the concentration procedure and thereby retain a larger percentage of the extractable carbonyls for chromatographic examination.

Ultraviolet spectra were obtained on the pigmented residues deposited in the still pot during the vacuum distillation. A major absorption band at 240 millimicrons was obtained for all the samples examined. No major differences in the spectra were discernible, discounting any significant relationship between spoilage and nature of the pigment.

Infrared spectroscopy studies confirmed the presence of carbonyls in the concentrates. Absorption characteristics in the region of 1700 cm⁻¹ (5.6 microns) indicated the presence of diones.

Viscosity changes in the homogenates were observed during
the storage studies. Decreasing viscosity with increasing storage time, probably as a result of proteolysis, suggests the need for further evaluation of these changes as a sign of incipient spoilage.

When studying metabolic components present at the microgram level, as in the case of carbonyls, it is desirable that the sample be as biologically free from contamination--bacterial and otherwise--as is technically feasible. Only under these conditions can it be assumed that the materials measured represent those actually derived from tissue metabolism. Even when ideal circumstances exist, certain foodstuffs are so susceptible to microbial activity that it is impossible to ascertain with any degree of certitude that the results obtained reflect metabolic processes inherent to the tissue under examination.

Shrimp are a classical example of such foodstuffs. Even when first removed from the water shrimp carry an indigenous microbial flora which cannot be completely removed without extensive tissue destruction and the consequent production of components not normally present. While it is not technically feasible to obtain a biologically-pure condition with shrimp, this state can be approximated by exercising caution in obtaining, handling, and preparing the sample for study.

In all of the experimental work considerable effort was expended to obtain samples which could be considered "biologically-pure". This minimized the determination of exogenous materials whose initial concentrations could vary significantly depending on the original source of the sample.
Shrimp which were less than 24 hours removed from the water when received at the laboratory were utilized when available. Damaged and black-spotted shrimp were discarded. After deheading, peeling, and thorough washing these samples invariably showed total bacterial counts less than 10,000 per gram.

During the period when Louisiana coastal waters were closed to shrimping, it was necessary to use shrimp which were obtained from trawlers off the Florida coast. These shrimp had been iced at least three days before receipt at the laboratory and invariably had high bacterial loads. Drain water from the ice of these shrimp had total bacterial counts up to 10 million per milliliter at 35°C, and the deheaded, unpeeled shrimp exhibited counts in excess of 10 million per gram. Peeling, deveining, and washing produced samples which usually had counts less than 100,000 per gram. Low trimethylamine nitrogen, ammonia nitrogen, and indole values for the original controls attested to a sample which approximated the "biologically-pure" condition.

The Scotchpak pouches used as storage containers exhibited total bacterial counts less than 100 per milliliter of sterile distilled water used in the determination. Water absorption through the film was negligible and permeability to oxygen from air was approximately 1.5 cc per 100 sq. in. of the film surface during a 24 hour period (63). This reduced oxygen availability to some extent and also reduced the loss of volatiles from the system.

Initial experiments were concerned with establishing carbonyl contents of the reagent used in the extraction, isolation,
and concentration procedures. With the exceptions of the n-pentane and ethanol used in the extraction of the tissue, the reagents employed were determined to be carbonyl-free.

It became evident during the course of the experimentation that impurities present in the solvent systems (n-pentane and 50 percent ethanol) were contributing to the total carbonyl values and were producing extraneous peaks in the chromatographic analysis. Solvent system checks were made by extracting 900 ml of 50 percent ethanol with n-pentane in the absence of shrimp tissue. Isolation and concentration of the impurities present in the resulting extracts were conducted according to the usual procedure. Carbonyl determinations indicated an average of 5 ppm to be present. Gas chromatographic analyses of the concentrates demonstrated the presence of a minimum of six components as shown in Figure 1.

To remove these interfering compounds, the solvent systems were subjected to a purification extraction in which 900 ml of 50 percent ethanol were extracted with n-pentane (four passages through the column). Following the final passage the level of liquid in the extractant boiling flask was reduced to approximately 100 ml and this material discarded.

Subjection of the purified solvents to the extraction, isolation, and concentration procedures resulted in the reduction of total carbonyl to less than 2 ppm and the elimination of most of the interfering component peaks as shown in Figure 5. Because of this reduction in concentration of the interfering compounds to insignificant levels, no further treatment beyond the purification
Figure 4
Chromatogram of Unpurified Solvent Systems

Figure 5
Chromatogram of Purified Solvent Systems
extraction was considered necessary.

To establish the efficiency of the extraction process, various concentrations of acetophenone were added to 500 ml of 50 percent ethanol and the total carbonyl determined after extracting and isolating the carbonyls. These data are shown in Table IV.

Known amounts of acetophenone were added to 300 g samples of peeled and deveined shrimp in order to ascertain recovery efficiencies under normal operating conditions. A sample to which no carbonyl was added served as the blank. These data are presented in Table V. High recoverability of the added carbonyl in both experiments demonstrated the suitability of the extraction procedure for the removal of carbonyls from shrimp tissue. Percentage of carbonyl recovered was surprising in view of the large number of steps involved in the procedure. It should be noted that the recovery efficiency of the more volatile components, e.g., acetaldehyde, would in all probability be somewhat less than that experienced for acetophenone.

Total carbonyl concentrations of fresh shrimp averaged 3.1 ppm. In general, carbonyl contents of shrimp stored in crushed ice increased with increasing length of storage as shown in Table VI. Carbonyl contents between different samples for the same period of storage showed some variation, but this usually could be related to the past history of the sample. Shrimp which were in good condition at the initiation of storage, as measured by minimum odor, firm texture, and low bacterial counts, exhibited low carbonyl values through 14 days' storage.

In one series of tests, shrimp were permitted to spoil at
### Table IV

**Recovery of Added Carbonyl from Solvent**

<table>
<thead>
<tr>
<th>Added carbonyl (in ppm)</th>
<th>Recovered carbonyl (in ppm)</th>
<th>Recovery efficiency (in percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.95</td>
<td>95</td>
</tr>
<tr>
<td>5.0</td>
<td>4.9</td>
<td>98</td>
</tr>
<tr>
<td>10.0</td>
<td>9.7</td>
<td>97</td>
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<tr>
<td>20.0</td>
<td>19.5</td>
<td>97</td>
</tr>
</tbody>
</table>
### Table V

**RECOVERY OF ADDED CARBONYL FROM SHRIMP HOMOGENATE**

<table>
<thead>
<tr>
<th>Added carbonyl (in ppm)</th>
<th>Recovered carbonyl* (in ppm)</th>
<th>Recovery efficiency (in percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.80</td>
<td>80</td>
</tr>
<tr>
<td>5.0</td>
<td>4.6</td>
<td>92</td>
</tr>
<tr>
<td>10.0</td>
<td>8.8</td>
<td>89</td>
</tr>
<tr>
<td>20.0</td>
<td>18.0</td>
<td>90</td>
</tr>
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</table>

*Average of two determinations
<table>
<thead>
<tr>
<th>Storage time (in days)</th>
<th>Total carbonyl** (in ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.1</td>
</tr>
<tr>
<td>7</td>
<td>3.4</td>
</tr>
<tr>
<td>14</td>
<td>3.5</td>
</tr>
<tr>
<td>21</td>
<td>9.2</td>
</tr>
<tr>
<td>28</td>
<td>6.2</td>
</tr>
</tbody>
</table>

*Samples stored at 5.5°C (10.2°C)

**Average of two determinations
room temperature (21°C to 30°C) and carbonyl determinations made after 24, 30, 48, and 72 hours. These results are presented in Table VII. Spoiled shrimp invariably showed higher carbonyl contents than their unspoiled counterparts. After 30 hours there was a trend toward decreasing carbonyl contents. This same trend can be noted in Table VI.

Several processes are operative during decomposition that could account for these observed decreases.

(a) Extensive proteolysis may create a more favorable diffusion gradient for the loss of volatiles from the system.
(b) Release of compounds capable of condensing with carbonyls may occur in the latter stages of decomposition. Basicity of the system would result in a condition catalytic for the occurrence of the condensations.
(c) Aldehydes may be oxidized to acids.
(d) Carbonyls may be used as precursors in microbial processes.
(e) Increase in pH may create an environment unfavorable to microbial activity responsible for carbonyl production.
(f) Carbonyl precursors in the tissue may be depleted.

Carbonyl generation from the precursors may be viewed as occurring through processes such as hydrolysis in which aldehydes are liberated from plasmalogens or α-ηεal phospholipids (88). Such hydrolytic reactions could readily be catalyzed by endogenous tissue enzymes or by exogenous microbial activity. It is equally feasible to consider the carbonyls as originating from microbial metabolism
### TABLE VII

**CHANGE IN CARBONYL CONTENT OF SPOILING SHRIMP**

<table>
<thead>
<tr>
<th>Spoilage time (in hours)</th>
<th>Total carbonyl** (in ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.4</td>
</tr>
<tr>
<td>24</td>
<td>8.0</td>
</tr>
<tr>
<td>30</td>
<td>17.0</td>
</tr>
<tr>
<td>48</td>
<td>15.6</td>
</tr>
<tr>
<td>72</td>
<td>11.7</td>
</tr>
</tbody>
</table>

*Temperature range of 24 to 30°C during studies
**Average of two determinations
leading to the formation of these materials as end products or as intermediates for other pathways.

Successful commercialization of gamma irradiation in the preservation of shrimp and other shellfish is directly related to the quality of the product at the time of irradiation. Bacterial load reduction is the most notable change observed in shrimp subjected to pasteurization doses. Selective action in reducing the pseudomonads, would appear to be the primary factor in increased storageability of the irradiated product. Other, less obvious but perhaps more significant, changes occur such as inactivation of tissue enzyme systems catalyzing proteolysis. Pasteurizing doses may also improve the quality of the product by catalyzing the formation of desirable flavor components. High doses may reduce bacterial counts even further but at the sacrifice of acceptability due to formation of off-odors and flavors. Novak et al. (72) have shown that shrimp exposed to doses in excess of 0.2 Mrad suffer loss in quality as revealed by organoleptic tests. To avoid the introduction of additional variables, irradiation studies in relation to carbonyl formation were restricted to the utilization of pasteurization doses.

Results of storage studies of carbonyl changes occurring in irradiated shrimp are shown in Table VIII. Analyses were also made for trimethylamine nitrogen (TMA-N), ammonia nitrogen (NH$_3$-N), indole, and microbial load to determine if any correlation existed between carbonyl concentrations and changes in the levels of these substances during storage. These results are graphically presented
<table>
<thead>
<tr>
<th>Storage time (in days)</th>
<th>Sample</th>
<th>Nitrogen (in mg per 100 g)</th>
<th>Indole (in ug per 100 g)</th>
<th>Total carbonyl (in ppm)</th>
<th>Total bacterial count (in colonies per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TMA</td>
<td>NH$_3$</td>
<td></td>
<td>20°C</td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>0.7</td>
<td>11</td>
<td>nil</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>1.0</td>
<td>11</td>
<td>nil</td>
<td>3.2</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>0.9</td>
<td>11</td>
<td>nil</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>1.0</td>
<td>15</td>
<td>nil</td>
<td>2.2</td>
</tr>
<tr>
<td>14</td>
<td>Control</td>
<td>9.1</td>
<td>37</td>
<td>nil</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>2.5</td>
<td>10</td>
<td>nil</td>
<td>2.0</td>
</tr>
<tr>
<td>21</td>
<td>Control</td>
<td>16.0</td>
<td>68</td>
<td>nil</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>1.0</td>
<td>20</td>
<td>nil</td>
<td>2.8</td>
</tr>
<tr>
<td>28</td>
<td>Control</td>
<td>22.0</td>
<td>108</td>
<td>10</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>2.1</td>
<td>18</td>
<td>nil</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*Samples stored at 5.5°C (±0.2°C)*
Shrimp employed in the experiments were of good quality. Control samples were spoiled at 21 days' storage. Irradiated samples initially exhibited a slight atypical odor which disappeared after 21 days' storage and was replaced by the more acceptable odor of fresh shrimp. They were considered to be of good quality at 28 days' storage.

Diminution of the irradiation odor during ice storage at 5.5°C is in harmony with results reported by Sinnhuber (103) of the decrease in irradiation flavor of ice-stored shrimp.

The data indicates that low dose gamma irradiation inhibits carbonyl formation in shrimp. Carbonyl values obtained for the irradiated samples remained essentially constant throughout the storage period, averaging 2.6 ppm. The slightly higher carbonyl value of the irradiated sample over that for the control at initiation of the experiment was not considered significant. Control samples displayed the general pattern of increasing after 11 days' storage and then falling off by the 28th day; however, the level was always higher than that observed for the original sample.

Low plate counts at 35°C were noted for the irradiated samples throughout the storage period. Those for control samples increased rapidly after 7 days. Counts at 20°C were higher than those at 35°C for both samples. Several-fold higher counts at the lower temperature were indicative of a high psychrophilic flora and of the temperature to which the shrimp were native. It is suggested that plate counts at 20°C could be of much greater value in evaluating incipient spoilage than those at 35°C.
Comparison of Carbonyl Content with Total Plate Counts of Gaʻema Irradiated Shrimp During Ice Storage
Figure 7

Comparison of Carbonyl Content with Trimethylamine and Ammonia Production of Gamma Irradiated Shrimp During Ice Storage
TMA-N and NH₃-N values correlated well with carbonyl values through 21 days' storage. The former continued to rise through the 28th day while carbonyl contents decreased. This data implies that carbonyl formation does not result primarily as an intermediate stage in the formation of TMA, ammonia, or indole.

TMA production parallels microbial growth in the samples and may be considered as resulting from bacterial reduction of trimethylamine oxide (TMAO) present in the tissue. This relationship has been confirmed in several studies with other fish products (91, 96). It has also been postulated to originate by enzymatically-catalyzed lactic acid reduction of TMAO (11).

Ammonia production also parallels bacterial growth. This substance may arise from many sources as the result of proteolytic activity (4, 6, 12).

Irradiation suppresses the formation of TMA and ammonia as well as carbonyls. Reduction in bacterial counts due to gamma irradiation is probably the main factor in the decreased formation of all three substances. It would appear from Figure 6 that their formation may be more dependent on the 35°C flora than that at 20°C, unless it is assumed that irradiation preferentially inactivates enzyme systems of the psychrophilic flora necessary for the generation of these substances. This assumption is supported in recent studies by Corlett (19) which showed that low dose gamma irradiation readily inactivates the aerobic psychrophilic Pseudomonas. If initial numbers are low, they may be completely eliminated in which case spoilage usually results from the more radiation resistant.
psychrotrophic Achromobacter, yeasts, and a variety of gram-positive rods.

Bacteriological studies associated with the carbonyl investigations verified these results for irradiated shrimp. Seven colonies were taken from total count plates at 20°C. These plates were from samples, irradiated and non-irradiated, which had been in ice storage for 28 days. Three of the isolates were from the non-irradiated sample and included an Aeromonas, a Pseudomonas, and a Micrococcus. Isolates from the irradiated sample included a yeast, a Flavobacterium, and two Achromobacter.

While plate counts are normally high in spoiled shrimp, low counts may not be assumed to classify shrimp as unspoiled. Conditions under which the product has been maintained must be known before the relevance of bacterial counts can be assessed. Storage of shrimp at temperatures below freezing results in reduced counts. This has also been proven in other fish species (53).

It is quite possible for spoiled shrimp with high bacterial loads prior to freezing to be classified as unspoiled after several months of frozen storage if bacterial counts alone are employed in the evaluation. Decomposition products such as indole and TMA would still be present in the tissues, and checks for these substances would reveal the inedible quality of the shrimp. High bacterial counts will sound a warning note, but they must be kept in proper perspective in relation to other analytical data which should be available.

Indole values were not measurable in any of the experiments.
until spoilage was evident as detected by odor and appearance. This test has practically no value as an indicator of incipient spoilage in shrimp but is characteristic of gross spoilage. TMA-N and NH$_3$-N values are more valuable in gauging marginal spoilage.

It appears from data collected in the course of these studies that spoilage is imminent when the level of TMA-N exceeds 15 mg per 100 g of shrimp and the NH$_3$-N level is in excess of 30 mg per 100 g of tissue.

Extractable-carbonyl contents fall into the category of TMA-N and NH$_3$-N as indices of incipient spoilage. Data obtained in these studies suggest that spoilage is a fact, or is at a threshold level, when the carbonyl content exceeds 5 ppm. Again, the carbonyl content alone should not be considered as conclusive evidence of shrimp decomposition. These values only become meaningful when considered in relation to those for TMA, ammonia, indole, and total bacterial plate counts.

Gas chromatographic analysis of the concentrates obtained in the decomposition studies revealed the presence of at least five carbonyls (Figures 8 and 9). Four of these were tentatively identified as acetaldehyde, acetone, 3-methyl-2-butanone, and diacetyl. These results are presented in Table IX. Comparative retention times for standard carbonyls used in establishing tentative identification are shown in Table X. The chromatograms indicated the presence of several other components but in such minute quantities as to preclude even tentative identification. Formation of acetaldehyde and diacetyl during shrimp decomposition is in accord with
Figure 8
Separation of Carbonyls in Shrimp Concentrate on Carbowax 20M

Figure 9
Separation of Carbonyls in Shrimp Concentrate on 1,2,3-tris-(2-cyanoethoxy) Propane
TABLE IX

RETENTION TIMES OF CARBONYLS IN SHRIMP

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Carbowax 20M (in minutes)</th>
<th>1,2,3-tris-(2-cyanoethoxy) Propane (in minutes)</th>
<th>Tentative identification of carbonyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>1.2</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>2</td>
<td>1.9</td>
<td>2.0</td>
<td>acetone</td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td>3.1</td>
<td>3-Me-2-butanone</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>4.0</td>
<td>diacetyl</td>
</tr>
<tr>
<td>5</td>
<td>6.3</td>
<td>5.5</td>
<td>???</td>
</tr>
<tr>
<td>Carbonyl</td>
<td>Retention time on: Carbowax 20M (in minutes)</td>
<td>1,2,3-tri-(2-cyanoethoxy) Propane (in minutes)</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>1.2</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>1.5</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>iso-Butyraldehyde</td>
<td>1.8</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>n-Butyraldehyde</td>
<td>2.9</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>3-Me-2-Butanone</td>
<td>3.5</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Diacetyl</td>
<td>4.9</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>2-Pentanone</td>
<td>5.4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>3-Pentanone</td>
<td>5.4</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>3-Me-2-Pentanone</td>
<td>5.7</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>l-Hexanone</td>
<td>8.1</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>2-Hexanone</td>
<td>8.6</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>2,4-Pentanedione</td>
<td>9.5</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Citronellal</td>
<td>13.2</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>
data obtained by Ota (77, 78) for other fish products.

Infrared data obtained from the concentrates validate the presence of diketo compounds such as diacetyl during shrimp decomposition.

Formaldehyde was not resolved by the chromatographic technique employed. Propionaldehyde, n-butyraldehyde, and iso-butyraldehyde—carbonyls implicated in the spoilage of other fish products—were not detected as components of shrimp decomposition.

Chromatograms of concentrates from irradiated unspoiled shrimp did not indicate the presence of acetone. Only in chromatograms of concentrates from spoiled, irradiated samples was this peak present suggesting that gamma irradiation temporarily inhibits the biochemical mechanism for its production. This peak was noted consistently on chromatograms of non-irradiated samples after 14 days' storage and was generally present after 7 days' storage. Acetone production in non-irradiated shrimp deserves further investigation as to its role in shrimp spoilage.

Variability in peak areas of the major components did not show any particular pattern in relation to storage time. It is very probable that a definitive relationship does exist, but must await improvements in the concentrating procedure before it can be determined.

Metabolic processes have been established for the production of acetaldehyde, acetone, and diacetyl in many foods, shrimp not included. 3-Methyl-2-butanone has been reported to be produced by E. coli (55). The metabolic source of this compound is unknown.
Acetaldehyde, acetone, and diacetyl are not considered toxic at the levels found in spoiling shrimp (104). Toxicological data for 3-methyl-2-butanone has not been reported in the literature.

While studies have not been conducted on the origin of these carbonyls in shrimp, plausible mechanisms which may be operative can be postulated.

(a) In general, aldehydes may originate from amino acids by a combination of decarboxylation and deamination.

\[
\begin{align*}
\text{CH}_2\text{R} \quad \text{pyridoxal phosphate} \quad \text{CH}_2\text{R} \quad \text{COOH} \\
\text{CHNH}_2 \quad + \quad \text{CH}_2\text{NH}_2 \quad (0) \quad \text{CHO} \\
\text{COOH} \quad + \quad \text{CO}_2 \quad + \quad \text{NH}_3
\end{align*}
\]

Free amino acid contents increase as decomposition progresses due to autolytic and bacterial proteolytic activity. Decarboxylation and deamination of these compounds would account for the initial increases in carbonyl and ammonia concentrations. This mechanism undoubtedly functions during shrimp spoilage.

(b) In general, ketones may be formed by the oxidation of unsaturated fatty acids according to the following reaction:

\[
\begin{align*}
\text{R'} \quad \text{R'=COOH} \\
\text{R-C=CH-R''-COOH} \quad (0) \quad \text{R-C=O} \quad + \quad \text{R''} \\
\text{COOH}
\end{align*}
\]

Literature references are nonexistent for the presence of branched-chain unsaturated fatty acids in shrimp tissue. These precursors could be formed from the metabolism of spoilage bacteria.

(c) Acetaldehyde can be produced in plants and certain
microbiological systems by the decarboxylation of pyruvic acid (9).

\[
\begin{align*}
&\text{COOH} \\
&\text{CHO} + \text{CO}_2
\end{align*}
\]

This necessitates the presence of the enzyme system pyruvic decarboxylase. It is not known whether shrimp tissue contains this enzyme system.

Acetaldehyde has also been reported to be produced by the scission of threonine through a dismutation type of reaction (52).

\[
\begin{align*}
&\text{COOH} \\
&\text{CHO} + \text{COOH}
\end{align*}
\]

Threonine levels increase in shrimp spoilage as do glycine levels, both as the result of proteolysis. This does not preclude the possibility of acetaldehyde arising in part via this mechanism.

Diacetyl can be cleaved by some pyruvate enzymes to yield acetaldehyde plus acetate (22).

\[
\begin{align*}
&\text{COOH} \\
&\text{COOH}
\end{align*}
\]

Since diacetyl has been tentatively shown to be present in
spoiled shrimp, it must be considered as a possible source of this carbonyl.

(d) Acetone can arise metabolically from acetoacetic acid by beta keto acid decarboxylation.

\[
\begin{align*}
\text{COOH} & \quad \text{C=O} & \quad \text{CO}_2 \\
\text{CH}_2 & \quad \text{CH}_3 \\
\text{C=O} & \quad \text{CH}_3 \\
\text{CH}_3
\end{align*}
\]

This reaction is catalyzed by the enzyme system acetoacetic decarboxylase. It is not known whether shrimp tissue contains this enzyme system. 

Clostridium acetobutylicum and Cl. butyricum contain this enzyme system but have not been implicated as a major group contributing to the normal decomposition of shrimp (78). Other groups of shrimp spoilage organisms could possess this system. Microbiological and/or autolytic activity involving this mechanism could readily account for the production of acetone.

(e) Diacetyl is produced in certain microorganisms, e.g., Acetobacter, by the oxidation of acetoin.

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{CHOH} & \quad \text{C=O} & \quad \text{H}_2\text{O} \\
\text{C=O} & \quad \text{C=O} \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]

Green (146) found that 49 percent of freshly caught shrimp contained Aerobacter spp. and 35 percent contained Acetobacter spp. The former are noted for their ability to produce
acetoin and the latter for their ability to oxidize the acetoin to diacetyl. It is highly probable that diacetyl production in shrimp occurs through this microbial mediated process.

(f) 3-Methyl-2-butanone could originate from the general mechanism for ketone production in the following manner:

 Unsaturated fatty acids are found in almost all fish products, including shrimp. Oxidation of these substances is a normal spoilage phenomenon. It is not known whether any branched-chain unsaturated acids similar to the structure above are present in shrimp. In view of the apparently rare occurrence of this compound as a metabolite in food products, further investigation is warranted to substantiate its presence in shrimp tissue.

Results of the investigation herein reported demonstrate the feasibility of quantitatively removing carbonyl compounds from shrimp. It was further established that carbonyl metabolism can be used as an index of shrimp decomposition when correlated with other objective and subjective measurements. Isolation of specific carbonyls produced during the decomposition process may prove to be the eventual key to unravelling the complex nature of the spoilage mechanism in not only this product but in all foods.
SUMMARY

Establishment of reasonable quality standards for irradiated and non-irradiated shrimp is imperative for the protection of both the general public and the shrimp industry. Objective tests must be developed which can detect incipient spoilage in both products. In the case of the irradiated product, acceptance by the Food and Drug Administration of recommended standards is contingent on demonstrating that toxic substances are not induced as a result of the irradiation treatment.

Implication of carbonyl compounds in the spoilage processes of other foods warranted an evaluation of their role in shrimp decomposition. Ascertaining the metabolic origin and fate of these compounds during transition from a fresh to a spoiled condition would be of immense importance in elucidating the biochemical mechanisms underlying shrimp decomposition.

Methodology was developed for the semiquantitative removal of extractable carbonyls from an alcoholic homogenate of shrimp using a batch-type, countercurrent, liquid-liquid extraction process. Isolation and concentration of the carbonyls was accomplished by fractional distillation and column chromatographic techniques.

Studies revealed that carbonyl contents of non-irradiated ice-stored shrimp increase with storage time, reaching a peak after 21 days' storage and then falling off. Carbonyl contents of fresh
shrimp were very low, averaging 3.1 ppm.

Carbonyl values for gamma irradiated (0.2 Mrad) shrimp remained essentially constant during a 28 day ice storage period, averaging 2.6 ppm. Spoiled irradiated shrimp exhibited increased carbonyl concentrations above those for unspoiled controls. There was no indication that gamma irradiation produced any carbonyl not present in the non-irradiated control.

Carbonyl contents correlated well with total bacterial plate counts at 20°C and 35°C and with trimethylamine nitrogen and ammonia nitrogen values through 21 days' storage for both non-irradiated and irradiated samples. It was evident from the data that carbonyls do not arise primarily as a result of trimethylamine and ammonia production. Total plate counts at 20°C were considered more significant in assessing quality than those at 35°C.

Gas chromatographic analysis of the concentrates showed the presence of at least five carbonyls. Four of these were tentatively identified as acetaldehyde, acetone, diacetyl, and 3-methyl-2-butanone. Plausible biochemical mechanisms for their origin are presented.

A valid evaluation of shrimp quality can be made only after consideration of all physical, chemical, microbiological, nutritional, and sensory measurements. Data obtained in these studies suggest that incipient spoilage is present when the following values are exceeded: extractable carbonyl—5 ppm; trimethylamine nitrogen—15 mg per 100 g of shrimp; ammonia nitrogen—30 mg per 100 g of shrimp; and total bacterial plate counts—50 million per gram.
at 20°C or 5 million per gram at 35°C.
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VITA

Patrick Delano Settoon was born February 15, 1934, in Amite, Louisiana. He received his elementary education in Ella Strickland Grammar School of Amite and was graduated from Amite High School in May of 1952.

The following September he entered Louisiana State University where he remained until January of 1954. He transferred to South-eastern Louisiana College where he served as an undergraduate laboratory teaching assistant from 1955 to 1957. During the summer of 1956, he was employed as a student trainee by Dow Chemical Company in Freeport, Texas. In May of 1957 he was graduated with a Bachelor of Science degree in chemistry.

In July of the same year, he was employed by Union Carbide Corporation as a chemist in their Special Problems Laboratory located at Seadrift, Texas. In September of 1958, he returned to Southeastern Louisiana College as an instructor of chemistry in charge of introductory chemistry laboratories and stockroom facilities.

In June of 1959, he enrolled in the graduate school at Louisiana State University. He served two years as a graduate research assistant in the Department of Agricultural Chemistry and Biochemistry, and in January of 1961, he received a Master of Science degree in biochemistry.
In September of 1961, he returned to his teaching duties at Southeastern. In June of 1963 he entered the Department of Food Science and Technology. In December of 1965, he was awarded a Science Faculty Fellowship by the National Science Foundation in order that he might complete requirements for the doctoral degree.

He was married to the former Barbara Ann Beaty of Austwell, Texas in September of 1956. He and Mrs. Settoon have been blessed with two sons, Patrick Richard and Randall Wayne, and a daughter, Terry Ethelyn, who is deceased.

He is presently on leave from Southeastern Louisiana College where he holds the rank of Associate Professor of Chemistry. He is a candidate for the degree of Doctor of Philosophy in food science and technology.
EXAMINATION AND THESIS REPORT

Candidate: Patrick Delano Setton

Major Field: Food Science and Technology

Title of Thesis: Carbonyl Metabolism as an Index of Shrimp Decomposition

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

July 10, 1967