THESIS

This is to certify that the

thesis entitled

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR RAPID ENDPOINT PROCESSING TEMPERATURE DETERMINATION IN TURKEY HAM; AND EFFECTS OF DELAYED BLEEDING ON RESIDUAL SERUM PROTEINS IN TURKEY MUSCLES

presented by

LISA DAWN DESROCHER

has been accepted towards fulfillment
of the requirements for

M.S. degree in Food Science

Louise M. Smith

Major professor

Date March 25, 1994

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR RAPID ENDPOINT PROCESSING TEMPERATURE DETERMINATION IN TURKEY HAM; AND EFFECTS OF DELAYED BLEEDING ON RESIDUAL SERUM PROTEINS IN TURKEY MUSCLES

By

Lisa Dawn Desrocher

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1994

ABSTRACT

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR RAPID ENDPOINT PROCESSING TEMPERATURE DETERMINATION IN TURKEY HAM; AND EFFECTS OF DELAYED BLEEDING ON RESIDUAL SERUM PROTEINS IN TURKEY MUSCLES

By

Lisa Dawn Desrocher

Enzyme-linked immunosorbent assays (ELISA) were developed to verify endpoint processing temperature (EPT) of turkey ham processed between 66.9° and 73.8°C. Lactate dehydrogenase (LDH), immunoglobulin G (IgG), pyruvate kinase (PK), and turkey serum albumin (TSA) were identified as potential EPT markers. Indirect competitive (IC) ELISAs were developed using commercial IgG polyclonal antibodies (pAbs) and pAbs raised in rabbits against TSA and PK. Sandwich and IC-ELISAs using LDH monoclonal and polyclonal antibodies were developed previously. As processing temperature increased, LDH, IgG, and TSA concentration curvilinearly (α=0.001) decreased in turkey ham, indicating these protein markers could be used in rapid assays to verify EPT. Processing temperature above 66.9°C did not affect PK concentration.

The effects of delayed bleeding on total pigment, color and residual serum protein concentration in turkey breast and thigh muscles were analyzed. Delayed bleeding had no effect on serum protein concentration in turkey breast and thigh muscle.

DEDICATION

This thesis is dedicated to my mother, Sharon,
who taught me the "science" of life.
I love you, Mom.

ACKNOWLEDGMENTS

The author expresses deep appreciation to Dr. Denise M. Smith for serving as major professor and for her support and guidance throughout this project.

Appreciation is also expressed to Dr. A.M. Booren and Dr. J.J. Pestka for their assistance and guidance as committee members. The author also wishes to thank Dr. J.L. Gill for his assistance in statistics.

Special thanks are extended to Dr. Mohamed Abouzied, Cheng-Hsin Wang, and Alicia Orta for their valuable counsel and assistance.

The author would like to thank Bil-Mar Foods, Inc. for providing the meat products used in this study.

Foremost, I want to thank my dear husband, Bill, for his love, encouragement and understanding throughout my graduate study. And to my daughter, Elisabeth, who's smile always made me smile.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The 1993 outbreak of hemorrhagic *E. coli* O157:H7 in the northwestern United States has significantly increased the awareness of foodborne illnesses among the general population. This increased awareness has led to severe scrutiny of the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) and the methodology used to monitor the safety of food, primarily cooked meat products. Title 9 of the Code of Federal Regulations (CFR) states minimum endpoint processing temperature (EPT) requirements for several pre-cooked meat products. Poultry products currently require an internal temperature of 71.1°C (uncured) and 68.3°C (cured). Although the USDA-FSIS uses several assays to monitor EPT in beef and pork products, there are no rapid assays to evaluate EPT in poultry products.

Immunoassays are an emerging technology in the field of food analysis. Enzyme-linked immunosorbent assays (ELISA) are continually being developed to replace standard conventional methodology in food safety. The application of immunoassays allows for a rapid, accurate, and highly sensitive assessment of components in a food system.

Endpoint processing temperature is used as a determinant of adequate thermal processing in meat products.

Determination of EPT can verify if a meat product has been sufficiently heated to destroy viable organisms that can cause foodborne diseases. Once a marker for adequate heat treatment is identified, an ELISA can be developed. The standardized ELISA can then be manipulated into a field test kit with minimal reagents and simple protocol.

The goal of this research was to develop an ELISA that can verify the EPT of a cured poultry product. The first objective was to identify potential EPT marker proteins in turkey hams processed to internal temperatures of 66.9°, 68.3°, 69.7°, 71.1°, 72.5°, and 73.9°C. The proteins selected for further study were lactate dehydrogenase, pyruvate kinase, immunoglobulin G, and turkey serum albumin. The second objective was to produce polyclonal antibodies to chicken muscle pyruvate kinase and turkey serum albumin. Anti-turkey immunoglobulin G polyclonal antibodies were available commercially. The anti-turkey lactate dehydrogenase polyclonal and monoclonal antibodies were produced previously (Wang et al., 1992; Abouzied et al., 1993). The third objective was to develop and standardize indirect competitive ELISA's utilizing anti-turkey serum albumin, anti-chicken pyruvate kinase, and anti-turkey immunoglobulin G polyclonal antibodies. Indirect competitive and sandwich ELISA's using anti-turkey lactate dehydrogenase antibodies were performed according to Wang et al. (1992) and Abouzied et al. (1993), respectively.

Application of ELISA's to verify EPT in turkey hams commercially processed was the fourth objective. The final objective was to determine the effect of delayed bleeding on residual blood in turkey breast and thigh muscle to verify that turkey serum albumin and immunoglobulin G concentrations do not vary in muscle tissue and thus can be used as EPT indicators.

LITERATURE REVIEW

The USDA-FSIS employs several assays to determine if meat products have met thermal processing requirements as stated in Title 9 of the Code of Federal Regulations (CFR). These requirements were established to ensure the destruction of viable organisms that can cause foodborne illnesses. However, due to increasing incidence and awareness of foodborne disease, the EPT requirements and current methodology for monitoring EPT are being questioned by both federal officials and consumers.

Title 9 of the CFR outlines thermal processing requirements for beef, pork, poultry and several other meat products. Section 318.17b of the CFR requires cooked beef and roast beef to be heat processed in any of 16 different time/temperature protocols with holding times or to 62.8° (145°F) with no holding time. Section 94.12 of the CFR requires pork products be processed to an internal temperature of 58.3°C (137°F) or subjected to freezing temperatures to ensure destruction of Trichinae (USDA-FSIS, 1986a). Poultry rolls require thermal processing to an internal temperature of 71.1°C (160°F) for uncured products and 68.3°C (155°F) for cured/smoked products as stated in section 381.150 of the CFR (USDA-FSIS, 1990). These requirements were established to protect against foodborne

illnesses caused by pathogens such as hemmorhagic *E. coli* O157:H7, *Salmonella, Campylobacter,* and *Staphylococcus*.

In January of 1994, the FDA published a new Food Code establishing new recommended internal cooking temperatures for hamburger patties, roast beef, poultry, and pork (FDA, 1994). For all poultry products, an EPT of 165°F (73.8°C) or above for 15 sec was recommended.

Foodborne Disease in the United States

Inadequately cooked meat products is the most commonly cited factor in foodborne disease outbreaks (Bean and Griffin, 1990). An outbreak is loosely defined as an incident where two or more persons have a similar illness from which food is implicated. Foodborne disease in the U.S. caused at least 6 million illnesses and 9,000 deaths in 1983 (Bean et al., 1990). This was the last year in which full estimates are available from the U.S. Centers for Disease Control and Prevention (CDC). The reporting of foodborne diseases started over 50 years ago. The CDC has traditionally followed three main objectives: Disease prevention and control, knowledge of disease causation, and administrative guidance. Early identification and removal of contaminated products is key in foodborne disease prevention and control. The CDC recently summarized the status of foodborne disease outbreaks in the U.S. from 1983

to 1987 (Bean et al., 1990). The 5 year summary for 1988 to 1993 is expected in late 1994. Bacterial pathogens caused the largest number of foodborne disease outbreaks (66%) and cases (92%), indicating that current methodology used to identify products of potential harm are inaccurate and unreliable.

A 1990 epidemiologic study found that persons with listeriosis were more likely to have eaten undercooked chicken or hot dogs that were not reheated (Wenger et al., 1990). The author suggested that concurrent culturing and analysis of products at the production site will help identify and control the contamination in ready-to-eat meat products.

The cost of foodborne disease to the U.S. is estimated at $8.4 billion annually (Todd, 1989). Salmonellosis and staphylococcal illnesses have an annual cost of $4.0 billion and $1.5 billion, respectively. Microbiological diseases from both bacterial and viral agents, represent 85% of all foodborne illness costs. The author suggests the main effort to prevent rising costs due to outbreaks should focus on prevention of microbiological contaminants. Prevention can be achieved through stricter control of processing requirements and improved methodology to assess the safety of foods.

Current USDA Methodology for EPT Determination

Although the increase in EPT requirements for meat products will help decrease the incidence of foodborne illnesses, the USDA-FSIS still needs to address the problem of accurately monitoring the EPT during heat processing to ensure the destruction of pathogens. The USDA-FSIS currently uses several assays to determine adequate processing in meat products as required by Title 9 of the CFR. The Coagulation Test is used for both beef and pork products that are processed to temperatures below 65°C (149°F)(USDA-FSIS, 1986b). The Bovine Catalase Test is used to assess adequate processing of rare roast beef and cooked beef (USDA-FSIS, 1989). The residual Acid Phosphatase Activity Method is used for canned hams, picnics, and luncheon meats, which require an EPT of 68.8°C (156°F)(USDA-FSIS, 1986c). There are no rapid assays available to determine the EPT achieved in poultry products.

The Coagulation Test measures the loss in protein solubility due to increasing temperature when a meat product is heat processed (USDA-FSIS, 1986b). The soluble muscle proteins are extracted and subsequently heated until the first sign of cloudiness appears. The temperature at which the reaction is observed is indicative of the EPT achieved during heat processing of the product. The EPT determined by the Coagulation Test may vary 8°-10°C from the actual EPT

achieved during processing (USDA-FSIS, 1986b). Although the Coagulation Test can determine if products have been grossly underprocessed, it cannot determine if products have been adequately processed (Townsend and Blankenship, 1989).

The principal of the Bovine Catalase Test is based on the reaction of residual catalase with oxygen to produce a foam (Eye, 1982). The volume of foam produced is indicative of the amount of residual catalase in the bovine product and is subsequently classified as strong, medium, weak, or no activity. The Bovine Catalase Test was recently revised by USDA-FSIS (1989). The assay was standardized to give a positive or negative response, rather than basing results on foam volume. A catalase-positive sample indicates that the meat product did not reach 62.8°C (145°F) during thermal processing. Bovine catalase activity is destroyed at 60.5°-61.1°C (141°-142°F), therefore identifying samples that are undercooked by 1.7°-2.3°C (3°-4°F). However, this particular method was found to be highly subjective (Stalder et al., 1991). When the authors attempted to validate the actual EPT with the Catalase Test, the results were dependent upon the person performing the assay.

The residual Acid Phosphatase Activity Method is used to determine if canned hams, picnics, and luncheon meats have been thermally processed to 68.8°C (156°F)(USDA-FSIS, 1986c). This enzymatic method assays water-soluble protein extracts for presence of acid phosphatase. A positive

enzymatic reaction produces a blue color that can be read spectrophotometrically at 610 nm. When comparing actual EPT achieved during processing and resultant acid phosphatase activity, the correlation is very low (Kormendy et al., 1987; Townsend, 1989).

Of all methods previously described none provide a rapid, accurate, and highly sensitive assay that can be performed either at the production site or in the field. The increasing incidence and severity of foodborne illnesses in the U.S. necessitates research to provide new, alternative methods to assess the safety of meat products.

Current Experimental Methods to Determine EPT

In recent years, research has focused on utilizing alternate methodology to determine EPT in meat products. New methods are designed to identify a change in a food component due to thermal processing.

Loss in protein solubility. The loss of protein solubility with increasing heat treatment has been used to identify adequate heat treatment of porcine and bovine muscle (Davis et al., 1985, 1987). Davis et al. (1985) evaluated the effects of heat treatment on porcine and bovine muscles. The amount of extractable water soluble proteins was determined by biuret method. The study illustrated the difficulty using soluble protein as an EPT

indicator (Davis et al., 1985). Extracts of water-soluble proteins from heat treated canned pork picnic shoulders were also evaluated using biuret method and isoelectric focusing on polyacrylamide gels (Davis et al., 1987). Loss of protein solubility proved to be useful in following increased heat treatment of a meat product, but not as an accurate indicator of EPT.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful method that provides a visual illustration of the loss of protein solubility with increased heat treatment. The potential for identification of EPT in meat products using SDS-PAGE has been investigated by many researchers (Lee et al., 1974; Caldironi and Bazan, 1980; Steele and Lambe, 1982). Lee et al. (1974) developed an SDS-PAGE method to measure the effects of cooking temperatures ranging between 65° and 90°C on bovine muscle. The authors state the method can determine EPT within ±5°C, with high accuracy and reproducibility. Protein extracts from rabbit, chicken, pig, horse, ox, and duck were analyzed for loss of protein solubility due to heat treatment using SDS-PAGE by Steele and Lambe (1982). Several bands showed a temperature-related decrease in intensity, but a specific EPT indicator protein was not identified.

Although SDS-PAGE is a well established technique, the

average duration of the assay is 3.5 to 8 hr depending on size of gel. In addition, subsequent quantitation of protein bands would require expensive instrumentation.

Enzymatic methods. Residual enzyme activity in thermally processed meat products has proven to be successful in ascertaining potential EPT markers. Klinger et al. (1982) studied the effect of temperature on 12 different enzymes in breast muscle as possible indicators of sufficient heating to destroy Velogenic-Viserotropic Newcastle Disease Virus in infected turkeys. The authors concluded that asparate-oxoglutarate aminotransferase (AST) was an acceptable EPT indicator for 70°C.

Several researchers have investigated lactate dehydrogenase (LDH) as an EPT marker in bovine and porcine muscle (Collins et al., 1991a,b; Stalder et al., 1991). Stalder et al. (1991) investigated the influence of pH, salt, phosphate, cooking temperature, muscle variation, carcass sex, and maturity on LDH activity. LDH activity was found to decrease sharply at 63°C. In porcine muscle, LDH markedly decreased in activity when heated to 68°C and above (Collins et al., 1991b).

Acid phosphatase (AP) (Kormendy et al., 1992) and pyruvate kinase (PK) (Davis et al., 1988) have also shown potential as EPT indicators in canned hams and a model cured pork product, respectively. Kormendy et al. (1992) modified the existing Residual Acid Phosphatase Assay currently used

by the USDA to monitor EPT in canned hams which require an EPT of 69°C (156°F). The authors improved the sensitivity of the assay by adequately blending the sample, using optimum pH and increased substrate concentration in teh assay. Pyruvate kinase was investigated by Davis et al. (1988) as an EPT indicator for cured pork. Pyruvate kinase activity was not detected in samples heated to 69.5° and 70°C. Transaminase activity as an EPT indicator in ground beef was reported by Townsend and Davis (1992). Transaminase activity was detected at 79.4°C and therefore was not a good indicator for ground beef which requires 68.3°C EPT.

Bogin et al. (1992) evaluated 12 enzymes as possible EPT indicators in turkey breast meat. Creatine kinase, malic dehydrogenase, lactic dehydrogenase, and isocitric dehydrogenase were shown to be prospective EPT markers. The effect of heat on several endogenous enzymes in turkey breast and thigh muscle was also reported by Hsu (1993). Lactate dehydrogenase and malate dehydrogenase (MDH) were recommended for further study as potential EPT indicators in breast muscle. None of the previously discussed studies used a commercial product thermally processed under simulated industrial conditions.

Alternative methods. The complexity of a meat system makes it imperative that an assay to determine adequate heat treatment be sensitive as well as accurate. The assessment

of heat treatment has been investigated using reverse phase high performance liquid chromatography (RP-HPLC)(McCormick et al., 1987), near infared spectroscopy (Isaksson et al., 1989; Ellekjaer and Isaksson, 1992), and differential scanning calorimetry (DSC)(Quinn et al., 1980; Wright and Wilding, 1984; Ellekjaer, 1992). Heat treatment of porcine muscles was evaluated by McCormick et al. (1987) using RP-HPLC. The authors concluded that the absorbance of LDH and PK fractions in water soluble protein extracts from porcine muscle, as determined by RP-HPLC, could be used to evaluate the extent of heat treatment in porcine muscle. Near infrared diffuse reflectance and transmittance were used to determine the EPT of beef heated between 50° to 85°C within 2.0°-2.1°C (Ellekjaer and Isaksson, 1992). Ellekjaer (1992) studied DSC as a method to evaluate heat treatment in beef. The DSC method was able to determine the EPT of beef samples heated between 50° to 72°C with a prediction error of 0.6°C. Although the sensitivity required for EPT determination may be achieved using the previously mentioned methodology, the assays are time consuming and require expensive equipment. The instrumentation also requires extensive training and technical expertise to interpret results and maintain standard conditions for subsequent assays.

Immunoassays. The need for a sensitive assay that is both rapid and accurate has led researchers to employ immunological methodology to determine appropriate heat

treatment in meat products. In addition, immunoassays are relatively easy to perform and inexpensive. Wang et al. (1992) developed an indirect (competitive) ELISA to determine EPT in turkey rolls using LDH as the EPT indicator protein. When turkey rolls were commercially processed between 68.3°C (155°F) to 72.1°C (161.8°F), LDH disappeared at 70.9°C (159.6°F). The disappearance was monitored using an enzyme assay and SDS-PAGE. Once identified as a potential EPT marker, polyclonal antibodies (pAb) were raised against turkey LDH. The pAb were used to develop and standardize an indirect competitive ELISA for turkey breast rolls. The LDH content, as determined by ELISA, significantly decreased as the heat treatment of the turkey breast rolls increased. A sandwich ElISA was also developed utilizing LDH monoclonal antibodies (mAb) and pAb (Abouzied et al., 1993) for determining EPT in turkey breast rolls. The assay proved effective in determining the EPT of turkey breast rolls processed to 71.1°C (160°F). The assay was also applied to turkey products purchased from a local grocer. The LDH sandwich assay was valid when applied to turkey breast rolls, but not for turkey ham products which require an EPT of 68.3°C (155°F). The effects of formulation, storage, and processing on the validity of the LDH sandwich ELISA for turkey breast rolls was investigated (Wang et al., 1993). The LDH concentration was found to differ in rolls processed to an internal temperature of

70.0°C (158°F) and 71.1°C (160°F); However, the extractable protein concentration and enzyme activity did not differ. Other factors such as salt concentration, cooking schedule, and casing diameter did not significantly influence LDH concentration. The LDH sandwich ELISA provides a rapid and highly sensitive assay that can accurately distinguish EPT within ±1.1°-1.2°C in turkey breast rolls.

Application of Immunoassays to Food Safety

The application of immunological assays for the detection of various meat species, non-meat proteins, microorganisms, bacterial toxins, drugs, anabolic hormones, pesticides, mycotoxins, and other contaminants have been well documented (Patterson et al., 1984; Berger et al., 1988; Breton et al., 1988; Dixon-Holland et al., 1988; Pestka, 1988; Janssen et al., 1990; Bhunia et al., 1991; Fukal, 1991; Samarajeewa et al., 1991; Andrews et al., 1992). The high specificity and sensitivity, ease of use, rapidity, and adaptability to automation make immunoassays the preferred method over other available methods. Immunoassays also provide for a reduction in assay time, small sample size, and up to 90% decrease in chemicals and equipment expense (Samarajeewa et al., 1991). In addition, commercial immunoassay kits are increasingly available to detect a wide variety of food components and contaminants.

Commercial kits allow for the same assay to be performed in different laboratories under identical standardized conditions.

Immunoassay Methodology

The basis for competitive binding immunoassays is the competition between "bound" antigen and "free" antigen for antibody binding sites. The antibody is defined as a glycoprotein which is produced during an immune response to a foreign material called an antigen or an immunogen. The high sensitivity of immunoassays is due to the antibody-antigen interactions which often have affinity constants exceeding 10^{-8} (Pestka, 1988). The high specificity of antibodies are due to their ability to differentiate slight chemical variations between target antigens. Radioimmunoassay (RIA) and ELISA are the most common immunoassays used in food analysis. The RIA is a useful tool but has several disadvantages. Use of an RIA requires disposal of radioactive waste, a scintillation counter, expensive and time consuming labeling of antigen with tritium, and gradual loss of antigen from isotope emissions. Comparatively, the ELISA is much easier and safer to perform. The ELISA is currently the most commonly used immunoassay in meat-product analysis (Fukal, 1991).

Development of an immunoassay starts with the

production of antibodies against the antigen of interest in a host animal. The antigen will not elicit an immune response if the molecular mass is below 10,000 daltons. In this case the antigen is called a hapten and must be conjugated to a larger carrier protein before it is rendered immunogenic. The initial immunization will include the antigen of interest emulsified with complete adjuvant (e.g. Freund's complete adjuvant or Titermax). The complete adjuvant contains mycobacterium that initiates a strong immune response in the host animal. Booster injections with antigen emulsified with incomplete adjuvant (without mycobacterium) follow the initial immunization until adequate antisera is produced. Both the complete and incomplete adjuvant contain oil (e.g. mineral oil) that encapsulates the antigen, allowing for a time-release effect and initial protection from macrophage. The antisera is assessed by means of titer, avidity, and specificity. The titer is arbitrarily designated as the maximum dilution that yields at least twice the absorbance of nonimmune serum at the same dilution. Avidity refers to the strength and stability of the antibody-antigen complex, whereas specificity is the ability of the antibody to react with various substances.

Antibodies for Immunoassay Applications

Antibodies are made by plasma cells and B-lymphocytes, the precursor for plasma cells, in response to a foreign substance (antigen) in the body. Antibodies are made up of two heavy and two light glycoprotein chains that are held together by covalent interchain disulfide bonds. Depending on methodology used for production, either monoclonal or polyclonal antibodies can be made. Monoclonal antibodies are produced from a single hybridoma cell line, whereas polyclonal antibodies originate from multiple lymphocyte clones.

Monoclonal antibodies. The typical immune response is heterogeneous as a result of different B-lymphocytes producing antibodies to the same antigen. However, each cell retains the distinctive capacity to secrete a specific monoclonal antibody against a single antigenic determinant. The antibodies produced from the cell, also known as a clone, are homogeneous. The limited lifespan of the B-lymphocytes makes it necessary to fuse the clone with a myeloma cell line to form a hybridoma. The resultant hybridoma allows for a pure and unlimited supply of monoclonal antibodies (mAb) (Galfre and Milstein, 1981).

The spleen is removed from an immunized animal (e.g. mouse) and B-lymphocytes are collected. The lymph nodes can also be used to harvest the B-lymphocytes, but the spleen is

most commonly used. The spleen cells are initially fused with a myeloma cell line through addition of polyethylene glycol which promotes membrane fusion. The best myeloma cell lines are derived from mutant cell lines of a B-lymphocyte tumor from BALB/c mice (Harlow and Lane, 1988). The resultant hybridomas are grown on selective medium known as hypoxathine-aminopterinthymidine (HAT). Only fused cells are able to grow in HAT media. Hybridoma technology allows the monoclonal antibody to be produced indefinitely in culture.

Monoclonal antibodies are mass produced by ascites formation or tissue culture. Ascites is intraperitoneal fluid extracted from a tumor induced by injecting hybridoma cells into the peritoneum of an experimental animal. Supernatant is the fluid resulting from the centrifugation of hybridomas grown up in tissue culture.

The advantages of monoclonal antibodies include homogeneity to a defined antigenic determinant and unlimited supply. In addition, mAb can be used to study functional domains of a molecule and low-affinity mAb can be selected for screening procedures. The disadvantages include expensive and time-consuming production and the need for well established tissue culture facilities. Monoclonal antibodies also may recognize an epitope shared by many antigens and the hybridoma cell lines are often lost due to instability and tissue culture contamination.

Polyclonal antibodies. Polyclonal antibodies (pAb) are produced by many different cell lines of B-lymphocytes and are able to react with different determinants on the same antigen. The resultant response is heterogeneous in nature. To produce versatile and high affinity pAb, the animal must be immunized with extremely pure antigen.

Rabbits are usually the animal of choice when producing polyclonal antibodies. The antibodies are purified using the ammonium sulfate precipitation method described by Hebert et al. (1973). The advantages of using pAb include their heterogeneous nature, high affinity for numerous epitopes on a single antigen, and high stability in frozen or lyophilized storage. In addition, purified polyclonal antibodies can recognize conformational as well as sequential determinants and are produced at lower cost than monoclonals. However, polyclonal antibodies cannot detect single epitopes, require a highly purified immunogen, and need to be produced in large batch sizes to ensure low variability.

Conjugated antibodies. Conjugated antibodies (polyclonal or monoclonal) are used in immunoassays to generate a rapid, quanitifiable signal. In addition, conjugated antibodies can provide signal amplification when assaying for minute quantities of antigen. Enzyme, avidin-biotin, colloidal gold, and fluorochrome-labeled antibody conjugates are currently utiltized in modern immunoassays

(Malik and Daymon, 1982; Brada and Jurgen, 1984; Wilchek and Bayer, 1988; Dutton, 1990).

Enzyme-labeled antibodies are widely used in immunoassays. Horseradish peroxidase and alkaline phosphatase are the most commonly used enzymes for labeling (Malik and Daymon, 1982). An antibody-enzyme conjugate is detected when substrate is added and a colored product forms. Biotin and avidin react to form a strong, stable complex that is highly specific. The avidin-biotin complex can replace conventional horseradish peroxidase conjugates in assays where endogenous peroxidase may interfere. Biotinylated antibody can be used either as the primary or secondary antibody in an immunoassay. Avidin labeled with enzyme will form a highly specific complex with the biotin-labeled antibody. Upon addition of substrate, the amount of bound enzyme can be determined. The sensitivity of the avidin-biotin technique can be significantly increased by preincubating a biotinylated enzyme with avidin or avidin derivative to form large complexes. These complexes are added to the wells and bind to any unoccupied biotin-binding sites, thus further amplifying the signal (Wilchek and Bayer, 1988).

Colloidal gold-labeled antibodies were introduced by Faulk and Taylor (1971). They conjugated gold particles to rabbit anti-*Salmonella* antibody for identification and localization of *Salmonella* antigen. This research led to

the development of colloidal gold based secondary labeling reagents. The high electron density of colloidal gold makes it an excellent choice for studies utlizing electron microscopy, light microscopy, and immunoblotting (Brada and Jurgen, 1984). Immunoblotting techniques using gold-labeled secondary antibodies can detect picogram levels of antigen after silver enhancement staining.

Fluorochrome-labeled antibodies have wide application in flourescent immunoassays, immunoblotting, and hybridoma screening among others. Fluorochromes absorb energy when exposed to light of a particular wavelength. The flourescence emitted from excited fluorochromes can be measured. The amount of fluorescence is relative to the amount of antigen bound by labeled antibody (Dutton, 1990).

Of all labeled antibodies previously discussed, the enzyme-labeled antibodies are used most often. They are sensitive, inexpensive and have a rapid reaction rate.

Enzyme-linked Immunosorbent Assays

Enzyme-linked immunosorbent assays can be divided into three basic categories: antibody capture, antigen capture and sandwich (two-antibody) assays (Harlow and Lane, 1988).

Antibody capture assays are also known as competitive binding assays. Competitive binding immunoassays can be either indirect or direct. An indirect competitive ELISA is

based on the competition between "bound" and "free" antigen for the binding site of the antibody. The antigen is bound to a solid phase (i.e. microtiter well), followed by the addition of "free" antigen and primary antibody. The wells are washed, leaving behind only bound primary antibodies. When the enzyme-labeled secondary antibody is added, it will bind to the primary antibody. As the amount of "free" antigen increases, the amount of bound primary and secondary antibody will decrease which results in decreased enzymatic color formation. The direct competitive ELISA has fewer steps than the indirect, by eliminating the need for a secondary antibody. In this case, the primary antibody is enzyme-labeled (Harlow and Lane, 1988).

The antigen capture ELISA is performed using antibody bound to the solid phase. Antigen is added to each well and the amount of antigen/antibody complexes can be quantitated after addition of enzyme-labeled secondary antibody (Harlow and Lane, 1988).

The sandwich (two-antibody) assay is generally a more sensitive assay and more widely used (Palomaki, 1991). Monoclonal or polyclonal antibodies are bound to the microtiter plate. The target antigen is added to the wells and binds to the antibody. After the unbound antigen is removed through a series of washing steps, either monoclonal or polyclonal primary antibody is added. After removal of the unbound primary antibody, anti-rabbit (pAb) or anti-

mouse (mAb) enzyme-labeled secondary antibody is added. Addition of the appropriate enzyme substrate to the wells will produce a color product that is relative to the amount of bound antigen.

Muscle Proteins

Muscle proteins are divided into three categories: myofibrillar, sarcoplasmic, and connective tissue. The myofibrillar fraction contains structural proteins that comprise the myofibrils in muscle. Sarcoplasmic proteins make up 30-35% of total muscle protein (Pearson and Young, 1990). Many enzymes necessary for normal functioning of the cell are sarcoplasmic proteins, with 70-80% being glycolytic in nature. The connective tissue proteins are insoluble and consequently not a good choice for an EPT indicator.

Myosin and actin are the two main proteins in the myofibrillar fraction. They account for 70% of total myofibrillar protein, with 50% attributed to myosin and about 20% to actin (Obinata et al., 1981). Other myofibrillar proteins include actinins, tropomyosin, and troponin A and B (Scopes and Penny, 1971). The extraction of myofibrillar proteins can be achieved using a KCl solution with an ionic strength of 0.3-0.5 μM. The amount of myofibrillar proteins in each individual animal is dependent upon age (Pearson and Young, 1990).

There are over 100 sarcoplasmic proteins that make up the cytoplasm and occupy about 25% of the cellular space. In addition to energy metabolism, the sarcoplasm is necessary for transport of oxygen and waste materials. The sarcoplasmic proteins are subdivided into four water-soluble fractions separated by differential centrifugation. These fractions include nuclear, mitochondrial, microsomal, and cytoplasmic (Pearson and Young, 1990).

Turkey Muscle Proteins as Potential EPT Markers

Determination of an EPT indicator protein involves a thorough investigation of potential markers endogenous to the muscle product. The EPT indicator should be present in high amounts prior to heat processing, easily identifiable, and illustrate degradation or inactivation at or near the EPT. Some of the proteins to be discussed have already been recognized as potential EPT markers, while others were specifically chosen for this study. The two sarcoplasmic proteins of interest in this study are lactate dehydrogenase and pyruvate kinase.

Lactate dehydrogenase. Lactate dehydrogenase (LDH) can exist in five different isoforms each composed of four subunits. Each subunit has a molecular mass of approximately 35,000 for a total molecular mass of 134,000 to 140,000 (Lehninger, 1975). All five isoforms catalyze

the reversible conversion of lactate to pyruvate in the presence of NADH or NAD^+. Lactate dehydrogenase isoforms consist of five different combinations of M and H polypeptide chains which exist as M_4, M_3H, M_2H_2, MH_3 and H_4 tetramers (Holbrook et al., 1975). All five isoforms exist in both white and red fiber types. However, the M containing isoforms are more predominant in white fiber types and H containing isoforms in red. Townsend and Davis (1991) and Townsend et al. (1993) determined LDH in light meat to be less heat stable than that in dark meat. Similar findings were observed by Wang (1994). Polyacrylamide gels stained with LDH specific stain showed only M_2H_2, MH_3, and H_4 isozymes present in turkey thigh rolls processed to 68.9°, 70.0°, and 71.1°C. These results indicated that M_4 and M_3H were less heat stable than the M_2H_2, MH_3, and H_4 isozymes.

Lactate dehydrogenase has been extensively studied as a potential EPT indicator in beef, pork and turkey muscle (McCormick et al., 1988; Collins et al., 1991a,b; Stadler et al., 1991; Wang et al., 1992; Abouzied et al., 1993; Wang et al., 1993). Lactate dehydrogenase as an EPT marker for cured poultry products has not been investigated.

Pyruvate kinase. Pyruvate kinase (PK) catalyzes the formation of pyruvate from phosphoenolpyruvate, the final reaction in glycolysis. The reaction forms 2 moles of ATP for each mole of glucose entering the glycolytic pathway.

The molecular mass of PK is approximately 237,000. SDS-PAGE yielded a subunit molecular mass of appproximately 57,000-59,000, suggesting that PK exists as a tetramer (Kiffmeyer and Farrar, 1991). The four known isoforms of PK are designated as L, M_1, M_2, and R. Pyruvate kinase has been identified as a potential EPT marker for canned picnic ham by Davis et al. (1988). Pyruvate kinase is present in relatively large amounts with high activity in muscle. The amount of PK in muscle is primarily based on the age of the animal.

Plasma Proteins

Plasma is the fluid portion of unclotted blood. The proteins that make up the plasma can be separated into several fractions as described by Cohn et al. (1946). The separation process depends on protein solubility in ethanol solution at -1° to -5°C with varying pH. Fraction I, II, and III are made up of fibrinogen, gamma globulins, and lipoproteins, respectively. Fraction IV is composed of various enzymes and proteins. Fraction V is approximately 95% pure albumin, which constitutes almost 50% of total protein in blood (Price and Schweigert, 1987). The use of animal serum proteins, especially albumin, have many significant applications in diagnostics, research, and vaccine manufacture (Peters, 1980).

Turkey Serum Proteins as Potential EPT Markers

Two plasma proteins of interest in this study are turkey serum albumin (TSA) and immunoglobulin G (IgG). Turkey serum albumin and IgG are the two most predominant proteins in the serum (Berne and Levy, 1988).

Turkey serum albumin. Albumin is the principal protein in the serum (Berne and Levy, 1988). Serum albumin has a molecular mass of 66,000 and serves various functions in the blood (Tietz, 1976). These functions include transport of poorly soluble compounds such as bilirubin, cortisol, and aldosterone. In addition, serum albumin binds toxic heavy metals, maintains osmotic pressure and provides a protein reserve in the plasma. Significant decreases in serum albumin are caused by protein loss due to infection and illness (Pearson and Young, 1990).

Immunoglobulin G. Immunoglobulin G constitutes 70% of serum immunoglobulins, making it the second most abundant serum protein (Berne and Levy, 1988). Immunoglobulin G is a monomer consisting of two heavy and two light chains covalently attached by disulfide bonds. The molecular mass of IgG is approximately 150,000. The heavy and light chains have molecular masses of 60,000 and 20,000, respectively. There are four subclasses of IgG which are completely cross reactive.

Residual Blood in Meat

The level of TSA and IgG in muscle prior to thermal processing is conditional upon the amount of residual blood in the musculature. The factors affecting the residual blood content in meat has been the focus of many investigations (Warriss, 1977; Warriss, 1978; Warriss and Leach, 1978; Williams et al., 1983; Gregory and Wilkins, 1989a,b, 1990; Fleming et al., 1991; Mohan Raj and Gregory, 1991). The vascular system of muscle consists primarily of small blood vessels. The degree to which the small blood vessels are dilated or constricted determines the amount of residual blood after exsanguation (Warriss, 1977).

The effects of slaughtering methods on residual blood in sheep were studied by Warriss and Leach (1978). The authors designated hemoglobin as the index for residual blood. Of all stunning methods, pithing, and bleeding-out positions examined, none had an effect on residual hemoglobin content of the muscle. In addition, there was no relationship between the residual hemoglobin content of muscle and amount of blood lost at slaughter. The authors concluded the degree of dilation of the small blood vessels and stress associated with stunning and exsanguation ensures minimal retention of blood, regardless of slaughter methodology.

The influence of delayed bleeding on sensory

characteristics of beef was investigated (Williams et al., 1983). The amount of blood retained in the carcass was greater when exsanguation was delayed after stunning; However, no differences were found in total pigment or hemoglobin in the muscle tissue. Tenderness, juiciness, flavor, shear values, and cooking loss were not affected by delayed bleeding.

Gregory and Wilkins (1989a,b,; 1990) studied the effects of slaughtering methods on bleeding efficiency and subsequent quality defects in broilers. Electrically stunned chickens in which cardiac arrest or ventricullar fibrillation (VF) was induced were observed. The authors concluded that none of the downgrading characteristics such as red wingtips, red necks, and hemmorhaging were attributable to electrical stunning. Bleeding efficiency was determined as the factor responsible for downgrading regardless if either cardiac arrest or VF occurred at stunning.

Consumers view pink color in cooked chicken breast meat as highly undesirable (Fleming et al., 1991). In addition to age, sex, strain and preslaughter factors, heme pigment concentrations markedly affect muscle color (Ahn and Maurer, 1989). Fleming et al. (1991) studied the effects of ice-slush-chilling versus air-chilling on levels of heme pigments and HunterLab values in chicken gizzard, breast, and thigh muscles. The authors indicated cytochrome c

levels increased when broilers were air-chilled, whereas HunterLab colorimetric analysis showed no significant differences.

Hemoglobin and total pigment are commonly used by most researchers as an index for residual blood. Hemoglobin is the predominant pigment (90-95%) in living animals. Sixty percent of total hemoglobin is lost by bleeding while the viscera retains 20-25%, carcass 15-20%, and musculature approximately 10% (Warriss, 1977). Warriss and Rhodes (1977) reported that only 0.3% of the total blood is retained in meat as sold by retailers. When determining total pigment, both myoglobin and hemoglobin are measured. The quantification of serum proteins as an index for residual blood has not been investigated.

METHODS AND MATERIALS

Experimental Design

This study consisted of five experiments. The first experiment was designed to identify possible protein markers for determining EPT. Turkey ham was processed to targeted internal temperatures, soluble proteins extracted and used to determine potential EPT protein markers. The second experiment involved the production of polyclonal antibodies (pAb). Polyclonal antibodies were made against the EPT protein markers, turkey serum albumin (TSA) and chicken muscle pyruvate kinase (PK). Anti-turkey Immunoglobulin G (IgG) was purchased from Sigma (St. Louis, MO 63178) and anti-turkey LDH monoclonal (D5E) and polyclonal antibodies (F) were produced previously (Wang et al., 1992; Abouzied et al., 1993). The third experiment utilized the results from the first two experiments to develop ELISA's. Indirect competitive ELISA's (IC-ELISA) were developed using anti-TSA, anti-PK, and anti-Turkey IgG pAb. An IC-ELISA and sandwich ELISA using LDH monoclonal and polyclonal antibodies (Wang et al., 1992; Abouzied et al., 1993) were also performed. The application of ELISAs for EPT verification in turkey ham was performed in the fourth experiment. The final experiment was conducted to evaluate

the effects of delayed bleeding on residual blood in turkey breast and thigh muscle. Serum protein levels, total pigment and colorimetric analysis was performed.

Identification of Potential Indicator Proteins

Processing of turkey ham. Formulated, uncooked turkey ham stuffed into semi-permeable casings were obtained from Bil-Mar Foods, Inc. (Zeeland, MI 49464) and transported to Michigan State University. The turkey ham was formulated using fresh, boneless, skinless turkey thigh meat, water, salt, sugar, sodium phosphate, ascorbic acid, citric acid, and sodium nitrite. Proximate analysis was performed and supplied by Bil-Mar Foods, Inc. Prior to smokehouse processing, the hams were 10.80 cm in diameter, 30.5 cm in length, and weighed an average of 2.67 kg. Turkey hams were processed in a microprocessor (Model PC-5809, Powis Corp., Oak Grove, MO 63080) controlled smokehouse (Drying Systems, Inc., Chicago, IL 60601) to predetermined target internal temperatures of 66.9, 68.3, 69.7, 71.1, 72.5, and 73.8°C using the smokehouse schedule listed in Table 1. Internal temperatures were monitored by placing RTD probes (Omega, Stamford, CT 06907) in the geometric center of the roll. Internal temperatures were monitored throughout the cooking and cooling cycle. When the internal temperature was within 1°C of the target temperature, the ham was removed and

Table 1. Smokehouse processing schedule for turkey ham[a]

Stage	Cycle time (min)	Internal temp.(°C)	Dry bulb (°C)	Wet bulb (°C)
1	60	(30.0±2.0)	60.0	60.0
2	120	(53.3±1.5)	65.6	65.6
3	60	(60.0±2.3)	71.1	71.1
4	60	(65.6±2.3)	76.7	76.7
5	(122±5)[b]	73.8	82.2	82.2

[a]Total cook time for turkey ham processed to an internal temperature of 73.8°C was about 7.1 hr.
[b]Values in parentheses indicate mean ± standard error of internal product temperature at the end of each cycle of cycle time to reach internal temperature of 73.8°C.

placed in ice slush for 1.5 hr to prevent post-cook rise. The cooled hams were stored overnight at 4°C prior to analysis. All instrumentation was calibrated using manufacturers guidelines. Processing was performed in triplicate using smokehouse cycles of about 7 hr.

Extraction of protein from turkey ham. A 3 cm diameter core was cut from the geometric center of a 2.54 cm slice of turkey ham. After mincing, 25 g of ham was extracted using 3 volumes (w/v) cold phosphate buffer saline (PBS, 0.15 M NaCl, 0.01 M Na phosphate buffer, pH 7.2) in a Waring blender for a total of 90 sec (30 sec on, 10 sec off, repeated three times). The homogenate was immediately centrifuged at 16,000 x g for 20 min at 4°C. The supernatant was collected after filtration (Whatman No. 1) and held at 4°C. The protein concentration was determined by biuret method using bovine serum albumin (BSA) as the standard (Gornall et al., 1949). The concentration was calculated from the BSA standard curve and expressed on a per gram meat basis.

Determination of pH. Ten grams of ham was minced and homogenized in a Waring blender with 90 mL of distilled water for 1 min. The pH of the homogenate was measured using a Corning 145 pH meter (Fisher Scientific, Itasca, IL 60143) at 25°C.

Electrophoresis. To determine potential protein markers, sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) was performed (Laemmli, 1970). A Mini-Protean Gel assembly (Bio-Rad Laboratories, Hercules, CA 94547) was used to form a 4% acrylamide stacking and 12% acrylamide resolving gel. The protein extracts were combined 1:1 or 1:4 with 0.0625 M Tris Sample buffer (pH 6.8), placed in boiling water and held for 15 min. Ten microliters (μl) of prepared extracts (1.5 μg/μl) were loaded on the gel using a glass syringe. High and low molecular mass markers (Sigma) were used to tentatively identify the unknown protein bands. Standard protein markers were used (TSA-A4650, PK-P6406, IgG-T8410 , LDH-L9887, Sigma) to verify the identity of protein bands which decreased in intensity as processing temperature of the ham increased. Electrophoresis was performed using Tris-glycine electrode buffer (0.025 M Tris, 0.192 M Glycine, pH 8.3), a current of 55 milliamps and a voltage of 200 for 45 minutes. The gels were stained with Coomassie Brilliant Blue R 250 (Sigma), which has a detection limit of 0.1 to 1 μg protien per band. Calculation of R_f values (Weber and Osborn, 1969) for TSA, IgG and PK standard protein markers were performed.

Determination of enzymatic activity. The enzymatic activity of LDH and PK was determined. An LDH diagnostic kit (DG 1340-K, Sigma) was used to determine the activity at 25°C. The activity was determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH:

$$NADH + pyruvate \xrightarrow{LDH} lactate + NAD^{+}$$

One unit (U) of LDH activity is expressed as 1 μmol NADH oxidized per minute at 25°C, pH 7.6, under specified conditions. Pyruvate kinase activity was determined using an assay adapted from Bergmeyer (1974)(Table 2). The activity was determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH:

$$PEP + ADP \xrightarrow{PK} pyruvate + ATP$$

$$pyruvate + NADH \xrightarrow{LDH} lactate + NAD^{+}$$

One unit (U) of PK activity was expressed as 1 μmole NADH oxidized per minute at 25°C, pH 7.6. The total volume of the assay was 2.98 mL. The buffer was held at 25°C and other reagents at 4°C. The first three reagents were added to a quartz cuvette and allowed to equilibrate for 1 min. The remaining reagents were added quickly, the cuvette was covered and inverted 3 times before placing in the spectrophotometer (Lambda 4B Perkin-Elmer, Norwalk, CT 06856). The decrease in absorbance was monitored for 3 min. The optimal range for activity was change of 0.02-0.04 Absorbance units/min. Extracts were diluted to acheive activity in this range. Pyruvate kinase activity was expressed as units per g meat.

Table 2. Pyruvate kinase enzyme assay procedure

Volume(mL)	Reagent	Concentration in assay
2.50	Triethanolamine buffer 0.1M, pH 7.6	85.6 mM
0.20	PEP[a] (3.75 mg/mL) in 0.05M $MgSO_4$/0.2M KCl	0.54 mM
0.20	ADP, neutralized with KOH, 30 mg/mL	4.7 mM
0.05	NADH, Na salt (10 mg/mL)	0.2 mM
0.01	LDH (5 mg/mL)	9.2 U/mL
0.02	Sample extract	

[a]Phosphoenolpyruvate

Production of Anti-TSA and Anti-PK Polyclonal Antibodies

Production of polyclonal antibodies in rabbits. Six White New Zealand female rabbits, six weeks old, were purchased (Hazelton Laboratories, Kalamazoo, MI 49001). The rabbits were housed by Michigan State University Laboratory Animal Care Services (LACS). The LACS personnel performed all immunizations and blood collection. The rabbits (3 per group) were subjected to the same immunization protocol. The immunization schedule and concentration of protein were the same as those commonly used for polyclonal antibody production. Rabbits A, B, and C were immunized with commercial TSA (A4650 Sigma) and rabbits D, E, and F were immunized with commercial chicken muscle PK (P4406 Sigma). Purity of TSA and PK was determined using SDS-PAGE, prior to injection, in which single bands were observed. The initial injection consisted of 500 μg antigen in 0.5 mL 0.85% NaCl (saline) emulsified with 0.5 mL Freund's complete adjuvant (Sigma). The antigen-adjuvant was subcutaneously injected at about 10 sites on the back of each rabbit. Each booster injection consisted of 500 μg of protein emulsified with Freund's incomplete adjuvant (Sigma) for a total volume of 1 mL per rabbit.

Conjugation of the protein to the adjuvant was carried out using two 5cc glass syringes connected using a 2.2 cm microemulsifying needle (18G-2 Perfektum, Popper & Sons,

Inc., New Hyde Park, NY 11040). Just prior to injection, a total of 4 mL of protein-adjuvant emulsion was made, by diluting 2000 μg protein in 2 mL saline. This fraction was drawn into one of the 5cc glass syringes. Two milliliter of either complete or incomplete adjuvant was drawn into the other syringe. The two syringes were connected via the microemulsifying needle. Caution was used to completely eliminate air bubbles in the syringes and needle. The plunger of each syringe was sequentially depressed to force the mixture through the needle. After 45 min of continuous action, the mixture was tested for complete conjugation by placing a droplet in 4°C PBS. If the droplet maintained a spherical shape, the conjugation was complete and ready for injection. If the droplet spread out in an irregular pattern, the emulsifying action was continued until conjugation was complete. Occasionally, additional adjuvant was required to form a proper emulsion. The booster injection schedule is listed in Table 3. Rabbits were bled at weeks 6, 10, and 15 from the marginal ear vein. At week 23, the blood was collected using cardiac puncture. Antibodies from each treatment group were purified using a modification of the ammonium sulfate method described by Hebert et al. (1973). After collection, the blood coagulated overnight at 4°C. The blood was centrifuged at 16,000 x g for 30 min at 4°C. The red blood cell-free supernatant (serum) was collected and volume recorded.

Table 3. Immunization schedule for polyclonal antibody production in rabbits

Time (weeks)	Immunization
0	initial
4	booster
8	booster
13	booster
21	final boost

Saturated ammonium sulfate adjusted to a pH of 7.6 using 10 N NaOH, was added drop by drop to the supernatant (with stirring) to a saturation of 33% (v/v) and stirred for an additional 30 min. After centrifugation at 16,000 x g for 30 min at 4°C, the supernatant was discarded. The pellet was dissolved and brought to original volume of serum using saline (0.85% w/v NaCl) filtered through a 0.2 μL filter. The ammonium sulfate saturation was repeated two more times. The final pellet was resuspended in 5 mL of filtered saline. The purifed serum was dialyzed for 3 days against PBS, pH 7.2, at 4°C, with buffer changes every 24 hr. The dialyzed serum was brought to original serum volume, aliquoted into vials and stored at -20°C.

Indirect ELISA was used for determining titer during the production of polyclonal anti-TSA and anti-PK antibodies. Removawell strip holders (Dynatech, Chantilly, VA, 22021) were fitted with Immunlon 4 Removawell strips (Dynatech). Plates were coated with the antigen (purified EPT protein markers) in 0.1 M carbonate buffer, pH 9.6 at a final concentration of 5 μg/mL and allowed to bind overnight at 4°C. The plates were washed 4 times in a manual ELISA plate washer (EL401, Fisher Scientific) with 0.01 M PBS, pH 7.2, containing 0.05% Tween 20 (Sigma)(PBST). Next, plates were blocked with 1.5% (w/v) gelatin (Sigma) in PBS (PBSG), by adding 300 μl of PBSG to each well and incubating for 30 min at 37°C. After incubation, the plates were washed 4

times with PBST and 50 µl of purified serum (polyclonal antibodies) serially diluted with 0.5% PBSG was added to each well and incubated at 37°C for 1 hr. Next, plates were washed 8 times with PBST, and 100 µl goat anti-rabbit IgG peroxidase conjugate (Organon Teknika, Durham, NC 27704) diluted 1:500 in 0.5% PBSG, was added to each well and incubated for 30 minutes at 37°C. After washing plates 8 times with PBST, bound peroxidase activity was determined by adding 100 µl of 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (A-1888 Sigma) (ABTS)-H_2O_2 substrate (11.0 mL citrate buffer, 1.0 mL ABTS stock solution, 8.0 µl H_2O_2) to each well (Pestka et al., 1982). Color development was inhibited by the addition of 100 µl stopping reagent (63 g citric acid monohydrate, 1 g sodium azide, in 1 l dH_2O). Absorbance was read using Vmax Kinetic Microplate Reader, (Model 1234, Molecular Devices Corporation, Menlo Park, CA 94025) at 405 nm. The titer was arbitrarily designated as the dilution at which the optical density reading for the immune serum (antibody) was twice that of the non-immune serum at the same dilution.

Sensitivity and specificity of anti-TBA and anti-PK polyclonal antibodies. Indirect competitive ELISA was used to determine sensitivity and specificity. The methodology is almost identical to that of the indirect ELISA, except that 50 µl antigen was added to each well simultaneously with 50 µl diluted pAb. The sensitivity of

antisera from each rabbit was determined using competitive antigen concentrations of 0.5, 1, 5, 10, and 50 μg/mL. The antisera from each rabbit was diluted 1:10,000. The results were expressed as binding inhibition (%) versus antigen concentration. Binding inhibition (%) is calculated by dividing the absorbance resulting from competition by the control (no competitive antigen) and converting to a percentage. The antisera from rabbit A (anti-TSA pAb) and rabbit D (anti-PK pAb) was used in all remaining experiments.

Cross-reactivity of anti-TSA pAb with serum albumin from bovine (P0033217, American Research Products Co., Solon, OH 44139), porcine (A2764, Sigma), chicken (A3014, Sigma), rabbit (A0639, Sigma), sheep (A3264,Sigma) was determined. Cross-reactivity of anti-chicken PK pAb with PK from porcine muscle (P2040, Sigma) and rabbit muscle (P1506, Sigma) was also determined. Competitive antigens were added to the microtiter wells in concentrations of 10, 100, 500, 1000, 2500, 5000, and 10,000 ng/mL. Antisera was diluted 1:10,000. Results were expressed as absorbance (404 nm) versus competitive antigen concentration (μg/mL).

Confirmation of indicator proteins by Western blot. Western blot was performed in a Mini Trans-Blot unit (Bio-Rad Laboratories). After running SDS-PAGE on the protein extracts, the gels were equilibrated in 4°C Transfer buffer (25 mM Tris, 192 mM glycine, and 20%(v/v) methanol, pH 8.3)

for 15 min. Filter paper, nitrocellulose membrane (0.45 μm, Schleicher & Schuell, Keene, NH 03431) and fiber pads were soaked in transfer buffer for 30 min. The gel holder cassette was assembled by placing a fiber pad flat against the surface. Next, saturated filter paper was placed on the pad, then the SDS-PAGE gel. The membrane was then placed on the gel, flooded with transfer buffer and air bubbles eliminated. Next the filter paper and fiber pad, completed the sandwich. The procedure was repeated for both gels. The cassettes were placed in the mini Trans-Blot electrode buffer chamber with the Bio-Ice cooling unit and transfer buffer. The proteins were transfered for 1 h at 100 V and current of 350 milliamps. After transfer the membranes were washed with PBST (2 min, 2 times), blocked for 30 min using 1.5% PBSG at room temperature, and washed with PBST (2 min, 2 times). Polyclonal antibodies against TSA, PK, IgG, and LDH diluted 1:3000 in 0.5% PBSG were incubated separately with a blocked membrane for 30 min at room temperature. After washing with PBST (1 min, 2 times), goat anti-rabbit IgG peroxidase conjugate diluted 1:2000 in 0.5% PBSG was added and incubated for 10 min. The bound peroxidase was determined after washing with PBST (2 min, 3 times), using substrate solution (24 mg 3,3', 5,5'-tetramethyl benzidine and 80 mg diocytl sulfosuccinate in 10 mL ethanol, 30 mL 0.1M citrate-phosphate buffer, pH 5.0 and 20 μl 30% H_2O_2). After the color developed, the reaction was stopped by

washing with distilled water.

Development of ELISAs

Indirect-competitive ELISA. Indirect competitive ELISA (IC-ELISA), as described previously, was used to determine the concentration of TSA, PK, IgG, and LDH in the protein extracts. The antigen used for the standard curve was added at concentrations of 625, 1250, 2500, 5000, 10,000 ng/mL. The heat-processed ham extracts were diluted 1:1, 1:5, 1:10, 1:100, and 1:1000 times using 0.5% PBSG. The raw protein extracts were diluted 1:5, 1:10, 1:100, 1:1000, and 1:10,000 times using 0.5% PBSG. Anti-TSA pAb was diluted 1:10,000, anti-PK pAb 1:5000, anti-IgG pAb 1:5000, and anti-LDH pAb 1:30,000. Results were quantitated using the standard curve and then converted to a nanogram per gram meat basis.

Sandwich ELISA. Sandwich ELISA was performed as described by Abouzied et al. (1993) for LDH protein marker only. The plate was coated with LDH monoclonal Ab at 1:350 dilution. The LDH pAb was diluted 1:1500. Turkey LDH was added at concentrations of 5, 10, 25, 50, 100, and 1000 ng/mL to prepare the standard curve. The heat-processed extracts were added at dilutions of 1:2, 1:5, 1:25, 1:100, 1:500, and 1:1000. The raw extract was added at the dilutions of 1:10, 1:100, 1:1000, 1:3000, 1:10000, and 1:30000. The results were quantitated using the standard

curve and then converted to a nanogram per gram meat basis.

Application of ELISAs for EPT Verification

To determine viable indicator proteins for EPT, protein extracts from heat-processed turkey hams were assayed using IC-ELISA or sandwich ELISA. Concentration of the indicator proteins in ham extracts were calculated from standard curves. The concentration of EPT marker proteins were expressed as ng per g meat. Four separate IC-ELISAs were performed, utilizing anti-TSA, anti-PK, anti-IgG, and anti-LDH polyclonal antibodies. A sandwich ELISA was performed using anti-LDH monoclonal and polyclonal antibodies.

Statistical Analysis

A randomized complete block design was used to calculate analysis of variance (ANOVA) for all experiments. The error mean square from ANOVA tables were analyzed using orthogonal polynomial contrasts to determine relationship (e.g. no relationship, linear, or curvilinear) between dependent variables and proccessing temperature. All calculations were performed using MSTAT (1989) statistical software. Three replicates of turkey ham at each temperature were analyzed.

Delayed Bleeding Study

Slaughter protocol. Sixteen birds were electrically stunned for 10 seconds at 41 V and 2.25 amps on a slaughter line at a rate of 33.5 birds per min at Bil-Mar Foods, Inc. Four control birds were cut with an automatic circular blade 14 sec after stun and bled. The second and third set of birds (4 each) had a 50 sec and 100 sec delay before cut, respectively, and subsequently bled. The last set of four stunned birds was not cut. All birds were processed commercially under USDA supervision and were chilled in ice for 10 hr separate from commercial product. The birds were manually deboned on site. The *Sartorius* (thigh) and *Pectoralis major* (breast) muscles were collected for the study. The remainder of the carcass was disposed of and not used for human consumption. The pH and temperature of each muscle was recorded immediatley after deboning using an internal probe (Solomat Partners Lt., Stamford, CT 06906). Muscles were transported to Michigan State University in ice, held at 4°C and analyzed the next day. Three replicates from each treatment were arbitrarily chosen for further study. Prior to analysis, breast and thigh muscles were ground twice through a 4 mm plate in a food grinder (Model K5-A Kitchen Aide, Troy, OH 45374).

HunterLab color analysis. Ground muscles were pressed into a 10 cm diameter plastic dish and immediately

analyzed utilizing a HunterLab colorimeter (DP-9000 Hunter Associates Laboratory, Inc. Reston, VA 22090). In HunterLab anaylsis, L* values depict lightness (+) to darkness (-), a* values redness (+) to green (-) and b* values yellowness (+) to blue (-). Black and white (X=82.09, Y=84.31, and Z=97.56) tiles were used as standards and the pink tile (X=49.0, Y=44.0, and Z=42.1) as the reference for L*, a*, and b* readings. The tiles were calibrated in 1992. Each muscle was read in triplicate with a 45˚ turn between readings.

Protein extraction and total pigment determination. Fifty grams of ground muscle was homogenized with 100 mL of 0.1 M potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 16,000 x g for 10 min. The procedure was repeated twice to remove all pigment. Supernatants were combined, filtered (Whatman No. 1), and protein content was determined by biuret method using BSA as the standard (Gornall et al., 1949). Protein concentration was expressed as milligram protein per gram meat.

Total pigment was determined using a modification of the alkaline hematin method described by Karlsson and Lundstrom (1991). Four milliliters of muscle extract was combined with 400 μL of 10% TritonX-100 (Sigma) and 250 μL of 5.0 N NaOH. Due to cloudiness, the method was modified by placing extract plus reagents in a 50˚C water bath for 10 min. The cloudiness disappeared and the samples were read

at 5

Perk

from

abso

to q

chlo

musc

extr

usin

desc

dilu

conc

The

1:10

prot

Stat

calc

The

orth

(e.g

depe

were

at 575 nm and 700 nm in a spectrophotometer (Lambda 4B Perkin-Elmer). The absorbance at 700 nm (A) was subracted from the absorbance at 540 nm (B) to eliminate background absorbance readings. The adjusted absorbance (B-A) was used to quantify total pigment using a standard curve with hemin chloride as the standard. Hemin content (total pigment) of muscle was expressed as μicrogram per gram meat.

Determination of serum proteins. Breast and thigh extracts were also evaluated for serum protein concentration using anti-whole turkey serum pAb (Sigma) in IC-ELISA, as described previously. The anti-whole turkey serum pAb was diluted 1:8000. The standard (TSA) was added at concentrations of 0, 100, 500, 1000, 5000, and 10000 ng/mL. The muscle extracts were added at dilutions of 1:10, 1:50, 1:100, 1:1000, 1:5000, and 1:10000. The content of serum proteins was expressed as μicrogram per gram meat.

Statistical Analysis

A randomized complete block design was used to calculate analysis of variance (ANOVA) for all experiments. The error mean square from ANOVA tables were analyzed using orthogonal polynomial contrasts to determine relationship (e.g. no relationship, linear, or curvilinear) between dependent variables and delayed bleeding. All calculations were performed using MSTAT (1989) statistical software.

Thr

tre

Three replicates of turkey breast and thigh muscle from each treatment were analyzed.

RESULTS AND DISCUSSION

Thermal Processing of Turkey Ham

Turkey ham was thermally processed to six target internal temperatures by monitoring product temperature throughout cooking and cooling cycle (Table 4). Post-cook rise was stopped by immediate submersion in an ice bath. The internal temperature was monitored every 30 sec for 3 min after placing ham in ice bath. No increase in internal temperature was observed. Internal inspection of post-processed turkey hams verified the placement of thermocouple in the geometric center of the ham.

Determination of pH and Proximate Analysis

The pH of the uncooked turkey ham was 6.5, whereas cooked samples averaged 6.6. Proximate composition of the turkey ham was provided by Bil-Mar Foods, Inc. (Table 5).

Determination of Potential EPT Indicator Proteins

Protein extraction. Extractable water-soluble protein content decreased linearly with increasing processing temperature at an α level of 0.005 (Table 6). At 73.7°C, the extractable protein is about 2 mg lower than at 66.8°C.

Table 4. Target and final internal temperatures of turkey ham after smokehouse processing[a]

Target Temp. (°C)	Actual Temp.[b] (°C)
66.9	66.8±0.1
68.3	68.3±0.1
69.7	69.7±0.2
71.1	71.1±0.1
72.5	72.2±0.1
73.8	73.7±0.1

[a]Total cook time for turkey ham processed to an internal temperature of 73.7°C averaged 7.1 hr
[b]Expressed as mean ± standard error of three replicate values

Table 5. Composition of a 85 g serving of turkey ham

Component	Percentage
Moisture	70-72
Protein	17-19
Fat	3-5
Salt	2.4-2.6
Nutrient	**Weight (mg)**
Iron	1.5
Calcium	5.7
Sodium	980
Thiamine	0.1
Riboflavin	0.2
Niacin	4.1
Cholesterol	2.3

Table 6. Effect of endpoint cooking temperature on extractable protein content in turkey ham

Temperature (°C)	Protein[ab] (mg/g meat)
66.8	8.6
68.3	8.6
69.7	7.9
71.1	6.8
72.2	7.2
73.7	6.9

[a]Expressed as mean of three replicate values
[b][Protein]/Temp. response is linear, α=0.005

Similarly, Wang et al. (1992) reported extractable protein content did not differ (P>0.05) in turkey breast rolls processed between 68.3° and 72.1°C. Extractable protein content was not indicative of EPT reached during thermal processing.

Electrophoresis. Electrophoretograms representative of extracts from turkey ham processed to EPT between 66.8° and 73.7°C are shown in Figures 1-3. The extract from uncooked (Raw) turkey ham contained 20 distinct bands. The intensity of bands at 66,000-Da and 35,000-Da decreased with increased heat treatment. The intensity of the 66,000-Da band did not markedly decrease between 66.8° and 68.3°C; However, at 73.7°C the band is barely visible. The 35,000-Da band decreased in intensity between 66.8° to 72.2°C, but was not detectable at 73.7°C. These bands were tentatively identified as TSA (66,000-Da) and LDH (35,000-Da) based on molecular mass (Figure 1). Wang et al. (1992) also observed decreases in 66,000-Da and 35,000-Da proteins in SDS-PAGE of extracts from turkey breast rolls processed between 68.3° and 72.1°C. The authors used LDH specific stain and Western blot analysis to positively identify the 35,000-Da protein as LDH, but did not conclusively identify the 66,000-Da band.

The heavy and light chains of IgG have molecular masses of 60,000-Da and 20,000-Da, respectively. The 66,000-Da

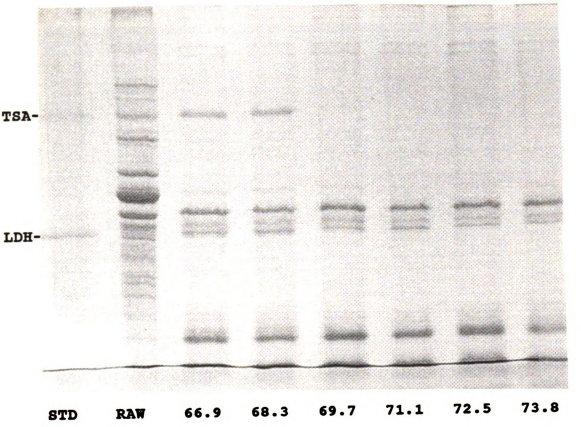

Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoretogram of extracts from first replicate of turkey ham thermally processed to six temperatures (°C). 10 μg and 20 μg of protein were loaded for raw and cooked samples, respectively. 5 μg of turkey serum albumin and turkey lactate dehydrogenase standard markers (STD) were loaded.

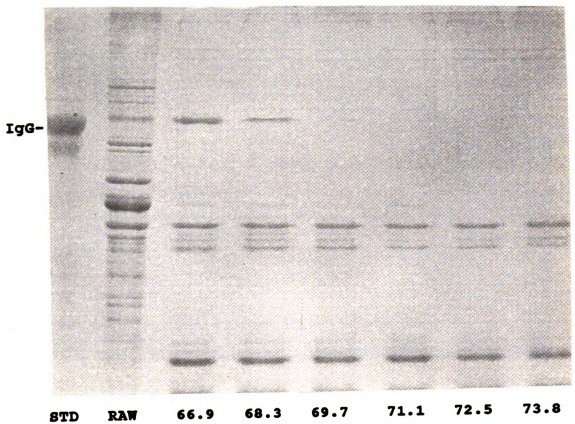

Figure 2: Sodium dodecyl sulfate polyacrylamide gel electrophoretogram of extracts from second replicate of turkey ham thermally processed to six temperatures (°C). 10 μg and 20 μg of protein were loaded for raw and cooked samples, respectively. 5 μg of chicken immunoglobulin G standard marker (STD) was loaded.

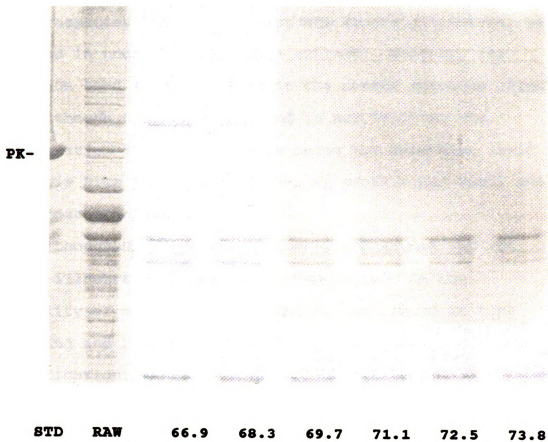

Figure 3: Sodium dodecyl sulfate polyacrylamide gel electrophoretogram of extracts from third replicate of turkey ham thermally processed to six temperatures (°C). 10 μg and 20 μg of protein were loaded for raw and cooked samples, respectively. 5 μg of chicken muscle pyruvate kinase standard marker (STD) was loaded.

protein may be the heavy chain of turkey IgG (Figure 2). The observed difference in relative mobility between the heavy chain of chicken IgG and turkey IgG may be attributed to intraspecies variation. Pyruvate kinase (57,000-Da) was observed in uncooked turkey ham extract; However, the 57,000-Da band is not visible in the cooked extracts (Figure 3). Although the 57,000-Da band is not visible, the concentration of protein may be below the detection limit of Coomassie Blue R 250 stain (0.1-1 μg protein per band) and thus undetectable.

Although SDS-PAGE is an established method and can simply illustrate the effect of temperature on the solubility of a variety of proteins, the method is time consuming and requires subsequent testing for quantification. Nevertheless, SDS-PAGE does provide a basis for identifying proteins for further study as potential EPT markers (Lee et al., 1974; Steele and Lambe, 1982).

Enzymatic analysis. The inactivation of LDH activity in response to increasing processing temperature of turkey ham was linear with an α level of 0.001 (Table 7). The LDH activity of uncooked turkey ham extracts was 210 U/g meat. After thermal processing of turkey ham to 72.2°C, LDH activity was 39 U/g meat. Similarly, Wang (1994) observed 275 U/g meat of LDH in uncooked uncured turkey thigh roll and 44 U/g meat of LDH in turkey thigh roll thermally processed to 72.2°C. Wang et al. (1992) reported 735 U/g

Table 7. Lactate dehydrogenase and pyruvate kinase activity (U/g meat) in turkey ham processed to internal temperatures between 66.8° and 73.7°C

Temp. (°C)	LDH[ab]	PK[ac]
66.8	59	0.03
68.3	59	0.02
69.7	46	0.02
71.1	40	0.02
72.2	39	0.02
73.7	31	0.02

[a]Expressed as mean of three replicate values
[b]LDH activity/Temp. response is linear, α=0.001
[c]PK activity/Temp. response, no relationship

meat LDH activity in uncooked turkey breast rolls and 1.2 U/g meat of LDH in turkey breast rolls thermally processed to 72.1°C. These results indicate LDH isoforms in thigh muscle are more heat stable than LDH isoforms predominantly found in breast muscle.

The PK activity of turkey ham extracts was not related to processing temperatures (Table 7). Pyruvate kinase activity in cooked turkey ham extracts was almost completely inactivated at 66.8°C. These results are in direct contrast to those found by Davis et al. (1988), who observed PK activity in pork heated to 68.6°C. The cured pork product, however, was studied in a model system and thermally processed in a 22 mm diameter glass tube and thus not indicative of a commercially prepared product.

Assessment of Anti-TSA and Anti-PK Polyclonal Antibodies

Titer determination. Polyclonal antibody production was completed in 24 weeks. Production was monitored by determining titers of antisera from rabbits A, B, C, D, E, and F (Table 8). All rabbits, except rabbit F, showed a 100-fold increase in titer from week 5 to week 9. At week 15, a 10-fold titer increase over week 9 was observed in rabbits immunized with TSA. The titers of rabbits immunized with PK showed no increase from week 9 to week 15, indicating that maximum titers were reached at week 9.

Table 8. Polyclonal antibody titers against turkey serum albumin (TSA) and pyruvate kinase (PK) in rabbits[a]

Week	Antibody Titer					
	TSA			PK		
	A	B	C	D	E	F
5	4.27×10^3	2.13×10^3	8.53×10^3	2.13×10^3	1.07×10^3	1.07×10^3
9	8.53×10^5	4.27×10^5	2.13×10^5	1.07×10^5	1.07×10^5	5.33×10^4
15	1.28×10^6	2.56×10^6	5.12×10^6	3.23×10^5	3.21×10^5	3.20×10^5

[a]Rabbits A, B and C immunized with TSA; Rabbits D, E and F immunized with PK

Similarly, Wang et al. (1992) produced pAb to chicken and turkey LDH in rabbits and ten weeks after initial immunization, antisera titers averaged $5.6x10^5$ and $7.5x10^5$ for rabbits immunized with chicken LDH and turkey LDH, respectively.

Sensitivity of antisera. Standard curves of antisera were prepared against TSA and chicken PK and analyzed by IC-ELISA (Figure 4 and 5). Anti-TSA pAb from rabbit A was used for the remainder of the study, because it showed the highest percent inhibition (60%) as compared to rabbit B (57%) and rabbit C (53%) in IC-ELISA. Anti-PK pAb from rabbit D was used for the remainder of the study, because it showed the highest percent inhibition (27%) as compared to rabbit E (23%) and rabbit F (18%). The percent inhibition in IC-ELISA of antisera from rabbits immunized with TSA was about 30% higher than PK-immunized rabbits and was attributed to lower sensitivity of anti-PK pAb versus anti-TSA pAb.

Specificity of antisera. The specificity of anti-TSA pAbs to turkey, chicken, rabbit, bovine, porcine and sheep serum albumin was determined using IC-ELISA. A decrease in absorbance of 0.8 units was observed when TSA increased from 10 to 10,000 ng/mL (Figure 6). Absorbance did not decrease with increasing concentration of serum albumins from all other species, except chicken, which indicated anti-TSA pAbs were not specific for rabbit, bovine, porcine or sheep serum

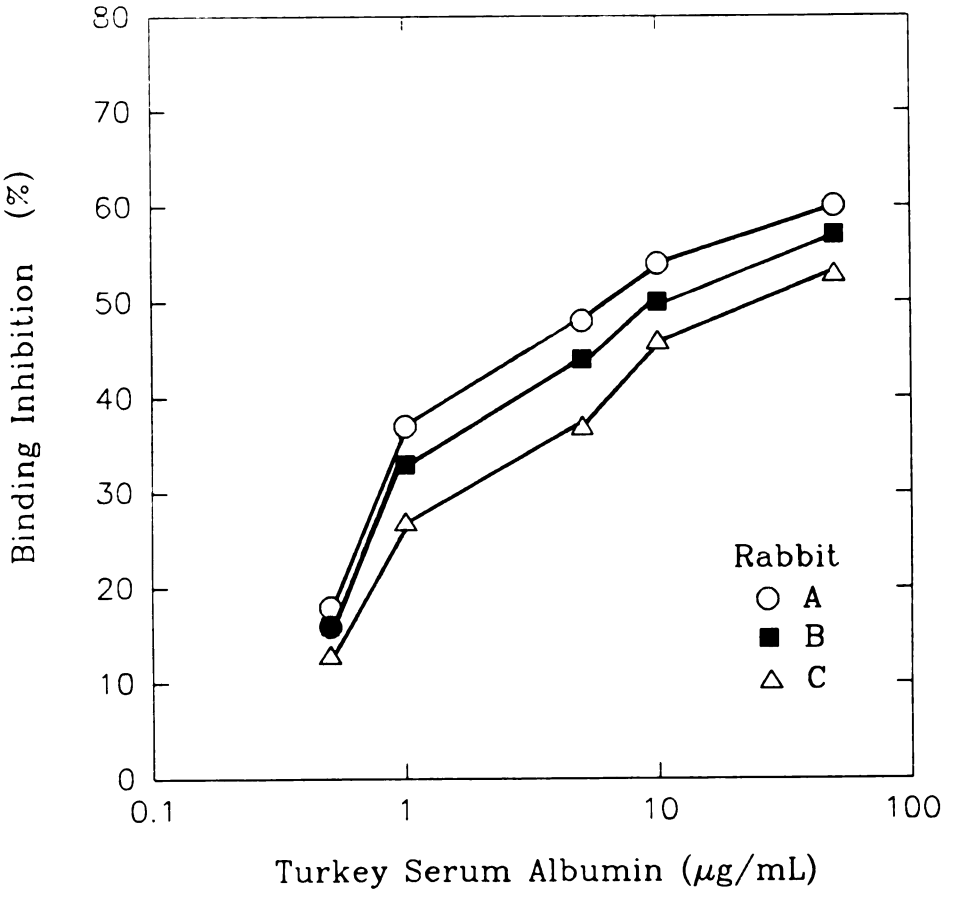

Figure 4: Standard curves of rabbit polyclonal antibodies prepared against turkey serum albumin. Turkey serum albumin was used as solid phase and as inhibitor in indirect-competitive enzyme-linked immunosorbent assay.

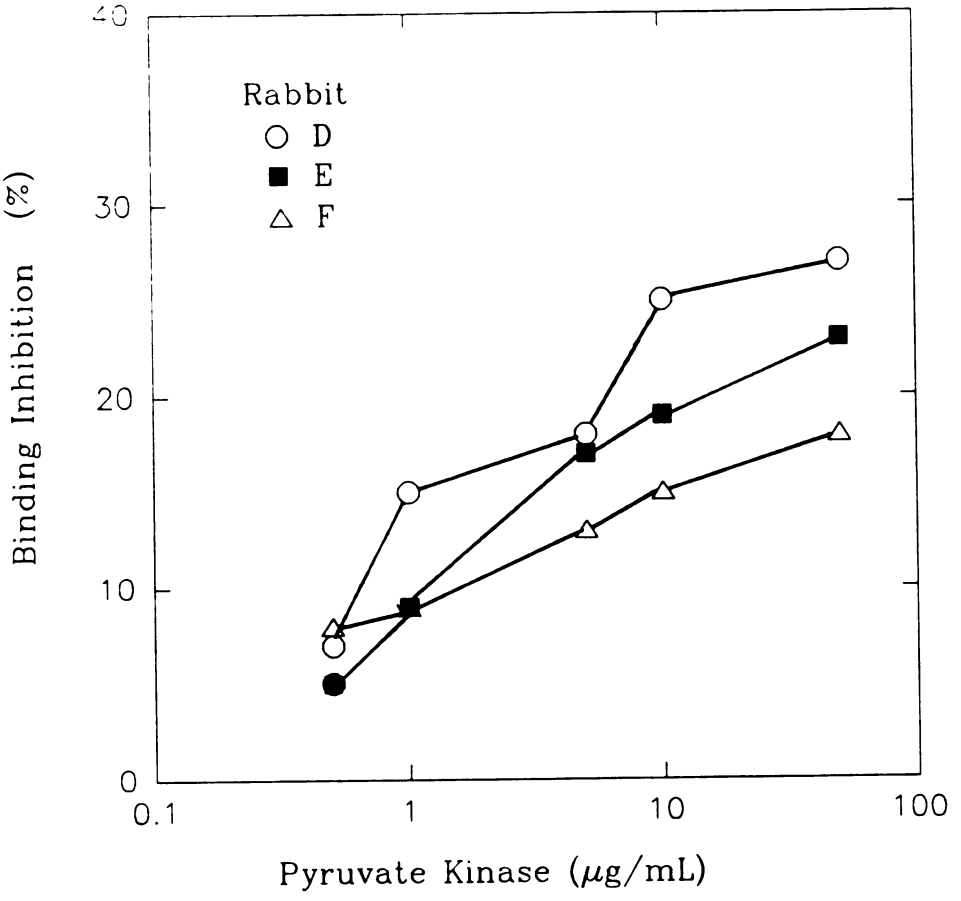

Figure 5: Standard curves of rabbit polyclonal antibodies prepared against chicken muscle pyruvate kinase. Chicken muscle pyruvate kinase was used as solid phase and as inhibitor in indirect-competitive enzyme-linked immunosorbent assay.

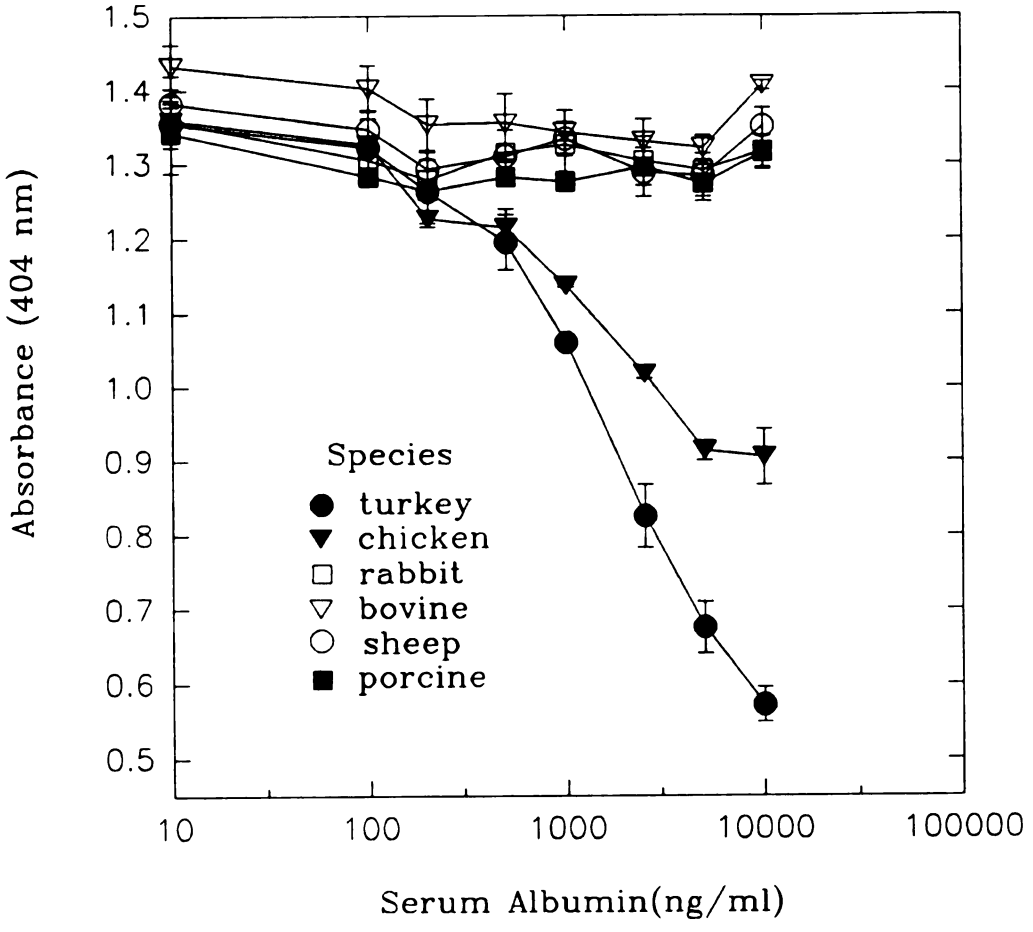

Figure 6: Cross-reactivity of anti-turkey serum albumin with serum albumin from various animal species. Antisera from rabbit A was used.

albumins. Anti-TSA antibodies cross-reacted with chicken albumin; However, the decrease in absorbance was only 0.5 units, which indicated a weaker interaction between anti-TSA pAb and chicken albumin.

The specificity of anti-PK antibodies to chicken, rabbit and porcine PK was determined using IC-ELISA (Figure 7). As chicken PK increased from 250 ng/mL to 10,000 ng/mL, absorbance decreased by about 0.2 units. However, a change in absorbance was not observed when concentrations of either porcine or rabbit PK increased.

Confirmation of EPT Indicators by Western Blot

Anti-LDH pAb positively identified turkey LDH standard marker and LDH in turkey ham extracts as a single band (Figure 8). The intensity of LDH in cooked ham extracts decreased with increasing temperature, but was still detectable at 73.7°C. The anti-TSA blot contained two major bands in TSA standard, uncooked and cooked ham extracts and several minor bands in uncooked and cooked ham extracts (Figure 9). The two major bands were designated as TSA-1 (top) and TSA-2 (bottom). The intensity of TSA-1 markedly decreased as processing temperature increased, but was not observed at 72.2° and 73.7°C. At 69.7°C, TSA-2 completely disappeared. Although the purity of TSA was established by SDS-PAGE prior to pAb production, the appearance of several

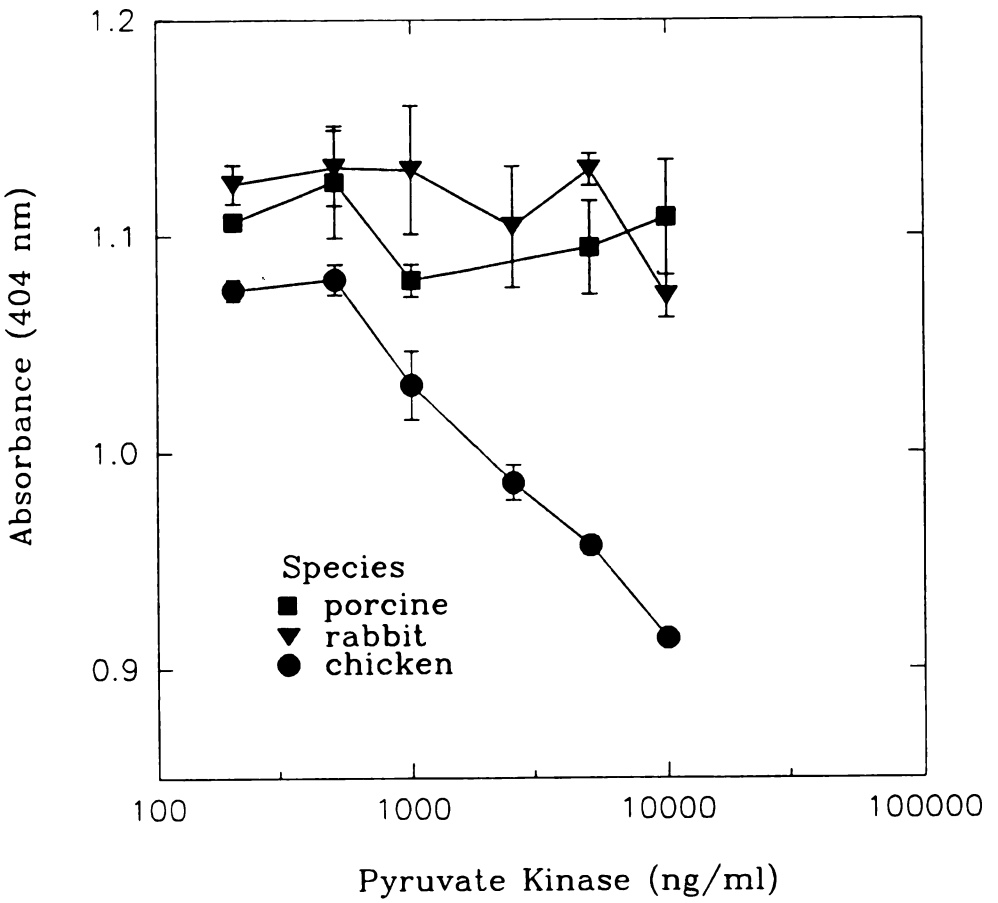

Figure 7: Cross-reactivity of anti-chicken muscle pyruvate kinase with pyruvate kinase from various animal species. Antisera from rabbit D was used.

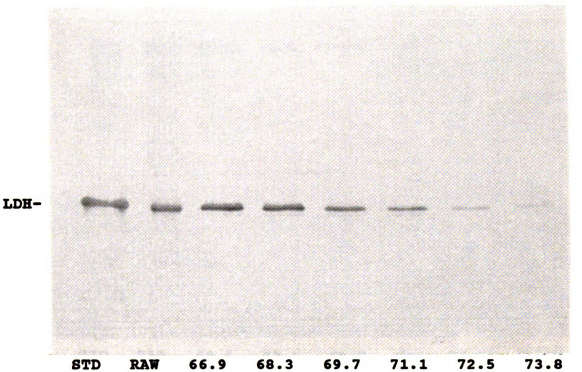

Figure 8: Western blot of extracts from turkey ham thermally processed to six temperatures (°C). 10 μg and 20 μg of protein were loaded for raw and cooked samples, respectively. 5 μg of lactate dehydrogenase standard marker (STD) was loaded. Anti-turkey lactate dehydrogenase antisera was used at dilution 1:3000.

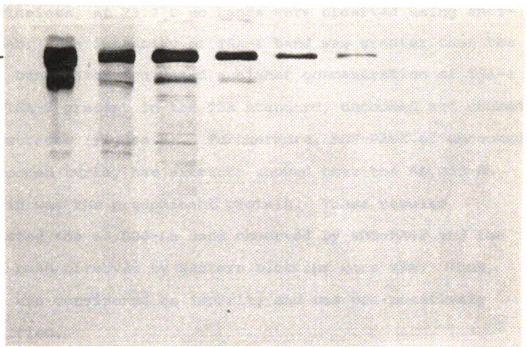

Figure 9: Western blot of extracts from turkey ham thermally processed to six temperatures (°C). 10 μg and 20 μg of protein were loaded for raw and cooked samples, respectively. 5 μg of turkey serum albumin standard marker (STD) was loaded. Anti-turkey serum albumin antisera from rabbit A was used at dilution 1:3000.

bands indicated the TSA standard was impure and anti-TSA pAbs were able to recognize other proteins besides TSA. Nevertheless, at 73.7°C no bands were observed using anti-TSA pAb. The intensity of TSA-1 band was greater than the TSA-2 band which indicated a higher concentration of TSA-1 than TSA-2 present in the TSA standard, uncooked and cooked ham extracts (Figure 9). Furthermore, SDS-PAGE of uncooked and cooked turkey ham extracts showed that the 66,000-Da protein was the predominant protein. These results suggested the 66,000-Da band observed by SDS-PAGE and the TSA-1 band observed by Western blotting were TSA. Thus, TSA-2 was considered an impurity and was not positively identified.

Anti-turkey IgG pAb identified the heavy chain in the chicken IgG standard and uncooked turkey ham extract (Figure 10). The heavy chain of IgG was not detected in cooked ham extracts; Therefore, the 66,000-Da band observed by SDS-PAGE was concluded to be TSA. Anti-PK pAb recognized the chicken PK standard and turkey PK in the uncooked extract in a single band, but PK was not observed in cooked ham extracts (Figure 11).

Development of ELISAs

Several trials were run to determine optimum conditions for ELISAs. The concentration of coating antigen, blocking

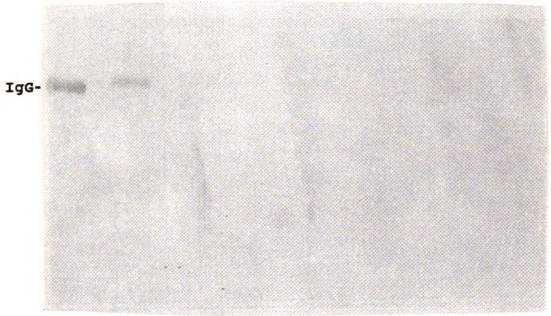

Figure 10: Western blot of extracts from turkey ham thermally processed to six temperatures (°C). 10 μg and 20 μg of protein were loaded for raw and cooked samples, respectively. 5 μg of immunoglobulin G standard marker (STD) was loaded. Anti-turkey immunoglobulin G antisera was used at dilution 1:3000.

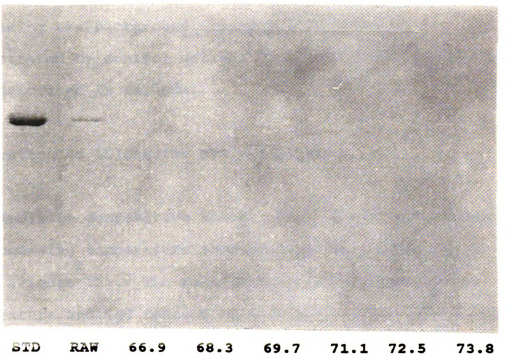

Figure 11: Western blot of extracts from turkey ham thermally processed to six temperatures (°C). 10 μg and 20 μg of protein were loaded for raw and cooked samples, respectively. 5 μg of chicken muscle pyruvate kinase standard marker (STD) was loaded. Anti-chicken muscle pyruvate kinase antisera from rabbit D was used at dilution 1:3000.

agent, blocking incubation time, dilution of antibody, and development time was determined for each ELISA. Optimization improved the sensitivity of the ELISA by decreasing the background absorbance (absorbance demonstrated by control wells). The optimized procedures were described in Methods.

Application of ELISAs for EPT Verification

Indirect competitive ELISA. The IgG content decreased as processing temperature increased as determined by IC-ELISA (Figure 12). The relationship between processing temperature and IgG content at dilution 1:1 was curvilinear at α=0.001. The IgG concentration in uncooked ham extracts was 290 μg/mL extract (880 μg/g meat), which decreased by 99.9% in extracts of turkey hams processed to 73.7°C (43 ng/mL extract). The relationship between processing temperature and IgG content at extract dilutions 1:5, 1:10, 1:100, and 1:1000 were linear at α levels of 0.005, 0.025, 0.05, and 0.1, respectively.

Turkey serum albumin content of ham extracts (1:1) curvilinearly decreased as processing temperature increased at an α level of 0.001 (Figure 13). The TSA content in uncooked turkey ham was 418 μg/mL extract (1,254 μg/g meat), which decreased by 98.9% in extracts of turkey ham thermally processed to 73.7°C (3 μg/mL). However, at dilution 1:5 and

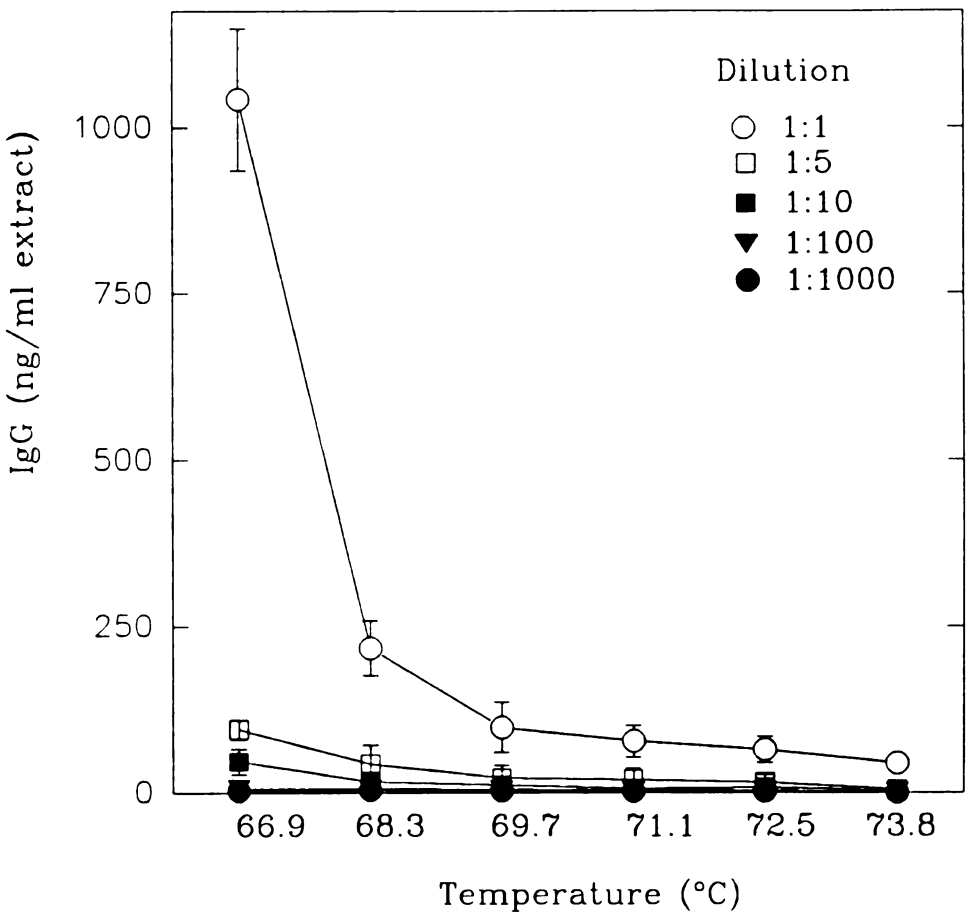

Figure 12: Indirect competitive enzyme-linked immunosorbent assay to determine cooking endpoint by measuring immunoglobulin G content in turkey ham cooked to six temperatures (°C). Data points are mean of three replicate values. Vertical bars indicate standard error of the mean. Data points without bars indicate error bars fit within point.

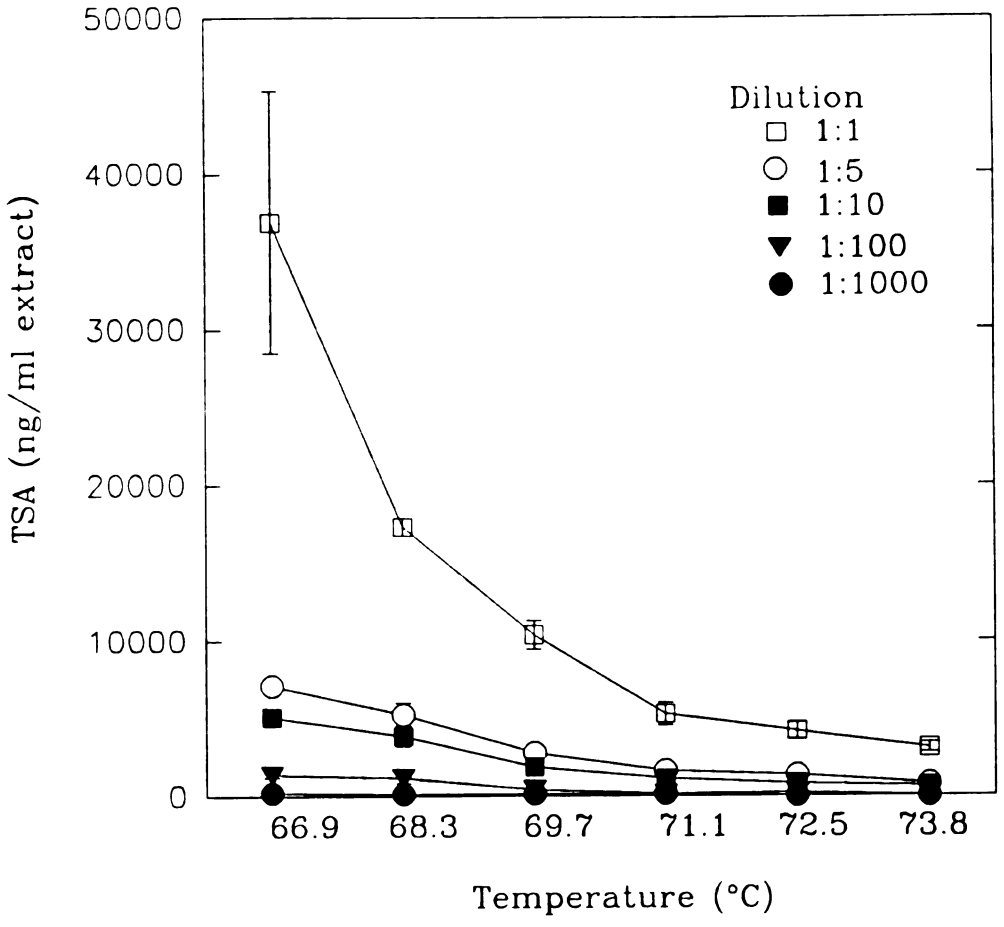

Figure 13: Indirect competitive enzyme-linked immunosorbent assay to determine cooking endpoint by measuring turkey serum albumin content in turkey ham cooked to six temperatures (°C). Data points are mean of three replicate values. Vertical bars indicate standard error of the mean. Data points without bars indicate error bars fit within point.

above, TSA content was still detectable at 73.7°C. The relationship between processing temperature and TSA content in cooked turkey ham extracts at dilutions 1:5, 1:10, 1:100, and 1:1000 were curvilinear at α levels of 0.001, 0.001, 0.001, and 0.005, respectively.

The anti-PK pAb IC-ELISA results were consistent with the results observed by SDS-PAGE, Western blotting and enzymatic analysis. Pyruvate kinase was detected in the uncooked ham extracts (15 μg/mL extract, 45 μg/g meat), but not detected in cooked ham.

The LDH content in turkey ham extracts (1:1) curvilinearly decreased as processing temperature increased at an α level of 0.001 as determined by IC-ELISA (Figure 14). The LDH content in uncooked turkey ham extracts was 160 μg/mL extract (480 μg/g meat), which decreased by 99.9% in extracts of turkey ham processed to 73.7°C (163 ng/mL extract). The relationship between processing temperature and LDH content in cooked turkey ham extracts at dilutions 1:5, 1:10, 1:100, and 1:1000 were curvilinear at α levels of 0.001, 0.001, 0.10, and 0.25, respectively.

Sandwich ELISA. The relationship between processing temperature and LDH content in cooked turkey ham extracts at all dilutions were curvilinear at an α level of 0.001 using sandwich ELISA (Figure 15). The LDH content of uncooked turkey ham was 67 μg/mL extract (201 μg/g meat), which decreased by 99.9% in extracts of turkey ham processed to

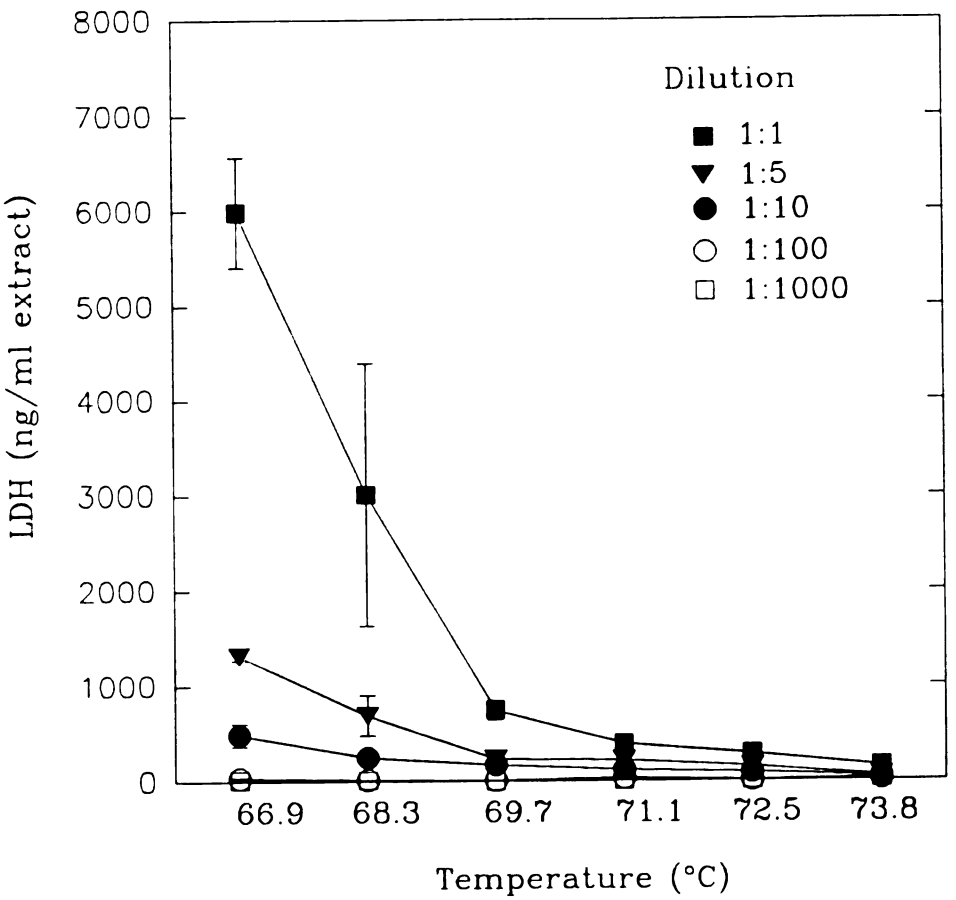

Figure 14: **Indirect competitive enzyme-linked immunosorbent assay to determine cooking endpoint by measuring lactate dehydrogenase content in turkey ham cooked to six temperatures (°C). Data points are mean of three replicate values. Vertical bars indicate standard error of the mean. Data points without bars indicate error bars fit within point.**

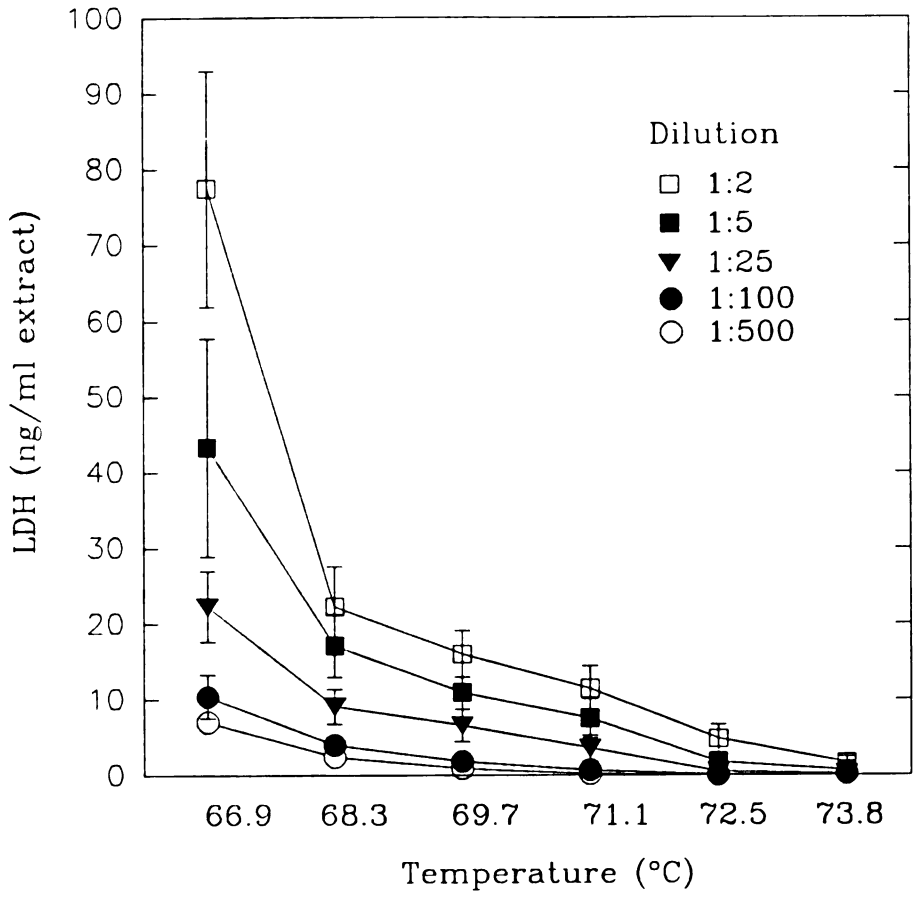

Figure 15: Sandwich enzyme-linked immunosorbent assay to determine cooking endpoint by measuring lactate dehydrogenase content in turkey ham cooked to six temperatures (°C). Data points are mean of three replicate values. Vertical bars indicate standard error of the mean. Data points without bars indicate error bars fit within point.

73.7°C (24 ng/mL extract).

Summary of ELISA results. Statistical analysis showed the strongest response (e.g. curvilinear at α=0.001) between processing temperature and all EPT markers (IgG, TSA, and LDH) at dilution 1:1 in cooked turkey ham extracts (Table 9).

The IgG concentration of turkey ham decreased from 3024 ng/g meat at 66.9°C EPT, to 129 ng/g meat at 73.8°C (Table 9). The USDA currently requires turkey ham to be processed to an internal temperature of 68.3°C (USDA-EPT), whereas the FDA recommended EPT for all poultry is 73.8°C or above, for 15 sec (FDA-EPT). The IgG concentration at 68.3°C (USDA-EPT) was 1,221 ng/g meat and decreased 90% (129 ng/g meat) at 73.7°C (FDA-EPT). Similarly, Wang (1994) observed an IgG concentration of 1,258 ng/g meat in extracts of uncured turkey thigh rolls processed to 68.9°C. Furthermore, the IgG concentration in uncooked turkey ham and uncured turkey thigh roll extracts were 880 µg/g meat and 658 µg/g meat, respectively. However, after thermal processing to 72.2-72.2°C, the IgG concentration in extracts of turkey ham and uncured turkey thigh rolls were 192 ng/g meat and 605 ng/g meat, respectively. The differences observed in IgG content between uncured and cured turkey thigh rolls thermally processed to 72.2-72.2°C may be attributed to standardization of the IC-ELISA or thermal processing schedule.

Table 9. Effect of endpoint temperature on indicator protein (ng/g meat) in turkey ham determined by indirect competitive enzyme-linked immunosorbent assay

Temp. (°C)	IgG[ab]	LDH[ab]	TSA[ab]
66.8	3024	17,967	111,177
68.3	1221	9027	51,852
69.7	618	2241	31,200
71.1	393	1176	15,888
72.2	192	840	12,576
73.7	129	489	9,324

[a]Expressed as mean of three replicate values
[b][IgG], [LDH], [TSA]/Temp. response is curvilinear, α=0.001

The TSA concentration of turkey ham decreased from 111 μg/g meat, at 66.9°C EPT, to 9.3 μg/g meat at 73.8°C (Table 9). The TSA concentration at 68.3°C (USDA-EPT) was 52 μg/g meat and decreased 83% (9.3 μg/g meat) at 73.7°C (FDA-EPT). These results indicated that TSA could be a potential EPT marker if the recommended EPT (73.8°C) in the 1994 Food Code is adopted as the required EPT for poultry or if raised further in the future.

The LDH concentration of turkey ham decreased from 17,967 ng/g meat, at 66.9°C EPT, to 489 ng/g meat at 73.7°C (Table 9). The LDH concentration at 68.3°C (USDA-EPT) was 9,027 ng/g meat and decreased 95% (489 ng/g meat) at 73.7°C (FDA-EPT). Indirect competitive-ELISA was also used by Wang et al. (1992) to determine the effects of processing temperature on LDH in turkey breast rolls. Similar to the results of this study, LDH content decreased as cooking temperature increased.

The LDH concentration, as determined by LDH sandwich ELISA, of turkey ham decreased from 1065 ng/g meat, at 66.9°C EPT, to 24 ng/g meat at 73.7°C at dilution 1:2 (Table 10). The LDH concentration at 68.3°C (USDA-EPT) was 330 ng/g meat and decreased 93% (24 ng/g meat) at 73.7°C (FDA-EPT). Similarly, commercially available turkey ham, with minimum USDA internal temperature requirements of 68.3°C, was reported to contain 225-360 ng LDH/g meat as determined by sandwich ELISA (Abouzied et al., 1993). These

Table 10. Effect of endpoint temperature on lactate dehydrogenase (LDH) (ng/g meat) in turkey ham as determined by sandwich enzyme-linked immunosorbent assay

Temp. (°C)	LDH[ab]
66.8	1065
68.3	330
69.7	240
71.1	165
72.2	60
73.7	24

[a]Expressed as mean of three replicate values
[b][LDH]/Temp. response is curvilinear, α=0.001

results are similar to LDH content of turkey ham processed between 68.3° (330 ng/g meat) and 69.7°C (240 ng/g meat)(Table 10), which indicated LDH sandwich ELISA results are reproducible and can accurately determine EPT in precooked turkey ham within 1.4°C. Wang (1994) reported 461 ng/g meat of LDH in uncured turkey thigh rolls thermally processed to 68.3°C (USDA-EPT) using sandwich ELISA. In addition, Wang (1994) found similar LDH concentrations in uncooked uncured turkey thigh rolls (201 μg/g meat), as found in uncooked turkey ham (210 μg/g meat). These results indicate that the sandwich ELISA produces the same results for either a cured or uncured turkey thigh roll. However, at 71.1°C, uncured turkey thigh roll and turkey ham had 305 ng LDH/g meat and 165 ng LDH/g meat, respectively. These differences observed in LDH content at processing temperatures above 70.0°C may be attributed to differences in processing schedule. The uncured turkey thigh had a total processing time of 6.5 hr; Whereas, turkey ham processed to 72.2°C had a total processing time of 7.0 hr.

The results of the LDH IC-ELISA and sandwich ELISA both indicated a 99.9% decrease of LDH between uncooked turkey ham and hams processed to 73.7°C, but the LDH content (ng/g meat) was different. At 73.7°C, LDH concentration in ham was 24 ng/g meat and 489 ng/g meat in sandwich ELISA and IC-ELISA, respectively. The lower LDH content by sandwich ELISA as compared to the IC-ELISA may be due to several

factors. The results of the IC-ELISA may be be affected by protein-protein interactions and high background absorbance.

The lower LDH content determined by sandwich ELISA as compared to the IC-ELISA may also be due to the differential binding abilities of anti-turkey LDH mAb and pAb to LDH isozymes that contain M form, which are less heat stable (Wang, 1994). Western blot analysis of native polyacrylamide gels of raw uncured turkey thigh roll extracts with anti-LDH pAbs showed three major bands (M_4, M_3H and M_2H_2 LDH isoforms) and one faint band (MH_3). Whereas, Western blot analysis of native polyacrylamide gels of raw uncured turkey thigh roll extracts with anti-LDH mAb D5E showed two major bands (M_4 and M_3H) and one faint band (M_2H_2). These results indicated that anti-turkey LDH pAbs only recognized isozymes containing M form and anti-turkey LDH mAb D5E mainly bound LDH isozymes with at least 2 subunits of M form (Wang, 1994). Thus, the lower LDH content determined by sandwich ELISA as compared to IC-ELISA may also be attributed to differential binding ability of anti-turkey mAb D5E to LDH isozymes. Furthermore, in polyacrylamide gels of cooked thigh roll extracts processed between 68.3° and 71.1°C stained with LDH specific stain, only the H_4, H_3M, and H_2M_2 LDH isozymes were observed, which indicated that H_4, H_3M and H_2M_2 were more heat stable than M_4 and M_3H LDH isozymes (Wang, 1994). Therefore, the LDH

sandwich ELISA was mainly quantifying LDH isozymes containing at least 2 subunits of M form in cooked turkey ham extracts; Whereas, the LDH IC-ELISA was quantifying all LDH isozymes containing M form in cooked turkey ham extracts.

Effect of Delayed Bleeding on Residual Blood

Breast muscle. Temperature averaged 44°F and pH averaged 5.9 in breast muscle, which indicated the birds did not suffer undue stress (Table 11). Delayed bleeding had no effect on extractable protein concentration. Hematin linearly increased (α=0.005) with increased delay time before cut; However, the content increased by only 4 μg/g meat.

As delay time increased, ground breast muscle showed a linear decrease in L* (lightness) and b* values (yellowness) (α=0.001), whereas a* values (redness) linearly increased (α=0.005). HunterLab L*, a*, and b* values of 37, 5, and 8, respectively, were observed in the control breast. Similarly, Ngoka et al. (1982) observed L*, a*, and b* values of 51, 8, and 12, respectively, in turkey breast muscle. In addition, Fleming et al. (1991) also observed L*, a*, and b* values of 41, 2, and 5, respectively, in chicken breast muscle. The slight differences in HunterLab values may be attributed to equipment standardization and

Table 11. Effect of delayed bleeding on turkey breast muscle

Delay (sec)	Protein[a] (mg/ g meat)	Hematin[b] (μg/ g meat)	HunterLab L*[c]	HunterLab a*[c]	HunterLab b*[c]	Serum proteins[d] (mg/ g meat)
Control	56	31	37.4	5.0	7.7	11
50	56	30	36.4	5.8	7.2	16
100	43	32	34.7	5.8	6.1	15
Stun	43	35	33.8	6.4	5.8	13

[a][Protein]/Delay response, no relationship
[b][Hematin]/Delay response is linear, α=0.005
[c]L*, a*, b*/Delay response is linear, α=0.001
[d][Serum protein]/Delay response is linear, α=0.025

breast muscle preparation. Although the color change in the breast had a strong relationship with delayed bleeding (α=0.001-0.005), the L*, b*, and a* HunterLab values changed by only 2.5, 1.4, and 1.8 units, repsectively.

Serum protein levels increased linearly (α=0.025) with delayed bleeding; However, the statistical significance is questionable at an α level of 0.025. The control breast had 11 mg serum protein/g meat and at 50 sec delay before cut, serum protein concentration increased to 16 mg/g meat. At 100 sec delay, 15 mg serum protein/g meat was observed, whereas the stunned but not cut (Stun) breast muscle had a serum protein content of 13 mg/g meat. These results indicated that the effects of delayed bleeding on residual serum proteins in breast muscles were minimal, as suggested by statistical analysis (α=0.025).

Thigh muscle. Temperature averaged 42°F and pH averaged 6.4 in thigh muscles, which indicated the birds did not suffer any undue stress (Table 12). The extractable protein concentration and hematin levels in turkey thigh were not affected by delayed bleeding. HunterLab values L*, a*, and b* of 32, 8, and 6, respectively, were observed in thigh muscle. Similarly, Fleming et al. (1991) reported L*, a*, and b* values of 37, 6, and 5, respectively, in chicken thigh muscle. HunterLab color analysis showed a very weak relationship between delayed bleeding and L* (α=0.025), a* (no relationship) and b* (α=0.025) values, indicating

Table 12. Effect of delayed bleeding on turkey thigh muscle

Delay (sec)	Protein[a] (mg/ g meat)	Hematin[a] (μg/ g meat)	HunterLab L*[b]	HunterLab a*[a]	HunterLab b*[b]	Serum proteins[b] (mg/ g meat)
Control	34	61	32.1	7.5	6.4	3.6
50	36	74	31.1	7.7	5.2	3.5
100	32	74	30.4	7.6	5.0	4.2
Stun	35	70	31.0	7.1	5.1	5.0

[a][Protein],[Hematin],a*/Delay response, no relationship
[b]L*,b*,[Serum protein]/Delay response is linear, α=0.025

delayed bleeding did not affect the pigment in turkey thigh muscle.

Residual serum proteins also show a very weak response (α=0.025) to delayed bleeding. The control thigh muscle had a serum protein concentration of 3.6 mg/g meat. Conversely, Wang (1994) determined a serum protein concentration of 36 mg/g meat in raw uncured turkey thigh rolls using a sandwich ELISA with anti-turkey serum pAbs capture antibodies and biotin labeled anti-turkey serum pAbs as detector antibodies. The difference between serum protein concentration in turkey thigh muscle and raw turkey thigh roll formulations was attributed to the higher sensitivity of sandwich ELISA with biotin-conjugated detector turkey serum polyclonal antibodies, as compared to IC-ELISA. The overall results for thigh muscle show no effect of delayed bleeding on variables tested.

Delayed bleeding had a minimal effect on color and hematin content in breast muscle, but not in thigh muscle. The effects in breast may be attributed to the position of the muscle relative to the jugular vein. The amount of blood in the jugular vein after stunning has been shown to be inversely related to the blood content of the spleen (Warriss and Leach, 1978). The spleen in a stunned animal is considerably contracted, which under normal functioning conditions contains one-seventh of total blood volume in sheep. Engorgement of the jugular vein with blood is

readily apparent in the neckflap after stunning (Gregory and Wilkins, 1990). Delaying the neck cut after stunning has been shown to cause haemorrhaging in the breast muscle (Mohan Raj and Gregory, 1991). In this study, the color of breast muscle was slightly darker and more red and blue due to delayed bleeding. In addition, hematin concentration increased in the breast muscle. Color and hematin concentrations were unaffected in thigh muscle. The serum protein concentration in both muscles showed a very weak linear response (α=0.025) to delayed bleeding, supporting the findings of Warriss and Leach (1978) who concluded the degree of dilation of the small blood vessels and stress associated with stunning and exsanguation in sheep ensures minimal retention of blood, regardless of slaughter methodology.

The weak response (α=0.025) of residual serum proteins to delayed bleeding, indicated that serum protein concentrations in musculature of turkeys would be unaffected by differences in slaughter methodology. Furthermore, these results suggested that concentrations of TSA and IgG (e.g. serum proteins) in turkey breast and thigh muscle would also be unaffected by slaughter methodology. The concentration of TSA and IgG in a precooked turkey breast or thigh products would therefore be dependent upon the effects of temperature, not slaughtering methodology; Thus, validating the use of IgG and TSA as potential EPT markers.

CONCLUSIONS

The ELISAs developed in this study can provide a rapid, highly sensitive, and accurate method for verification of EPT never before developed for cured poultry products. The 1994 Food Code states the recommended EPT for poultry products processed by food service and retailers is 73.7°C. Of all assays performed, the LDH sandwich and IgG IC-ELISA show the highest potential to accurately determine EPT of 73.7°C (165°F) in turkey ham. The TSA IC-ELISA may have potential for determining adulteration of precooked meat products with turkey parts or blood, due to heat stable nature of TSA up to 73.7°C. In addition, if EPT requirements were further increased, TSA may be a potential marker at temperatures higher than 73.7°C. Pyruvate kinase is not recommended as an EPT marker in any turkey product processed above 66.8°C, due to inactivation at this temperature or lower.

The next logical step in verifying an ELISA for EPT determination, is to apply the LDH sandwich and IgG IC-ELISA to several cured poultry products. The cured poultry products should vary in formulation, composition, size, cooking schedule, packaging and storage temperature. After validation in a defined product category, the ELISA should

be adapted into a commercial immunoassay kit system. The commercial ELISA kit will permit rapid assessment of the actual EPT turkey products reached during processing. The manifestation of a commercial ELISA kit will enable retailers and food service facilities to verify adequate EPT in cured poultry products.

Delayed bleeding was found to have a minimal effect on residual blood in both breast and thigh muscle. The color and hematin concentrations in the breast were only slightly affected by delayed bleeding, which may be attributed to localization of the blood in the jugular vein, not in the musculature. Prior to this research, serum proteins had not been used as an index for residual blood in the musculature. Delayed bleeding had no effect on residual serum protein concentration in both breast and thigh muscle; Thus, serum protein concentrations in turkey breast and thigh products prior to thermal processing would not be affected by slaughter methodology. Therefore, the concentration of TSA and IgG in thermally processed turkey breast or thigh meat would be dependent upon the effects of heating, not slaughtering methodology and could be used as potential EPT markers for adequate thermal processing.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abouzied, M.M., Wang, C.H., Pestka, J.J. and Smith, D.M. 1993. Lactate dehydrogenase as safe endpoint cooking indicator in poultry breast rolls: development of monoclonal antibodies and application to sandwich enzyme-linked immunosorbent assay (ELISA). J. Food Protect. 56(2):120-124,129.

Ahn, D.U. and Maurer, A.J. 1989. Effects of added pigments, salt, and phosphate on color, extractable pigment, total pigment, and oxidation-reduction potential in turkey breast meat. Poultry Sci. 68:1088-1099.

Andrews, C.D., Berger, R.G., Mageau, R.P., Schwab, B. and Johnston, R.W. 1992. Detection of beef, sheep, deer, and horse meat in cooked meat products by enzyme-linked immunosorbent assay. J. Assoc. Off. Anal. Chem. Intl. 75(3):572-576.

Bean, N.H. and Griffin, P.M. 1990. Foodborne disease outbreaks in the United States, 1973-1987:pathogens, vehicles, and trends. J. Food Protec. 53:68-69.

Bean, N.H., Griffin, P.M., Goulding, J.S. and Ivey, C.B. 1990. Foodborne disease outbreaks, 5-year summary, 1983-1987. J. Food Protec. 58(8):711-728.

Berger, R.G., Mageau, R.P., Schwab, B., and Johnston, R.W. 1988. Detection of poultry and pork in cooked and canned meat foods by enzyme-linked immunosorbent assays. J. Assoc. Off. Anal. Chem. 71:406-409.

Bergmeyer, H.U. 1974. Methods of Enzymatic Analysis, 4th ed. Plenum, New York, NY.

Berne, R.M. and Levy, M.N. 1988. Physiology, 2nd ed. The C.V. Mosby Company, St. Louis, MO.

Bhunia, A.K., Ball, P.H., Fuad, A.T., Kurz, B.W., Emerson, J.W. and Johnson, M.G. 1991. Development and characterization of a monoclonal antibody specific for *Listeria monocytogenes* and *Listeria innocua*. Infect. Immun. 59(9):3176-3184.

Bogin, E., Israeli, B.A. and Klinger, I. 1992. Evaluation

of heat treatment of turkey breast meat by biochemical methods. J. Food. Protect. 55(10):787-791.

Brada, D. and Jurgen, R. 1984. "Golden blot" detection of polyclonal and monoclonal antibodies bound to antigens on nitrocellulose by protein A-gold complexes. Anal. Biochem. 142:79-83.

Breton, C., Thanh, L.P. and Paraf, A. 1988. Immunochemical identification and quantification of ovalbumin additive in canned mushrooms. J. Food Sci. 53(1):226-230.

Caldironi, H.A. and Bazan, N.G. 1980. Quantitative determination of low-salt soluble proteins patterns of bovine muscles cooked at different temperatures. J. Food Sci. 45:901-904.

Cohn, E.J., Srong, L.E., Hughes, W.L., Mulford, D.J., Ashworth, J.N., Melin, M. and Taylor, H.L. 1946. Preparation and properties of serum and plasma proteins IV: A system for the separation into fractions of protein and lipoprotein components of biological tissues ans fluids. J. Am. Chem. Soc. 68: 459-463.

Collins, S.S., Keeton, J.T., and Smith, S.B. 1991a. Lactate Dehydrogenase Activity in Bovine Muscle as a Potential Heating Endpoint Indicator. J. Agric. Food Chem. 39: 1291-1293.

Collins, S.S., Keeton, J.T. and Smith, S.B. 1991b. Lactate Dehydrogenase Enzyme Activity in Raw, Cured and Heated Porcine Muscle. J. Agric. Food Chem. 39: 1294-1297.

Davis, C.E., Lyon, B.G., Reagan, J.O. and Townsend, W.E. 1985. Time temperature heating effect on biuret-positive water-extractable porcine and bovine muscle proteins. J. Food Protect. 48(3):215-220.

Davis, C.E., Lyon, B.G., Reagan, J.O. and Townsend, W.E. 1987. Effect of heating on water soluble biuret-positive compounds of canned cured pork picnic shoulder. J. Food. Protect. 50(8):681-684.

Davis, C.E., Searcy, G.K., Blankenship, L.C. and Townsend, W.E. 1988. Pyruvate kinase activity as an indicator of temperature attained during cooking of cured pork. J. Food Protect. 51(10): 773-777.

Dixon-Holland, D.E., Pestka, J.J., Bidigare, B.A., Casale, W.L., Warner, R.L., Ram, B.P., and Hart, L.P. 1988.

Production of sensitive monoclonal antibodies to aflatoxin B1 and aflatoxin M1 and their application to ELISA of naturally contaminated foods. J. Food Protect. 51:201-204.

Dutton, A.H. 1990. Double fluorescence immunoblotting studies. Meth. Enzymol. 184:343-353.

Ellekjaer, M. R. 1992. Assessment of maximum cooking temperatures of previously heat treated beef. Part 2: Differential scanning calorimetry. J. Sci. Food Agric. 60:255-261.

Ellekjaer, M. R. and Isaksson, T. 1992. Assessment of maximum cooking temperatures in previously heat treated beeef. part 2. Near infrared spectroscopy. J. Sci. Food Agric. 59:335-343.

Eye, J.G. 1982. A rapid procedure for the detection of underprocessing of roast beef. Annual Meeting of the Food Research Institute. Madison, WI. May 25.

FDA. 1994. Recommendations of the U.S. Public Health Service Food and Drug Administration, Food Code. Section 3-401.11. Food and Drug Administration, Washington, DC.

Faulk, W.P. and Taylor, G.M. 1971. An immunocolloid method for the electron microscope. Immunochem. 8:1081-1083.

Fleming, B.K., Froning, G.W. and Yang, T.S. 1991. Heme pigment levels in chicken broilers chilled in ice slush and air. Poultry Sci. 70:2197-2200.

Fukal, L. 1991. Modern immunoassays in meat-product analysis. Die Nahrung. 35(5):431-448.

Galfre, G. and Milstein, C. 1981. Preparation of monoclonal antibodies: strategies and procedures. Meth. Enzymol. 73:3-47.

Gornall, A.G., Bardawill, C.J. and David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-766.

Gregory, N.G. and Wilkins, L.J. 1989a. Effect of slaughter methods on bleeding efficiency in chickens. J. Sci. Food Agric. 47:13-20.

Gregory, N.G. and Wilkins, L.J. 1989b. Effect of ventricular fibrillation at stunning and ineffective bleeding on carcase quality defects in broiler

chickens. Brit. Poultry Sci. 30:825-829.

Gregory, N.G. and Wilkins, L.J. 1990. The role of electrical stunning, bleeding and plucking efficiency on the downgrading of chicken carcasses. Vet. Record. 127:331-333.

Harlow, E. and Lane, D. 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory, New York.

Hebert, G.A., Pelham, P.L. and Pittman, B. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. Appl. Microbiol. 25(1):26-36.

Holbrook, J.J., Liljas, A., Steindal, S.J. and Rossmann, M.G. 1975. The Enzymes. Academic Press: New York, NY.

Hsu, Y.C. 1993. Characterization of enzymes suitable as endpoint temperature indicators in turkey muscle. M.S. Thesis, Texas A&M University, College Station.

Isaksson, T., Ellekjaer, M.H.R. and Hildrum, K.I. 1989. Determination of the previous maximum temperature fo heat-treated minced meat by near infared reflectance spectroscopy. J. Sci. Food Agric. 49:385-387.

Janssen, F.W., Hagele, G.H., Voorpostel, A.M.B. and deBaaij, J.A. 1990. Myoglobin analysis for determination of beef, pork, horse, sheep, and kangaroo meat in blended cooked products. J. Food Sci. 55:1528-1530.

Karlsson, K. and Lundstrom, K. 1991. Meat pigment determination by a simple and non-toxic alkaline haematin method-(alternative to the Hornesy and the cyanometmyoglobin methods). Meat Sci. 29:17-24.

Kiffmeyer, W.R. and Farrar, W.W. 1991. Purification and properties of pig heart pyruvate kinase. J. Protein Chem. 10(5):585-591.

Klinger, I.B., Bogin, W.E., Israeli, B. and Hornstein, K. 1982. A verification test for the heating grade of turkey breast meat. Internal report; Ministry of Agriculture Veterinary Services and Animal Health, Kimrov Veterinary Institute. Beit Dagan, Israel.

Kormendy, L., Rehasi, E. and Fetter, I. 1987. Determination of the extent of heat treatment in canned hams by use of the phosphatase test. J. Biol. Chem. 260:4653-4660.

Kormendy, L., Zsarnoczay, G. and Mihalyi, V. 1992. A new, modified acid phosphatase assay for determining the extent of heat treatment in canned hams. Food Chem. 44: 367-375.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature. 227:680-685.

Lee, Y.B., Rickansrud, D.A., Hagberg, E.C. and Briskey, E.L. 1974. Application of SDS-acrylamide gel electrophoresis for determination of the maximum temperature to which bovine muscles have been cooked. J. Food Sci. 39:428-429.

Lehninger, A.H. 1975. Biochemistry, 2nd ed. Worthington. Baltimore, MD.

Malik, N.J. and Daymon, M.E. 1982. Improved double immunoenzyme labelling alkaline phosphatase and horseradish peroxidase. J. Clin. Path. 35:1092-1094.

McCormick, R.J., Reeck, G.R. and Kropf, D.H., Hunt, M.C. and Kastner, C.L. 1987. Effect of heating temperature and muscle type on porcine muscle extracts as determined by reverse phase HPLC. J. Food Sci. 52: 1481-1485.

McCormick, R.J., Reeck, G.R. and Kropf, D.H. 1988 Seperation and identification of porcine sarcoplasmic proteins by Reversed-phase HPLC and polyacrylamide gel electrophoresis. J. Agric. Food Chem. 36: 1193-1196.

Mohan Raj, A.B. and Gregory, N.G. 1991. Efficiency of bleeding of broilers after gaseous or electrical stunning. Vet. Record. 128:127-128.

MSTAT. 1989. Microcomputer statistical program version c. Michigan State University, East Lansing.

Ngoka, D.A., Froning, G.W., Lowry, S.R. and Babji, A.S. 1982. Effects of sex, age, preslaughter factors and holding conditions on the quality characteristics and chemical composition of turkey breast muscles. Poultry Sci. 61:1996-2003.

Obinata, T., Maruyama, K., Sugita, H., Kohama, K. and Ebashi, S. 1981. Dynamic aspects of structural proteins in vertebrate skeletal muscle. Muscle Nerve. 4:456-460.

Palomaki, P. 1991. Simultaneous use of poly- and monoclonal

antibodies as enzyme tracers in a one-step enzyme immunoassay for the detection of hepatitis B surface antigen. J. Immunol. Methods. 145:55-63.

Patterson, R.M., Whittaker, R.G., and Spencer, T.L. 1984. Improved species identification of raw meat by double sandwich enzyme-linked immunosorbent assay. J. Sci. Food Agric. 35:1018-1023.

Pearson, A.M. and Young, R.B. 1990. Muscle and Meat Biochemistry. Academic Press, New York, NY.

Pestka, J.J., Lee, Y.K., and Chu, F.S. 1982. Reactivity of aflatoxin B2a antibody with aflatoxin B1 modified DNA and related metabolites. Appl. Envrion. Microbiol. 44:1159-1165.

Pestka, J.J. 1988. Enhanced surveillance of foodborne mycotoxins by immunochemical assay. J. Assoc. Anal. Chem. 71:1075-1081.

Peters, T.R., Jr. 1980. Albumin, An Overview and Bibliography. Research Products Division, Miles Laboratories, Inc., Elkhart, IN.

Price, J.F. and Schweigert, B.S. 1987. The Science of Meat and Meat Products, 3rd ed. Food and Nutrition Press, Inc. Westport, CT.

Quinn, J.R., Raymond, D.P. and Harwalker, V.R. 1980. Differential scanning calorimetry of meat proteins as affected by processing treatment. J. Food Sci. 45:1146-1149.

Samarajeewa, U., Wei, C.I., Huang, T.S. and Marshall, M.R. 1991. Application of immunoassays in the food industry. Crit. Rev. Food Sci. Nut. 29:403-434.

Scopes, R.K. and Penny, I.F. 1971. Subunit sizes of muscle proteins as determined by sodium dodecyl sulphate gel electrophoresis. Biochem. Biophys. Acta. 236:409-415.

Stalder, J.W., Smith, G.L., Keeton, J.T. and Smith, S.B. 1991. Lactate dehydrogenase activity in bovine muscle as a means of determining heating endpoint. J. Food Sci. 56:895-898.

Steele, P. and Lambe, W.J. 1982. SDS-gradient gel electrophoretic separation of muscle polypeptides for the estimation of maximum cooking temperatures in meat. J. Food Protect. 45:59-62.

Tietz N. 1976. Fundamentals of Clinical Chemistry. W.B. Saunders Company, Philadelphia, PA.

Todd, E.C. 1989. Preliminary estimates of costs of fooodborne disease in the United States. J. Food Protect. 52(8):595-601.

Townsend, W.E. 1989. Stability of residual acid phosphatase activity in cured/caned picnic samples stored at -34°C for 15 and 36 months. J. Food Sci. 54:752-753.

Townsend, W.E. and Blankenship, L.C. 1989. Methods for detecting processing temperatures of previously cooked meat and poultry products-a review. J. Food Protect. 52:128-135.

Townsend, W.E. and Davis C.E. 1991. Influence of thermal processing conditions on lactate dehydrogenase (LDH) activity in ground beef and chicken. Abstract, presented at 51st Annual Meeting of the Inst. of Food Technologists, Dallas, TX, June 2-5.

Townsend, W.E. and Davis, C.E. 1992. Transaminase (AST/GOT and ALT/GPT) activity in ground beef as a means of determining end-point temperature. J. Food Sci. 57:555-557.

Townsend, W.E., Searcy, G.K., Davis, C.E., Wilson, Jr. R.L. 1993. Endpoint temperature (EPT) affects N-acetyl-β-D-glucosaminidase activity in beef, pork and turkey. J. Food Sci. 58:710-712.

USDA-FSIS. 1986a. Cooking requirements for pork and pork products. Animal and plant products. (Revised January 1, 1992) Code of Federal Regulations, part 94.12; Title 9; Office of the Federal Register, National Archives and Records, GSA: Washington, DC. Chapter 3. p.440-441.

USDA-FSIS. 1986b. Determination of internal cooking temperature (Coagulation). Revised Basic Chemistry Laboratory Guidebook. (Revised March, 1986) No. 3.019:3-35. Science Chemistry Division. Food Safety and Inspection Service, Washington, DC.

USDA-FSIS. 1986c. Determination of internal cooking temperature (Acid phosphatase activity). Revised Basic Chemistry Laboratory Guidebook. No. 3.018:3-49. Science Chemistry Division. Food Safety and Inspection Service, Washington, DC.

USDA-FSIS. 1989. Performing the catalase enzyme test. A

self-instruction guide. Food Safety and Inspection Service, Technical Services Training Division, Washington, DC.

USDA-FSIS. 1990. Cooking requirements for poultry rolls and certain other poultry products. Animal and plant products. (Revised January 1, 1992) Code of Federal Regulations, part 381.150; Title 9; Office of the Federal Register, National Archives and Records, GSA: Washington, DC. Chapter 3. p.491-492.

Wang, C.H., Abouzied, M.M., Pestka, J.J., and Smith D.M. 1992. Antibody development and enzyme-linked immunosorbent assay (ELISA) for the protein marker lactate dehydrogenase to determine safe cooking endpoint temperatures of turkey rolls. J. Agric. Food Chem. 40:1671-1676.

Wang, C.H., Booren, A.M., Abouzied, M.M., Pestka, J.J. and Smith, D.M. 1993. ELISA determination of turkey roll endpoint temperature: effects of formulation, storage, and processing. J. Food Sci. 58:1258-1261.

Wang, C.H. 1994. Enzyme-linked immunosorbent assay for assurance of safe endpoint processing temperatures in poultry products. Ph.D. Dissertation. Michigan State University, East Lansing.

Warriss, P.D. 1977. The residual blood content of meat-A review. J. Sci. Food Agric. 28:457-462.

Warriss, P.D. and Rhodes, D.N. 1977. Haemoglobin concentrations in beef. J. Sci. Food Agric. 28:931-933.

Warriss, P.D. 1978. Factors affecting the residual blood content of meat. Meat Sci. 2:155-159.

Warriss, P.D. and Leach, T.M. 1978. The influence of slaughter method on the residual blood content of meat. J. Sci. Food Agric. 29:608-610.

Wenger, J.D., Swaminathan, B., Hayes, P.S., Green, S.S., Pratt, M., Pinner, R.W., Schuchat, A. and Broome, C.V. 1990. Listeria monocytogenes contamination of turkey franks: evaluation of a production facility. J. Food Protect. 53(12):1015-1019.

Wilchek, M. amd Bayer, E.A. 1988. The avidin-biotin complex in bioanalytical applications. Anal. Biochem. 171:1-32.

Williams, J.C. Vimini, R.J., Field, R.A., Riley, M.L. and Kunsman, J.E. 1983. Influence of delayed bleeding on sensory characteristics of beef. Meat Sci. 9:181-190.

Wright, J.D. and Wilding, P. 1984. Differential scanning calorimetric study of muscle and its proteins: myosin and its subfragments. J. Sci. Food Agric. 35:357-372.