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PROTEOLYTIC DEGRADATION PRODUCTS AS INDICATORS OF QUALITY IN MEAT AND FISH.

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ABSTRACT

Assessment of freshness and quality of meat and fish is a major activity of both food regulatory agencies and the food industry. Various methods are used for measuring fish and meat quality, each with its particular advantages and limitations. However, methods based on monitoring the products of proteolysis have received relatively little attention. The objective of the present study was to identify specific protein and peptide products of proteolysis as indicators of freshness and quality during chilled storage of fresh fish and meat.

Samples of meat and fish were subjected to chilled storage; at intervals of 0, 2, 4, 8, 12 and 16 days, samples were subjected to protein and peptide extraction, and separation of individual sarcoplasmic and myofibrillar proteins by SDS and native electrophoresis. These extracted proteins along with acid soluble nitrogen (ASN) were separated by RP-HPLC, fractions were collected and identified by electrospray ionization mass spectrometry (ESI-MS).

RP-HPLC separated at least thirty fractions from the ASN extract of fresh fish. ESI-MS revealed the presence of at least twenty-five polypeptides with molecular weights (MW) ranging from 2 to 32 kDa. The relative area % of the polypeptides with MW 32.8 kDa and 42.8 kDa decreased during the storage while polypeptides of MW of 10.9 kDa and 16.7 kDa increased during storage. Changes in polypeptides of MW 12, 34.2 and 42.8 kDa was also observed. The sarcoplasmic protein extracted from ground and whole meat contained at least 12 polypeptides with MW ranging from 11 to 42 kDa. The relative area % of polypeptide of MW of 35.7 kDa decreased during storage. The results suggest that changes in proteins and polypeptides of MW 10.9, 12, 16.7, 32.8, 34.2 and 42.88 kDa in fish and 35.7 kDa in meat could serve as indicators of spoilage.

RESUME

Déterminer la qualité et la fraîcheur de la viande et du poisson est un gros problème à la fois pour l' industrie alimentaire et les agences gouvernementales. Plusieurs méthodes sont utilisées pour arriver à cette fin, chaqu' une avec ses avantages et ses inconvenients. Toutefois, les méthodes basées sur le suivit des produits de protéolyse n' ont reçu que peu d' attention. L' objectif de cette étude était d' identifier des protéines et des peptides spécifiquement produits par la protéolyse et pouvant servir d' indice de fraîcheur et de qualité pendant l' entreposage au froid de la viande et du poisson.

Les échantillons de viande et de poissons furent entreposés au froid durant différentes périodes (0, 2, 4, 8, 12 et 16 jours). L' extraction des peptides et des protéines fut ensuite réalisée sur ces échantillons. La séparation des protéines sarcoplasmiques et myofibrillaires par SDS et native électrophorése fut réalisée. Les protéines extraites et la fraction azotée soluble dans l' acide furent séparées par chromatographie liquide à haute pression (HPLC) en phase inverse, les fractions furent collectées et identifiées par spectrophotométrie de masse en ionistion d' electrospray (ESI/MS).

La HPLC en phase inverse permit la séparation d' au moins 25 fractions à partir de la fraction azotée soluble dans l' acide obtenues des extraits de poissons. La ESI/MS revela la présence d' au moins 25 polypeptides avec des poids moléculaires variant de 2 à 32 kDa. Le pourcentage de surface relative des polypeptides de 32.8 et 42.8 kDa de poids moléculaire decrut durant l' entreposage. Des variations au niveau des peptides de 12, 34.2 et 42.8 kDa de poids moléculaire furent également observées. Les protéines sarcoplasmiques extraites de la vainde hachée et entiére contenaient au moins 12 polypeptides dont le poids moléculaire variait de 11 à 42 kDa. Le pourcentage de surface relative du polypeptide de 35.7 kDa de poids moléculaire décrut durant l' entreposage. Ces resultats sugérèrent que les variations en protéines et en polypeptdes de poids moléculaire égal à 10.9, 12, 16.7, 32.8 et 42.8 kDa pour le poisson et 35.7 kDa pour la viande pouvaient servir d' indicateurs pour la dégradation de ces produits.

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1. INTRODUCTION

Meat and fish are major protein food commodities in many parts of the world. The chronic shortage of proteins of high biological value in developing countries, along with the relatively high cost of meat and fish provides the justification for research on the quality and freshness and therefore the preservation and spoilage of these commodities.

Loss of freshness of food in general, is the result of undesirable changes in odour, colour, flavour, texture or appearance of food when loss of freshness reaches a certain degree, spoilage results. Spoilage of meat and fish can be caused by several factors including: rodent, insect, tissue enzymes, non enzymatic chemical changes, physical effect and the action of microorganisms. Tissue enzymes affect microbial deterioration of meat and fish by altering their components so they are more readily available for microbial use. In addition, meat and fish are good substrates for microorganisms because they have (a) high moisture content, (b) high levels of nitrogenous components and minerals, and (c) favorable pH and accessory growth factors.

Protein hydrolysis by microorganisms and by autolysis in meat and fish can produce a variety of odour and flavour defects; some psychotropic spoilage bacteria are strongly proteolytic and cause undesirable changes. Lipid oxidation is associated with the deterioration of the quality of meat and fish products particularly in the presence of unsaturated lipids (Schaich 1980). The spoilage of wholecuts of meat or fish under refrigeration is primarily a surface phenomenon resulting in sliminess, lipids, off-odour and taste, discolouration and souring. During storage, souring can result from action of endogenous enzymes, fatty acid or lactic acid formation by bacteria and proteolysis without putrefaction. Crystallization of water

and cryo-concentration of solutes ultimately causes disintegration of muscle membrane systems in meat and fish allowing mixing of tissue enzymes and substrates followed by reactions resulting in pigment and lipid oxidation.

Assessment of overall quality of meat and fish is a major concern for regulatory food laboratories. In order to determine freshness of meat and fish many physical, chemical, biochemical, sensory and microbiological methods have been proposed. The traditional sensory methods are subjective and costly because they require specially trained personnel. Microbiological assessment and chemical methods do not measure early postmortem deterioration; they measure bacterial spoilage instead of loss of freshness caused by autolysis shortly after death (Luong et al., 1991). It is important, therefore, for methods to be developed to provide indicators of freshness and deterioration in addition to spoilage.

The fact that proteins (a) represent a substantial non-water component of meat and fish and (b) are substrates for degradative processes during storage, provides the basis for investigating products of proteolysis as indicators of spoilage. Non-protein nitrogen (NPN) is commonly used as an indicator for spoilage. Peptides can be significant compounds of NPN, depending on the nature of the initial degraded protein. There is little information in the published literature on the quantities and types of peptides which results during degradation of meat and fish proteins. The objective of the present work was to determine whether specific proteins or peptides or groups of products of proteolysis can be identified as indicators of freshness in fish and meat.

2. LITERATURE REVIEW

2.1. Definition of Spoilage

Meat spoilage has been defined as "any single symptoms or group of symptoms of overt microbial activity, manifest by changes in meat odour, flavour, or appearance" (Gill, 1986). In general, food spoilage is the result of undesirable change in odour, colour, flavour, texture or appearance. Deterioration of food during storage has been attributed to both autolytic as well as bacterial changes (Dainty et al., 1983). In muscle tissue autolytic changes include some proteolytic action on muscle and connective tissue and hydrolysis of fat (Anders, 1989). Preliminary hydrolysis of proteins by meat and fish enzymes facilitates the ability of the microorganisms to penetrate and grow in the tissue (Anders, 1989). The actual spoilage of fleshy food has been attributed mainly to the action of contaminating bacteria; gram negative organisms have been recognized as the predominant spoilers (Hobbs, 1983).

In general, food products can be categorized into the following three basic groups: (i) perishable foods (eg. meat and fish), (ii) partially processed foods that are processed to increase the shelf life and are generally refrigerated, and (iii) fully processed food, that have a long shelf life and are usually held at room temperature.

Like meat, fish may be spoiled by autolysis, oxidation, bacterial activity or by combination of these. Fish is considered more perishable than meat because of more rapid autolysis by fish enzymes and because of the less acid conditions which favour microbial growth. To understand meat and fish spoilage fully it is necessary to know (i) the types of bacteria and their proportions within the microflora, (ii) the relative contributions of various

factors affecting the growth of spoilage bacteria and (iii) the chemical changes produced during growth and the end products responsible for off-odour and off-flavour.

2.2. Factors Affecting Spoilage

Most biological systems experience direct deterioration as a result of the following types of activities; physical (by foreign bodies), chemical (water activity, gaseous conditions, pH), biological (microorganism, bacteria, spores, protozoa, insect), or biochemical (enzymatic, which are natural catalysts present in food). In addition, food deterioration is indirectly influenced by environmental factors, i.e., temperature, light and time. These factors can operate either alone or in combination; however, one of them can terminate useful shelf life before the others become serious (Lambert et al., 1991). Furthermore, the availability of essential nutrients, such as amino acids and sugars and certain lipids can also effect spoilage (Gill and Newton, 1980).

2.2.1. Initial Contamination

The inner tissues of healthy animals slaughtered under good hygienic conditions are relatively free from contamination (Gill, 1979). Bacteria found on meat originate from the skin of the animal, from faecal material, soil, water or air. The spoilage in meat and fish is associated with a particular level of bacteria (Hobbs, 1987). The lower the initial contamination the longer it takes for bacteria to achieve spoilage level. Greer (1989) reported that the shelf life of beef steak with an initial load of 1×10^2 was longer than 3 d at 7°C, while beef steak with an initial load of 6×10^4 cm^{-2} exhibited slime in 2 d at 0 °C; with an initial load of 1×10^7, the refrigerated shelf life was less than 1 d.

2.2.2. Nutrients

The interaction between microorganism and nutrient influence both microbial spoilage population and spoilage pattern (Lambert et al., 1991). The level of protein or fat is unchanged during the onset of rigor and is not a substrate for microorganism attack prior to the onset of spoilage (Gill and Newton, 1980). Several studies have shown that in meat the initial microbial growth occurs at the expense of low molecular weight soluble compounds formed during postmortem glycolysis (Gill, 1986). These compounds include glucose, glucose-6-phosphate and lactic acid (Eskin, 1990).

Pseudomonas species preferentially use glucose as substrate at the surface of meat (Gill and Newton, 1978), and then later use amino acids as substrate with the consequent production of sulfides, esters and acid (Gill, 1986 and McMeeking, 1982). However, when oxygen is limiting, glucose concentration is considered to have a limiting effect on the microbial spoilage pattern. Depletion of glycogen supplies occurs in muscle of live animals as a result of stress, starvation, or fright prior to slaughter. This can lead to a higher pH and low concentration of low molecular weight substances, this favouring spoilage.

2.2.3. pH and Acids

Microorganisms are significantly affected by the pH of growth medium because of the absence of a mechanism for adjusting their internal pH (Corlett and Brown, 1980). Not only are the rates of growth of microorganisms affected by pH, but also the rates of survival during processing (Jay, 1992). Foods with low pH values (below 4.5) usually are not readily spoiled by bacteria and are more susceptible to spoilage by yeast and molds and therefore, are microbiologically more stable than neutral foods (Gill, 1986). Growth of most bacteria is

favoured by neutral pH although some, such as those that form acid, are favoured by moderate acidity while others, such as proteolytic active bacteria in the white of stored eggs, can grow in alkaline media. Inhibition of microorganisms by acids depends on strains, incubation, temperature, types of acid, and concentration. At the same pH value, for example, inhibition of *L. monocytogenes* by various acids follows the order; acetic > lactic > citric > malic > hydrochloric. However, storage temperature has an affect; in the presence of equal concentration at 10°C, the order is: malic > citric > acetic > lactic > hydrochloric while at 25 and 35°C, the order was citric > malic > lactic > acetic > hydrochloric (Jay, 1992). The initial pH may be suitable or restrictive for the growth of bacteria, however growth of competitive bacteria or the growth of the organism itself could alter the pH so that it becomes more favourable or unfavourable for growth (Glass and Doyle, 1991).

2.2.4. Water Activity (a_w)

Microorganisms have an absolute demand for water, for without water no growth can occur. Water activity is considered as a limiting factor of microbial growth as it determines the osmotic stress (Franks, 1991). Each microorganism has a critical a_w below which growth cannot occur. For example pathogenic bacteria cannot grow at $a_w < 0.086$, yeast and molds are more tolerant, but show no growth at $a_w < 0.62$ (Hayes, 1992). On the other hand most bacteria can grow at a_w of 0.85 to 0.98. Factors affecting the a_w requirement of microorganisms includes type and strain of microorganism, substrate, pH, availability of oxygen, temperature, types of solute, and presence of inhibitory substances (Tapia de Daza et al., 1991). Chirife (1994) reviewed the a_w requirements for growth of several pathogenic bacteria and suggested that minimal a_w allowing growth was independent of the type of

dissolved solute, typically sodium chloride, sucrose, glucose, or potassium chloride Frank (1991) stated that a_w did not predict the relative usefulness of additives for antimicrobial stabilization. The concept of water dynamics has been proposed to replace the use of a_w values to predict microbial stability of concentrated and intermediate moisture food systems However, Chirife and Buera (1994) showed that water dynamics does not permit prediction of microbial stability of food with confidence

2.2.5. Temperature

Temperature is considered the most important environmental factor influencing both the rates of chemical reactions linked to the processes of microbial growth and the spoilage bacteria population (Lambert et al., 1991). Four physiological groups of bacteria may be distinguished on the basis of their temperature relationships A great deal of confusion surrounds the use of terms psychrophile and psychrotroph in the literature Morita (1975) pointed out that the term psychrophile has been used incorrectly and suggest that this terminology be restricted to organisms with optimum temperature of 15 °C or less, a maximum of 20 °C and a minimum of 0 °C The term psychrotroph refers to organisms which can grow at refrigeration temperature, but have temperature optima greater than 20 °C The temperature will determine which spoilage microorganism is predominant For example *Pseudomonas* maintain a growth rate advantage over competing psychrotrophs and mesophiles at temperature approaching 20 °C When the temperature is closer to optimum for mesophyllic temperature growth (30 °C), *Pseudomonas* are replaced by mesophyllic stains of *Acinetobacter* and *Enterobacteriaceae* as the dominant aerobic spoilage population (Gill and Newton, 1980) Unlike psychrophile, psychrotroph growth would decrease as a result of a

small decrease in temperature (below 5 °C) (Hanus and Morita, 1968). Therefore, this can affect the development of psychrotrophs which are the organisms of concern in the spoilage of refrigerated meat and fish.

The effect of temperature on microbial growth cannot be considered in isolation; other factors (e.g., a_w) in combination with temperature ultimately determine the spoilage of a food stored under particular set of conditions (Mossel, 1971). A decrease in temperature below 0 °C lowers the water activity of stored meat and fish; hence, spoilage above 0 °C is the result of bacteria, while in the frozen state, yeasts and moulds are the predominant spoilage organisms (Jay, 1992).

2.2.6. Atmospheric Conditions

The shelf life of meat and fish can be extended at refrigeration temperature (< 4°C). Spoilage will occur due mainly to aerobic, psychrotrophic bacteria, unless additional control measures are taken to modify the environmental storage condition (Lambert et al., 1991). Two major innovations in packaging systems are modified and controlled atmospheric packaging, (MAP and CAP systems, respectively). MAP systems alter the atmosphere surrounding the product with one packaging process, while CAP systems continuously control the composition of the atmosphere surrounding the product (Gill, 1986). For meat packaging, the lower the oxygen transmission rates the better the end product quality. This is not the case with fish and fish product (Jay, 1992); oxygen is a critical component when applying MAP to fresh fish since certain fish are naturally contaminated with *C. botulinum*, type E, (anaerobic bacteria). The spoilage which develops on chill stored meats is determined to a large extent by the storage atmosphere (Jay, 1992). *Pseudomonas spp.* are typical spoilage

organisms in red meat, poultry and fish. Carbon dioxide (CO_2) significantly inhibits *Pseudomonas spp.* growth, allowing anaerobic bacteria to flourish. The growth rates of *Lactobacillus* and *Brocothrix thermosphacta* are much slower and therefore, spoilage is retarded.

2.3. Predominant Microorganisms

Microbial growth is considered to be the most important factor that determines the keeping quality of fresh meat and fish (Lambert et al., 1991). The organisms of significance in the spoilage of aerobically refrigerated meat and fish are gram negative psychrotrophic organisms (Hobbs, 1987) particularly *Pseudomonas*. Psychrotrophic bacteria are found

Table 1. Some Microbial Defects of Red Meat and Fish

Meat	Fish
Acinetobacter	*Acinetobacter*
Aeromonas	*Aeromonas* (fresh water)
Alcaligenes	*Alcaligenes*
Bacillus	*Bacillus*
Campylobacter	*Brevibacterium* (fresh water)
Clostridium	*Corynebacterium*
Escherichia	*Flavobacterium*
Flavobacterium	*Lactobacillus* (fresh water)
Lactobacillus	*Micrococcus*
Leuconostoc	*Moraxella*
Micrococcus	*Pseudomonas*
Moraxella	*Sarcina*
Proteus	*Serratia*
Pseudomonas	*Streptococcucs* (fresh water)
Salmonella	*Vibrio*
Sarcina	
Streptococcucs	
Strreptomyces	

primarily in the genera *Pseudomonas*, *Flavobacterium*, *Achromobacter* and *Alcaligenes*, although *Micrococcus*, *Lactobacillus*, *Enterobacter*, *Arthrobacter* also contain psychrotrophic species (Palumbo, 1985 and Hayes, 1992). The relative spoilage potential of bacteria depends on which groups or species predominate and on their ability to form malodorous compounds, such as H_2S, volatile amines, esters and acetone (McMeeking, 1982).

The types of microorganisms which contaminate meat and fish vary considerably because of the differences in meats and fish. Common spoilage and pathogenic microorganisms associated with meat and fish are shown in Table 1. The flora of living fish depends on the microbial content of the waters in which they live. The bacteria on fish from northern waters are primarily psychrophiles, while fish from tropical water carry more mesophiles. Fresh water fish carry fresh water bacteria, which include the common genera found in salt water plus species of *Aeromonas*, *Lactobacillus*, *Brevibacterium*, *Alcaligenes* and *Streptococcus*.

2.4. Proteolytic Enzymes

Proteases are a group of enzymes that can hydrolyse muscle and connective tissue protein and in turn, induce tissue softening which affects its marketability (An et al., 1995); they are classified according to their source (animal, plant, microbial), their catalytic action (endoprotease/exoprotease) (Table 2 and 3) and the nature of the catalytic site (neutral, acidic and alkaline) (Ward, 1983). Proteolytic enzymes occur naturally in meat and fish tissue and can also be secreted by microorganisms (Hisano et al., 1989). The utilization of exogenous proteins by bacteria requires the enzymatic degradation of protein to peptides and amino acid before cellular uptake. The enzymes involved in this action are mainly extracellular proteinases

Table 2. Some Characteristics of Exopeptidase Proteolytic Enzymes

Proteolytic Enzymes	MW (kDa)	Optimum pH	Cellular Distribution	Inhibitors
Aminopeptidase				
Leucine aminopeptidase	150	7.8-8.0	Cytosolic	Puromycin
Arylamidase (Cathepsin II)			Cytosolic	
Neutral	257	~ 7.0	Cytosolic	
Basic	105	~ 8.0	Cytosolic	
Acidic	---	~ 6.0	Cytosolic	
Carboxypeptidase				
Cathepsin A	100	5.5	Lysosomal	
Cathepsin B2	52	5.5-6.0	Lysosomal	
Dipeptidase				
Prolinase	300	8.0-8.8	Cytosolic	
Prolidase	108	7.5-8.2	Cytosolic	
Glycyl-leucine dipeptidase	---	7.8-8.0	Cytosolic	
Glycyl-glycine dipeptidase	---	7.0-7.5	Cytosolic	
Dipeptidyl peptidase				
Dipeptidyl amino peptidase I (Cathepsin C)	200	5.0-6.0	Lysosomal	
DAP II	130	4.5-5.5	Lysosomal	
DAP III	80	7.0-8.5	Cytosolic	Puromycin
DAP IV	250	~ 7.5	Microsomal	

Source: Asghar and Bhatti, 1987; Alicia, 1986 and Etherington, 1984.

Table 3. Some Characteristics of Enopeptidase Proteolytic Enzymes

Proteolytic Enzymes	MW (kDa)	Optimum pH	Cellular Distribution	Inhibitors
Serine (alkaline) proteinase				
Myofibrillar serine protease	25	8.3-9.0	Cytosolic	Diisopropyl fluorophosphate (DIFP)
Myosin cleaving protease	26	7.5-9.5	Cytosolic	Phenyl methyl sulfonyl fluoride (PMSF)
Myofibrillar protease	30.8	9.5	Cytosolic	Alpha-halogenyl carbonyl
Group specific protease	24	9.0-10.5	Cytosolic	
Cytosolic protease	25	9.5	Cytosolic	
ATP-activated alkaline protease	550	7.8	Cytosolic	
Trypsin	23.5	7.0-8.5	Digestive	
Chymotrypsin	25.3	6.7-8.5	Digestive	
Elastase				
Cysteine (thiol) proteinase				Dipyridyl disulfides
Cathepsin B1	25	3.0-6.5	Lysosomal	L-tosyl-amino-2 phenylethyl ketone (TPCK)
Cathepsin H	28	5.0-7.0	Lysosomal	5,5'-dithiobis (2-nitrobenzoic acid)
Cathepsin L	24	3.0-6.5	Lysosomal	Heavy metals
Cytosol insulin-glucagen protease	80	5.0-7.0	Lysosomal	p- Chlorobenzenoate
mM-μM- CAF	110	7.5	Cytosolic	N-ethyl maleimide
Papain	23	7	Plant	Antipain, Leupeptin, Iodoacetamide
Carboxyl proteinase				Pepstatin
Cathepsin D	42	2.5-5.0	Lysosomal	Diazoactylnorleucine methyl ester
Cathepsin E	100	2.0-3.5	Lysosomal	ß- bromophenacyl bromide
Chymosin	30	2.0-3.0	Digestive	Trimethyloxonium fluoroborate
Pepsin	36-41	2.0-3.0	Digestive	Epoxy (p-nitrophenoxy propane)
Metalloproteinases				Chelating agents
Collagenase	33	7.5	---	Cynides
Carboxypeptidase A				EDTA
Thermolysin				O-phenanthroline
Gelatinase				8-hydroxyquinoline

Source: Asghar and Bhatti, 1987; Alicia, 1986 and Etherington, 1984.

(Lee and Chang, 1990). All bacteria have intracellular proteinases, but only a limited number have extracellular proteinases (Ward, 1983). Many bacterial species are known to produce extracellular proteinases; for example, many of the species of *Bacillus* (Ward, 1983), *Pseudonomas* (Hisano et al., 1989), *Clostridium* (Kalchayanand et al., 1989), and *Proteus* (Baeza et al., 1990) considered as proteolytic bacteria.

2.4.1. Carboxyl Proteinases

Generally, for all carboxyl proteinases there is considerable homology for sequence of amino acid residues in the active site (Tange and Wong, 1987). Carboxyl proteinases are recognized by the acidic amino acid residues at the active site and their preferred cleavage sites contain adjacent hydrophobic amino acids. Examples of common carboxyl proteases are: pepsin, chymosin and cathepsin D (Table 3). Pepstatin A and diazoacetyl-DL-norleucine methyl ester (DAN) can inhibit the activity of carboxyl proteinases, due to the acetylation of the hydroxyl group of inhibitors (Zhu et al., 1990).

Pepsin refers to a wide spectrum enzyme catalyzing the hydrolysis of a number of different kinds of peptide bonds, with a high preference for hydrophobic amino acids. Pepsin also catalyses transpeptidation reactions (Makinen et al., 1985). The optimum pH for pepsin activity is between pH 2 and 3. Like pepsin, chymosin possesses optimum enzymatic activity at low pH value and is an endoproteinase. However, unlike pepsin, chymosin is stable up to pH 6.5, while pepsin is generally denatured at pH 5.0 (Ward, 1983).

Cathepsin D is considered to be a major enzyme in the proteolytic events contributing to meat tenderization by its action on myosin (Dutson, 1983). Myofibrillar proteins were found to be susceptible to hydrolysis by cathepsin D at pH 5.5 (37^0C); these include a proteins

named titin (Wang et al., 1979) and connectin (Maruayama et al., 1980). Lasalle et al. (1983) considered titin and connectin to be the same protein. Myosin (both heavy and light chains) was rapidly degraded by Cathepsin D (Zeece and Katoh, 1989) while Kirschke and Barret (1987) reported that the enzyme did not degrade mature collagen or gelatin. Cathepsin D activity under normal temperature aging of meat (0-4^{0}C) has not been observed (Bandsman and Zandis, 1988); there is very little or no activity at 15^{0}C and maximum activity occurs at 45^{0}C and pH 5.5 (Zeece and Katoh, 1989). Activity of Cathepsin D can be inhibited by microbial peptides, pepstaine and diazo compounds. Cathepsin D is active over the pH range 3 to 5.

The microbial carboxyl proteases are widely distributed in molds and yeasts, but seldom found in bacteria; their optimum pH is at 2.5-5 and they are sensitive to acid proteinase inhibitors (Zhu et al., 1990). Like pepsin, microbial carboxyl proteinases exhibit specificity toward α-amino groups of hydrophobic amino acids (Ward, 1983). Matsubara and Feder (1971) have proposed that microbial carboxyl proteinases can be classified either as pepsin-like enzymes or rennin-like enzymes.

2.4.2. Serine Proteinases

Trypsin and chymotrypsin are common examples of serine proteases (Table 3). Although the mechanism of catalysis for all serine proteases is similar, there are different specificities toward the peptide bond depending on the presence of basic, aromatic, or hydrophobic amino acids (Trevor, 1991). For example, during digestion trypsin acts as a very specific endo-proteinase, only the peptide bonds whose carbonyl groups are donated by basic side chains of lysine and arginine are hydrolyzed (Trevor, 1991). On the other hand,

chymotrypsin catalyses the hydrolysis of the peptides whose carbonyl groups are donated by aromatic amino acids.

Morihara and Tsuzuki (1971) classified the serine microbial proteinases into 4 main groups: trypsin-like proteinases, alkaline proteinases, Myxobacter α-lytic proteinases and staphylococcal proteinases. Microbial trypsin-like proteinases exhibit specificity for carboxyl groups which are donated by amino acids with basic side chains. The second group shows a specificity toward aromatic or hydrophobic residues. The third group exhibits specificity for small aliphatic residues; the acidic residues at the carboxyl side act as a specific splitting point for the fourth group (Ward, 1983).

2.4.3. Metallo Proteinases

Carboxypeptidases, aminopeptidases, gelatinases, prolidases, and collagenases belong to a group of enzymes which contain divalent metal ions in their active site (Table 3). The enzymes can be inactivated by metal chelating agents, such as EDTA, 1,10-phenathnoline, glycyltrosine, and sulfhydryl-blocking agents (Table 3).

Carboxypeptidases contain one atom of zinc per mole, and are exopeptidases, since they catalyse the hydrolysis of peptide bonds in sequence (particularly peptides with aromatic and branched side chain amino acid) at the C-terminal end of protein and polypeptides. The carboxypeptides enzyme catalyses the further hydrolysis of chymotrypsin and trypsin produced polypeptides to release some important essential amino acids for assimilation. Therefore, the composition and range of peptides produced is controlled sequentially by the ordered action of pepsin, the serine proteinases and carboxyl peptidases.

Collagenase hydrolyses collagen and is considered important in the characterization

of deteriorated meat (Hisano et al., 1989). Collagenase has been found in many vertebrates and microorganisms. Hisano et al. (1989) isolated two kinds of collagenolytic enzymes from *Pseudomonas spp* and concluded that the purified enzyme shows both collagenolytic and caseinolytic activity. Collagenases have also been found in *Clostridium histoliticum*, *Vibrio arginolyticus*, *Vibrio* B-30 and *Streptomyces spp*. (Endo et al., 1987).

The microbial metallo proteinases can be divided into four groups: neutral and alkaline proteinases, and Myxobacter proteinases I and II, (Hisano et al., 1989). The neutral proteinases show specificity toward hydrophobic amino acid residues, while alkaline proteinases exhibit very broad specificity; Myxobacter proteinases I are specific for small amino acid molecules at either side of the splitting point (Ward, 1983).

2.4.4. Cysteine Proteinases

According to Goll et al. (1983 b), the major cysteine proteinases are Cathepsin B, L and H (Table 3). These enzymes show optimum pH in the range of 5.5 to 6.0 (Kirschke and Barret, 1987) (Table 3). The mode of action of these enzymes is considered to be similar to that of serine proteinases (Trevor, 1991); however, with cysteine proteinases, the nucleophilic attack on the carbonyl carbon atom of the peptide is by polarized -SH group. As a group, cysteine proteinases are inhibited by chemical reagents which react with -SH group such as Hg^{+2}, 4-Chloromeruriben Zoate, N-ethyl maleimide and iodoacetic acid (Robinson, 1987). Bojork and Karin, (1990) reported that the cysteine proteinase inhibitor cystatin (from chicken egg white) bond to cysteine proteinases actinidin, chymopapain A, ficin and papain; the inhibition was reversible. Bandsman and Zandis (1988), suggested that cysteine proteases and carboxyl type proteinases are responsible for myosin degradation in muscle.

Papain, a mixture of proteinases obtained by tapping the unripe fruit of papaya while still on the plant, is a cysteine proteinase used industrially to convert protein molecules into simpler peptides (Baeza et al., 1990).

The microbial cysteine proteinases have neutral pH optima and are sensitive to cysteine inhibitors. They are classified into two groups based on specificity: Clostrtripain which exhibits stringent specificity against basic amino acids at the carboxyl side and streptococcal proteinases show broader specificity (Ward, 1983).

2.5. Quality and Freshness

Freshness is a property of fish and meat that have a considerable influence on their quality; it is the most important criterion for judging the quality of most fish and meat products (Howgate, 1982). Loss of freshness, followed by spoilage, is a complex combination of microbiological, chemical, and physical processes (Pedrosa and Regenstein, 1990). Usually some specific property of the spoilage process is measured and used as an index of the stage of spoilage (Connell et al., 1976). However, quality is determined by several factors, some being more important than others (Pedrosa and Regenstein, 1990).

2.5.1. Sensory (Organoleptic Methods)

As meat and fish spoil, there is a sequence of changes that are readily detectable by the human senses (sight, touch, smell and taste). Sensory methods are considered the most satisfactory way of assessing the freshness of meat and fish products (Connell et al., 1976). These methods are quick and often nondestructive unless the samples are being cooked and most importantly, can be closely related to the criteria the consumer uses in evaluating food acceptability (Pedrosa and Regenstein, 1990). Therefore, sensory methods offer the most

appropriate means of determining the expectation of the consumer (Connell et al., 1976). However, sensory methods have some degree of subjectivity. The same quality measured in different places, at different times, or by different inspectors, could be estimated to have a different quality (Morin, 1989).

2.5.2. Physical Methods

Many tests have been developed to monitor the physical changes that take place in meat and fish. Refractive index, textural changes, electrical conductivity, optical tests, surface tension, viscosity, drip, and internal friction are some physical tests reviewed by Gould (1971). The Torrymeter is widely used to measure spoilage in fish, it measures changes in the dielectric properties of fish as spoilage proceeds (Connell et al., 1976). This method is based on the difference between the ratio of two electrical resistances of the cell tissue as measured by electrical currents of different frequencies (Pedrosa and Regenstein, 1990). The disadvantages of an earlier instrument were resolved by measuring the power factor of intact fish. The power factor decreases uniformly as the fish and meat spoils (Cheyne, 1975). Since the power factor is dependent on temperature, an automated correction thermistor in the apparatus gives readings corrected to temperature. In general, most physical tests have not shown satisfactory correlation with freshness of meat and fish (Pedrosa and Regenstein, 1990).

2.5.3. Physico-Chemical Methods

Physico-chemical tests used to determine the freshness of meat and fish include pH measurement, the titratable alkalinity (TA), and extract release volume (ERV).

2.5.3.1. pH

Yamanaka (1990) studied the use of pH as a potential index for freshness of fish, and found an initial decrease followed by increase at the onset of decomposition. Consequently, pH might be useful as an index for the stage of initial decomposition only. Relatively small increase in pH, along with the fact that the change is not usually uniformly distributed, makes direct pH measurement unsuitable for detecting incipient spoilage (Shelef and Jay, 1970).

2.5.3.2. Titrable Alkalinity/ Acidity (TA)

The TA method is based on the fact that as meat undergoes refrigerator spoilage, there is a gradual increase in the production of alkaline substances by the spoilage flora. The level of these substances was measured by titrating filtered aqueous extract of meat and fish to pH 5.00 (Shelef and Jay, 1970). The TA value is affected by both acids produced by microorganisms and amino acid metabolism leading to production of ammonia (Sutherland et al., 1976).

2.5.3.3. Extract Release Volume (ERV)

The measurement of ERV is a simple objective method for assessing freshness of refrigerator-stored meat and fish. It was introduced in 1964 (Jay, 1964) and has since been applied to pork (Borton et al., 1968), seafood products (Shelef and Jay, 1971), and vacuum-packaged meat (Patterson and Gibbs, 1977).

2.5.3.4. Lactic Acid Determination

Changes in other constituents like glucose, glycogen, and lactic acid are also examined as freshness indicators of meat and fish (Sutherland et al., 1976). Lactic acid determination is based on the addition of $CuSO_4$ and solid calcium hydroxide to aqueous meat and fish extract to remove substances which might interfere with lactic acid determination. Lactic acid

can be estimated by the method of Barker and Summerson (1941) which is based on converting lactic acid into acetaldehyde by treatment with concentrated sulphuric acid and the consequent determination of acetaldehyde by its color reaction with p-hydroxydiphenyl in the presence of cupric ions, read at 560nm. Sutherland et al. (1976) reported that there is no correlation of lactic acid concentration with the proportion of lactic acid bacteria in the microflora, maybe due to the further metabolism of lactic acid by the remainder of the microflora. Lactic acid and carbohydrate determination is therefore not a useful method for evaluating the overall bacteriological condition of packaged meat and fish.

2.5.4. Fat and Lipid Oxidation

Fat oxidation is an important deterioration reaction causing flavour, colour, and textural changes associated with rancidity (Pedrosa and Regenstein, 1988). Lipid hydrolysis is a common postmortem feature in meat and fish. The major products are free fatty acid (FFA) and glycerol. Hydrolysis is caused either by lipolytic activity of fish and meat or by bacterial lipases during meat and fish storage (Pedrosa and Regenstein, 1988). A variety of procedures have been proposed for estimating lipid oxidation in meat and fish. Among the most widely used methods are determination of thiobarbituric acid, free fatty acid and peroxide value.

2.5.4.1. Thiobarbituric Acid (TBA)

The thiobarbituric acid (TBA) test is one of the more commonly used methods for the detection of lipids oxidation (Gray, 1978). A red pigment is formed as result of a condensation reaction of two molecules of acidified TBA with one molecule of malonaldehyde, a secondary product in the oxidation of polyunsaturated fatty acids (Figure 1).

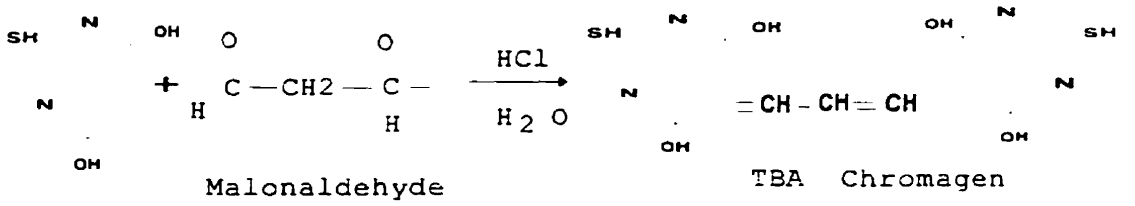


Figure 1. Proposed TBA reaction (Gray, 1978)

TBA measures deterioration in both extractable and non-extractable lipids (Kirk and Sawyer, 1991). The TBA test is based on measurement of the absorbance of the red pigment at 532nm, however, other pigments (notably a yellow pigment) with maximum absorbance have been reported at 450 nm (Gray, 1978). TBA can also react with compounds (e.g., sucrose and acetaldehyde) other than those found in lipid oxidizing systems to produce the characteristic red pigment. In complex biological materials such as animal tissue, poor correlation between TBA value and other indicators of oxidation have been observed, due to the competition between protein (Cysteine-arginine) and TBA for malonaldehyde (Buttkus and Rose, 1972). In addition, Tarladgis et al. (1962) reported that the structure of TBA was altered by acid and heat treatment and by the presence of peroxides.

2.5.4.2. Peroxide Value

The primary products of lipid oxidation are hydroperoxides (Figure 2). There are at least three methods to determine peroxide value; these are the iodometric, ferric thiocyanate and 2-6-dichlorophenolindophenol methods. The iodometric method is widely used, it is based on the reaction of potassium iodine with sodium thiosulphate, with chloroform as a solvent (Kirk and Sawyer, 1991). According to Gray (1978), two principal sources of error are (i) the

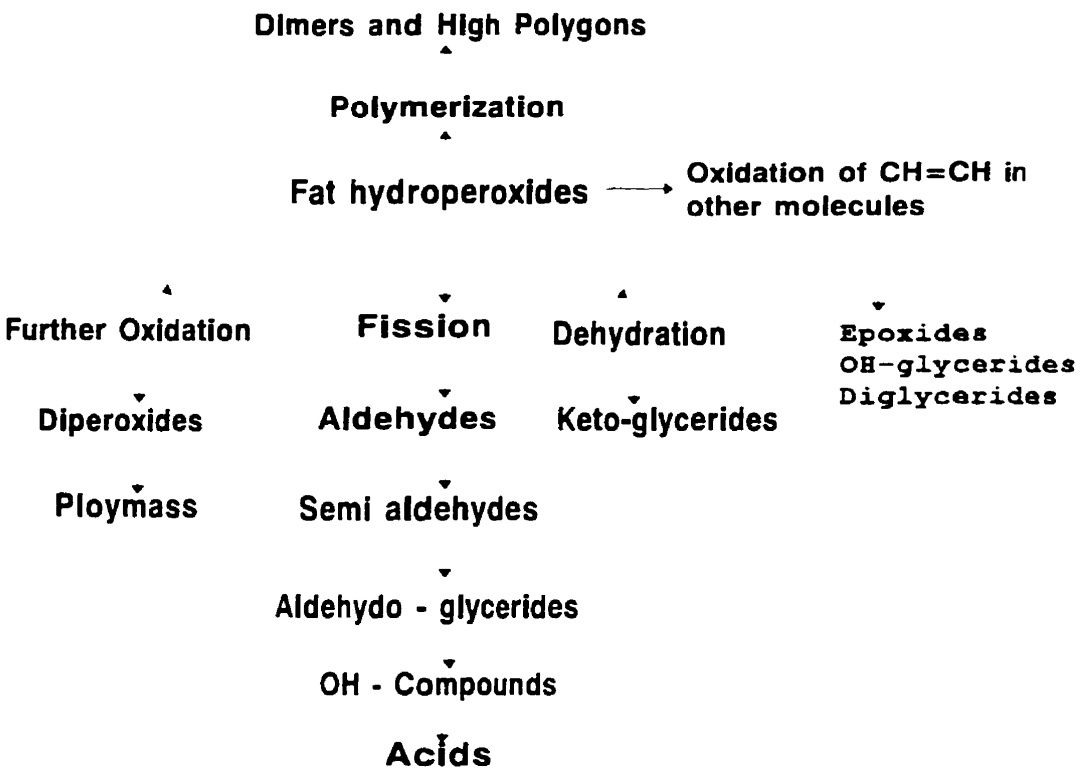


Figure 2. Some pathways of decomposition of fat hydroperoxide (Gray, 1978)

absorption of iodine at unsaturated bonds of fatty material, and (ii) the liberation of iodine from potassium iodide by oxygen present in the solution to be titrated. In addition, due to their transitory nature, peroxides are intermediate products in the formation of carbonyl and

hydroxy compounds (Figure. 2). Consequently, determination of peroxides concentration as a measure of lipid oxidation, has certain limitations (Gray, 1978).

2.5.5. Microbial Methods

The number of microorganisms found in meat and fish can be a reliable indicator of the state of freshness of meat and fish (Pedrosa and Regenstein, 1990). The two most widely used types of microbiological methods are the standard plate count (SPC) and the selective plate count. A SPC test gives a comparative measure of the overall degree of contamination for which the temperature of incubation should always be quoted since the type (psychrophilic or mesophilic) and rates of bacterial growth critically depend on temperature (Connell et al., 1976). The selective methods utilize a special media that selectively favors the growth of a particular group of organisms such as pathogenic bacteria (Pedrosa and Regenstein, 1990).

Microbial methods for assessment of freshness have certain disadvantages. Firstly, not all species of bacteria present on meat and fish causes spoilage. As spoilage proceeds, the number of spoilage organisms as a proportion of the total bacteria population changes. Secondly, most bacteriological tests require two to three days to complete. Thirdly, as various new methods of preserving meat and fish are tested, the level of bacteria that would indicate problems might change. In the extreme case, the spoilage, of even fresh meat and fish, would no longer be basically microbiological (Pedrosa and Regenstein, 1990).

2.5.6. Nucleotide Degradation Products

2.5.6.1. Hypoxanthin (Hx) Tests

Hypoxanthin (Hx) is a normal constituent of fish, although it is present in very low concentration in the living animal. It is the end product of the series of enzymatic breakdown

reaction of ATP (Pedrosa and Regenstein, 1990). After death, hypoxanthine increases in most species and can thus be used to discriminate between different degrees of good quality of meat and fish (Howgate, 1982). An enzymatic test method, uses the enzyme xanthine oxidase to convert hypoxanthine to xanthine and then to uric acid (Figure 3) which is measured spectrophotometrically at 290 nm(Jahus et al., 1976).

2.5.6.2.K-Value

As fish and meat age, adenosine triphosphate (ATP) breaks down (Figure 3) mainly to ADP to IMP to I to Hx (hypoxanthine) to xanthine to uric acid (Pedrosa and Regenstein, 1988). Certain degradation products of ATP are indicators of fish freshness. For example, inosine monophosphate (IMP), has flavour enhancing properties, while hypoxanthine, has a bitter flavour, which is associated with the taste of spoiled fish (Morin, 1989). Generally, the reaction involved in converting ATP to IMP in the early stage after death is rapid, while the IMP to inosine step is rate limiting (Pedrosa and Regenstein, 1988).

The measurement of intermediates for the degradation of ATP at any given time, gives an estimation of freshness of the fish (Morin, 1989). In particular, determination of the total concentration of hypoxanthine and IMP is considered a reliable indicator of fish freshness, hypoxanthine or inosine alone cannot be used as universal indicator of fish (Pedrosa and Regenstein, 1988).

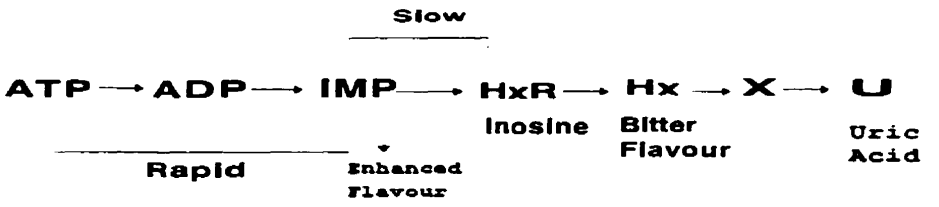


Figure 3. Known degradation pathway of ATP (Morin, 1989 and Pedrosa and Regenstein, 1981)

The K-value (Equation 1) is the ratio of inosine plus hypoxanthine, to the total amount of all degradation products of ATP (Luong et al., 1991).

$$Kvalue = \frac{[HxR]+[Hx]}{[ATP]+[ADP]+[AMP]+[IMP]+[HxR]+[Hx]} X100 (Equation 1)$$

(HxR is inosine; Hx is hyoxanthine; ATP, ADP, AMP are adenosine tri-phosphate, adenosine diphosphate, adenosine mono phosphate, respectively; IMP is inosine mono-phosphate)

Since ATP, ADP, AMP generally disappear quickly after death, Equation 1 can be simplified to Equation 2 (Ohashi et al., 1991).

$$Kvalue = \frac{[HxR]+[Hx]}{[IMP]+[HxR]+[Hx]} x100 (Equation 2)$$

Loung et al. (1989) developed a technique for HxR, and Hx and IMP determination in fish homogenate. A polarographic probe was attached to a temperature controlled reaction chamber in which metabolites in fish muscle were enzymatically degraded by xanthine oxidase, nucleoside phosphorylase, or nucleotidase to uric acid and peroxide (Figure. 4) which where detected by a polarographic electrode.

a) Fish metabolites + Enzyme (nucleoside phosphorylase NP)

$$[H \times R] + [Hx] \longrightarrow 2[Hx]$$

b) Fish metabolites + Enzyme (NP + nucleotidase)

$$[IMP] + [HxR] + [Hx] \longrightarrow 3\,[Hx]$$

Figure 4. Enzymatic reaction which involves in nucleotide determination by polarographic probe

2.5.7. Polyamines

In fish, free histidine is abundant and is easily converted to the corresponding amine, histamine (Hm), by decarboxylation of bacterial enzymes (Yamanaka, 1990). Polyamines other than Hm can be also formed from amino acids by the action of bacterial enzymes (Yamanaka, 1990). Ingestion of foods which contain high levels of histamine and other

biogenic amines cause clinical symptoms, such as histamine poisoning (Taylor, 1983). Yamanaka (1990) found that the histamine content in sardines after 1 day of storage at 20°C was 104 mg/100 g which exceeded the level (100 mg/100g) required to cause allergy-like food poisoning, although the fish was acceptable organoleptically.

There are several reports on the increase of histamine (Hm), cadaverine (Cad), putrescine (Put), tyramine (Tym), tryptamine (Tpm) and agmatine (Agm) concentration in stored fish (Yamanaka, 1990) and beef (Edward et al., 1985 and Sayem-El Daher and Simard, 1985). These polyamines can serve as a quality index for fish and meat.

Naguib et al. (1995) summarized different methods used for determination biogenic amines: (a) a bioassay method based on the observation of guinea pig ileum contraction which has not yet been applied to foods. (b) a colorimetric assay using a ninhydrin, or Pauly's diazo reagent, in which colour was measured spectrophotometrically, but was not considered to be satisfactory (Taylor, 1983). (c) a fluorometric assay based on conversion of amines to stable and strongly fluorescent products by reaction with reagents such as dansyl chloride. (d) the current official method (AOAC, 1975) which uses phthaladehyde for derivatization of histamine resulting in fluorescence which is measured spectrophotofluorometry or densitometry. Lerke and Bell (1976) reported that the official method is slow and time consuming and lack sensitivity at very low levels; it is used only for histamine determination. (e) an enzymatic isotope assay based on the incubation of tissue sample with trace amount of (^{3}H) histamine and (^{14}C)-S-adenosylmethionine in the presence of the enzyme histamine methyl transferase (Arnold and Brown, 1978). (f) a gas chromatography (GC) method based on derivatization of polyamines to form perfluoropropionyl products (Staruszkiewics and

Bond, 1981). (g) HPLC after derivatization with phthalaldehyde and mercaptoethanol which convert polyamines into fluorescent derivatives (Yamanaka, 1990).

HPLC, GC, and enzymatic isotope assays are among the more recent techniques which offer some advantages in accuracy and flexibility, but are costly. Naguib et al. (1995) reported that separation of biogenic amines could be obtained by using two dimensional TLC with two solvent systems providing simple and inexpensive means to separate and detect biogenic amines.

Pedrosa and Regenstein (1990) suggested that since hypoxanthine peaked at ten days and declined after 15 days of storage and polyamines did not begin to develop after 8 days after storage, the combined analysis of hypoxanthine and polyamines could reflect the freshness and incipient spoilage phases of fish and more adequately evaluate overall quality than either procedure alone.

2.5.8. Trimethylamine (TMA)

TMA is produced during fish spoilage, although a small amount may be derived from the intrinsic enzymes in fish. It is mainly derived from bacterial action (Figure 5) of trimethylamine oxide (TMAO), a natural compound of marine fish, by bacterial enzyme triaminooxidase (TAO) (Pedrosa and Regenstein, 1990). TMA is one of the major components associated with smell of spoiled marine fish. A ratio of TMAO/TMA has been proposed as an objective index of raw fish quality (Pan, 1989).

Several procedures have been proposed for estimating the quantity of TMA present in fish. Among the most widely used methods are: steam distillation (Hjorth-Hansen and Bakken, 1947), the Beatly and Gibbons (1936) modification of Conway microdiffusion

method, the Dyer method (Ritchner et al., 1941) and chromatographic methods (GC and HPLC) (Wong et al., 1967). Other methods include the use of specific TMA electrodes and the automatic methods (Chang et al., 1976). The Dyer determination of TMA is often used; it is a colorimetric method that is based on treating of fish muscle extract with formic acid to fix any ammonia (NH_3) present, followed by liberation of ammonia by adding alkali. The TMA is then extracted with toluene, reacted with picric acid and estimated colorimetrically at 410 nm (Shewan et al., 1971); however, Dyer method is tedious and expensive (Chang et al.,

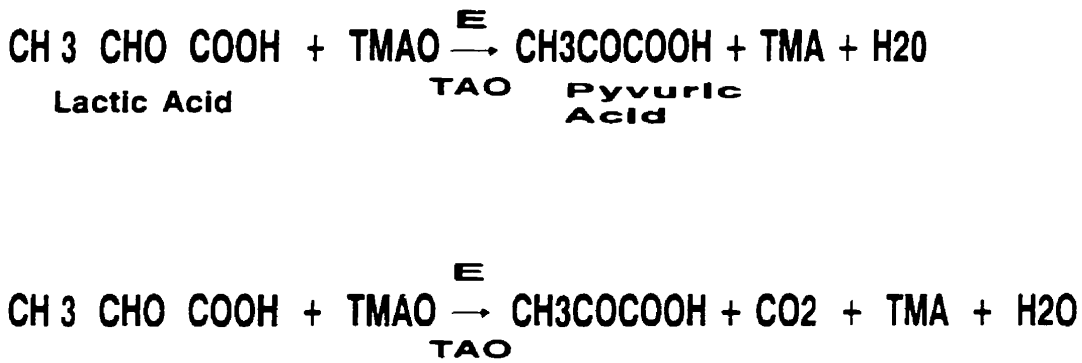


Figure 5. Reduction of Trimethylamine oxide and Formation of Trimethylamine

1976). Although TMA method is used frequently, it is not considered suitable for evaluation the freshness of fish stored for less than 6 days in ice (Pedrosa and Regenstein, 1990)

2.5.9. Protein Degradation Products

2.5.9.1. Total Volatile Bases (TVB) (Nitrogen) (TVB-N)

In meat and fish, as spoilage approaches, there is formation of volatile bases which are comprised mainly of ammonia, trimethylamine (TMA) and dimethylamine (DMA) in amounts that depend on the species and the degree of spoilage (Pedrosa and Regenstein, 1990). As a result, TVB is used as an alternative to measure TMA content. However, the TVB content is not a sensitive index of freshness because of its high variability; the test is usually reserved for meat and fish near the limits of acceptance (Howgate, 1982).

The Conway and Byrne, (1933) method for the microdetermination of TVB is widely used. The methods for TVB are based on (a) TCA extraction/microdiffusion, (b) MgO with direct distillation, (c) ethanol extraction/direct distillation (d) MgO with steam distillation, (e) TCA extraction / steam distillation and (f) $MgSO_4$ extraction / steam distillation. Botta et al. (1984) evaluated the accuracy, precision, suitability as an index of spoilage, speed and cost of determination of six different methods of determining TVB and concluded that the methods were similar in their suitability as an index of spoilage.

2.5.9.2. Non-Protein Nitrogen (NPN)

NPN is defined as "peptides that are too small to be precipitated and filtered, free amino acid (AA), amides and other nonpolymeric nitrogen (N) constituents of the plant or animal product" (Imafidon and Sosulski, 1990). Determination of NPN is often performed by two methods. Firstly, extracting biological materials with TCA; the proteins are precipitated by TCA leaving only low molecular weight compounds (amino acids, amides, peptides and polypeptides) in solution (Wolf et al., 1994). Optimum concentration of TCA is established

by examining the solubility of nitrogen compounds as a function of TCA concentration and determining the concentration that gives minimal values for the nitrogen extracted (Wolf et al., 1994). Secondly, NPN can be determined by measuring the total nitrogen content of the ultrafiltration from ethanol extracts (25%, V/V) of finely ground food products. Bell (1963) carried out a comparative study for determining NPN by twenty methods and concluded that none of the methods studied completely avoids the main sources of error (hydrolysis of protein and adsorption of NPN onto protein) in determining NPN yet dialysis and gel filtration techniques appeared to achieve separation most closely relative to NPN.

Imafidon and Sosulski (1990) extracted NPN from 20 primary food products and concluded that despite the comparatively high N contents of the animal products, including beef and fish, the NPN values were less than 2.5 mg/g of sample or 3.5 % of the total N. Glutamic acid and glutamine were the principle free amino acids in most food products. Of the NPN constituents, the concentration of free low molecular weight peptides decreased whereas the concentration of free amino acid increased during the early spoilage period (DeMasi et al., 1990). Espe et al. (1992) indicated that NPN and TVN values increased in stored fish due to the autolytic activity of endogenous enzymes present in fish. Amino acid composition, however is not effected during storage.

2.5.9.3. Water Soluble Nitrogen (WSN) and Total Nitrogen (TN)

The ratio of water soluble nitrogen to total nitrogen (WSN:TN) content of meat and fish is commonly used as an index of spoilage. Nitrogen is usually determined by the Kjeldahl procedure which both laborious and time consuming. Alternatively WSN can be measured according to Lowry et al. (1951). This method measures only tyrosine components of the

protein but, by using bovine serum albumin as a standard, the result can be expressed in terms of TCA-soluble protein (Aksnes, 1988). WSN can be measured by the reaction of a homogenate with 12%(W/V) TCA to precipitate protein and the assay of the supernatant with TNBS (Gallagher et al., 1994).

2.5.9.4. Free Amino Acid

As protein breakdown proceeds in fish and meat spoilage, increasing quantities of soluble amino acids are produced. Reagents that react specifically with α-amino groups have been employed to measure free amino acid in meat and fish, including nihydrin (Reimerdes and Klostermeyer, 1976), 2,4,6-trinitrobenzenesulphonic acid (TNBS) (McKellar, 1984), o-phthalaldehyde (Church et al., 1983), and fluorescamine (Kwan et al., 1983). Most of these methods require protein to be removed by precipitation with trichloroacetic acid (TCA).

Free amino acids are highly correlated with flavor development in meat and fish. These amino acids have been reported as precursors of sour, sweet and bitter taste (Kato et al., 1989). On the other hand, amino acid degradation to amines would affect the health of the consumers if the biogenic amines are formed (Córdoba et al., 1994). In muscle, major NPN initially present originates from peptide nitrogen, while amino acid nitrogen is the dominant source during storage (Ventanas et al., 1992). Levels of tryptophan and glutamic acid increase during meat storage; changes were increased as the bacterial load and storage temperature increased

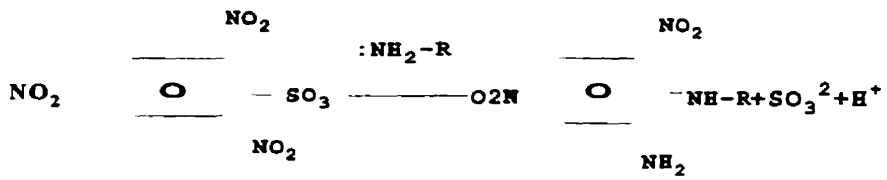


Figure 6. Reaction of TNBS with amino acid

(Gardner, 1966). In fish, glutamic acid, B-alanine and L-methyl-histidine rose during chill storage, while alanine, lysine and leucine decreased during storage (Hodgkiss and Jones, 1955). Jiang and Lee (1985) have proposed the use of free amino acid content as an indicator of freshness in fish, since muscle having higher free amino nitrogen content also had greater protein denaturation.

2.6. Electrophoresis

The term electrophoresis is used to describe the migration of a charged particle under the influence of an electric field. Proteins are charged at pH other than their isoelectric point (PI) and thus will migrate in an electric field in a manner dependent on their charge density, size and relative hydrophobicity (Dun, 1989). The major advantages of electrophoresis are: 1) it permits the analysis of partially purified sample, 2) it eliminates the losses and, 3) it is a commonly used technique that can be performed in most laboratories (Charbonneau, 1993).

Animal muscle protein can be separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) such that samples containing different proteins or different amounts of the same protein can be distinguished by examination and comparison of the resulting protein profiles (Savage et al., 1995). Studies have indicated that SDS-PAGE is a time consuming, but sensitive method of detecting molecular changes in stored fish and meat muscle tissue (LeBlance and LeBlance, 1989). SDS-PAGE has gained wide application in monitoring the proteolytic degradation of meat myofibrillar proteins (Olson et al., 1977; Penny, 1980; Bechtel and Perrish, 1983; Kooharaie et al., 1984 a,b; Zeece et al., 1986; Xiong and Anglemier, 1989; Crouse et al., 1991 and Negishi et al., 1996) as well as sarcoplasmic proteins (Hay et al., 1973; Dainty et al., 1975; Harrington and Henahan, 1982; Yates et al., 1983; Bechtel and Parrish, 1983; Xiong and Anglemier, 1989; McCormik et al., 1988; Wang et al., 1996 and Park et al., 1996).

SDS-PAGE often has been used as an aid in the species identification of fish and fish products (Mackie, 1969, 1972, 1980; Laird et al., 1982; Nakagawa et al., 1988b; An et al., 1988 and Huang et al., 1995), to monitor fish myofibrillar (Gill et al., 1979; Yowell and Flurkey, 1986) and sarcoplasmic protein degradation (Yowell and Flurkey, 1986; Nakagawa et al., 1988a and Park et al., 1996). Moreover, SDS-PAGE were used to examine changes in protein solubility (Xiong and Brekke, 1989), meat composition and palatability (Xiong et al., 1996) and digestibility (Kim and Barbeau, 1991).

2.7. High Performance Liquid Chromatography

Reverse-phase high performance liquid chromatography (RP-HPLC) is a rapid method for separating amino acids, peptides and proteins based primarily on hydrophobicity (Regnier

and Gooding, 1980 and Lemieux and Amiot, 1989). Howard and Martin (1950) were the first to describe the reversed partition process as a reversed-phase chromatography; the term reversed-phase implies that the stationary phase is a non-polar liquid and the mobile phase is a more polar solvent. RP-HPLC has received considerable attention for proteins and peptides due to its versatility, short analysis time, effective separation, high resolution, column stability, economy and low toxicity of its common solvents, and the ease of gradient elution (Little et al., 1991 and Polo et al., 1992). The main disadvantages of RP-HPLC in particular and HPLC in general are their availability, cost and the experience needed to use them (Naguib et al., 1995).

RP-HPLC has been used for a variety of different applications including the separation of peptide hormones (O'Hare and Nice, 1979), the characterization of proteolysis (Fullmer and Wasserman, 1979 and Cliffe et al., 1991), the optimization of protein synthesis (Lobley and Lovie, 1979), the measurement of protein concentration (Shimizu et al., 1989), evaluating protein cross-linking and biodegradability (Kim and Haering, 1994) and the separation of globin chains (Petrides et al., 1980). RP-HPLC is considered the standard procedure currently employed in the routine monitoring for domic acid in mussel extract (Smith et al., 1995). Moreover quantitative and qualitative determination of biogenic amines (Yen and Hsieh, 1991) and nucleotide degradation products (Loung et al., 1991) is performed by RP-HPLC.

Chromatographic separation of bovine, rabbit, chicken, porcine and fish sarcoplasmic and myofibrillar proteins have been reported using ion-exchange (Fujimaki and Deathereroge, 1964 and Rampton et al., 1965), affinity elution (Scopes, 1977) and size exclusion chromatography (Klotz et al., 1978; Mozersky et al., 1981; Schreurs et al., 1983; Davis and

Anderson, 1984; LeBlance and LeBlance, 1989 and Murch et al., 1992). RP-HPLC was used to separate and identify fish (Ashoor and Knox, 1985; Osman et al., 1987; Ashoor et al., 1988 and Armstrong et al., 1992) and meat sarcoplasmic and myofibrillar proteins(Grandier-Vazeille and Tetaert, 1984; Libera et al., 1984 and McCormick et al., 1988). These studies were performed either to monitor proteolysis or to differentiate species.

Although monitoring meat and fish proteolysis by RP-HPLC is not well established, monitoring proteolysis of cheese by RP-HPLC has been used during the last decade (Cliffe et al., 1991). For example. Lee and Warthesen (1996) used RP-HPLC to separate and determine peptide and amino acids that cause bitterness in cheese while many researchers have monitored proteolysis during the ripening of cheese (Minagwa et al., 1989; Altemueller and Rosenberg, 1996; and Jin and Park, 1996).

2.8. Mass Spectrometry

Mass spectrometry (MS) is a microanalytical technique that provide characteristic information regarding the structure and molecular weight of the analyte. It is based on transferring energy into neutral molecule to induce ionization. The ionized molecules then explodes into a variety of fragment ions. In principle, the fragment ions are unique for each compound and can be used as a chemical fingerprint to characterize the sample (Watson, 1985).

There are four common types of mass spectrometer detectors: magnetic, quadruple, time of flight and fourier transform. In the magnetic sector mass spectrometer ions, are transfered from their source into discrete ions beamed by a process of momentum dispersion and direction focusing . Magnetic scanning is advantageous because the entire mass range may

be scanned while the instrument maintains optimum accelerating potential. However, if scan speeds exceeds a certain rate (0.1 sec/decade) the magnetic field can change significantly during the ion transit period, causing ions to follow a distorted path to the detector (Holland et al., 1983). Consequently, this type of spectrometer is not recommended when fast scan rates are needed.

Quadruple mass spectrometer (QMS) employs a combination of direct current (DC) and radio frequency (RF) potentials as a mass filter. The DC/RF ratio is adjusted so that only ions of specific M/Z (mass to charge ratio) avoid collision with rods and successfully traverse the quadruple filter to reach the detector. Furthermore, the ratios that controls the mass scale can be changed rapidly, with good response, and are well stabilized throughout the mass range (Caprioli and Bruner, 1993). Therefore QMS is more capable of maintaining quality output at higher pressure than other conventional MS; this may be an advantage in chemical ionization (CI), GC-MS or LC-MS application.

The operating principle of the time of flight (TOF) mass spectrometer involves measuring of the time needed for an ion to arrive at a detector located 1 to 2 m from the sources (Harrington, 1960). All the ions will receive the same kinetic during acceleration, but the velocity of movement will differ due to differences in masses, thus separation will occur. The fact that TOF instrument has an electron multiplier and that can be scanned rapidly, make it suitable for GC-MS application.

Fourier transform mass spectrometry (FT-MS) is based on the original technique called ion cyclotron resonance (ICR). ICR is based on the principle that ions in a magnetic field move in circular orbits with a characteristic cyclotron frequency, Wc, that depends only

on the M/Z value of the ion.

$$Wc = ZeB/2\prod m$$

If energy is provided to these ions at a frequency equal to their process frequency, the ions will absorb the energy and move to orbits of larger radius and become chorent. The ions will transmit a complex RF signal to the excited chip. These signals contain the frequency components characteristic of all of the ions present.

2.9. LC-MS of Peptides and Proteins

The MS is probably the ideal detector for liquid chromatography (LC), as it is capable of providing both structural information and quantitative analysis of the various components of mixture separated by HPLC, with minimum handling losses and analysis time. The main constraint imposed with direct coupling of LC and MS is the radical difference between the LC environment (solute at room temperature) and the MS electron impact source requirement (gas phase at relatively high temperature and in a very high vacuum) (Caprioli and Bruner, 1993). Different interface techniques have been explored to overcome direct LC-MS coupling problems. Some of these technique are moving wire/belt system, a 1% split of the LC effluent into MS (Covery et al., 1991), a vacuum nebulizing coupled with Micro-LC, a particle beam coupling device (Caprioli and Bruner, 1993), and electrospray (Loo et al., 1989)

To generate ions from so-called non-volatile or thermally labile molecules, such as peptides and proteins, soft ionization is required. Field desorption (FD), desorption chemical ionization (Cotter, 1980), ^{252}Cf plasma desorption(^{252}Cf-PD), laser desorption (Karas and Hillenkamp, 1988), fast atom bombardment (FAB) (Rinehart, 1982) and secondary ion MS (LSIMS) are considered soft ionization techniques, producing stable even electron molecular

ions (MH^+). The main advantage of these techniques is direct MS analysis without the necessity of chemical derivatization to improve volatility and stability of the molecule of interest (peptides).

3. MATERIAL AND METHODS

3.1. Materials

Eighteen Carp fish (*Cyprinus carpio*) were purchased fresh from a local shop (Waldmanplus; Montreal, Canada). The fish were immediately placed on ice in an insulated box and transported to the laboratory, they were then immersed and covered with wet ice in an insulated container and kept in a refrigerated room (5 ± 1°C). Three fish were selected at random after 0, 2, 4, 8, 12 and 16 days of storage in the laboratory for microbial and protein analysis. The average weight of fish was 1380 g (min. 1000 g, max. 1800 g).

3.2. Microbiological Analysis

Fish removed from storage was washed with running tap water and flesh was removed using a flamed, sterile knife and forceps. The underlying muscle was aseptically removed, 10 g of muscle was placed in sterile stomacher bags (Seward 4000, England) containing sterile pre-measured 90 ml of 0.1 % peptone diluent (Difco, MI, USA); this mixture was pummelled for 1 min in a stomacher 400 (Seward Medical, England) and 1 ml of the homogenate was transferred into a sterile screw-cap dilution bottles containing 9 ml of 0.1 % peptone diluent. Decimal dilution series were prepared for each sample.

Total aerobic bacteria counts were determined by placing 1 ml of the original homogenate and 1 ml from each dilution in sterile petri dishes(AOAC 1990). Plate count agar (PCA, Difco) heated to 50 °C was poured into the inoculated plates and swirled to distribute the sample evenly. The plates were left to dry, then incubated at 37 ± 1 °C; colonies were counted after incubation for 48 h.

Psychrotrophic and anaerobic bacteria were enumerated by spreading 0.1 ml of

original sample homogenate and 0.1 ml from each dilution on prepoured(AOAC 1990), dried PCA (Difco, USA) with a sterile bent glass rod. Psychrotrophic plates were incubated at 5 ± 1 °C for 7 days. Anaerobic plates were placed in an anaerobic jar equipped with a palladium catalyst (Fisher Scientific, Montreal, Canada) and hydrogen and carbon dioxide generating kits (BBL, USA) were activated to flush the jar. To insure complete anaerobic condition, anaerobic indicator strips (Difco, USA) were used. Incubation of anaerobic plates was done at 37 ± 1 °C for 48 h. Colonies were counted after incubation period.

3.3. pH Measurement

On each sampling day, pH of the three sampled fish was measured according to Dhananjaya and Stroud (1994). For each fish, triplicate samples of flesh (10 g) were macerated in a beaker using glass rod. Whatman filter paper (No.4) was inserted into the macerate and the beaker was stored at 5 °C for 30 min. The pH was measured with a Fisher digital pH/ion meter (Model 750 Accument, Pittsburgh, PA, USA) equipped with a flat combination electrode.

3.4. Extraction of Acid Soluble Nitrogen (ASN)

Acid Soluble Nitrogen (ASN) in fish was extracted according to the method of (DeMasi et al., 1990) with some modifications. Macerated flesh (5 g) was mixed with 100 ml 0.1 N HCL; the mixture was blended for 1 min in a Oster blender at high speed. The extract was held at 4 °C for 24 h and then centrifuged at 6500 RPM (Avanti Centrifuge, Model J-25I,Beckman, CA, USA) for 10 min. at 4 °C. The supernatant was filtered (Whatman No. 4 filter paper), and refrigerated.

3.5. Determination of Protein Concentration

The method of Lowry et al. (1951) as modified by Hartree (1972) was used to determine protein content in ASN, sarcoplasmic and myofibrils extracted from fish and meat. Bovine serum albumin (BSA, egg white extract, Sigma, St. Louis, MO) was used as standard.

3.6. Preparation of Fish Myofibrillar and Sarcoplasmic Protein Fractions

The method used was that of Dyer et al. (1950) as modified by Machado and Sgarbiere (1991) and is shown in Figure 7. Myofibrils were solubilized with 1.1 M KI in 0.1 M potassium phosphate, (pH 7.4). All extraction procedures were carried out at approximately 4 °C. A homogenized fish sample (10 g) was blended at high speed (Oster Blender) in 166.5 ml buffer solution (5% NaCl in 0.02 M $NaHCO_3$, pH 7.4) for 2 min. Care was taken to prevent the final temperature after blending from exceeding 5 °C. The mixture was centrifuged at 4000 RPM for 30 min. at 4 °C to separate insoluble material and extracted protein. The supernatant (salt extract) which consists of sarcoplasmic and myofibrillar protein was retained. The pellet, which consists mainly of stroma, collagen and denatured proteins, was discarded. The myofibrillar was precipitated by 10 fold dilution of salt extract with distilled water (4 °C) and the mixture was held at 4 °C for 24 h.

The diluted salt extract was centrifuged at 10000 X g (7500 RPM) for 15 min at 4 °C. The supernatant (sarcoplasmic protein) was filtered through 2 layers of cheese cloth and kept for further analysis. The precipitate (myofibrillar protein) was resuspended in 15 volumes of 1.1 M KI in 0.1 M potassium phosphate buffer, (pH 7.4). The mixture was blended for 30 s. at high speed followed by centrifugation at 1000 X g for 15 min and the supernatant was retained as myofibrillar protein fraction.

10 g homogenized fish + 166.5 ml 5% Nacl in 0.02 M $NaHCO_3$, (pH7.4), 4 °C

▼

In Blender with baffle plate, blend for 2 min. at high speed

▼

Centrifuge (4000 RPM, 30 min., 4 °C)

▼ Supernatant
dilute 10 fold with water (4°C), stored at 4°C for 24 h.

▼ Residue
Stroma, Collagen and Denatured Protein

▼

Centrifuge (10000 X g, 15 min., 4 °C)

▼ Pellet
Myofibrils

▼ Supernatant
Sacroplasmic protein

▼

Resuspend in 15 vol. of 1.1 M KI in 0.1 M KPO_4, pH7.4, centrifuge at 4000 RPM for 15 min. at 4 °C

▼

Myofibrillar protein

Figure 7. Procedure utilized for extracting sarcoplasmic and myofibrillar proteins from fish

3.7. Preparation of Meat Myofibrillar and Sarcoplasmic Protein Fractions

The method used was that of Molina and Toldiá (1992) and is outlined in Figure 8. All extraction procedures were carried out at approximately 4 °C. A homogenized meat sample (3 g) was homogenized for 90 sec. using Polytron (Polytron®, PT-MR 3000, Kinematic, AG, Littau, Switzerland) in 30 ml phosphate buffer solution (0.03 M, pH 7.4). Care was taken to prevent the final temperature after blending from exceeding 5 °C. The mixture was centrifuged at 4000 RPM for 15 min. at 4 °C to separate sarcoplasmic proteins. The supernatant (sarcoplasmic protein) was filtered through 2 layers of cheese cloth and kept for further analysis. The precipitate (myofibrillar, stroma, collagen and denatured proteins) was resuspended in 10 volumes of 1.1 M KI in 0.1 M potassium phosphate buffer, (pH 7.4). The mixture was blended for 30 s. at high speed followed by centrifugation at 4000 RPM for 15 min and the supernatant was retained as the myofibrillar protein fraction.

3 g Homogenized meat + 30 ml 0.03 Phosphate buffer, pH 7.4, 4°C

▼

Homogenized for 90 sec. using polytron at 20,000 RPM

▼

Centrifuge (4000 RPM, 15 min., 4 °C)

▼ Pellet
Myofibrils

▼ Supernatant
Sarcoplasmic proteins

▼

Resuspend in 10 vol. of 1.1 M KI in 0.1 M KPO_4, pH 7.4, centrifuge at 4000 RPM for 15 min., 4 °C

▼ Supernatant
Myofibrillar Proteins

▼ Residue
Stroma, Collagen and
Denatured Protein

Figure 8. Procedure Utilized for Extracting Sarcoplasmic and Myofibrillar Proteins from Meat

3.8. High Performance Liquid Chromatography (HPLC)

HPLC analysis was carried out with a Hewlett-Packard (HP) liquid chromatograph model 1090 equipped with a binary high-pressure solvent delivery system, an auto-injector and diode array detector (Model 1040 A). Spectral and chromatographic data were stored on disc using HP model 85 B computer and a model 9012 twin disc driver. 1 ml of acid soluble nitrogen (ASN) was centrifuged (14000 RPM, 4 min, Eppendorf Centrifuge, Model 415 C), the supernatant was filtered (0.45 μ, Millipore, USA), and injected into a C_{18} reversed phase column (5 mm, 25 X 0.46 cm, Vydac Co., USA) operated at room temperature. The following conditions were used to elute the sample at flow-rate of 1 ml min^{-1} from the column: - Solvent A, 0.1 % trifluoroacetic acid (TFA) in water (V/V, pH 2); and solvent B, 0.1 % TFA in acetonitrile (V/V); solvent B was increased linearly from 10 - 70% over 60 min. The fractions which were separated by RP-HPLC were collected manually, evaporated (Speed-vac concentrator, Savant, Hicksville, NY, USA) and stored at -18 °C until they were analyzed by mass spectrometry.

3.9. Mass Spectrometry (MS)

A SCIEX API III- triple-quadrupole mass spectrometer equipped with a standard atmospheric pressure ionization (API) source (Sciex, Inc., Thornhill, Ontario, Canada) was used to sample ions produced from an ion spray interface(Alli et al., 1993 and 1994). Liquid samples were introduced to the electro-spray source (ES) by continuous infusion from a syringe pump (Model 22, Harvard Apparatus, South Natick, MA, USA), through a fussed silica capillary (100 μm i.d.) at flow rate of 1-2 μL min^{-1}. The ions were detected by scanning the first quadrupole of the mass spectrometer and the scans were monitored between *m z*

700-1700 in 5 s. Between 15 and 20 scans were averaged to obtain representative spectra. The abundances of the ions are reported as percentages relative to the peak that was most intense. The detector was a Chevron type multichannel plate multiplier (3025, Galileo Electro-optics, Strurbidge, MA) used in the pulse-counting mode. Signals were recorded with a 100-MH_2 pulse counter (SR430, Stanford Research system, Sunny-vale, CA) which incorporated internal amplitude discrimination. All data were transferred to Macintosh computer over Apple File Exchange software (Apple computer, Inc., Cupertino, CA). Postacquisition analyses were conducted on the Macintosh using the Passages Software package (Version F, Passage Software, Inc., Ft. Collins, CO).

The instrument *m z* scale was calibrated with singly-charged ammonium adduct ions of polypropylene glycol ($1x10^{-4}$M in 50/50 methanol / H_2O 2mM ammonium formate, NH4oAC) under unit *m z* resolution. Before molecular weight determination of the HPLC fractions, the mass spectrometer accuracy was first checked with 1 mg/ml lysozyme in 10% acetic acid (Sigma, L-6876 grade 1, St. Louis, MO). Figure 9 shows the interpreted mass spectra of the lysozyme solution; the expected MW of 14.305 kDa was confirmed. The potential on the sampling orifice of the instrument was set at +30 V during calibration and was raised to +100 V to enhance ion signal for protein.

3.10. Electrophoresis

3.10.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis of myofibrillar protein was performed according to the method of Fritz et al. (1989). All the chemicals used for electrophoresis were purchased from Bio-Rad

Actual peak	Intensity	Pred. peak	Charge	Compound mass
1431.60	353,842	1431.56	10	14,305.92
1590.60	5,259,990	1590.51	9	14,306.33
1789.20	1,483,380	1789.20	8	14,305.54

Avg. compound mass 14,305.93 Std. Deviation: 0.40
3 Estimates of compound mass

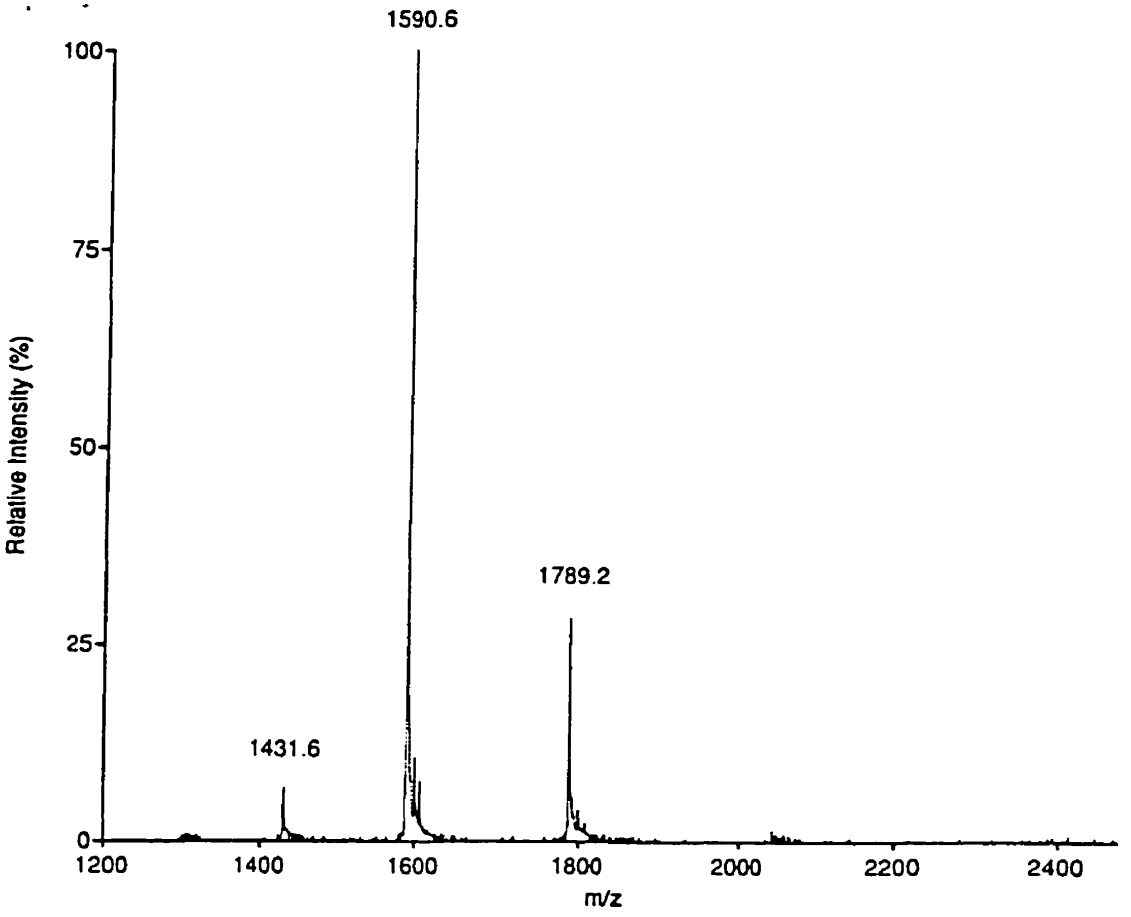


Figure 9. Representative of Typical Interpreted ESI-Mass Spectra of Standard Lysozyme. Compound Mass is 14,305 D.

(Mississauga, Ontario, Canada, electrophoresis grade) except for urea and thiourea urea which were brought from Sigma. All other chemical unless specifically noted, are reagent grade.

3.10.1.1. Sample Preparation

Freeze dried myofibrillar protein extract was diluted to a final protein concentration of 1.4 mg/ml with water. The diluted protein solutions were mixed 2:1 with sample buffer containg 3% (W/V) SDS, 0.7 M 2-Mercaptoethanol, 25 mM Tris-HCL (pH 6.8), 8 M Urea, 2 M Thiourea and 0.35 mg/ml Bromophenol-blue. The protein mixture were heated in boiling water bath for 5 min. and allowed to cool to room temperature before application to the gel.

3.10.1.2. Gel Concentration and Preparation

The final composition of the separation gel was 10% (W/V) acrylamide and 0.05% (W/V) bis. The ratio of acrylamide to bis was (200:1), so the gel contained 10.05 % total (T%) acrylamide + bis with a cross-linking (C%) of 0.5%. It also contained 0.1% (W/V) SDS, 10% (V/V) glycerol and 0.75 M Tris-HCl (pH 8.6). Polymerization was catalysed by the addition of 0.067% (W/V) ammonium persulfate (APS) and 0.067% N,N,N',N' - tetramethylethylene-diamine (TEMED). The stacking gel contained 3.9% (W/V) acrylamide, 0.1% (W/V) bis, 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS. Polymerization was catalysed by the addition of 0.05% (W/V) APS and 0.1% (V/V) TEMED. Slab gels were prepared between 7 x 8 cm glass plates. After polymerization(45~60 min.) the area above the separating gel was dried with filter paper, a comb was mounted on top of the gel and the stacking gel was poured. The comb formed 10 sample wells. Soluble protein (25 µL) and molecular weight markers (5 µL) were loaded into the sample wells using a Hamilton syringe.

The correlation of molecular weight with relative mobility was obtained with the following standard proteins (Bio-Rad. broad standard): Myosin (MW 200 kDa). ß-galactosidase (116.5 kDa). Phosphorylase B (97.4 kDa). Serum albumin (66.2 kDa). Ovalbumin (45 kDa). Carbonic anhydrase (31 kDa). Trypsin inhibitor (21.5 kDa). Lysozyme (14.4 kDa) and Aprotinin (6.5 kDa). Molecular weights (MW) of the specific protein bands were estimated from the log MW-relative migration distance plot that was generated using the protein standards (Figure 10).

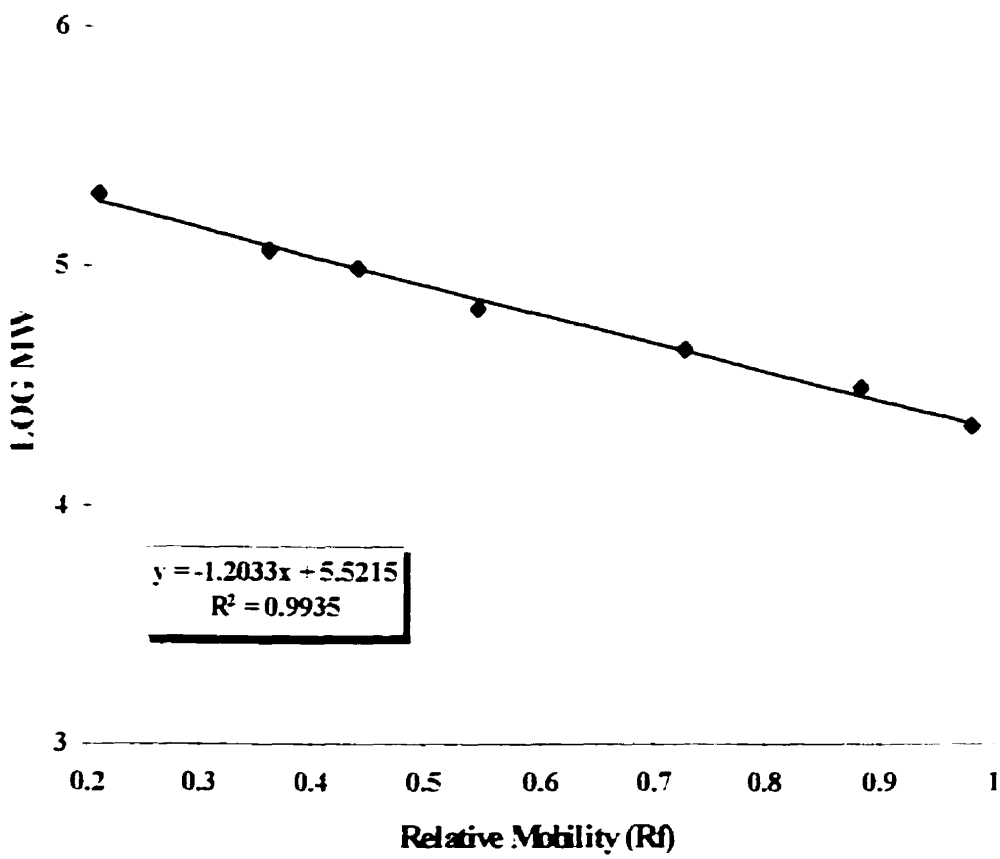


Figure 10. Plot of Log MW vs relative mobility of standard proteins

3.10.1.3. Electrophoresis Condition

The running buffer contained 50 mM Tris-HCl (pH 8.3), 0.384 M glycine and 0.1% (w/v) SDS. In order to improve the mobility and the resolution of the high molecular weight proteins, 10 mM 2-Mercaptoethanol was added to the upper buffer chamber. Electrophoresis was performed at a constant current of 15 milliamps (mA). The voltage, current and power limits for the electrophoresis run were 400 V, 30 mA and 15 W respectively. The temperature of the electrophoresis unit was maintained at 20 °C, by immersion the bottom part of the unit in water bath at 12 °C. Electrophoresis was terminated when the tracking dye front (Bromophenol blue) reached the bottom of the gel; this took 2.1 h.

3.10.1.4. Protein Fixing, Staining and Destaining

After electrophoresis, the gels were removed from glass plates and placed in a fixing solution that contained 20% (V/V) methanol, 10% (V/V) acetic acid for 2 h, and were then transferred to a washing solution (10% acetic acid, and 20% (V/V) ethanol for 1 h. (Claeys et al., 1995). Protein bands were stained by immersion of the gel for 18 h in 10% (V/V) acetic acid, 20% (V/V) methanol and 0.1% (W/V) Coomassie Brilliant Blue R_{250}. Destaining was carried out by storing of the gel in the fixing solution until the background colour was completely removed. The destained gels were stored in a 7% acetic acid until they were photographed.

3.10.2. Native Electrophoresis

3.10.2.1. Sample Preparation

Freeze dried sarcoplasmic extract (0.03g) was diluted with 0.6 ml sample buffer to give a final protein concentration of 1.4 mg/ml (3%). The sample buffer contained 0.3 M Tris-

HCL (pH 8.8), 10% glycerol and 25 mg/ml bromophenol blue.

3.10.2.2. Gel Concentration and Preparation

Native electrophoresis was performed using the method of Davis (1964) with some modifications. The separation gels consisted of 7.7% T (total) polyacrylamide 0.65% C (cross linkage) and 0.375 M Tris-HCL (pH 8.9). The ratio of acrylamide to bis was 37.5:1 Polymerization was catalysed by the addition of 0.03% (V/V) TEMED and 0.07% (W/V) APS. The stacking gels contained 3% (W/V) acrylamide, 0.75% (W/V) bis (monomer:Bis, 4:1) and 0.0617 M Tris-HCl (pH 6.7). Polymerization was initiated by the addition of riboflavin 0.0005% (W/V) and 0.0575 % (V/V) TEMED. Sucrose (20% W/V) was added to increase the gel density. Gel preparation and sample application is similar to that described in section 3.10.1.2.

3.10.2.3. Electrophoresis Conditions

Native electrophoresis was performed by using a running buffer that contained 4.95 mM Tris and 0.0376 M glycine (pH 8.3). A constant current of 10 mA per gel was used. The time of electrophoresis was 1.1 h. The electrophoresis power limits and temperature is similar to that described in section 3.10.1.3.

3.10.2.4. Protein fixing, Staining and Destaining

The method was similar to that described in section 3.10.1.4. with exception that washing step with ethanol was eliminated (SDS was not part of the gel composition)

3.11. Statistical Analysis

The Statistical Analysis System (SAS Institute Inc., Cary, NC) was used for analysis of Least significant difference (LSD) of means using completely randomized block design.

Whenever the LSD test was found to be significant, NSK and Tukey's tests were performed between the means to confirm the differences. In all cases, results were considered significantly different when $P < 0.05$.

4. RESULTS AND DISCUSSION

A. Proteolysis of Carp Fish

4.A.1. Bacterial Plate Count

Table 2 shows results of mesophilic, psychrotrophic and anaerobic plate counts; the data is plotted in Figure 11. Mesophilic and anaerobic counts found in this study were similar to those reported by Gelman et al. (1990), who also worked with carp fish; however, the psychrotrophic counts reported by these researchers were lower than that found in our study. A likely explanation for this difference is that psychrotrophic bacteria favours cold environment; the work of Gelman et al. (1990) was carried out on carp fish harvested from tropical water, while the carp fish used in our study were from cold water. The initial microbial load (log 4.43) in our samples of carp fish confirms their fresh state.

Table 4. Counts of Mesophilic, Psychrotrophic and Anaerobic Bacteria of Carp Fish

	Mean of Log_{10} CFU/g Storage Time (day)					
	0	2	4	8	12	16
Mesophilic	4.38^{b} (0.03)	3.19^{d} (0.10)	2.56^{e} (0.25)	3.71^{c} (0.16)	4.34^{b} (0.19)	5.86^{a} (0.06)
Psychrotrophic	4.42^{c} (0.09)	3.23^{e} (0.27)	3.24^{e} (0.12)	3.87^{d} (0.08)	4.66^{b} (0.19)	6.14^{a} (0.12)
Anaerobic	4.54^{b} (0.22)	2.26^{e} (0.03)	2.77^{d} (0.30)	3.54^{c} (0.14)	4.59^{b} (0.22)	5.31^{a} (0.31)

Figures in parentheses represent standard deviation of six replicates
Means within rows marked with the same superscript are not significantly different (P>0.05)

In our study, no attempt was made to eviscerate the fish since previous work has

confirmed that this step is unnecessary. Although Shewan (1962) recommended gutting to extend storage life of chilled fish, many workers found it of a marginal value (Hoffman et al. ,1974 and Maia et al., 1983). Moreover, in a sensory evaluation study (Gelman et al., 1990), gutted carp fish did not show a significant difference when compared to whole carp fish. On the contrary, whole carp fish remained in a slightly better condition than gutted fish for the

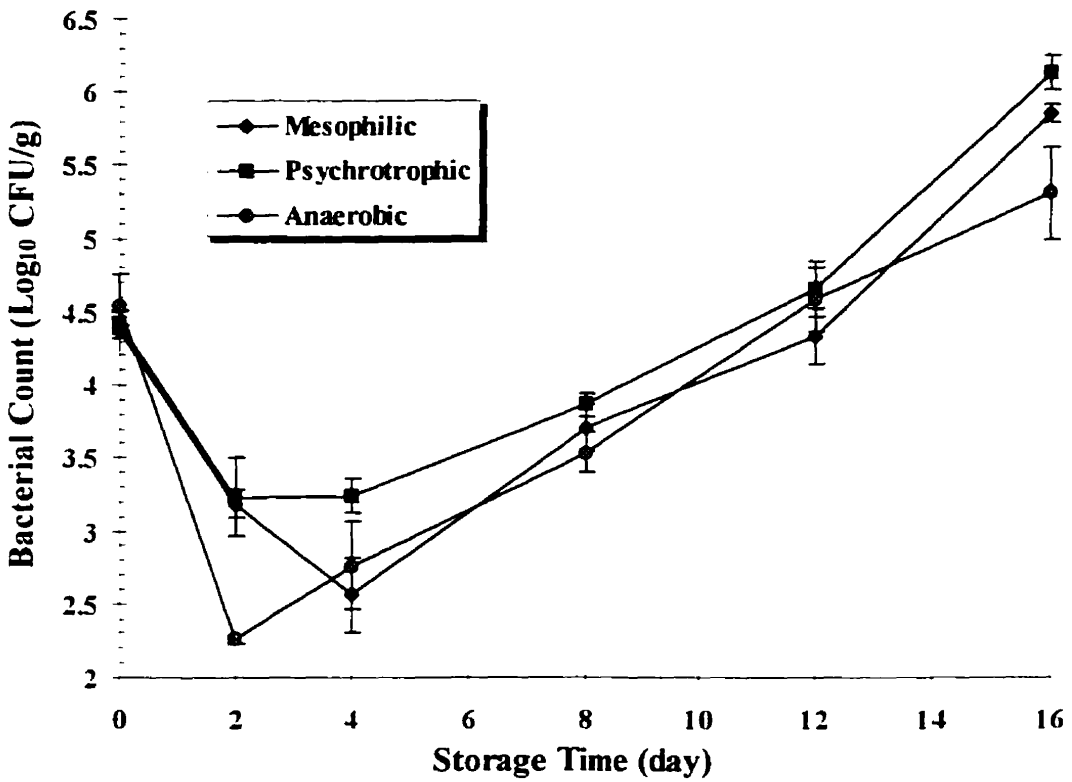


Figure 11. Mesophilic, Psychrotrophic and Anaerobic Bacterial Count of Stored Carp Fish

same storage period.

Statistical analysis demonstrated that there was no significant difference (P> 0.05) between fish samples, for mesophilic (p= 0.47), psychrotrophic (p= 0.34) and anaerobic bacteria (p=0.30) for the same storage period. Differences in microbial count between fish samples stored for the same period were explained by Lasta et al. (1995) in terms of the heterogeneity of different samples and random distribution of bacteria contamination; however this type of difference was not observed in our study.

The three groups of bacteria (mesophilic, anaerobic and psychrotrophic) showed a decrease in counts during the first 2 days of storage; however, the rate of decrease was different among the three groups (Figure 11). The psychrotrophic count decreased from log 4.42 to log 3.23 during the first 2 days, then increased to log 3.24 (day 4) to and finally to log 6.14 (day 16). The mesophilic bacteria count also decreased from log 4.38 to log 2.56 during the first 4 days. Anaerobic bacteria showed the greatest decrease from log 4.54 to log 2.26 after 2 days then increased to log 2.77 (day 4) and finally to log 5.31 (day 16). Although mesophilic bacteria showed the same decrease pattern as psychrotrophic bacteria after 2 days in storage (Figure 11), mesophilic bacteria did not cease the decrease in day 2 but decreased from log 3.19 (day 2) to log 2.56 (day 4). On day 8, mesophilic bacteria count recovered from log 2.56 (day 4) to log 3.71 (day 8) and finally increased to log 5.86 (day 16).

The decrease in bacterial number during the first 2-4 days of storage in our study was also observed by Gelman et al. (1990). This rapid decrease is the result of the 'cold shock' phenomenon. Rose (1968) suggested that 'cold shock' resulted from release of cell constituents from bacteria following the freezing of certain membrane lipids after sudden

chilling, with consequent development of 'holes' in the membrane. Based on this hypothesis the three bacterial groups showed considerably different responses to 'cold shock'. The difference in bacterial response to 'cold shock' is due to 'lipid solidification', a concept proposed by Gaughram (1947), Allen (1953) and Byrne and Chapman (1964) who suggested that bacteria increase their rate of synthesis of unsaturated fatty acid at the expense of saturated fatty acid in response to low temperature, which apparently decreases the melting point of membrane lipids. In psychrotrophic bacteria, 59 to 72% of the lipid is in the unsaturated form (Jay, 1992); this could explain the lowest observed 'cold shock' effect this group (Figure 11). Another reason for the differences in bacterial response to low temperature storage has been suggested by Farell and Rose (1968) who related the minimum growth temperature of an organism to the inhibition of substrate uptake by inactivating permease-protein to changes in the molecular architecture of the cytoplasmic membrane, and a shortage of energy required for the active transport of solutes.

The results from this study show that carp fish stored aerobically at temperature 0-2 °C up to 2 weeks have the capacity to support bacterial growth to a level which did not result in noticeable deterioration. This is in agreement with previous reports which indicate that sensory spoilage was detectable only when bacterial counts of 10^6 to 10^8 / g are reached (Gray and Sorhang, 1983; Gill, 1986 and Gelman et al., 1990).

4.A.2. pH

The pH changes are shown in Figure 12. The initial pH (day 0) was 6.41. During storage the pH decreased to 6.31 at day 2 then increased to 6.97 and 7.11 after day 8 and 10, respectively. An initial reduction in pH was also observed with haddock and herring

(Dhananjaya and Stroud, 1994) and with tilapia fillets (Reddy et al., 1995). This decrease is due mainly to the accumulation of lactic acid as a result of glycolysis (Ashie et al., 1996). During storage a slow increase in pH was reported in Pacific cod (Reppond and Collins,

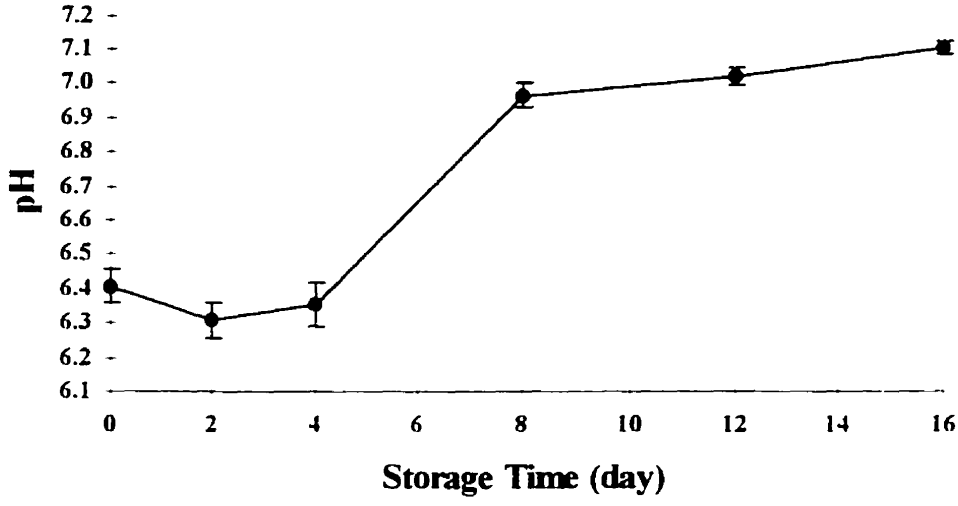


Figure 12. Changes in pH During Storage of Carp Fish

1983), mackerel (Ryder et al., 1984), orange roughly (Scott et al., 1986) and sardines (El Marrakchi et al., 1990), these authors attribute the increase in pH to the production of volatile bases.

Changes in pH which occur during storage of fish have been shown to be variable and dependent on fish species, sample handling, analysis method and lack of replication (Bilinski et al., 1983; Gelman et al., 1990; El Marrackchi et al., 1990 and Bennour et al., 1991). Several researchers have found that changes in pH are insignificant and consequently, have questioned the value of using pH as an index of spoilage. (El Marrakchi et al., 1990 and

Bennour et al., 1991).

It can be proposed that changes in pH during storage was significant under controlled experimental condition, this supports the notion that post-harvest handling affects the pH changes which occur during storage (Ashie et al., 1996).

4.A.3. Changes in Protein Content Extracted from Carp Fish

Changes in protein content of soluble proteins is shown in Figure 13. The average initial content of sarcoplasmic, myofibrillar and ASN in carp fish was 0.1, 1.2 and 3.8 g/L, respectively. Similar results were reported by Machado and Sgarbieri (1991). Protein content of ASN increased significantly at day 2 (5.1 g/L) and kept constant throughout the storage period. No significant changes was observed in the solubility of sarcoplasmic and myofibrillar during storage.

Several reports have indicated that increased storage time (Owusu-Ansah and Hultin, 1986), freezing (Matthews et al., 1979) and draying (Raghunath et al., 1995) reduce the extractability of fish proteins as a result of protein denaturation. On the other hand, several other reports indicated that changes in protein content during storage are insignificant (Xiong and Brekke, 1989 and Lin and Park 1996). Salm et al. (1983) suggested that myofibrillar protein solubility in KI buffer increased with ageing of beef. These reports and our results demonstrate the difficulty associated with the use of protein solubility as an indicator of quality.

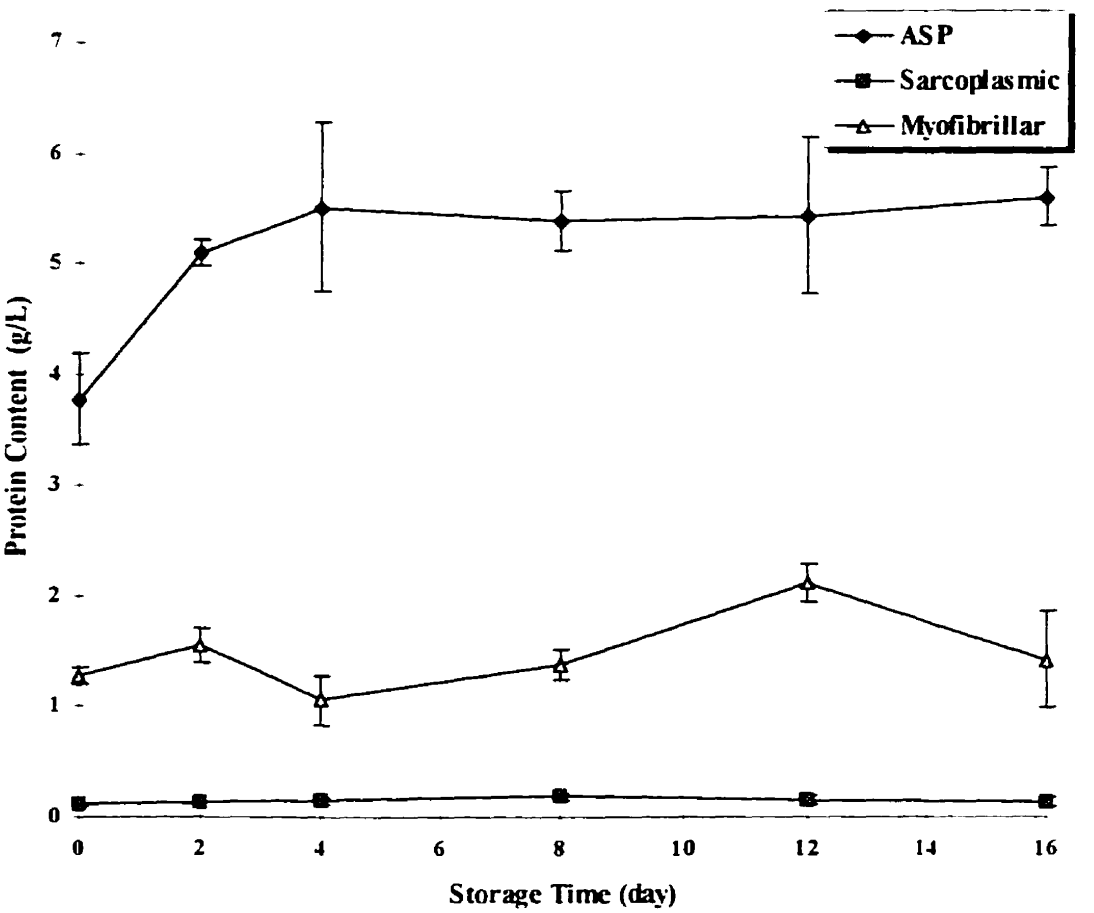


Figure 13. Changes in the Protein Content of Extract Sarcoplasmic, Myofibrillar and Acid Soluble Nitrogen (ASN) Extracted from Carp Fish

4.A.4. Electrophoresis

The effect of chill storage on the proteolysis of myofibrillar and sarcoplasmic proteins during 16 days of storage was examined by native and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Since the region of 30-50 kDa is associated with postmortem changes in myofibrillar and sarcoplasmic proteins are located (Claeys et al., 1995) and since Huang et al. (1995) reported that 10% polyacrylamide in reducing and non reducing form were able to produce protein profiles for species identification of 12 fish, 10% polyacrylamide was selected to be used in this study.

4.A.4.1. Myofibrillar Proteins

4.A.4.1.1. SDS-PAGE

Figure 14 shows the electrophoretogram (SDS-PAGE) of myofibrillar proteins extracted from carp fish stored at 0-2 °C. 12-14 bands were observed (A-L, Figure 14) and band A and G were considered to be major fractions. Table 5 summarizes the observed changes relative to day 0, and the estimated MW of the separated proteins.

It is important to mention that technical difficulties were faced in extracting myofibrillar protein at day 12, consequently, monitoring proteolytic degradation in these proteins was not possible.

As illustrated in Figure 14 and summarized in Table 5, major protein degradation took place after day 2. This is in agreement with previous studies in carp fish (Watab et al., 1990). The electrophoretogram of myofibrillar proteins for the storage period 0 and 2 days were similar with the exception that band Y (MW = 300 kDa) was of much lower intensity after day 2 (Figure 14). In general, no evident postmortem alterations in large MW proteins were

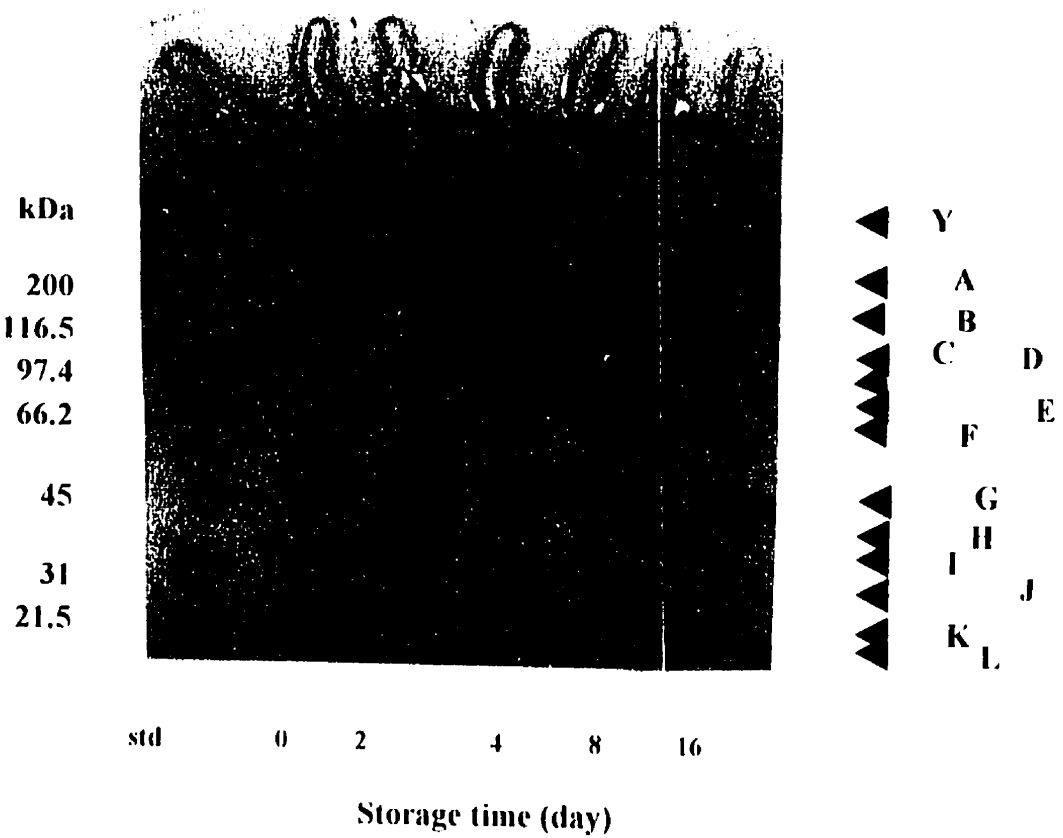


Figure 14. SDS-PAGE of myofibrillar proteins extracted from carp fish after 0, 2, 4, 12 and 16 days of storage. The labels Y-L are identified in Table 5. Numbers at left represent molecular weight of standard protein (MW X 10^3)

Table 5. Changes in Myofibrillar Protein Components of Carp Fish During Storage; (SDS-Electrophoresis)

Band	Calculated MW (kDa)	Presumptive Identity*	Storage period (day)				
			0	2	4	8	16
Y	300 - 320		P			xxx	xxx
A	200	Myosin	P	. .	. .	. .	. .
B	158.7	M-protein	P	. .	. .	. .	
C	114	C-protein	P	. .	. .	. .	
D	93.8	Alpha- actinin	P	. .	. .	. .	
E	81.7		NP	. .	. .	. .	
F	73.1		NP	. .	. .	. .	
G	45.0	Actin	P	. .	. .	. .	. .
H	42.1	Troponin - T	P	. .	. .	. .	. .
I	31.9	Tropomyosin	P	. .		. .	
J	25.6	MLC 1	P	. .		. .	
K	21.6	Troponin - I	P	. .		. .	xxx
L	18.3	MLC 2	P	. .		. .	xxx

Changes noted are relative to day 0

- . . No change in band intensity
- Band weakening
- xxx Band disappeared
- ↑ Increase in band intensity
- NP Not present in gel
- P Present in gel

* According to Dias et al., (1994), Haard N.F. (1995)

observed; thus, myosin heavy chain (band A, MW=200 kDa), M-protein (band B, 158.7 kDa) and C-protein (band C, 114 kDa), alpha actinin (band D, 94 kDa) actin (band G, 45 kDa) and troponin-T (band H, 42.1 kDa) did not show any detectable changes during storage. Ragnarsson and Regenstein (1989) relate the stability of these proteins to their abundance in a muscle. The electrophoresis gel is usually overloaded with these proteins so that it would be much harder to detect decrease in their intensity. On the contrary, reduction in band intensity of low MW proteins occurred. After day 2, there was a reduction in the intensity of band I (31.9 kDa), band J (25.6 kDa), band K (21.6 kDa) and band L (18.3 kDa). The presummed identity of these separated proteins are tropomyosin, myosin light chain1, troponin-I and myosin light chain 2. Similar changes were also reported by many researchers (Zeece et al., 1986; Busconi et al., 1989 and Crouse et al., 1991).

Proteolytic changes observed in our study can be related mainly to autolytic activity rather than bacterial action, since bacterial count was relatively low (Table 4). During the first week, carp fish had pH in the range of 6.4 - 7 which was neither optimal for mammalian CAF (pH 7.5) (Zeece et al., 1986) nor for cathepsin D (pH 4) (Schwartz and Brid, 1977), but optimal for neutral proteolytic enzymes (Yu and Lee, 1986). This was supported by Makinodan et al. (1979) who suggested that neutral proteases (tentatively called a sub-endopeptidase) were responsible for fish deterioration. Moreover, Maeda et al. (1992) found that CAF enzymes from carp fish (also called calpain) have optimal activity at pH 6.8 and Penny et al. (1980), reported that CAF enzymes can retain as much as 25-28% of their maximum activity at low temperature as 5 °C; therefore, the involvement of CAF enzymes in the pervious observed proteolysis cannot be excluded. In addition, Busconi et al. (1989)

suggested that endogenous serine proteinase (trypsin-like serine proteinase) is responsible for fish muscle degradation during ice storage.

4.A.4.1.2. Native Electrophoresis

Figure 15 shows the electropherogram (native) of myofibrillar protein samples at 0, and after 2, 4, 8, 12 and 16 days of storage. Table 6 summarize the changes and relative mobility of the separated proteins. The most apparent changes were: (a) the rapid decrease in intensity of band Y (Rm = 0) which rapidly decreased by day 2 and completely disappeared by day 4 and (b) a gradual decrease in intensity of bands F (Rm= 0.82), G (Rm=0.91), and H (Rm= 0.96) during the storage period. The presumptive identity of these proteins is unknown.

4.A.4.2. Sarcoplasmic Proteins

4.A.4.2.1. SDS-PAGE

Electrophoretograms of the sarcoplasmic proteins conditions are shown in Figure 16; the estimated mean molecular weights and changes in the separated protein are shown in Table 7. The major sarcoplasmic proteins (11 proteins) separated in this study, with molecular weights ranging from 100 to 23 kDa, were comparable to proteins found in sarcoplasm of carp fish by Nakagawa et al. (1988 a and b) and of red snappers by Huang et al. (1995). The most apparent changes were as follows: (a) gradual increase in intensity of bands F (Creatine kinase) and G (Aldolase), (b) gradual decrease in bands A (~ 140 kDa), C (alpha-actinin), D (BSA), E (~ 53 kDa) and H (glyceraldehyde-3-phosphate dehydrogenase, GAPDH).

The appearance of myofibrillar protein, α-actinin (arrow C), in sarcoplasmic electrophoregram was not expected. The release of α-actinin from myofibrillar extract to sarcoplasmic extract was also proposed by Yamamoto et al. (1979) and Xiong et al. (1996).

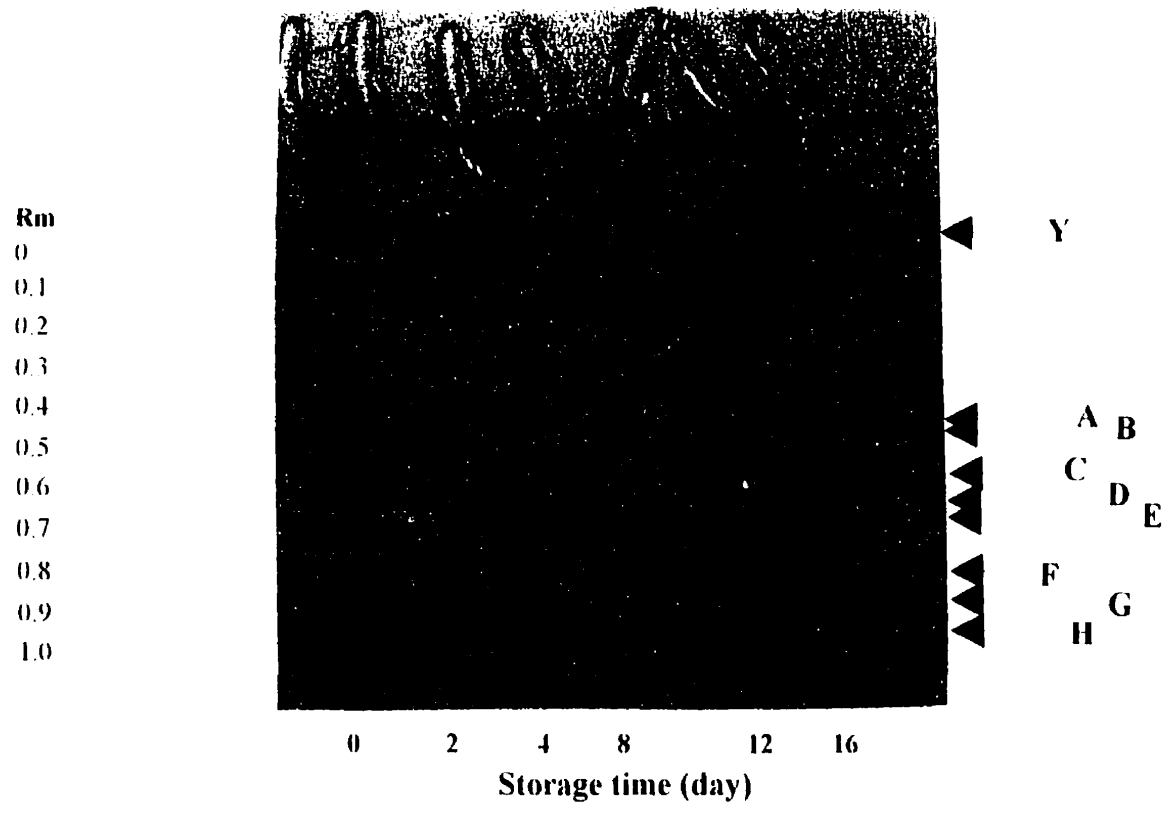


Figure 15. Native-PAGE of myofibrillar protein extracted from carp fish after 0, 2, 4, 8, 12 and 16 days of storage. The labels Y-H are identified in Table 6. Number at left represent proteins relative mobility (Rm.), where Rm. of bromophenol blue band = 1

Table 6. Changes in Myofibrillar Protein Components of Carp Fish During Storage; (Native-Electrophoresis)

Band	Relative Mobility*	Storage period (day)				
		0	2	4	8	16
Y	0	P	· ·	xxx	xxx	xxx
A	0.45	P	· ·	· ·	· ·	· ·
B	0.51	NP	↑	↑	↑	
C	0.58	NP	↑		xxx	xxx
D	0.64	P	· ·	↓	· ·	xxx
E	0.67	P	· ·	↓	↓ ·	xxx
F	0.82	P	↓	↓	· ·	xxx
G	0.91	P	↓	· ·	· ·	xxx
H	0.96	P	↓	· ·	xxx	xxx

Changes noted are relative to day 0

· ·	No change in band intensity
↓	Band weakening
xxx	Band disappeared
↑	Increase in band intensity
NP	Not present in gel
P	Present in gel

* Mobility of bromophenol blue band = 1

Kimberly and Flurkey (1986) suggested that myofibrillar protein could be resolubilized by electrophoresis denaturing buffer. Moreover, Goll et al. (1977) indicated that myofibrillar proteins are soluble in water once they have been extracted and some are extractable with water.

The disappearance of bands D (67 kDa) and A (140 kDa) and the gradual increase in band intensity of creatin kinase (band F) and aldolase (band G) can be used as potential indicators for the state of freshness of chilled carp fish.

4.A.4.2.2. Native Electrophoresis

Figure 17 shows the electropherogram (native) of the sarcoplasmic extract at 0 and after 2, 4, 8, 12 and 16 days of storage. Changes in fractions and relative mobilities of separated sarcoplasmic proteins are summarized in Table 8. In general, the intensity of separated bands remained stable throughout the storage period; only minor changes were observed in bands B (creatine kinase), E (parvalbumin 1), and F (parvalbumin 3) which completely disappeared in day 16. Bands D, E, and F are most likely parvalbumins, the presence of which in carp fish was initially reported by Konosu et al. (1965) and later identified by Nakagawa et al. (1988 a); these proteins in fish may constitute upto 20-30% of the sarcoplasmic fraction (Schwimner, 1995). Although cathepisns can degrade sarcoplasmic proteins (Drabikowski et al., 1977), no signs of degradation were observed in native electrophoretogram of sarcoplasmic extract (Figure 17). Moreover, Goll et al. (1983 b) stated that CAF, originally found in the sarcoplasmia, has very little effect on the sarcoplasmic proteins. From results obtained we can conclude that native electrophoresis is not applicable to monitor protein degradation in carp fish.

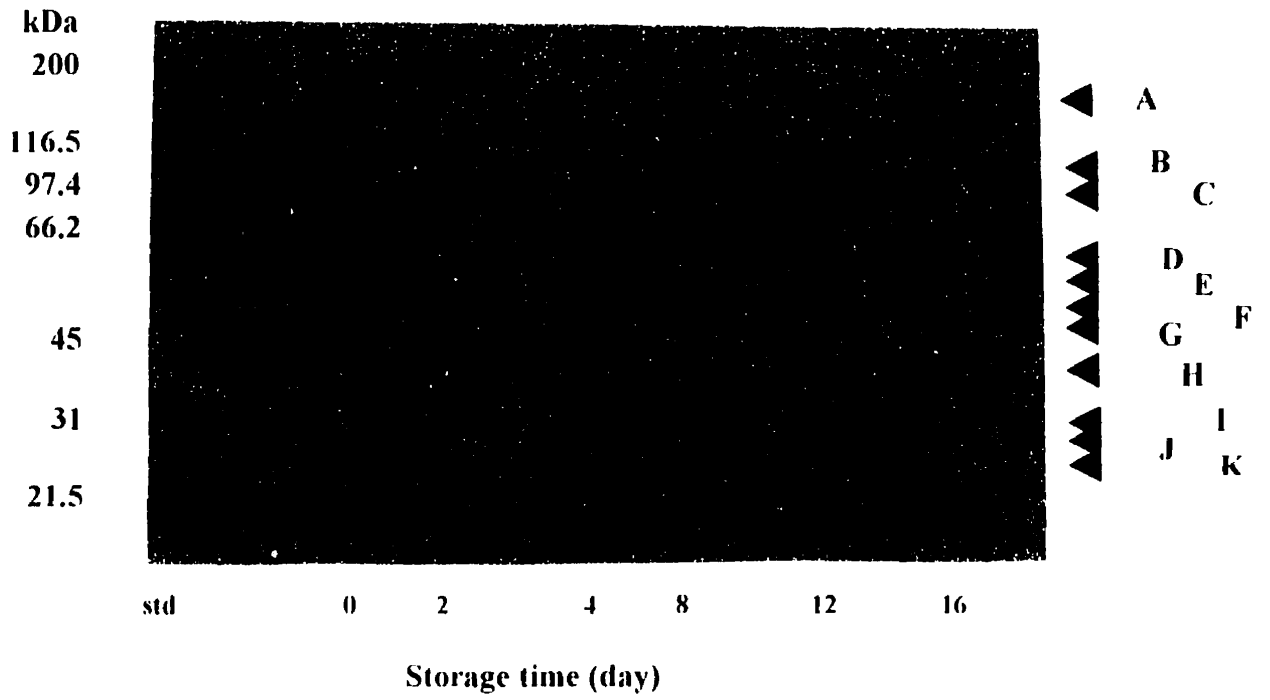


Figure 16. SDS-PAGE of sarcoplasmic proteins extracted from carp fish after 0, 2, 4, 8, 12 and 16 days of storage. The labels A-K are identified in Table 7. Numbers at left represent molecular weight of standard proteins (MW X 10^3)

Table 7. Changes in Sarcoplasmic Protein Components of Carp Fish During Storage; (SDS-Electrophoresis)

Band	Calculated MW (kDa)	Presumptive Identity*	Storage period (day)					
			0	2	4	8	12	16
A	140		P	↓	↓	xxx	xxx	xxx
B	104	Ca^{2+}-ATPase	P	↓		↓	↓	↓
C	96	Alpha-actinin	P	↓	↓		xxx	xxx
D	67.0	BSA	P	↓	↓	xxx	xxx	xxx
E	53		P	↓	↓		xxx	xxx
F	43.0	Creatine kinase	P	↓	↓	↓	↑	↑
G	39.7	Aldolase	P	↑	↑		↑	↑
H	36.5	GAPDH	P	↓	↓			xxx
I	27.4		P	↓	↓	↓	↓	↓
J	25.0		P	↓	↓	↓	↓	↓
K	23.0	TPI	P	↔	↔	↔	↔	↔

Changes noted are relative to day 0

↔ No change in band intensity

↓ Band weakening

xxx Band disappeared

↑ Increase in band intensity

NP Not present in gel

P Present in gel

*According to Hashimoto et al., (1979), Nakagawa et al., (1988a,b), Xiong and Anglemier (1989), Ushio and Watabe (1994) and Wang et al., (1996).

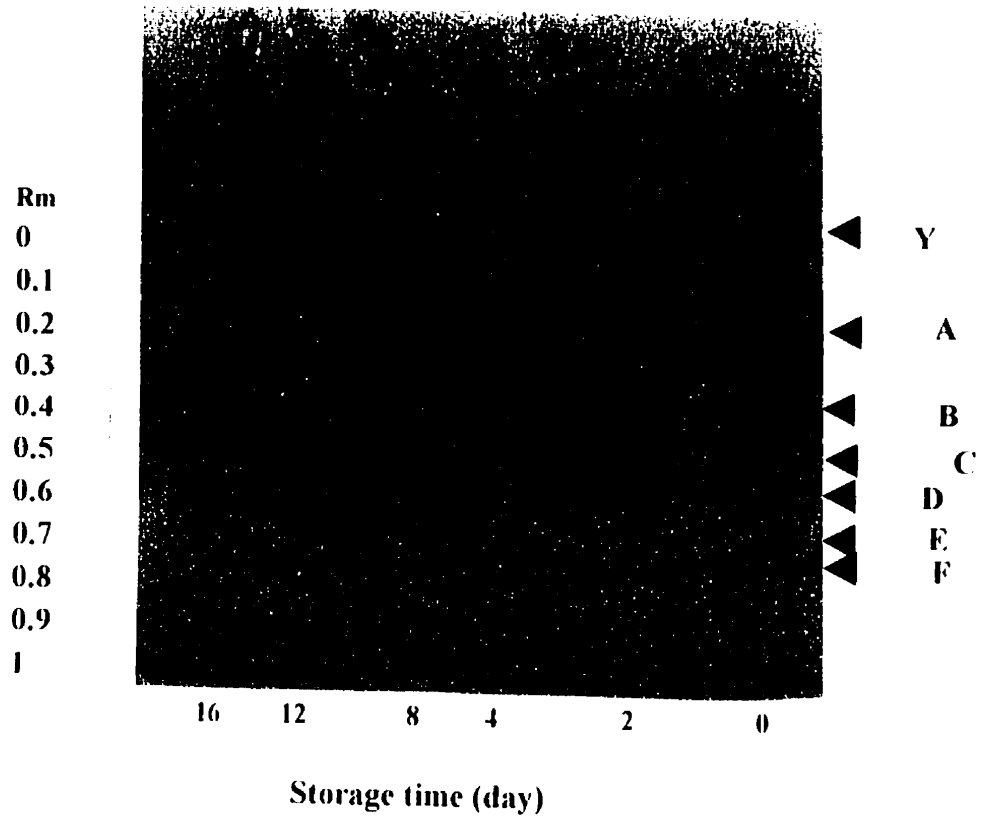


Figure 17. Native-PAGE of sarcoplasmic proteins extracted from carp fish after 0, 2, 4, 8, 12 and 16 days of storage. The labels Y-F are identified in Table 8. Number at left represent proteins relative mobility (Rm.), where Rm. of bromophenol blue band = 1

Table 8. Changes in Sarcoplasmic Protein Components of Carp Fish During Storage; (Native-Electrophoresis)

Band	Relative Mobility*	Presumptive Identity**	Storage period (day)					
			0	2	4	8	12	16
Y2	0		P	. .	. .		. .	. .
A	0.22		P	. .	. .	. .	. .	. .
B	0.45	Creatine kinase	P	. .	↑	. .	. .	xxx
C	0.57	Myoglobin	P	. .	. .	. .	. .	
D	0.65	Parvalbumin 1	P	. .	. .	. .	↑	
E	0.76	Parvalbumin 2	P	. .	. .	. .	. .	xxx
F	0.80	Parvalbumin 3	P	. .	. .	. .	. .	xxx

Changes noted are relative to day 0

. . No change in band intensity

↓ Band weakening

xxx Band disappeared

↑ Increase in band intensity

NP Not present in gel

P Present in gel

* Mobility of bromophenol blue band = 1

**According to Hashimoto et al., (1979), Nakagawa et al., (1988a,b), Xiong and Anglemier (1989), Ushio and Watabe (1994) and Wang et al., (1996)

4.A.5. Liquid Chromatography Mass spectrometry

Figure 18 shows a representative chromatogram obtained from RP-HPLC separation of extracted acid soluble nitrogen (ASN). Base line separation was not achieved because of the complexity of the chromatogram. The region of the chromatogram corresponding to retention time 33 min to 38 min was different for the various storage periods. These are shown in Figure 19. Table 9 shows the changes in the relative area % (RA%) of the fractions in the 33-38 min retention time period; these data are plotted in Figure 20 to show fractions which change during storage, and in Figure 21 to show fractions which show no or little change during storage.

Table 10 shows the molecular weights of ASN fractions in the 33-38 min retention time range. These were determined by electrospray ionization-mass spectrometry (ESI-MS). Representative interpreted mass spectra of these fractions are shown in Figures 22 A-J. A brief description of each of these fractions is provided.

4.A.5.1. Acid Soluble Nitrogen Fractions which Show Changes During Storage

4.A.5.1.1. Fraction A

The molecular weight (MW) of fraction A as determined by ESI-MS was 32,811 D (Table 10), this fraction remained constant until day 8 (Figure 19). After day 8, the MW of fraction A was 32,837 D a slight increase of 26 D. The RA% of fraction A decreased substantially after day 8 and this fraction was not detected after day 12 and completely disappeared in day 16 (Table 9). The decrease and disappearance of fraction A (MW 32.8 kDa) after day 12 can be related to the decrease and disappearance of band H (MW 36.5 kDa) in the sarcoplasmic extract separated by SDS-PAGE (Table 7), the presumed identity of this

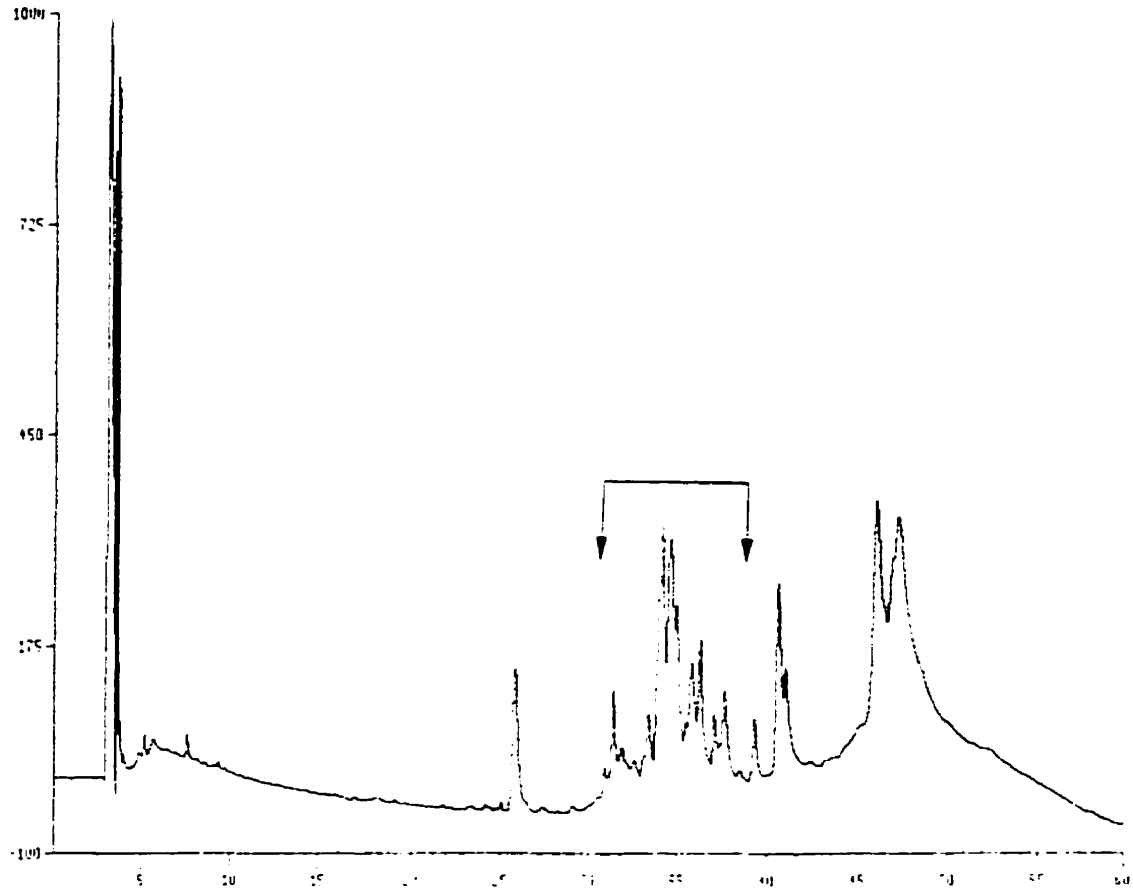


Figure 18. Representative of RP-HPLC Separation of ASN Extracted from Carp Fish

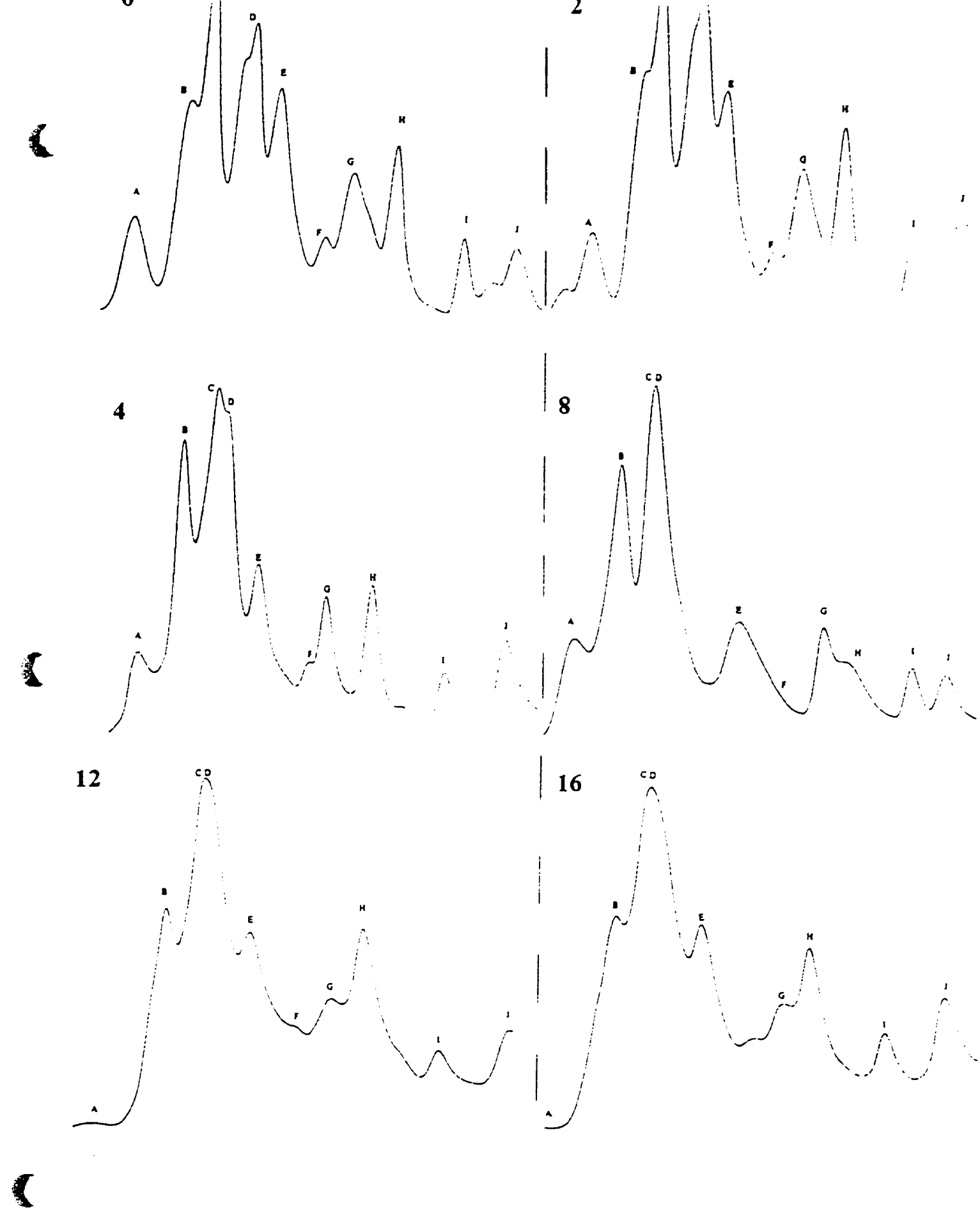


Figure 19. Section of the ASN Chromatograms Corresponding to Retention Time Range of 33 min. to 38 min.

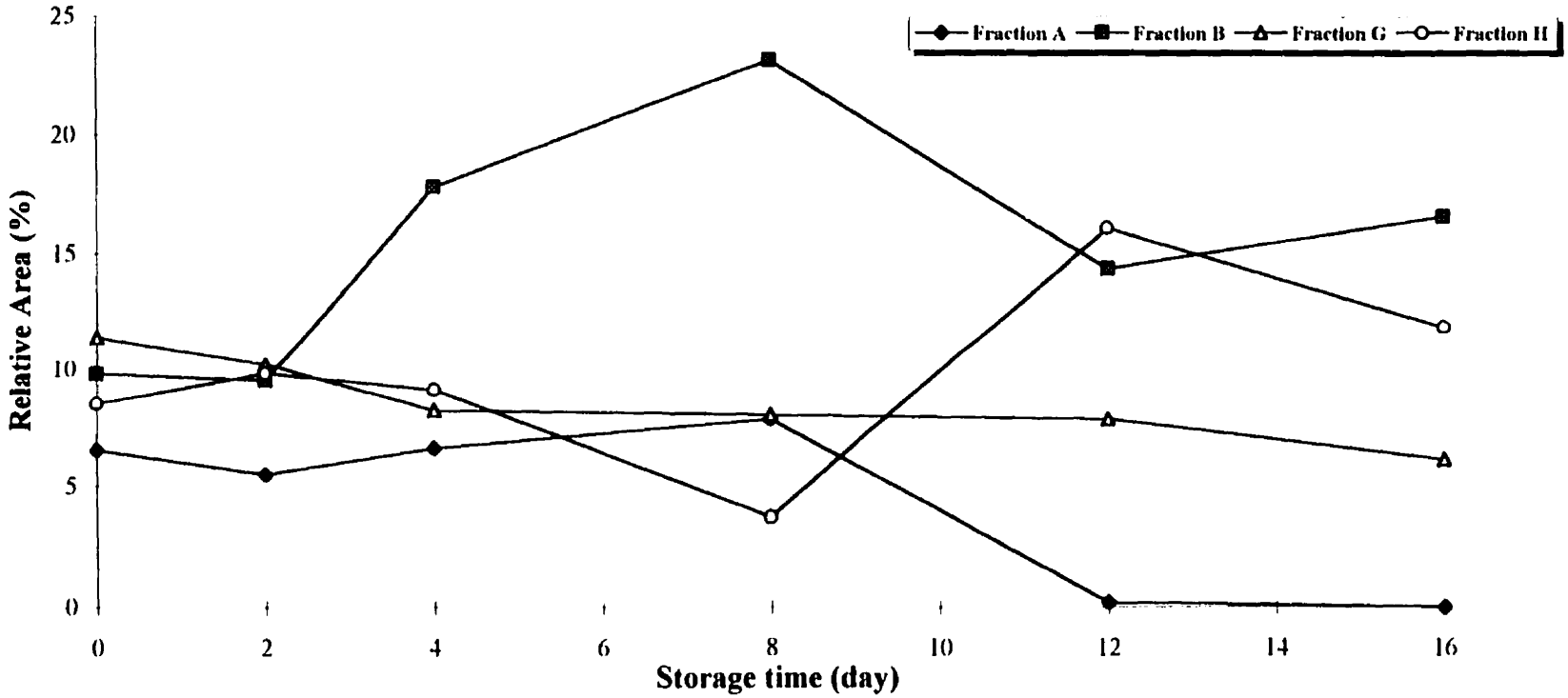


Figure 20. Fractions which Showed Changes in Relative Area (%) of Acid Soluble Nitrogen During Storage of Carp Fish

Table 9. Relative Area (%) of Separated Acid Soluble Nitrogen During Chilled Storage

Storage Time (Day)	Fraction										
	A	B	C	D	C+D	E	F	G	H	I	J
	Relative Area (%)										
0	6.5	9.8	18.2	20.3	38.2	15.7	3.3	11.4	8.5	2.6	3.6
2	5.5	9.5	16.7	22.6	39.3	13.9	2.5	10.2	9.8	3	6.4
4	6.6	17.8			35.6	13 5	2.8	8.2	9.1	2.3	4.3
8	7.8	23.1			35.6	13.3	2.4	8	3.7	2.9	3.2
12	0.2	14.3			36.4	16	3.6	7.8	16	2.4	3.3
16	0	16.5			39.2	15.6	3.2	6.1	11.8	2.8	4.8

Table 10. Molecular Weights of Acid soluble Nitrogen Fractions Separated by HPLC

Fraction label	Storage Time (day)					
	0	2	4	8	12	16
A	32,811 ± 5.00	32,811 ± 5.00	32,811 ± 3.40	32,837 ± 5.00	ND	ND
B	10,995 ± 1.52	10,995 ± 1.52	10,993 ± 1.04	10,994 ± 1.42	10,994 ± 0.26	10,993 ± 2.50
	10,898 ± 1.19	10,898 ± 1.19	10,897 ± 1.00	10,897 ± 1.35	10,898 ± 1.52	ND
C	11,482 ± 1.00	11,482 ± 1.00	11,482 ± 1.02	11,482 ± 1.02	11,482 ± 0.89	11,482 ± 1.02
D	11,511 ± 1.13	11,511 ± 1.13	11,511 ± 1.20	11,511 ± 1.47	11,511 ± 0.70	11,511 ± 1.52
E	42,857 ± 5.00	42,857 ± 5.00	42,861 ± 4.50	------	32,738 ± 4.00	32,739 ± 3.93
F	11,958 ± 1.52	11,958 ± 1.52	11,956 ± 1.66	11,956 ± 1.88	11,956 ± 1.32	11,956 ± 1.80
	12,015 ± 4.20	ND	ND	ND	ND	ND
G	42,842 ± 9.13	42,842 ± 9.13	42,842 ± 3.00	42,846 ± 0.70	42,839 ± 10.0	42,846 ± 5.96
H	16,751 ± 1.25	16,751 ± 1.25	16,746 ± 4.38	16,750 ± 2.43	16,749 ± 1.05	16,750 ± 1.74
I	34,251 ± 2.31	34,251 ± 2.31	34,245 ± 4.00	34,248 ± 8.00	33,498 ± 5.00	33,502 ± 10.00
J	42,054 ± 5.00	42,054 ± 5.00	42,052 ± 10.00	42,052 ± 10.00	42,052 ± 10.00	42,052 ± 10.00

ND = Not Detected

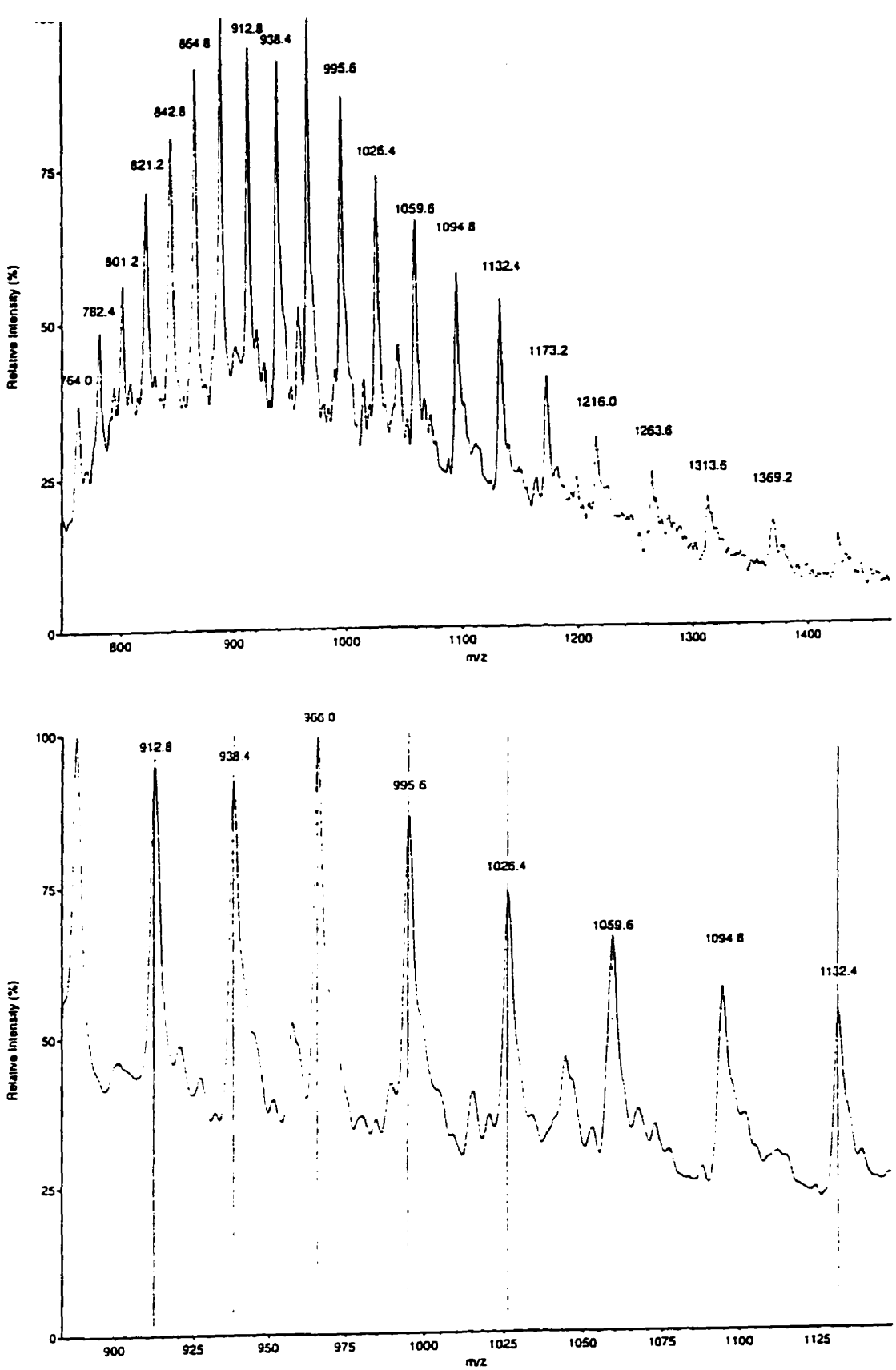


Figure 22-A. Representative of Typical Interpreted ESI-Mass Spectra of Fraction A. Component Mass is 32,811 D

band is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 7). The difference in MW of GAPDH from SDS-PAGE (36.5 kDa) and ESI-MS (32.8 kDa) could be related to the fact is that SDS-PAGE has limited accuracy range (Creighton, 1982).

4.A.5.1.2. Fraction B

Interpretation of mass spectra of fraction B (Figure 22-B1 and B2) revealed the presence of two components having MW of 10,995 D and 10,898 D, which differ by a mass of 97 D. This difference is accounted for loss of proline in peptide chain (Feng et al., 1991). Both protein components were detected until day 12; at day 16 the component with MW 10,898 D was not detected (Table 10). Figure 20 shows changes in the RA% of fraction B during the storage period; the RA % increased from 9.5 (day 2) to 17.8% (day 4) and 23.5% (day 8) then decreased to 14.3% (day 12) and 16.5% (day 16). These changes suggest that initially, this fraction was formed from the breakdown of a larger protein followed by its breakdown by bacterial and autolytic activity in late stages of storage. Results from SDS - PAGE indicated that a large sarcoplasmic protein (140 kDa) separated by SDS-PAGE (Figure 16, Table 7) disappeared during the early storage period. The complete disappearance of band A could be responsible for the increase in RA% at day 8. In the same sense, absence of the precursor (band A) could be the cause for decline in RA% at day 12 and 16. From the present data we suggest that peak B may serve as an indicator for carp fish freshness.

4.A.5.1.3. Fraction H

The MW of fraction H as determined to be 16,750 kDa (Figure 22-H). This MW remained constant until the experiment was terminated (Table 10). Table 9 and Figure 20 show the changes in RA% of this fraction; the RA% increased from 8.5% (day 0) to 16% (day

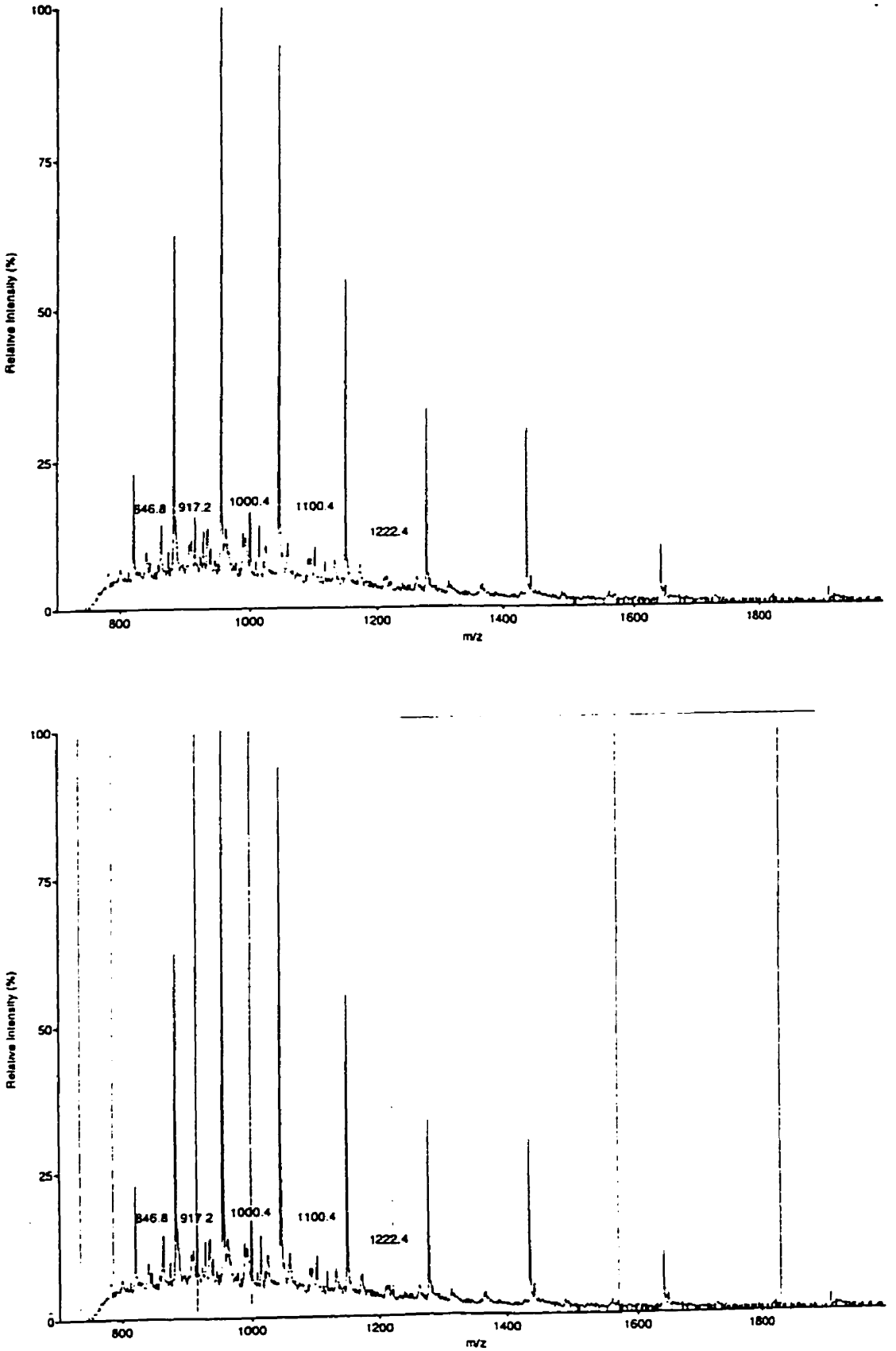


Figure 22-B1. Representative of Typical Interpreted ESI-Mass Spectra of Fraction B1. Component Mass is 10,995 D

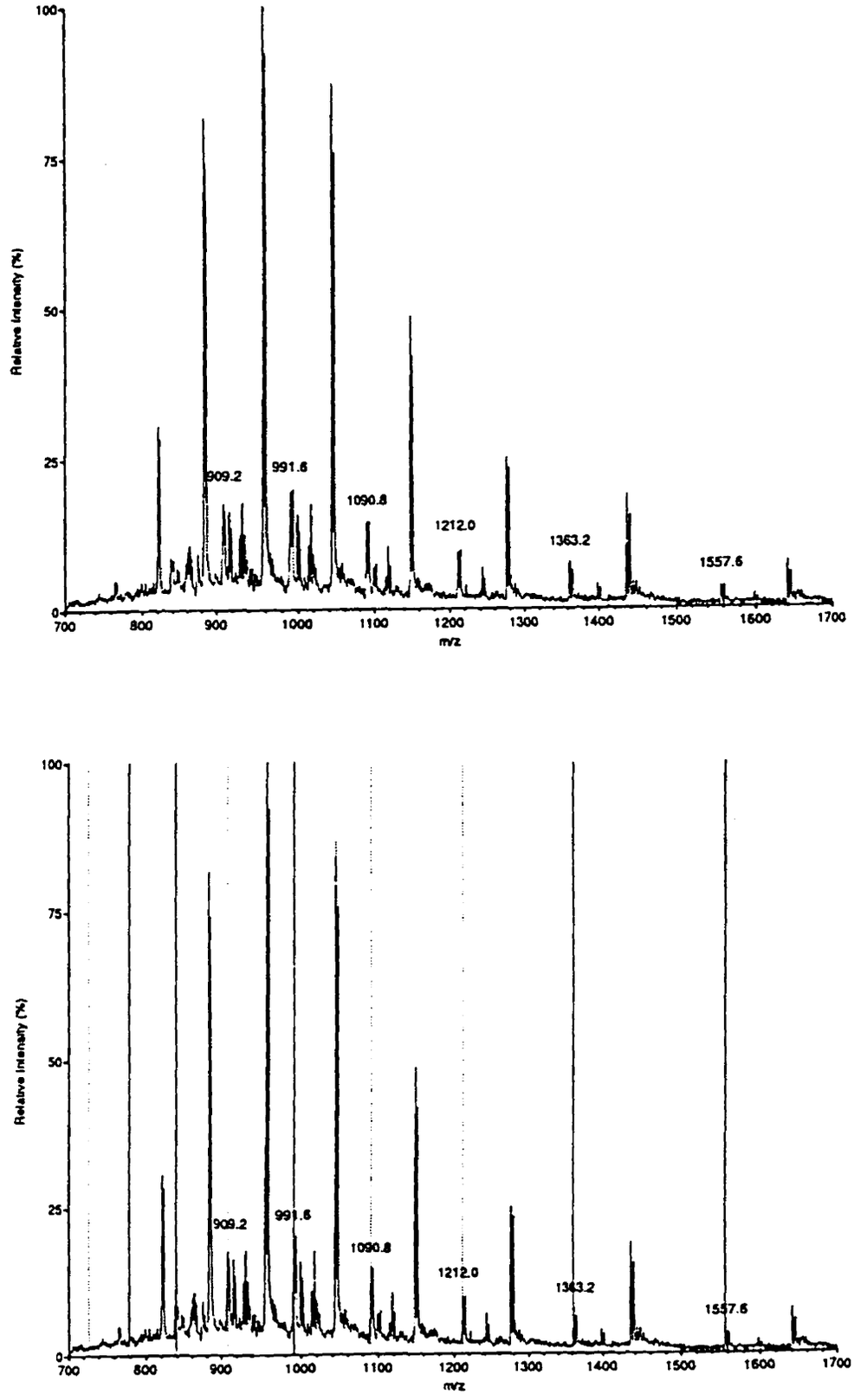
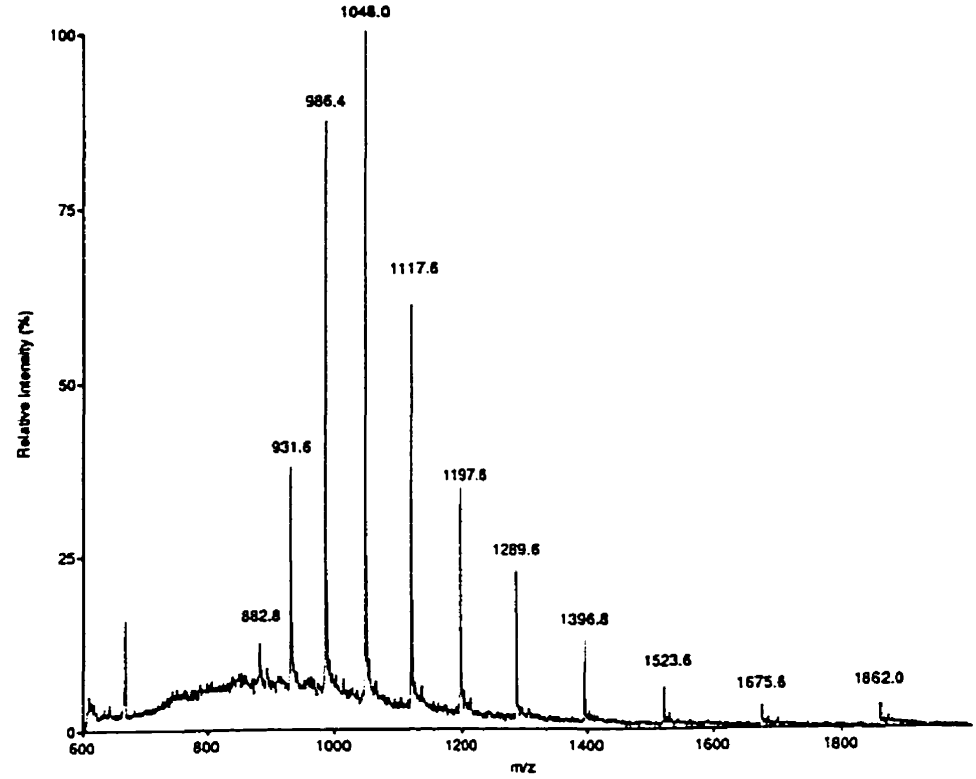


Figure 22-B2. Representative of Typical Interpreted ESI-Mass Spectra of Fraction B2. Component Mass is 10,898 D



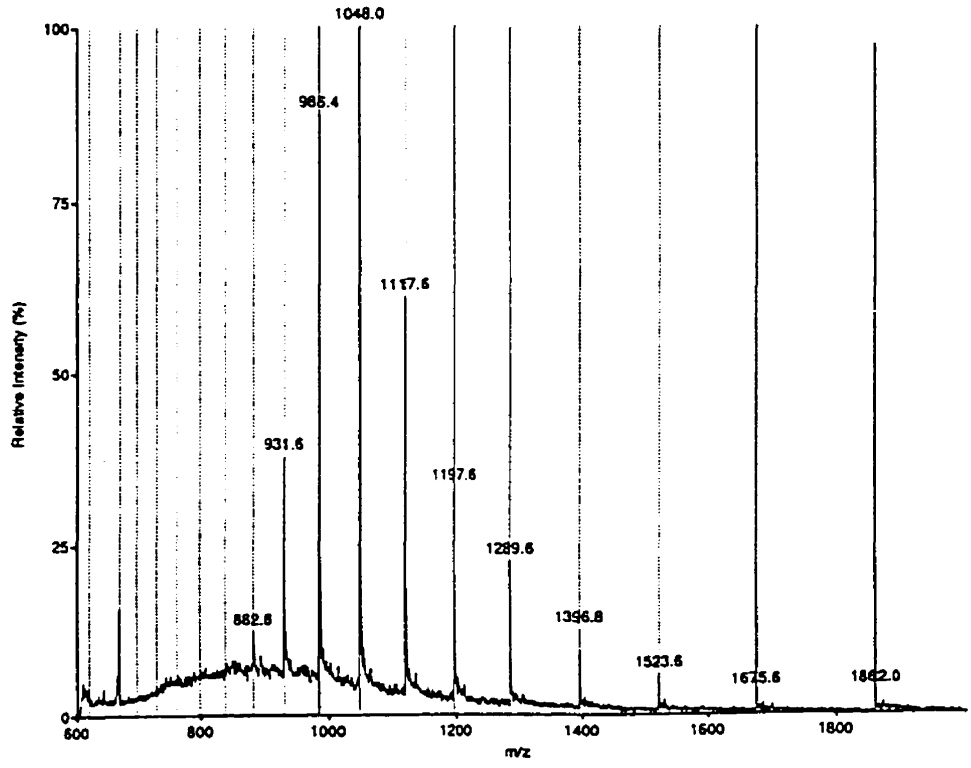


Figure 22-H. Representative of Typical Interpreted ESI-Mass Spectra of Fraction H. Component Mass is 16,751 D

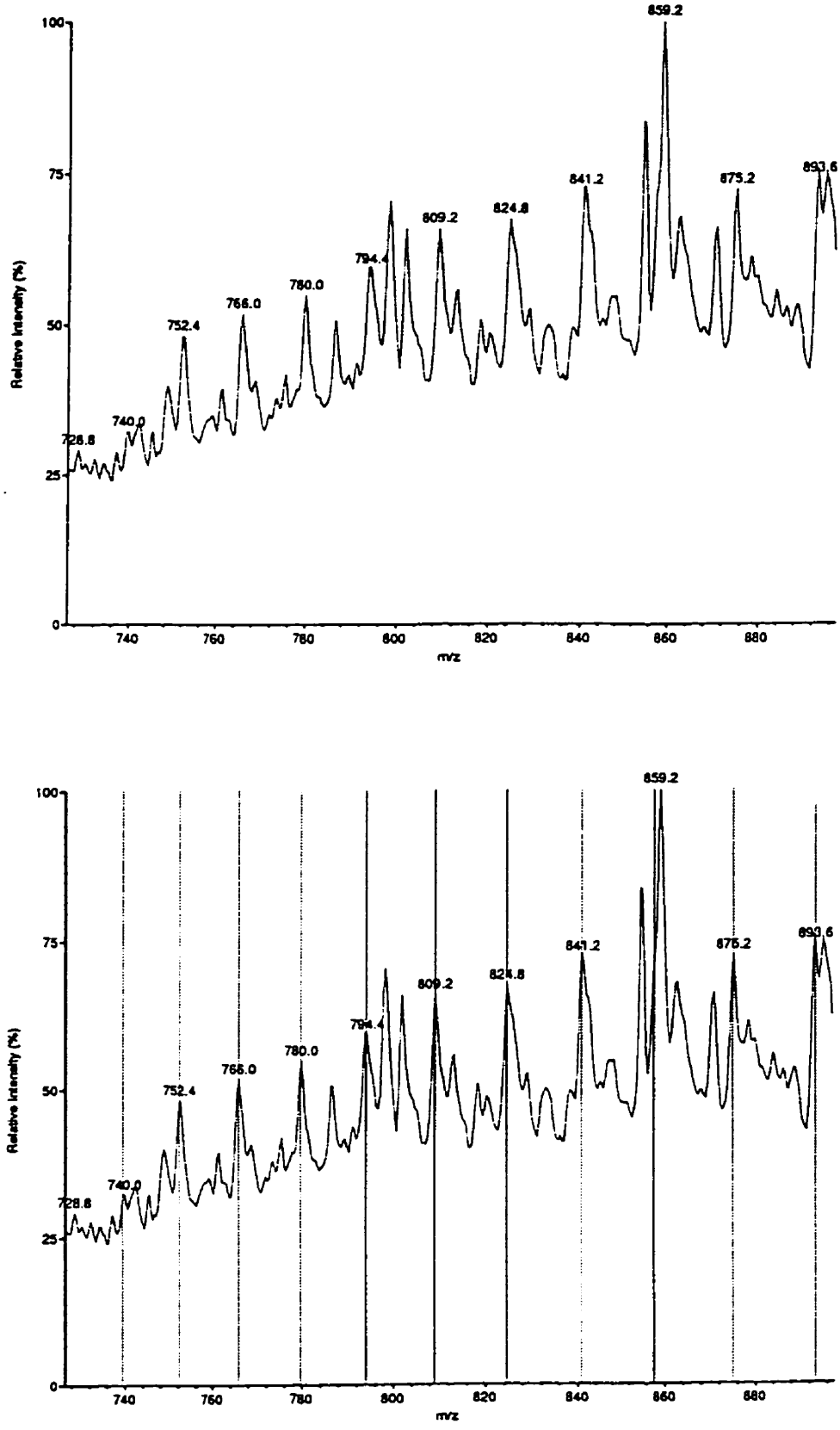


Figure 22-G. Representative of Typical Interpreted ESI-Mass Spectra of Fraction G. Component Mass is 42,842 D

12, then decreased to 12% (day 16).

4.A.5.1.4. Fraction G

The interpreted mass spectra of peak G (Figure 22-G) revealed the presence of a single protein component with an average MW of 42,842 D. During storage, there was a steady decrease in the RA% of peak G (Figure 20, and Table 9); the RA% decreased from 11.4% (day 0) to 6.1% (day 16). The changes associated with fraction G (RP-HPLC) could not be related to changes in any bands in the sarcoplasmic protein separated either in native or SDS-PAGE (Tables 7 and 8). Band F, a sarcoplasmic protein (creatine kinase) separated by SDS-PAGE has the same MW (43 kDa) as fraction G, yet no evident decrease in band intensity was observed.

In summary, interpretation of the mass spectra (Table 10) and changes in RA% of fractions A, B, G, and H (Figure 20) suggest the following: 1) fraction A was subjected to considerable proteolytic activity, since it completely disappeared after the 16 day storage period. 2) fraction B and H were subjected to proteolysis after the 8th day of storage. 3) fraction G was subjected to a relatively slow rate of proteolysis.

4.A.5.2. Fractions which Showed No or Little Changes During Storage

4.A.5.2.1. Peak C and D

The chromatographic results of peaks C and D are shown in Figure 19. Interpreted mass spectra of peak C and D (Figure 22-C and D) revealed the presence of proteins with a constant MW of 11,482 and 11,511 D respectively. The retention characteristic of fraction C and D changed dramatically after day 2 such that these two fractions could not be separated from each other (Figure 19). Consequently a combined RA% for fraction C and D was

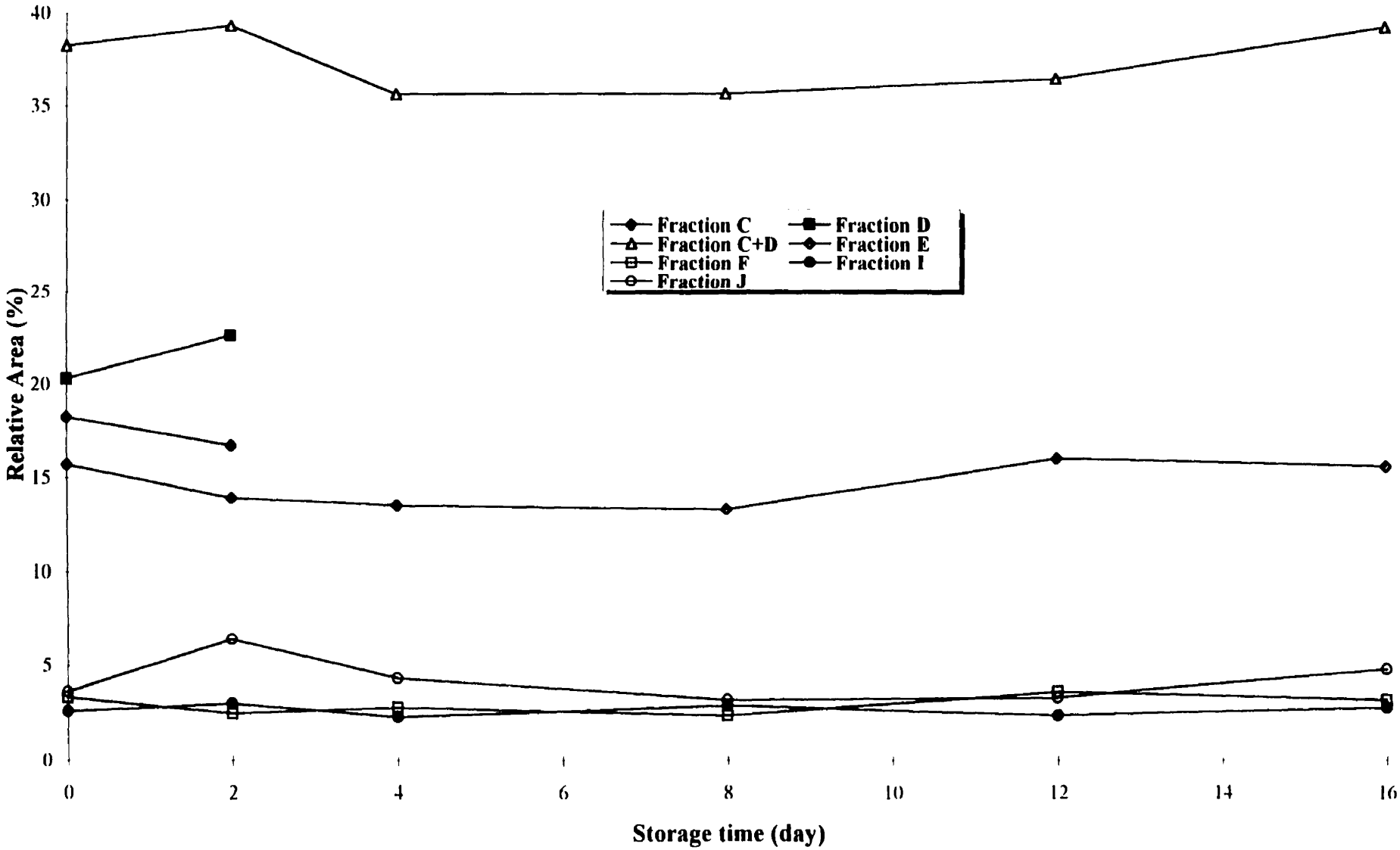
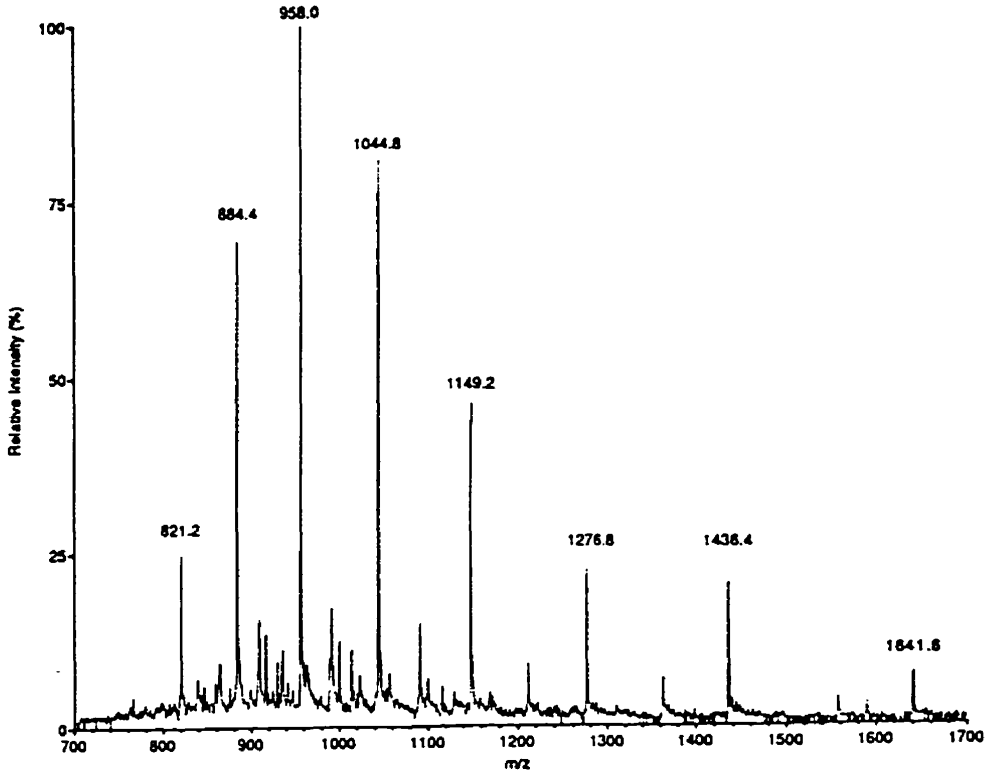


Figure 21. Fractions which Showed No Changes in Realtive Area (%) of Acid Soluble Nitrogen During Storage of Carp Fish



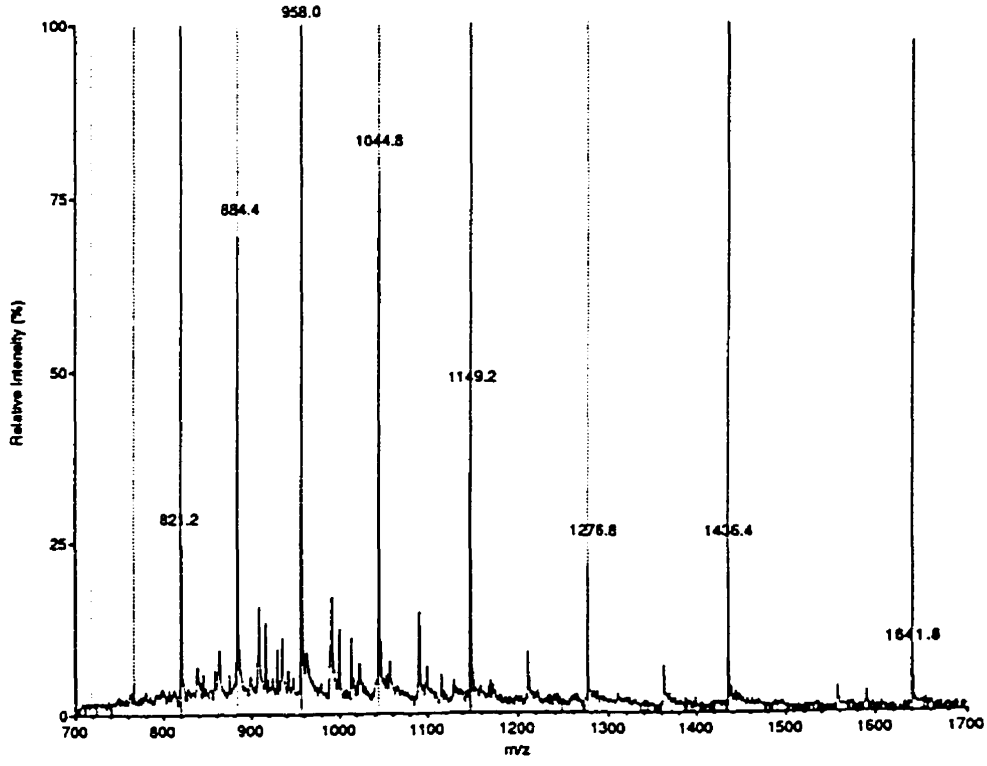


Figure 22-C. Representative of Typical Interpreted ESI-Mass Spectra of Fraction C. Component Mass is 11,482 D

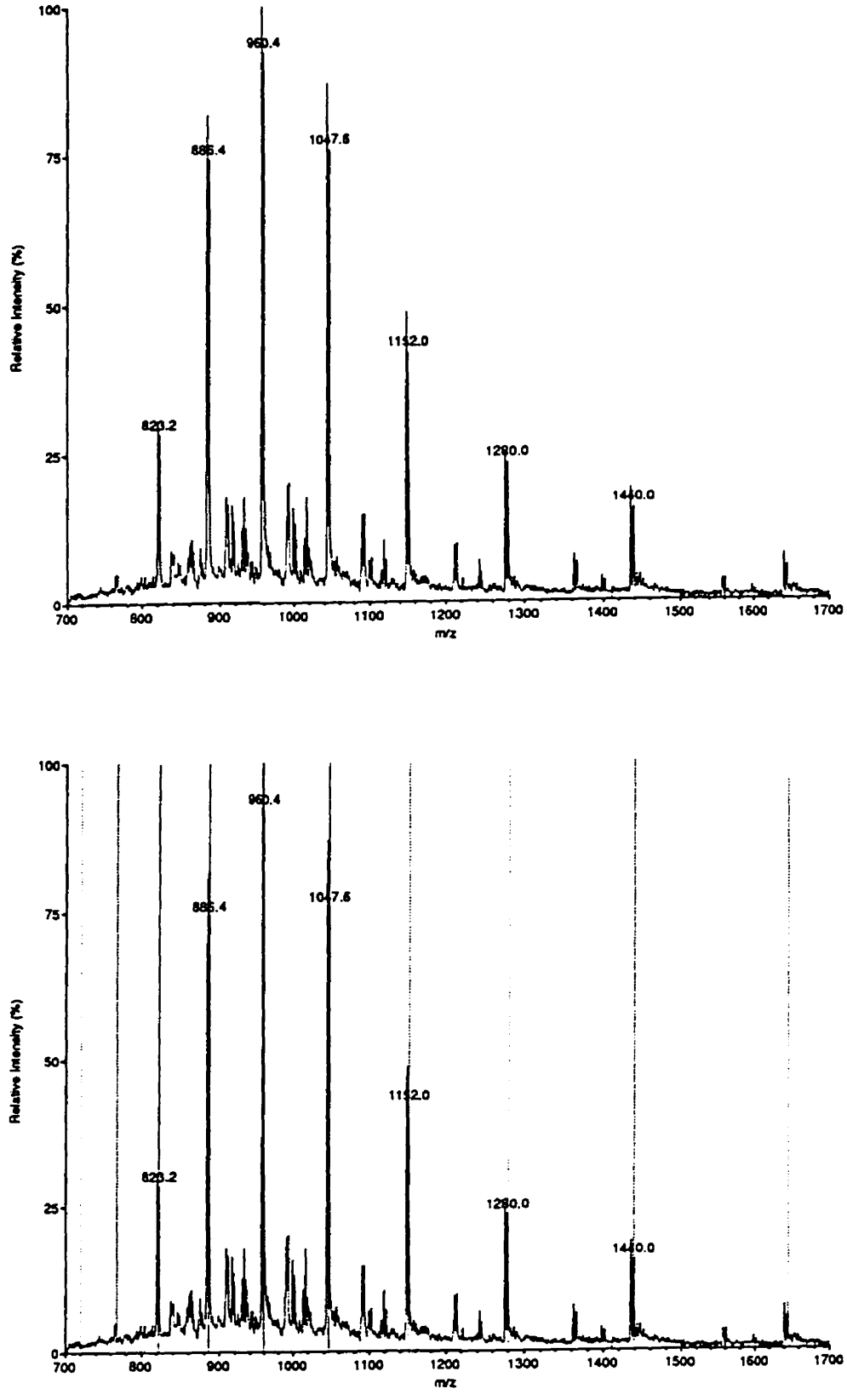


Figure 22-D. Representative of Typical Interpreted ESI-Mass Spectra of Fraction D. Component Mass is 11,511 D

considered (Figure 21, Table 9). In summary, no changes in RA% of peaks C and D were observed (Figure 21), yet changes in retention characteristic are a promising freshness indicator for carp fish.

4.A.5.2.2. Fraction E

Interpreted mass spectra of fraction E revealed the presence of a single protein with a MW of 42,857 D (Figure 22-E), however, after day 8, ESI-MS indicated that the MW of this fraction was 32,738 D (Table 10). No new peak was observed with a MW corresponding to this difference (10 kDa). It is possible that fragmentation of the 42,738 D component occurred resulting in the 32,738 D component. In conclusion, changes in MW, not in RA%, were observed in peak E. Therefore, these changes could serve as indicator of freshness in chilled carp fish.

4.A.5.2.3. Fraction F

Interpreted mass spectra of fraction F (Figure 22-F) revealed the presence of two proteins having a MW of 11,958 D and 12,015 D in the fresh fish; the stored fish showed the presence of the 11,958 D component only (Table 10). There was no change in the RA% of fraction F during storage (Figure 21). Further work needs to be carried out to determine if the presence of the MW component of 12,015 D is always present only in the fresh fish.

4.A.5.2.4. Fraction I

The interpreted mass spectra of fraction I (Figure 22-I) revealed the presence of single protein component with a MW of 34, 251 D; the RA% of this fraction is presented in Figure 21. The results in Table 10 show that there was a slight decrease in the MW of fraction I from 34,251 D to 33,500 D between day 8 and 12 of the storage period.

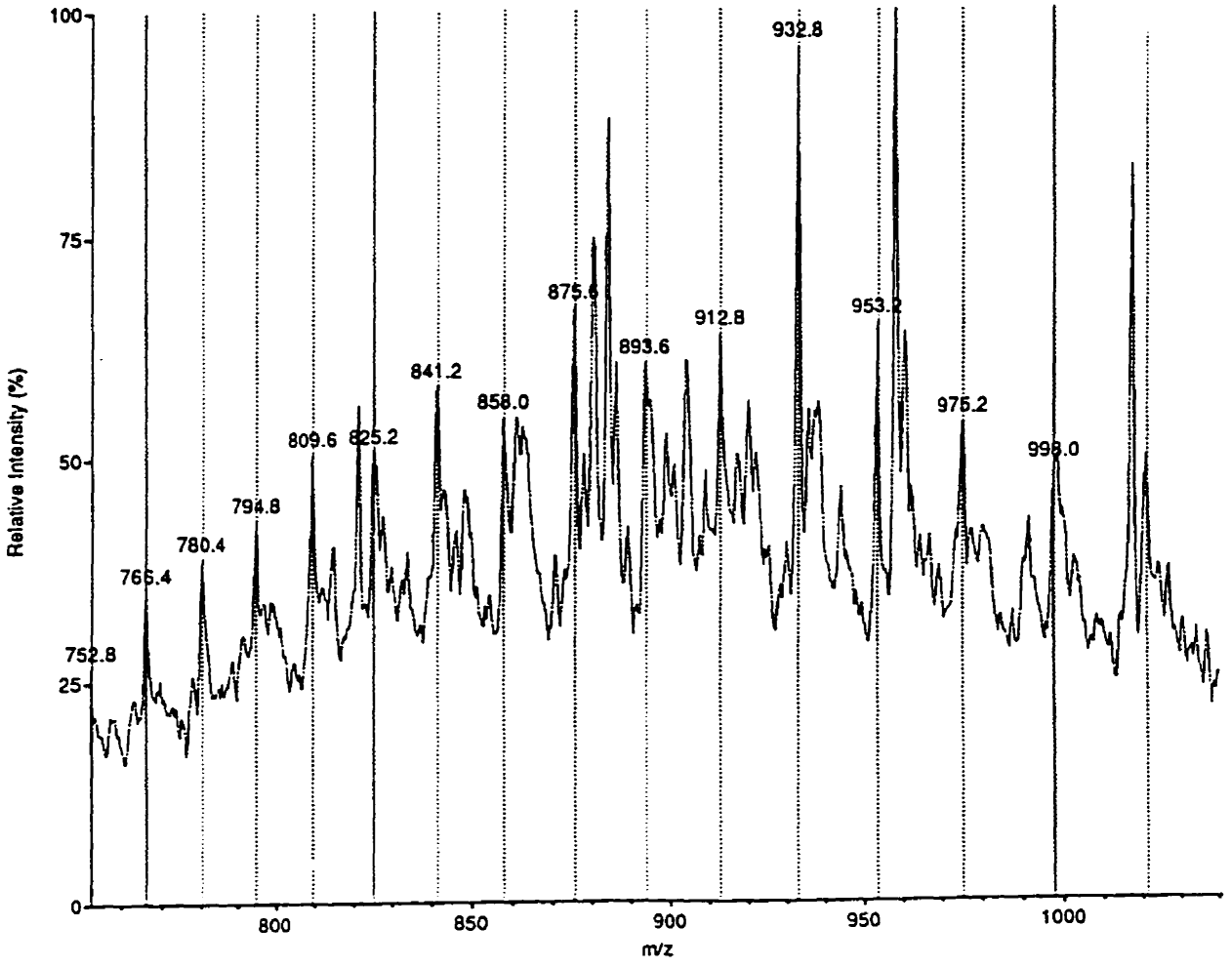


Figure 22-E. Representative of Typical Interpreted ESI-Mass Spectra of Fraction E. Component Mass is 42,857 D

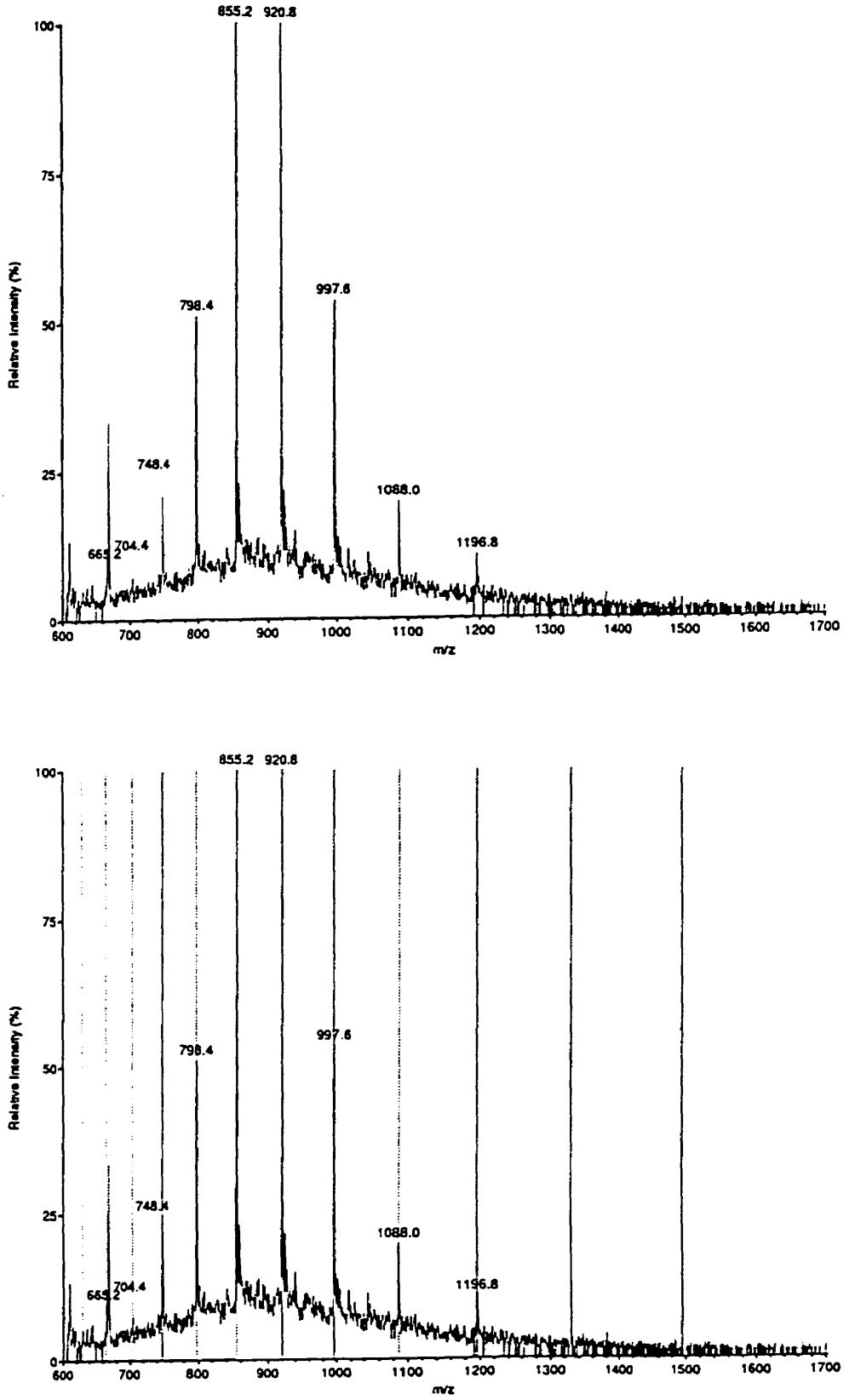


Figure 22-F1. Representative of Typical Interpreted ESI-Mass Spectra of Fraction F1. Component Mass is 11,958 D

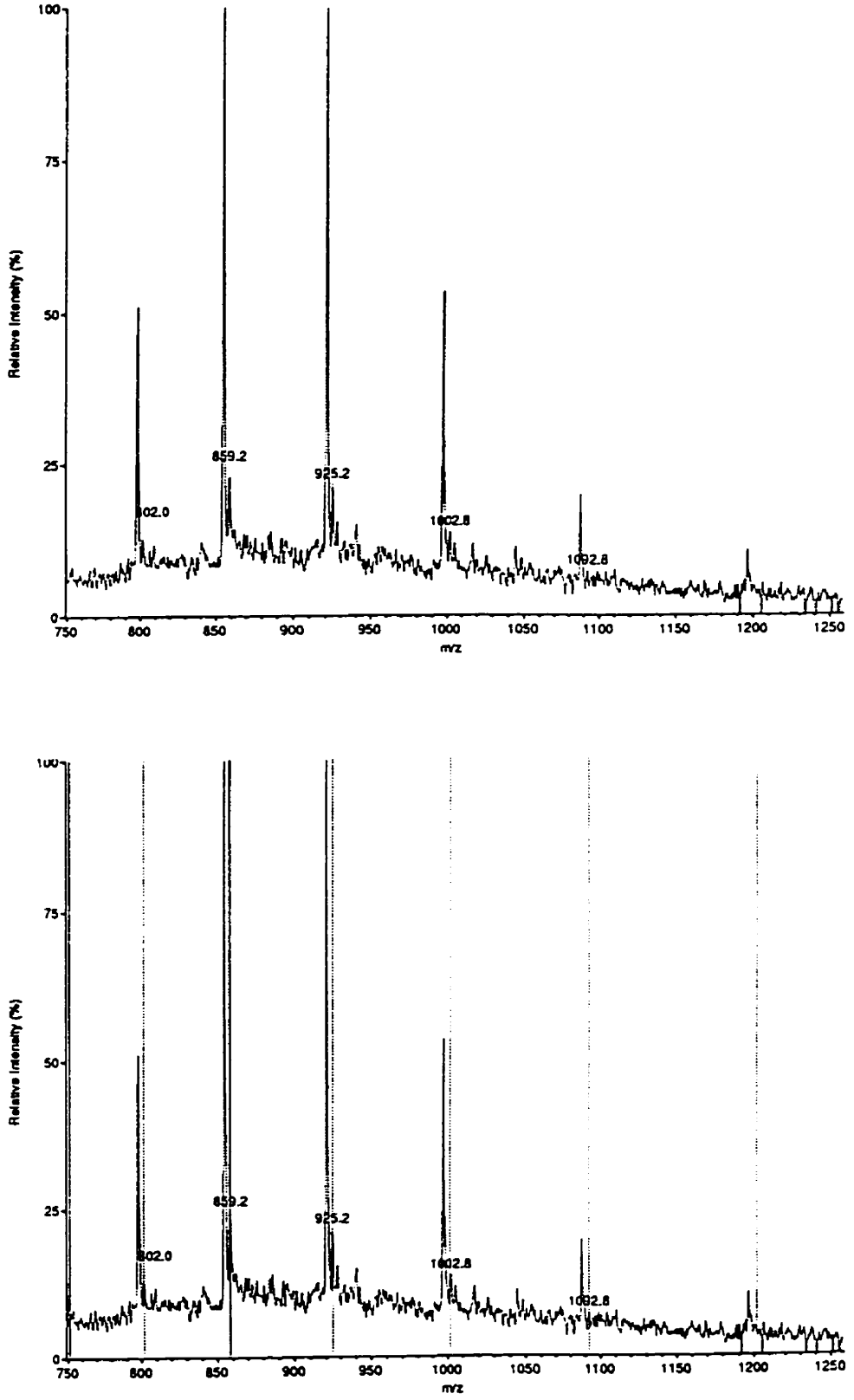


Figure 22-F2. Representative of Typical Interpreted ESI-Mass Spectra of Fraction F2. Component Mass is 12,015 D

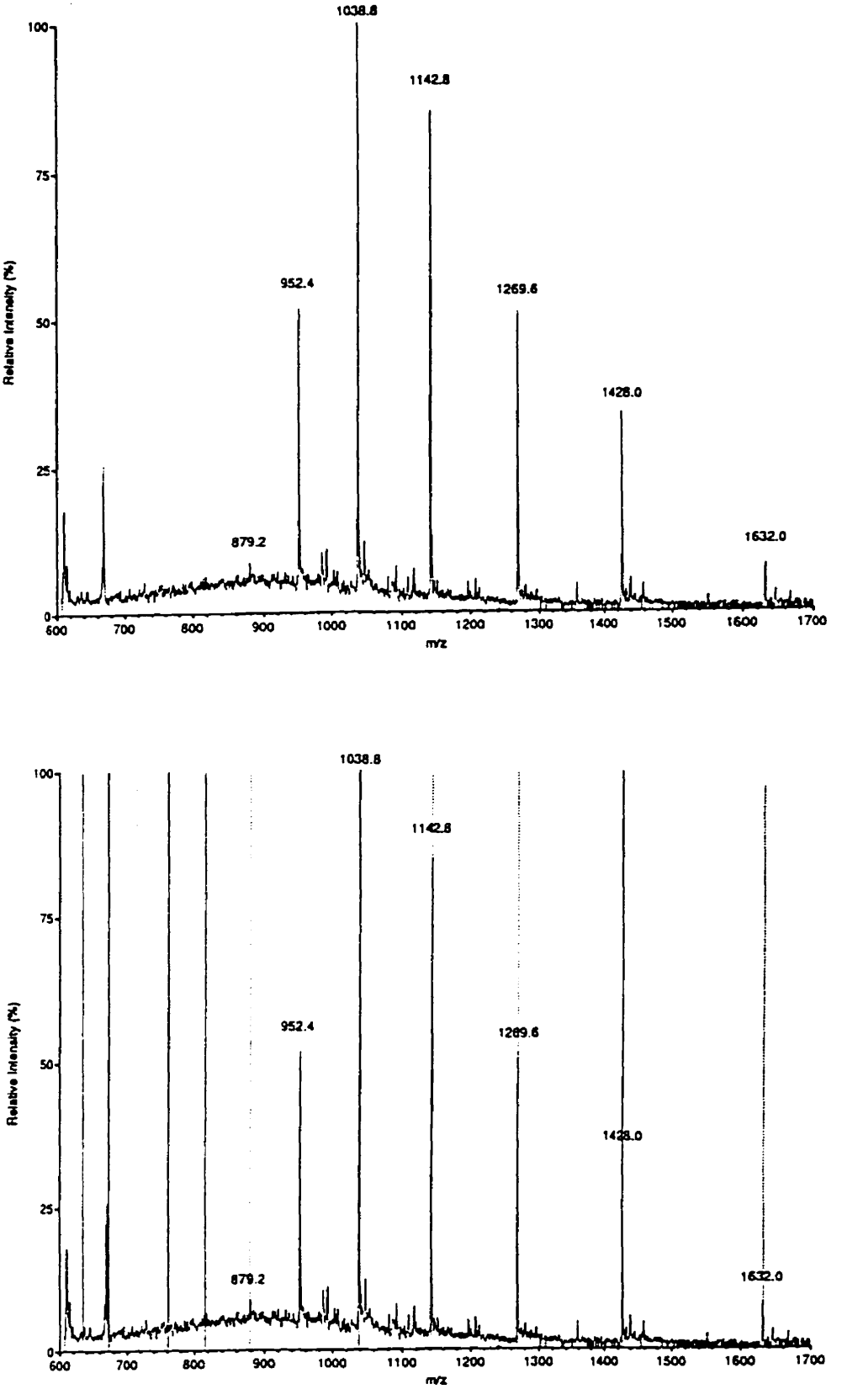


Figure 22-I. Representative of Typical Interpreted ESI-Mass Spectra of Fraction I. Component Mass is 34,251 D

4.A.5.2.5. Peak J

The interpreted mass spectra of peak J revealed the presence of a single protein component with MW of 42,054 D (Figure 22-J); the RA% of this fraction is plotted in Figure 21. This fraction did not show any changes in RA% or in MW during the storage period.

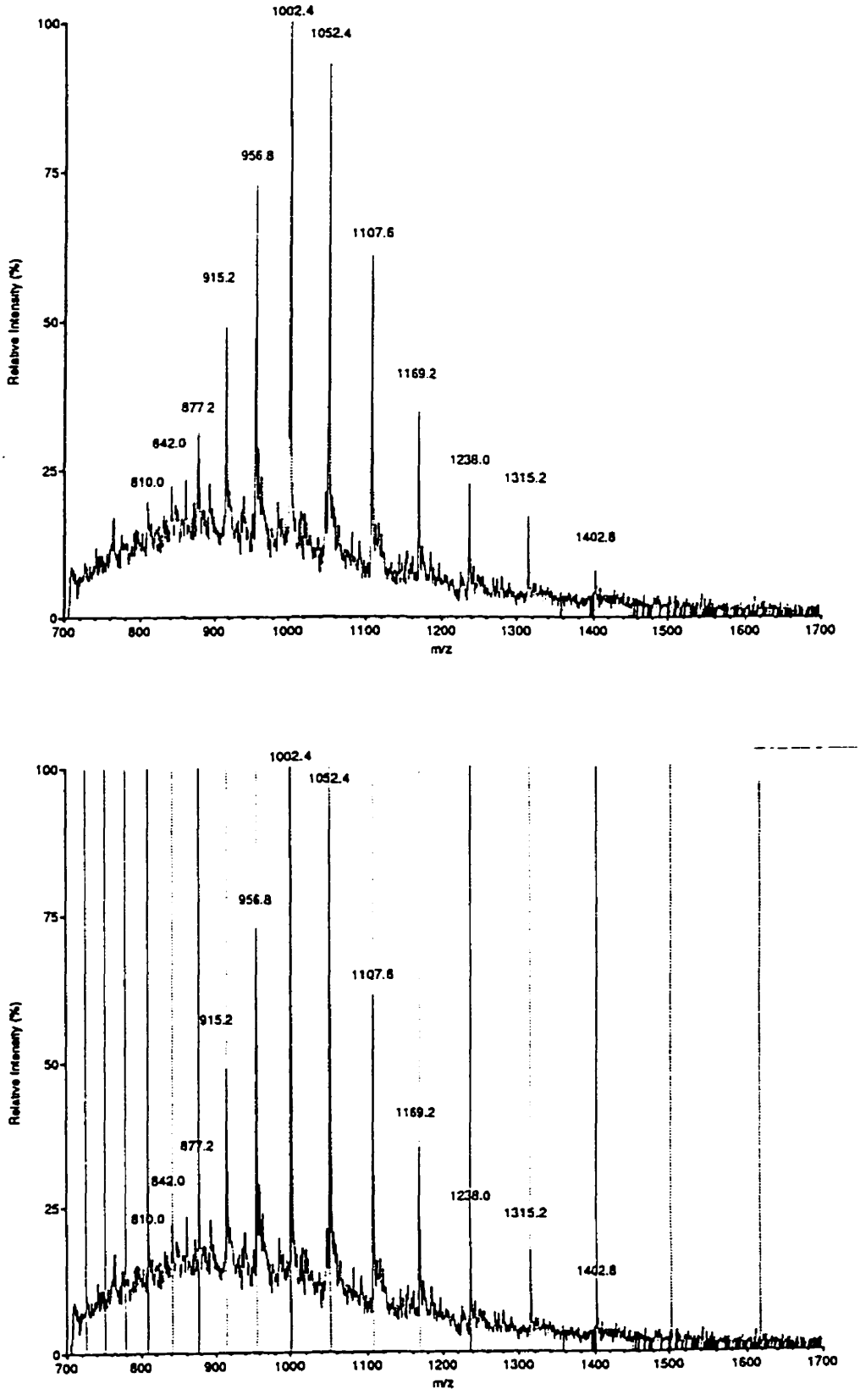


Figure 22-J. Representative of Typical Interpreted ESI-Mass Spectra of Fraction J. Component Mass is 42,054 D

B. Proteolysis of Ground and Whole Meat

4.B.1. Bacterial PlateCount

4.B.1.1. Whole Meat

Table 11 shows the population of mesophilic, psychrotrophic and anaerobic bacteria of whole meat; the data is plotted in Figure 23. The average initial counts of mesophilic and psychrotrophic bacteria was 3.6×10^3; the aerobic plate counts of freshly dressed beef carcasses ranged from 10^3 to 10^5 CFU/g (Grau, 1986). The mesophilic count decreased from log 3.71 to log 2.99 at day 0 and 2, respectively. This decrease can be attributed to the 'cold shock' phenomena (Section 4.1). Psychrotrophic count did not change significantly during the first 4 days of storage (Figure 23). Mesophilic and psychrotrophic bacteria showed identical growth rates during the first 4 days of storage. The psychrotrophic count is due primally to *pseudomonas spp.* which is a relatively minor component of initial mixed population (Perez de Castro et al., 1988), but eventually predominates over aerobic spoilage flora (Seymour et al., 1994). Davidson et al. (1973) demonstrated that *pseudomonas spp.* accounted for 91% of spoilage microbial population of chilled stored meat. This, along with the fact that *pseudomonas* can grow in mesophilic conditions, but have optimum growth in psychrotrophic conditions (Jay, 1987), can explain the increase in psychrotrophic and mesophilic counts. Similar observations have been reported by Prieto et al. (1991) in lamb and by Seymour et al. (1994) in ground beef.

Nortjé et al. (1990) reported that anaerobic bacteria were not important in the shelf life of aerobically chilled whole meat. Table 11 shows that the anaerobic count did not exceed log 4 throughout the storage period. Kraft (1992) demonstrated that off-odour and slime were

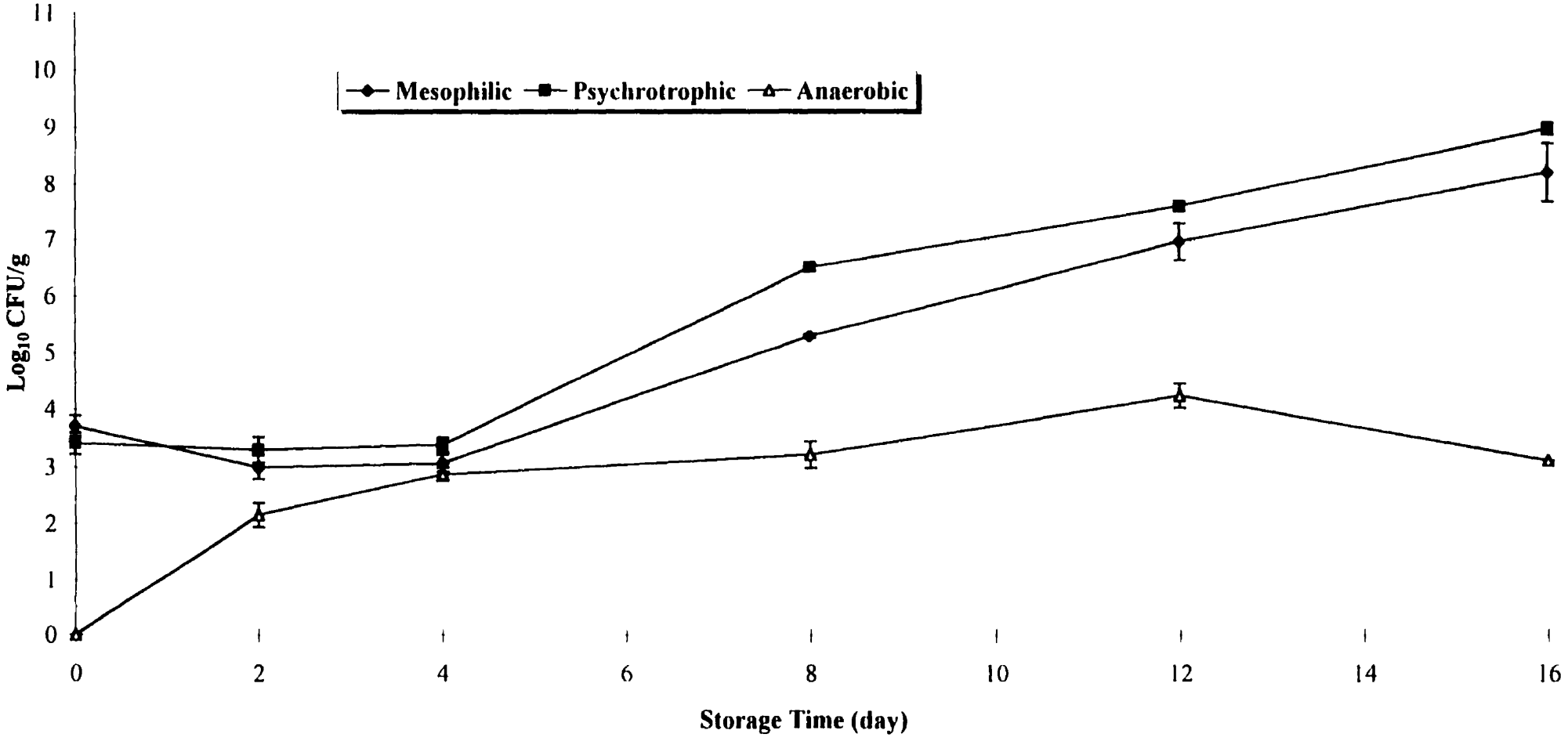


Figure 23. Mesophilic, Psychrotrophic and Anaerobic Bacterial Counts of Chilled Whole Meat

evident on aerobically packaged meat when the level of bacteria reached counts of 10^7 and

Table 11. Mesophilic, Psychrotrophic and Anaerobic Bacterial Count of Chilled Whole Meat Stored Under Aerobic

	Bacterial Count (Log_{10} CFU/g)Count Storage Time (day)					
	0	2	4	8	12	16
Mesophilic	3.71^{d} (0.18)	2.99^{e} (0.21)	3.06^{e} (0.15)	5.28^{c} (0.04)	6.95^{b} (0.33)	8.20^{a} (0.53)
Psychrotrophic	3.41^{d} (0.19)	3.29^{d} (0.23)	3.38^{d} (0.13)	6.49^{c} (0.06)	7.58^{b} (0.09)	8.99^{a} (0.10)
Anaerobic	0^{e} (0)	2.15^{d} (0.21)	2.87^{c} (0.12)	3.20^{b} (0.23)	4.23^{a} (0.21)	3.11^{b} (0.0)

Figures in parentheses represent standard deviation of six replicates
Means within row marked with the same superscript are not significantly different ($P > 0.05$).

Table 12. Mesophilic, Psychrotrophic and Anaerobic Bacterial Count of Chilled Ground Meat Stored Under Aerobic

	Bacterial Count (Log_{10} CFU/g)Count Storage Time (day)					
	0	2	4	8	12	16
Mesophilic	5.68^{e} (0.08)	5.64^{e} (0.24)	6.12^{d} (0.20)	7.66^{c} (0.06)	8.93^{b} (0.07)	9.15^{a} (0.09)
Psychrotrophic	4.46^{e} (0.06)	5.63^{d} (0.21)	6.41^{c} (0.28)	8.86^{b} (0.06)	9.91^{a} (0.09)	10.06^{a} (0.11)
Anaerobic	4.88^{f} (0.14)	5.77^{e} (0.12)	6.32^{d} (0.07)	7.07^{c} (0.14)	8.46^{b} (0.07)	9.12^{a} (0.19)

Figures in parentheses represent standard deviation of six replicates
Means within row marked with the same superscript are not significantly different ($P > 0.05$).

10^{8} CFU/g, respectively. Therefore, whole meat samples have an estimated shelf life of 12 days (Figure 23).

4.B.1.2. Ground Meat

Aerobic and anaerobic bacteria counts of ground meat are shown in Table 12; the data is plotted in Figure 24. The initial averaged count of mesophilic and psychrotrophic was log 5. This count is within the acceptable levels of American (1- 10 x 10^6) (Westhoff and Felstein, 1976) and Canadian guidelines (10^7) (Pivnik et al., 1976). Significant increase in mesophilic and psychrotrophic count was observed. The 'cold shock' phenomena were not observed. Mates (1983) indicated that the grinding process of meat distribute bacteria normally present on the surface of meat throughout the product and create ideal conditions for their multiplication. This explains the higher initial count in ground meat. Psychrotrophic and mesophilic count in ground meat were identical during the first 4 days of storage. After day 4 the changes in psychrotrophic and mesophilic count was similar to that of whole meat.

The initial count of anaerobic bacteria in ground meat samples was log 4.88 (Figure 24). This count increased significantly during the storage period (Figure 24). Mackey and Kerridge (1988) indicated that the count of anaerobic bacteria is useful to evaluate the hygiene and public health concern of ground meat. Gill and Newton (1978) indicated that aerobically, there is no inhibitory interaction between different bacterial species until the maximum cell density (10^9-10^{10}) is reached, this was true in our experiment in which aerobic (mesophilic and psychrotrophic) and anaerobic counts increased linearly throughout the storage period.

4.B.2. pH

The changes in pH of whole and ground meat are shown in Figure 25. Both meat

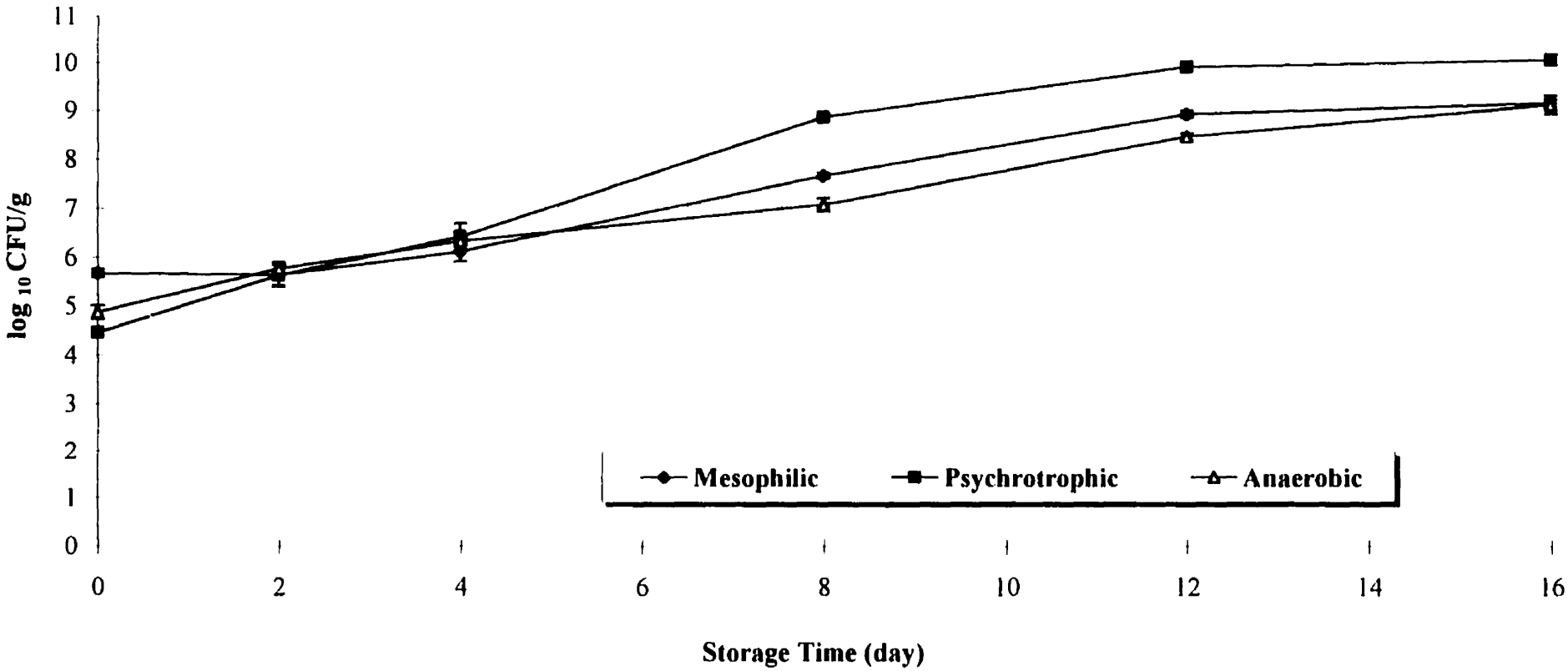


Figure 24. Mesophilic, Psychrotrophic and Anaerobic Bacterial Counts of Chilled Ground Meat

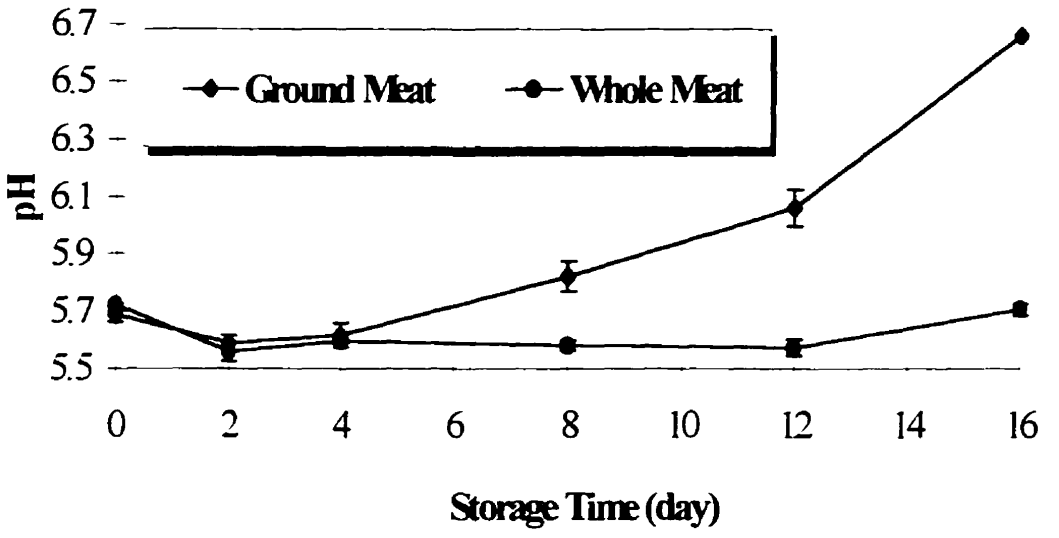


Figure 25. Changes in pH of Ground and Whole Meat

samples had initial pH of 5.70 (Table 13). This is similar to results reported previously for ground meat (Seymour et al., 1994) and for whole meat (Koohmaraie et al., 1988b). Xiong et al. (1996) reported that a random sampling of 21 cattle carcasses gave a range of pH values from 5.30- 5.40.

Table 13. Changes in pH Value of Chilled Whole and Ground Meat During Storage

	Storage Time (days)					
	0	**2**	**4**	**8**	**12**	**16**
Ground Meat	5.69^{c}# (0.025)	5.59^{c} (0.029)	5.62^{c} (0.041)	5.82^{c} (0.052)	6.06^{b} (0.065)	6.89^{a} (0.394)
Whole Meat	5.72^{a} (0.007)	5.56^{b} (0.033)	5.59^{b} (0.007)	5.58^{b} (0.017)	5.57^{b} (0.029)	5.71^{a} (0.02)

Means of pH value corresponding to six replicates
Means marked with the same superscript are not significantly different ($P > 0.05$).

Whole meat samples showed a slight decrease in pH over the first 2 days then remained unchanged until day 12, but by day 16 the pH increased to the initial value of 5.71 (Figure 25). The initial decrease in pH observed in whole meat was not observed in ground meat. The pH of ground meat did not change from the initial value until day 12 after which the pH increased significantly. Seymour et al. (1994) indicated that changes in meat pH are dependent on the number of microorganisms present, their metabolic activity, the amount of available glucose and glycogen metabolised, the buffering capacity of the muscle protein and the available oxygen.

4.B.3. Electrophoresis

4.B.3.1. Myofibrillar Proteins

4.B.3.1.1. Native Electrophoresis

Native electrophoresis of myofibrillar proteins of whole and ground meat after 0, 2, 4, 8, 12 and 16 days of storage are illustrated in Figures 26 and 27, respectively. Table 14 summarizes the calculated relative mobility and changes in band intensity of myofibrillar proteins of whole and ground meat samples. The four protein bands indicated in Figure 26 and 27 were the major component of myofibrillar protein; the presumptive identities of these proteins are unknown. To our knowledge, this is the first report of successfull separation of myofibrillar protein using native electrophoresis. The native electropherogram (Figures 26 and 27) indicates that no appreciable protein degradation occurred in the myofibrillar proteins from whole or ground meat during storage period.

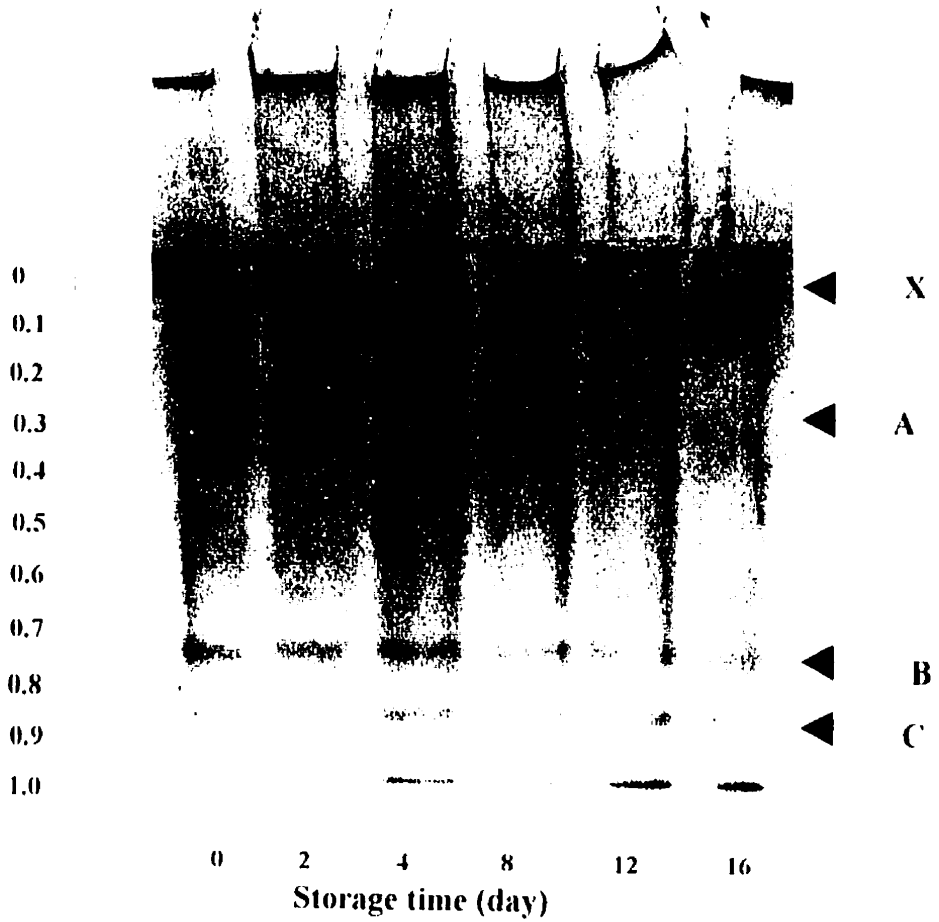


Figure 26. Native-PAGE of myofibrillar protein extracted from whole meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels X-C are identified in Table 14. Number at left represent proteins relative mobility (Rm.), where Rm. of bromophenol blue band = 1

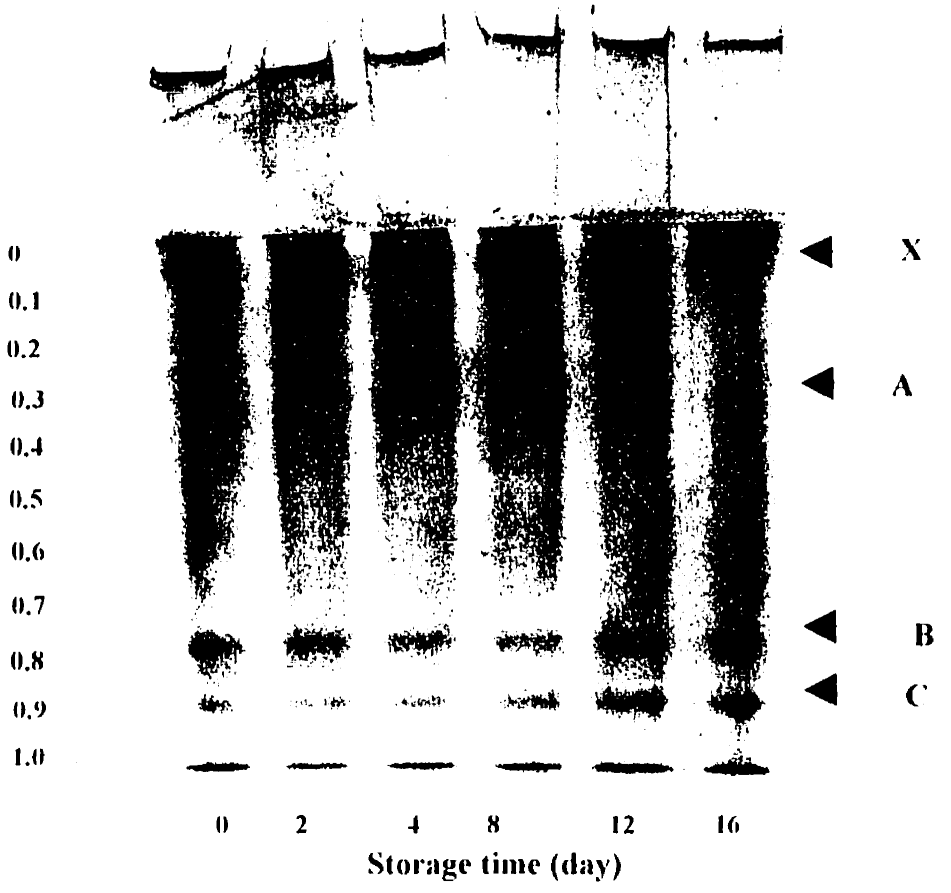


Figure 27. Native-PAGE of myofibrillar protein extracted from ground meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels X-C are identified in Table 14. Number at left represent proteins relative mobility (Rm.), where Rm. of bromophenol blue band = 1

Table 14. Changes in Myofibrillar Protein Components of Ground and Whole Meat During Storage; (Native-Electrophoresis)

Band	Rm*	Storage period (day)					
		0	2	4	8	12	16
X	0	P	→	→	→	→	→
A	0.25	P	→	→	→	→	→
B	0.75	P	→	→	→	→	→
C	0.88	P	→	→	→	→	→

→ No change in band intensity
Band weakening
xxx Band disappeared
↑ Increase in band intensity
NP Not present in gel
P Present in gel

* Mobility of bromophenol blue band = 1

4.B.3.1.2. SDS-Electrophoresis

4.B.3.1.2.1. Whole meat

SDS-electrophoresis of myofibrillar protein extracted from whole meat stored after 0, 2, 4, 8, 12 and 16 days of storage are shown in Figure 28; table 15 summarizes the changes in band intensity and estimated molecular weight (MW) of myofibrillar proteins from whole meat. SDS-electrophoresis revealed differences in myofibrillar protein fraction at certain storage periods. The following postmortem changes were observed: (a) The presence of two bands (X and Y) migrating slower than myosin at day 16; band X and Y gave MWs of 285 and 277 kDa, respectively. (b) At day 12, band A (MW 201 kDa, identified as myosin heavy chain) started to degrade with complete disappearance at day 16. (c) The formation of bands B and C at day 12. (d) Band L (MW 48.6 kDa, identified as actin) started to degrade at day 12 and disappeared at day 16. (e) The gradual appearance of bands M and N. (f) The intensities of band D (MW 158.7 kDa, identified as M-protein) and band E increased at days 12 and 16. (g) Band G (MW 103 kDa, identified as α- actinin) did not show any detectible changes except for a slight decrease in intensity at day 16. (h) Band K (MW 59 kDa, presumed identity desmin) (Goll, 1983 a and Koohmaraie et al., 1984 a) disappeared completely at day 8. (i) Bands P and Q (MW 37.8, MW 35.8, identified as α and β tropomyosin, respectively) disappeared completely at day 12. (j) Band F (MW 108.9 kDa, identified as C-protein) did not show any detectable change throughout the storage period. (k) At day 16, the intensities of bands H, I and S (MW 89.7, MW78 and MW 31 kDa) and troponin-T increased, while the intensities of band J (MW 66 kDa, identified as BSA), bands U, T, and V (MW 24.9, MW 28.2, MW 21.7 kDa, identified as troponin-I, myosin light chain

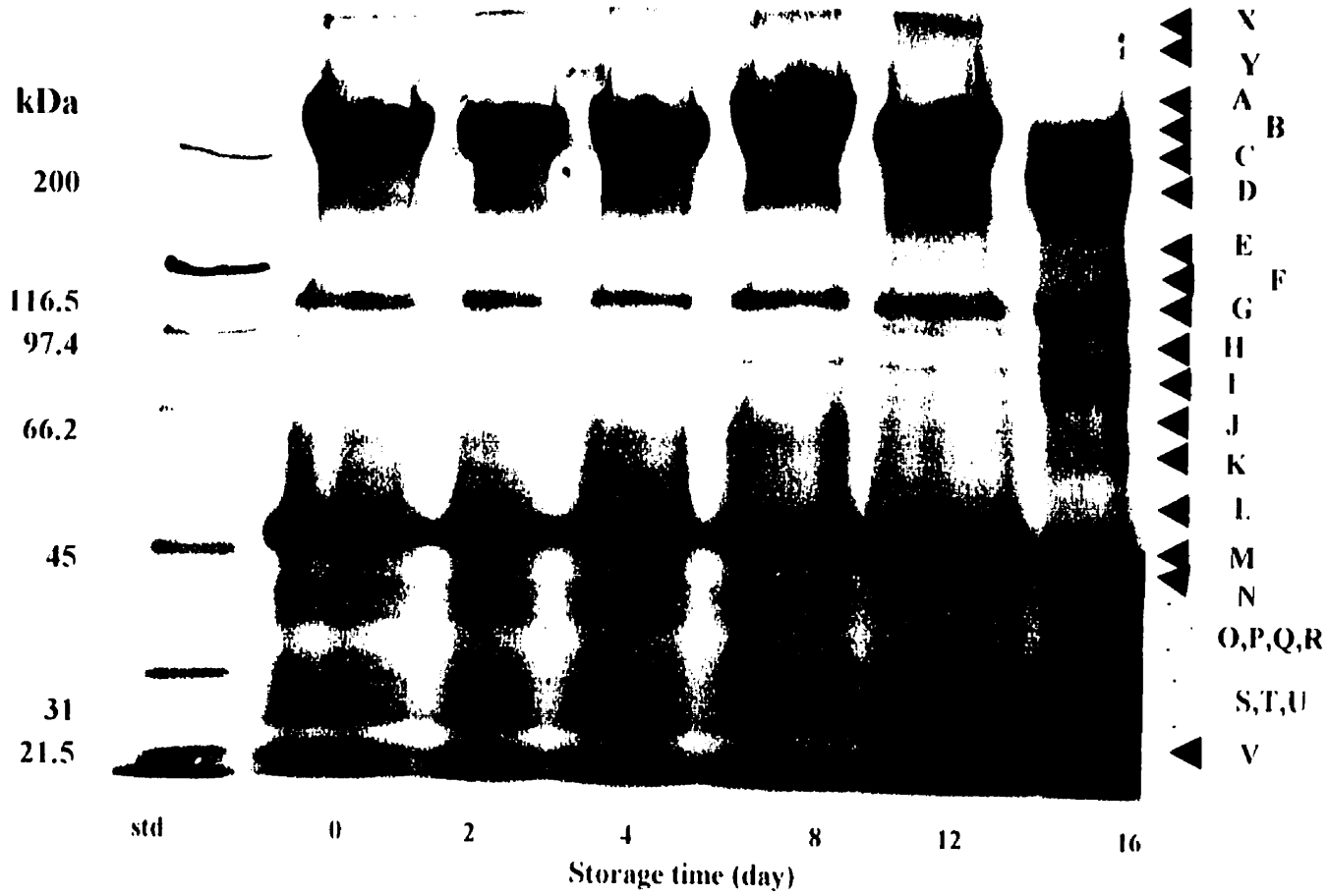


Figure 28. SDS-PAGE of myofibrillar proteins extracted from whole meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels X-V are identified in Table 15. Numbers at left represent molecular weight of standard protein (MW X 10^3)

Table 15. Changes in Myofibrillar Protein Components of Whole Meat During Storage: (SDS - Electrophoresis)

Band	Calculated MW (kDa)	Presumptive Identity*	Storage period (day)					
			0	2	4	8	12	16
X	285		NP	NP	NP	NP	NP	·
Y	277		NP	NP	NP	NP	NP	·
A	201	Myosin	P	—	—	—	–	–
B	195.5		NP	NP	NP	NP	·	—
C	173		NP	NP	NP	NP	·	·
D	158.7	M-Protein	P	—	—	—	·	—
E	129		P	—	—	—	·	—
F	108.9	C-Protein	P	—	—	—	—	—
G	103.0	Alpha-actinin	P	—	—	—	—	×××
H	89.7	95 kDa	P	—	—	—	—	·
I	78.0		P	—	—	—	—	·
J	66.0		P	—	—	—	—	–
K	59.0	Desmin	P	—	—	×××	×××	×××
L	48.6	Actin	P	—	—	—	—	×××
M	45.9		NP	NP	NP	NP	·	—
N	43.5		NP	NP	NP	NP	·	—
O	39.5	Troponin-T	P	—	—	—	–	·
P	37.8	Tropomyosin A	P	—	—	—	×××	×××
Q	35.8	Tropomyosin ß	P	—	—	—	×××	×××
R	32.9		NP	NP	NP	NP	NP	·
S	31.0	30 kDA	P	—	—	—	—	·
T	28.2	MLC 1	P	—	—	—	—	×××
U	24.9	Troponin-I	P	—	—	—	—	×××
V	21.7	MLC 2	P	—	—	—	—	×××

— No change in band intensity
– Band weakening
××× Band disappeared
· Increase in band intensity
NP Not present in gel
P Present in gel

* According to Goll. (1983); Xiong and Anglemier. (1989); Koohmaraie et al.,(1991, 1992); An et al., (1994); Kamin-Belsky et al., (1996) and Xiong et al., (1996)

1 and myosin light chain 2, respectively) decreased. These changes were consistent with the findings of other researchers (Koohmaraie et al., 1984a and b; Xiong and Anglemier, 1989; Ikeuchi et al., 1980; Etherington 1984 and Xiong et al., 1996), with exception that troponin-T was not found to decrease during storage. Olson et al. (1977) suggest that a 30 kDa protein was from degradation of troponin-T (band O); this could not be confirmed in our study Xiong and Anglemier (1989) reported that it was uncertain whether the appearance of 30 kDa protein was due to troponin-T degradation. Band B (α-myosin) and band C (β-myosin), could be the result of myosin degradation at day 12 and 16; Porzio and Pearson (1979), Robbins et al. (1979), An et al. (1994) and Kamin-Belsky et al. (1996) reported the formation of the same bands (B and C) as a result of myosin degradation and suggested that their formation is the result of autolytic changes.

Two endogenous proteinases systems have been proposed to be responsible for proteolytic degradation of myofibrillar proteins (Etherington et al., 1984). The first system is a calcium dependent neutral protease system (CAF, mM-CAF and μM-CAF); these enzymes have maximal proteolytic activities at neutral pH and are capable of degrading minor myofibrillar protein (Schreurs et al., 1996). The second system is composed of aspartic proteinases (cathepsin D), cysteine proteinases (cathepsin B, H and L) and cystatins (Ouali, 1992); these enzymes have maximal proteolytic activities at low pH and are capable of degrading myosin and actin and a large number of other myofibrillar proteins.

Zeece et al. (1986), Koohmaraie et al. (1986, 1987, 1988a,b,c, 1989, 1990, and 1992) and Uytterhaegen et al. (1994) suggested that CAF was responsible for changes observed during postmortem storage. Okitani et al. (1981), Matsukura et al. (1981), Mikami et al.

(1987) and Ouali et al. (1987) have shown that lysosomal proteases are responsible for myofibrillar protein degradation. Etherington et al. (1987) suggested that there was no relationship between muscle proteolysis and the activity of either CAF or lysosomal enzymes. In our study, the muscle pH of 5.7 is not optimal for CAF or for lysosomal enzymes (Yu and Lee, 1986); it is more likely that cysteine proteases (cathepsin B1, H and L), which have been shown (Etherington, 1981) to be active within the range of postrigor muscle pH of 5.0-6.0, are responsible for observed proteolytic changes; however, contribution of CAF enzymes (μM-CAF) cannot be excluded, since they retain as much as 25-28% of their maximum activity at 5 °C and pH 5.5-5.8 (Koohmaraie et al., 1986 and Xiong and Anglemier, 1989).

4.B..3.1.2.2. Ground Meat

Figure 29 shows the electrophoresis of myofibrillar protein isolated from ground meat; table 16 summarizes the changes in band intensity and estimated molecular weight (MW) of isolated myofibrillar protein fractions. The electrophoretic pattern of ground meat samples was generally similar to that of whole meat samples. At day 12, there was considerable loss of the following fractions: (a) Band A, E, F, J, K, R and S (MW 196.6, MW 101, MW 86.9, MW 57.7, MW 47, MW 24.8 and MW 22 kDa, identified as myosin, α-actinin, 95 kDa protein, desmin, actin, troponin-I and myosin light chain 2, respectively). (b) The intensity of band X, Y, B, G, L, C and D (MW 279, MW 255, MW 180, MW 84.4, MW 41.9, MW 160 and MW 105 kDa, respectively) increased at day 12. (c) Band N and O (MW 37.3, MW 35.2 kDa, identified as α and β tropomyosin) were slowly but gradually disappeared during storage. These results are in good agreement with reports by Xiong and Anglemier (1989) and Lin and Park (1996).

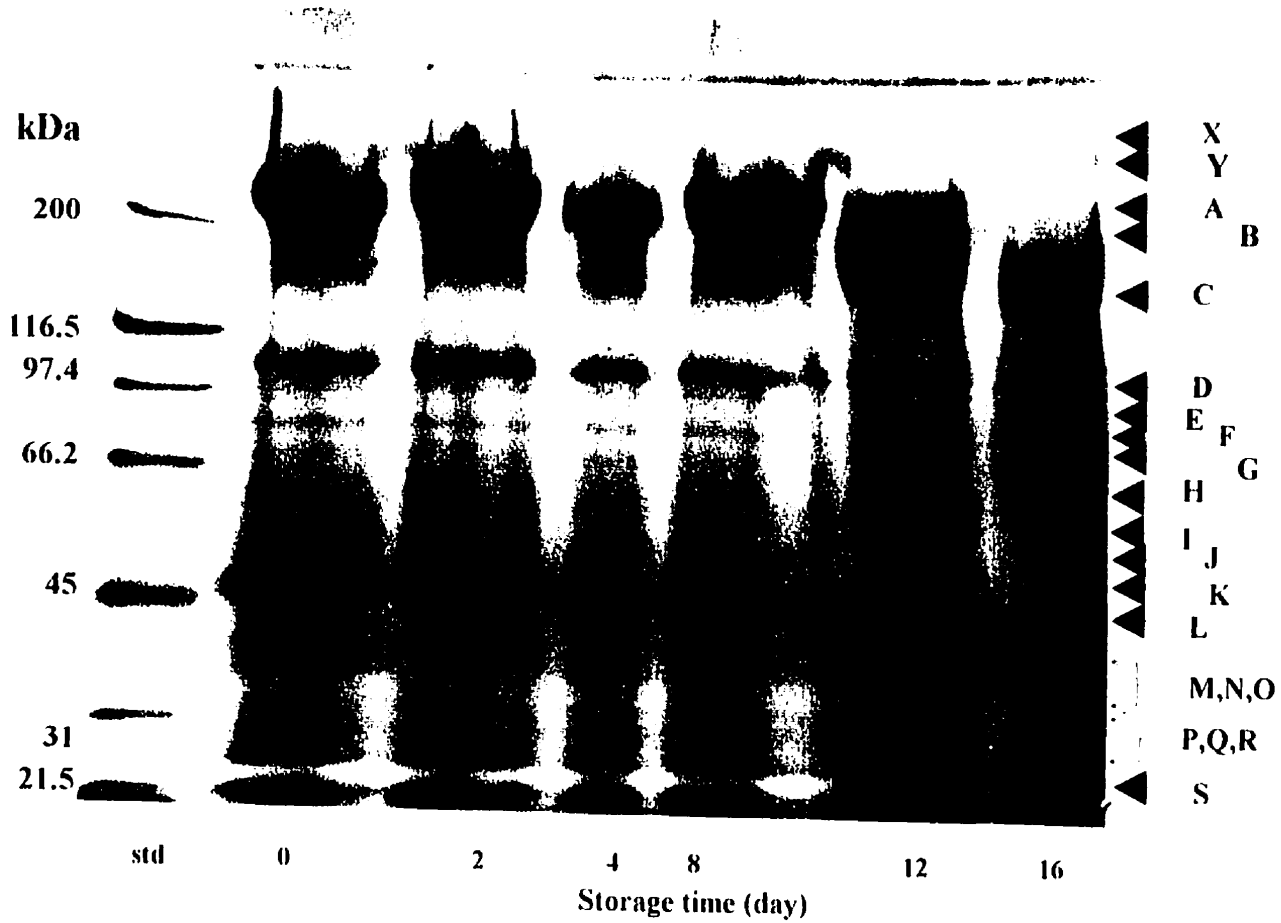


Figure 29. SDS-PAGE of myofibrillar proteins extracted from ground meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels X-V are identified in Table 16. Numbers at left represent molecular weight of standard protein (MW X 10^3)

Table 16. Changes in Myofibrillar Protein Components of Ground Meat During Storage: (SDS - Electrophoresis)

Band	Calculated MW (kDa)	Presumptive Identity*	Storage period (day)					
			0	2	4	8	12	16
X	279		NP	NP	NP	NP	·	——
Y	255		NP	NP	NP	NP	NP	·
A	196.6	Myosin	P	——	—	—	—	xxx
B	180		NP	NP	NP	NP	·	——
C	160	M-Protein	P	——	——	——	·	·
D	105.0	C-Protein	P	——	——	——	——	·
E	101	Alpha-actinin	P	——	——	——	xxx	xxx
F	86.9	95 kDa	P	——	——	——	xxx	xxx
G	84.4		NP	NP	NP	NP	——	·
H	77.3		P	——	——	——	xxx	xxx
I	66.8		P	——	——	——	——	xxx
J	57.7	Desmin	P	——	——	——	xxx	xxx
K	47.0	Actin	P	——	——	——	xxx	xxx
L	41.9		NP	NP	——	·	·	——
M	38.4	Troponin-T	P	——	——	——	·	——
N	37.3	Tropomyosin A	P	——	——	——	—	xxx
O	35.2	Tropomyosin ß	P	——	——	——	—	xxx
P	30.4	30 kDa	P	——	——	——	·	——
Q	28.6	MLC 1	P	——	——	—	——	——
R	24.8	Troponin-I	P	——	——	——	xxx	xxx
S	22.0	MLC 2	P	——	——	——	xxx	xxx

—— No change in band intensity

— Band weakening

xxx Band disappeared

· Increase in band intensity

NP Not present in gel

P Present in gel

* According to Goll. (1983); Xiong and Anglemier. (1989); Koohmaraie et al.,(1991, 1992); An et al., (1994); Kamin-Belsky et al., (1996) and Xiong et al., (1996)

It has been proposed that the rapid changes observed in ground meat when compared to whole meat can be attributed to the grinding process (Hamm, 1977). This along with high initial microbial loads in ground meat compared to whole meat samples. Our results suggest that the grinding process and high initial microbial load in the ground meat did not affect myofibril proteolysis. Crouse et al. (1991) reported that most of the changes in refrigerated meat result from autolytic enzymes during the first 14 days of storage. Beyond this period, microbial proteases are responsible for changes in myofibrillar proteins. The differences we observed in myofibrillar degradation between ground and whole meat samples at day 12 and 16 can be attributed to changes in pH and bacterial composition.

4.B.3.2. Sarcoplasmic Proteins

4.B.3.2.1. Native Electrophoresis

The electrophoretograms of sarcoplasmic proteins isolated from whole meat and ground meat and are shown in Figure 30 and 31, respectively. Seven protein bands (Table 17) were considered as the major components of sarcoplasmic protein. Band C (Rm 0.22, identified as creatin kinase) and band D, E and F (Rm 0.25, Rm 0.43 and Rm 0.51, identified as paravalbumin 1, 2 and 3, respectively) were the only identified proteins in sarcoplasmic extract. The native electrophoretograms of sarcoplasmic protein extracted from whole and ground meat (Figures 30 and 31) were essentially identical suggesting that no appreciable postmortem protein degradation occurred.

4.B.3.2.2. SDS-Electrophoresis

SDS-PAGE of sarcoplasmic protein isolated from ground and whole meat is shown in Figures 32 and 33. Proteins with MW 24-35 kDa and 60-160 kDa were well separated,

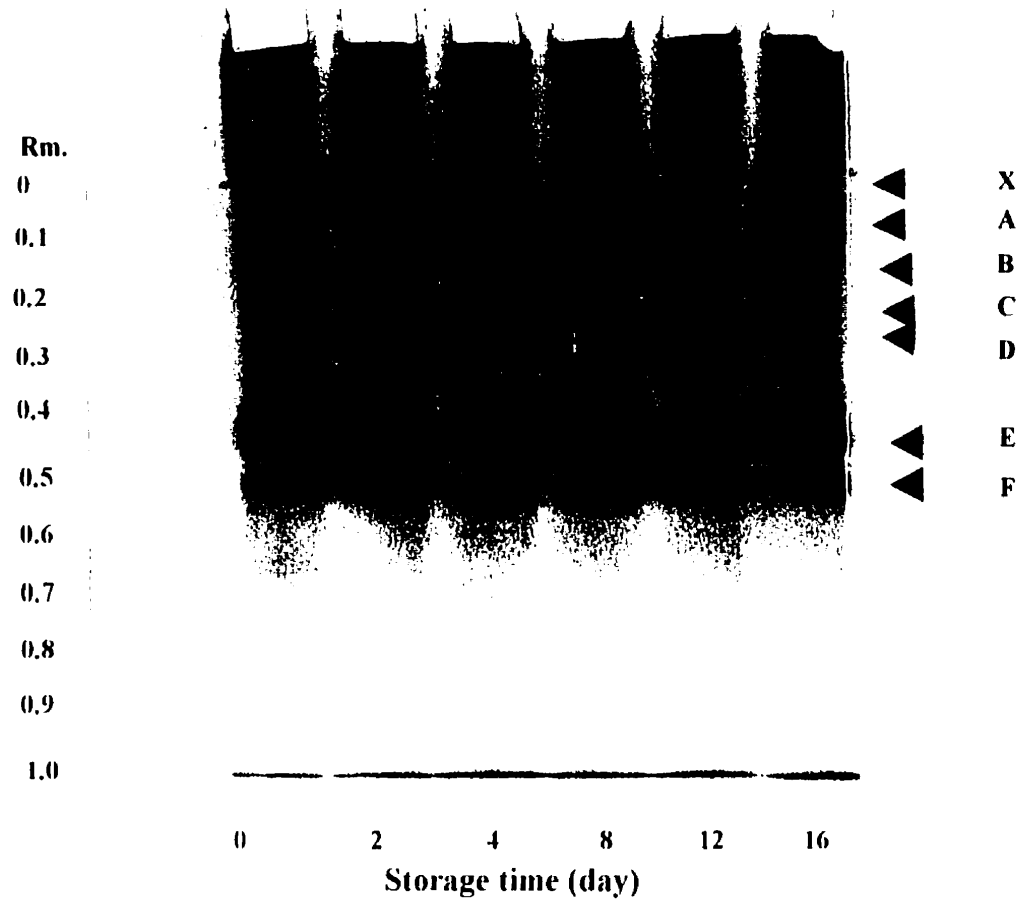


Figure 30. Native-PAGE of sarcoplasmic proteins extracted from whole meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels X-F are identified in Table 17. Number at left represent proteins relative mobility (Rm.), where Rm. of bromophenol blue band = 1

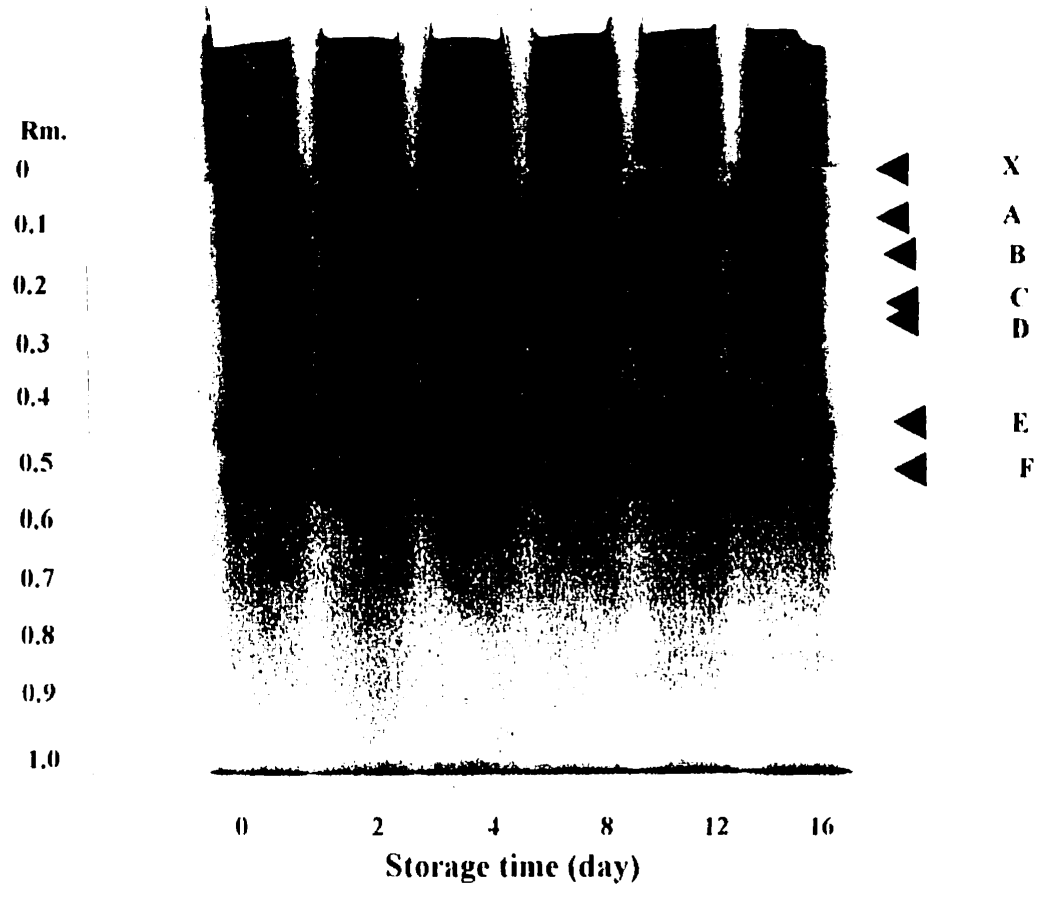


Figure 31. Native-PAGE of sarcoplasmic proteins extracted from ground meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels X-F are identified in Table 17. Number at left represent proteins relative mobility (Rm.), where Rm. of bromophenol blue band = 1

Table 17 . Changes in Sarcoplasmic Protein Components of Ground and Whole Meat During Storage; (Native Electrophoresis)

Band	Rm*	Presumptive Identity**	Storage period (day)					
			0	2	4	8	12	16
X	0		P	· ·	· ·	· ·	· ·	· ·
A	0.10		P	· ·	· ·	· ·	· ·	· ·
B	0.17		P	· ·	· ·	· ·	· ·	· ·
C	0.22	Creatine kinase	P	· ·	· ·	· ·	· ·	· ·
D	0.25	Parvalbumin 1	P	· ·	· ·	· ·	· ·	· ·
E	0.43	Parvalbumin 2	P	· ·	· ·	· ·	· ·	· ·
F	0.51	Parvalbumin 3	P	· ·	· ·	· ·	· ·	· ·

· · No change in band intensity
Band weakening
xxx Band disappeared
| Increase in band intensity
NP Not present in gel
P Present in gel

* Mobility of bromophenol blue band = 1

** According to Dias et al., (1994); Haard, (1995); and Wang et al., (1996)

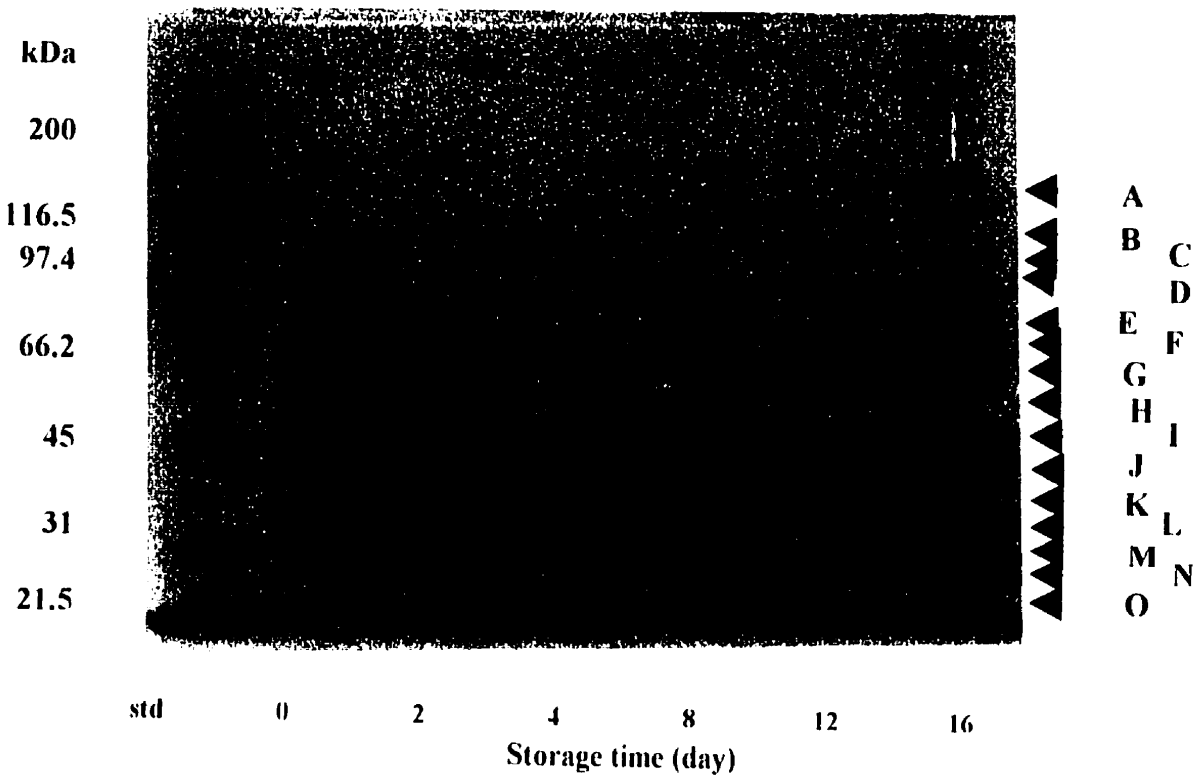


Figure 32. SDS-PAGE of sarcoplasmic proteins extracted from ground meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels A-O are identified in Table 18. Numbers at left represent molecular weight of standard protein (MW X 10^3)

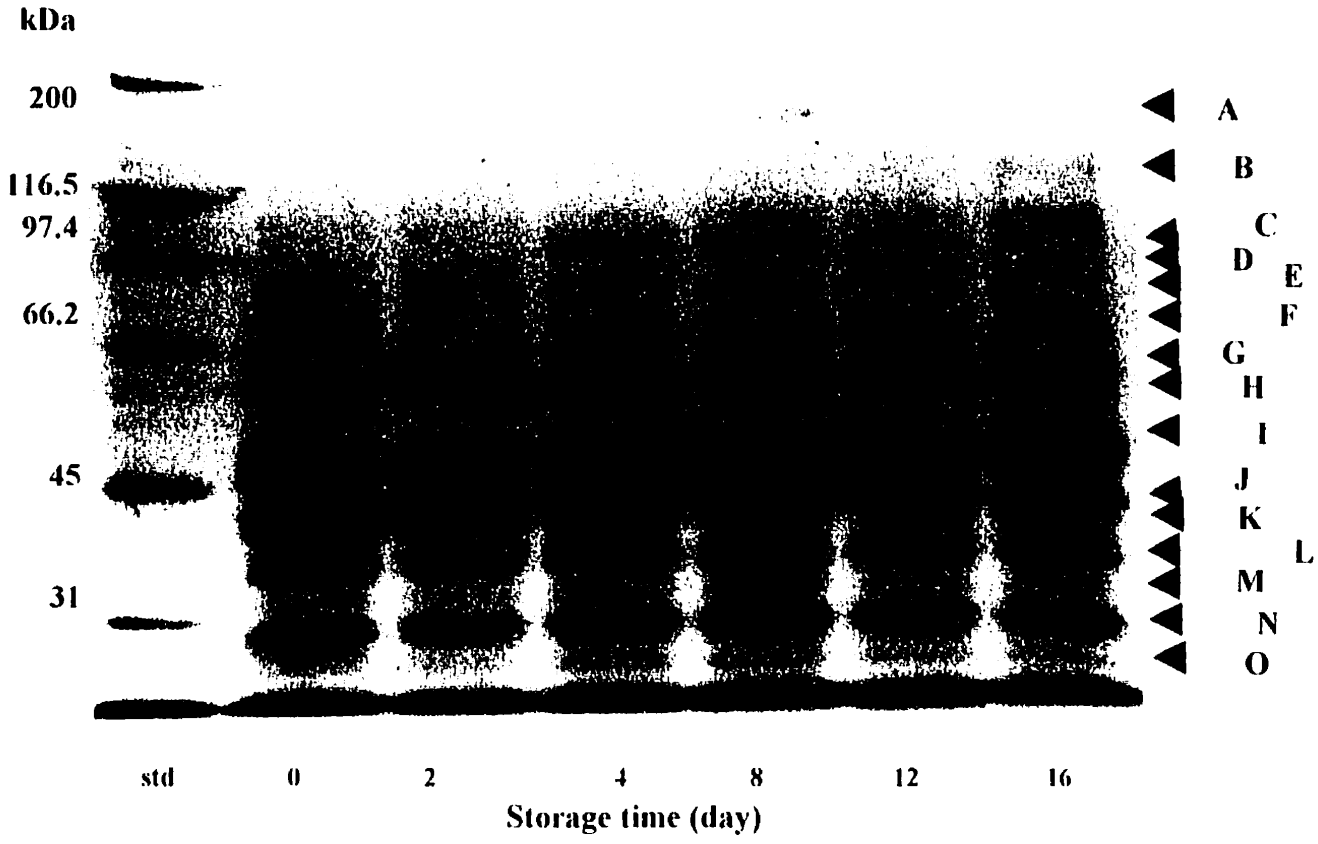


Figure 33. SDS-PAGE of sarcoplasmic proteins extracted from whole meat after 0, 2, 4, 8, 12 and 16 days of storage. The labels A-O are identified in Table 19. Numbers at left represent molecular weight of standard protein (MW X 10^3)

Table 18. Changes in Sarcoplasmic Protein Components of Ground Meat During Storage; (SDS-Electrophoresis)

Band	Calculated MW (kDa)	Presumptive Identity*	Storage period (day)					
			0	2	4	8	12	16
A	161.1		P	· ·	· ·	· ·	· ·	|
B	140.0		NP	NP	NP	NP		|
C	109.6		NP	NP	NP	· ·	|	|
D	102.2	Ca^{2+} ATPase	P	· ·		xxx	xxx	xxx
E	98.7	Alpha-actinin	P	· ·	· ·	· ·	· ·	· ·
F	77.3		P	· ·	· ·	· ·	· ·	· ·
G	67.2	BSA	P	· ·	· ·	· ·	· ·	· ·
H	62.6		P	· ·	· ·	· ·	· ·	· ·
I	52.6		P	· ·	· ·	· ·	· ·	· ·
J	44.2		P	· ·	· ·	· ·	· ·	· ·
K	41.2	Creatine kinase	P			xxx	xxx	xxx
L	35.8	Aldolase	P	· ·	· ·	· ·	· ·	|
M	33.4	GAPDH	P	· ·	· ·	· ·	· ·	· ·
N	28.5		P	· ·	· ·	· ·	· ·	|
O	24.4	TPI	P	· ·	· ·	· ·	· ·	· ·

· · No change in band intensity

Band weakening

xxx Band disappeared

\| Increase in band intensity

NP Not present in gel

P Present in gel

* According to Dias et al., (1994), Haard, (1995), and Wang et al., (1996)

Table 19. Changes in Sarcoplasmic Protein Components of Whole Meat During Storage; (SDS-Electrophoresis)

Band	Calculated MW (kDa)	Presumptive Identity*	Storage period (day)					
			0	2	4	8	12	16
A	155.7		NP	NP	· ·	· ·	· ·	· ·
B	135.9		NP	NP	NP	NP	· ·	↑
C	108.0		NP	NP	NP	· ·	↑	↑
D	100.9	Ca^{2+} ATPase	NP	NP	· ·	· ·		⋏⋏
E	93.0	Alpha-actinin	P	· ·	· ·	· ·	· ·	· ·
F	74.9		P	· ·	· ·	· ·	· ·	· ·
G	65.4	BSA	P	· ·	· ·	· ·	· ·	· ·
H	60.3		P	· ·	· ·	· ·	· ·	· ·
I	48.5		P	· ·	· ·	· ·	· ·	· ·
J	41.2	Creatine kinase	P	· ·	· ·	· ·	· ·	· ·
K	39.0	Aldolase	P				⋏⋏	⋏⋏
L	34.1	GAPDH	P	· ·	· ·	· ·	· ·	· ·
M	29.8		P	· ·	· ·	· ·	· ·	· ·
N	26.7		P	· ·	· ·	· ·	· ·	· ·
O	24.0	TPI	P	· ·	· ·	· ·	· ·	· ·

· · No change in band intensity

Band weakening

⋏⋏ Band disappeared

↑ Increase in band intensity

NP Not present in gel

P Present in gel

* According to Dias et al., (1994), Haard, (1995), and Wang et al., (1996)

while those major proteins with MW between 35-60 kDa clustered together (Figures 32 and 33). The electrophoretic patterns of ground and whole meat were similar; in both there was a noticeable increase in intensity of bands A, B, and C (MW 155.7-161.1, MW 135.9-140 and MW 108-109.2 kDa, respectively) and gradual disappearance of band K and D (MW 39-41.2, MW 100.9-102.2 kDa) (Tables 18 and 19). This decrease was noticeable earlier (day 8)in ground meat (Figure 32 and Table 18), when compared to whole meat (day 12) (Figure 33 and Table 19); similar degradation patterns were observed for band D (Table 18 and 19) The disappearance of band K and D correlate well with formation of high MW proteins (band A, B, and C). Although CAF is present in sarcoplasm and have the ability to degrade sarcoplasmic proteins (Drabikowksi et al., 1977 and Erikson et al., 1983) its effect was not pronounced in our experiment; similar results were reported by Goll et al. (1983 a) and Xiong and Anglemier (1989).

4.B.4. Changes in Protein Content Extracted from Whole and Ground Meat

Changes in protein content of soluble proteins is shown in Figure 34. The average initial content of sarcoplasmic, myofibrillar and ASN in ground and whole meat was 4 8, 9 7 and 8.2 g/L, respectively. There was a trend for myofibrillar protein content to increase in whole meat after day 4 and after day 8 in ground meat however, this increase was not statistically significant ($P > 0.05$). Similar results were reported by Salm et al (1983) Lopez-Bote et al. (1989) reported that the use of sarcoplasmic protein solubility, rather than solubility of total protein, was a useful indication of muscle quality

Several reports have indicated that increased storage time (Miller et al., 1980), freezing (Awad et al., 1968) and postmortem aging (Lan et al., 1993) reduce the extractability

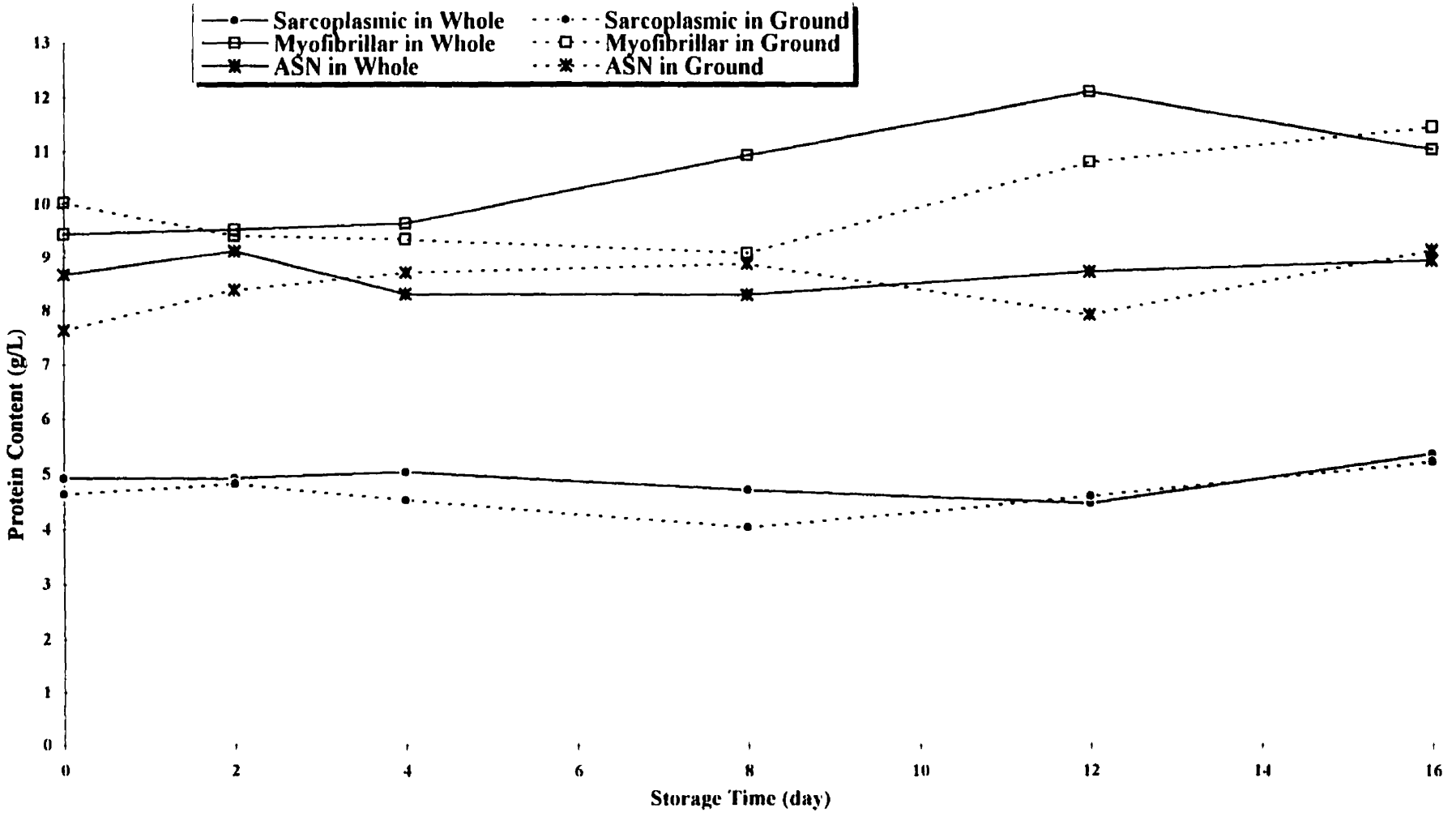


Figure 34. Changes in the Protein Content of Sarcoplasmic, Myofibrillar and Acid Soluble Nitrogen (ASN) Extracted from Ground and Whole Meat

of muscle proteins as a result of protein denaturation. On the other hand, several other reports indicated that changes in protein content during storage are insignificant (Xiong and Brekke, 1989 and Lin and Park, 1996). Salm et al. (1983) suggested that myofibrillar protein solubility in KI buffer increased with ageing of beef. These reports demonstrate the difficulty associated with protein solubility as an indicator of quality.

4.B.5. Liquid Chromatography - Mass Spectrometry

The chromatograms obtained from HPLC analysis of sarcoplasmic proteins isolated from ground and whole meat is shown in Figures 35 and 36, respectively; at least 12 peaks having MWs ranging from 11 to 42 kDa were identified by electrospray ionization-mass spectrometry (ESI-MS). A representative, interpreted mass spectra of typical ESI-MS of the major peaks are presented in Figure 37 a, b, c,d,e. The results suggest that proteolytic changes were observed in sarcoplasmic protein extracted from ground and whole meat. Changes in RA % of protein with a MW of 35.7 kDa are shown in Figure 38. These data indicated that RA % of this protein decreased progressively at day 12 and completely disappeared at day 16 (Figure 38); this decrease was more pronounced in whole meat when compared with ground meat. Since the bacterial load in whole meat was lower than that of ground meat (Tables 9 and 10), the decrease in RA % of this protein in whole meat can be attributed to the autolytic changes rather than microbial degradation.

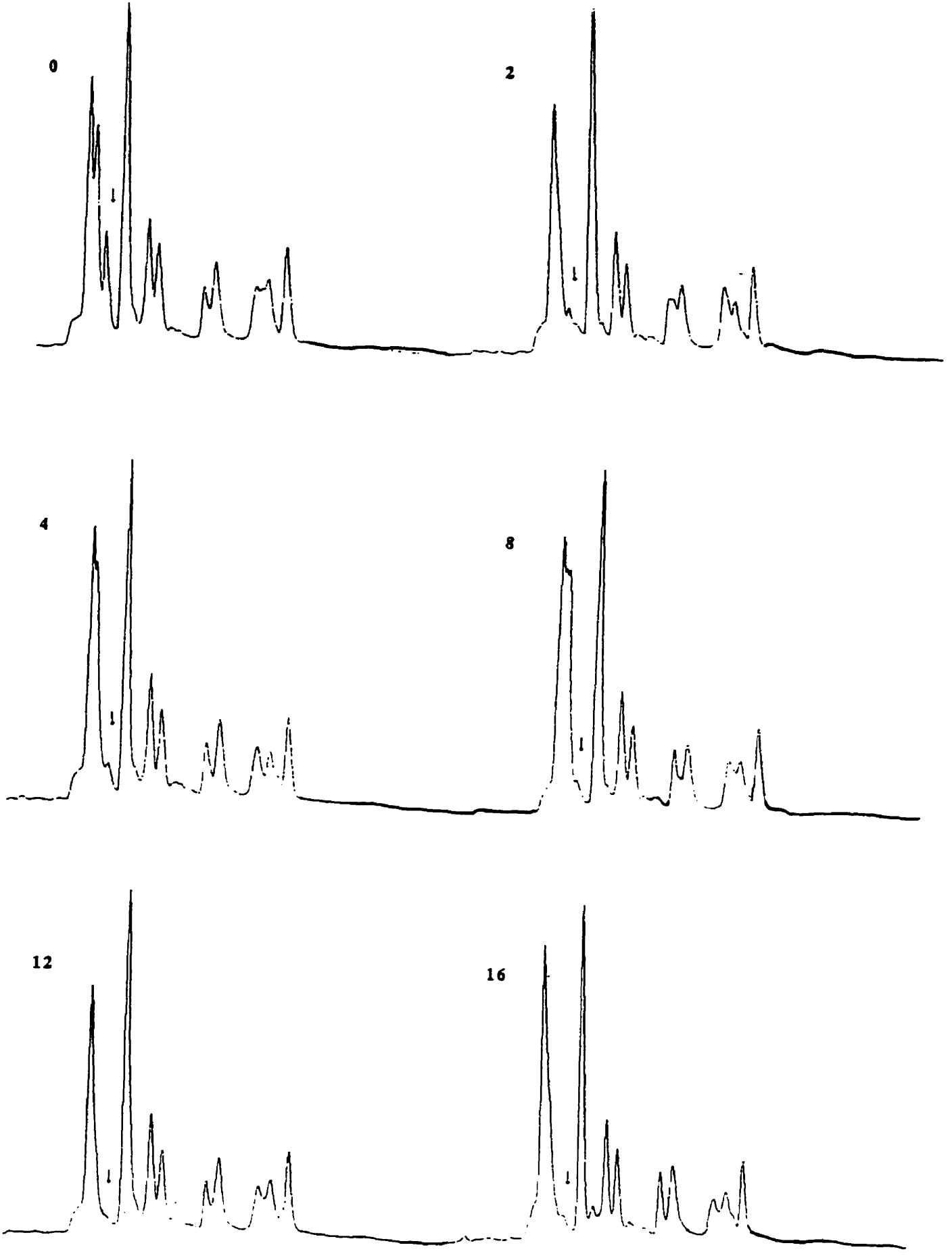


Figure 35. RP-HPLC Chromatogram of Sarcoplasmic Proteins Extracted from Whole Meat During 16 Days of Storage

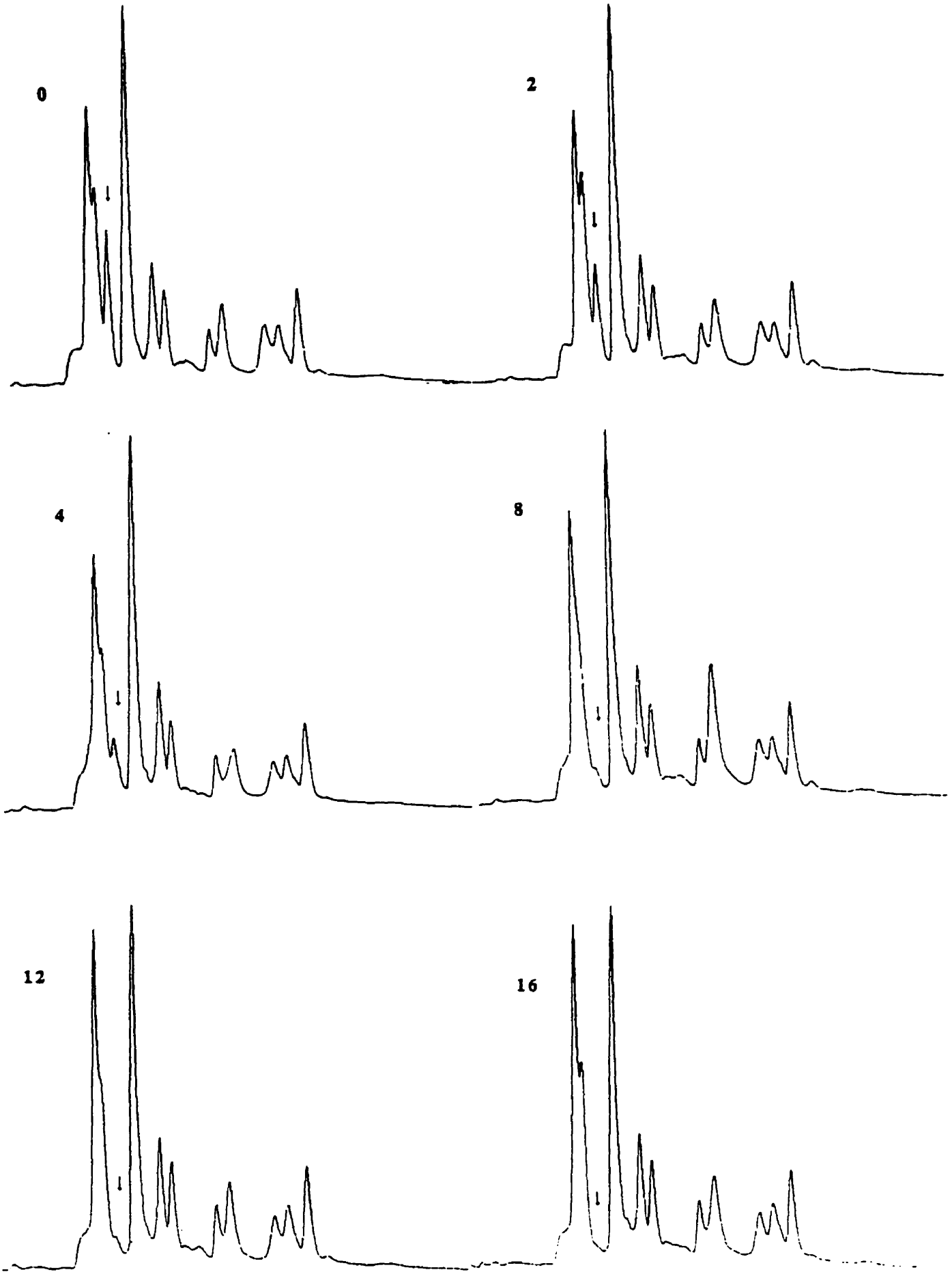


Figure 36. RP-HPLC Chromatogram of Sarcoplasmic Proteins Extracted from Ground Meat During 16 Days of Storage

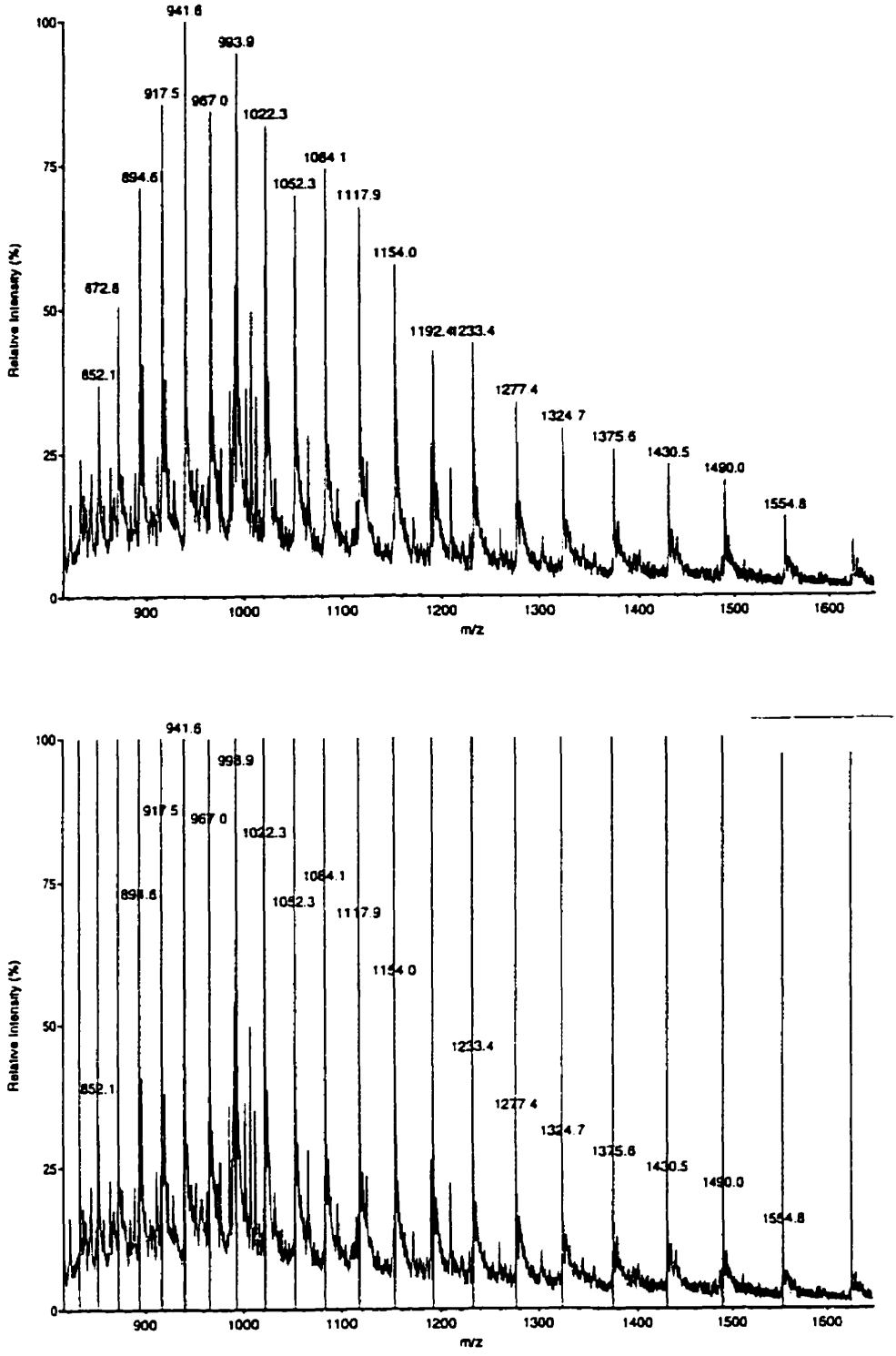


Figure 37-A. Representative of Typical Interpreted ESI-Mass Spectra of Fraction A. Component Mass is 35,741 D

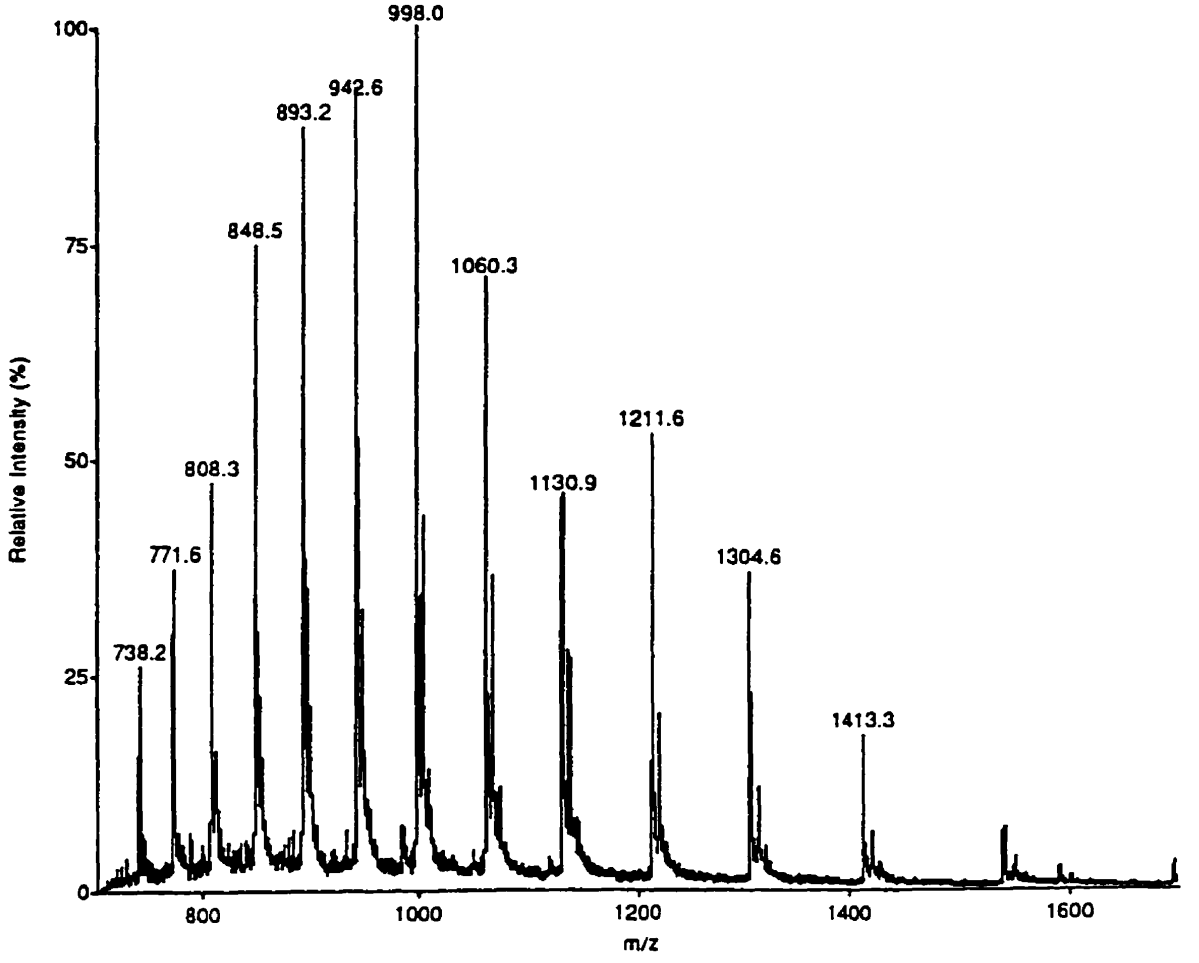


Figure 37-B. Representative of Typical Interpreted ESI-Mass Spectra of Fraction B. Component Mass is 16,950 D

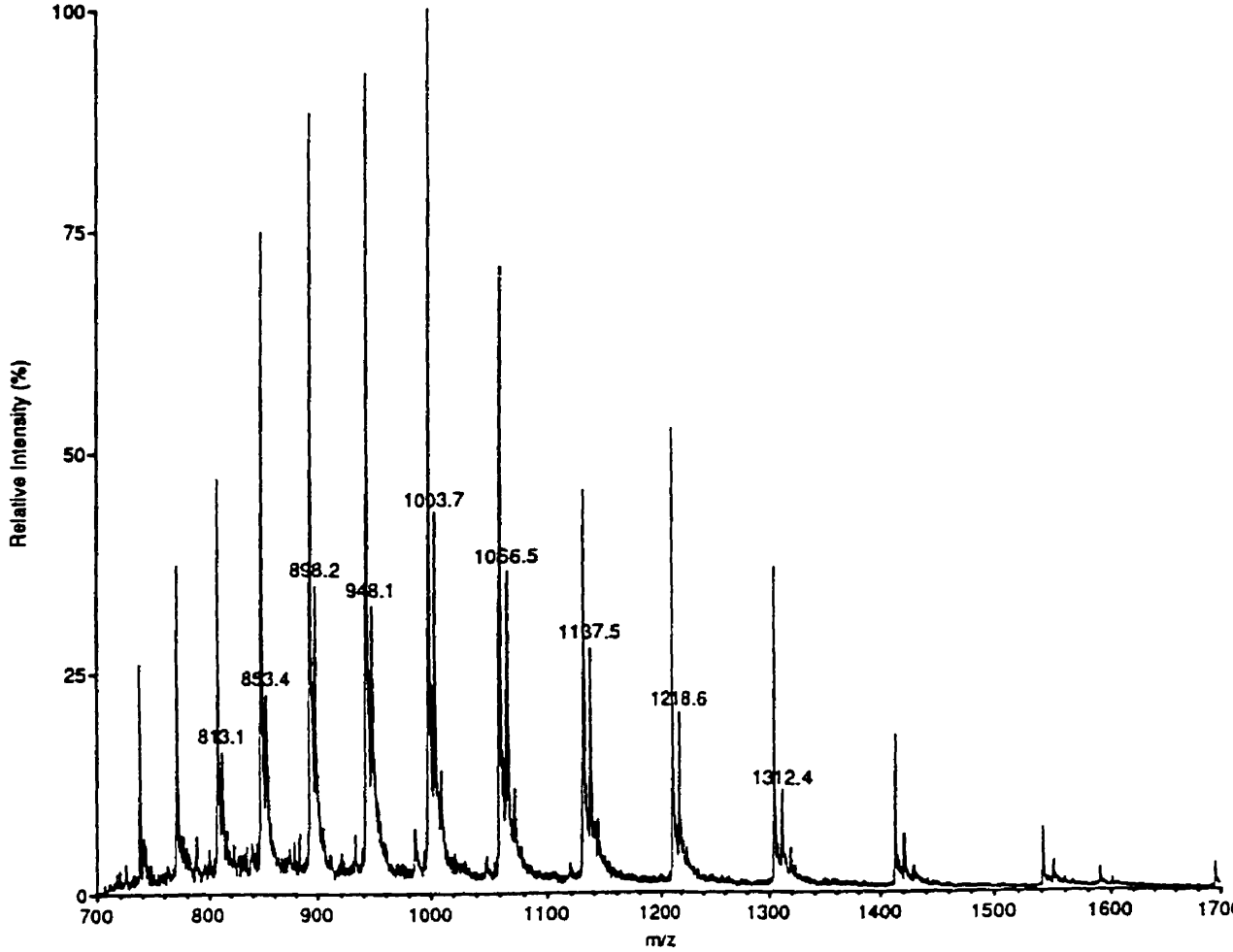


Figure 37-C. Representative of Typical Interpreted ESI-Mass Spectra of Fraction C. Component Mass is 17,047 D

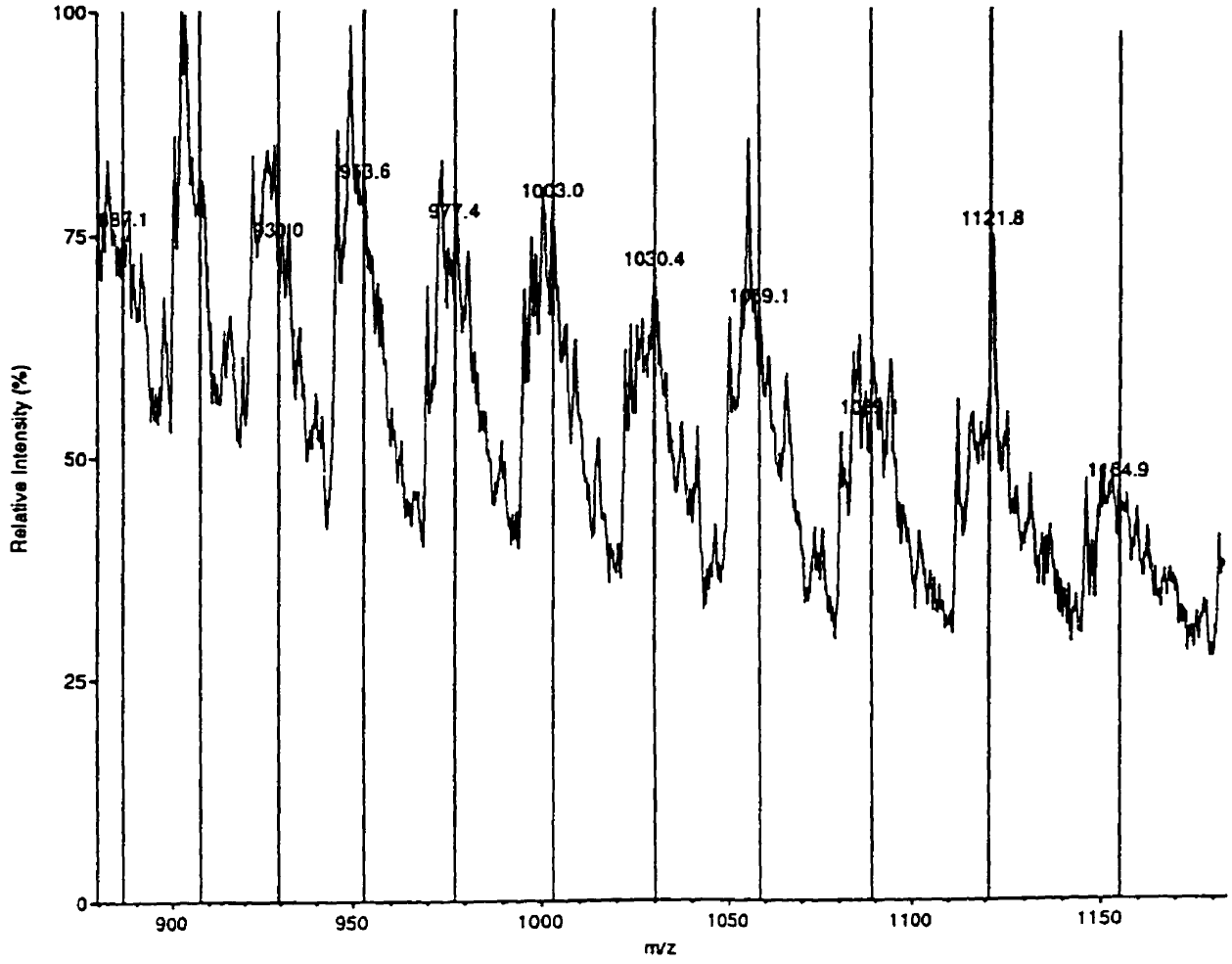


Figure 37-D. Representative of Typical Interpreted ESI-Mass Spectra of Fraction D. Component Mass is 37,800 D

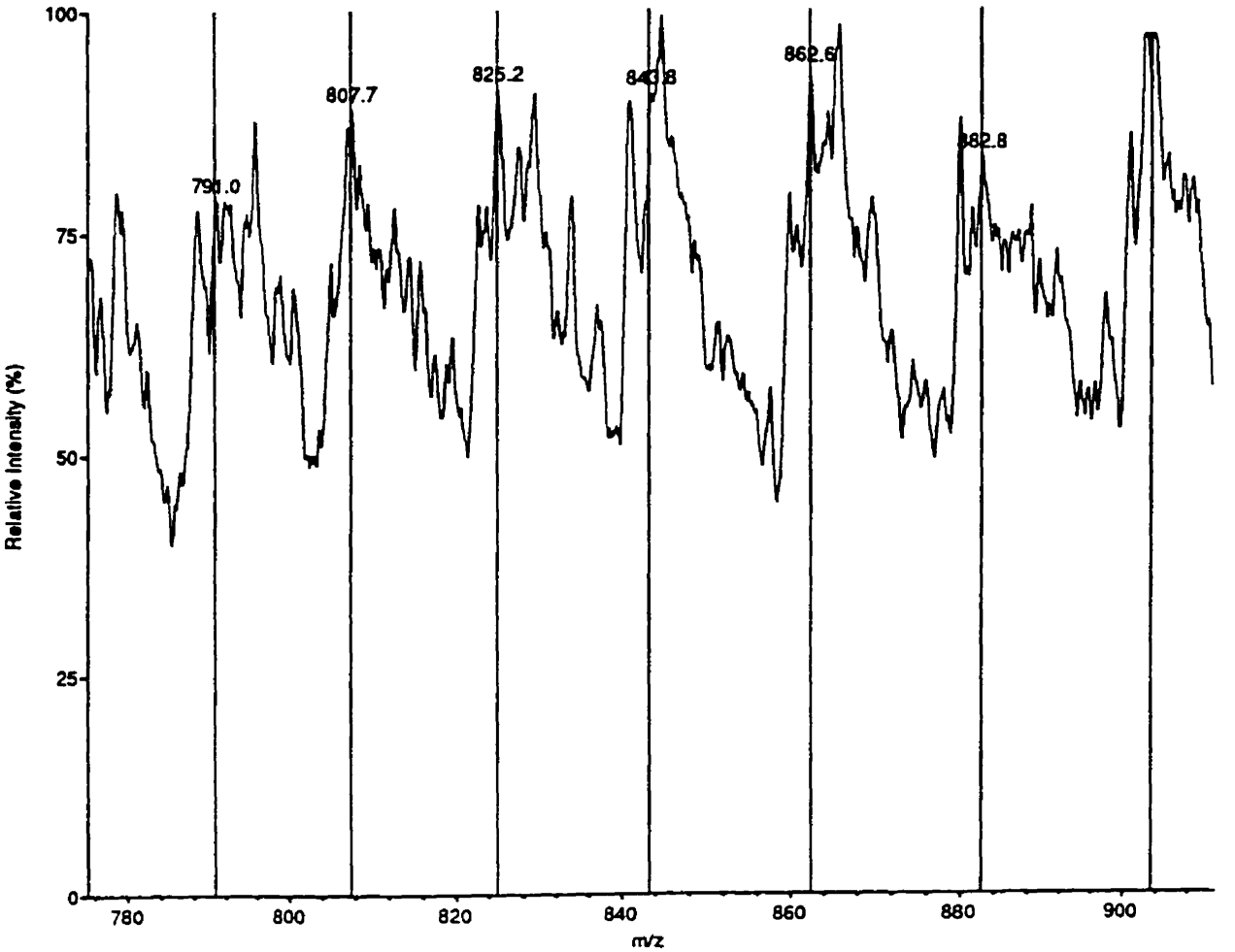


Figure 37-E. Representative of Typical Interpreted ESI-Mass Spectra of Fraction E. Component Mass is 37,917 D

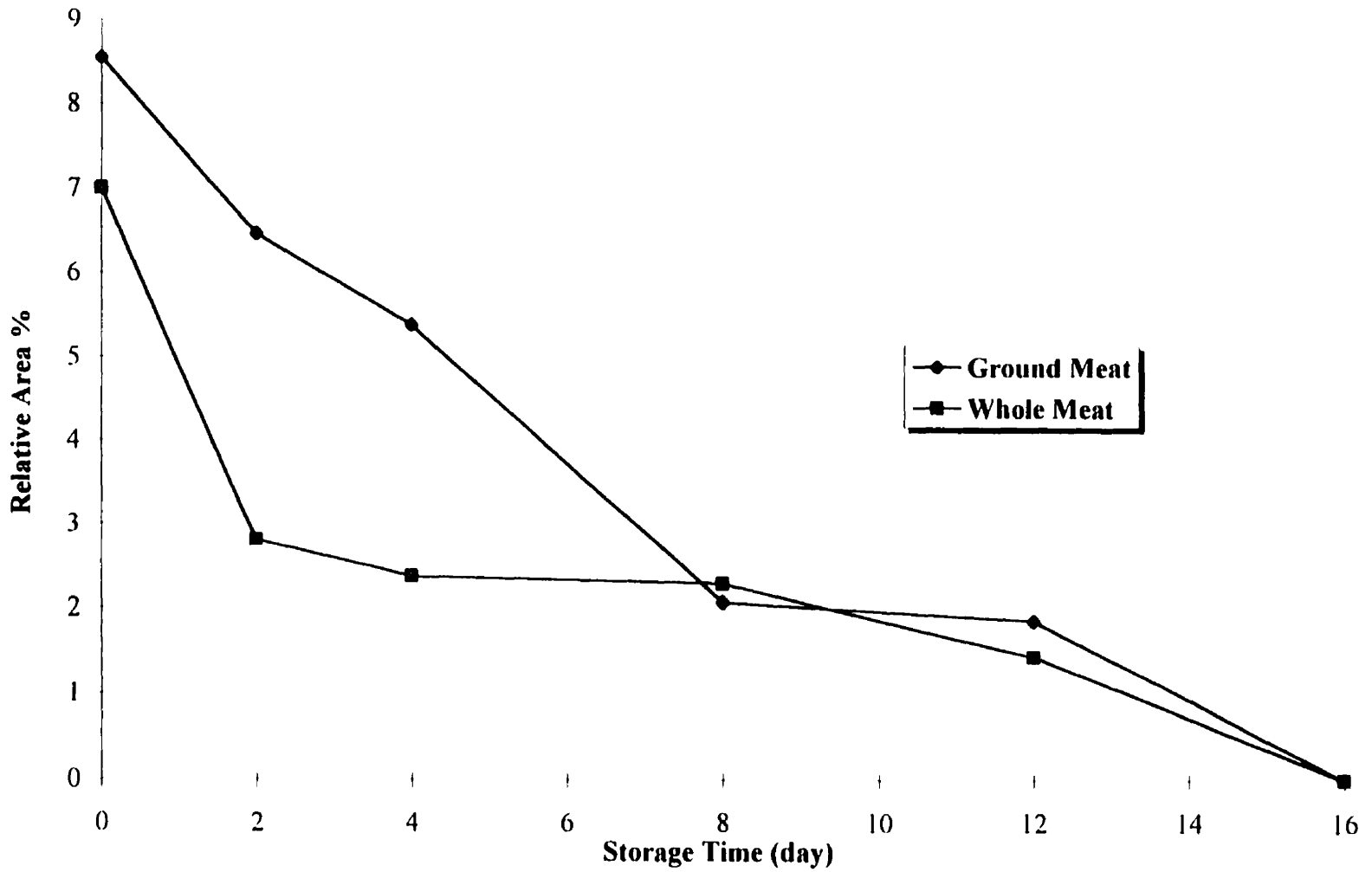


Figure 38. Changes in the Relative Area % of Protein 35.7 kDa Separated from sarcoplasmic Extract of Ground and Whole Meat

5. GENERAL CONCLUSIONS

1. Microbiological analysis indicated that carp fish stored aerobically at temperature 0-2 °C for 16 days supported bacterial growth to a level which did not result in noticeable deterioration.

2. Changes in carp fish pH during storage was significant under controlled experimental conditions.

3. Major changes in carp fish myofibrillar took place after day 2 of storage. No changes in large MW proteins were observed, however, reductions in band intensity of low MW proteins identified as tropomyosin, myosin light chain 1, troponin-I and myosin light chain 2 occurred. These changes can be related to autolytic activity rather than bacterial action since the bacterial counts in carp fish were relatively low.

4. The disappearance of bands D (66 kDa) and A (140 kDa) and the gradual increase in band intensity of band F (creatin kinase) and band G (aldolase) were the major changes in sarcoplasmic proteins; they are potential indicators for the state of freshness of chilled carp fish.

5. In carp fish, changes in relative area (%) of the polypeptides with MW 10.9, 16.7, 32.8 and 42.8 kDa and changes in MW of polypeptides with MW 12, 34.2 and 42.8 kDa were observed.

6. No significant changes in pH were observed in whole meat, and only at day 12 and 16 for ground meat. This can be attributed to the buffering capacity of meat muscle proteins.

7. Changes in band intensity of high myofibrillar proteins MW identified as myosin, actin and desmin of ground and whole meat were observed; these changes were more noticeable in ground meat samples. It appears that the proteolytic activity of cysteine proteases could be responsible for myofibrillar degradation in meat, since optimal pH of these enzymes is within muscle pH.

8. Changes in band intensity of a sarcoplasmic protein with MW of 39-41 kDa was identified as potential indicator of freshness in ground and whole meat samples.

9. In general, native electrophoretograms of sarcoplasmic and myofibrillar proteins in fish and meat (ground and whole) revealed no significant changes in band intensity of separated proteins. Thus, proteolysis cannot be monitored by native electrophoresis.

10. No significant changes in content of soluble proteins, either in fish or in meat (ground and whole) was observed. Therefore, changes in protein extractibility were not identified as potential indicators of freshness in fish and meat.

11. Change in RA % of sarcoplasmic protein with a MW of 35.7 kDa extracted from ground and whole meat was observed, with a progressive decrease until day 12 and complete disappearance at day 16; this decrease was more noticeable in whole meat. Since the bacterial load in whole meat was lower than that of ground meat, then rapid decrease in RA % in whole meat can be attributed more to autolytic changes than bacterial changes.

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IMAGE EVALUATION
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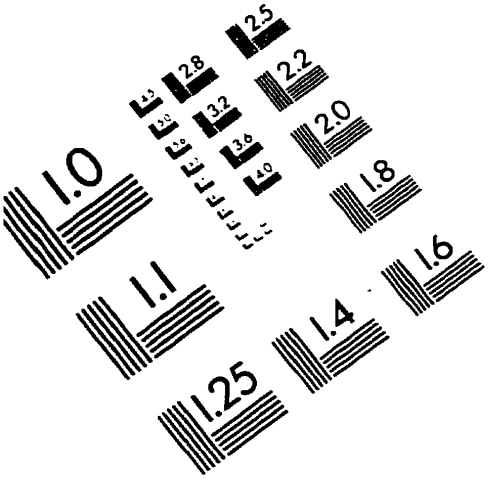

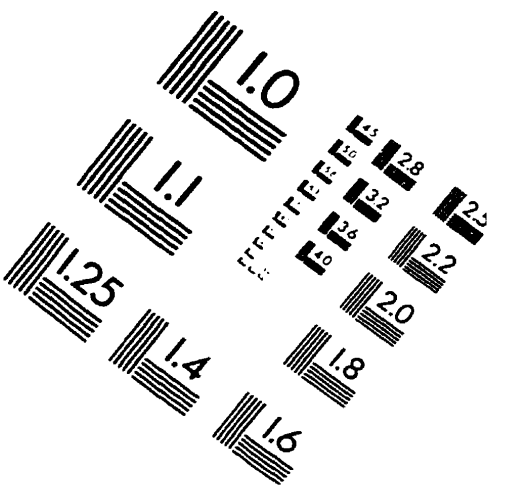

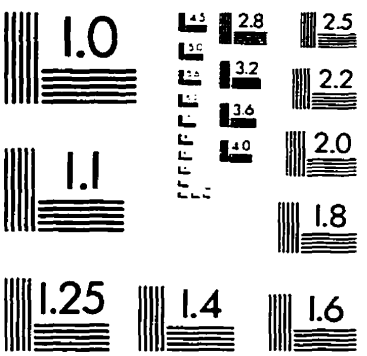

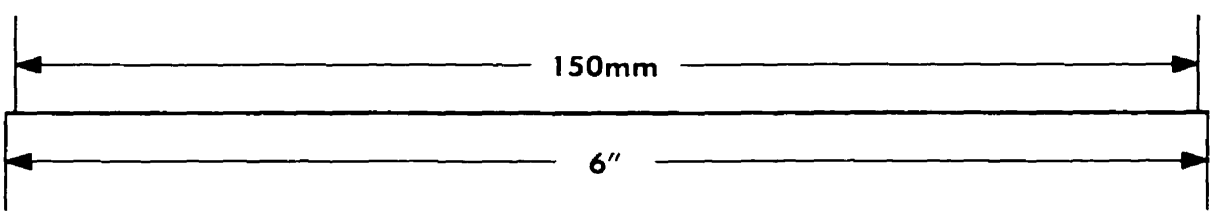

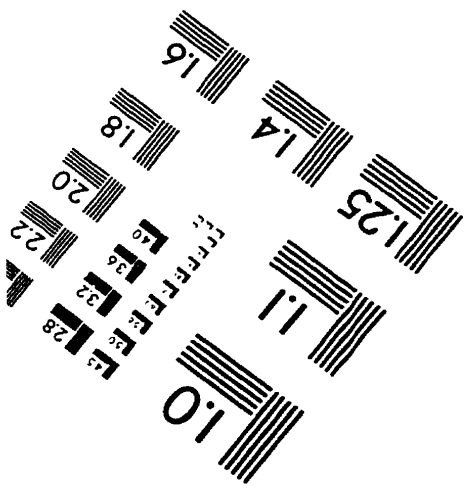

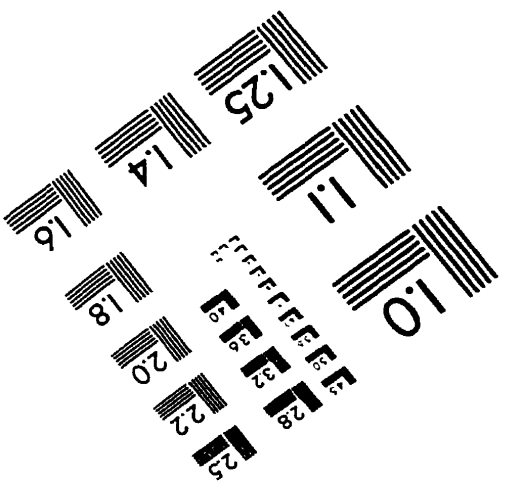

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