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Carolyn Felicity Ross

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James J Pestka

Major professor

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VERIFICATION OF A MONOCLONAL ANTIBODY-BASED ELISA FOR MONITORING LIPID OXIDATION IN CHICKEN MYOFIBRILS

By

Carolyn Felicity Ross

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ABSTRACT

VERIFICATION OF A MONOCLONAL ANTIBODY-BASED ELISA FOR MONITORING LIPID OXIDATION IN CHICKEN MYOFIBRILS

By

Carolyn Felicity Ross

Lipid oxidation is a major quality deterioration problem in all muscle foods, affecting both the organoleptic value and toxicological status of the food. Detection of lipid oxidation needs to be accomplished in a very specific, sensitive and rapid manner. The standard method for measuring lipid oxidation is the 2-thiobarbituric acid reactive substances (TBARS) assay. While this assay is simple, criticisms arise from its lack of specificity. Hexanal, a secondary product of lipid oxidation, has often been used as an indicator of lipid oxidation. Headspace-gas chromatography has typically been used to measure hexanal; however, this method is expensive and tedious. As a sensitive, rapid and reproducible alternative, an enzyme-linked immunosorbent assay (ELISA) has been previously described for the detection of hexanal in meat products. The objective of this study was to verify the use of the monoclonal ELISA for monitoring lipid oxidation in chicken protein through the quantification of hexanal.

A solid-phase microextraction (SPME) method combined with gas chromatography/mass spectrometry (GC/MS) was developed and evaluated for its suitability in the detection of hexanal in chicken myofibrils. Results indicated that recovery of hexanal from spiked myofibrils was 95% using a 5 min sampling time, with a

total analysis time of about 12 min/sample. Using SPME, the limit of detection for hexanal in myofibrils was 10 ppb with a coefficient of variation ranging from 1 to 13%, and a working linear response from 10 ppb to 10 ppm (r^2=0.995). The ability of the hexanal-specific monoclonal CI-ELISA to monitor lipid oxidation in freeze-dried chicken protein was compared to the thiobarbituric acid reactive substances assay (TBARS) and the solid-phase microextraction- gas chromatography/mass spectrometry (GC/MS-SPME) method for hexanal. Protein extraction and solubilization procedures were optimized to maximize the formation of hexanal-protein adducts and percent extractable protein. Freeze-dried myofibrils (MF) with added methyl linoleate (0.6 mmol/g protein) were stored for 5 days at 50°C at a_w of 0.30 and 0.75. Hexanal was measured by GC/MS-SPME and CI-ELISA, and malonaldehyde was measured by TBARS. Lipid oxidation reached a maximum after 4 days of storage at both water activities, then decreased. At low water activity, 34.7 and 39.7 ppm hexanal were detected by GC/MS-SPME and CI-ELISA respectively, after 4 days of storage. The CI-ELISA was well correlated with the GC/MS-SPME (r=0.78) and TBARS (r=0.87) methods. The correlation of the hexanal-specific CI-ELISA to both GC/MS-SPME and TBARS verified the ability of the CI-ELISA to be used as an index of lipid oxidation, offering the convenience for use in a kit to be utilized within a food processing facility.

My Days Go On

I praise Thee while my days go on;
I love Thee while my days go on;
Through dark and dearth, through fire and frost,
With emptied arms and treasure lost,
I thank Thee while my days go on

~ Elizabeth Barrett Browning

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CHAPTER 1

INTRODUCTION

Lipid oxidation has long been recognized as a leading cause of quality deterioration in muscle foods. Changes in quality are manifested by adverse changes in flavor, color, texture and nutrient value. Lipid oxidation is often the decisive factor in determining the useful processing and storage life of both fresh and frozen meat products (Frankel, 1993). In addition, consumer acceptance of meat products depends to a major extent on flavor quality, and flavors generated by lipid oxidation are generally undesirable (Ramarathnam et al., 1991). All muscle foods are susceptible to lipid oxidation; however, the muscle foods of most concern are those with high concentrations of unsaturated fatty acids, such as chicken. As reported by the USDA (2001), chicken consumption has surpassed pork and beef, at 82 pounds of chicken consumed per person per year. Chicken consumption is expected to continue to increase over the next several years (National Chicken Council, 2001).

The production of off-flavor and odors were considered to be the most important consequences of lipid oxidation in the past; however, increased attention is now being given to the health risks that lipid oxidation may impose. In animal studies, thermally oxidized lipids were found to be precursors of colon cancer (Yang et al., 1998; Corpet et al., 1990). The genotoxicity and cytotoxicity of the secondary oxidation products, malonaldialdehyde and 4-hydroxynonenal, has been established through several studies

(Esterbauer, 1993; Bird et al., 1982). Research has also linked the formation of these secondary products to disease states such as atherosclerosis (Kondo et al., 2001; Salomon et al., 2000), autoimmunity (Khan et al., 2001), and cancer (Bruce et al., 1993). Due to health concerns, measuring the extent of lipid oxidation is becoming increasingly important due to the possible health risks associated with the consumption of lipid oxidation products.

From the perspective of the meat industry, detection of lipid oxidation needs to be accomplished in a very specific, sensitive and rapid manner. While organoleptic evaluation provides the most useful consumer information, it is time consuming and often has low reproducibility (Frankel, 1993). Chemical methods attempt to improve on these limitations but ultimately, the results of chemical methods must support sensory measurements. The most commonly used method for measuring lipid oxidation is the 2-thiobarbituric acid reactive substances test (TBARS), which was originally thought to measure only malonaldehyde, a secondary product of lipid oxidation. Criticisms of TBARS arise from its lack of sensitivity and its specificity to malonaldehyde.

The measurement of hexanal, another secondary product of lipid oxidation, has been used as an indicator of lipid oxidation. Hexanal is the dominant oxidation product of linoleic acid, being generated by both the 9 and 13-hydroperoxide of linoleate. Due to its low odor threshold, hexanal is also a major contributor to the rancid odors and flavors associated with oxidized meat (Shahidi and Pegg, 1994). Gas chromatography was used to establish strong correlations (0.995) between hexanal content, sensory scores and TBARS values in a number of meat products (Spanier et al., 1992; Ahn et al., 1998). Gas

chromatography has many drawbacks, including cost, time and the need for trained personnel. The majority of studies in the area of lipid oxidation have employed the traditional headspace GC techniques as a standard against which other methods are evaluated. Solid-phase microextraction (SPME) is a new adsorption technique that has gained widespread acceptance in volatile analysis during recent years (Steffen and Pawliszyn, 1996). SPME involves the extraction of analytes from the headspace above a sample onto a fused silica fiber; the analytes are then desorbed into a GC for analysis. While SPME combined with gas chromatography/mass spectrometry has been used for volatile analysis in various fruits and vegetables, its application has been limited in meat products. In the area of lipid oxidation, SPME has recently been employed for the detection of aldehydes in turkey (Brunton et al., 2000) and sunflower oil (Keszler et al., 1998). SPME overcomes the difficulties associated with traditional GC techniques, including lengthy analysis time, use of solvents, and equipment dedication.

Hexanal has been detected using both polyclonal (Smith et al., 1999) and monoclonal antibodies (Zielinski et al., 2001) in a competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) . The advantages of a CI-ELISA over conventional techniques have been well described in the literature as rapid, reproducible, and highly sensitive (Samarajeewa et al., 1991). In addition, the CI-ELISA offers the convenience for use in a self-contained kit, which can be easily used within a food processing facility to monitor products as a routine component of a quality assurance program. This kit would also allow analyses in different laboratories to be performed under standard conditions in different laboratories.

Though the use of a monoclonal-based CI-ELISA for detection of lipid oxidation seems ideal, previous studies still have not established its use in a food system. The overall goal of this project is to verify the use of a monoclonal CI-ELISA for monitoring lipid oxidation in chicken, through the quantification of hexanal. To achieve this goal, the following objectives were investigated:

1) develop and optimize a GC/MS method for hexanal detection
2 optimize extraction procedures to maximize formation of hexanal-protein adducts and percentage extractable protein on preparation for the CI-ELISA
3) optimize the monoclonal CI-ELISA for hexanal detection
4) conduct an accelerated oxidation study in which results from the monoclonal CI-ELISA are correlated with results from the GC/MS and TBARS

CHAPTER 2

LITERATURE REVIEW

2.1 Mechanism of Lipid Oxidation

The process of lipid oxidation, as outlined by Asghar et al. (1988), proceeds via a free radical mechanism termed autoxidation and involves initiation, propagation and termination steps. Initiation is the abstraction of a hydrogen atom from the methylene carbon in the side chain of an unsaturated fatty acid to form a lipid free radical. Propagation involves the molecular rearrangement of the lipid free radical followed by a reaction with molecular oxygen (in an aerobic system) to form the hydroperoxy radical. The hydroperoxy radical can remove hydrogens from other fatty acid side chains, thereby propagating the reaction. Termination occurs when two radicals react to form nonpropagating (non-reactive) intermediates. The major primary products of this reaction, hydroperoxides, are relatively unstable and decompose into a wide range of carbonyl compounds, hydrocarbons, ketones and other materials that contribute to the flavor deterioration of the food.

The decomposition of lipid hydroperoxides involves further reactions and the production of low molecular weight compounds such as alkanes, alkenes, aldehydes, ketones, alcohols, esters and acids. Homolysis of lipid hydroperoxides to hydroxy and alkoxy radicals is followed by cleavage of the fatty acid chain adjacent to the alkoxy radicals. Cleavage, or scission, occurs via one of two routes. In the first scission route,

an unsaturated aldehyde and an alkyl radical are formed, which can react with a hydroxyl radical to form an alcohol. In the second scission route, a saturated aldehyde and vinyl radical are formed, which in turn can react with a hydroxyl radical to form an aldehyde. Alternative pathways of hydroperoxide decomposition are further oxidation, yielding epoxides, cyclic and bicyclic peroxides, or condensation into dimers and polymers, which may oxidize and decompose into volatile breakdown products (Ladikos and Lougovois, 1990). Aldehydes are the most important breakdown products as they possess low threshold values and are the major contributors to the development of rancid off-flavors and odors (Ladikos and Lougovois, 1990).

The interactions of hydroperoxides and their secondary products with proteins or amino acids have a considerable impact on flavor stability during processing, cooking and storage. The form of these interactions is determined by the point at which the proteins enter the lipid oxidation reaction chain, when either the radicals or secondary prod .cts predominate (Ladikos and Lougovois, 1990). Radical induced cross-linking of proteins may be responsible for most nutritional losses, including lysine (up to 71%) and histidine (up to 57%) in a whey protein-methyl linoleate system (Nielsen et al., 1985). At high temperature (160°C), hydroperoxides begin to decompose spontaneously and the radical concentration can become relatively high, creating a greater likelihood of radical-radical interactions. In addition, color changes in beef during lipid oxidation are due to the loss of oxygen from oxymyoglobin (bright-red), together with an electron transfer, to produce metmyoglobin (brown).

Lipid oxidation in foods may be initiated by a number of mechanisms, however,

the majority of biological lipid peroxidation reactions involve transition metal ions present within the system (Kanner, 1994). This can occur through the homolysis of weak bonds, such as the O-O bond in a peroxide (Kaur and Perkins, 1991). The classic example is the reduction of hydrogen peroxide by iron (II), known as the Fenton reaction. The resulting hydroxyl free radical is extremely reactive and is capable of removing a hydrogen atom from an unsaturated lipid (Kubow, 1993). Following initiation, metals may also play a role in the propagation of lipid oxidation by catalyzing the breakdown of lipid hydroperoxides. The source of these metal catalysts in food is controversial; however, heme and various iron proteins including hemoglobin and myoglobin, both in muscle tissues, have been suggested as sources of iron (Gutterridge and Halliwell, 1990). Ferric heme pigments have been implicated as the major prooxidants in tissue lipid oxidation (Love and Pearson, 1971). In heated pork, heme proteins were reported to have a greater prooxidant effect than inorganic iron (Monahan et al., 1993). All muscle foods contain iron and are therefore susceptible to lipid oxidation.

Another important internal factor influencing lipid oxidation is the unsaturated fatty acid composition of the food (Shahidi and Pegg, 1994). In general, the reaction rate of autoxidation increases as the number of double bonds increases. The oxidation of oleic, linoleic, linolenic and arachidonic acid, the main unsaturated fatty acids in animal lipids, gives rise to a number of different hydroperoxides, which in turn leads to the formation of a large number of volatile compounds (Ladikos and Lougovois, 1990). Oleic acid oxidation generates octanal, nonanal and decanal, while the oxidation of linolenic and arachidonic acids generate propanal and hexanal, respectively. Linoleic

acid oxidation produces two hydroperoxides, which both undergo scission to produce hexanal (Ladikos and Lougovois, 1990).

In addition to internal factors, a number of external factors, including storage temperature, time, light, oxygen availability and water activity influence the rate of lipid oxidation. The term "warmed-over flavor" (WOF) was first introduced by Tims and Watts (1958) to describe the rapid onset of rancidity in meat products during refrigerated storage and can develop within 48 hours post-processing. While this term was originally used to describe the development of rancid off-odors and flavors in cooked, refrigerated meat, research has indicated that WOF also occurs in raw meat (Jakobsen and Bertelsen, 2000; Gray et al., 1996). Recently, the term WOF has been questioned on the basis that it does not adequately describe the flavor changes in cooked meat during storage (Gray et al., 1996). The term, meat flavor deterioration, has been developed to better describe the chemical reactions that contribute to the overall increase in oxidative off-flavors w th a simultaneous deterioration of the desirable meat flavor (Spanier et al., 1992).

Heat affects lipid oxidation through the decrease of activation energy required for oxidation; the fraction of total collisions that can lead to the reaction increases rapidly with increasing temperature (Gillespie et al., 1989). High temperatures also accelerate the break down of hydroperoxides to free radicals, which propagate lipid oxidation and the development of off-flavors (Kanner, 1994). Increased light and oxygen availability (Jakobsen and Bertelsen, 2000) have also been identified as factors that increase the rate of lipid oxidation.

In model lipid systems, research has shown that oxidation rate depends strongly

on water activity (Karel, 1980). At very low water activity, oxidation proceeds very rapidly. Increasing the water activity to a_w of 0.30 retards lipid oxidation, as this water activity is near the monolayer moisture content. The monolayer represents a theoretical moisture content where the protein binds one molecule of water per polar group on the surface of the protein (Kinsella and Fox, 1986). More recently, the monolayer has been defined as the first water molecules which interact with functional groups of solutes (Chinachoti, 2000). The protective effect of water is believed to occur by reducing the catalytic activity of metal catalysts, by quenching free radicals, by promoting non-enzymatic browning and/or by impeding the access of oxygen to the food. At higher water activities, the rate of oxidation increases again as a result of increased mobilization of the catalysts present (Nawar, 1985). These factors, both internal and external, influence the rate of lipid oxidation and by extension, the production of primary and secondary lipid oxidation products.

2.2 Toxicity of Lipid Oxidation Products

The production of off-flavors and odors were considered to be the most important consequence of lipid oxidation in the past; however, increased attention is now being given to the health risks that lipid oxidation may impose. Therefore, considerable interest exists in the role played by lipid oxidation in human disease and toxicology. While there has been no direct evidence implicating meat lipid hydroperoxides as a possible health hazard, there are numerous studies that report the toxic effects of oxidized fats in animals. Two studies (Yang et al., 1998; Corpet et al., 1990) reported thermally oxidized lipids to be precursors of colon cancer. In both studies, the aberrant crypt foci (ACF) assay was

used as a short-term test of colon carcinogens based on early putative steps in colon carcinogenesis. In the Corpet et al. (1990) study, rats were fed oxidized beef tallow (20% of the diet) for 100 days while in the Yang et al. (1998) study, rats were fed oxidized beef tallow for 28 or 100 days. Results from both studies indicated that oxidized beef tallow produced more ($p<0.05$) ACF than did the control diet. Furthermore, Fouad et al. (1995) conducted a histological and pathological study that demonstrated the effect of thermally oxidized lipids on the development of cirrhosis of the liver in rats fed oxidized butter oil for 100 days at 1.05 mL per day.

Several studies have been conducted examining the toxicity of aldehydes, secondary products of lipid oxidation. Bird et al. (1982) examined the chronic toxicity of malonaldehyde in female mice. Malonaldehyde, administered in the drinking water for one year at levels of 0.1, 1 and 10 mg/g body weights, resulted in a dose-dependent ($p<0.05$) increase of neoplasms and liver lesions. A study conducted by Yao (1979, reported that 24-hour exposure of mammalian cells to 20 mm malonaldehyde produced detectable cytotoxicity. Esterbauer et al. (1993) also reported toxicity of prepared 4-hydroxynonenal in primary cultures of rat hepatocytes at a concentration of 100 mm following 48-hour incubation. Micronuclei frequency, chromosomal aberrations and sister-chromatid exchanges were also observed following incubation.

Hydroperoxides readily decompose, leading to the production of secondary compounds. Due to health concerns, measuring the extent of lipid oxidation in foods is becoming increasingly important due to the possible health risks associated with the consumption of these secondary lipid oxidation products.

2.3 Measurement of Lipid Oxidation

Lipid oxidation is a complex process and a number of methods exist by which oxidative changes can be measured. Sensory methods provide the most useful information related to consumer acceptance of the food; however, these methods are time-consuming and display poor reproducibility (Frankel, 1991). Chemical methods include the measurement of hydroperoxides, the colorless, tasteless and odorless primary products of lipid oxidation. The breakdown of these products yields aldehydes, ketones and esters, and other low molecular weight compounds, which impart rancid and other off-flavors to the meat and the toxic effects mentioned above.

Lipid oxidation methods have been divided into those that measure primary change and those that measure secondary change (Gray and Monahan, 1992). Methods that measure primary change quantify the loss of reactants (unsaturated fatty acids or oxygen) or the formation of primary lipid oxidation products (hydroperoxides, conjugated dienes). A new method of hydroperoxide determination in dark chicken meat was proposed by Grau et al. (2000). This method consisted of the peroxide-mediated oxidation of ferrous ions in an acidic medium containing the dye xylenol orange; the dye bound to the ferric ions in the chicken and produced a blue-purple complex that was measured spectrophotometrically. Results indicated that this method was highly specific for hydroperoxides and showed a linear relationship between volume of meat extract and absorbance. Peroxide is a common measurement of lipid oxidation; however, because peroxides decompose to secondary products relatively quickly, this value can result in an underestimation of the degree of oxidation. These methods are thought to be more suited

to measure low levels of oxidation in uncooked products stored at low temperature (Frankel, 1991).

In situations where oxidation occurs at an accelerated rate, as in cooked meats, an accumulation of secondary products quickly occurs. Lipid oxidation is best assessed through the measurement of these secondary products (Frankel, 1991). Secondary changes include the formation of carbonyls and hydrocarbons, and the formation of aldehydes such as malonaldehyde, measured by the 2-thiobarbituric acid reactive substances assay (TBARS) or the increasingly popular HPLC, and hexanal, usually measured by gas chromatography.

TBARS is one of the oldest and most commonly used methods for assessing lipid oxidation in foods (Ladikos and Lougovois, 1990). This method is based on the spectrophotometric determination of extracted malonaldehyde, a minor product of oxidation, and can be performed either on the food product or on a steam distillate of the food. In this assay, one mole of malonaldehyde reacts with 2 moles of thiobarbituric acid to form a pink complex, which can then be measured spectrophotometrically at 532 nm. The extent of lipid oxidation is reported as a TBARS value, or milligrams of malonaldehyde equivalents per kilogram of sample. TBARS measurements have been reported to correlate well with sensory scores of oxidized flavors in muscle foods and WOF development (St. Angelo et al., 1987; Igene et al., 1985).

Due to the simple procedure and high correlation with sensory scores (Igene et al., 1979), many studies have relied on TBARS for lipid oxidation detection. In several recent studies in chicken (Tang et al., 2001a; Tang et al., 2001b; Alasnier et al., 2000;

Sista et al., 2000; Li and King, 1996), freeze-dried beef (Wilkinson et al., 2001), and beef (Jakobsen and Bertelsen, 2000), TBARS was the principal method used for lipid oxidation assessment. Criticisms of the TBARS method include wide variations in procedures between laboratories, leading to large differences in results. Several methods, such as direct heating of the sample with the TBA solution (Sinnhuber and Yu, 1958) and distillate reaction with the TBA solution (Tarladgis et al., 1960) require a heating step, which may cause the formation of additional lipid oxidation products. The major disadvantage of the TBARS reaction is that it is not specific for malonaldehyde (Gray and Monahan, 1992). Other lipid oxidation compounds, or compounds not related to lipid oxidation, can react with thiobarbituric acid to form colored complexes that also absorb at 532 nm, thereby leading to an overestimation of the extent of lipid oxidation (Gray and Monahan, 1992). Sun et al. (2001) reported that in freeze-dried beef stored at 49°C, propional, butanal and 5-hydroxymethyl-2-furfural all produced strong TBARS. For these reasons, the TBARS procedure may be used to assess the extent of lipid oxidation in general, rather than to quantify malonaldehyde.

2.4 Hexanal

Due to the limitations of TBARS, the measurement of hexanal, a six-carbon aldehyde, has become a popular indicator of lipid oxidation. Hexanal, one of the secondary products formed during the oxidation of linoleic acid, dominates among the volatile aldehydes. Hexanal is the only aldehyde that arises from both the 9 and 13 hydroperoxides of linoleate, and from other unsaturated aldehydes formed during the oxidation of linoleate (Shahidi and Pegg, 1994). Dupuy et al. (1987) reported that in

cooked beef, hexanal, pentanal, nonanal and total volatiles increased following five days of storage at 4°C. Specifically, hexanal increased from 0.1 to 15 ppm in white meat and 0.9 to 11 ppm in dark meat during storage. In comparison, in white meat, pentanal increased from 0.07 to 0.8 ppm and 0.03 to 0.9 ppm in dark meat. Another study found hexanal to increase in cooked white chicken meat from 0.064 ppm at Day 0 to 0.314 ppm following 15 days of storage at 4°C (Ajuyah et al., 1993).

In order for any chemical method to be adopted as a valid measure of lipid oxidation, the method must support sensory measurements (Gray, 1978), as odor and flavor evaluations relate directly to consumer acceptance (Frankel, 1991). A strong correlation of 0.995 between hexanal content and TBARS values was reported in ground pork (Shahidi et al., 1987). Flavor acceptability decreased as hexanal and TBARS number increased. Correlations between hexanal, TBARS and sensory scores were also reported in cooked roast beef (Dupuy et al., 1987), ground beef (St. Angelo et al., 1987), cooked pork patties (Ahn et al., 1998), cooked beef patties (Spanier et al., 1992), and chicken nuggets stored at -20°C for 6 months (Lai et al., 1995).

In sensory tests, hexanal is often described as "painty", "grassy" and "fishy" (Brewer and Vega, 1995). Sensory tests using descriptive analyses and category scales identified the detectable odor threshold for hexanal to range between 2.8 and 36.8 ppm in lean beef (Brewer and Vega, 1995) and between 5 and 10 ppm in cereal (Fritsch and Gale, 1977). Hexanal was also reported to correlate well to the development of WOF and sensory scores in refrigerated cooked beef (Kerler and Grosch, 1996; St. Angelo et al., 1987; Dupuy et al., 1987). Du et al. (2001) employed a dynamic purge-and-trap GC

technique for hexanal detection in irradiated cooked chicken meat, in addition to using TBARS and a 16-member trained sensory panel. Although correlations were not published, similar trends were observed between the hexanal, TBARS and sensory methods. Based on these studies, hexanal determination has shown to be a robust and accurate means of assessing lipid oxidation. Several researchers have suggested that hexanal represents the lipid oxidation of meat better than any other volatile component (Brunton et al., 2000; Ahn et al., 1998).

2.5 Volatile Analysis

Gas chromatography has been used extensively for the separation and identification of volatile lipid oxidation products (Gray, 1978). Traditionally, the two major gas chromatography methods used for volatile analysis in food are static headspace and dynamic purge-and-trap techniques (Frankel, 1991). With the static headspace method, the sample is closed in a vessel and equilibrated; the headspace is then sampled and injected directly into the GC. Using static headspace, a correlation of 0.856 was reported between TBARS and hexanal in broiler breast patties (Su et al., 1991). Static headspace GC was also employed for the measurement of volatile compounds in cooked chicken (Ang and Young, 1989) and beef (Wu et al., 1998). While static headspace techniques are simple and rapid, insufficient quantities of hexanal are often obtained, leading to a decrease in sensitivity (Miller and Stuart, 1999; Reineccius, 1996).

To overcome the low sensitivity of the static headspace technique, dynamic headspace or purge-and-trap techniques utilize an adsorbent material, such as Tenax, to collect volatiles over a period of time. The volatiles are then applied directly to the

column. The use of dynamic headspace GC for the analysis of lipid oxidation volatiles has been widespread, including cooked chicken (Ang et al., 1994; Ajuyah et al., 1993), beef (Spanier et al., 1992) milk products (Park and Goins, 1992) and irradiated pork (Ahn et al., 2001; Ahn et al., 2000). More recently, a correlation of 0.98 was reported between TBARS and dynamic headspace GC in the detection of lipid oxidation in cooked chicken thigh (Zielinski et al., 2001). While these dynamic headspace techniques are extremely sensitive, disadvantages include excessive preparation time. Some methods require as long as 10 hours to trap volatiles (Ahn et al., 2001). Additional disadvantages include difficulty of use, equipment dedication and inconvenience for analyzing large number of samples quickly (Steffen and Pawliszyn, 1996).

2.6 Solid-Phase Microextraction (SPME)

Solid-phase microextraction (SPME) is a new adsorption analytical technique that has gained widespread acceptance in many areas during recent years. SPME overcomes the difficulties experienced with headspace methods, including high cost, excessive preparation time and difficulty of use (Steffen and Pawliszyn, 1996). SPME was also reported to be more sensitive to flavor volatiles and allow for higher recovery than traditional static headspace techniques (Miller and Stuart, 1999; Garcia et al., 1998).

In SPME, analytes are first adsorbed from a sample (by headspace extraction) onto the stationary phase, a fused silica -poly(dimethylsiloxane)-coated fibre (Figure 2.1). The analytes are then thermally desorbed into the injection port of a GC for analysis (Arthur and Pawliszyn, 1990). Whether the analytes migrate into the coating of the fiber or remain at its surface depends on the diffusion coefficient of the analytes (Gorecki et al.

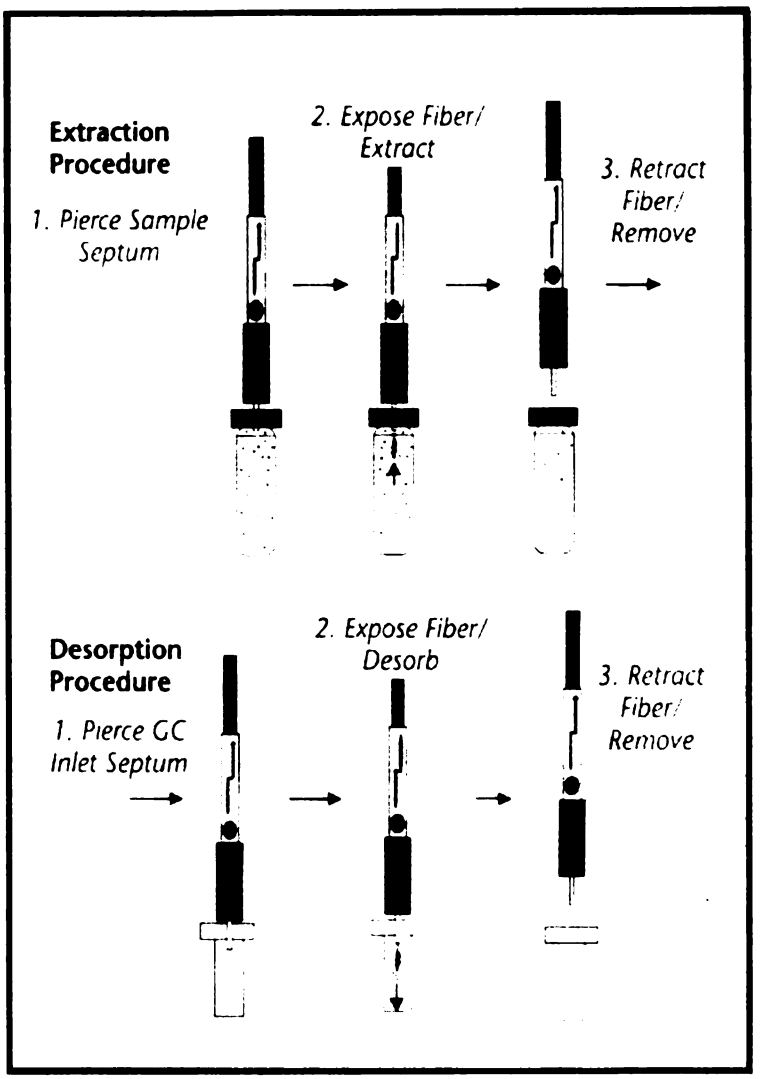

Figure 2.1. Solid-phase microextraction-extraction process. Adapted from Supelco - The Reporter 19:1 (2001)

1999). During analysis of headspace by GC-SPME, two equilibria are reached by the analytes: between the matrix and the headspace, and between the headspace and the coating of the fiber (Zhang and Pawliszyn, 1993). Using SPME, 17 analytes commonly found in orange juice flavor volatiles, were calculated to be within the same range as those reported using headspace techniques, with high reproducibility (below 10% relative standard deviation) (Steffen and Pawliszyn, 1996). In addition to being used for volatile detection in food, SPME has also been employed for other uses, including the analysis of volatiles in wire coating varnishes (Hinz et al., 1999), tobacco (Clark and Bunch, 1997) and pesticides (Betran et al., 2000; Chen et al., 1998).

SPME has successfully been applied to the analysis of a number of products. The use of SPME for wine characterization based on aroma profiles has been demonstrated by many researchers. While the use of SPME and GC provides identification of known compounds, the use of GC/MS can provide tentative identification of unknown compounds based on a spectral library of identified compounds. Garcia et al. (1998) used GC/MS-SPME for the varietal characterization of 90 German wines using aroma profiles or aromagrams. Vas et al. (1998) reported similar findings in the screening of wine, finding that SPME was capable of distinguishing between different wine types and wines produced in different regions. In the comparison of SPME with solvent extraction, the researchers also found that GC-SPME was more sensitive. In the detection of pesticides in wine, the GC/MS-SPME was reported to compare favorably to an ELISA for the pesticide procymidone. A collaborative study coordinated by The American Chemical Society found a correlation of 0.995 between the GC/MS-SPME detection of

procymidone in wine and their ELISA results. Other researchers have used GC-SPME for the detection of pesticides in wine including Corrcia et al.(2001) and Vitali et al. (1998). Researchers have also used GC-SPME for the verification of the decaffeination process in a variety of products (Yang et al., 1997) and for the analysis of flavor volatiles present in vanilla (Sostaric et al., 2000).

The use of GC/MS-SPME in cereals and grains have been limited in the past but is becoming a more popular technique. Grimm et al. (2001) used GC-SPME for the detection of the volatile compound 2-acetyl-1-pyrroline in rice and found that GC-SPME compared favorably to the traditional solvent extraction technique. In oats, Sides et al. (2001) used GC-SPME to survey changes in the volatile profile induced by processing. Both the trained taste panel and SPME reported similar findings and were able to differentiate between oats processed under various heating conditions. Biswas and Staff (2001) used GC/MS-SPME with thermogravimetric analysis in distiller grains to follow volatile changes during processing.

Detection of off-flavors and product characterization have been the predominate uses of SPME in the dairy industry. In milk, SPME was employed for shelf-life prediction of processed milk through the quantification of volatile bacterial metabolites (Marsili, 2000; Marsili, 1999). A number of researchers have used SPME for the characterization of cheese based on volatile profiles, finding the GC-SPME patterns distinctly different among cheese varieties (Peres et al., 2001; Chin et al., 1996). SPME has also been employed for the detection of free fatty acids and lactones in cheese (Wijesundera et al., 1998) and acetaldehyde in milk (van Aardt et al., 2001). In both

studies, the limit of detection of GC-SPME was greater than using solvent extraction and similar to sensory scores.

SPME and GC/MS have been used for volatile flavor compound analyses in many fruits and vegetables. In tomato and strawberry fruit (Song et al., 1998), the flavor compounds detected and their relative abundance determined using GC/MS-SPME were reported to compare favorably to those values published using traditional methods. Steffen and Pawliszyn (1996) reported similar findings in orange juice. GC-SPME has been used to follow growth stage and stage of ripeness in sweet oranges (Alonzo et al., 2001) and cantaloupe (Beaulieu and Grimm, 2001). The volatiles in strawberry drink (Siegmund et al., 2001) and apple fruit (Song et al., 1997) have also been studied using GC-SPME. In vegetables, GC-SPME was employed to detect pesticide residues and compared with the traditional solvent extraction method, it showed a strong agreement (Volante et al., 2000).

In the meat industry, the use of SPME for many applications has been growing. Dry-cured hams have been analyzed for volatiles using SPME-GC/MS, while smoked hams have been analyzed for N-nitrosodibutylamine and N-nitrosobenzylamine using SPME and GC-thermal energy analysis (Sen et al., 1997). For both compounds, high recoveries were achieved (86% and 70%, respectively), and the GC-SPME method showed a strong correlation with the conventional solvent method (r^2=0.97). In dry-cured Iberian ham, Ruiz et al. (1998) used GC-SPME to assess the influence of extraction time and temperature on the volatile profile. Results indicated a time and temperature effect on the chromatographic area of extracted compounds at 40 and 60°C at 20, 40 and 60

min. In most of the compounds studied, the largest peak areas were found in samples extracted at 60°C for 60 min. Heating can release bound hexanal, improving hexanal recovery. However, some volatile compounds, such as hexanal, are thermally generated and heating a sample produces additional hexanal. In a variety of meat products, smoked chicken, ham and corned beef, GC-SPME analyses for lipid oxidation products were compared to the analyses of supercritical fluid extraction and traditional purge and trap GC (Snyder et al., 1998). Results from each method showed the pattern of oxidation products formed to be similar and consistent, but GC-SPME was able to measure compounds at lower concentrations than supercritical fluid extraction. GC-SPME has also been used for volatile analysis in pork (Elmore et al., 2000) and as an indicator of food safety, to measure volatile compounds produced by bacteria in processed poultry (Arnold and Senter, 1998).

Recently, SPME fibers have been used for the quantitative measurement of hexanal in cooked turkey (Brunton et al., 2000). A strong correlation between SPME/GC and TBARS (r^2 of 0.994) was reported when measuring lipid oxidation in cooked turkey during storage. Currently, this is the highest reported correlation between hexanal and TBARS. In previous studies in muscle foods, lower correlations were found between hexanal and TBARS when using different methods. Using a headspace method, a correlation of 0.856 was reported in broiler breast patties (Su et al., 1991). When a steam distillation method was used for hexanal quantification, a correlation of 0.93 was reported in pork patties (Ahn et al., 1998). Due to this high correlation, SPME GC/MS is a promising method for quantification of lipid oxidation through measurement of hexanal.

2.7 Immunoassays

The increased regulation of foodstuffs requires analytical methods which are easy to perform, sensitive, and specific (Fukal, 1991). Immunoassays, including radio immunoassays (RIA), and enzyme-linked immunosorbent assays (ELISA), employ the specificity of an antibody and corresponding antigen to permit rapid detection or measurement of the antigen or hapten. Because the ELISA protocol can be carried out by unskilled analysts using laboratory-prepared or commercially available reagents or standardized kits, the highly sensitive and specific ELISA continues to gain popularity (Fukal, 1991).

Currently the most widely used immunoassay format is the ELISA (Swaminathan and Feng, 1994). Three common types of ELISAs are the competitive direct, sandwich and indirect. In the competitive direct ELISA, the sample and an enzyme-linked competitor are added to an antibody-coated solid support. A substrate is added which will bind to the enzyme-linked competitor and the reaction is stopped. As this assay is based on competition for antibody binding sites, free antigen concentration is inversely related to antibody-bound enzyme conjugate (Pestka, 1988). The sandwich ELISA begins with an antibody-coated solid support followed by antigen addition. The antigen and antibody are allowed to react and after washing, the enzyme-conjugated secondary antibody is added. The final step is substrate addition and the measurement of the colored reaction product. In the indirect ELISA, a sample containing primary antibody is allowed to bind to the antigen-coated solid support (Figure 2.2). After any free antibody is washed away, the enzyme-conjugated secondary antibody is added to bind to the primary antibody. The

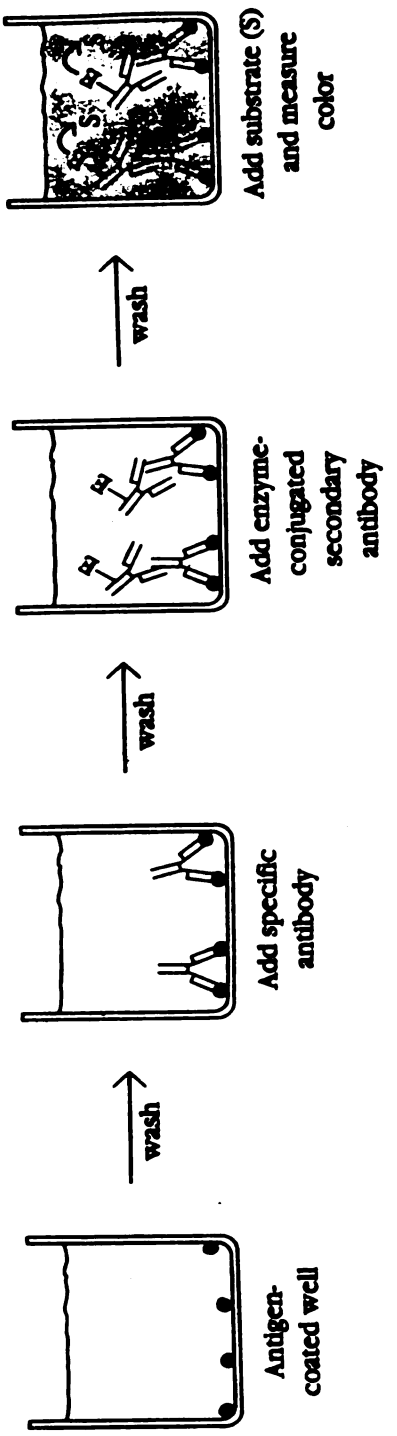

Figure 2.2. Diagram of indirect competitive ELISA. An antigen-coated well is prepared, followed by the addition of the monoclonal antibody and sample. Enzyme-conjugated secondary antibody and substrate are then added. Adapted from Kuby (1997)

substrate for the enzyme is added and the colored reaction product is measured.

Production of both polyclonal and monoclonal antibodies begins with the introduction of the antigen into the host. In order to elicit an immune response, the compound must be immunogenic. Small molecules alone, generally less than 5000 daltons, are non-immunogenic and must be conjugated to a larger protein; this conjugate is then large enough to elicit a response (Fukal, 1991). Following injection of the antigen, the host produces a number of polyclonal antibodies, each specific to the different epitopes on the antigen. For most research and therapeutic purposes, the polyclonal antibodies produced are not specific enough and a monoclonal antibody specific for a single epitope on the antigen is preferred (Kuby, 1997).

Direct purification of a monoclonal antibody from a polyclonal antibody is not feasible. In 1975, Koehler and Milstein devised a method for preparing monoclonal antibodies. In this method, antibody-producing spleen cells are isolated from the host and fused with a myeloma cell in order to produce a hybrid cell. This cell, called a hybridoma, possesses immortal growth properties and secretes the antibody. The resulting clones of the hybridomas can be cultured indefinitely, with regular harvesting and subsequent purification of the antibody (Kuby, 1997).

2.8 ELISAs in use

ELISAs have found specific applications in the meat industry, including adulteration detection, thermal processing of food proteins, and food safety. To detect species adulteration in ground meat products, a monoclonal antibody was produced specific to a heat stable protein in cooked mammalian meats (Hsieh et al., 1998).

Adulteration of the chicken with as little as 0.5% pork, beef, horse and lamb could be detected using the monoclonal antibody in the ELISA. In order to detect adulteration of sausage with soy protein, Medina (1988) employed a polyclonal antibody to soy protein. Using this antibody in an ELISA, soy concentrations of less than 2% were detected in sausage. A sandwich ELISA using polyclonal antibodies for the indicator protein lactate dehydrogenase was developed to verify endpoint processing temperatures in turkey roll (Wang et al., 1993). Lactate dehydrogenase concentration, determined by the ELISA, differed in turkey rolls processed to 70°C and 71.1°C, while lactate dehydrogenase activity and extractable protein results showed no difference between those temperatures. The use of LDH as an endpoint for heating was also studied in ground beef with similar results (Wang et al., 1995). In the area of meat food safety, the ELISA format has been adopted for the detection of bacterial toxins, including *Salmonella* and *Staphylococcal* enterotoxins (Morissette et al., 1990) and other contaminants of meat, such as drugs, pesticides and hormones (Fukal, 1991).

In the medical field, antibodies have also been produced against many compounds, including several aldehydes. Israel et al. (1986) produced antibodies to acetaldehyde, a biochemical marker of alcohol consumption that has been shown to form adducts with a number of proteins (Donahue et al., 1983). The antibodies reacted specifically with erythrocyte protein-acetaldehyde but not with the control erythrocyte proteins. Specifically, the adduct formed between the lysine group on the protein and the acetaldehyde was recognized by the antibody. No recognition was observed when adducts were prepared with the amino acids valine and tyrosine. Both stable (non-

reduced) and unstable adducts (Schiff bases) were formed when acetaldehyde bound to protein. Using sodium cyanoborohydride as the reducing agent, Klassen et al. (1994) and Thiele et al. (1994) raised monoclonal antibodies capable of detecting reduced acetaldehyde-protein adducts formed with the N-ethyl lysine group on the protein.

In medicine, the cytotoxicity of several products of lipid peroxidation has prompted researchers to develop antibodies for their rapid detection, replacing such traditional methods as TBARS, spectrophotometry and high performance liquid chromatography (Raharjo and Sofos, 1993). Antibodies have been raised to 4-hydroxy-2-nonenal protein adducts (Uchida et al., 1995). This aldehyde is believed to be largely responsible for cytopathological effects observed during oxidative stress and has been implicated in Parkinson's disease and cardiovascular disease. In a study of people afflicted with Parkinson's Disease and using appropriate controls, researchers successfully used the 4-hydroxy-2-nonenal antibody to detect the presence of oxidative stress within the nigral neurons in the brain in those affected with the disease (Yoritaka et al., 1996). Another research group produced antibodies to a 2-oxo-nonanal protein adduct (Salomon et al., 2000). Using an ELISA, the concentration of 2-oxo-nonanal were elevated in renal failure and atherosclerosis patients when compared to those in healthy volunteers.

Antibodies have also been produced against malonaldehyde, an aldehyde thought to play a role in the development of atherosclerosis (Swets et al., 2001; Kondo, 2001). Results from a study in mice by Khan et al. (2001) indicated that an early appearance of anti-malonaldehyde antibodies in the blood showed increased oxidative stress in the

animal. In another study, malonaldehyde antibodies were used to determine lipid peroxidation damage following the administration of the industrial chemical aniline (Khan et al., 2000). Rats were gavaged with different doses of aniline and a dose-response relationship was evident, with increased concentrations of malonaldehyde in the blood observed as the dose increased.

A food allergy study also employed 4- hydroxynonenal and malonaldehyde antibodies in the examination of the increased allergenicity of roasted peanuts over raw peanuts (Chung and Champagne, 2001). The authors postulated that an increase in IgE binding of peanut allergy sufferers was attributed to increased levels of protein-bound adducts in the blood, such as malonaldehyde and 4-hydroxynonenal. For this study, antibodies to adducts of 4- hydroxynonenal and malonaldehyde were produced, and the ability of these antibodies to bind to IgE from serum samples of patients with peanut allergies was studied. No binding between IgE and either 4- hydroxynonenal or malonaldehyde was observed, indicating the limited involvement of 4- hydroxynonenal and malonaldehyde in the allergenicity of peanuts.

Recently, a CI-ELISA using polyclonal (Smith et al., 1999) and monoclonal antibodies (Zielinski et al., 2001) was developed for the detection of hexanal. While some compounds are antigenic and can elicit an antibody response alone, smaller molecules such as hexanal rarely act as antigens. These molecules are referred to as haptens and require conjugation with a large protein molecule, such as chicken serum albumin, to become antigenic and capable of inducing an antibody-antigen response. For hexanal, conjugate production involves the reaction of the carbonyl group in hexanal with

a free amino group to form an unstable Schiff base (Figure 2.3). These bases readily dissociate when exposed to dialysis, gel filtration or treatment with weak acids or bases. The Schiff bases can be stabilized however, by reduction to secondary amines using sodium cyanoborohydride, a strong reducing agent, resulting in a stable conjugate (Jentoft and Dearborn, 1979). Reductive alkylation, through the addition of sodium cyanoborohydride, appears highly specific for amino groups of the protein, primarily lysine (Means and Feeney, 1968).

Both the polyclonal and monoclonal CI-ELISAs for hexanal were sensitive and showed potential for use in a meat system to follow lipid oxidation. The limit of detection of the polyclonal antibody was 7.4 ng hexanal/mL, with coefficients of variation ranging from 4 to 13.9 % (Smith et al., 1999). The antibody was able to detect hexanal conjugated to several proteins, including bovine and chicken serum albumin, but did not recognize free hexanal. In an accelerated oxidation study in a chicken thigh muscle homogenate system, the polyclonal CI-ELISA for hexanal was found to correlate v ith the dynamic headspace GC (r^2 = 0.79) method and with lipid oxidation determined by TBARS (r^2= 0.72). While the polyclonal antibody was hexanal sensitive, its also displayed cross reactivity to pentanal (11.8%) and heptanal (86.3%) when conjugated to chicken serum albumin.

In order improve the specificity of the ELISA, monoclonal antibodies to hexanal were developed. The monoclonal antibody did not recognize free hexanal but did recognize hexanal when conjugated to a protein. The sensitivity of the monoclonal antibody was determined to be 1 ng hexanal/mL and had coefficients of variation ranging

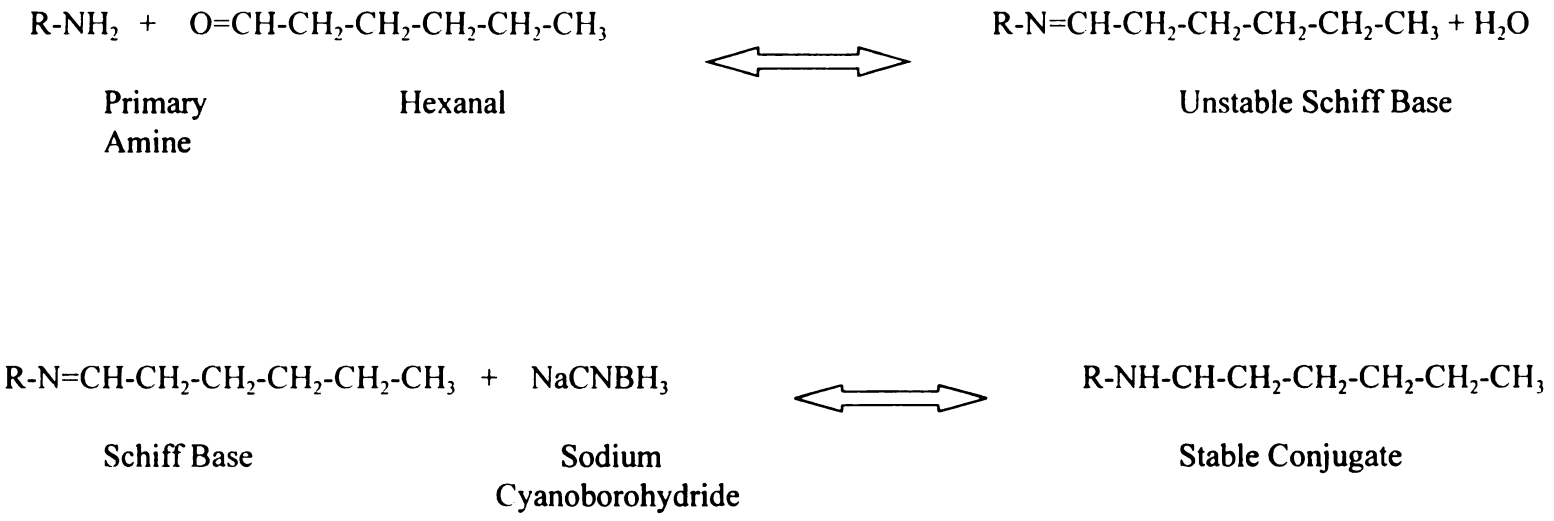

Figure 2.3. Reaction in the formation of a Schiff base between a primary amine and hexanal: 1) unstable conjugate (Schiff Base) 2) stable conjugate

from 4.15 to 7.71 % (Zielinski et al., 2001). The monoclonal antibody displayed moderate cross-reactivity with other aldehydes, reacting at 76.6%, 45.0% and 37.9% to modified heptanal, 2-t-hexenal and pentanal, respectively. In the detection of hexanal in an oxidized chicken thigh homogenate accelerated oxidation study, hexanal contents determined using the CI-ELISA were not different from values determined using dynamic headspace GC except at one time point ($r^2 = 0.66$). These initial studies indicated that the use of hexanal specific monoclonal antibody was feasible for accurate assessment of lipid oxidation.

CHAPTER 3

SOLID-PHASE MICROEXTRACTION FOR THE MEASUREMENT OF HEXANAL IN CHICKEN MYOFIBRILS

3.1 ABSTRACT

Solid-phase microextraction (SPME) combined with gas chomatography/mass spectrometry (GC/MS) was evaluated for its suitability in the detection of hexanal in chicken myofibrils. Recovery of hexanal from spiked myofibrils was 95% using a 5-minute sampling time, with a total analysis time of ~12 min/sample. Hexanal partitioning into the chicken myofibrils was also examined. Using SPME, the limit of detection for hexanal in myofibrils was 10 ppb with a coefficient of variation ranging from 1 to 13%, and a working linear response from 10 ppb to 10 ppm (r^2=0.995). Freeze-dried chicken myofibrils with added methyl linoleate (0.6 mmol/g of protein) were stored at 50°C at water activities of 0.30 and 0.75. Samples were removed at two time intervals, in Trial 1, Day 0, 3, 6 and 9 and Trial 2, H 0, 12, 27 and 50. Hexanal was measured by SPME coupled with gas chromatographic separation and time-of-flight mass spectrometry (GC/MS-SPME). Samples were also analyzed using the thiobarbituric acid reactive substances assay (TBARS) and a conjugated diene assay (CD). In both trials, sampling time had an effect on lipid oxidation ($p<0.001$). In the 50 h study, differences in oxidation were observed between the two water activities. A strong correlation (r=0.975 and 0.938) existed between SPME and the TBARS assay in both the 9 day and

50 h studies. SPME offers a sensitive, rapid and solvent-free method of detecting hexanal as an indicator of lipid oxidation in chicken myofibrils.

3.2 INTRODUCTION

Lipid oxidation has long been recognized as a leading cause of quality deterioration in muscle foods and is often the decisive factor in determining food product storage life (Frankel, 1993). All muscle foods are susceptible to lipid oxidation; however, the muscle foods of most concern are those with high concentrations of unsaturated fatty acids, such as chicken (Tamura et al., 1991). The oxidative deterioration of food lipids involves autoxidative reactions of unsaturated fatty acids, accompanied by various secondary reactions. Hydroperoxides, the major initial products, are colorless, tasteless and odorless. The degradation of hydroperoxides into a complex mixture of low molecular weight alkanes, alkenes, aldehydes and ketones lead to oxidative rancidity and the objectionable off-odors and flavors characteristic of rancid food. Consumer acceptance of meat products depends to a major extent on flavor quality, and the flavors generated by lipid oxidation are generally undesirable (Ramarathnam et al., 1991). Associated changes in quality are manifested by deterioration in texture, nutritional quality and the production of potentially toxic compounds (Kanner, 1994).

From the perspective of the meat industry, detection of lipid oxidation needs to be accomplished using a very specific, sensitive and rapid method. A wide spectrum of lipid oxidation tests exists, ranging from simple organoleptic evaluations to more complex chemical methods. The most commonly used method for measuring lipid oxidation is the 2-thiobarbituric acid reactive substances test (TBARS), which was originally thought to

quantify only malonaldehyde, a secondary product of lipid oxidation (Ladikos and Lougovois, 1990). Criticisms of this test arise from its lack of sensitivity and specificity to malonaldehyde. Other lipid oxidation compounds can react with thiobarbituric acid to form colored complexes, leading to an overestimation of the extent of lipid oxidation. For this reasons, the TBARS procedure may be used to assess the extent of lipid oxidation in general, however, it should be complemented with sensory scores and a valid analytical procedure (Gray and Monohan, 1992).

Hexanal has become a popular indicator of lipid oxidation in foods. Hexanal, one of the secondary products formed during the oxidation of linoleic acid, dominates among the volatile aldehydes. Hexanal is the only aldehyde that arises from both the 9 and 13-hydroperoxide of linoleate, and from other unsaturated aldehydes formed during the oxidation of linoleate (Shahidi and Pegg, 1994). Dupuy et al. (1987) reported that in cooked chicken, all aldehydes increased following 5 days of storage at 4°C, with hexanal showing the greatest increase from 0.1 to 15 ppm in white meat. Hexanal, often described as "painty", "grassy" and "fishy", contributes notably to undesirable off-odors created during lipid oxidation and has a low odor threshold. In sensory tests, the detectable odor threshold of hexanal in lean ground beef was reported to be 5.9 ppm (Brewer and Vega, 1995), and between 5 and 10 ppm in cereal (Fritsch and Gale, 1977).

Gas chomatography (headspace GC) was used to establish strong correlations between hexanal content, sensory scores and TBARS values in a number of meat products, including chicken (Ahn et al., 2000), beef (Spanier et al., 1992; St. Angelo et al., 1987), irradiated pork (Ahn et al., 2001) and ground pork (Shahidi et al., 1987).

Several GC techniques have been employed for the analysis of hexanal, including static headspace techniques and dynamic purge-and-trap techniques. Using a static headspace method, a correlation (r^2) of 0.856 was reported between TBARS and hexanal in broiler breast patties (Su et al., 1991). In pork patties, using a steam distillation method, a correlation of 0.93 was reported between hexanal and TBARS (Ahn et al., 1998) and a correlation of 0.97 in chicken thigh was found between a TBARS and a dynamic headspace method (Zielinski et al., 2001). Static headspace techniques are simple and rapid, however lack sensitivity as insufficient quantities of hexanal are often obtained (Reineccius, 1996). To overcome the decreased sensitivity of the static method, dynamic headspace or purge-and-trap techniques utilize an adsorbent material to collect volatiles over a period of time. While these dynamic headspace techniques are extremely sensitive, disadvantages include excessive preparation time, difficulty of use, and inconvenience when analyzing a large number of samples quickly (Steffen and Pawliszyn, 1996).

Solid-phase microextraction (SPME) is a new adsorption technique that has gained widespread acceptance in analysis of volatile compounds as it overcomes the difficulties experienced with traditional headspace methods (Steffen and Pawliszyn, 1996). SPME involves the extraction of analytes directly from a sample, or from the headspace above a sample in a closed vial, onto a fused silica fiber coated with a polymeric liquid phase. Following equilibration, the fiber containing the analytes is removed from the vial and the analytes are desorbed in the injection port of a GC for analysis. Using GC-SPME, analytes were calculated to be within the same concentration

range as those reported using headspace techniques, and the SPME method displayed high reproducibility (Steffen and Pawliszyn, 1996). SPME has been used for defection of volatile compounds in a number of products, including coffee (Yang and Peppard, 1994), tomato and strawberry fruit (Song et al., 1998), vanilla extract (Sostaric et al., 2000), cheese (Peres et al., 2001), oats (Sides et al., 2001), milk (van Aardt et al., 2001), and meat products such as cooked pork (Elmore et al., 2000) and ham (Sen et al., 1997; Ruiz et al., 1998). Recently, SPME fibers have been used for the quantitative measurement of hexanal in cooked turkey as an indicator of lipid oxidation. Strong correlations (r^2= 0.994) between SPME/GC and TBARS were reported (Brunton et al., 2000).

The primary objective of this study was to develop a method for the application of SPME combined with gas chomatography/mass spectrometry (GC/MS) for the detection of hexanal in chicken myofibrils as an indicator of lipid oxidation. The specific objectives were to 1) develop a precise and accurate GC/MS SPME method for the quantification of hexanal, 2) study the partitioning of hexanal between chicken myofibrils and headspace, and 3) compare hexanal concentration in chicken myofibrils determined by GC/MS SPME to malonaldehyde concentration determined by TBARS and establish a correlation between the two.

3.3 MATERIALS AND METHODS

3.3.1 Preparation of Chicken Myofibrils

The procedure used for myofibril isolation was described by Smith (1987). Chicken breast was obtained from a local supermarket and ground in a Hobart

Kitchen-Aid food grinder with a 4.5 mm plate (Model KF-A, Troy, OH). The ground chicken was blended for 90 s in 4 volumes of 0.05 M potassium phosphate buffer (0.1 M NaCl, pH 7.0) at maximum speed in a Waring blender (Model 1120, Winsted, CT). The slurry was stirred for a two-1 h intervals at 4°C. Following each interval, the myofibrils were centrifuged at 2000 x g for 15 min. The final pellet was washed twice in 10 volumes of distilled water, and centrifuged at 6000 x g to precipitate the protein. Protein content in the final pellet was determined using the Bradford Protein Assay (Bradford, 1976). If required for an experiment, methyl linoleate (ML) (Sigma Chemical Co., St. Louis, MO) was stirred in at 0.6 mmol/g of protein. The spiked myofibrils are referred to as chicken myofibrils-ML. The myofibrils were freeze-dried (Labconco, Kansas City, MO), vacuum packaged in polyethylene-laminated pouches (Butcher and Pack Supply, Detroit, MI) and stored at –80°C until needed.

3.3.2 GC/MS-SPME Procedure

A SPME fiber assembly (57310-U, Supelco Co., Bellefonte, PA) coated with 100 μm polydimethylsiloxane (PDMS) was used to absorb volatiles. The fiber assembly was inserted into a fiber holder for manual sampling (5733OU, Supelco) and preconditioned at 250°C for 1 h prior to initial use. All SPME samplings were conducted at 22°C. For all experiments, the fiber was introduced into the headspace of the clear glass screw top vial (Supelco), through a Mininert valve screw cap fitted for the vial (33301, Supelco). The sampling time of the fiber to the headspace was 5 min, after which, the fiber was retracted. The desorption time in the glass-lined, splitless injection port of the GC (HP-6890, Hewlett Packard Co., Wilmington, DE) was 2 min.

A Supelcowax -10 capillary GC column (15 m x 100 μm, film thickness of 0.25 mm; 24077, Supelco) and helium carrier gas at a flow rate of 0.6 mL/min were used for volatile detection. The first 20 cm of the column was cooled with liquid nitrogen tc cryofocus the volatiles from the fiber. Oven temperature was increased from 40°C to 250°C at a rate of 60°C/min, and held for 3 min. The flow was maintained at 0.6 mL/min, the GC/MS transfer line was at 200°C and the inlet temperature was 250°C. Volatiles were detected using time of flight mass spectrometry (TOFMS), with electron impact ionization (Pegasus II, Leco Corp., St. Joseph, MI) and an ionization energy of 70 eV. Mass spectra were collected at a rate of 40 spectra/second over the mass range of m/z 33 to 250. Data were analyzed using LECO deconvolution software and identification of hexanal was authenticated by comparison with reference spectra found in the National Institute for Standards and Technology (NIST) Mass Spectral Library (Search Version, 1.5). Total analysis time was ~12 min/sample.

The hexanal vapor standard was prepared by diluting liquid hexanal (Sigma) 10 times in squalene (Sigma) to a concentration of 83 ng/mL. An aliquot of the diluted hexanal (0.2 μL) was applied to a piece of filter paper and placed into a 4.4-L Erlenmeyer flask, fitted with a Mininert valve (Supelco). The flask was sealed and the hexanal was allowed to vaporize to provide a headspace concentration of 0.9 ppm. The hexanal vapor standard was analyzed as above. The peak area of the standard was determined. Peak identities were confirmed using mass spectroscopy at m/z 56. Hexanal concentrations were expressed in ppm (μL hexanal/L).

3.3.3 Limit of Detection and Response Linearity of GC/MS-SPME

The linearity of the response was calculated. Vapor phase hexanal standards of 10, 5, 1, 0.1, 0.01 and 0.001 ppm were prepared in squalene. To prepare a standard curve, an aliquot of each standard (1.5 mL) was added to a 15 mL clear glass screw top vial (27162, Supelco) sealed with a Mininert valve (33301, Supelco) and allowed to equilibrate for 10 min. Each standard was then sampled and analyzed using the procedure described above. The concentration of the standard was plotted against the resulting hexanal peak area. The coefficient of variation of each standard was determined using 6 replicates. For each standard, the mean response and standard deviation were calculated, and from those values, the coefficient of variation was determined (Kvanli, 1988).

The limit of detection of the GC/MS-SPME was estimated as the concentration of analyte to produce a signal that was 10x that of the noise (Song et al., 1997). To determine the limit of detection, a series of hexanal standards were prepared, ranging from 1 ppb to 10 ppm. Six replicates of each standard was tested using the procedure described above. The limit of detection was considered the concentration at which the hexanal signal became less than 10x that of the noise.

3.3.4 Hexanal Loading and Repeated Sampling of Spiked Chicken Myofibrils

Recovery of hexanal from spiked myofibrils was determined by incubating chicken myofibrils over 0.01, 0.1, 1 and 10 ppm hexanal standards (Figure 3.1a). Each standard was tested in triplicate. Briefly, 1.5 mL of each hexanal standard was added to a 15-mL screw top vial (27162, Supelco) containing 2 g of glass beads (33201, Fisher Chemicals, Pittsburgh, PA). A 0.03 g sample of myofibrils was weighed in a 4-mL screw

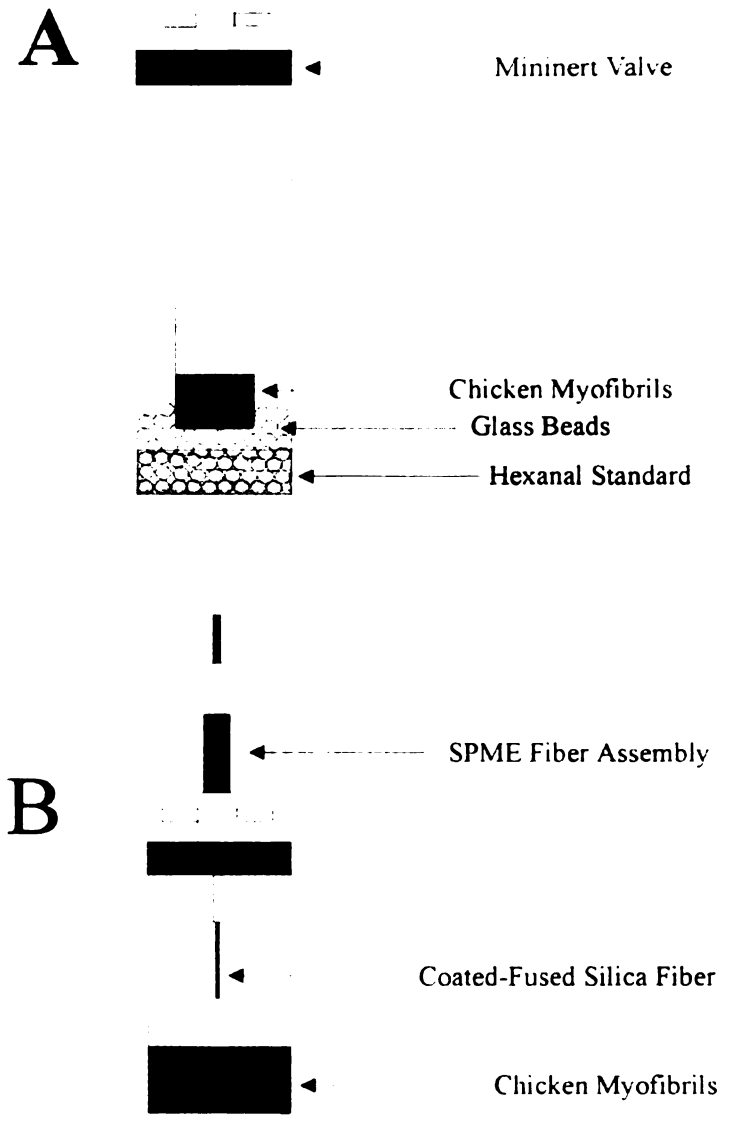

Figure 3.1. Hexanal loading and recovery from chicken myofibrils. A) Hexanal loading: 0.03 g of myofibrils incubated over 1.5 mL of hexanal standard B) Recovery of hexanal from spiked myofibrils following 24 hours of incubation

top vial (27111, Supelco), and these vials were nested into the glass beads in the larger 15-mL vials. A Mininert valve (33301, Supelco) was used to seal the 15-mL screw top vial and allow for sampling. At hourly intervals, the headspace in the vial was sampled using SPME and GC/MS analysis was conducted. Following 24 h of incubation, the myofibrils were removed from the small vials, placed in new 4-mL screw top vial, sealed with a Mininert valve (33300, Supelco) and sampled hourly by GC/MS-SPME using the procedures described above (Figure 3.1b). Briefly, the SPME fiber was exposed to the headspace above the myofibrils for 5 min, after which, the fiber was desorbed into the injection port of the GC/MS. GC/MS analysis was conducted and recovery of hexanal from the myofibrils was determined.

3.3.5 Hexanal Partitioning

Hexanal standards from 0.01 ppm to 10 ppm were prepared in squalene. Each standard was tested in triplicate. Aliquots of 1.5 mL of each standard were added to 15-mL screw top vials containing glass beads. The vials were sealed with a Mininert valve and allowed to equilibrate for 24 h at ambient temperature. To a second set of vials, 1.5 mL of each standard was added to a 15-mL glass vial with glass beads. Small 4-mL screw top vials containing 0.03 g myofibrils were nested into the glass beads; the vial was sealed with a Mininert valve and allowed to equilibrate for 24 h. Following 24 h of incubation, each vial was sampled using the GC/MS-SPME procedure described in Section 3.3.2. The coefficients of variation were determined for each standard, and overall. Differences in hexanal partitioning were calculated by dividing the hexanal peak area of the standards alone by the hexanal peak area of the standards incubated with

myofibrils.

3.3.6 Accelerated oxidation study

Freeze-dried chicken myofibrils, spiked with 0.6 mmol/g of protein, prepared as described in Section 3.3.1, were used for two separate oxidation trials. For both trials, the samples were stored at 50°C, an accelerated storage temperature typical of military provision storage studies (Sun et al., 2001). Two storage chambers were used for each time point at each water activity, and replicate determinations were made for each sample. A 0.5 g and 0.05 g sample of myofibrils were weighed out, evenly distributed on an aluminum weigh dish and transferred to a 1-L Teflon chamber sealed with a screw lid (Berghof of America, Coral Spring, FL). Each chamber held one-0.5 g and one-0.05 g sample myofibril sample. The samples were pre-weighed to account for any changes in weight that may have occurred upon storage in either high or low water activity

At the bottom of the Teflon chambers were 10 mL of saturated salt solutions. The water activities used represented the maximum and minimum lipid oxidation rate observed by Smith et al. (1990) in freeze-dried chicken myofibrils. Saturated salt solutions used for humidity control were sodium chloride (a_w=0.75) and magnesium chloride (a_w=0.30) and were prepared at 50°C using the procedure described by Labuza (1984). In each trial, the myofibrils were separated into two groups (for each a_w) and incubated in the dark. For all time points, prior to removal of the myofibril sample, the chamber headspace was sampled with the SPME fiber through a septum in the lid of the chamber. Following sampling of the headspace in the chamber, myofibrils were removed and sampled.

In the first trial, samples were removed and analyzed at day 3, 6, and 9. In the second trial, samples were removed and analyzed at 12, 27 and 50 h. At each sampling time, 0.05 g of myofibrils was removed and assayed using the conjugated diene (CD) assay. Also at each time, 0.5 g of myofibrils was transferred to a 15-mL glass screw top vial, sealed with a Mininert valve and the hexanal in the headspace was analyzed following equilibrium using GC/MS-SPME as described in Section 3.3.2. The sample that was analyzed using GC/MS-SPME was then analyzed using the TBARS assay.

3.3.6.1 Conjugated Diene Assay (CD)

Conjugated dienes were quantified using the method of van Ruth et al. (2000) except with a smaller sample size. In this method, 0.05 g of myofibrils were mixed with 5 ml cyclohexane and centrifuged for 3 min at 1500 x g, after which the absorbance of the supernatant was read at 234 nm. Absorbances were calculated as hydroperoxides in mmol per g protein, using a molar absorptivity of 26,000 for linoleate peroxides (AOCS, 1992). Results were expressed as mmol/g protein. Each sample was tested in triplicate.

3.3.6.2 Thiobarbituric Acid-Reactive Substances Assay (TBARS)

The TBARS procedure of Tarladgis et al. (1960) was used with modifications of a decreased sample size and the addition of antioxidant. Specifically, following accelerated oxidation, myofibrils (0.5 g) were homogenized for 1 minute with 48.75 mL of distilled water containing 100 ppm Tenox 2 antioxidant (Eastman Chemical Company, Roebuck, SC). Hydrochloric acid (4 N), glass beads and 3 sprays of antifoam (Arthur H. Thomas Company, Philadelphia, PA) were added, and using the highest possible heat on the distillation apparatus, 15 mL of distillate was collected. A 5 mL sample of distillate was

reacted with 5 mL of 0.02 M thiobarbituric acid, and held in a boiling water bath for 35 min. Upon removal and cooling for 10 min, absorbance of the solution was read at 532 nm. Each sample was tested in triplicate.

The standard used for TBARS determination of malonaldehyde was tetramethoxypropane (TEP); under acid conditions, TEP hydrolyzes to yield malonaldehyde (Sinnhuber and Yu, 1957). The malonaldehyde standard curve was prepared using 0 to 9 x 10^{-6} M tetramethoxypropane (TEP). Total recovery was established through the addition of each TEP standard to 0.5 g of chicken myofibrils and recovery of malonaldehyde following the TBARS procedure. The TBARS number was calculated by multiplying the absorbance of the sample by a constant (Tarladgis et al., 1960). The constant was determined as follows:

$$K(\text{distill}) = \frac{\text{conc in mol/5 mL of distillate}}{\text{optical density}} \times \text{MW of MA} \times \frac{10^7}{\text{wt of sample}} \times \frac{100}{\%\ \text{recovery}}$$

The value of the first term from the standard curve was found to be 3.09 x 10^{-8}. With a molar extinction coefficient of 10^7 (Sinnhuber and Yu, 1958), a sample size of 0.5 g and an average 75.8% recovery, the K value was calculated to be 58.7. Results of the TBARS assay, often expressed as mg MA/kg sample, were converted to ppm (w/w).

3.3.6.3 Accelerated Oxidation Study Data Analysis

Accelerated oxidation study statistical analyses were preformed using SAS (Version 6.1; SAS Institute, Inc., Cary, NC). A two-way ANOVA on the log transform of the CD, TBARS and GC/MS-SPME data was conducted. Pairwise differences were

tested and correlation coefficients were determined between GC/MS-SPME, TBARS and CD. Measurements for each method were taken in triplicate and were all included in the analyses. The significance level used in these analyses was defined as $p<0.05$.

3.4 RESULTS AND DISCUSSION

3.4.1 Validation of the GC/MS-SPME Procedure

The response of the GC/MS-SPME procedure to hexanal concentration was linear over the five hexanal standards tested ($r^2=0.995$) (Figure 3.2). The limit of detection for this method was 0.01 ppm (10 ppb). This value is comparable to the 7 ng hexanal/g (7 ppb) protein limit of detection reported in turkey by Brunton et al. (2000), and to the limit of quantification of 36 ng hexanal/g (36 ppb) sample reported by Song et al. (1998) in tomato and strawberry fruit .

The precision of the GC/MS-SPME, as reflected in the coefficients of variation of hexanal peak areas, was found to range between 1 and 13%, with the majority of responses falling below 10%. The higher coefficient of variation was found to be at the lowest hexanal concentration. Elmore et al. (1997) made a similar observation in their studies, in which compounds with small peak areas had a higher coefficient of variation. Gutheil and Bailey (1992) also reported greater coefficients of variation at lower concentrations of hexanal. The precision of this GS/MS-SPME method was comparable to the SPME method developed for orange juice, in which coefficients of variation for 17 common flavor volatiles ranged from 1 and 18%, with the majority of volatiles falling below 10% deviation (Steffen and Pawliszyn, 1996). Song et al. (1997) reported coefficients of variation of hexyl acetate, 1-butanol, 2-methylbutyl acetate and alpha-

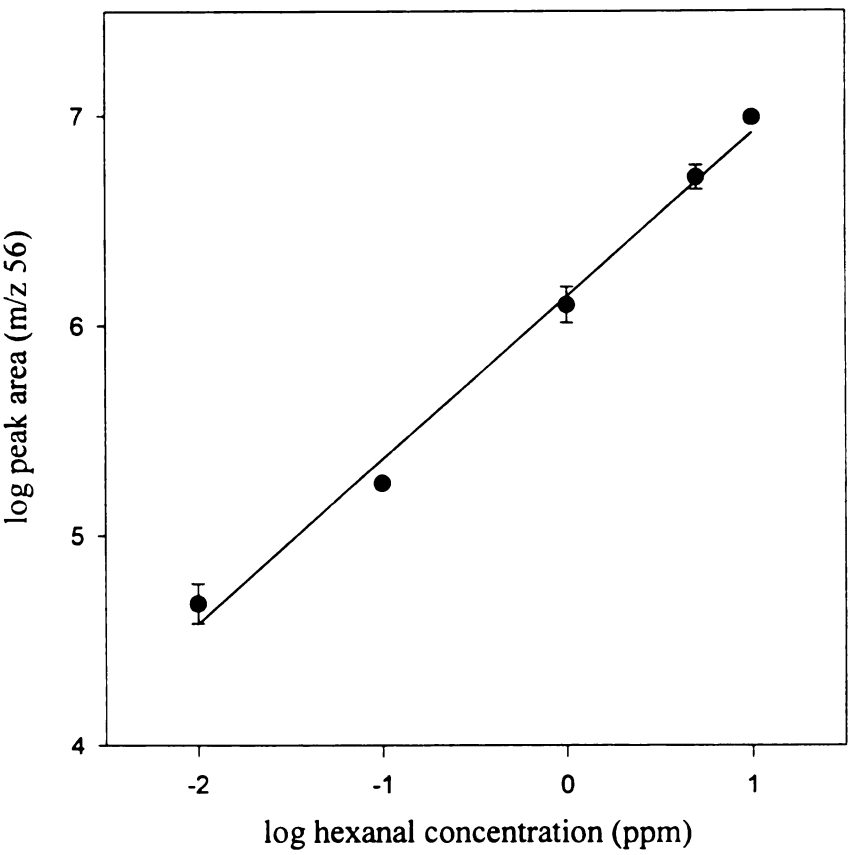

Figure 3.2. Linearity of GC/MS-SPME response to hexanal concentrations (r^2=0.995). Standards were prepared in squalene and sampled using SPME. Line represents a fitted regression line (y=963142x + 168333.2). Determinations were made in triplicate.

farnesene to range from 2 to 10% in apple fruit.

3.4.2 Hexanal Loading and Repeated Sampling of Spiked Chicken Myofibrils

The headspace concentration of hexanal increased during the first 4 h of incubation of the chicken myofibrils with the hexanal standards (Figure 3.3). The headspace concentration of hexanal in the vial increased for each of the four concentrations tested. Following 4 h of incubation, an equilibrium within the chamber was reached, and maintained up to 24 h.

Following 24 h of incubation with hexanal standards, the chicken myofibrils were removed and sampled to determine the amount of hexanal that the myofibrils had sorbed during the incubation time. Recovery of hexanal from the spiked myofibrils was greatest with no equilibration following transfer to the 4 mL vial, and with a 5-min SPME sampling time (Figure 3.4). Recovery of hexanal from myofibrils at the first sampling time ranged from 91 to 95.3%. Due to equilibration in hexanal between the myofibrils and headspace, hexanal concentration decreased with repeated sampling as it was desorbed from the chicken myofibrils into the headspace and removed from the system. Hexanal was removed from the headspace in the vial with repeated sampling, ranging from 29.2% to a 56.7% loss in hexanal over 6 sampling times. An even greater percentage of hexanal might have been removed from the system had sampling continued beyond 6 h. Volatile materials are generally held by solid substrates and the extent of volatile loss as a function of time at a given set of conditions is mainly determined by the energies of the interaction between the volatile and the substrate (Saleeb and Schenz, 1978).

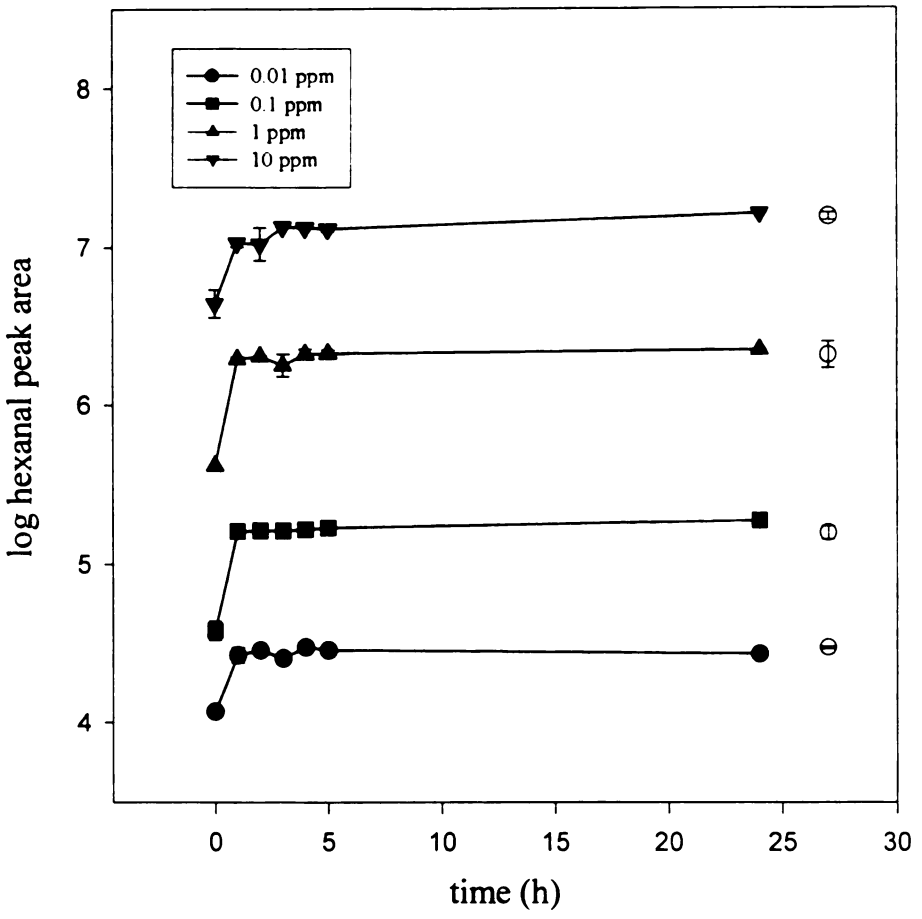

Figure 3.3. Steady state equilibrium of chicken myofibrils with hexanal loading. Myofibrils (0.03 g) were incubated over 1.5 mL hexanal standard (0.01 to 10 ppm hexanal) prepared in squalene. The headspace above the myofibrils in the chamber was sampled every hour up to 5 hours and following 24 hours of incubation. Unfilled shapes represent the first desorption of hexanal from spiked chicken myofibrils following incubation. Determinations were made in triplicate.

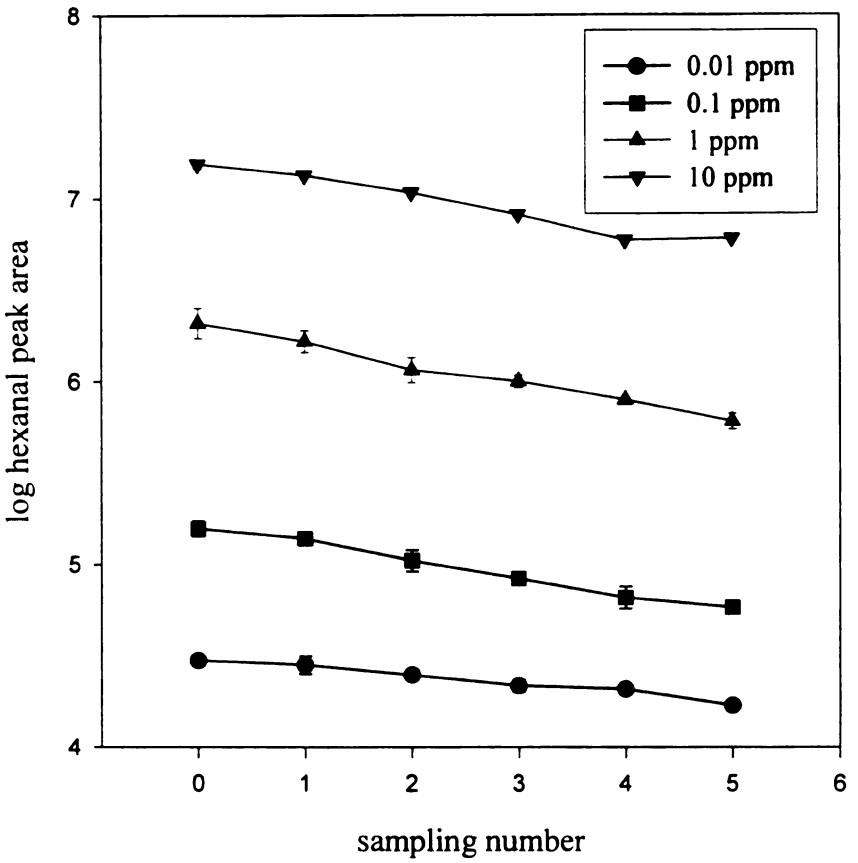

Figure 3.4. Effect of repeated sampling on headspace above chicken myofibrils (0.03 g) incubated over 1.5 mL of 0.01 to 10 ppm hexanal standard prepared in squalene. Headspace was sampled 6 times over 5 hours. Determinations were made in triplicate.

3.4.3 Hexanal Partitioning Between Headspace and Myofibrils

Hexanal partitioning was examined by comparing the hexanal concentration found in the vial headspace above the chicken myofibrils following 24 h of incubation to the hexanal concentration of standard alone following 24 h of incubation (Table 3.1). For all standards, a low coefficient of variation was reported between peak areas, ranging from 1.6 to 7.8% in hexanal standard (mean of 5.16%), and in hexanal plus myofibrils, ranging from 1.1 to 5.5 (mean of 1.77%). The hexanal standards incubated with myofibrils had a lower coefficient of variation than the hexanal standards alone. Gutheil and Bailey (1992) reported similar findings in their study examining the binding of hexanal to myosin and actin. The linearity of the hexanal standard curve was unaffected by the presence of myofibrils, with a correlation coefficient of 0.998 with hexanal standard and 0.999 with hexanal standard plus myofibrils.

Hexanal partitioned between the headspace and the chicken myofibrils. The slopes of the standard curves prepared from hexanal standard only, and hexanal standard plus myofibril, displayed little variation; a greater variation was observed between intercepts of the two standard curves. The standard curve prepared from hexanal standard plus myofibril had a lower intercept, indicating decreased detection of hexanal in the system. This may be attributed to the partitioning of the hexanal into the myofibrils, removing it from the system and from detection using the GC/MS-SPME.

Addition of the myofibrils to the vials containing the hexanal standard reservoir caused a reduction in headspace hexanal. This reduction may be accounted for by the physical and chemical bonding of hexanal to the myofibrils, as the myofibrils present in

Table 3.1. Log peak area of hexanal alone and hexanal incubated with chicken myofibrils following equilibrium. Hexanal standards (1.5 mL) were either incubated alone in a 15-mL vial or with chicken myofibrils (0.03 g) for 24-h at ambient temperature and sampled.

Hexanal concentration (ppm)	Hexanal log peak area following 24-h incubation	% CV	Hexanal plus myofibril hexanal log peak area following 24-h incubation	% CV	Percent loss in hexanal due to the addition of myofibrils in incubation vial
0.01	4.5 ± 0.031	7.1	4.4 ± 0.05	1.1	19.3
0.1	5.3 ± 0.034	7.8	5.25 ± 0.02	5.5	10.6
1	6.4 ± 0.02	4.1	6.39 ± 0.003	0.62	3.3
10	7.2 ± 0.007	1.6	7.19 ± 0.004	0.90	1.8

the vial became a secondary repository for the hexanal. The concentration of the hexanal standard impacted the percentage of hexanal retained by the myofibrils versus the percentage detected in the headspace. At 0.01 ppm standard, a greater percentage difference (19.3%) was observed between the hexanal peak area of the standard alone compared to the hexanal peak area of the standard plus chicken myofibrils. This indicated that a higher percentage of the hexanal present in the system was being retained by the myofibrils. As hexanal concentration was increased, the percentage concentration of hexanal retained by the myofibrils decreased. In 10 ppm hexanal, a 1.8% difference existed between the standard alone and the standard plus the myofibrils. Thus, while the myofibrils still sorbed hexanal and removed it from the system, the percentage of hexanal removed from the system was lower at higher hexanal concentrations. This may be due to the saturation of the limited number of hexanal binding sites (O'Keefe et al., 1991).

The partitioning of hexanal vapors into the chicken myofibrils may be attributed to specific binding and sorption. When a matrix sorbs a compound, the molecules are adsorbed or absorbed in the free volume which is always present in the amorphous regions (Hernandez-Munoz et al., 1999). While several studies in the packaging area have examined the relationship between hexanal and its sorption into different polymer films (Hernandez-Munoz et al., 1999; Hernandez-Munoz et al., 1998), few studies have studied the sorption of hexanal by a food matrix.

Aldehyde binding has been studied in numerous protein slurries, including soy glycinin and beta-conglycinin (O'Keefe et al. 1991), soy protein (Aspelund and Wilson,

1983), lysozyme (Karel et al., 1975), bovine serum albumin and textured vegetable protein (Franzen and Kinsella, 1974), whey-based and egg-based fat replacers (Schirle-Keller et al., 1994), and myoglobin (Lynch and Faustman, 2000). Gremli (1974) conducted a study using a high vacuum-shell freezing system to determine whether different flavor compounds reacted reversibly or irreversibly with soy protein. In a 5% soy protein solution, 37-44% of hexanal was found to be reversibly bound, while less than 5% was irreversibly bound. Franzen and Kinsella (1974) studied the binding of carbonyl flavor compounds to soy proteins using headspace gas chomatography. Results showed that 10-21% of the hexanal added to the soy protein solution was retained by the protein solution. In a later study, Gutheil and Bailey (1992) examined the relationship of hexanal with the proteins myosin and actin. Their studies were conducted with high concentrations of hexanal, ranging from 100 to 1600 ppm. Using these concentrations, myosin was reported to bind less than 10% of the hexanal. When studying the binding of actin to hexanal (200 to 1000 ppm), an average of 25 to 30% of the added hexanal bound to the protein. In determining binding of hexanal to protein, the published studies all used a hexanal solution, while this study was the first to examine binding using hexanal vapors.

3.4.4 Accelerated Oxidation Study

3.4.4.1 Hexanal in Headspace

During both the 9-day and 50-h oxidation study, the concentration of hexanal increased in the chamber headspace above the myofibrils (Figure 3.5). The hexanal concentration during the 9-day study at both a_w showed a plateau at day 6. In the 9-day

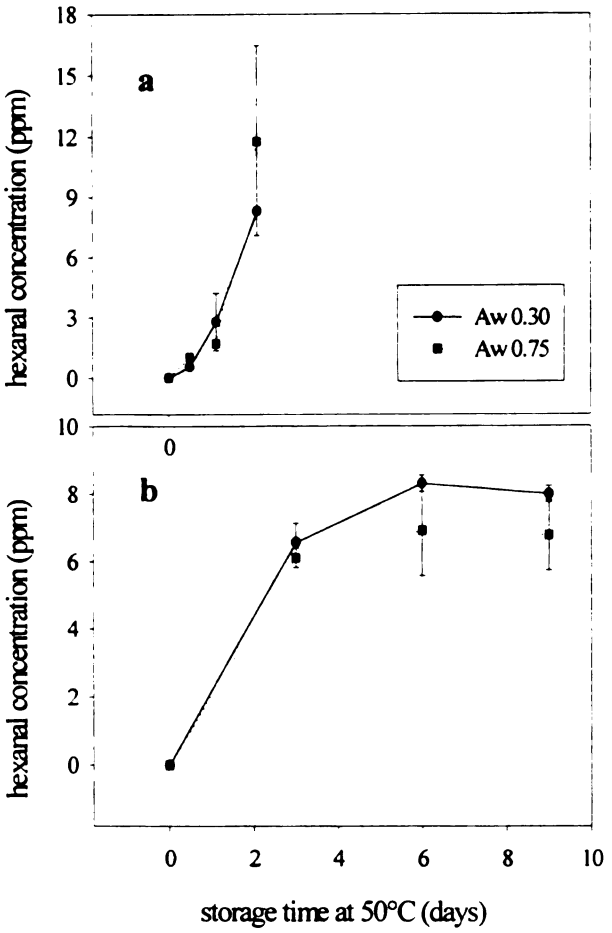

Figure 3.5. Hexanal formation in chicken myofibrils containing 0.6 umol methyl linoleate/g protein as affected by storage time at 50°C at a_w of 0.30 and 0.75. The headspace above the myofibrils was sampled using GC/MS-SPME for 50 hr storage period (a) and 9 day storage period (b). Error bars represent standard deviation.

storage study, the maximum hexanal concentration in the chamber (8.3 ppm) was reached at day 6 at an a_w of 0.30. A similar trend was seen at a_w of 0.75, with a maximum hexanal concentration of 6.9 ppm also at day 6. During the 50-h oxidation study, the maximum hexanal concentration for both water activities was reached at 50 h of storage. At a_w of 0.30, the hexanal concentration was 8.3 ppm , whereas the hexanal concentration was 11 ppm at a_w of 0.75.

Equilibrium is reached when the rate of volatiles moving out of a sample equals that of volatiles moving back into the sample (Taylor, 1998). Combinations of physicochemical parameters such as the partition and mass transfer coefficients, together with dynamic factors, determines the relative distribution of the volatile compound between the food and air phases (Marin et al, 1999). In a complex food matrix, true equilibrium is rarely achieved and only under certain storage conditions. When foods undergo a dynamic process, such as lipid oxidation, the gas phase is often not constrained and a true equilibrium is not attained (Taylor, 1998).

3.4.4.2 Effect of Water Activity on Lipid Oxidation during Accelerated Studies

In the 9-day lipid oxidation study, no differences ($p<0.05$) were observed between myofibrils stored at a_w 0.30 and a_w 0.75. Lipid oxidation measured by TBARS proceeded very slowly at a_w of 0.30, changing little over the 50 h storage period. Hexanal and conjugated diene data indicated that oxidation proceeded at the low water activity, but not as rapidly as it did at the high water activity.

Lipid oxidation proceeded very slowly in myofibrils stored at an a_w of 0.30, indicating that this water activity is near the monolayer value. Theoretically, a food is

least susceptible to lipid oxidation when stored at relative humidities corresponding to monolayer moisture content. The monolayer represents a theoretical moisture content at which the protein binds one molecule of water per polar group (Kinsella and Fox, 1986). Smith et al. (1990) also made a similar finding in chicken myofibrils treated with methyl linoleate. Following 3 weeks of storage at 20°C, these researchers found that TBARS values were lower at a_w of 0.85 and 0.43

In myofibrils stored at a_w of 0.75, hexanal and TBARS increased ($p<0.05$) at almost all time points over the 50 h storage period, indicating an increase in oxidation. Smith et al. (1990) observed that lipid oxidation increased most rapidly when myofibrils with added methyl linoleate were stored at a_w of 0.65.

3.4.4.3 Nine-Day Accelerated Oxidation Storage Trial

The formation of conjugated dienes during storage at 50°C confirmed that lipid oxidation proceeded in the myofibrils. Conjugated diene formation was affected by water activity, storage time, and the interaction of the two factors ($p<0.05$). The conjugated diene concentration reached a maximum at day 3, decreased at day 6 and did not change at day 9, indicating the usual pattern of primary oxidation product formation during lipid oxidation (Figure 3.6). Orlien et al. (2000) reported a similar pattern of conjugated diene formation in rapeseed oil stored at 25°C for 25 days.

Chicken myofibrils contained increasing amounts of TBARS and hexanal over the first 6 days of storage, followed by a decrease in both products at day 9. Hexanal and TBARS were not affected by water activity ($p<0.05$). TBARS increased over time, but no differences were observed between sample day 3 and 9. The maximum concentration

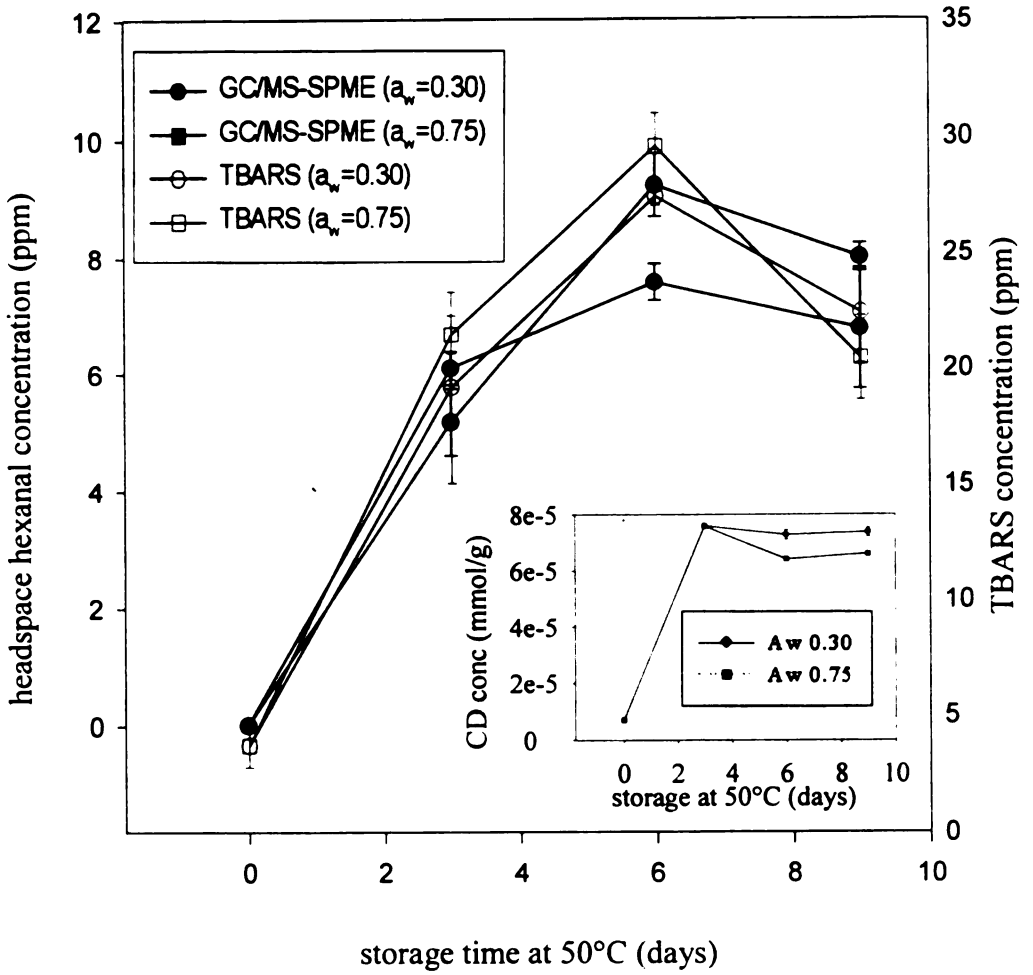

Figure 3.6. Comparison of the headspace hexanal concentration determined by GC/MS and TBARS concentration of chicken myofibrils stored for 9-days at different water activities at 50°C. Chicken myofibrils contained 0.6 umol methyl linoleate/g protein. Inset: Effect of water activity and length of storage at 50°C on the production of conjugated dienes (CD) during the same 9-day storage period. Determinations were made in triplicate. Error bars represent standard deviations.

of TBARS was reached at day 6. In myofibrils stored at a_w of 0.75 and 0.30, TBARS of 29.6 ppm and 27.4 ppm, respectively, were observed. TBARS declined at day 9 to concentrations of 20.4 and 22.4 ppm for a_w of 0.75 and 0.30, respectively. Hexanal concentration increased over time, reaching a maximum at day 6 of 7.6 at 0.75 a_w and 9.2 ppm at a_w 0.30. No differences ($p<0.05$) were reported between sample day 6 and 9.

The minor decrease in hexanal was attributed to the decomposition of hexanal to hexanoic acid (Shahidi and Pegg, 1994). The results from the GC/MS confirmed this increase in hexanoic acid over storage time. The decrease in TBARS is attributed to increased polymerization of the secondary products (Wen et al., 1997)

At day 6 of storage, the concentrations of TBARS observed were in general agreement with those values presented by other researchers. Rhee et al. (1996) followed oxidation in cooked chicken thigh stored at 4°C for 6 days. TBARS values were reported to increase from 0.5 to 14 ppm (w/w) by the last day of storage. In turkey thigh, oxidation was followed over 8 days of storage at 4°C, during which TBARS values increased from 0.5 to 11 ppm (w/w) (Whang and Peng, 1987). In roast chicken white meat, Dupuy et al. (1987) reported hexanal concentrations of 14.6 ppm (w/w) following 5 days of storage at 4°C. Brunton et al. (2000) found that in a turkey homogenate stored at 4°C for 6 days, the maximum malonaldehyde and hexanal concentrations reached were 7 and 4 ppm (w/w) respectively. Few studies have followed lipid oxidation in freeze-dried meats stored at high temperatures (Wilkinson et al., 2001; Sun et al., 2001).

Correlations between the methods used to measure lipid oxidation are listed in Table 3.2. As conjugated dienes are a measure of primary oxidation, poor correlations

Table 3.2. Correlation coefficient matrix (r) between methods used to measure the extent of lipid oxidation in freeze-dried chicken myofibrils over 9-day storage at 50°C. Tests used were GC/MS SPME to measure hexanal, TBARS, and CD to measure conjugated dienes.

Method	GC/MS-SPME	TBARS
--	--	--
TBARS	0.974[a]	--
CD	0.343	0.557

[a] Significant at $p<0.05$

were observed between conjugated diene concentration with the methods used to quantify secondary products, hexanal (0.343) and malonaldehyde (0.557). Other researchers have reported low correlations between CD and secondary product measurements, including Aubourg (1999) who reported a correlation of 0.57 in fish stored at -30°C for 1 year.

A strong correlation (r) of 0.974 ($p<0.05$) was established between hexanal and TBARS, when both water activities were averaged. Using linear regression, individual correlation coefficients of 0.954 and 0.976 were calculated for a_w of 0.30 and 0.75, respectively.

3.4.4.4 Fifty-Hour Accelerated Oxidation Storage Trial

Conjugated diene concentration increased ($p<0.05$) during the 50-h study, indicating that lipid oxidation was proceeding (Figure 3.7). Water activity, sampling time and the interaction of the two had an effect ($p<0.001$) on conjugated diene formation.

During the early stages of oxidation, differences between myofibrils stored at high versus low water activity were not detected using TBARS, whereas differences in hexanal concentration were detected. TBARS were ($p<0.05$) affected by water activity, time and the interaction of the two factors. TBARS values were not affected ($p<0.05$) by water activity at 12 and 27 h of storage. At 50 h of storage, water activity affected ($p<0.05$) TBARS formation.

Hexanal production during storage was affected by water activity and sample time ($p<0.05$). At a_w of 0.75, lipid oxidation increased ($p<0.05$) at all time points. At a_w of 0.30, differences in hexanal concentration were observed between all time points except at h 27. Hexanal concentration reached 7.5 ppm at a_w of 0.75 and 3.9 ppm at a_w of 0.30

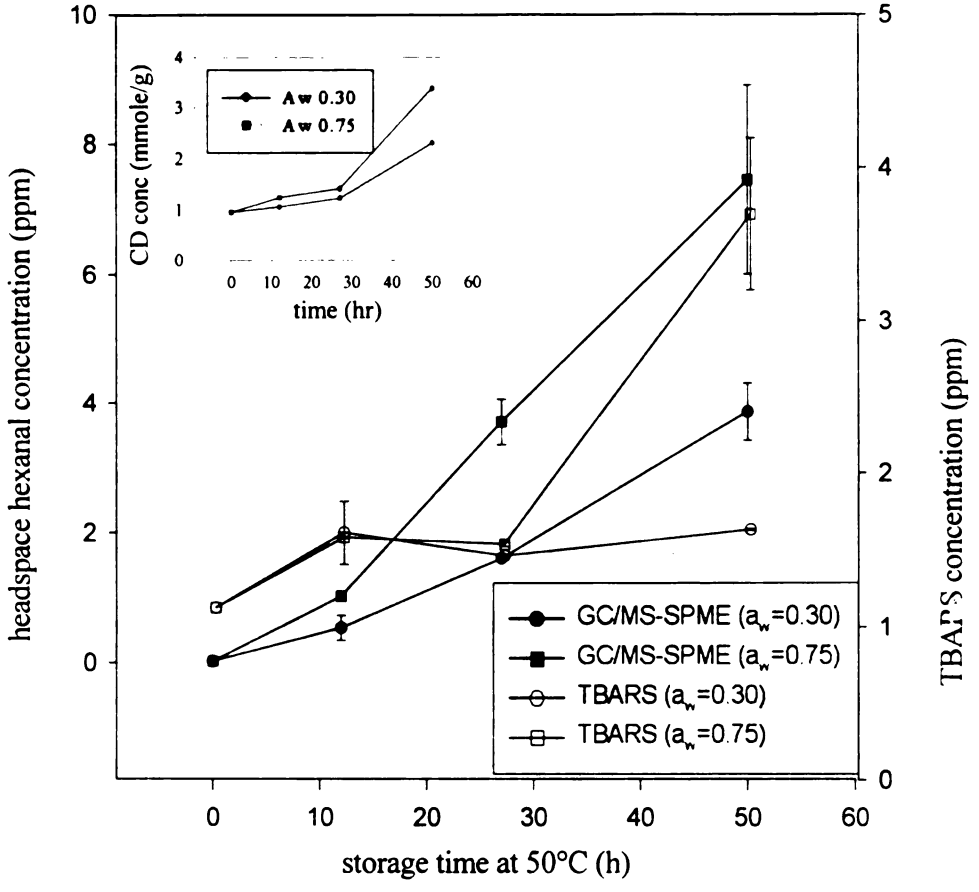

Figure 3.7. Comparison of the headspace hexanal concentration determined by GC/MS and TBARS concentration of chicken myofibrils stored for 50-h at different water activities at 50°C. Chicken myofibrils contained 0.6 umol methyl linoleate/g protein. Inset: Effect of water activity and length of storage at 50°C on the production of conjugated dienes (CD) during the same 50-h storage period. Determinations were made in triplicate. Error bars represent standard deviations.

after 50 h of storage.

Correlations were established between hexanal, TBARS and conjugated diene concentration during the 50 h storage trial (Table 3.3). Hexanal formation was correlated to TBARS ($r=0.938$) and conjugated dienes ($r=0.975$) ($p<0.05$). The correlation (r) of TBARS to conjugated diene formation was0.952 ($p<0.05$). Correlations (r) between hexanal and TBARS were found to be 0.60 and 0.894 for a_w of 0.30 and 0.75, respectively (Figure 3.9). The conjugated diene concentration increased as the secondary product concentration increased, resulting in high correlations.

The increased sensitivity of the GC/MS-SPME at 12 h and 27 h compared to the small differences in TBARS numbers at these same sample times resulted in a lower correlation than reported in the 9-day study. Shahidi et al. (1987) used hexanal analysis and sensory evaluation as indicators of oxidative stability and flavor acceptability in cooked ground pork Following two days of storage at 4°C, little difference between TBARS values were reported whereas differences were detected using hexanal analysis and sensory evaluation. The researchers went on to suggest that hexanal content is a better measure of oxidative state than TBARS in meats during the early stages of storage.

3.4.5 SPME Applications in Lipid Oxidation Detection

In order to validate a novel lipid oxidation assessment method, it needs to be compared to the classical TBARS method. Using this GC/MS-SPME method, a strong correlation was achieved between hexanal and TBARS. Shahidi et al. (1987) reported a strong correlation (r^2 of 0.995) between TBARS and hexanal content quantified using purge and trap GC in a slurry of stored cooked ground pork. In cooked beef, pork and

Table 3.3. Correlation coefficient matrix (r) between methods used to measure the extent of lipid oxidation in freeze-dried chicken myofibrils over 50-hr storage at 50°C. Tests used were GC/MS SPME to measure hexanal, TBARS and CD to measure conjugated dienes.

Method	GC/MS-SPME	TBARS
--	--	--
TBARS	0.938[a]	--
CD	0.975[a]	0.952[a]

[a] Significant at $p<0.05$

turkey burgers held at 4°C for 7 days, correlations between TBARS and hexanal concentration, were 0.98, 0.99 and 0.97 respectively (Wen et al., 1997). The researchers quantified hexanal using hexane extraction and separation by reversed-phase high performance chomatography. Nielsen et al. (1997) published a correlation of 0.98 between TBARS and GC-SPME in minced pork over a 6-day storage time at 4°C. Brunton et al. (2000) reported a strong correlation between SPME/GC and TBARS (r^2=0.994) when measuring lipid oxidation in cooked turkey during storage over 6 days at 4°C.

The use of SPME for analysis of volatile compounds, including those volatiles generated during lipid oxidation, has been growing. Many different SPME procedures have been published, varying in fiber use, equilibration time, and sampling temperature. For detection of hexanal in chicken myofibrils, the GC/MS-SPME method outlined in this study employed a 22°C (room temperature) sampling temperature. Other SPME procedures have been published describing the use increased temperatures in order to maximize desorption. Ruiz et al. (1998) studied the effect of extraction time and temperature on volatile detection in ham observing that the chomatographic area of hexanal increased with longer extraction times and higher extraction temperature (60°C). The researchers suggested that the increase in chomatographic area may be attributed not only to the enhancement in extraction, but also to the formation of hexanal during extraction due to the higher temperature. In their studies of lipid oxidation in minced pork, Nielsen et al. (1997) employed a SPME method that involved sampling the pork for 15 min at 45°C. Brunton et al. (2000), in measuring lipid oxidation in cooked turkey

during storage, employed a 20 min elevated sampling temperature (40°C). While these elevated temperatures enhance extraction of the hexanal from the meat sample, one must exercise caution as hexanal can be thermally generated. Because of the high sensitivity of the GC for hexanal detection, it is important to minimize hexanal production during sampling, which would require the use of a low sampling temperature as was used in this study.

3.5 CONCLUSIONS

The development of objectionable flavors and odors generated by lipid oxidation have detrimental consequences on food quality and consumer acceptability. Hexanal content has been reported to be a sensitive and reliable indicator for the evaluation of the oxidative status of meat products. Traditional methods of hexanal analysis by gas chomatography, while sensitive, are labor intensive, often requiring dedicated equipment. A precise and accurate GC/MS-SPME method for the rapid quantification of hexanal was described in this study. Over long-term storage of chicken myofibrils at 50°C, this hexanal analysis method was found to correlate well with TBARS. In a short term study of chicken myofibrils, the GC/MS-SPME method for hexanal appeared to be more sensitive than TBARS during the initial stages of lipid oxidation. As early detection of lipid oxidation is crucial in the food industry, this method shows promise as a fast and accurate alternative to the traditional methods of hexanal analysis.

CHAPTER 4

VERIFICATION OF A MONOCLONAL ANTIBODY-BASED ELISA FOR MONITORING LIPID OXIDATION IN CHICKEN

4.1. ABSTRACT

Hexanal, a secondary product of lipid oxidation, has often been used as in indicator of lipid oxidation. Headspace-gas chromatography has typically been used to measure hexanal; however, this method is expensive and involved. As a sensitive, rapid and reproducible alternative, a monoclonal antibody was developed for the detection of hexanal. The objective of this study was to compare the ability of a monoclonal Competitive Indirect ELISA (CI-ELISA), thiobarbituric acid reactive substances assay (TBARS) and a solid-phase microextraction- gas chromatography/mass spectrometry (GC/MS-SPME) method for monitoring lipid oxidation in freeze-dried chicken protein. Protein extraction and solubilization procedures were optimized to maximize the formation of hexanal-protein adducts and percent extractable protein. Freeze-dried myofibrils (MF) with added methyl linoleate (0.6 mmol/g protein) were stored at 50°C at water activities (a_w) of 0.30 and 0.75. Samples were removed each day over a 5 day storage period. Hexanal was measured by GC/MS-SPME, TBARS and the CI-ELISA. Lipid oxidation occurred more rapidly, and higher concentrations of hexanal and TBARS were observed, when MF were stored at the higher water activity ($p<0.05$). Lipid oxidation reached a maximum after 4 days of storage at both a_w, then decreased. At an a_w

of 0.30, 34.7 and 39.7 ppm hexanal were detected by GC/MS-SPME and CI-ELISA respectively, after 4 days of storage. The CI-ELISA was well correlated with the GC/MS-SPME (r=0.78) and TBARS (r=0.87) methods. The correlation of the hexanal-specific CI-ELISA to both GC/MS-SPME and TBARS verified the ability of the CI-ELISA to be used as an index of lipid oxidation, offering the convenience for use in a kit to be utilized within a food processing facility.

4.2. INTRODUCTION

Lipid oxidation is a major quality deterioration problem in all muscle foods, affecting both the toxicity and organoleptic quality of the food. The oxidative deterioration of muscle lipid involves the oxidation of the unsaturated fatty acid via a free radical mechanism (Melton, 1983). In addition to the formation of hydroperoxides or primary products of oxidation, other types of reactions may occur (Gray, 1978). The peroxides break down to low molecular weight compounds, including alkanes, alkenes, ketones, aldehydes and alcohols (Gray and Monahan, 1992). The carbonyl compounds present have the greatest impact on flavor and odor development in rancid foods owing to their low odor threshold (Shahidi and Pegg, 1994). Based on sensory tests, the aldehyde hexanal was found to have an odor threshold of 5.9 ppm in lean ground beef (Brewer and Vega, 1995) and between 5 and 10 ppm in cereal (Fritsch and Gale, 1977). The production of off-flavors and odors were considered to be the most important consequence of lipid oxidation in the past; however, increased attention is now being given to the health risks that lipid oxidation may impose. Numerous studies have reported the toxic effects of oxidized fats in animals (Yang et al., 1998; Corpet et al.,

1990). Primary and secondary lipid oxidation products have also been linked to diseases, such as atherosclerosis (Khan et al., 2000) and Parkinsons's disease (Uchida et al., 1995).

Lipid oxidation is a complex process and a number of methods exist by wh.ch oxidative changes can be measured. Sensory methods provide the most useful information related to consumer acceptance of the food; however, these methods are time-consuming and display poor reproducibility (Frankel, 1993). While primary products have been used as indicators of lipid oxidation in food products, such as cooked meat, where oxidation occurs at an accelerated rate, the measurement of secondary lipid oxidation products is considered a more appropriate index of lipid oxidation (Gray and Monahan, 1992).

One of the most commonly used methods to assess lipid oxidation is the 2-thiobarbituric acid reactive substances assay (TBARS) (Menton, 1983). In this assay, the TBA reagent reacts primarily with malonaldehyde, a relatively minor product of oxidation, to form a red-colored complex with an absorption maximum at 532 nm (Gray and Monahan, 1992). The concentration of this complex is then used as an objective measure for the evaluation of the oxidative state of cooked meats. While the TBARS assay is a relatively rapid and simple procedure and has been highly correlated to sensory scores (Igene et al., 1979), it has been criticized primarily for its lack of specificity Aldehyde products other than malonaldehyde may react with thiobarbituric acid (TBA) to produce yellow (455 nm), orange (495 nm) and red pigments (532 nm) (Sun et al., 2001). Other compounds such as sucrose and compounds in woodsmoke have also been reported to react with the TBA reagent (Dugan, 1961).

Hexanal is a dominant oxidation product of linoleic acid and has been successfully used to follow lipid oxidation in a number of products. As hexanal arises from both the 9- and 13-hydroperoxides of linoleate, it predominates among volatile aldehydes formed during oxidation (Shahidi and Pegg, 1994). Strong linear relationships were reported between hexanal content, sensory scores and TBARS in cooked pork (Shahidi et al., 1987), chicken (Ahn et al., 2000) and beef (Spanier et al., 1992). Hexanal analysis by conventional analysis techniques such as static headspace gas chromatography (GC) lack sensitivity, while more sensitive dynamic methods are tedious and difficult to perform (Reineccius, 1996; Steffen and Pawliszyn, 1996). Recently, solid-phase microextraction (SPME) has been introduced as an alternative to traditional methods (Steffen and Pawliszyn, 1996). Direct extraction and sorption of hexanal onto a coated silica fiber is followed by analyte desorption into the injection port of a GC. Because this method is fast, inexpensive and simple to use, SPME has gained popularity in food analysis. In the area of lipid oxidation, SPME has recently been employed for the detection of aldehydes in turkey (Brunton et al., 2000) and sunflower oil (Keszler et al., 1998). Headspace chromatographic methods, such as SPME and traditional GC techniques, require the volatization of hexanal for quantification. However, in food systems, covalent binding between hexanal and proteins in the food may affect accurate hexanal quantification (O'Keefe, 1991).

The increased regulation of foodstuffs requires analytical methods which are easy to perform, sensitive and specific (Fukal, 1991). Since the introduction of immunoassays, extensive research efforts have focused on their development (Samarajeewa et al., 1991).

The development of immunoassay kits has allowed rapid and sensitive detection of mycotoxins and microorganisms in the field, with good agreement to standard quantification methods (Samarajeewa et al., 1991). The development of a commercially available ELISA kit for hexanal detection would require several modifications, but does show promise for development. The food industry would gain immense benefits from a hexanal ELISA kit because such kits are easy to operate, convenient, and require less technical skill compared with conventional detection assays (Samarajeewa et al., 1991).

Recently, a Competitive Indirect-ELISA (CI-ELISA) using polyclonal (Smith et al., 1999) and monoclonal antibodies (Zielinski et al., 2001) was developed for the detection of hexanal. Hexanal is a hapten and non-immunogenic, and thus requires conjugation with a large protein molecule, such as chicken serum albumin, in order to become antigenic. Hexanal-conjugate production was accomplished through the formation of Schiff bases via the lysine group on the protein, followed by stable conjugate formation using the strong reducing agent, sodium cyanoborohydride (Jentoft and Dearborn, 1979; Means and Feeney, 1968). Both the polyclonal and monoclonal CI-ELISAs for hexanal were sensitive and showed potential for use in a meat system to follow lipid oxidation (Zielinski et al., 2001). In an accelerated oxidation study in a chicken thigh homogenate system, the polyclonal CI-ELISA for hexanal was found to correlate with dynamic headspace GC ($r = 0.89$) and with lipid oxidation determined by TBARS ($r = 0.85$). While the polyclonal antibody was hexanal sensitive, it displayed cross reactivity to pentanal (11.8%) and heptanal (86.3%) modified with chicken serum albumin.

To improve the specificity of the CI-ELISA, monoclonal antibodies to hexanal were developed (Zielinski et al., 2001). The monoclonal antibody displayed moderate cross-reactivity with other aldehydes, reacting 76.6%, 45.0% and 37.9% to modified heptanal, 2-t-hexenal and pentanal, respectively. In the detection of hexanal in an oxidized chicken thigh homogenate accelerated oxidation study, hexanal contents determined using the monoclonal CI-ELISA were not different from values determined using dynamic headspace GC except at one time point r = 0.81). These initial studies indicated that the use of hexanal specific monoclonal antibodies was feasible for accurate assessment of lipid oxidation in a meat product.

The primary goal of this study was to verify the use of a monoclonal CI-ELISA for monitoring lipid oxidation in chicken through the quantification of hexanal. In order to achieve this goal, specific objectives were to 1) optimize the monoclonal CI-ELISA for hexanal detection, 2) optimize protein extraction procedures for the formation of hexanal-protein adducts, 3) conduct an accelerated oxidation study to correlate results from CI-ELISA, the novel GC/MS-SPME method for hexanal, developed in the previous study, and TBARS.

4.3. MATERIALS AND METHODS

4.3.1. Materials

Hybridoma media was composed of: Dulbecco's Modified Eagle Medium (DMEM; Sigma Chemicals, St. Louis MO), fetal bovine serum (FBS; Gibco BRL, Rockville, Maryland), NCTC-109 (Gibco BRL), and penicillin-streptomycin solution (100 U/mL) (Sigma Chemicals). Dimethyl sulfoxide was purchase from Sigma

Chemicals and tissue plastic ware were purchased from Corning Laboratory Science Co. (Corning, NY).

Microtiter wells (Immunolon-2 Removawells) were from Dynatech Laboratories (Alexandria, VA). Goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (GAM-IgG HRP) was purchased from Cappel Laboratories (West Chester, PA), and 3,3',5,5'-tetramethyl-benzidine (TMB; T 8665) was purchased from Sigma Chemicals Co. Fungal protease AS-L (Lot 9025-49-4), alkaline protease (Lot 3-0130-000; Protease L-FG) and papain (Lot 3-6950-510; Liquipanol T-100) were all purchased from the Enzyme Development Corporation (New York, NY). The fungal protease had activity of 40,000 HUT/g, alkaline protease had activity of 560,000 Delft units/g, and papain had an activity of 100 to 115 T.U./mg.

Endoproteinase lys-C and trypsin were purchased from Sigma Chemicals. One unit of endoproteinase lys-C activity was defined as the amount of lys-C that would hydrolyze 1.0 μmol of N-p-tosyl-Gly-Pro-Lys-p-nitroanilide per minute at pH 7.7 at 25°C. Trypsin had activity of 1000 to 1500 BAEE units/mg solid. BioRad protein dye was from BioRad Laboratories (Hercules, CA). Tenox 2 antioxidant was obtained from East Chemical Company (Roebuck, SC). Hexanal, chicken serum albumin (CSA), bovine serum albumin (BSA), picrylsulfonic acid and all other materials were purchased from Sigma Chemicals.

4.3.2. Optimization of Extraction Procedures to Solubilize Myofibrils

Myofibrils (15 mg/ mL) were mixed with 10 mL of 0.6 M KCl, 0.05 M potassium phosphate (adjusted to desired pH), 200 ppm antioxidant and incubated for 2 h in a 50°C

shaking water bath. Following incubation, solutions were filtered through 2 layers of cheesecloth. An aliquot of the filtrate was centrifuged 30 min at 20,000 x g (Morr et al., 1985), and protein content of the supernatant was determined using the Bradford protein assay (1976) described in Section 4.3.7.

4.3.2.1 Effect of KCl on CI-ELISA

A 0.6 M KCl buffer is commonly used to solubilize myofibrils (Smith et al., 1990). BSA-hex standards containing between 3 and 69 ng hex/mL were prepared using different dilutions of the extraction buffer (0.6 M KCl, 0.05 M potassium phosphate, pH 9.0). These standards were analyzed using the CI-ELISA to determine the effect of KCl on the assay.

4.3.2.2 Use of Proteases to Solubilize Myofibrils

In the first experiment, papain concentrations used were 0.05 and 0.15% (w/w) at pH 5.5, alkaline protease concentrations were 0.05 and 0.15% (w/w) at pH 9.5, and fungal protease concentrations were 0.05 and 0.5 % (w/w) at pH 7.4. Myofibrils (~5 mg/mL) were mixed with 10 mL of pH-adjusted extraction buffer (0.6 M KCl, 0.05 M potassium phosphate), and the procedure described in Section 4.3.2 was followed.

The ability of alkaline protease and a lysine-specific protease, endoproteinase lys-C, to solubilize myofibrils and maximize protein-hexanal conjugate formation was also compared. For these studies, endoproteinase lys-C (3 units) was diluted 100, 250, 500, 1000 and 2000 times in extraction buffer adjusted to pH 7.7. The concentrations of alkaline protease studied were 0.20%, 0.60% and 1 % (w/w), using 0.6 M KCl extraction buffer at pH 9.0. After the addition of protease to the myofibrils (0.15 mg/mL), the

solubilization procedure described in Section 4.3.2 was followed.

These samples were then subjected to the conjugation-reduction reaction. Briefly, 8.75 mL of each sample was mixed with 246 μL hexanal and 1 mL of 800 mM sodium cyanoborohydride dissolved in 0.1 N NaOH. Final reactant concentrations were 200 mM hexanal and 80 mM of sodium cyanoborohydride . Samples were vortexed and held on ice with periodic vortexing for 1 h. Samples were dialyzed overnight in 2 L of buffer with 2 changes of PBS, after which the CI-ELISA was performed (Section 4.3.6.1).

Due to the expense of the endoproteinase lys-C, another protease with a lysine cleavage site, trypsin, was studied. The endoproteinase lys-C concentration used was 1000x dilution of 3 units (based on the experiment above). The trypsin concentration used was 0.25% (w/w) (manufacturer suggestion). Myofibrils (15 mg/mL) or BSA were mixed with 10 mL of 0.6 M KCl, 0.05 M potassium phosphate (pH 7.7) (Section 4.3.2). The CI-ELISA was performed on each sample (Section 4.3.6.1). Percentage recovery of hexanal was determined through comparison of spiked hexanal with recovered hexanal.

4.3.2.3 Trypsin Optimization and Inactivation

The parameters for trypsin optimization were pH (8.0 and 9.0), incubation time (2, 4, 5.5 h) and trypsin concentration (0.05, 0.1 and 0.25%). Specifically, myofibrils or BSA (30 mg/mL) were mixed with trypsin, and brought to 9 mL with extraction buffer (0.6 M KCl, 0.05 M potassium phosphate buffer). Hexanal (246 μL) and 1 mL of 800 mM $NaCNBH_3$ in 0.1 N NaOH were added and the protein-hexanal solution was incubated at 50°C in a shaking water bath for the appropriate incubation time (Section 4.3.2). The protein-hexanal conjugates were analyzed using the TNBS assay to determine

the loss of reactive groups (Section 4.3.5).

Inactivation of trypsin by heat was examined. BSA (30 mg/mL) was mixed with 0.25 % (w/w) trypsin, and brought to 9 mL with buffer (0.6 M KCl, 0.05 M phosphate buffer, pH 9.0). Hexanal (246 μL) and 1 mL of 800 mM $NaCNBH_3$ in 0.1 N NaOH were added and the solution was incubated at 50°C in a shaking water bath for 2 h. Following incubation and filtering, the mixture was heated at 100, 90 and 80°C for 5 min. The solution was centrifuged for 15 min at 20,000 x g and the supernatant used in the CI-ELISA. Standard curves ranging from 3 to 69 ng hex/mL were constructed using each treatment (100, 90 and 80°C, and unheated) and compared to the BSA-hex standard curve prepared with no trypsin.

4.3.3. Growth and Maintenance of Hybridomas

To generate a fresh supply of antibody, hybridomas were grown up each year. The media used was Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS), 1% NCTC-109 and penicillin-streptomycin solution (100 U/mL). Hybridomas were previously frozen in 1 mL aliquots at 2.66 x 10^6 cells/mL in 15% dimethyl sulfoxide (v/v) (DMSO) in FBS. These were resuspended in 10 mL of media and centrifuged for 2 min at 200 x g. The pellet was resuspended in 10 mL of fresh media and applied in 1 mL aliquots to a 24-well microtiter plate. The hybridomas were incubated at 37°C with 5% CO_2, and when adequate growth was observed, the cultures were split and grown in 75 mL tissue culture flasks. The hybridomas were expanded gradually and split. Upon reaching the desired volume of hybridomas, an aliquot was removed, mixed with 15% DMSO, in FBS and frozen in liquid nitrogen. The remaining

hybridomas were grown until less than 20% viability was observed. Supernatant from the hybridoma culture was collected and the antibody was purified by precipitation with 70% ammonium sulfate and dialyzed for 2 days against phosphate buffered saline (PBS, 0.1 M sodium chloride, 0.01 M sodium phosphate, pH 7.4) at 4°C (Zielinski, 1998). Purified antibody was allocated and frozen at -80°C.

4.3.4. Protein-Hexanal Conjugation for Standard Preparation

The protein-hexanal conjugates were prepared using the procedure described by Smith (1997). Briefly, 0.15 g CSA and BSA was mixed with 8 mL of PBS. A 246 μL aliquot of hexanal was added, and the mixture was diluted to 9 mL. Following vortexing, 1 mL of 800 mM sodium cyanoborohydride dissolved in 0.1 N NaOH was added to reduce Schiff bases. Final reactant concentrations were 15 mg/mL protein, 200 mM hexanal and 80 mM sodium cyanoborohydride. The mixture was held on ice for 60 min with periodic vortexing, followed by overnight dialysis at 4°C with PBS. The degree of modification of the protein was assessed using the trinitrobenzenesulfonic acid assay (TNBS) described in Section 4.3.5. Conjugates were diluted to a concentration of 1 mg/mL with PBS. Protein concentration was determined using the Bradford protein assay (described in Section 4.3.7). The protein solution was allocated and frozen at −20°C.

4.3.4.1 Use of Dialysis during Protein-Hexanal Conjugation

The necessity of a dialysis step was examined in order to minimize assay time. For dialysis studies, 30 mg/mL BSA was added to 9 mL of PBS. Hexanal (246 μL) was added; the reduction reaction was completed by adding 1 mL of 800 mM sodium

cyanoborohydride dissolved in 0.1 N NaOH. Final reactant concentrations were 200 mM hexanal and 80 mM sodium cyanoborohydride. The solution was held on ice for 60 min with periodic vortexing. Portions of the conjugate solutions were dialyzed at 4°C overnight with 2 changes of 2 L of PBS (pH 7.4), while the other half of the conjugate solutions were not dialyzed. Standard solutions of 3 to 69 ng hexanal/mL were prepared from the dialyzed and non-dialyzed samples and analyzed using the CI-ELISA. Standards curves from both treatments were constructed and compared as described in Section 4.3.9.

4.3.5. Trinitrobenzenesulfonic Acid Assay (TNBS)

The degree of modification of the protein was determined by measuring the loss of reactive groups by the TNBS acid assay described by Habeeb (1966) and modified by Smith et al. (1999). In this assay, a 0.25 mL aliquot of protein sample (0.15 mg/mL) was added to 1 mL of 4% $NaHCO_3$, 0.75 mL 0.5 M PBS buffer (pH 7.4) and 1 mL of 0.1 % TNBS (picrylsulfonic acid). The solution was vortexed and incubated at 40°C in a circulating water bath for 2 h. Following incubation, 1 mL of 10% SDS was added to each sample, followed by the addition of 0.5 mL 1N HCl. The solution was vortexed and the absorbance was read at 335 nm. The concentration was determined from a standard curve of leucine solutions ranging from 10 to 300 μm. Results were expressed as moles of reactive amino groups per mole protein.

4.3.6 CI-ELISA Optimization

4.3.6.1 Indirect Competitive ELISA (CI-ELISA)

The indirect competitive ELISA procedure for hexanal was adopted and modified

from Zielinski (1998). Microtiter plates were coated overnight at 4°C with 100 µL of hexanal-chicken serum albumin (CSA-hex) conjugate (1 mg/mL), prepared in 0.1 M carbonate buffer (pH 9.5). Following incubation, plates were washed 4 times with PBS-Tween (0.05% Tween-20), pH 7.2. To minimize non-specific binding, plates were blocked using 300 µL of PBS-casein (0.5% casein, pH 7.2) for 30 min at 37°C. Plates were then washed 4 times with PBS-Tween. Purified monoclonal antibody (50 µL of 1.6 µg protein/mL in PBS, pH 7.2), together with 50 µL of CSA-hex or sample, were added to each well and incubated for 60 min at 37°C. Plates were washed 6 times with PBS-Tween. Secondary antibody, GAM-IgG HRP diluted 1:500 in 0.5% casein in PBS was added to each well at 100 µL. The plates were incubated for 30 min at 37° and then washed 8 times with PBS-Tween prior to substrate addition. Visualization was accomplished using 100 µL of the substrate 3,3',5,5'-Tetramethyl-benzidine (TMB). Following a 10 min incubation at room temperature, the absorbance of each well was measured at 650 nm using a THERMOmax plate reader (Molecular Devices, Menlo Part, CA).

On each plate, a standard curve from 14 to 1000 ng CSA-hex/mL (3 to 69 ng hex/mL) was constructed. From the standard curve and using a 4-parameter curve fit, the concentration of CSA-hex in each unknown sample was determined using SoftMa: software (version 2.34). Using the concentration of CSA-hex (ng/mL) determined from the standard curve and the formula below, the concentration of hexanal (ng hex/mL) in the sample was calculated.

4.3.6.2 Calculation of Hexanal Concentration from the CI-ELISA

Hexanal quantification involved conjugation between the lysine groups on the protein and hexanal. The standard curve was constructed using CSA, thus a calculation was made to convert CSA concentration to hexanal concentration based on the moles of lysine modified per mol of CSA. The following formula was used to convert protein concentration to hexanal concentration:

$$\frac{\mu g\ protein}{mL} \times \frac{46\ mol\ lysine}{1\ mol\ protein} \times \frac{1\ mol\ protein}{66{,}300\ g} \times \frac{1\ gram}{10^6\ mg} \times \frac{10^6\ mmol}{1\ mol} = \frac{\mu mol\ lysine}{mL}$$

The μmol of lysine are multiplied by the percent modification to account for differences in lysine modification during conjugation. Since one mole of lysine will bind one mole of hexanal, the following formula describes the conversion from μmol/mL of hexanal to ng/mL of hexanal:

$$\frac{\mu mol\ hexanal}{mL} \times \frac{100\ g\ (MW\ of\ hexanal)}{1\ mol} \times \frac{1\ mol}{10^6\ mmol} \times \frac{10^9 ng}{1\ g} = \frac{ng\ hexanal}{mL}$$

The mol of lysine in each protein were determined using amino acid sequences from the National Center for Biotechnology and Information (CBI; www.ncbi.nlm.nih.gov). The mole percent of lysine was multiplied by the molecular weight of the protein to obtain the molecular weight contributed by lysine. This was divided by the molecular weight of lysine to obtain the moles of lysine per mole of protein. The molecular weight used for CSA was 66,300 daltons and for BSA, 66,000 daltons. CSA contains 47 lysine groups, while BSA contains 42 lysine groups.

4.3.6.3 Precision of the CI-ELISA

Intra- and inter-assay precision of the CI-ELISA was determined using the method described by Deshpande (1996). Standard solutions of 0, 3.4, 6.7, 34.4 and 68.8, 171.9, 343.8, and 687.5 ng hex/mL were prepared from CSA-hex conjugates, corresponding to 100, 96, 87, 79, 66, 45, 27, 8, 3 and 1 percent of the maximum binding respectively. The intra-assay variability was determined using 8 replicate wells and the inter-assay variability was determined using 8 replicate wells from 13 plates. The average, standard deviation, and coefficient of variation, an indicator of variability, were determined for all replicates and at each concentration.

4.3.7. Bradford Protein Assay

In this assay, 10 µL of diluted sample was added to a 96-well microtiter plate. A 200 µL aliquot of BioRad protein dye was added to each well and mixed. Following 5 min of incubation, wells were read at 590 nm. Protein concentration of the supernatant was calculated using a BSA standard curve, prepared using concentrations from 100 to 1000 µg BSA/mL. Percent soluble protein was calculated by dividing the resulting protein concentration by the starting protein concentration.

4.3.8. Accelerated Oxidation Study

Myofibrils with 0.6 mmol methyl linoleate/g of protein were prepared as described in Section 3.3.1. Freeze-dried myofibrils were separated in 2 subsamples and incubated in the dark in desiccators containing saturated salt solutions. Samples were incubated at 50°C, a temperature typical of military provision storage studies (Sun et al., 2001). Saturated salt solutions used for humidity control were sodium chloride (a_w=0.75)

and magnesium chloride (a_w=0.30) (Labuza, 1984). Duplicate desiccators for each water activity were used. Six beakers (80 mL) each containing 3 g of myofibril were prepared and placed in each desiccator.

Sample beakers were removed and myofibrils analyzed at Day 0, 1, 2, 3, 4, and 5 of storage. Hexanal was determined by GC/MS-SPME, using the procedure described in Section 3.3.2. The same sample was then used for TBARS analysis as outlined in Section 3.3.6.2.

To prepare samples for CI-ELISA, 0.3 g myofibrils were mixed with 0.25 % trypsin (w/w), and 50 ppm Tenox-2 antioxidant in 10 mL of 0.6 M KCl in 0.05 M potassium phosphate buffer, pH 9.0. Sodium cyanoborohydride was added to a final reactant concentration of 80 mM. The solution was incubated for 2 h at 50°C, after which it was filtered through 2 layers of cheesecloth. The trypsin was inactivated by heating the filtrate for 5 min at 90°C. Following cooling on ice, the solution was centrifuged at 20,000 x g for 15 min. Appropriate dilutions (10 or 100 times) of the supernatant were made and the CI-ELISA was conducted.

4.3.9. Data Analysis

In order to assess the effect of dialysis and proteases on the CI-ELISA, comparisons were made to the CSA-hex standard curve prepared with PBS. Values falling within two standard deviations of the mean of the control standard curve (the measurement error) were not considered to be markedly different, indicating that the treatment was not detrimental to the standard curve (Deshpande, 1996). Each treatment level was analyzed in triplicate. The limit of detection of the CI-ELISA was determined

as the lowest concentration of hexanal which was greater than 2x the measurement error of 0 ng hexanal/mL.

For the accelerated oxidation study, data were analyzed using SAS (Version 6.1; SAS Institute, Inc., Cary, NC). A two-way ANOVA was performed, and significance was defined as $p<0.05$. Data were log transformed in order to ensure homogeneity of variance. Pairwise differences were tested using Tukey's adjusted differences test to control the pairwise error. Correlations were made between TBARS, GC/MS-SPME and ELISA.

4.4 RESULTS AND DISCUSSION

4.4.1 CI-ELISA Optimization

4.4.1.1 Limit of Detection and Reproducibility

Determination of hexanal concentration from the CSA-hexanal standard curve required the conversion formula described in the materials and methods section (Section 4.3.6.2). The limit of detection of the CI-ELISA was 1 ng hexanal/mL, supporting the same value published by Zielinski et al. (2001). Compared to the polyclonal antibody limit of detection of 7.4 ng/mL (Smith et al., 1999), the monoclonal antibody was shown to be more sensitive. In general, the limit of detection of ELISAs is low, often in the ng/mL range (Samarajeewa et al., 1991). In ELISAs for both lactate dehydrogenase (Wang et al., 1995) and a staphylococcal enterotoxin (Morissette et al., 1990), the minimum detection limit was 10 ng/mL. The sensitivity of immunoassays for mycotoxins in food is higher, with limits of detection as low as 0.1 ng aflatoxin/mL (Samarajeewa et al., 1991).

The working range of the CI-ELISA, as demonstrated by the percentage binding response, ranged from 3 to 69 ng/mL corresponding to concentrations of 14 to 1000 ng CSA-hexanal/mL (Figure 4.1). This working range was comparable to the working range determined by Zielinski et al. (2001) of 1 to 50 ng hexanal/mL. The working range of the hexanal polyclonal antibody was found to range to 740 ng hexanal/mL (Smith et al., 1999).

The reproducibility of the CI-ELISA within the assay (intra-assay) and between assays (inter-assay) was calculated (Table 4.1). Intra-assay coefficients of variation ranged from 4.2 to 5.8%, while inter-assay coefficients of variation were slightly higher, ranging from 5.0 to 6.2%. The inter-assay precision had slightly higher coefficients of variation than the intra-assay precision, which is common as variables are introduced day to day and run and run (Deshpande, 1996). These coefficients of variation for the hexanal CI-ELISA were similar to those published by other researchers. A previous study using monoclonal-based CI-ELISA for hexanal reported coefficients of variation ranging from 4.2 to 6.4% intra-assay, and 5.1 to 7.7% inter-assay (Zielinski, 1998). In an ELISA of malonaldehyde-modified low density lipoproteins, Swets et al. (2001) reported intra- and inter-assay coeffficients of variation of 3.3 and 10.6% respectively. Similarly, Salomon et al. (2000) reported intra-assay coefficients of variation from 2 to 8%, and an inter-assay coefficient of variation of 5%.

4.4.1.2 Effect of Dialysis

The original protein-hexanal conjugation procedure employed overnight dialysis of the conjugate to remove excess sodium cyanoborohydride (Zielinski, 1998). To

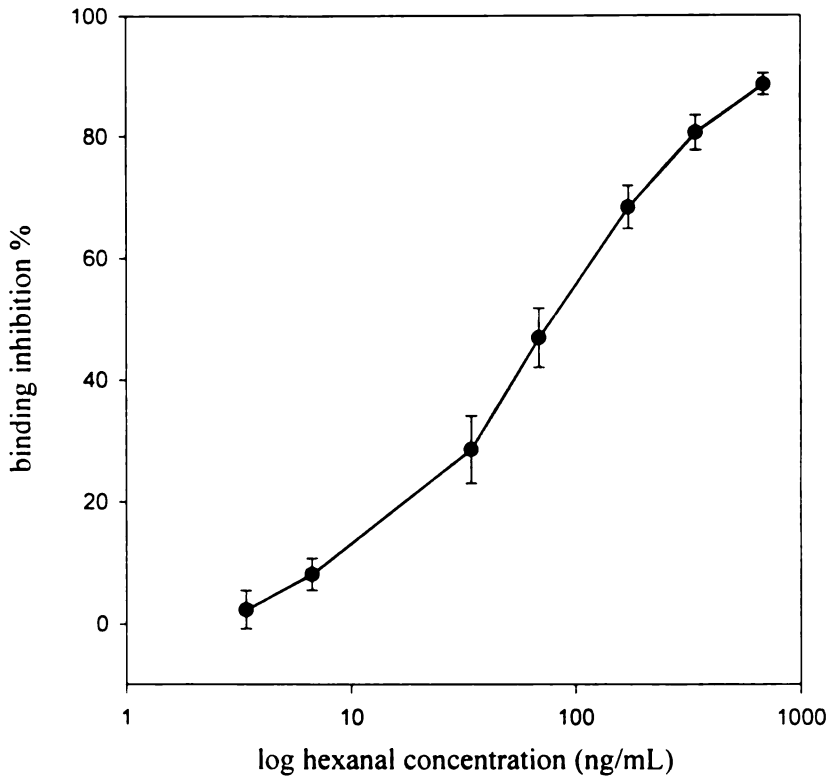

Figure 4.1. The percent binding inhibition produced in the CI-ELISA from concentrations of hexanal. A stock solution of 1mg/mL CSA was prepared, reduced, dialyzed and diluted. Standards between 0 and 690 ng hex/mL were prepared. Each point represents 6 replicates (r^2=0.975).

Table 4.1. Reproducibility of the CI-ELISA as described by the coefficient of variation for intra-assay and inter-assay reproducibility.

Hexanal concentration (ng/mL)	Intra-assay[a] coefficient of variation (%)	Inter-assay[b] coefficient of variation (%)
0	4.2	5.0
50	5.2	6.2
100	4.9	5.1
250	5.8	5.8
500	5.1	5.2
1000	5.5	5.7

[a] - Intra-assay precision was calculated using 8 replicate wells
[b] - Inter-assay precision was calculated using 8 replicate wells from 13 different plates

minimize assay time, the necessity of the dialysis step was evaluated through comparison of standard curves prepared with dialyzed and non-dialyzed BSA (Figure 4.2). No effect of dialysis was seen on the standard curve, indicating that the sodium cyanoborohydride did not interfere with the antibody reaction during the CI-ELISA, either due to the nature of the chemical or the sample dilution prior to analysis. Dialysis was eliminated from the conjugation procedure, considerably decreasing assay time.

4.4.2 Optimization of Extraction Procedure

4.4.2.1 Effect of Extraction Buffer

A major problem facing the application of monoclonal antibodies in food research is the solubilization of the sample (Paraf, 1992). Myofibril solubility is also decreased during storage, as observed by Smith et al.(1990) and Xiong and Brekke (1989), thus the low solubility of the freeze-dried myofibrils was a significant hurdle.

The ionic strength and the pH of the solubilization buffer are important factors in maximizing solubility. Several studies have been conducted varying the characteristics of the solubilization buffer, including employing phosphates (Xiong et al., 2000), urea (Lin and Park, 1998), and low ionic strengths (Stanley et al., 1994). In preliminary experiments, none of these buffers were found to be effective on myofibril solubilization. The traditional method to solubilize myofibrillar proteins is through the use of a buffered 0.6 M KCl solution (Smith et al., 1990; Xiong and Brekke, 1989). The effect of KCl concentration on the antibody was assessed. Standards in different dilutions of 0.6 M KCl, 0.05 M phosphate buffer were prepared (Figure 4.3). Only the standard curve prepared using undiluted 0.6 M KCl in 0.05 M phosphate buffer was found to fall outside

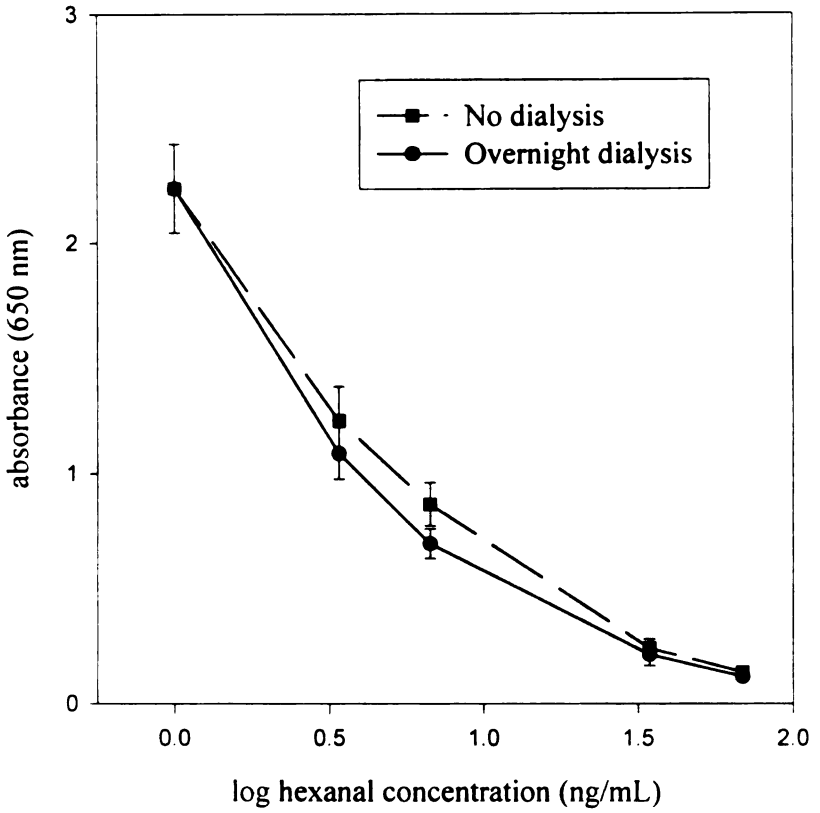

Figure 4.2. Effect of overnight dialysis following conjugation/reduction on the CI-ELISA standard curve. The CSA-hex conjugate (1 mg/mL) was prepared, reduced and dialyzed (control was not dialyzed) and standards from 3 to 69 ng hex/mL were prepared. Each point represents three replicates.

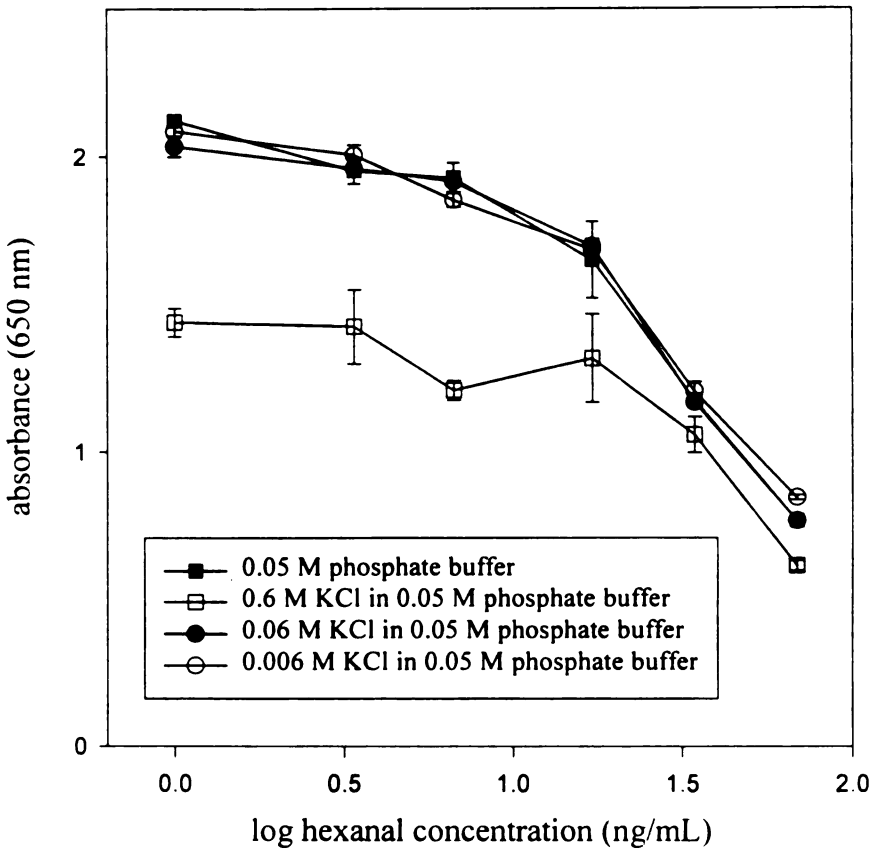

Figure 4.3. Effect of the addition of different concentrations of 0.6 M KCl in 0.05 M potassium phosphate on the CI-ELISA standard curve. From a stock of 1 mg/mL CSA-hex, standards from 3 to 69 ng hex /mL were prepared in different dilutions 0.6 M KCl in 0.05 M potassium phosphate. The control standard curve was prepared in 0.05 M phosphate buffer. Determinations were made in triplicate using the CI-ELISA.

the measurement error of the control standard curve, indicating that the undiluted buffer did have an effect on the standard curve. Dilutions of the buffer above 10 x did not have an effect on the CI-ELISA standard curve. Therefore, the minimum sample dilution for CI-ELISA was considered to be 10x.

4.4.2.2 Effect of Proteases

The use of proteases was evaluated to further increase myofibril solubility. In the first study, papain, fungal and alkaline protease were assessed under their optimum conditions as described by the manufacturer (Table 4.2). Alkaline protease at 0.2 and 1.0% were the most effective concentration, increasing protein solubility by about 4-fold. Alkaline protease has been used in previous studies to increase the solubility of spray-dried lactalbumin (Ennis and Harper, 1986) and cooked chicken thigh (Zielinski, 1998).

Because of its effectiveness at increasing protein solubility, alkaline protease was compared to endoproteinase lys-C in the next study (Table 4.3). Available lysine groups are important during the conjugation reaction as lysine is the amino acid responsible for the formation of Schiff bases (Jentoft and Dearborn, 1979). Endoproteinase lys-C is a lysine specific enzyme. It hydrolyzes peptide bonds on the carboxyl side of lysine, and may leave more available lysine for conjugation (Sorensen et al., 1995). In hexanal spiked chicken myofibrils, endoproteinase lys-C was found to be approximately twice as effective at increasing protein solubility and hexanal recovery than alkaline protease. Both greater protein solubility and hexanal recovery indicated the effectiveness of the protease at protein digestion.

Due to the expense of the endoproteinase lys-C, trypsin was evaluated as an

Table 4.2. The effect of papain, fungal protease and alkaline protease on the solubility of chicken myofibrils. Myofibrils (0.15 g/mL) were mixed with 10 mL of extraction buffer and protease and incubated at 50°C for 2 hours in a shaking water bath. Following straining through cheesecloth, % soluble protein was determined. Determinations were made in triplicate.

Protease	pH	Concentration[a]	% Soluble Protein[b]
Papain	5.5	0	5.8 ± 1.7
	5.5	0.05	5.5 ± 0.39
	5.5	0.15	5.1 ± 0.01
Fungal Protease	7.4	0	5.3 ± 0.89
	7.4	0.05	5.2 ± 1.2
	7.4	0.5	4.9 ± 1.1
Alkaline Protease	9.5	0	3.9 ± 0.9
	9.5	0.2	16.0 ± 1.8
	9.5	1	18.7 ± 2.5

a - Enzyme concentration expressed as % of total protein in chicken myofibrils
b – Theoretical maximum protein recovery was 0.03 g/mL

Table 4.3. Effect of alkaline protease and endoproteinase lys-C on the soluble protein and hexanal detection in chicken myofibrils. Myofibrils (15 mg/mL) were mixed with 10 mL of extraction buffer and protease and incubated in a 50°C circulating water bath for 2 hours. Following straining through cheesecloth, soluble protein was determined. Myofibrils were conjugated to 200 mM hexanal, Schiff bases reduced, and hexanal concentration was determined using the CI-ELISA; the amount of spiked hexanal was compared to the amount of recovered hexanal. Determinations were made in triplicate.

Protease	pH	Concentration[a]	% Soluble Protein[b]	% Hexanal Recovery[c]
alkaline protease	9.5	0	3.6 ± 1.0	11.3 ± 0.05
	9.5	0.05	26.5 ± 5.8	17.7 ± 0.12
	9.5	0.15	14.8 ± 2.1	24.0 ± 0.11
endoproteinase Lys-C	7.7	0	4.5 ± 0.71	13.3 ± 0.52
	7.7	0.03	38.8 ± 4.4	58.6 ± 0.74
	7.7	0.012	35.6 ± 2.2	54.5 ± 0.66
	7.7	0.006	32.4 ± 5.6	63.9 ± 5.6
	7.7	0.003	39.4 ± 3.6	59.9 ± 0.58
	7.7	0.0015	30.6 ± 0.1	53.3 ± 0.89

a – Alkaline protease concentration expressed as weight protease/ g protein in chicken myofibrils. Endoproteinase-Lys-C concentration expressed as dilution of 3 units.
b – Theoretical maximum protein recovery was 0.03 g/mL
c - Theoretical maximum hexanal recovery was 7.51 mg hexanal/mL

alternative protease as it also cleaves at the lysine group (Kunitz and Northrop, 1948). In BSA, the addition of either trypsin or endoproteinase lys-C had little impact on the percentage soluble protein and the percentage recovery of hexanal (Table 4.4). However, in chicken myofibrils, the addition of either trypsin or endoproteinase lys-C increased protein solubility by 9.1-fold and 7.9-fold, respectively. Similarly, hexanal recovery was markedly increased in chicken myofibrils when either enzyme was added during the extraction process. Therefore, trypsin was selected as the protease for protein solubilization.

4.4.3 Optimization of Trypsin Digestion

The effectiveness of trypsin on improving protein solubility and protein-hexanal conjugation was optimized by varying incubation times, pH of extraction buffer (0.6 M KCl in 0.05 M phosphate buffer) and trypsin concentration (Table 4.5). For BSA (data not shown), the degree of protein modification determined using the TNBS assay and percent soluble protein were all greater than 95% for each trypsin optimization parameter tested. Greater percent modification indicated the loss of reactive groups, consequently, the increase in the formation of conjugates. The percent modification of chicken myofibrils at pH 8.0 at varying trypsin concentrations and incubation times was similar to modification at pH 9.0 under the same conditions; at pH 8.0, modification increased from 69.2% to 91.7%, while at pH 9.0, modification increased from 67.5% to 89.3%. Soluble protein showed a greater increase under pH 9.0, increasing from 56% to 96.9% over the range of conditions. At pH 8.0, solubility increased from 55.2% to 85.4% over the parameters tested. The final extraction conditions selected were a 2 h incubation time at

Table 4.4. Effect of trypsin and endoproteinase lys-C on protein solubility and hexanal recovery in spiked chicken myofibrils. Myofibrils (0.6 g) or BSA were mixed with 20 mL of buffer (pH 7.7). Treatments included the addition 0.25% (w/w) trypsin or 0.005% (v/v) Endoproteinase Lys-C. Samples were incubated at 50°C for 2 hours in a shaking water bath, strained through cheesecloth, and soluble protein determined. The conjugation and reduction reactions were performed, and hexanal concentration was determined using the CI-ELISA. Recovery of hexanal was determined by comparing the amount of hexanal used for spiked and the amount determined using the CI-ELISA. Determinations were made in triplicate.

Sample	% Soluble Protein[a]	% Recovery of Hexanal[b]
BSA	91.2 ± 7.2	91.3 ± 5.5
BSA + Lys-C	95.3 ± 2.2	77.5 ± 7.1
BSA + trypsin	94.7 ± 0.6	80.5 ± 6.2
Myofibrils	4.8 ± 1.1	5.7 ± 2.8
Myofibrils + Lys-C	38.7± 0.51	38.4 ± 9.9
Myofibrils + trypsin	43.9 ± 1.3	48.7 ± 10.6

a – Theoretical maximum protein recovery of 0.03 g/mL
b – Theoretical maximum hexanal recovery was 7.51 mg hexanal/mL

Table 4.5. Protein solubility as affected by trypsin concentration (0.05, 0.1 0.25 % w/w), pH level (8.0 and 9.0), and incubation time at 50°C (2, 4, 5.5 hours). Myofibrils (0.15g/mL) were mixed with 10 mL of 0.6 M KCl in 0.05 M phosphate buffer, trypsin, and hexanal, making a final concentration of 200 mM hexanal. Solutions were conjugated, reduced and incubated at 50°C for the appropriate time. Solutions were analyzed for % soluble protein using Bradford Protein Assay, and % modification using the TNBS Assay. Determinations were made in triplicate.

trypsin conc %	incubation time (hrs)	% modification of protein		% soluble protein[a]	
w/w		pH 8.0	pH 9.0	pH 8.0	pH 9.0
0	2	68.2 ± 3.5	66.8 ± 1.4	55.2 ± 2.1	55.2 ± 1.0
0	4	69.2 ± 2.7	66.8 ± 1.7	55.2 ± 1.9	55.4 ± 1.8
0	5.5	70.2 ± 3.1	68.9 ± 1.3	55.2 ± 2.0	57.4 ± 2.1
0.05	2	71.2 ± 1.1	68.6 ± 0.9	55.2 ± 2.3	73.5 ± 1.9
0.05	4	71.4 ± 1.4	69.2 ± 1.2	73.5 ± 2.1	79.7 ± 2.0
0.05	5.5	71.2 ± 2.4	70.5 ± 1.3	75.9 ± 2.4	79.5 ± 1.9
0.1	2	70.2 ± 3.2	71.5 ± 1.2	78.0 ± 1.9	89.6 ± 2.0
0.1	4	72.2 ± 2.9	71.8 ± 1.8	78.0 ± 1.5	89.2 ± 1.4
0.1	5.5	73.2 ± 1.4	83.7 ± 2.1	80.2 ± 1.2	91.9 ± 1.2
0.25	2	87.9 ± 0.9	88.5 ± 1.7	90.0 ± 3.0	99.1 ± 3.1
0.25	4	90.5 ± 0.8	87.2 ± 1.3	85.0 ± 2.8	95.6 ± 1.9
0.25	5.5	96.8 ± 1.0	92.3 ± 1.1	81.2 ± 2.6	96.1 ± 2.2

a - Theoretical maximum protein recovery of 0.03 g/mL

pH 9.0 buffer (0.6 M KCl in 0.05 M phosphate buffer), with a trypsin concentration of 0.25% (w/w). In the enzymatic hydrolysis of shrimp using trypsin, Simpson et al. (1998) listed similar optimal condition; 0.25% trypsin at pH 8.2 for 2.5 to 3 h at 40°C.

Heat was used to inactivate the trypsin prior to the CI-ELISA in order to prevent proteolysis of the antibody. Depending on the purity, trypsin can be either irreversibly or reversibly denatured by temperature or pH (Kunitz and Northrop, 1948). Crude trypsin preparations are completely and permanently inactivated at temperatures above 70°C while solutions of purified trypsin may be heated to boiling for a short time without a permanent loss in activity (Kunitz and Northrop, 1948). In these studies, pure trypsin was used, necessitating a higher temperature of inactivation.

BSA was used for these studies, as BSA is soluble without the aid of trypsin and it simplified the protein-conjugation procedure. The BSA showed that the CI-ELISA was still valid with the addition of trypsin and still recognized the hydrolyzed protein. Heating at 90 and 100° both had a similar effect on the standard curve and were not markedly different from standard curve prepared from the non-trypsin control (Figure 4.4). Low absorbances of the CI-ELISA standard curve indicated the proteolysis of the antibody by the trypsin. A heating time of 90°C for 5 min was selected for the remaining studies in order to minimize the harsh effect of heat on the protein. The optimized procedure for the CI-ELISA is summarized in Figure 4.5.

With Western Blot analysis, Zielinski (1998) showed that the antibody displayed recognition of the hexanal modified salt soluble protein but showed no recognition of the native salt soluble protein. Also, the hexanal modified protein did not migrate as far

Table 4.5. Protein solubility as affected by trypsin concentration (0.05, 0.1 0.25 % w/w), pH level (8.0 and 9.0), and incubation time at 50°C (2, 4, 5.5 hours). Myofibrils (0.15g/mL) were mixed with 10 mL of 0.6 M KCl in 0.05 M phosphate buffer, trypsin, and hexanal, making a final concentration of 200 mM hexanal. Solutions were conjugated, reduced and incubated at 50°C for the appropriate time. Solutions were analyzed for % soluble protein using Bradford Protein Assay, and % modification using the TNBS Assay. Determinations were made in triplicate.

trypsin conc %	incubation time (hrs)	% modification of protein		% soluble protein[a]	
w/w		pH 8.0	pH 9.0	pH 8.0	pH 9.0
0	2	68.2 ± 3.5	66.8 ± 1.4	55.2 ± 2.1	55.2 ± 1.0
0	4	69.2 ± 2.7	66.8 ± 1.7	55.2 ± 1.9	55.4 ± 1.8
0	5.5	70.2 ± 3.1	68.9 ± 1.3	55.2 ± 2.0	57.4 ± 2.1
0.05	2	71.2 ± 1.1	68.6 ± 0.9	55.2 ± 2.3	73.5 ± 1.9
0.05	4	71.4 ± 1.4	69.2 ± 1.2	73.5 ± 2.1	79.7 ± 2.0
0.05	5.5	71.2 ± 2.4	70.5 ± 1.3	75.9 ± 2.4	79.5 ± 1.9
0.1	2	70.2 ± 3.2	71.5 ± 1.2	78.0 ± 1.9	89.6 ± 2.0
0.1	4	72.2 ± 2.9	71.8 ± 1.8	78.0 ± 1.5	89.2 ± 1.4
0.1	5.5	73.2 ± 1.4	83.7 ± 2.1	80.2 ± 1.2	91.9 ± 1.2
0.25	2	87.9 ± 0.9	88.5 ± 1.7	90.0 ± 3.0	99.1 ± 3.1
0.25	4	90.5 ± 0.8	87.2 ± 1.3	85.0 ± 2.8	95.6 ± 1.9
0.25	5.5	96.8 ± 1.0	92.3 ± 1.1	81.2 ± 2.6	96.1 ± 2.2

a - Theoretical maximum protein recovery of 0.03 g/mL

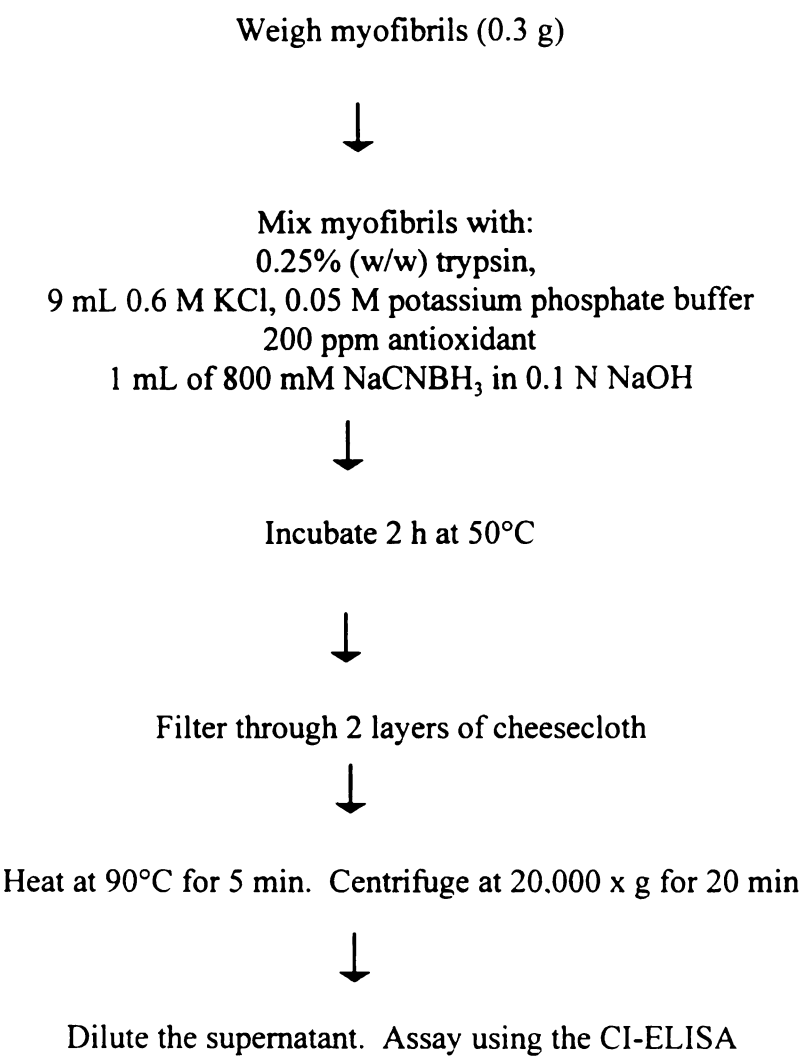

Figure 4.5. Flow chart of the optimized CI-ELISA method

through the gel as the unmodified protein, consistent with the higher molecular weight of the modified protein due to the addition of hexanal.

4.4.4 Accelerated Oxidation Study

Hexanal, measured by GC/MS-SPME and CI-ELISA, and TBARS increased in the chicken myofibrils during the 5 days of storage at 50°C. Over the 5 day study, lipid oxidation occurred in myofibrils stored at a_w 0.30 (Figure 4.6) and myofibrils stored at a_w 0.75 (Figure 4.7). For both a_w, changes in hexanal production between each preceding sample day were detected by the CI-ELISA. The GC/MS-SPME method detected significant differences in hexanal production between all sample days at both a_w except at day 3. TBARS did not differ from the preceding day at day 1 and 5 for either a_w ($p<0.05$).

The a_w of storage was also a factor in the progression of oxidation. For both water activities and all methods, lipid oxidation proceeded slowly initially, reaching a maximum at day 4 of storage. Both CI-ELISA and TBARS showed differences in hexanal and malonaldehyde production, respectively, between the two water activities from day 2 to 5 ($p<0.05$). GC/MS-SPME data demonstrated differences in hexanal production between water activities at day 2, 3 and 4 ($p<0.05$).

The three methods were highly correlated (Table 4.6). CI-ELISA and GC/MS-SPME had an overall correlation (r) of 0.78, while CI-ELISA and TBARS had an overall correlation of 0.87. The CI-ELISA and GC/MS-SPME were more strongly correlated in myofibrils stored at 0.30 a_w than those stored at 0.75 a_w. The decreased correlation may be attributed to lower solubility of the myofibrils stored at 0.75 a_w. At a_w 0.30, the CI-ELISA and GC/MS-SPME had a correlation of 0.95, while at a_w 0.75, the correlation was

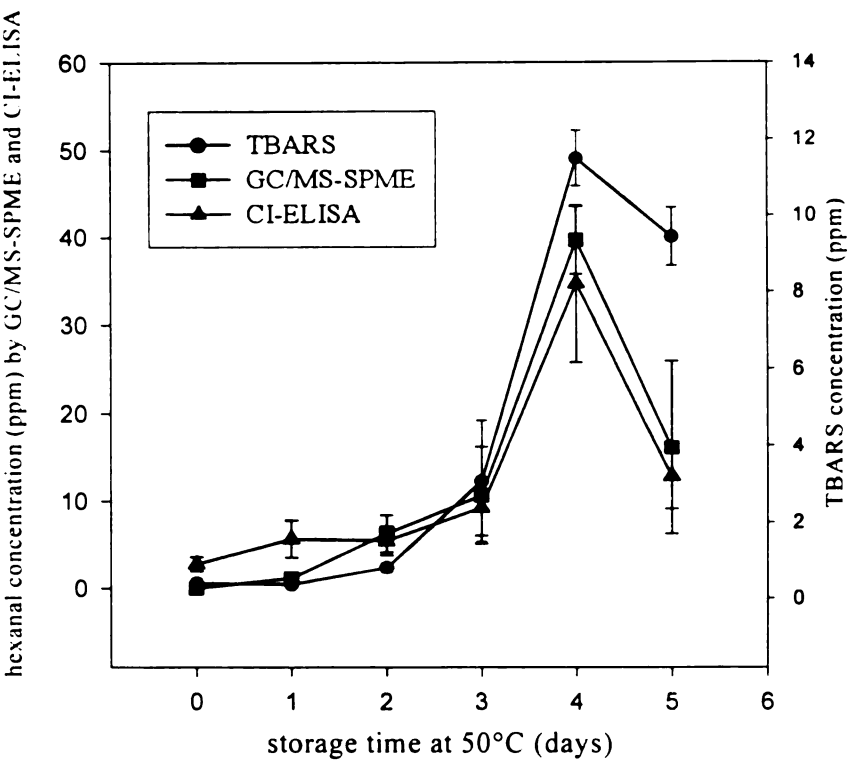

Figure 4.6. Comparison of the hexanal concentration determined by GC/MS-SPME and CI-ELISA, and TBARS of chicken myofibrils stored for 5 days at 50°C. GC/MS-SPME quantified headspace hexanal concentration and CI-ELISA quantified hexanal content within the myofibrils. Chicken myofibrils contained 0.6 umol methyl linoleate/g protein and were stored at a_w of 0.30. Determinations were made in triplicate.

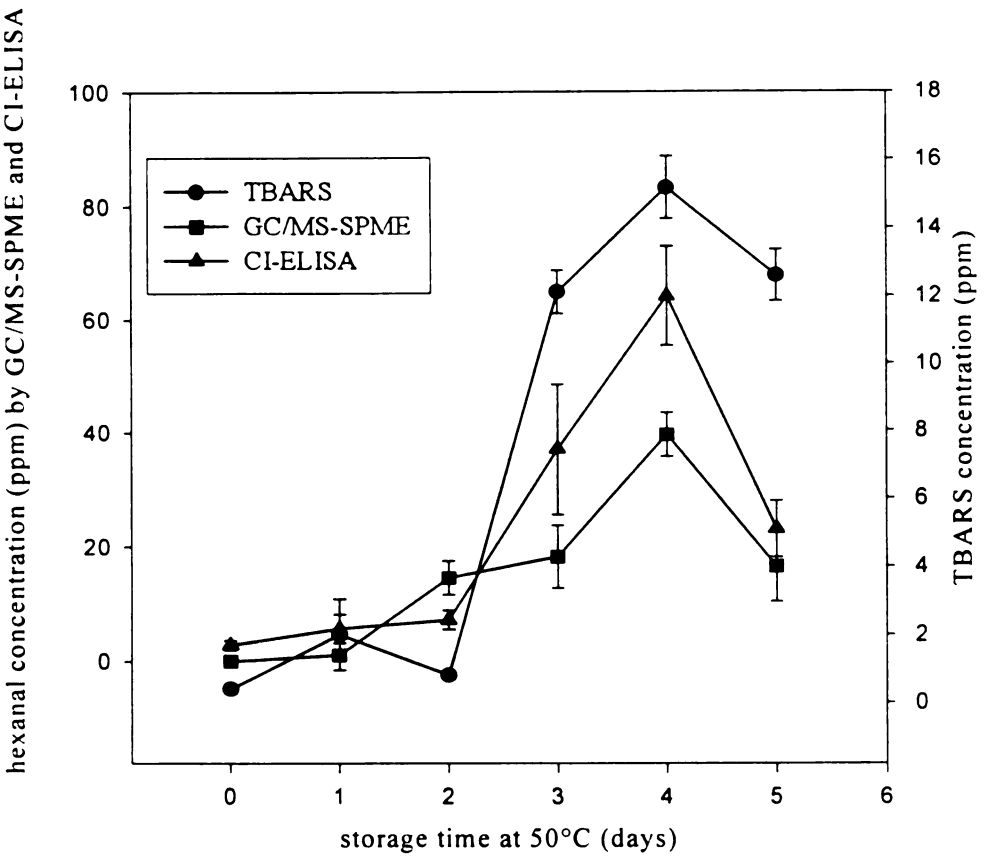

Figure 4.7. Comparison of the hexanal concentration determined by GC/MS-SPME and CI-ELISA, and TBARS of chicken myofibrils stored for 5 days at 50°C. GC/MS-SPME quantified headspace hexanal concentration and CI-ELISA quantified hexanal content within the myofibrils. Chicken myofibrils contained 0.6 umol methyl linoleate/g proteinand were stored at a_w of 0.75. Determinations were made in triplicate.

Table 4.6. Correlation coefficient (r) matrix between methods used to measure the extent of lipid oxidation in freeze-dried chicken myofibrils over 5 days of storage at 50°C at 2 water activities (a_w 0.30 and 0.75). Methods used were GC/MS-SPME and CI-ELISA to measure hexanal and TBARS.

Method	GC/MS-SPME			CI-ELISA		
	overall	**a_w=0.30**	**a_w=0.75**	**overall**	**a_w=0.30**	**a_w=0.75**
CI-ELISA	0.775[a]	0.947[a]	0.730[a]		- - -	
TBARS	0.838[a]	0.937[a]	0.836[a]	0.865[a]	0.848[a]	0.861[a]

[a]Significant at $p<0.05$

0.73. These correlations were similar to those reported by Zielinski et al. (2001) in the quantification of hexanal in a chicken thigh homogenate system using a hexanal CI-ELISA. These researchers reported a correlation (r) of 0.81 between a dynamic headspace GC method and the monoclonal CI-ELISA, 0.97 between hexanal determined using a dynamic headspace GC method and TBARS, and 0.77 between TBARS and the CI-ELISA.

While several other researchers made reference to the TBARS method in the development of their aldehyde ELISA (Khan et al., 2001; Kondo et al., 2001), no other studies were found in which correlations between the two methods were determined. For other substances, studies have reported correlations between GC and ELISA data. To verify an ELISA developed for the detection of 13-hydroxyoctadecadienoic acid, Spindler et al. (1997) compared ELISA and GC results, concluding that the two methods were comparable. One of the few studies to report correlations between GC-SPME and immunoassays identified metolachlor in water samples (Gaynor et al., 1996). A correlation (r^2) of 0.80 was reported between GC-SPME and an ELISA technique.

The CI-ELISA and the GC/MS-SPME displayed a high correlation quantifying different forms of hexanal. While both methods quantify hexanal, the GC/MS-SPME method quantifies only volatile hexanal found in the headspace, while the CI-ELISA detects chemically and physically bound hexanal (Jennings, 1977). Hexanal binding has been studied in a number of protein systems, including soy protein in which 37 to 44% of hexanal was found to be reversibly bound, while less than 5 % was irreversibly bound (Gremli, 1974). Myosin was reported to bind less than 10% of the hexanal while in actin,

an average of 25 to 30% of the added hexanal bound to the protein (Gutheil and Bailey, 1992). This hexanal-protein binding may complicate quantification by the GC/MS-SPME, which measures only volatile hexanal, neglecting the hexanal physically or chemically bound in the sample.

Preliminary studies (data not shown) showed that the addition of excess CSA to the hexanal-myofibril system did not result in additional hexanal detection by the CI-ELISA, indicating enough binding sites on the myofibrils were available for free hexanal. The number of lysine groups in myosin and actin, both components of chicken myofibrils, were 209 and 285, respectively (NCBI; www.ncbi.nlm.nih.gov). Due to the large number of available lysine groups in the myofibrils, an adequate number of binding sites was available for conjugate formation.

4.5 CONCLUSION

Lipid oxidation is a major determinant in the consumer acceptance of food due to the off-odor and flavors that are generated during the oxidative process. The current methods used to quantify lipid oxidation, including those used to quantify hexanal, have many limitations including low sensitivity and labor intensiveness. The use of a CI-ELISA for the quantification of hexanal as an indicator of lipid oxidation would provide a fast and easy-to-use alternative to the traditional methods. This study described the use of a CI-ELISA for the detection of hexanal as an indicator of lipid oxidation in freeze-dried chicken myofibrils. The CI-ELISA assay was optimized, as was the trypsin solubilization of the myofibrils. Over long-term 50°C storage of chicken myofibrils at two water activities, the CI-ELISA was found to correlate well with the GC/MS-SPME method, and

appeared to be more sensitive than the TBARS method. In addition the advantages outlined above, the CI-ELISA also offers convenience for use in a kit, which can easily be utilized within a food processing industry.

CHAPTER 5

CONCLUSIONS

1. We successfully developed a rapid and sensitive GC/MS-SPME method for the detection of hexanal as an indicator of lipid oxidation. By using a short sampling time and a low sampling temperature, we were able to minimize hexanal production during sampling. We also achieved high recovery of hexanal from spiked myofibrils. In an accelerated oxidation study, the GC/MS-SPME detected differences in hexanal between myofibrils stored at two water activities at 50°C over 50 hours; this method also had a strong correlation with TBARS.

2. We optimized protein extraction and hexanal conjugate formation in chicken myofibrils. Through the use of trypsin, a lysine specific enzyme, we were able to solubilize the protein and expose the lysine for hexanal conjugation. As trypsin remained active during the CI-ELISA, trypsin inactivation via heating was required.

3. We validated the use of the monoclonal CI-ELISA for the detection of hexanal during lipid oxidation. The CI-ELISA was able to detect differences in hexanal concentration in chicken myofibrils stored at two

water activities over a 5-day storage period at 50°. The CI-ELISA also correlated well with both GC/MS-SPME and TBARS. The CI-ELISA proved to be a viable alternative for industry to detect lipid oxidation, and has potential for use in a commercial kit.

CHAPTER 6

FUTURE RESEARCH

The use of the CI-ELISA showed promise for hexanal detection in chicken myofibrils. Because the hexanal CI-ELISA is a novel method, no sensory studies have yet been conducted relating CI-ELISA to sensory data. To support the use of the CI-ELISA as a viable indicator of lipid oxidation, sensory evaluation needs to be included in future studies (Gray and Monahan, 1992).

To continue pursuing application of the CI-ELISA in the meat industry, the next stage of the project is to study a commercial meat product. Appropriate extraction and conjugation procedures need to be developed and optimized for different products, including whole muscle and comminuted meat, fresh and frozen products. In order to provide a viable alternative for industry, hexanal detection using the CI-ELISA needs to be completed in one working day (Fukal, 1991). To meet this criteria, various aspects of the assay could be examined, including the length of incubation time of protein with trypsin, and CI-ELISA incubation times. Alternate methods of trypsin inactivation could also be examined, including the use of a water-soluble trypsin inhibitor such as

In order to maximize its usefulness to the industry, the CI-ELISA would need to be adopted for commercial ELISA use in a kit. Before the CI-ELISA can be modified for kit development, a number of questions need to be addressed (Pestka, 1988):

1. What are the limits of detection and sensitivity range of the ELISA? Are

these values consistent with the needs of the user?

2. Are practical sampling protocols described in the assay kit?
3. Are the protein extraction and protein-hexanal conjugate procedures maximized?
4. How may samples can be processed in a given period of time?
5. How stable are the assay components, specifically, the primary and secondary antibody?

Quality control guidelines would also need to be established. The odor threshold of hexanal in lean ground beef is reported to range between 2.4 and 36.8 ppm (Brewer and Vega, 1995). Using these values as a reference, the acceptable limit of hexanal in a given product would need to be determined by the company.

With increased numbers of test kits becoming available on the market, the establishment of guidelines for ELISA kits has become necessary. The Official Methods Board Task Force on Test Kits and Proprietary Methods has described certain requirements pertaining to test kits: the need to include appropriate controls to the users, need to consider false positive/negative results, sensitivity and specificity in relation to intended use and the necessity to have a specific approach (Samarajeewa et al., 1991).

The number of commercially available ELISA kits available on the market continues to increase (Swaminathan and Feng, 1994). Currently, commercial ELISA kits are available for the detection of malonaldehyde and nonenal, although the use of these kits is targeted to the medical profession. An ELISA kit for the detection of lipid oxidation would be beneficial to the food industry.

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