BROWNING REACTION IN DEHYDRATED
SHORT-FINNED SQUID (ILLEX ILLECEBROSUS)

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BROWNING REACTION IN DEHYDRATED
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Illex illecebrosus

BY

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Abstract

The market value of Atlantic short-finned squid, *Illex illecebrosus* for certain importing countries is adversely affected by its greater propensity to undergo browning reaction than other available genera and species of squid. Browning reaction adversely affects the quality of certain dried and shredded squid. Canadian squid exports are, therefore, not competitive with exports of other genera and species of squid. The present study provides evidence supporting the hypothesis that a Maillard type reaction is responsible for the browning reaction in dried squid. This evidence includes the finding of the following precursors of Maillard browning in squid mantle: glucose, ribose, glucose-6-P, ribose-5-P and free amino acids, notably proline and taurine.

ACKNOWLEDGEMENT

Table of Contents

List of Tables

List of Figures

List of Abbreviations

DWB	Dried Weight Basis
F-6-P	Fructose-6-Phosphate
FWB	Fresh Weight Basis
G-1-P	Glucose-1-Phosphate
G-6-P	Glucose-6-Phosphate
HPLC	High Performance Liquid Chromatography
Mass.	Massachusetts
MS	Massachusetts squid
Nfld	Newfoundland
NS	Newfoundland squid
PCA	Perchloric acid
μg	microgram
μl	microliter
U. V.	Ultra Violet
R. I.	Refractive Index
R-5-P	Ribose-5-Phosphate

Chapter 1

INTRODUCTION

Canada is a country endowed by the Benevolent Power with rich and bountiful natural resources. Its natural resources are so rich and bountiful that, some, especially the marine resources, are underutilized. One example of an underutilized resource is squid.

The short-finned squid, *Illex illecebrosus* (Leseur), is a cephalopod, an exclusively marine group of mollusks. There are about 350 species of squid that inhabit the oceans and seas of the world (Ampola, 1975). Since a long time ago this "head-footed" animal fascinated the imagination of man. Before man contrived jet propulsion, the cephalopods had been jetting in primeval seas, and were masters in using smokescreens as camouflage, before man's armies thought of them. They used "ink" (a brown or black viscous fluid) which is ejected as effective screen for escaping from their enemies.

For centuries, squid has been considered as an important and palatable source of food for countries bordering the Mediterranean Sea and the Orient (Ampola, 1975). Their relative abundance and long standing popularity as as gourmet delight make them very appealing especially among Orientals. Also,

Spanish and Latin American people like squid. Ommastrephid squid, *Illex illecebrosus* does not appeal to the majority of Canadian local consumers, but for foreign consumers especially from Asian countries, it is considered as a delicacy. It could be exported in bulk due to its abundance, thereby generating income which would increase the dollar reserves of the country.

Experts estimated that the annual potential production of oceanic squids probably lies in the range of 90 to 280 million metric tons. (Ampola, 1975). Table 1-1 shows Newfoundland squid catch and landings in metric tons from 1978-1983. Squid exports from Canada have increased in the late 1970's due to increased catch and improved foreign market demands. Table 1-2 shows Canadian frozen squid exports from 1978-1983. Various problems have been encountered with Canadian squid by importing countries (Haard, 1982). One of these problems is excessive browning of dried squid and products prepared from dried squid. Other problems encountered with Atlantic short-finned squid by foreign importers include the texture and shredability of dried products and the bland flavor of the frozen product (Haard, 1983). *Illex illecebrosus* does command a relatively low price compared to other sources of squid in foreign markets. Canadian squid exports are, therefore, not competitive with exports of most other genera and species of squid, due to the problems associated with the processing of *Illex illecebrosus*. This present study was undertaken to determine the nature of browning reaction in dried squid by examining the reaction mechanism. It is hoped that the fundamental information will eventually provide a basis for technologists to develop means of controlling browning reaction in squid products.

Table 1-1: Squid Landing and Value, Newfoundland
1978-1983

Year	Landings (Metric tons)	Value ($)
1978	44,058	8,103,690
1979	86,069	19,328,652
1980	33,978	3,283,952
1981	17,464	2,504,012
1982	11,160	2,102,667
1983	5	787

From: Fisheries Statistics & Systems Branch
Department of Fisheries & Oceans
St. John's, Newfoundland, Canada
1978-1973

Table 1-2: Canadian Exports of Frozen Squid Product

Year	Type of Product	Unit (Metric Ton)	Value $
1978	Squid, whole, round and tubes	7,200	8,010.00
1979	Squid, whole, round and tubes	9,808	9,407.00
1980	Squid, whole, round and tubes	65,981	62,531.00
1981	Squid, whole, round and tubes	5,047	4,855.00
1982	Squid, whole, round and tubes	742	1,130.00
1983	Squid, whole, round and tubes	1,606	2,224.00
Total		90,384	88,157.00

From: Trade of Canada, Exports By Commodities.
   Department of Fisheries and Oceans
   St. John's, Newfoundland Canada
   1978-1983

1.1. Types of Browning Reaction

The interaction of reducing sugars and amino acid residues of proteins to produce insoluble substances having brown discoloration is referred to as Maillard browning reaction. Generally, there are four mechanisms of browning reactions as shown in Table 1-3 (Eskin *et al.*, 1971).

Table 1-3: Mechanism of Browning Reaction[1]

Mechanism	Requires Oxygen	Requires Amino Group	pH Optimum
Maillard Browning	-	+	Alkaline
Caramelization	-		Slightly acid
Ascorbic Acid Oxidation	+		Slightly acid
Phenolase	+		Slightly acid

[1] From Eskin *et al.*, 1971.
(+) indicates requirement.
(-) indicates no absolute requirement.

Browning reactions are also classified as enzymic and nonenzymic browning.

1.1.1. Enzymic browning

Enzymic browning usually occurs in fruits and vegetables like potatoes, apples and bananas, especially when they are exposed to abnormal conditions when there is a cut, when it is bruised and peeled or a disease is attacking it. When fruits and vegetables are cut or bruised, the injured tissue browns easily due to the conversion of dihydroxyl phenolic compounds to brown melanins. Enzymic browning also occurs in certain marine food products notably in shrimp, crab and lobster.

Polyphenol oxidase or phenolase (0-diphenol-oxygen oxidoreductase, EC 1.10.3.1) is the enzyme generally responsible for this type of browning reaction. The reaction is catalyzed by the copper prosthetic group of this enzyme in the presence of molecular oxygen. Bendall and Gregory (1963) believe that copper is monovalent in the case of mushroom phenolase and divalent in the case of the potato enzyme. Phenolase is classified as an oxidoreductase, and the function of oxygen is to act as hydrogen acceptor. This enzyme occurs in fungi other than mushroom and in some animal tissues.

1.1.1.1. Mechanism of Enzymic Browning

The greatest amount of research on phenolase has been undertaken on the mushroom enzyme (Smith and Krueger, 1962; Bouchilloux et al. 1963; Jolley and Mason, 1965), closely followed by work on the potato enzyme (Patil and Zucker, 1965). Later work involves an understanding of isoenzymes of phenolase and of the intracellular location of the enzyme with respect to particulate and supernatant forms (Craft, 1966; Constantinides and Bedford, 1967). The

phenolase complex can give rise to two types of reaction: the phenol hydroxylase or cresolase activity and the polyphenol oxidase or catecholase activity. This is illustrated by the oxidation of L-tyrosine which is the most abundant phenolic compound in potato tubers (Schwimmer and Burr, 1967) and the concentration of which appears to be the limiting factor determining the rate of enzymic browning (Mapson et al., 1963).

Thus for reaction (i) the substrate is a monophenol, and for reaction (ii) the substrate is a diphenol. Reaction (ii) is followed by removal of hydrogen to form a red compound dopachrome (5,6-quinone indole-2 carboxylic acid) which contains a heterocyclic ring derived from the closure of the amino-carboxylic acid side chain. Dopachrome subsequently undergoes polymerization to form products called melanins.

Catechol, being an o-diphenol, is readily attacked by phenolase, and only the catecholase type of reaction is exhibited.

Quinone formation from catechol is enzyme and oxygen dependent. Once this has taken place, the subsequent polymerization reactions occur spontaneously and no longer depend upon the presence of phenolase or oxygen. Joslyn and Ponting (1951), in a review article, have summarized these chemical reactions as far as were then known. This appears to be the most widely held theory to account for the formation of the brown melanins.

The first reaction is thought to be a secondary hydroxylation of the o-quinone or of excess o-diphenol.

The resultant trihydroxybenzene compound interacts with o-quinone to form hydroxyquinones.

Hydroxyquinones undergo polymerization and are progressively converted to red and red-brown polymers, and finally to the brown melanins which appear, for example, at a point of mechanical injury to a potato tuber, or at the cut surface of an apple (Eskin *et al.*, 1971).

1.1.2. Involvement of Lipid in Browning Reactions

Nonenzymic browning involving amino groups may include two types: sugar-amino or Maillard reaction and lipid-amino reaction (Jones, 1962). There are three main types of browning reactions in lipid-amino reaction.

1. Oxidation of unsaturated oils to form active carbonyl compounds.

2. Oxypolymerization of carbonyl compounds to form brown pigments.

3. Reaction between carbonyl groups and basic nitrogeneous constituents to form brown pigments.

During the course of oxidation of the fatty acid components (reaction 1) compounds are produced which are capable of interacting with proteins or amino groups. These fatty acid components are aldehydes, peroxides and ketohydroxyl and epoxy groups.

Accordingly, antioxidants and pro-oxidants such as copper ions, haemoglobin as well as oxygen can indirectly affect the rate of browning. Inhibition of browning occurs when the carbonyl-amino reaction is blocked by acetylating the amino groups or sulfiting the carbonyls. Reactions 2 and 3

.r tlined by Jones (1962) are competitive processes. Treatment which block carbonyl-amino browning favored oxypolymerization, which can result in a darker color than carbonyl-amino browning. On the other hand, antioxidant treatment favors carbonyl-amino browning. For different reasons, less discoloration is also obtained when pro-oxidant is present. The presence of pro-oxidants appears to favor reaction 1 at the expense of reaction 2.

1.1.3. Caramelization

When sugars are heated above their melting point they darken to brown coloration. This process, called caramelization, is favored by acidic or alkaline conditions and results in changes in flavor. If the process is not carefully controlled, this reaction can lead to the production of burnt, unpleasant and bitter products. It is, therefore, very important to control this reaction in order to retain the pleasant taste and quality of caramel especially in the production of sweets and candies.

The chemical composition is very complex and little understood. Caramels produced from different sugar sources show similarity in composition. Heyns and Klier (1968) carried out a series of studies on groups of different mono-, di-, and polysaccharides and found that the volatile products were produced. The authors concluded that a common pathway is involved for the acidic and alkaline degradation of sugars.

1.1.4. Ascorbic Acid Browning

Ascorbic acid is an important precursor of browning reactions in certain food products. The reactions of ascorbic acid in fruit juices and concentrates are very much dependent on pH and concentration of the juice. The browning process is inversely proportional to the pH over a range of 2.0-3.5 (Braverman, 1963). Those juices having higher pH tend to be less susceptible to browning, like orange juice at a pH of 3.4. Ascorbic acid browning in citrus products, does not appear to involve amino acids to any great extent. However, in the case of dehydrated vegetables where ascorbic acid is involved, it appears that additional reactions contribute to discoloration.

1.1.4.1. Mechanism of Ascorbic Acid Browning

The mechanism of ascorbate browning is complex, although a possible pathway from the decomposition of ascorbic acid to furfural accompanied by liberation of carbon dioxide is outlined by Eskin et al. (1971).

Lalikainen et al. (1958) observed that the amount of carbon dioxide evolved under aerobic condition was far greater than could be accounted for by the quantity of ascorbic acid present. Even under anaerobic conditions the amount was still much higher than expected from the pigment formation. These results indicated that ascorbic acid was not the sole source of carbon dioxide, and indicated the participation of other constituents.

Ranganna and Setty (1968) implicated the Strecker degradation in an ascorbic acid-amino acid interaction in dehydrated cabbage. The reaction occurs

at low moisture content and the browning observed during the reaction in a simple model system was similar to the browning of the dehydrated cabbage. The authors postulated that the formation of dehydroascorbic and diketogulonic acids from ascorbic acid occurred during the end of the drying process and the carbonyl thus generated interacts with free amino acids, nonenzymically, to produce a red-to-brown discoloration. The pH of cabbage of 5.2 is optimum for this reaction. The reaction did not proceed at a pH below 3.5.

1.1.5. Protein-Lipid Interaction

Products of lipid oxidation can interact with various food constituents including proteins (Kwon et al., 1965). Investigations of Buttkus (1967) demonstrated the interaction of myosin, with malonaldehyde and measured the extent of the interaction of the number of free amino groups in the protein molecule. Observations on the reaction were made at different temperatures: +20°C, -20°C, and 0°C. The results indicated that at +20°C almost 60% of the free amino group of lysine were rendered unavailable after 4 days, 40% having interacted after 8 h. The reaction rate was lower at 0°C than at 20°C. However, at -20°C the reaction proceeded at approximately the same rate as that observed at +20°C. The molecules in the reaction mixture are concentrated due to the freezing of pure ice crystals (Grant et al., 1966). Further work by Buttkus (1967) demonstrated that storing a mixture of malonaldehyde and myosin at -20°C for 6 days resulted in the participation of other amino acid residues in myosin with the aldehyde. The order of reactivity was myosin residues with aldehyde and methionine followed by lysine then tyrosine and last was arginine.

Further studies were carried out on lysine availability in herring meals. Lea *et al.* (1960) reported a 12% loss of lysine when oxidized fish meal was processed at 100°C for 30 h and this was accompanied by an increase in bound lipids. However, no losses were reported when fresh herring meal were similarly processed in the presence of nitrogen gas. Lea *et al.* (1958) carried out an earlier study and found out that the availability of lysine was reduced by 4% in herring meal when stored in air at 25°C for several months, whereas no losses were incurred in lysine availability when stored at similar condition under nitrogen. Based on the results, it indicated that when lipid oxidation is facilitated, interactions of amino acid and oxidized products of lipid results in the loss of amino acid availability and browning reactions.

1.1.6. Maillard Browning Reaction

1.1.6.1. Background

A series of papers published by L.C. Maillard in 1912 and 1917 outlined the reactions which can occur between sugars and amino acids. These studies provide the foundation in food technology for our understanding of the most prevalent nonenzymatic browning phenomena (Adrian, 1982). Other early investigators also wrote about the existence of such a reaction, but Maillard was the first to systematically study the causes, factors and manifestations of the browning reaction. It was Maillard who foresaw the importance of browning reaction in the food-industry and related fields, and up to now his name is related with this kind of reaction.

Food technology and nutritional sciences were in their early stage of

development during the time of Maillard's studies. Probably, this explains why Maillard's papers were ignored by many for several decades. For example, in 1912 thiamine had been known for one year only; while methionine and threonine were not yet identified. It was only after World War II that the significance of Maillard's work to the quality of processed foods was fully recognized. It was proven that almost all foodstuffs were more or less affected by the Maillard reaction. Products of sugar and milk industries suffer considerably from the effect of the reaction due to the fact that the commercial value of the product is lowered by browning. Sugar and milk products must have a white color in order to have the maximum value. On the other hand, there are food products which rely directly or indirectly on the resulting brownings, especially the roasting phase. These foodstuffs include biscuit, breads, cookies, malt beer, chocolate, peanuts, etc.

From the nutritional point of view, Maillard reaction damages the foodstuffs because of the destruction of certain amino acids during heating and prolonged storage. On the other hand food technologists study the manifestations of the reaction of sugars and amino acids on consumer's acceptability of foods, while the nutritionists and physicians were concerned about the physiological consequences of the Maillard reaction. The reaction also captivated the attention of chemists who tried to define the mechanism and factors that control the reaction.

According to workers like Eskin *et al.* (1971), browning reactions in foods are important in view of their effects on the alteration of appearance. The

proximate lowering of the nutritive value of the food material with browning is another significant problem.

There are many articles and reviews relating the Maillard reaction to the handling and storage of foods, in particular dehydrated foods (Reynolds, 1969; Gottschalk, 1952). The Maillard reaction usually causes undesirable effects, such as large changes in solubility and color. However, some of the side reactions are responsible for the production of desirable as well as undesirable flavors and odors (Hodge, 1953).

1.1.6.2. Mechanism of Maillard Browning

The mechanism of Maillard browning appears to follow a common pathway for many foods in which this reaction is found to occur (Hodge, 1953; Ellis, 1959; Reynolds, 1965). The primary step involves a condensation reaction between the C-amino groups of amino acids or proteins and the carbonyl groups of reducing sugars, known as the "carbonylamino" reaction. The initial condensation product is a Schiff's base which undergoes cyclization to the corresponding N-substituted glycosylamine, the latter compound being in equilibrium in aqueous solution. The existence of the condensation product was demonstrated by Partridge and Brimley (1952) using ion exchange chromatography.

1.2. Factors Influencing Maillard Reaction

1.2.1. Presence of Sugars in Fish and Shellfish Muscle

Several workers like Hochachka et al. (1975) established that carbohydrate is the storage form of energy in the squid mantle muscle, although under some circumstances it is clear that proline and perhaps other amino acids can be utilized for catabolic purposes.

In addition to free sugars, a variety of sugar phosphates can be found in the muscle of fish and shellfish. Tarr (1949) indicated the presence of 2-61 mg% of fructose -1,6-diphosphate, phosphoglyceric acid, glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and pentose phosphate in muscle from several species of fish. Yamanaka et al. (1974) detected tenths of milligrams of G6P and F6P in the fresh muscle of skipjack. The analysis for G1P, G6P and F6P was also performed in hard clam and squid (Tsuchiya, 1962). Inositol, a sugar alcohol, is also known to be distributed widely in the muscle of fish and mollusk (Tsuchiya, 1962) and 5-10 mg% of inositol was also detected in crab muscle (Hayashi et al., 1979).

1.2.2. Amino-Sugar Browning With Model System

In Maillard browning, the most significant reaction involved is amino acid interacting with sugars. Kamata and Sakurai (1957) used 1% of either glycine, tryptophan, arginine or glutamic acid to react with 1% of either glucose, sucrose or xylose in distilled water at 30°C for 30 days. There was no browning. Addition of 10%-20% NaCl to the above system did not enhance the color development. The browning took place only when the concentration of amino acids and sugars

was increased to 5-10%. When 10% of glycine was heated with 10% of different sugars, they found that xylose gave the maximum browning intensity followed by arabinose, fructose, glucose, maltose and sucrose, respectively. Likewise, alanine, arginine, histidine, leucine, serine, cystine and sodium glutamate gave a high browning intensity with xylose followed by lysine and threonine. Kamata and Sakurai (1959) also found that glucose promoted the amino acid-pentose reaction whereas sucrose and maltose did not.

Nagayama (1962) compared the intensity of the browning reaction with glucose or ribose and various amino acids, in phosphate buffer, pH 6.8 in heated and sealed tubes at 115°C for 1 h. He observed that among the four amino acids tested (arginine, lysine, histidine and glycine), lysine showed the greatest browning intensity with either sugars followed by glycine, histidine and arginine. The degree of brown discoloration in the glucose-lysine system in phosphate buffer at pH 6.8 was proportional to the concentration of lysine. An increase in phosphate concentration was found to increase the browning intensity of the glucose-lysine system.

1.2.3. Effects of Temperature, pH, Buffers and Moisture Content

1.2.3.1. Temperature

It has been found that temperature affects Maillard browning reaction. Lea and Hannan (1949) reported a decrease in the free amino nitrogen for a casein glucose system which conformed to the Arrhenius equation over a temperature range of 0-9°C, where a linear relationship existed between the rate of reaction and temperature over this range.

1.2.3.2. pH

This carbonylamino reaction may occur in either acidic or alkaline media, but generally, alkaline conditions favor the process. Lea and Hannan (1949) and Underwood et al. (1959) demonstrated an increase in reaction rate with a rise in pH. They discovered that foods of high acidity are less susceptible to the reaction.

1.2.3.3. Buffer

Investigations carried out by Saunders and Jervis (1966) using sodium phosphate and sodium citrate with glucose-glycine indicated that buffering capacity plays an important role in these reactions. It appears that acidic products formed during the course of the reaction are buffered by these salts, and this allows the reaction to proceed. Acidic conditions are unfavorable for the browning process.

1.2.3.4. Moisture content

Maillard browning process proceeds in aqueous systems and complete dehydration of the reactants results in a rapid halt in the process. Lea and Hannan (1950) recorded the optimum moisture content level for a casein-glucose system and observed the loss in free amino groups to be greatest at a moisture content of 13% which corresponds to a level at which the reactants are still in the presence of free water. It appears that the reaction is favored at low moisture content due to the concentration of solute molecules which are involved in the reaction.

1.3. Chemical Composition of Squid Muscle as it Relates to Precursors of Browning

1.3.1. Carbohydrates

Dark-fleshed fish generally contain much more glycogen (about 1% in the muscle) than white-fleshed ones. A large amount of glycogen (1-8%) is also deposited in the body of mollusks (Ikeda, 1980). Several workers like Hochachka *et al.* (1975) established that carbohydrate is the storage form of energy in the squid mantle muscle, although under some circumstances it is clear that proline and perhaps other amino acid can be utilized for catabolic purposes.

As so elegantly pointed out by Goudsmit (1972) unusual polysaccharides are not uncommon in mollusk tissues and special care must be taken for their isolation and identification. The unique properties of squid mantle polysaccharides explain why they have often been missed by previous researchers and may explain why they are difficult to visualize in electron micrographs, even if, under favorable conditions, glycogen-like granules are readily observed.

1.3.2. Free Amino Acids

Mollusks normally lie between fishes and crustaceans with regard to free amino acid content. Generally mollusks are rich in taurine, proline, glycine, alanine, and arginine, but their levels fluctuate considerably from species to species which is in contrast to those of crustaceans. According to Konosu and Yamaguchi (1982) the content of glycine varied from 14-55 mg/100 g of raw muscle in scallops and was 10 mg/100 g in certain squid.

1.3.3. Lipids

Fatty acids occur in all fish partly as triglycerides and partly as phospholipids. Other classes of lipids such as glyceryl esters or wax esters also occur in a few species. Some other substances that may be associated with fish oils include hydrocarbons, sterols, vitamins and pigments. Gordon (1982) stated that as other compounds closely related to cholesterols were discovered, they were grouped under the term sterol. When dealing with mollusks, the term seems more appropriate, and no matter what term is used, mollusks have a diversity of sterols. These lipid components are not found in other foods.

Different studies have been made relating lipid oxidation to the quality of seafood. Oxidation is most important, particularly in the deterioration of frozen fish products causing flavor (Banks, 1939), color (Jones, 1962) and possibly textural changes (Sikorski et al., 1976). One certain effect of the oxidation of lipid in lean muscle of fish like cod is the introduction of odors, often called cold-storage flavours. The compounds most responsible for this are unsaturated carbonyls (McGill et al., 1977). Studies on model systems containing phospholipids and proteins show that oxidized phospholipid will react with protein (Labuza, 1971). Protein denaturation may result from lipid oxidation by co-oxidation of the sulfydryl groups or by carbonyl cross-linking of free amino groups (Hardy, 1979).

1.3.4. Homarine

Betaine compounds are minor components in muscle extract of fish, but are major compounds in extracts of crustaceans and mollusks. Homarine (N-methyl picolinic acid betaine) was first isolated by Hoppe-Seyler from the muscle of lobster (Hirano, 1975). Later, the homarine content of different marine invertebrates was determined by different authors (Hayashi *et al.*, 1978; Beers, 1967; Gasteiger *et al.*, 1960; Hirano, 1975).

1.3.4.1. Homarine Participation in Browning Reaction

Hirano, (1975) further elucidated the relationship between browning and homarine degradation of irradiated squid. It was found out that by addition of homarine, browning reaction remarkably increased with the increase of temperature to $80^{\circ}C$. Based upon the results of the model system of glucose-glycine, it was concluded that the radiation degradation and the resulting yellowish coloration of homarine may contribute to the browning of irradiated squid. Also the study indicated that irradiated homarine accelerates Maillard browning of glucose-glycine in the model system.

1.4. Browning in Marine Products

Browning occurs in different marine products, for example the browning of canned skipjack meat. The mechanism of browning of processed marine products depends upon the raw materials used, the process employed and storage. Yamanaka *et al.* (1975) found that glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) are mainly responsible for the orange discoloration of canned skipjack meat. This was further supported by Hasegawa *et al.* (1976) who stated

that when the sugar phosphates accumulated via glycolysis, the Maillard-type of browning reaction took place during canning at a high temperature. Yamanaka (1975) elucidated the cause of accumulation of glucose-6-phosphate and fructose-6-phosphate as follows: In the meat which gave a strong orange colour on cooking the ATP concentration decreased rapidly during thawing and disappeared completely after thawing. In normal meat, however, the ATP concentration decreased slowly. In the former, meat glycogen decreased extensively, but lactic acid increased only slightly with a small fall of pH. The NAD content was 60-80 mg/100g in normal meat, while it was 1-2 mg/100 g in the meat liable to orange discoloration. Anserine, creatine and histidine were the main amine components participating in this amino-sugar reaction (Ikeda, 1980).

Another case of browning in marine products is the one wherein browning is caused by oxidation of lipid. Various studies were made on the mechanism of the discoloration, or so called rusting, caused by the oxidation of lipids of marine products. Ikeda (1980) stated that this mechanism is so complicated that there has been little agreement on it. However, it has been widely accepted that carbonyl compounds from oxidized lipids are involved in the reaction (Matsuto et al., 1967). Rusting may be regarded as a type of browning induced by amino-carbonyl reactions. Little has been done on this type of work and no further elucidation has been made on how these nitrogeneous compounds participated in the development of rusting. Fugimoto and Kaneda (1973), however, presumed that aldol condensation, in the presence of volatile bases as catalysts, might serve as the early stage of the mechanism. On the other hand, Nakamura et al. (1973)

reported that the non-volatile basic nitrogeneous compounds made a greater contribution to the browning than the volatile ones.

1.5. Purpose of the Study

Squid harvested in Canada *Illex illecebrosus* have a greater tendency to undergo browning in dried products than other sources of squid (Haard, 1982). *Illex* is sometimes rated lower than other sources of squid in the Japanese market for a variety of reasons (Haard, 1983). For example, it is said to have less flavor for direct consumption; it is less desirable for "shiokora" manufacture because of its higher pigment content and texture; and it is less desirable for dried seasoned products because of poor shredability and its tendency to undergo browning reactions during storage and because of poor extensibility for rolled products.

In view of the relatively low market value of Canadian squid the prime purpose of the study was to find out what causes browning reaction in dried squid *Illex illecebrosus*. It was hypothesized that sugar and amino acids are precursors of Maillard browning reaction in squid products.

The squid muscle content of various chemical constituents like sugars, phosphorylated sugars, amino acids and homarine in squid was investigated because they are known to be potential precursors or catalysts of browning reactions, (Hirano,1975; Nagayama,1961; Tarr,1966; and Yamanaka, *et al.* 1974 The browning of *Illex illecebrosus* caught in Newfoundland waters was evaluated by comparison of results with those obtained with *Illex illecebrosus* captured off the coast of Massachussetts. The latter squid were shown to exhibit somewhat

less browning on dehydration than the same species caught off the coast of Newfoundland.

Chapter 2

MATERIALS AND METHODS

2.1. Biological Specimen

Squid, *Illex illecebrosus* (Lesueur), belongs to:
Phylum: Mollusca

Class : Cephalopoda

Order : Decapoda

Family: Ommastrephidae

Two sources of *Illex illecebrosus* were used in the study. The two sources were captured in Massachusetts and Newfoundland waters. Fresh frozen squid samples were obtained from Massachusettes in Nov., 1983 and stored at -70°C. For Newfoundland squid, fresh frozen sample were obtained from Conception Bay in 1982 and stored at -70°C prior to analyses. Both sources of squid were rapidly frozen in liquid nitrogen prior to the onset of rigor mortis.

Prior to freezing, they were cleaned with all the heads and entrails removed.

Frozen squid were employed because fresh squid was not readily available in Newfoundland during the 1983-84 fishing seasons.

2.2. Chemicals

Free sugar standards for HPLC was purchased from Supelco, Inc., Pennsylvania. The mixture consisted of fructose, glucose, ribose, sucrose, maltose and lactose, each at a concentration of 10 mg/ml.

Individual sugar standards were also purchased from Sigma Chemical Company, St Louis, MO., and included fructose, glucose, ribose, sucrose, maltose and lactose. Glucose-6-phosphate, ribose-5-phosphate and fructose-6-phosphate. The following chemicals were also obtained from Sigma Chemical Co.: dicyclohexylamine, orcinol reagent, perchloric acid (PCA), potassium hydroxide (KOH), copper reagent, arseno-molybdate reagent, molybdate solution and phosphoric acid.

Phosphorylated sugars standards were also purchased from Boehringer-Mannheim, Ontario, Canada. They were: glucose-6-phosphate, ribose-5-phosphate, fructose-6-phosphate, glucose-1-phosphate and ribose-1-phosphate. L-ascorbic acid test combination for the determination of L-ascorbic acid in foodstuffs, glucose/fructose UV method test combination and sucrose/glucose UV method test combination and hydrochloric acid were also purchased from Boehringer-Mannheim, Ontario, Canada.

HPLC grade water, acetonitrile and iso-propanol were purchased from Fisher Scientific Limited, Halifax. Sulfosalicylic acid was purchased from L.T. Baker Chemical Co., Phillipsburg N.J.

2.3. Methods and Procedures

2.3.1. Phosphorylated Sugars

Phosphorylated sugars were determined by HPLC with the use of C_{18} Radial Pak A column. The standards were prepared at a concentration of 10 mg/ml of glucose-6-phosphate, ribose-5-phosphate, fructose-6-phosphate, glucose-1-phosphate and ribose-1-phosphate following the method used by Cotter (1982) of Waters Associates, Inc. Milford, Massachusetts. The sample size injected was routinely 5-10 ul. Both U.V. and R.I. (refractive index) detection were used to monitor the column eluant. Ultraviolet absorption at 214 nm at 0-2 A range and for the R.I. sensitivity was normally at 8X or 4X to get the full scale for the sample. The flow rate was 1.5 ml/min, and the chart speed was 0.25 cm/min. The mobile phase used was 1 % dicychlohexylamine adjusted to pH 6.0 using phosphoric acid. The solvent was heated at 50°C for 15 minutes prior to filtration and degassing using a Gelman Inst. Co. Filtration Unit - Michigan with a millipore Type HA membrane (0.45 um). Squid extract samples were routinely injected after the standards were analyzed on the same day. Unknown peaks were tentatively identified by comparing the retention time with the authentic standards.

2.3.1.1. Sample Extraction

Phosphorylated sugars were obtained from squid mantle by the extraction method described by Tarr and Leroux (1966) modified in some aspects like tissue weight and solvent volume. A squid muscle sample (5 g) was blended with 8 ml of 0.6 M perchloric acid (PCA) at 0 °C. The blended material was filtered immediately on a sintered glass funnel with suction, the filter cake was washed with 2 ml of 0.6M PCA, and the filtrate was neutralized to pH 7.5 with 10 N KOH after bringing it to 25 ml final volume with 0.6 M PCA. The filtrate was again filtered by using the Multifit Interchangeable Syringe and sample clarification kit for organic samples (Waters Associates, Inc.). A 10 μl sample was then injected into a Radial Pak C18 column. The extract containing phosphorylated sugars was hydrolyzed with 0.1N PCA for 30 min at 20°C to convert phosphorylated sugars to their corresponding free sugars. The free sugars thus formed were analyzed by the procedure outlined in section 2.3.4. The analysis was based on fresh weight basis at 30% moisture.

2.3.2. Reducing Sugar Determination

2.3.2.1. Nelson and Somogyi Method

Total reducing sugar as glucose was determined by using the method described by Nelson and Somogyi (1970). A 5 g sample of squid mantle muscle was blended in a Waring Blendor with 10 ml of water and boiled at 100°C. The homogenate was cooled to 20-25°C and then acetonitrile was added bringing the volume up to 25 ml.

2.3.3. Test for Pentose

Pentose sugars in squid extract were determined by the qualitative method described by Joslyn (1970). Squid sample extract (2.3.2.1) was mixed with 0.2% orcinol in 30% hydrochloric acid containing ferric ions, 30 ppm. It was heated in a 100°C water bath for about 10 min. A positive reaction gives a green colour. The test detects pentoses whether as free sugars or as glycosides.

2.3.4. Reducing Sugar Determination by HPLC

Free sugars in squid muscle extract were determined by an HPLC method based on the work of Conrad and Palmer (1976). The free sugars were extracted from 5 g of squid muscles which were blended in a Waring Blendor and then mixed with about 15 ml of hot water boiled at 100°C in a volumetric flask. The mixture was cooled and brought to 25 ml with acetonitrile. The sample was filtered through a 0.45 um no.1 millipore filter (Millipore Corp., Bedford, Mass.). Before injecting it to the HPLC injector it was again filtered by using Multifit Interchangeable Syringe and sample clarification kit-organic of Water Associates Inc., Milford, Mass. Standard reducing sugars were prepared at a concentration of 10 mg/ml of glucose, ribose, maltose, fructose, lactose in 40% acetonitrile and injected at 10 μl.

The column use for this analysis was a Waters Carbohydrate Analysis, 3.9 mm x 30 cm (Water Associates Inc., Milford, Mass.). The flow rate was 1.5 ml/min and the chart speed was 0.25 cm/min. The eluant used was 85% acetonitrile and 15% water, heated at 50°C for 15 min and then filtered and

degassed prior to chromatography. Standard curves were prepared from known free sugars and used to estimate the concentration of corresponding unknown peaks contained in the squid extract.

2.3.5. Glucose-Enzymic Determination

2.3.5.1. Preparation of Sample

5 g of squid muscle was blended in a Waring Blendor, in liquid afterwards extracted with 25 ml of water, heated to 60°C, and, filtered on Whatman No. 1 paper, To the extract was added 5 ml Carrez-1-1 solution (3.60 g potassium hexacyanoferrate -11, $K_4[Fe(CN)_6]$ x 3 H_2O/100 ml, 5 ml x 7 H_2O/100 ml and 10 ml 0.1m NaOH , adjusted to room temperature, and brought up to a 25 ml final volume with water and filtered.

2.3.6. Glucose/Fructose/Sorbitol-Enzymatic Determination

Preparation of sample was the same as described in section 2.3.5.1. Results for determination, or methods and procedures followed from Method of Enzymatic Food Analysis (1980) Boehringer Mannheim GmbH, Biochemica, 6800 Mannheim, 31 W. Germany Cat. No. 724831.

2.3.7. Ascorbic Acid Determination-Enzymatic Method

Sample preparation was the same as with that described in section 2.3.5.1. Method and procedure were followed from Method of Enzymatic Food Analysis, (1980), Boehringer Mannheim GmbH, Biochemica Cat No, 409677.

2.3.8. Amino Acid Determination

The free amino acid content of squid muscle was determined. For sample extraction, 5 g of muscle from frozen or dried samples of Newfoundland and Massachusetts squid was blended in a Waring Blendor. Blended samples were placed in a volumetric flask and water was added bringing the volume to 25 ml. It was centrifuged for 20 min at 2000 rpm. The supernatant was sent for free amino acid analysis to the amino acid analyzer, Beckman 121 MB as described separately in Beckman 121 MB application note MB-TB-017, of Memorial University of Newfoundland operated by Mr. Doug Hall and Sonia Bansfield. The supernatant was deproteinized with 10% sulfosalicylic acid (SSA). To 1.00 ml of sample, 0.25 ml of 10% sulfosalicylic acid (SSA) and 0.75 ml citrate buffer (pH 2) were added. From the clarified extract 50 μl was injected to the amino acid analyzer, a Beckman Model 121-MB connected to a Waters QA-1 System Integrator.

2.3.9. Homarine Determination

The homarine content of squid muscle was estimated by HPLC. A Beckman D.U. Spectrophotometer with a Beckman Compuset D.U.-8 Wavelength Scan was also used to scan the fraction corresponding to the peak supposed to be homarine collected from HPLC.

Squid extracts were applied to a Waters Carbohydrate Analysis column using 85% acetonitrile and 15% water as eluant at a flow rate of 1.5 ml/min. Homarine standards were prepared at 0.25 to 2.0 μg/ml from a 10 mg/ml stock

solution and were injected at 10 ul. The effluent was monitored by UV absorbance at 214 nm. Analysis was made by comparing the peak area of the sample peaks to that of the standards.

The unknown peak from squid extract having the retention time of standard homarine was scanned with a Beckman DU-8 Spectrophotometer with Scanning Compuset. The spectrum of the unknown peak was compared to that of authentic homarine.

Samples employed for homarine analyses were obtained by homogenizing 5 g of mantle muscle with 15 ml of distilled water, boiling the homogenate for 5 min, cooling the homogenate to 20-25°C, addition of acetonitrile to a final volume of 25 ml and filtration of the homogenate on No. 1 Whatman filter paper.

2.4. Browning Reaction in Squid

A model system for browning was set up by mixing a 0.1M solution of either glucose, ribose, fructose, maltose and lactose sugar standard with an equal volume of a 0.1M solution of either arginine, lysine, histidine and glutamic acid standards. The reaction system consisted of 2 ml of standard sugar and 2 ml of standard amino acid. These mixtures were incubated at 37°C and were observed until brown color was visually apparent. A 2.00 ml sample of squid extract (section 2.3.2.1) was also incubated at the same temperature. Also, 2.00 ml squid extract with equal volumes of each of the sugar standards and amino acid standards were also incubated at 37°C and observed for browning reaction.

Phosphorylated sugar standards like glucose-6-phosphate, ribose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate and ribose-1-phosphate were prepared at 0.1 M concentration in water and mixed with the 0.1 M amino acid standards were also incubated for browning reaction.

Squid extracts were also mixed with 0.1 M homarine and were incubated at 37°C, and evaluated for brown coloration.

The browning reaction of various sugars and amino acids was based on Hoshiba's method (1983).

A mixture of 0.1 M sugar and 0.1 M amino acid at pH 5 was heated at 120°C for 5 min with 30 ppm Fe and kept at 37°C for 5 days.

Chapter 3

INSTRUMENTATION

3.1. HPLC-High Performance Liquid Chromatography

Chromatography is a method of separating and analyzing mixtures of chemical substances based on chromatographic adsorption (Hamilton and Sewell, 1981). Chromatography involves a stationary phase (column packing) and mobile phase (eluent or carrier). One or both of these phases can be solid, liquid or gas. The advantages of HPLC over other forms of liquid chromatography are:

1. The HPLC column can be used many times without regeneration.

2. The resolution achieved on such column far exceeds that of other methods.

3. The technique is less dependent on the operator's skill, and reproducibility is greatly improved.

4. The instrumentation of HPLC leads itself to automation and quantitation.

5. Analysis times are generally much shorter.

6. Preparative liquid chromatography is possible on a much larger scale.

HPLC has some advantages over Gas Chromatography (GLC) because in GLC the mobile or carrier phase is a gas and the sample must be vaporized for analysis. This often requires the use of elevated temperature for the analysis of

liquid and solid and can result in thermal degradation of the sample. In HPLC the mobile phase is a liquid, and the sample can be simply injected if it is a liquid or solubilized if it is a solid and analyzed at ambient temperatures.

Since carbohydrates are polar and difficult to vaporize, they must be derivatized for analysis by GLC. This can lead to the presence of unreacted materials, side products and difficulties in quantitation. In HPLC, in contrast, the carbohydrates do not need to be modified but can simply be directly analyzed by chromatography.

For sample collection by GLC, the effluent is a vapor and must be trapped and cooled in dry ice or liquid nitrogen. The derivatized sugar must be converted to free sugar for subsequent identification. In HPLC the effluent is already a liquid and the underivatized components can be collected directly. About 50 μg of an individual sugar is readily determined via HPLC with a refractive index detector. This is an adequate sensitivity for most food analyses. Sensitivity can be improved by using larger injection volumes; however this may adversely affect the resolution of peaks in certain samples.

3.2. Z-Module-Radial Compression Separation System of Waters Associates

The Z- Module is installed onto an LC system at the same position as any standard column, and remains as an integral part of the system. The Radial-Pak cartridge, however, can be conveniently exchanged as the application requires.

Operation involved inserting a cartridge into the module, tightening the inlet connector, applying compression by manually cranking a ratchet handle which advances the compression chamber (sleeve) over the cartridge, and tightening the outlet connector.

The compression chamber is an extremely precise stainless steel sleeve, which presses tightly over the cartridge causing uniform radial compression throughout the bed. This produces a homogenous packing inside the cartridge, eliminating channels or voids in the packing material which can effect efficiency and reproducibility.

3.3. Chart Recorder-Servogor 120

The SE 120 is of compact construction and is available in single and dual channel version. In this experiment a dual version channel was used. The decadic gradation of the fullscale values with intermediate ranges allows all values from 1 mV up to maximum 100 V to be recorded over the full scale. Recording are made on roll charts by means of refillable, ink pens with exchangeable capillary or fibre-type-tips, as well as with disposable felt pens.

The basic principle of operation is a potentiometric measurement. A D.C. motor displaces the slider of the servo-potentiometer (sealed round pot) until the balancing voltages equals the amplified or attenuated according to the selected range-input voltage. The slider of the servo-potentiometer is mechanically linked with the pen carriage.

3.4. Series R-400 Differential Refractometer of Waters Associates

The refractive index detector is widely used and popular LC detector because of its universal detection capability. All solutes influence RI, therefore, the detector is sensitive to all compounds in a solvent. By choosing the solvent properly, the refractive index detector becomes a nearly universal detector. It had the following advantages:

1. All sample components are detectable regardless of the other properties (Universal Detector).

2. For polymer distributions, an accurate distribution is obtained which is sensitive to the concentration of weight of polymer in the eluting solvent. When the degree of polymerization is greater than 10 monomer units, the RI is a measure of the weight of polymer per unit volume and is practically independent of the molecular weight of the material being measured.

The operating principle of R-400 was the conventional differential technique which measures the deflection of a light beam as it passes through a partition at an angle to the beam. This principle has been used reliably for more than 100 years to detect small changes in refractive index. Since the deflection phenomena takes place at the surface of the cell partition, an extremely small cell containing only 10 μl of liquid can be used to detect changes that occur in small liquid volume.

The principle of operation involved was that the R-400 directly measures the deflection of a light beam due to the difference in refractive index between the sample and reference liquids.

3.5. Model 680-Automated Gradient Controller

The Waters model 680 is an interacting microprocessor-based pump controller that can operate and monitor up to three waters solvent delivery pumps (Model 45 or Model 6000A, or a combination). The automated isocratic and binary or ternary gradient operations may be overidden manually at any time for complete operator control, or allowed to run unattended according to the operator's programmed instructions.

3.6. Series 440-Absorbance Detectors

Detectors are designed to monitor the eluent stream from a chromatographic column and detect the presence of sample molecules through optical absorbance, at a wide range of specific wavelengths in the ultraviolet and visible spectrum. The model operates at various fixed wavelengths; 254 nm and 214 nm were used in this study. The controls and parts are common to a large degree among the Series 440 detector with functional differences incurring in the options and application available to the operator.

The principle of operation is to distinguish the presence of sample molecules in the solvent stream on the basis of UV absorbance. The vast majority of organic compounds absorb light in some part of the ultraviolet and visible spectrum, whereas a variety of solvents exist that transmit nearly all light in the visible and as well as into the ultraviolet spectrum. Unfortunately, sugars do not exhibit appreciable absorbance in the UV-visible range.

3.7. Model 6000A-Solvent Delivery System

Model 6000A is designed specifically to pump solvents for liquid chromatography applications. As a high performance pump, it was characterized by pumping a constant flow of solvent at working pressures up to 6000 psi (420 bars) and at a flow rates that are accurate and can be repeated from 0.1 to 9.9 ml/min in 0.1 ml/min increments.

3.8. M-45 Solvent Delivery System

As a high performance pump the M-45 is characterized by pumping a constant flow of solvent at working pressures up to 300 bars (4500 psig) and at flow rates that are accurate and can be repeated from 0.1 to 9.9 ml/min in 0.1 ml/min increments.

The constant flow is achieved by using a pair of reciprocating pistons. Each pumping chamber consists of pumping head cavity in which a plunger slides back and forth through a support assembly. The reciprocating action of the pistons causes one plunger to draw in solvent while the other expells solvent. In this manner the pumping chambers alternately supply the solvent.

3.9. Model U6K Universal Liquid Chromatograph Injector

This U6K injector is a specifically designed injector which enables the user to load samples and make injections at system pressure up to 6000 psi (420 bars) without interruption of the solvent flow. It can accomodate the full range of analytical and preparative injections, from a fraction of a μl to 2 ml, without making any changes to the injector.

Reproducible sample loading is performed at atmospheric pressure, while solvent is simultaneously being delivered to the system at full pressure. When an injection is made, an integral chart recorder automatically marks the injection point on the chromatogram. The automatic chart marking capability is standard when using this injector with a detector which has a remote chart marking circuit (such as model 440 absorbance detector and R400 - Refractive Index). A chart marker accessory can be used for marking the sample injection point when the detector does not have a remote chart marking circuit. Peak spreading is comparable to that of a conventional system injector.

3.10. Beckman DU-8 UV/VIS Spectrophotometer

This spectrophotometer of Beckman Instruments, Inc. USA was used in determining certain sugars contents by the enzymatic test method.

3.10.1. Wavelength Scanning with the DU-8 Spectrophotometer

Wavelength Scan Composet[TM] Module which was also from Beckman Instruments, Inc. USA is inserted into the DU-8 Spectrophotometer. A series of automatic operations are involved like self-diagnostic checks and the establishment of the default conditions.

Chapter 4

RESULTS

4.1. Reducing Sugar Content of Squid Mantle Muscle

The method employed for the analysis of total reducing sugars in squid muscle was by Nelson and Somogyi method, (1970). wherein sugar containing free or potentially free aldehyde as ketone group will reduce metal ions in an alkaline medium. Although the reaction is complex and non-stoichiometric, under carefully controlled conditions the amount of reduction is proportional to an amount of reducing sugar. Cupric ions are reduced to cuprous oxide and this in turn quantitatively reduced the arsenomolybdous acid which can be determined spectrophotometrically.

Tables A-1 to 18 and Figures A-1 to 20 are found in the Appendix section. Table A-1 shows the results of the analysis. Reducing sugar content in the sample was determined from a standard curve obtained with authentic glucose (Figure A-1). The concentration was computed based on the standard curve. The total reducing sugar in squid muscle is expressed as glucose equivalents.

4.2. Pentose Analysis

Three tests were made in squid samples from both Newfoundland and Massachusetts squid. Table A-2 shows that the test was positive for the presence of pentose sugars in the squid extracts.

4.3. Free Sugar Analysis By High Pressure Liquid
Chromatography

The free sugars of glucose and ribose were determined by HPLC analysis as described in experimental section 2.3.4. Sample chromatograms of standard sugars are shown in Figures A-2 to A-7. As can be seen from A-3, the retention times of glucose and ribose were 7.66 $\pm$ 0.03 and 4.04 $\pm$ 0.03 min, respectively, for the chromatographic conditions.

The reproducibility of peak area obtained for chromatograms of standard sugars (10 μl injection) is shown in Table A-3. For the muscle extract of Nfld and Mass squid the retention time, area peak and concentration is shown in Table A-4. The first peak is a solvent peak marked (X). The basis of comparison is made on the retention time and area of the authentic standard.

4.3.1. Influence of Standard Sugar Concentration on Retention Time
and Peak Area

The relationship between sugar concentration and R. I. detector response for 10 μl injections of standard glucose and ribose solutions is shown in Figure A-8 and A-9. A linear relationship was observed between the amount of sugar in the standard and peak area. The correlation coefficients (r) for linear regression analysis were 0.98 and 0.97 for glucose and ribose respectively.

4.3.2. Chromatography of Frozen Squid Extracts

Figure A-10 shows the chromatographic separation of Newfoundland squid sample extract. Figure A-11 shows chromatographic separation of Massachusetts squid sample extracts. The first peak is a solvent peak marked (X) and different sugars are marked (R) for ribose, (G) for glucose, (G-6-P) for glucose-6-phosphate. and (R-5-P) for ribose-5-phosphate. Both samples contained components which have retention times comparable to glucose and ribose. Assuming those peaks correspond to pure glucose and ribose, the concentration of these sugars in squid mantle muscle were calculated from the standard curves (Table A-4). The peaks (Table A-3) at 7.66 ± 0.03, 4.04 ± 0.03, 5.47 ± 0.03, 7.09 ± 0.17, 8.49 ± 0.16 and 8.77 ± 0.02 min retention time did not exhibit absorbance at 214 nm and this is consistent with their apparent identity as sugars.

4.3.3. Chromatography of Dried Squid

Figures A-12 and A-13 show the chromatographic separations of extracts of freshly dried mantle from squid collected in Newfoundland and Massachusetts. The first peak is a solvent peak. The chromatographic effluent was monitored by refractive index and by absorbance at 214 nm. The results show that peaks eluted with a retention time corresponding to both glucose and ribose; however, the peaks did not exhibit absorbance at 214 nm. The latter observation indicates that the peaks may partly or entirely consist of components other than free sugars, since ribose and glucose show no appreciable absorbance at 214 nm at these concentrations.

After dried squid were stored for 3 months at 20°C, no peaks corresponding in retention time to glucose and ribose were detected (Table A-5).

4.4. Phosphorylated Sugar Analysis - HPLC

Phosphorylated sugar analysis was made by using HPLC as described in 2.3.1.

The retention time of four standards are shown in Table A-6. Figure A-16 and Figure. A-17 show a chromatographic analysis of Newfoundland and Massachusetts squid extracts. The first peak is a solvent peak. It appears that there are 3 peaks, two of which had retention times corresponding to ribose-5-phosphate and glucose-6-phosphate

Table A-6 shows phosphorylated sugar standard analysis by HPLC. Table A-7 shows squid sample extract analysis for phoshorylated sugars by HPLC. It shows the peak areas for triplicate 10 μl injections of phosphorylated sugars, and the estimated concentration of phosphorylated sugars in both Newfoundland and Massachusetts squid mantle. The first peak is a solvent peak.

4.5. Glucose, Enzymic Analysis

The principle of this analysis of glucose is that glucose is phosphorylated to glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine-5-triphosphate (ATP) (1).

(1) Glucose + ATP ----HK--------> G-6-P + ADP

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G-6-P-DH), G-6-P is oxidized by nicotinamide adenine dinucleotide phosphate (NADP) to glyconate-6-phosphate with the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH)(2).

(2) $G-6-P + NADP^+$ ------G6P-DH---------> gluconate-6-phosphate + $NADPH + H^+$
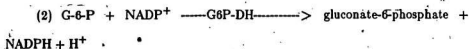
The amount of NADPH formed in the reaction is stoichiometric with the amount of glucose. The increase in NADH is measured by means of the absorbance at 330 nm.

The results of analysis of squid muscle extracts are shown in the Table A-8. It appears that Newfoundland squid contained more glucose than those of Massachusetts squid. The total glucose by this method will include glucose-6-phsphate.

4.6. Glucose/Fructose/Sorbitol, Enzymic Analysis

The principle of this method is that glucose and fructose are phosphorylated to glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) by the enzyme hexokinase (HK) and adenosine-5-triphosphate.

1. Glucose + ATP ------HK-----> G-6-P + ADP

2. Fructose + ATP ------HK-----> F-6-P + ADP
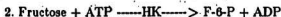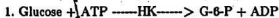
The results of the determination is shown in Table A-9.

There appeared to be more glucose + glucose-6-P in mantle muscle from Newfoundland than the Massachusetts squid. But, with the fructose and sorbitol the results were negative. With the forgoing result it is apparent that squid did not contain detectable fructose and sorbitol.

4.7. Amino Acid Analysis

Table A-12 and A-13 show the free amino acid content of Newfoundland and Massachusetts squid extracts.

Another free amino acid which is seldom found in meat and fish muscle is in considerable amount in squid is proline. This amino acid is reactive with carbonyl sugars, so it might affect the browning reaction in squid. Squid muscle also contained a high level of taurine. Almost all amino acids react with sugars, but there are only a few which are active reactants in Maillard browning like lysine, alanine, arginine, tryptophan, histidine, taurine and proline. The relatively high amount of these amino acids in dehydrated squid might be the reason why dehydrated squid *Illex illecebrosus* exhibits excessive browning. Table A-14 shows visual observation of amino acid plus squid sample extract. Free amino acids which are active reactants in Maillard Browning, according to Konosu *et al.* (1982), are shown in Table A-15.

4.8. Model System for Browning Reaction

Mixtures of ribose or glucose with certain amino acids, notably lysine, alanin, arginine, histidine, and trypsin, exhibited extensive browning after incubation for 4 weeks. Browning was evident approximately 1 week earlier with glucose-amino acid mixtures than it was with ribose-amino acid mixtures as shown in Tables A-14 and A-15. Browning was much less with lactose and sucrose (non-reducing sugars) than with maltose.

4.9. Homarine Analysis

Standard solutions of homarine were eluted from a carbohydrate analysis column with a retention time of 9.14 ± 0.17 min. as shown in Table A-16. Figures A-18 and A-19 show Newfoundland and Massachusetts squid sample extracts exhibited two peaks in which the first peak corresponds to the solvent peak and the second peak is homarine. By HPLC analysis the fraction which corresponds to the retention time of standard homarine was collected and scanned in the Beckman Spectrophotometer using the Compuset DU-8 Wavelength Scanning. A standard was also scanned to compare with this sample. Figure A-20 shows the absorbance vs wavelength scan of the standard and of the fractions from squid extract having a retention time of 9.14 ± 0.17 min. (Table A-16) which corresponds to homarine.

The unknown samples were similar to the standard in exhibiting a maximum absorbance at 275 nm in the ultraviolet range. Accordingly it would appear that the peak is a homarine.

The estimated homarine content in Newfoundland and Massachusetts squid were 79.2 ± 5.1 and 51.3 ± 6.6 mg% on fresh weight basis of squid mantle muscle respectively (Table A-17).

Addition of homarine to squid extract ($5 \mu l$ of homarine plus 5 μl of squid extract) increased the browning rate in a model system (Table A-18).

4.10. Ascorbic Acid Analysis

As stated by Eskin *et al.* (1971) ascorbic acid can participate in certain types of non-enzymic browning reactions. Based on this statement the possibility of the presence of ascorbic acid in squid would contribute to its browning reaction, so an analysis for ascorbic acid was made.

Analysis of ascorbic acid in squid extract by an enzymic method, did not reveal the presence of detectable levels. Accordingly, it was concluded that ascorbic acid does not contribute to browning reactions in squid.

4.11. Results for Browning Reaction in Squid

With the model system of 0.1 M sugar and 0.1 M amino acids, incubated at $37^{\circ}C$, it was found out that after 4 weeks, brown coloration occurred. Similar results were observed with the squid sample example extract for both Newfoundland and Massachusetts squid. In samples to which sugar was added it was found that brown coloration occurred after 3 weeks. Accordingly, these studies indicate that the somewhat greater concentrations of reducing sugars, free amino acids and homarine in the extracts from squid caught in Newfoundland are

consistent with the greater tendency to undergo browning at 37°C. It is possible that other components, not identified in this study, may also contribute to browning reactions in dried squid.

Chapter 5

DISCUSSIONS

5.1. Analysis of Reducing Sugar by Nelson and Somogyi Method

Tarr (1966) found that the addition of substances possessing a free aldehyde group (sugar, sugar phosphates, aldehydes, reductones, etc.) to leached flesh of fish which was subsequently heated caused browning reaction. The reducing sugar content for muscle from Newfoundland squid was 68.1 mg% whereas Massachusetts squid muscle contained was 67.5 mg% (Table A-1). It appears that this method underestimated the amount of reducing sugar in squid as shown by analysis of individual sugars. This may be due to the non-stoichiometry of the reaction.

5.2. Pentose Test Analysis

From the results shown in Table A-2 it is apparent that pentose sugars are present in squid. In the first result the color was slightly green. In the second result the color was green in both Newfoundland and Massachusetts squid samples. This may be due to the increase of heating time during the second test to 10 min from the original 5 min. The ribose content in squid is 37.6 mg% in Newfoundland and 40.3 mg% of squid mantle muscle in Massacusetts while the ribose-5-phosphate is 33.7 mg% and 54.2 mg% of squid mantle muscle in the same

order (Table A-4 and A-7) as shown by HPLC. It would appear that ribose sugars are primarily responsible for the positive pentose test.

5.3. Free Sugar Analysis, HPLC

It has been established by earlier workers that glucose occurs in the muscle of fish and shellfish. Early work by Tarr (1954) indicated that it was probably absent from muscle of living fish (liquid nitrogen frozen), but the technique used for its separation was probably not quite as sensitive as the methods now available, and very small concentrations could have been present but was not detected.

The amount of free sugars identified in *Illex* muscle in this study is typical of amounts reported in other marine organisms. Cod muscle contain 3-32 mg % glucose (Jones and Burt, 1960), crab muscle contains 3-86 mg % glucose with traces of ribose (Hayashi *et al.* 1979); lingcod contains 73.4 mg % ribose and 18.8 mg % glucose (von Tigerstrom and Tarr, 1965), and haddock contains 23 mg % glucose and 26 mg % ribose (Thompson *et al. 1980.*

5.4. Phosphorylated Sugar Analysis - HPLC

Phosporylated sugars are active precursors of browning in fish products like skipjack tuna (Yamanaka *et al.* 1974). *Illex* muscle contains 49-51 mg % G-6-P and 34-54 mg % R-5-P. Storey and Storey (1972) reported that live *Loligo pealii* contains trace quantities of G-6-P, F-6-P, F1,6-P (less than 1 mg %). It is likely that phosphorylated sugars accumulate in squid muscle rapidly after death of the animal (Yamanaka *et al.* 1974). Thompson *et al.* (1980) reported that haddock

muscle contains 49 mg % G-6-P and 129 mg % ribose phosphate. In general, glucose phosphate sugar content decreases after death (Burt and Stroud, 1966), while the pentose phosphate content increases transiently after death and then decreases (Tarr, 1966). In squid glucose-6-phosphate and ribose-5-phosphate were present in the frozen sample with a concentration of 59.4 and 33.7 mg% of mantle muscle in Newfoundland and 48.6 and 54.2 mg% mantle muscle in Massachusetts squid.

5.5. Glucose/Fructose/Sorbitol, Enzymic Analysis

Based on the results shown from Table A-10 comparing the concentrations of reducing sugars of both Newfoundland and Massachusetts squid extracts by the different methods used, discrepancies are noticable. The main discrepancy is that less total reducing sugars were detected by Nelson-Somogyi method than the results obtained for the individual sugars. The Nelson-Somogyi metdod determined the total free reducing sugars, which may include glucose or ribose and etc., but the result is expressed in glucose. When glucose and glucose-6-P estimated by HPLC is added together, the sum is similar to that obtained by enzymatic determination of glucose + glucose-6-P. Table A-11 shows a summary of the sugar analysis.

5.6. Amino Acid Analysis

The free amino acid content of *Illex* muscle was very high compared to that of other genera of squid (Konosu and Yamaguchi, 1982). *Oligo sloani, pacificus,* commonly used as drying squid in Japan contain 936 mg % amino acids, where as *Illex* muscle contained 3398 to 3675 mg % total free amino acids.

Saitsithi (1967) however, found lysine to give the greatest browning intensity among the twenty-one amino acids tested in his work with fish sauce. He claimed that lysine was detected in all the samples throughout the fermentation period. Aside from lysine, methyl histidine and alanine were found to be actually involved in non-enzymic browning. Based on the results obtained it was found that although almost all amino acids took part in the browning reaction, there are only 5 amino acids which actively take part in the reaction. They are lysine, alanine, arginine, tryptophan and histidine. Other amino acid proline and taurine which are also present in great amount and are active with reducing sugars, might also be a factor in the reaction.

5.7. Homarine Analysis

As elucidated by Hirano (1975), on the relation between browning and homarine degradation of irradiated squid, homarine can contribute the browning reaction. With his model system of a glucose - glycine solution, he found that browning reaction was increased when the dose of irradiation was raised, and heating temperature was increased to 80°C. With the addition of homarine in the solution the reaction was further enhanced. When squid sample extract was added with homarine, it was discovered that the length of time required for browning reaction to occur lessened; the usual time of 3 weeks was reduced to 2 weeks. The retention time of the homarine standard was 9.14 $\pm$ .17 min (Table A-16), while for Newfoundland and Massachusetts were 9.14 $\pm$ .41 and 9.19 $\pm$ min respectively (Table A-17). The estimated homarine content in Newfoundland and Massachusetts squid were 79.2 and 51.3 mg% of mantle muscles in the same order (Table A-17).

5.8. General Discussions

Due to the non-stoichiometricity the method of Nelson and Somogyi the amount of reducing sugar or phosphorylated sugars was not determined to its maximum value. Independent analyses of glucose, ribose, glucose-6-phosphates and ribose-5-phosphates yielded 43.4 and 37.6 mg% of muscle of glucose and ribose and 59.4 and 33.7 mg% of muscle of G-6-P and R-5-P in Newfoundland squid. For Massachusetts squid the yield was 37.5 and 40.3 mg% of mantle muscle and 48.6 and 54.2 mg% of mantle muscle in the same order. Whereas the Nelson-Somogyi method indicated a total reducing sugar content of 68.1 and 67.5 mg% of mantle muscles for Newfoundland and Massachusetts squid.

Enzyme analysis revealed that Newfoundland and Massachusetts squid contained 92.4 and 84.6 mg% of glucose plus glucose-6-phosphate, respectively. These data are comparable to the results obtained by HPLC. Unfortunately, enzyme methods were not available for the determination of ribose and ribose-phosphate.

Amino acid analysis was done using portions samples taken from different parts of 3 squid samples for Newfoundland and Massachusetts. The major amino acids found in Illex are proline and taurine, both are active precursors of Maillard browning.

Summing up the results, the mantle muscle of *Illex* contains precursors of Maillard browning. The reducing sugars identified in muscle tissue were glucose, ribose, G-6-P and R-5-P, and the major amino acids in *Illex* are proline and

taurine. Squid muscle also contained homarine, a compound previously shown to accelerate browning in irradiated squid. Although, there does not appear to be information available on the reducing sugar content of the other species of squid, the amount of reducing sugar found in this study did not appear to be very great, when compared to amounts reported in the muscle tissue of other marine organisms. The finding of extraordinary high levels of taurine and proline, may be sufficient information to explain why *Illex* is more susceptible to browning than other species commonly used for processing throughout the world. These are the reactants in Maillard browning so, it may be safe to conclude that it is Maillard browning that is occurring in dehydrated squid.

Chapter 6

CONCLUSIONS

1. The mantle muscle of *Illex* contains precursors of Maillard browning reaction. The reducing sugars identified are glucose, ribose, G-6-P and R-5-P and major amino acids especially proline and taurine.

2. Although, this work had not demonstrated the statistical difference of glucose content in Newfoundland and Massachusetts squids, we obtained higher values of glucose in Newfoundland than in Massachusetts, which may causee browning.

3. Ribose was present in both Massachusetts and Newfoundland squid extracts but the former has more ribose content than the latter. This reducing sugar is also a reactant in Maillard browning reaction.

4. Glucose-6-phosphate and ribose-5-phosphate were the only phosphorylated sugars found in the squid samples, and they are important precursors of Maillard browning reaction.

5. Ascorbic acid was not present in the squid extracts so it did not partake in the browning reaction.

6. Amino acids were present in the squid extract and some of them reacts with free sugars like glucose and ribose to produce the browning reaction.

7. Homarine was present in squid extracts and its presence increased the browning reaction.

8. Under visual observation it was noticed that Newfoundland squid exhibit a faster browning reaction than the Massachusetts squid.

Chapter 7

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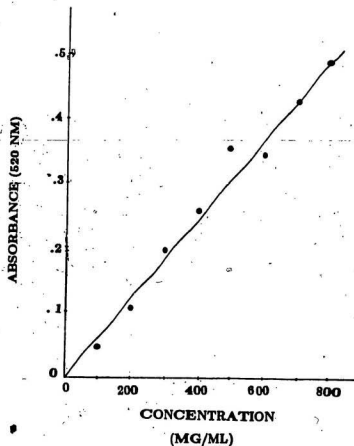
Appendix A

Figure A-1:   Glucose Standard Curve

Glucose standard curve based on Nelson and Somogyi method.
Method as described under 2.3.2.1.
Total reducing sugar was expressed in glucose equivalent.
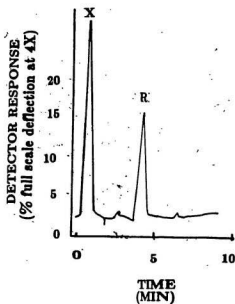Data are averages of triplicate determinations.

Figure A-2: Chromatogram of Ribose by HPLC

Sample: Ribose standard.
Sample Size: 10 µl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
Attenuation: 4X

Figure A-3: Chromatogram of Glucose by HPLC

Sample: Glucose standard.
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
Attenuation: 4X

Figure A-4: Chromatogram of Fructose by HPLC

Sample: Fructose standard.
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
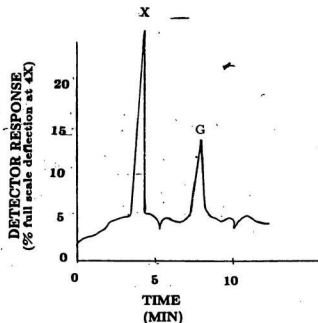Detector: R-400 Differential Refractometer.
Attenuation: 4X

Figure A-5: Chromatogram of Sucrose by HPLC

Sample: Sucrose standard.
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
Attenuation: 4X

Figure A-6: Chromatogram of Maltose by HPLC

Sample: Maltose standard.
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
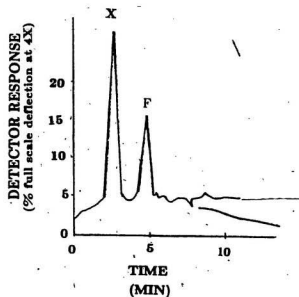Attenuation: 4X

Figure A-7: Chromatogram of Lactose by HPLC

Sample: Lactose standard.
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
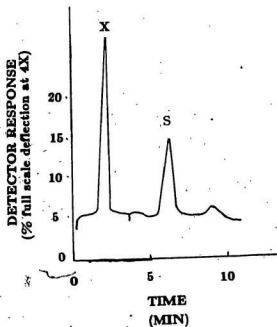Detector: R-400 Differential Refractometer.
Attenuation: 4X

Figure A-8: Glucose Standard Curve by HPLC

10μl injection of standard glucose was made to Waters
Carbohydrate Analysis Column. (3.9 mm x 30 cm). Detector used
was R-400 Differential Refractometer.
Data are averages of triplicate determinations.
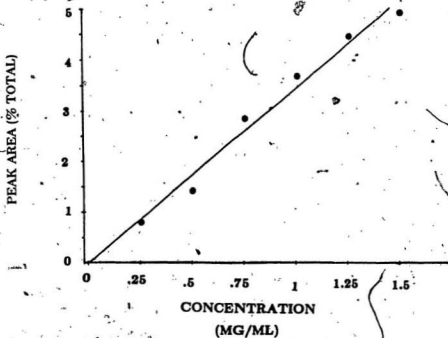
Figure A-9: Ribose Standard Curve by HPLC

10μl of ribose standard was made to Waters Carbohy-
drate Analysis Column, (3.9 mm x 30 cm). Detector used
was R-400 Differential Refractometer.
Data are averages of triplicate determinations.
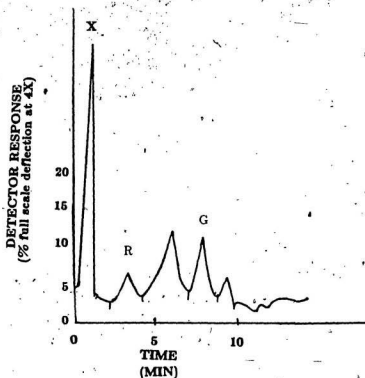
**Figure A-10: Chromatogram of Newfoundland Squid
Extract by HPLC**

Sample: Newfoundland Squid Sample Extract (frozen mantle).
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
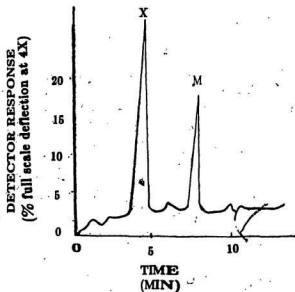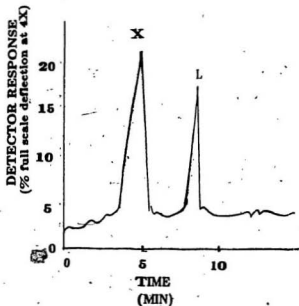Detector: R-400 Differential Refractometer.
Attenuation: 4X
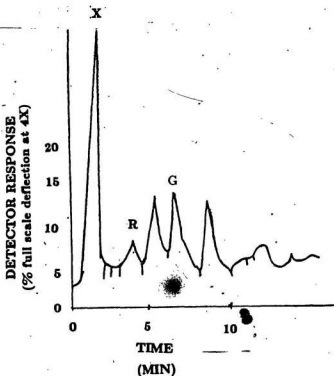
Figure A-11: Chromatogram of Massachusetts Squid Sample by HPLC

Sample: Massachusetts squid sample extract (frozen mantle).
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
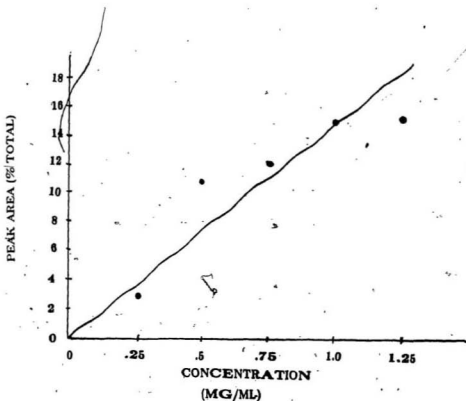Detector: R-400 Differential Refractometer.
Attenuation, 4X.

Figure A-12: **Chromatogram of Dried Mantle of Newfoundland Squid**

Sample: Dried mantle extract of Newfoundland squid.
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
Attenuation: 4X.

Figure A-13: Chromatogram of Dried Mantle of Massachusetts Squid by HPLC

Sample: Dried mantle extract of Massachusetts squid.
Sample Size: 10 μl.
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column, 3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
Attenuation: 4X.

Figure A-14: Glucose-6-P Standard Curve by HPLC

$10 \mu l$ injection of G-6-P standard was made to C18
Radial Pak A Column, using R-400 Differential
Refractometer as detector. The flow rate was
1.5 ml/min.
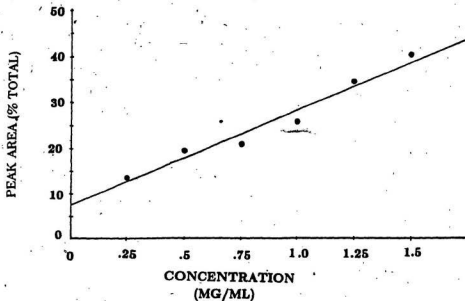
Figure A-16:   Ribose-5-P Standard Curve by HPLC

10μl injection of R-5-P standard was made to C18
Radial Pak Å Column, using R-400 Differential
Refractometer as detector. The flow rate was
1.5 ml/min.
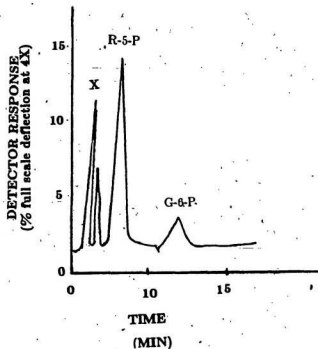
Figure A-16: Chromatogram of Newfoundland Squid for Phosphorylated Sugars by HPLC

Sample: Newfoundland squid sample extract (frozen mantle).
Sample Size: 10 μl.
Eluant: 1% dicyclohexylamine adjusted to pH 6.0 using
    phosphoric acid.
Column: C18 Radial Pak A Column.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
Attenuation: 4X.
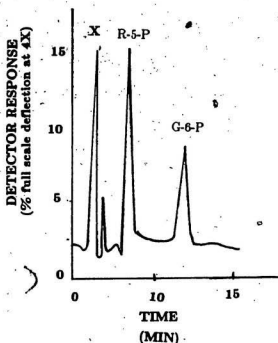
Figure A-17: Chromatogram of Massachusetts Squid for Phosphorylated Sugars by HPLC

Sample: Massachusetts squid sample extract (frozen mantle).
Sample Size: 10 μl.
Eluant: 1% dicychlohexylamine adjusted to pH 6.0 using phosphoric acid.
Column: C18 RADIAL PAK A Column.
Flow Rate: 1.5 ml/min.
Detector: R-400 Differential Refractometer.
Attenuation: 4X.
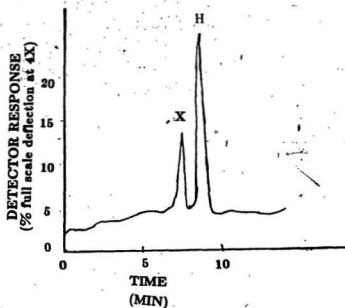
Figure A-18: Chromatogram of Newfoundland Squid
Extract for Homarine by HPLC

Sample: Newfoundland squid sample extract (frozen mantle).
Sample Size: 10 μl
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column,
3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
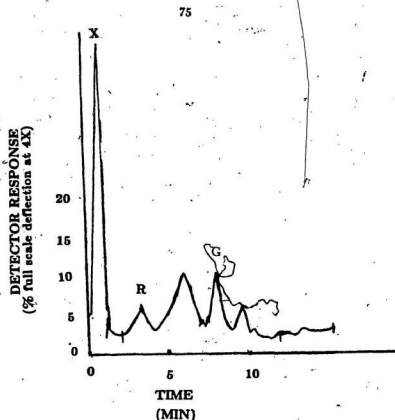Detector: R-400 Differential Refractometer
Attenuation: 4X
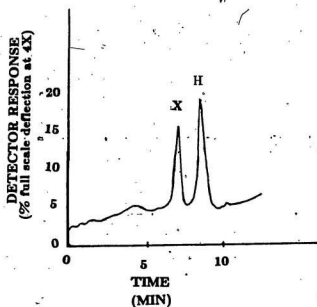
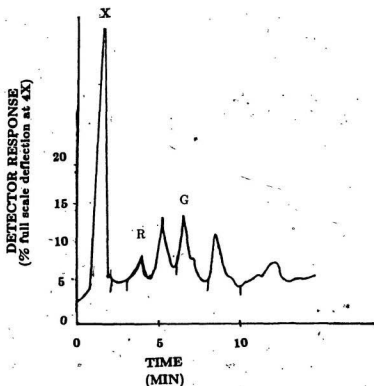Figure A-19: Chromatogram of Massachusetts Squid
Extract for Homarine by HPLC

Sample: Massachusetts squid sample extract (frozen mantle).
Sample Size: 10 μl
Eluant: 85% acetonitrile and 15% HPLC water.
Column: Waters Carbohydrate Analysis Column,
    3.9 mm x 30 cm.
Flow Rate: 1.5 ml/min.
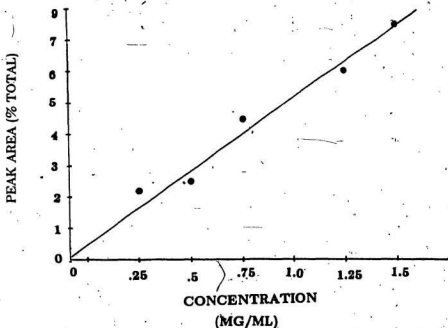Detector: R-400 Differential Refractometer
Attenuation: 4X
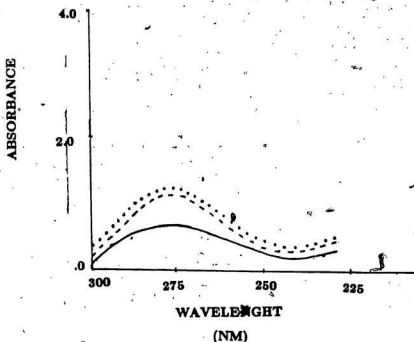
Figure A-20: **Wavelength Scanning of Standard Homarine and Fractions Collected From Squid Extract**

10 μl of homarine and fraction collected were scanned separately in Beckman Spectrophotometer using Compuset DU-8 Wavelength Scanning.

Legend:

———— : Homarine standard.

------------- : Massachusetts squid sample extract.

.... : Newfoundland squid sample extract.

Table A-1: Reducing Sugar Determination by Nelson and Somogyi Method

Sample[1]	Component (mg%[2])
Newfoundland squid	68.1 ± 7
Massachusetts squid	67.5 ± 5

[1] Sample preparation as described under 2.3.2.1.

[2] Data are averages of triplicate injections from 3 squid sample extracts; values following mean are standard deviations. Based on weight of mantle muscle.

Table A-2: Pentose Test

Date	Squid	Color
Feb. 17,1984	Newfoundland	Slightly green
Feb. 17,1984	Massachusetts	Slightly green
Feb. 20,1984	Newfoundland	Green
Feb. 20,1984	Massachusettes	Green

Green color showed that pentose sugar was present in
both sources of squid. The presence of pentose sugars were
determined by the method described in method section 2.3.3.

Table A-3: Reducing Sugars Analysis Standard by HPLC

Standard[1] (10 mg/ml)	Retention Time[2] (min)	Area Count
Glucose	7.66 ± 0.03	2026.90 ± 3.21
Ribose	4.04 ± 0.03	1825.80 ± 5.46
Fructose	5.47 ± 0.03	3061.56 ± 4.45
Sucrose	7.09 ± 0.17	3776.90 ± 6.11
Maltose	8.49 ± 0.16	2493.90 ± 9.02
Lactose	8.77 ± 0.02	3503.60 ± 4.46

[1] 10 μl was injected to Waters Carbohydrate Column, 3.9 mm x 30 cm per sugar standard.

[2] Data are averages of triplicate determinations; value following mean is standard deviation.

Table A-4: Squid Sample Extract Analysis by HPLC for Reducing Sugars

Peak No.	Retention Time min	Component mg%[1,2]	Identity Based on Reducing Sugar
NS-1	4.05 + 0.40	37.6 + 2.6	Ribose
NS-3	7.66 + 0.04	43.4 + 1.6	Glucose
MS-1	4.05 + 0.05	40.3 + 1.7	Ribose
MS-3	7.67 + 0.03	37.5 + 2.1	Glucose

[1] Data are averages of triplicate determinations; value following mean is standard deviation.

[2] Sucrose, maltose, lactose, and fructose were not detectable. HPLC of squid extracts for free sugars showed two peaks which were not considered to be sugars on the basis of their retention time and absorbance at 214 nm.
Based on weight of mantle muscle.

NS---Newfoundland Squid.
MS---Massachusetts Squid.

Table A-5: HPLC Analysis on Frozen and Dried Squid Extract for Reducing Sugar

Squid Sample	Retention Time[1] Glucose	Ribose	Time (mon.)	Apparent Sugar (mg%) Glucose	Ribose
NS	7.66 ± .04	4.05 ± .40	Frozen[2]	43.4 ± 1.6	37.6 ± 2.6
MS	7.67 ± .03	4.05 ± .05	Frozen	37.5 ± 2.1	40.3 ± 1.7
NS	7.69 ± .63	4.13 ± .92	Freshly Dried[3]	42.9 ± .5	16.1 ± 0.6
MS	7.57 ± .35	4.08 ± .72	Freshly Dried	37.2 ± 1.2	26.2 ± 4.0
NS	7.63 ± .21	4.09 ± .14	1	42.6 ± 2.2	15.1 ± 1.8
MS	7.62 ± .18	4.03 ± .43	1	40.8 ± 3.2	15.7 ± 4.4
NS	7.67 ± 1.7	4.17 ± .23	2	41.4 ± 2.4	8.6 ± 1.0
MS	7.69 ± .07	4.08 ± 1.2	2	39.6 ± 1.9	11.3 ± 2.3
NS	n.d.	n.d.	3	n.d.	n.d.
MS	n.d.	n.d.	3	n.d.	n.d.

[1] Data are averages of triplicate injections from squid muscle extracts, value following mean is standard deviation.
[2] Based on fresh weight of mantle muscle.
[3] Based on dried weight of mantle muscle.
NS---Newfoundland Squid.
MS---Massachusetts Squid.

Table A-6: Phosphorylated Sugars Standard Analysis by HPLC

Standard (10 mg/ml)	Retention Time	Area Count
G-6-P	14.52 ± 0.08	1590.09 ± 142.92
R-5-P	7.86 ± 0.05	2247.70 ± 252.08
F-6-P	18.15 ± 0.05	7635.86 ± 357.29
Maltose P	13.95 ± 0.05	1196.12 ± 166.24

10 μl was injected to Waters Carbohydrate Analysis
   Column, 3.9 mm x 30 cm per sugar standard.
Data are averages of triplicate determinations;
   values following mean are standard deviations.

Table A-7: Squid Sample Extract Analysis of Phosphorylated Sugars by HPLC

Peak No.	Retention Time (min)	Component (mg%²)	Identity Based on Phosphorylated Sugar
NS-1	7.87 ± 0.05	33.7 ± 8.2	Ribose-5-P
NS-2	14.55 ± 0.55	59.4 ± 9.3	Glucose-6-P
MS-1	7.82 ± 0.07	54.2 ± 1.1	Ribose-5-P
MS-2	14.56 ± 0.04	48.6 ± 1.2	Glucose-6-P

1 Data are averages of triplicate determinations; values
   following mean are standard deviation.
2 Fructose-6-P, glucose-1-P, glucose-1-6-DP, and ribose-1-5-DP
   were not detectable. HPLC analysis of phosphorylated sugars
   revealed two peaks which corresponded to the retention times
   of standard R-5-P and G-6-P.
   Based on weight of mantle muscle.
NS---Newfoundland Squid.
MS---Massachusetts Squid.

Table A-8: Glucose, Enzymic Analysis of Squid Sample

Sample[1]	Component $(mg\%^{2})$
NS	92.4 ± 2.2
MS	84.6 ± 6.1

[1] Sample preparation as described under 2.3.5.1.

[2] Data are averages of triplicate determinations; values following mean are standard deviations. Based on weight of mantle muscle.

NS---Newfoundland Squid.

MS---Massachusetts Squid.

Table A-9: Glucose/Fructose/Sorbitol Enzymic Method Analysis of Squid Sample Extract

Sample[1]	Sugar	Component (mg%[2])
NS	Glucose	88.2 ± 6.1
MS	Glucose	80.4 ± 6.7
NS	Fructose	n.d.[3]
MS	Fructose	n.d.
NS	Sorbitol	n.d.
MS	Sorbitol	n.d.

[1] Sample preparation as described under 2.3.5.1..

[2] Data are averages of triplicate determinations, values following mean are standard deviations. Based on weight of mantle muscle.

[3] n.d.: not detectable

NS---Newfoundland Squid.
MS---Massachusetts Squid.

Table A-10: Comparative Data for Sugar Determination of Different Methods Used in the Analysis of Maillard Browning

Analysis	Method	Component (mg%)	
		Newfoundland	Massachusetts
Pentose	Pentose Test	Positive	Positive
Total Sugar Expressed in Glucose Equivalent	Nelson and Somogyi	68.1 ± 7	67.5 ± 0.5
Glucose + G-6-P	Enzymic Method	92.4 ± 2.2	84.6 ± 6.1
Glucose + G-6-P	Enzymic Method	88.2 ± 2.8	80.4 ± 4.8
Glucose	HPLC	43.4 ± 1.6	37.5 ± 2.1
Ribose	HPLC	37.6 ± 2.6	40.3 ± 1.7
G-6-P	HPLC	59.4 ± 9.3	48.6 ± 1.2
R-5-P	HPLC	33.7 ± 8.2	54.2 ± 1.1

Data are averages of triplicate determinations; values
following mean are standard deviations.
Based on weight of mantle muscle.

Table A-11: Summary of Sugar Analysis

Sugar	Method	Component (mg%)	
		Newfoundland	Massachusetts
Total Sugar	Nelson & Somogyi	68.1 ± 0.7	67.5 ± 0.5
Glucose +G-6-P	Glucose Enzymatic Analysis	92.1 ± 2.2	84.6 ± 6.1
Glucose +G-6-P	Glucose/Fructose /Sorbitol Enzymatic Analysis	88.2 ± 2.8	80.4 ± 4.8
Glucose	HPLC	43.4 ± 1.6	37.5 ± 2.1
Ribose	HPLC	37.6 ± 2.6	40.3 ± 1.7
G-6-P	HPLC	59.4 ± 9.3	48.6 ± 1.2
R-5-P	HPLC	33.7 ± 8.2	54.2 ± 1.1
Glucose +G-6-P	HPLC	102.8	86.1

Data are averages of triplicate determinations;
values following mean are standard deviation.
Based on weight of mantle muscle.

Table A-12: Amino Acid Analysis of Newfoundland Squid

Amino Acids	mg%
Alanine	257.88 ± 86.72
Arginine	88.62 ± 36.52
Aspartic	13.83 ± 1.82
Cysteic	3.29 ± 0.73
Cystine	56.29 ± 18.79
Glutamic	66.29 ± 19.38
Glycine	119.58 ± 70.02
Histidine	56.42 ± 21.49
Hydroxylysine	0.25 ± 0.43
Hydroxyproline	1.33 ± 0.33
Isoleucine	30.46 ± 11.30
Leucine	59.33 ± 29.26
Lysine	31.67 ± 15.20
Methionine	37.96 ± 14.77
Phenylalanine	35.38 ± 10.69
Proline	1287.25 ± 398.92
Serine	48.42 ± 18.57
Taurine	1353.08 ± 538.51
Threonine	50.83 ± 17.82
Tryptophan	5.00 ± 1.32
Tyrosine	27.83 ± 7.27
Valine	40.17 ± 15.43
1-Methylhistidine	1.08 ± 1.20
3-Methylhistidine	4.67 ± 1.37
Total	3674.92 ± 732.10

Based on fresh weight basis

Data are averages of triplicate determinations;
value following mean is standard deviation.

Table A-13: Amino Acid Analysis of Massachusetts Squid

Amino Acids	mg%	
Alanine	138.00	$\pm$ 101.56
Arginine	175.62	$\pm$ 101.59
Aspartic	9.50	$\pm$ 6.63
Cysteic	2.79	$\pm$ 0.38
Cystine	64.42	$\pm$ 19.67
Glutamic	43.17	$\pm$ 8.39
Glycine	48.63	$\pm$ 4.12
Histidine	54.72	$\pm$ 8.52
Hydroxylysine	0.00	
Hydroxyproline	1.79	$\pm$ 0.40
Isoleucine	26.67	$\pm$ 5.01
Leucine	55.29	$\pm$ 11.2
Lysine	34.00	$\pm$ 11.12
Methionine	43.21	$\pm$ 6.89
Phenylalanine	26.96	$\pm$ 7.06
Proline	1367.12	$\pm$ 528.22
Serine	45.25	$\pm$ 5.88
Taurine	1137.08	$\pm$ 290.34
Threonine	50.20	$\pm$ 6.87
Tryptophan	6.25	$\pm$ 3.03
Tyrosine	21.46	$\pm$ 7.10
Valine	42.38	$\pm$ 8.77
1-Methylhistidine	3.88	$\pm$ 2.13
3-Methylhistidine	3.71	$\pm$ 1.45
Total	3397.67	$\pm$ 588.71

Data are averages of triplicate determinations;
value following mean is standard deviation.
Based on weight of mantle muscle.

Table A-14: Visual Observation on Squid Muscle Extracts + Sugars + Amino Acids

Squid Sample + Sugar + Amino Acid (0.1M with 30 ppm Fe^{+++})	Brown Color Time (Week)			
	1	2	3	4
NS	-	-	+	++
MS	-	-	-	+
NS + Glucose	-	-	++	+++
NS + Ribose	-	-	++	++
NS + Fructose	-	-	-	+
MS + Glucose	-	-	+	++
NS + Ribose	-	-	+	++
NS + Lysine	-	-	+	+++
NS + Alanine	-	-	+	++
NS + Glutamic acid	-	-	-	+
MS + Lysine	-	-	-	++
MS + Alanine	-	-	+	++
MS + Glutamic acid	-	-	-	+

- : no browning evident
+ : browning evident
++: brown
+++: dark brown
NS—Newfoundland Squid.
MS—Massachusetts Squid.

Table A-15: Amino Acids Which Are Active Reactants in Maillard Browning[1]

Amino Acids	I.illecebrosus Nfld	Mass	T.rhombus	L.Kensaki	L.chinensis	O.pacificus
Alanine	258	138	59	261	482	75
Arginine	89	177	568	702	225	99
Histidine	56	55	8	5	30	140
Lysine[2]	32	34	9	35	22	9
Methionine[2]	38	43	3	1	22	12
Proline	1287	1137	238	200	248	479
Taurine[2]	1353	1137	238	200	248	0
Tryptophan	5	6	-	-	3	2
Total Active	3188	2957	885	1533	1992	816
Very Active	1423	1214	250	236	295	23

[1] Data for species other than *I. illecebrosus* from Konosu and Yamaguchi (1982).

[2] Very Active amino acids.

Data are in (mg%) based on weight of mantle muscle.

Table A-16: Homarine Standard Determination by HPLC

Homarine Standard	Retention Time Minute	Average Area %
Homarine	9.14 ± .17	100

Data are averages of triplicate determinations;
value following mean is standard deviation.
10 μl of homarine standard was injected to
Waters Carbohydrate Analysis Column, 39 mm x
30 cm using a flow rate of 1.5 ml/min.
Homarine preparation as described under 2.3.9.

Table A-17: Homarine Concentration of Squid Muscle Extract
by HPLC

Squid Sample	Retention Time[1] (min.)	Area Peak (10 μl injection)			Component (mg% of mantle muscle)
		1	2	3	
NS	9.14 ± .41	211290	216030	238310	79.2 ± 5.1
MS	9.19 ± .88	149400	160270	122830	51.3 ± 6.6

[1]: Data are average of triplicate determinations; values
following mean are standard deviation.

Table A-18: Effect of Homarine in Squid Extract Samples

Squid Sample Extract + Homarine (0.1M conc. with 30 ppm Fe)	Time (Week)			
	1	2	3	4
NS squid extract	-	-	-	+++
MS squid extract	-	-	-	++
NS + Homarine	-	-	+	+++
MS + Homarine	-	-	+	++

- : no browning evident
+ : browning evident
++ : slight brown
+++: dark brown
NS---Newfoundland Squid.
MS---Massachusetts Squid.