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ELISA for Monitoring Lipid Oxidation in Chicken Myofibrils through Quantification of Hexanal−Protein Adducts

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The objectives of this study were to optimize a monoclonal competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) for hexanal detection, optimize solubilization and alkylation procedures for the formation of hexanal-protein adducts, and compare the ability the 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. Freeze-dried myofibrils with added methyl linoleate (0.6 mmol/g of protein) were stored at 50 °C at two water activities (a_w = 0.30 and 0.75) for 5 days. Hexanal was measured by GC/MS-SPME and CI-ELISA, and malonaldehyde by TBARS. At an a_w of 0.30, 34.7 and 39.7 μg of hexanal/g of myofibril were detected by GC/MS-SPME and CI-ELISA, respectively, after 4 days of storage. At an a_w of 0.75, 39.8 and 61.1 μg of hexanal/g of myofibril 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.

KEYWORDS: Lipid oxidation; hexanal; immunoassay; chicken

INTRODUCTION

Lipid oxidation is a major quality deterioration problem in all muscle foods, affecting both the toxicity and the organoleptic quality of the food. Changes in organoleptic quality are manifested by adverse changes in color, texture, nutrient value, and flavor. The production of off-flavors and odors was 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 (*1*, *2*). The genotoxicity and cytotoxicity of the secondary oxidation products malonaldehyde and 4-hydroxy-nonenal have been established through several studies (*3*, *4*). Research has also linked the formation of these secondary products to disease states such as atherosclerosis (*5*), autoimmunity (*6*), Alzheimer's disease (*7*), retinal disease (*8*), and cancer (*9*).

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 (*10*). Chemical methods attempt to improve upon these limitations. The most commonly used method for measuring lipid oxidation is the 2-thiobarbituric acid reactive substances assay (TBARS) (*11*). Although the TBARS assay is a relatively rapid and simple procedure and has been highly correlated to sensory scores (*12*), it has been criticized primarily for its lack of specificity as products other than malonaldehyde may react with thiobarbituric acid (TBA) (*13*).

Hexanal is a dominant oxidation product of linoleic acid and has been successfully used to follow lipid oxidation in a number of products. Due to its low odor threshold, hexanal is also a major contributor to rancid odors and flavors associated with oxidized meat (*14*). Strong linear relationships were reported between hexanal content, sensory scores, and TBARS in cooked pork (*15*), ground chicken (*16*), and beef (*17*). Hexanal has been quantified using a number of methods. Conventional hexanal analysis techniques such as static headspace gas chromatography (GC) lack sensitivity, whereas more sensitive dynamic methods are tedious and difficult to perform (*18*, *19*). In addition,

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headspace chromatographic methods require the volatization of hexanal for quantification. In food systems, hexanal volatization may be hindered due to covalent binding between hexanal and proteins in the food and thus may affect accurate hexanal quantification (*20*−*22*).

Recently, both polyclonal (*23*) and monoclonal (*16*) antibodies have been used in a competitive indirect-ELISA (CI-ELISA) to detect hexanal. These antibodies were produced to hexanal−protein conjugates prepared by reacting hexanal via Schiff base reactions with the lysine side chains (*23*). Hexanal conjugates to different proteins were recognized by the antibodies, whereas free hexanal was not recognized. In addition to hexanal, some antibody cross-reactivity was observed with pentanal and heptanal conjugates, but these compounds are also lipid oxidation products. Both polyclonal and monoclonal antibody-based CI-ELISAs were sensitive, but the latter displayed higher specificity for hexanal, with a limit of detection of 1 ng of hexanal/mL (*16*). These initial studies suggested that hexanal-specific monoclonal antibodies could be used to detect hexanal in food systems.

The advantages of a CI-ELISA over conventional techniques have been well described in the literature as rapidity, reproducibility, and high sensitivity (*24*). 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. The main challenge facing the application of the CI-ELISA is solubilization of the sample prior to analysis (*25*). Often it is necessary to extract and purify the protein of interest from the sample, and this must done without the denaturation of the protein.

The primary goal of this study was to verify the use of a monoclonal CI-ELISA for hexanal to monitor lipid oxidation in freeze-dried chicken. Freeze-dried chicken myofibrils were selected as a lipid-free system in order to model the quantification of hexanal during lipid oxidation. To induce lipid oxidation, myofibrils were spiked with methyl linoleate (ML) at a ratio of 1:5.0, equal to the ratio of fat to protein typical in chicken meat (*26*). The specific objectives were to (1) optimize the monoclonal CI-ELISA for hexanal detection in freeze-dried chicken, (2) optimize solubilization and alkylation procedures for the formation of hexanal−protein adducts in freeze-dried chicken, and (3) conduct an accelerated oxidation study using a freeze-dried chicken protein model system to correlate results from CI-ELISA, gas chromatography−mass spectrometry−solid-phase microextraction (GC/MS-SPME) method for hexanal, and a TBARS assay.

MATERIALS AND METHODS

Materials. 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′-tetramethylbenzidine (TMB; T 8665) was purchased from Sigma Chemical Co. (St. Louis, MO). Bio-Rad protein dye was from Bio-Rad Laboratories (Hercules, CA). Tenox-2 antioxidant was obtained from East Chemical Co. (Roebuck, SC), and antifoam was purchased from Arthur H. Thomas Co. (Philadelphia, PA). Hexanal, chicken serum albumin (CSA), bovine serum albumin (BSA), picrylsulfonic acid, trypsin (1000−1500 BAEE units/mg of solid), ML, tetramethoxypropane (TEP), squalene, and all other materials were purchased from Sigma Chemical. The monoclonal antibodies to hexanal−CSA were the same as those described by Zielinski et al. (*20*). All other chemicals were of reagent grade or better.

Preparation of Chicken Myofibrils. Chicken breast was obtained from a local supermarket, ground in a Hobart Kitchen-Aid food grinder with a 4.5 mm plate (model KF-A, Troy, OH), and isolated in 0.05 M potassium phosphate buffer (0.1 M NaCl, pH 7.0) (*27*). The myofibrils were washed twice in 10 volumes of distilled water and centrifuged at 6000*g* to precipitate the protein. Protein content in the final pellet was determined using the Bradford protein assay (*28*). If required for an experiment, ML was stirred in at 0.6 mmol/g of protein. 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.

Conjugate Preparation. The protein−hexanal conjugates used in the CI-ELISA were prepared by reductive alkylation (*23*). Briefly, 0.15 g of CSA or BSA was mixed with 8 mL of 0.1 M NaCl and 0.01 M sodium phosphate buffer (PBS), pH 7.4. Hexanal (246 μL) was added, and the mixture was diluted to 9 mL. One milliliter of 800 mM $NaCNBH_3$ 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 $NaCNBH_3$. Mixtures were held on ice for 60 min, followed by overnight dialysis at 4 °C with PBS (pH 7.2). Conjugates were diluted to 1 mg/mL with PBS and frozen at −20 °C.

Protein modification was determined by measuring the loss of reactive amino groups after conjugation by the TNBS acid assay (*23*) using a leucine standard curve. Results were expressed as moles of reactive amino groups per mole of protein, and modification was expressed as the percentage decrease in TNBS-reactive amino groups based on the reactive amino groups in the unmodified protein.

Optimization of Extraction Procedures To Solubilize Myofibrils. Extraction parameters were varied to find conditions to maximize protein solubility. Extraction parameters were pH (8.0 and 9.0), incubation time (2, 4, and 5.5 h), and trypsin concentration (0.05, 0.1, and 0.25% [w/w]). Specifically, myofibrils or BSA (0.15 g) were mixed with trypsin and brought to 9 mL using 0.6 M KCl and 0.05 M potassium phosphate extraction buffer. Alkylation was performed as described above, and the solution was incubated at 50 °C in a shaking water bath for the appropriate incubation time. The solution was filtered through two layers of cheesecloth. Protein modification was assessed using the TNBS assay (*23*). Protein in the filtrate was measured using the Bradford protein assay. Percent protein solubility was calculated using the original protein concentration and the protein concentration following treatment with trypsin.

Experiments were performed to determine the heating conditions necessary to inactivate trypsin. BSA−hexanal conjugates were prepared as described above using 0.25% trypsin (w/w) in 0.6 M KCl and 0.05 M phosphate extraction buffer, pH 9.0, and incubated at 50 °C in a shaking water bath for 2 h. Solutions were heated in a water bath at 80, 90, or 100 °C for 5 min, with heating times starting upon immersion of the tubes in the water. The solution was centrifuged for 15 min at 20000*g* and the supernatant used in the CI-ELISA. Concentration curves constructed using BSA−hexanal conjugates subjected to each treatment (100, 90, and 80 °C and unheated) were compared to a standard curve prepared from BSA−hexanal with no added trypsin. Curves falling within 2 standard deviations of the standard curve were considered to be acceptable (*29*).

CI-ELISA. The CI-ELISA procedure for hexanal was modified from that of Zielinski (*30*). Microtiter plates were coated overnight at 4 °C with 100 μL of hexanal−chicken serum albumin (CSA−hex) conjugate (1 μg/mL), prepared in 0.1 M carbonate buffer, pH 9.5. Following incubation, plates were washed four times with PBS, pH 7.2, containing 0.05% Tween 20 (PBS-T). To minimize nonspecific binding, plates were blocked using 300 μL of PBS, pH 7.2, containing 0.5% casein (PBS-casein) for 30 min at 37 °C. Plates were then washed four times with PBS-T. Purified monoclonal antibody (50 μL of 1.6 μg of protein/mL in PBS, pH 7.2), together with 50 μL of CSA−hex or sample, was added to each well and incubated for 60 min at 37 °C. Plates were washed six times with PBS-T, and 100 μL of GAM−IgG HRP diluted 1:500 in PBS−casein was added to each well. The plates were incubated for 30 min at 37 °C and then washed eight times with PBS-T. Visualization was accomplished using 100 μL of TMB. Following a 10 min incubation at ambient temperature, the absorbance of each well

was measured at 650 nm using a THERMOmax plate reader (Molecular Devices, Menlo Park, CA).

A standard curve was prepared using CSA−hex at 0, 50, 100, 250, 500, and 1000 ng/mL. Hexanal concentration (ng/mL) was calculated from the moles of modified amino groups in CSA−hex conjugates (*30*). The concentration of hexanal was plotted against binding inhibition. Results of the CI-ELISA were expressed as micrograms of hexanal per gram of myofibril. The intra-assay variability was determined using 8 replicate wells, and the inter-assay variability was determined using 8 replicate wells from 13 different plates (*29*).

Accelerated Lipid Oxidation Study. Freeze-dried myofibrils containing ML were stored at 50 °C in the dark in desiccators at water activities of 0.75 and 0.30. Saturated salt solutions used for humidity control were sodium chloride ($a_w = 0.75$) and magnesium chloride ($a_w = 0.30$) (*31*). Duplicate desiccators were used for each water activity. Six 80-mL beakers, each containing 3 g of myofibrils−ML, were placed in each desiccator. At days 0, 1, 2, 3, 4, and 5 of storage, beakers were removed; myofibrils were analyzed for TBARS, and hexanal was analyzed by GC/MS-SPME and CI-ELISA.

To prepare for CI-ELISA, 0.3 g of myofibrils and 75.0 mg of trypsin (0.25% w/w) were mixed with 10 mL of 0.6 M KCl and 0.05 M potassium phosphate buffer, pH 9.0, containing 50 ppm of Tenox-2 antioxidant. Sodium cyanoborohydride was added to a final reactant concentration of 80 mM, and the reactants were incubated for 2 h at 50 °C. Trypsin was inactivated by heating the filtrate for 5 min at 90 °C. Following cooling on ice, the solution was centrifuged at 20000*g* for 15 min. Appropriate dilutions of the supernatant were made, and the CI-ELISA was performed.

GC/MS-SPME Procedure. An SPME fiber assembly (57310-U, Supelco Co., Bellefonte, PA) coated with 100 μm poly(dimethylsiloxane) (PDMS) was inserted into a fiber holder (57330-U, Supelco) and preconditioned at 250 °C for 1 h prior to initial use. The fiber was inserted through a Mininert valve (33301, Supelco) into the headspace of a screw-top valve (27342 Supelco) containing 0.5 g of myofibrils at ambient temperature. Sampling time was 5 min at ambient temperature (23 °C), which was sufficient to permit the establishment of near equilibrium for hexanal (data not shown). Following sampling, the fiber was retracted and 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 × 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. The GC/MS-SPME procedure described by Goodridge et al. (*32*) was used. Specifically, the first 20 cm of the column was cooled with liquid nitrogen to cryofocus the volatiles. The oven temperature was increased from 40 to 250 °C at a rate of 60 °C/min and held for 3 min. The GC/MS-SPME transfer line was 200 °C, the inlet temperature was 250 °C, and the injection temperature was 250 °C. Volatiles were detected using time-of-flight mass spectrometry 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/s over the mass range of *m*/*z* 33−250. Hexanal was identified by comparison of retention times and mass spectra to authenticated hexanal and by comparison of the mass spectra to that of hexanal in the National Institute for Standards and Technology Mass Spectral Library (search version 1.5). Hexanal data were analyzed using LECO deconvolution software (Pegasus version 1.33). Total analysis time was ∼12 min/sample.

The concentration of hexanal in myofibrils was determined on the basis of a hexanal vapor standard and expressed as micrograms of hexanal per gram ofmyofibril. The hexanal vapor standard was prepared by diluting liquid hexanal (Sigma) in squalene (Sigma) to a concentration of 83 mg/L. An aliquot of the diluted hexanal 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 mg of hexanal/L. The hexanal vapor standard was analyzed by GC/MS-SPME as above, and the peak area of the standard was determined.

TBARS. The TBARS procedure of Tarladgis et al. (*11*) was followed except that a smaller sample size was used (0.5 g) and myofibrils were homogenized in distilled water containing 100 ppm of Tenox 2 antioxidant. The distillate was reacted with 0.02 M TBA, and the absorbance of the solution was read at 532 nm. A malonaldehyde standard curve was prepared using (0−9) × 10^{-6} M tetramethoxypropane (TEP), and total recovery was determined through the addition of each TEP standard to 0.5 g of chicken myofibrils. The TBARS number was expressed as milligrams of malonaldehyde per kilogram of myofibril. Each sample was tested in triplicate.

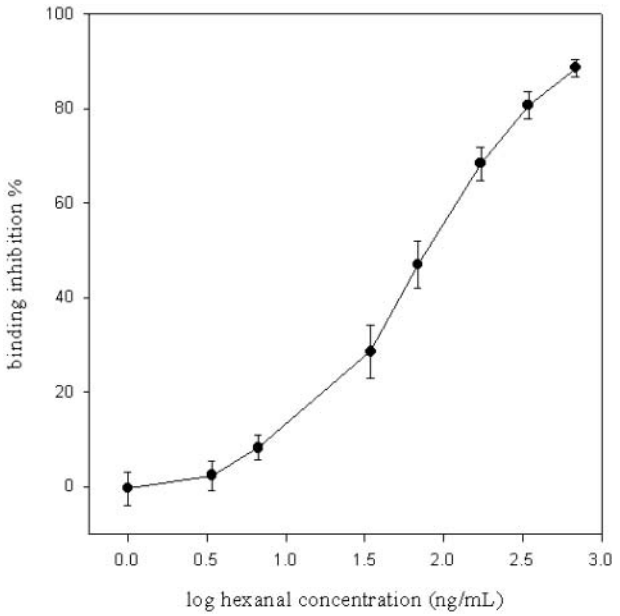

Figure 1. Standard curve of hexanal-modified chicken serum albumin in a CI-ELISA. CSA–hexanal standards containing between 0 and 690 ng of hex/mL were prepared. Each point represents six replicates ($r^2 = 0.975$). Error bars represent standard error of the mean (SEM).

Statistical Analysis. For the optimization of myofibril solubilization, one-way analysis of variance was used to compare multiple groups, with the dependent variable of protein solubility or protein modification, and incubation time and trypsin concentration as factors. Significant effects among individual means were subsequently separated using post hoc tests for multiple comparison (Student−Neuman−Keuls method) with significance defined as $p < 0.05$. 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 $p < 0.05$. The main factors were water activity and storage time. 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.

RESULTS AND DISCUSSION

CI-ELISA Limit of Detection and Reproducibility. The limit of detection of the CI-ELISA was 1 ng of hexanal/mL. The working range of the CI-ELISA, as demonstrated by the percentage binding response, ranged from 3 to 69 ng of hexanal/mL corresponding to concentrations of 14−1000 ng of CSA−hexanal/mL (**Figure 1**). This working range was comparable to the working range determined by Zielinski et al. (*20*) of 1−50 ng of hexanal/mL.

The reproducibility of the CI-ELISA was evaluated. Intra-assay coefficients of variation ranged from 4.2 to 5.8%, whereas inter-assay coefficients of variation ranged from 5.0 to 6.2% (**Table 1**). The inter-assay precision had slightly higher coefficients of variation (CV) than the intra-assay precision, which is common as variation is introduced by day and run (*29*). Zielinski et al. (*16*) reported a similar precision in the CI-ELISA for hexanal.

Table 1. Reproducibility of the CI-ELISA As Described by the Coefficient of Variation (CV) for Intra-assay and Inter-assay Reproducibility

hexanal concn (ng/mL)	intra-assay[a] CV (%)	inter-assay[b] CV (%)
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.

Table 2. Trypsin Concentration, pH, and Incubation Time at 50 °C Influenced Percent Solubility and Protein Modification during Solubilization and Alkylation of Chicken Myofibrils in Preparation for the CI-ELISA[a]

trypsin concn (%) (w/w)	incubation time (h)	modification of protein (%)		soluble protein (%)	
		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	85.5 ± 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] Values are expressed as mean ± standard error of the mean.

Optimization of Myofibril Solubilization and Alkylation Procedures. A major challenge facing the application of CI-ELISAs in food research is the solubilization of the sample prior to analysis (*25*). Solubility of the freeze-dried myofibrils is low and decreases during storage (*33, 34*). Trypsin, a lysine-specific protease, was evaluated for its ability to improve myofibril solubility and the efficiency of the conjugation reaction. The myofibril solubilization and alkylation procedure was optimized by varying the incubation time, pH of extraction buffer, and trypsin concentration. The formation of myofibril−hexanal conjugates was indicated by the loss of reactive amino groups expressed as an increase in percent modification. Protein modification ranged from 66.8.2 to 70.2% without trypsin (**Table 2**) and increased with increasing trypsin concentration. For both pH levels, incubation time was not found to increase modification of protein or soluble protein percent ($p < 0.05$). Trypsin concentration was found to significantly affect both modification of protein and soluble protein percent ($p < 0.05$), with the greatest increase in both factors seen with the addition of 0.25% trypsin. At pH 8.0, percent modification increased from 68.2% without trypsin to 87.9% with 0.25% trypsin using a 2 h incubation period. At pH 9.0 with a 2 h incubation period, protein modification increased from 66.8% without trypsin to 88.5% with 0.25% trypsin.

Freeze-dried myofibrils were ~55.0% soluble in 0.6 M KCl and 0.05 M or potassium phosphate extraction buffer, pH 8.0 or 9.0, prior to trypsin treatment. The percent soluble protein increased as the trypsin concentration increased and reached maxima of 90.0 and 99.1% solubility at pH 8.0 and 9.0, respectively, using 0.25% (w/w) trypsin and a 2 h incubation period. Similarly, Simpson et al. (*35*) found that 0.25% trypsin at pH 8.2 for 2.5−3 h at 40 °C was optimal for the enzymatic hydrolysis of shrimp protein.

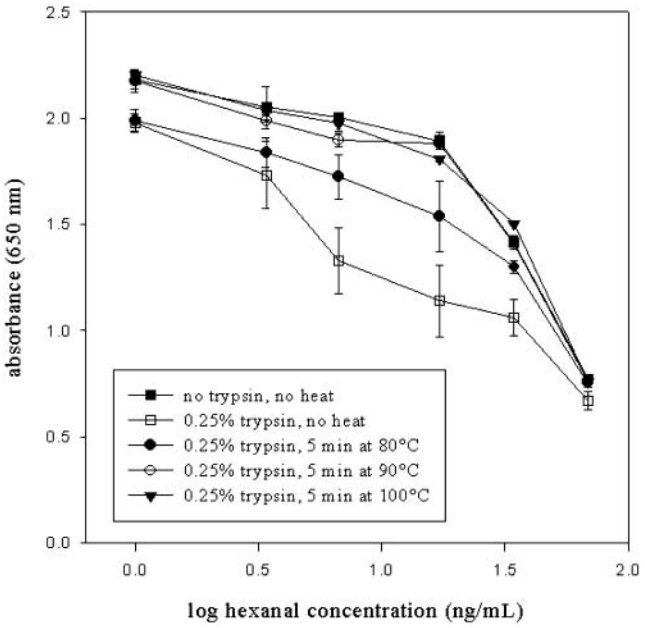

Figure 2. Standard curve of hexanal-modified bovine serum in the CI-ELISA. Hexanal-modified BSA (0.3 g) was incubated with or without 0.25% trypsin at 50 °C for 2 h and then heated at 80, 90, or 100 °C to inactivate trypsin prior to performance of the CI-ELISA. Determinations were made in triplicate. Error bars indicate standard error of the mean.

BSA was used for heat inactivation studies as it is soluble without the aid of trypsin and simplified the alkylation procedure. The concentration curves prepared from BSA−hexanal conjugates heated to 90 and 100 °C to inactivate trypsin were similar to the standard curve prepared from the same conjugates but without trypsin (**Figure 2**), indicating the antibody recognized the hydrolyzed protein conjugates. The absorbances of the curves prepared from the BSA−hexanal conjugates heated to 80 °C were lower than that of the control BSA−hexanal curve. These low absorbances were attributed to proteolysis of the antibody by trypsin that was not completely inactivated by heating.

The final solubilization and alkylation conditions selected were a pH 9.0 extraction buffer and 0.25% (w/w) trypsin. A 2 h incubation period was selected as this was the shortest incubation time tested and no significant effect of incubation time was observed ($p < 0.05$). Under these conditions, myofibrils displayed a high percentage of soluble protein and percent protein modification while minimizing the incubation time. Trypsin was inactivated by heating at 90 °C for 5 min after the conjugation reaction was complete.

Accelerated Oxidation Study. Lipid oxidation, as measured by hexanal concentration and TBARS, increased when myofibrils were stored at $a_w = 0.30$ (**Figure 3**) and $a_w = 0.75$ (**Figure 4**) for 5 days in the accelerated oxidation study. Lipid oxidation proceeded more quickly at $a_w = 0.75$, but reached a maximum at day 4 of storage at both a_w values. Both CI-ELISA and TBARS detected differences in hexanal and malonaldehyde production, respectively, due to a_w from days 2, 3, 4, and 5 ($p < 0.05$). GC/MS-SPME detected differences in hexanal concentration due to a_w at days 2, 3, and 4 ($p < 0.05$). Both the CI-ELISA and TBARS showed greater sensitivity than the GC/MS-SPME at the higher a_w.

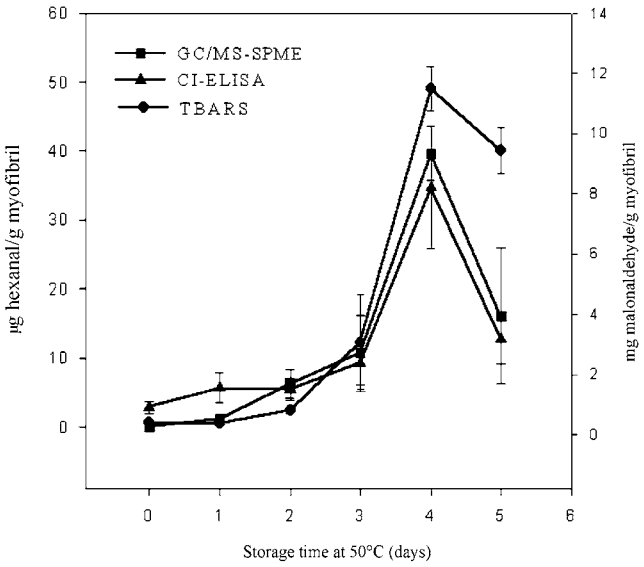

Figure 3. Hexanal concentration determined by GC/MS-SPME and CI-ELISA and malonaldehyde concentration determined by TBARS of chicken myofibrils stored for 5 days at 50 °C and a water activity of 0.30. Chicken myofibrils contained 0.6 mmol of methyl linoleate/g of protein. Determinations were made in triplicate. Error bars indicate standard error of the mean.

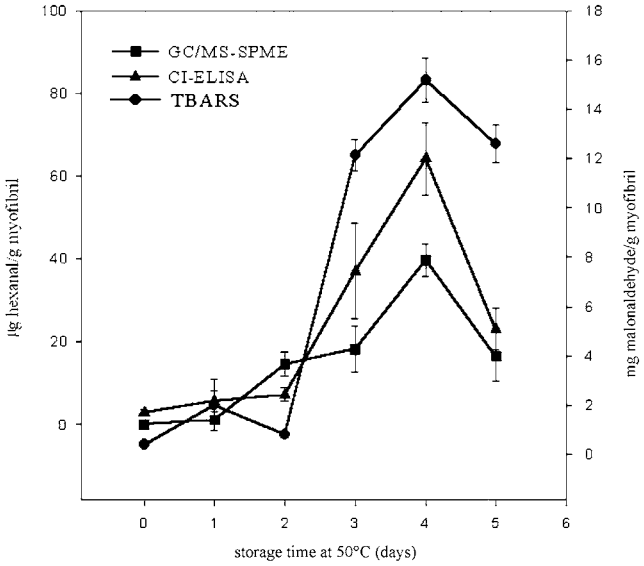

Figure 4. Hexanal concentration determined by GC/MS-SPME and CI-ELISA and malonaldehyde concentration determined by TBARS of chicken myofibrils stored for 5 days at 50 °C and a water activity of 0.75. Chicken myofibrils contained 0.6 mmol of methyl linoleate/g of protein. Determinations were made in triplicate. Error bars indicate standard error of the mean.

The three methods of lipid oxidation analysis were highly correlated (**Table 3**). CI-ELISA and GC/MS-SPME had a correlation (*r*) of 0.78, whereas CI-ELISA and TBARS had a correlation of 0.87. The CI-ELISA and GC/MS-SPME were more strongly correlated in myofibrils stored at $a_w = 0.30$ than those stored at $a_w = 0.75$. At $a_w = 0.30$, the CI-ELISA and GC/MS-SPME had a correlation of 0.95, whereas at $a_w = 0.75$, the correlation was 0.73. As GC/MS-SPME quantified free hexanal and CI-ELISA quantified bound hexanal, the higher correlation between the two methods at a_w 0.30 may indicate a more even distribution of hexanal between the bound and free forms. These correlations were similar to those reported by

Table 3. Correlation Coefficient (*r*) Matrix between Methods Used[a] To Monitor Lipid Oxidation in Freeze-Dried Chicken Myofibrils over 5 Days of Storage at 50 °C at Two Water Activities (a_w = 0.30 and 0.75)

	GC/MS-SPME			CI-ELISA		
method	overall	$a_w = 0.30$	$a_w = 0.75$	overall	$a_w = 0.30$	$a_w = 0.75$
GC/MS-SPME				0.78[b]	0.95[b]	0.73[b]
CI-ELISA	0.78[b]	0.95[b]	0.73[b]			
TBARS	0.84[b]	0.94[b]	0.84[b]	0.87[b]	0.89[b]	0.86[b]

[a] GC/MS-SPME and CI-ELISA were used to measure hexanal, and TBARS was used to measure malonaldehyde. [b] Significant at $p < 0.05$.

Zielinski et al. (*16*) of lipid oxidation in a chicken thigh homogenate system. These researchers reported a correlation (*r*) of 0.81 between a dynamic headspace GC method and the monoclonal CI-ELISA for hexanal and a correlation of 0.77 between TBARS and the CI-ELISA. Whereas several other researchers made reference to the TBARS method in the development of their aldehyde ELISA (*5*, *6*), no studies were found in which correlations between the two methods were reported. Spindler et al. (*39*) found a good correlation between an ELISA and GC methods for 13-hydroxyoctadecadienoic acid.

The CI-ELISA and GC/MS-SPME displayed a high correlation even though different forms of hexanal were measured. The GC/MS-SPME method quantified volatile hexanal found in the headspace (*37*, *38*). The CI-ELISA detected chemically bound hexanal formed through the alkylation procedure (*39*). The solubilization and alkylation procedures were designed to bind free hexanal to the myofibrils prior to analysis.

Hexanal binding has been studied in a number of protein systems. In soy protein, 37−44% of hexanal was found to be reversibly bound, whereas <5% was irreversibly bound (*40*). Myosin was reported to bind <10% of the hexanal, whereas actin bound 25−30% of the added hexanal (*41*). Hexanal−protein binding may complicate quantification by the GC/MS-SPME method, which measures only volatile hexanal, neglecting the hexanal physically or chemically bound in the sample. In these studies, the concentration of bound hexanal in the protein system was less than the concentration of free hexanal. In our study, the concentration of bound hexanal detected in the chicken myofibrils by CI-ELISA was similar to the concentration of free hexanal detected by GC/MS-SPME, suggesting the solubilization and alkylation procedure used to bind free hexanal to the myofibrils was successful.

The time required to complete the CI-ELISA was less dependent on the number of samples when compared to the time requirements of both TBARS and GC/MS-SPME. Using the CI-ELISA, the analysis of 5, 10, and 50 samples would take ~300 min. In comparison, to analyze 5, 10, and 50 samples by TBARS, time requirements would be 75, 95, and 300 min, respectively. Using the GC/MS-SPME procedure 100, 200, and 1000 min would be required for the analysis of 5, 10, and 50 samples.

The use of a CI-ELISA for the quantification of hexanal provides an alternative to chemical or chromatographic methods for monitoring lipid oxidation in foods. During 50 °C storage of freeze-dried chicken myofibrils at two water activities, the CI-ELISA was found to correlate well with the GC/MS-SPME method for hexanal analysis and appeared to be more sensitive than the TBARS method. Future studies include the adaptation of this assay for use in fresh chicken systems. With additional modification, the CI-ELISA might be developed into a commercially available kit for rapid field use.

LITERATURE CITED

(1) Yang, C. M.; Kendall, C. W.; Stamp, D.; Medline, A.; Archer, M. C.; Bruce, W. R. Thermally oxidized dietary fat and colon carcinogenesis in rodents. *Nutr. Cancer* **1998**, *30*, 69−73.

(2) Ammouche, A.; Bitam, R. F.; Bellal, M. M. Effect of ingestion of thermally oxidized sunflower oil on the fatty acid composition and antioxidant enzymes in rat liver and brain in development. *Ann. Nutr. Metab*. **2002**, *46*, 268−275.

(3) Esterbauer, H. Cytotoxicity and genotoxicity of lipid oxidation products. *Am. J. Clin. Nutr*. **1993**, *57*, 779S−785S.

(4) Bird, R. P.; Draper, H. H.; Valli, V. E. O. Toxicological evaluation of malonaldehyde: A 12-month study of mice. *J. Toxic. Environ. Health* **1982**, *10*, 897−905.

(5) Kondo, A.; Muranaka, Y.; Ohta, I.; Notsu, K.; Manabe, M.; Kotani, K.; Saito, K.; Maekawa, M.; Kanno, T. Relationship between triglyceride concentrations and LDL size evaluated by malondialdehyde-modified LDL. *Clin. Chem*. **2001**, *47*, 893−907.

(6) Khan, M. F.; Wu, X.; Ansari, G. A. Anti-malondialdehyde antibodies in MRL +/+ mice treated with trichloroethene and dichloroacetyl chloride: Possible role of lipid peroxidation in autoimmunity. *Toxicol. Appl. Pharmacol*. **2001**, *170*, 88−92.

(7) Sidell, K. R.; Montine, K. S.; Picklo, M. J.; Olsen, S. J.; Amarnath, V.; Montine, T. J. Mercapturate metabolism of 4-hydroxy-2-nonenal in rat and human cerebrum. *J. Neuropathol. Exp. Neurol*. **2003**, *62*, 146−153.

(8) Schutt, F.; Bergmann, M.; Holz, F. G.; Kopitz, J. Proteins modified by malondialdehyde, 4-hydroxynonenal, or advanced glycation end products in lipofuscin of human retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci*. **2003**, *44*, 3663−3668.

(9) Barrera, G.; Pizzimenti, S.; Serra, A.; Fazio, V. M.; Canuto, R. A.; Dianzanti, M. U. Effect of bioactive aldehydes on cell proliferation and c-myc expression in HL-60 human leukemic cells. *Cancer Detect. Prev.* **2000**, *24*, 244−251.

(10) Frankel, E. N. In search for better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends Food Sci. Technol*. **1993**, *4*, 220−225.

(11) Tarladgis, B. G.; Watts, B. M.; Younathan, M. T.; Dugan, L. R. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Am. Oil Chem. Soc*. **1960**, *37*, 44−48.

(12) Igene, J. O.; Pearson, A. M.; Merkel, R. A.; Coleman, T. H. Effect of frozen storage time, cooking and holding temperature upon extractable lipids and TBA values of beef and chicken. *J. Anim. Sci*. **1979**, *49*, 701−707.

(13) Sun, Q.; Faustman, C.; Senecal, A.; Wilkinson, A. L.; Furr, H. Aldehyde reactivity with 2-thiobarbituric acid and TBARS in freeze-dried beef during accelerated storage. *Meat Sci*. **2001**, *57*, 55−60.

(14) Shahidi, F.; Pegg, R. B. Hexanal as an indicator of the flavor deterioration of meat and meat products. In *Lipid Oxidation in Food*; St. Angelo, A. J., Ed.; ACS Symposium Series 500; American Chemical Society: Washington, DC, 1994; pp 105−119.

(15) Shahidi, F.; Yun, J.; Rubin, L. J. The hexanal content as an indicator of oxidative stability and flavor acceptability in cooked ground pork. *Can. Inst. Food Sci. Technol. J*. **1987**, *20* (2), 104−106.

(16) Zielinski, T. L.; Smith, S. A.; Pestka, J. J.; Gray, J. I.; Smith, D. ELISA to quantify hexanal-protein adducts in a meat model system. *J. Agric. Food Chem*. **2001**, *49*, 3017−3023.

(17) Spanier, A. M.; Miller, J. A.; Bland, J. M. Lipid oxidation: Effect on meat proteins. In *Lipid Oxidation in Food*; St. Angelo, A. J., Ed.; ACS Symposium Series 500; American Chemical Society: Washington, DC, 1992; pp 105−110.

(18) Reineccius, G. A. Instrumental means of monitoring the flavor quality of foods. In *Chemical Markers for Processed and Stored Foods*; Lee, T. C., Kim, H. J., Eds.; American Chemical Society: Washington, DC, 1996; pp 241−251.

(19) Steffen, A.; Pawliszyn, J. Analysis of flavor volatiles using headspace solid-phase microextraction. *J. Agric. Food Chem*. **1996**, *44*, 2187−2193.

(20) O'Keefe, S. F.; Wilson, L. A.; Resurreccion, A. P.; Murphy, P. A. Determination of the binding of hexanal to soy glycinin and β-conglycinin in an aqueous model system using a headspace technique. *J. Agric. Food Chem.* **1991**, *39*, 1022−1028.

(21) Estevez, M.; Morcuende, D.; Ventanas, S.; Cava, R. Analysis of volatiles in meat from Iberian pigs and leans pigs after refrigeration and cooking by using SPME-GC-MS. *J. Agric. Food Chem.* **2003**, *51*, 3429−3435.

(22) Kim, G. Y.; Lee, J. H.; Min, D. B. Study of light-induced volatile components in goatís milk cheese. *J. Agric. Food Chem.* **2003**, *51*, 1405−1409.

(23) Smith, S. A.; Pestka, J. J.; Gray, J. I.; Smith, D. M. Production and specificity of polyclonal antibodies to hexanal-lysine adducts. *J. Agric. Food Chem.* **1999**, *47*, 1389−1395.

(24) Samarajeewa, U.; Wei, C. J.; Huang, T. S.; Marshall, M. R. Application of immunoassay in the food industry. *Crit. Rev. Food Sci. Nutr.* **1991**, *29*, 403−434.

(25) Paraf, A. A role of monoclonal antibodies in the analysis of food proteins. *Trends Food Sci. Technol*. **1992**, *3*, 263−267.

(26) USDA. *Composition of Foods, Poultry Products*; Agriculture Handbook 805; U.S. Department of Agriculture: Washington, DC, 1979.

(27) Smith, D. M. Functional and biochemical changes in deboned turkey due to frozen storage and lipid oxidation. *J. Food Sci.* **1987**, *52*, 22−27.

(28) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.

(29) Deshpande, S. S. Assay development, evalulation and validation. In *Enzyme Immunoassay: From Concept to Product Development*; Chapman and Hall: New York, 1996; pp 275−359.

(30) Zielinski, T. L. Development and characterization of a monoclonal antibody based ELISA for monitoring lipid oxidation. Msc. Thesis, Michigan State University, East Lansing, MI, 1998.

(31) Labuza, T. P. Determination of moisture sorption isotherms. In *Moisture Sorption: Practical Aspects of Isotherm Measurement and Use*; American Association of Cereal Chemists: St. Paul, MN, 1984; pp 64−66.

(32) Goodridge, C. F.; Beaudry, R. M.; Pestka, J. J.; Smith, D. M. Solid-phase microextraction-gas chromatography for quantifying headspace hexanal above freeze-dried chicken myofibrils. *J. Agric. Food Chem*. **2003**, *51*, 4185−4190.

(33) Smith, D. M.; Noormarji, S. H.; Price, J. F.; Bennink, M. R.; Herald, T. J. Effect of lipid oxidation on the functional and nutritional properties of washed chicken myofibrils stored at different water activities. *J. Agric. Food Chem*. **1990**, *38*, 1307−1312.

(34) Xiong, Y. L.; Brekke, C. J. Changes in protein solubility and gelation properties of chicken myofibrils during storage. *J. Food Sci*. **1989**, *54*, 1141−1146.

(35) Simpson, B. K.; Nayeri, G.; Yaylayan, V.; Ashie, I. N. A. Enzymatic hydrolysis of shrimp meat. *Food Chem*. **1998**, *61*, 131−138.

(36) Spindler, S. A.; Clark, K. S.; Blackburn, M. L.; Bull, A. W.; Reddy, R. G. Occurrence of 13(S)-hydroxyoctadecadienoic acid in biological samples. *Prostaglandins* **1997**, *54*, 875−880.

(37) Jung, D. M.; Ebeler, S. E. Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. *J. Agric. Food Chem*. **2003**, *51*, 200−205.

(38) Toso, B.; Procida, G.; Stefanon, B. Determination of volatile compounds in cows' raw milk using headspace GC-MS. *J. Dairy Res*. **2002**, *69*, 569−577.

(39) Jennings, W. G. Objective measurements of flavor quality: General approaches, problems, pitfalls and accomplishments. In

Flavor Quality: Objective Measurements; Scanlan, R. A., Ed.; American Chemical Society: Washington, DC, 1977.

(40) Gremli, H. A. Interaction of flavor compounds with soy protein. *J. Am. Oil Chem. Soc*. **1974**, *51*, 95A−97A.

(41) Gutheil, R. A.; Bailey, M. E. A method for determining binding of hexanal by myosin and actin using equilibrium headspace sampling gas chromatography. In *Food Science and Human Nutrition: Developments in Food Science 29*; Charalambous, G., Ed.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1992; pp 783−815.

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