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Monoclonal Antibody-Based Sandwich Enzyme-Linked Immunosorbent Assay for the Detection of Mammalian Meat in Meat and Feed Products

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MONOCLONAL ANTIBODY-BASED SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF MAMMALIAN MEAT IN MEAT AND FEED PRODUCTS

By

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ABSTRACT

Detection of mammalian tissue in non-mammalian meat or feed products is important for enforcement of food-labeling laws and prevention of the spread of transmissible spongiform encephalopathies (TSEs). This study was conducted to develop a monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (ELISA) for rapid detection of raw, cooked (100°C, 30 min) and autoclaved (121°C/1.2 bar, 30 min) mammalian meats (beef, deer, elk, horse, lamb and pork) adulterated in non-mammalian meat (chicken, duck and turkey) and soy-based feed products, and to assess the performance of the assay. This assay utilized a pair of MAbs against thermal-stable skeletal muscle protein, troponin I (sTnI). MAb 6G1, specific to mammalian and poultry sTnIs, was used as the capture antibody and horseradish peroxidase (HRP) conjugated MAb 8F10, specific to mammalian sTnI, was used as the detection antibody. The assay conditions that were optimized include: the dilutions of the capture antibody and the detection antibody, the selection of the antibody buffer, the incubation time for antigen-antibody binding, and the dilutions of the adulterated meat and feed samples. The optimized assay achieved a detection limit of 0.05% (w/w) for raw, 0.50% (w/w) for cooked and 1.00% (w/w) for autoclaved beef in turkey ($P \leq 0.05$); 0.50% (w/w) for pork in chicken mixtures (raw, cooked and autoclaved) ($P \leq 0.05$); and 0.50% (w/w) for bovine meat meal in soy-based feed mixtures ($P \leq 0.05$). The fat content (0 − 30%, w/w) of the meat samples did not significantly affect the assay signals ($P \geq 0.05$). As the temperature and time of the heat treatment of the meat samples increased, the reactivity of this assay decreased slightly. However, the assay was still adequate to analyze samples subjected to the most severe heat treatment (132°C/2.0 bar, 120 min). This MAb-based sandwich ELISA is the first assay suitable for rapid, sensitive and reliable detection of undeclared mammalian proteins in meat and feed products, regardless of the extent of heat processing.

INTRODUCTION

Meat adulteration has been reported in several countries (Chemistry Centre of Western Australia 1999; Ministry of Agriculture, Fisheries and Food (MAFF) 1999; the United States (US) Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) 2001; Odumeru 2003). This issue not only involves economic fraud, which violates the food-labeling laws, but is also a concern for religious taboos that ban the consumption of specific meat species and individual moral aversions to particular meat species. It also, to some extent, relates to food safety with regard to individual allergies, contamination with food borne pathogens, and the spread of the fatal transmissible spongiform encephalopathies (TSEs).

Since the United Kingdom (UK) government verified the first case of bovine spongiform encephalopathy (BSE), the TSE disease in cattle, in 1987 (Anon 2000), there have been about 183,000 cases of BSE confirmed in more than twenty countries, with more than 98% of the cases being reported in Europe (OIE 2003). In late 2003, the first apparent case of BSE in the US was proved in a cattle farm in Washington State (Centers for Disease Control and Prevention (CDC) 2004). BSE, commonly known as "mad cow disease", has had a huge impact on the beef industry worldwide. As a result, the consumption of beef and lamb has been decreasing while poultry has shown a progressive rise (MAFF 2000; Sorenson and others 2003).

It has been reported that both human new variant Creutzfelt-Jakob disease (nvCJD) and BSE belong to the family of fatal TSE diseases, and they share the same infection mechanism, namely the abnormal prion protein (PrP^{sc}), which aggregates in cytoplasmic vesicles in the brains of infected individuals and animals and is highly resistant to heat (Irani and Johnson 2003). Several studies have reported that PrP^{sc} failed to completely inactivate after treatment at 121ºC for 60 min or after even more severe heat treatments (Brown and others 1982, 1990; Taylor and others 1994; Appel and others 2001).

Several studies have testified that the causative agents of BSE in cattle and nvCJD in humans share a common prion strain (Hill and others 1997; Irani and Johnson 2003). Furthermore, strong experimental evidence has shown a direct link between nvCJD and BSE (Collinge and others 1996; Bruce and others 1997; Cousens and others 1997; Taylor 2002). Up to December 2003, 153 cases of human nvCJD had been confirmed around the world

(CDC 2004b). In 2002, the first US nvCJD patient died in Florida (Wiersma and others 2002).

Animal by-products have been used as nutritional supplements in livestock feedstuffs for more than 100 years. A study reported that the yields of milk of cows fed with raw soybeans and animal by-product proteins (45.5 kg/d) were greater than those of cows receiving raw soybeans alone (43.2 kg/d) (Grummer and others 1994). However, due to several significant changes in the manufacturing processes of feedstuffs that took place in the 1970s, such as mechanical systems that permitted continuous flow production and solvent extraction of fats, PrP^{sc} entered the livestock feed chain. The recycling of animal-by products caused a spread of the prion disease in cattle that subsequently affected the human food chain.

To prevent all the meat and feed problems mentioned above, and thus minimize any heath risks to livestock and humans, the legislatures of many countries have enacted very strict laws. In the US, the FDA published final regulations in 1993 requiring comparable nutrition labeling requirements for multi-ingredient and heat processed meat and poultry products (FDA 1993). To avoid and reduce the danger of prion diseases, in 1994 the European Union (EU) banned the use of proteins originating from mammalian tissues for feeding ruminants, as did the US in 1997 (European Commission (EC) 1994; FDA 1997). In 2000, the EU further strengthened the regulations to ban the addition of processed animal proteins to feedstuffs for all animals raised for the production of food (EC 2000; EC 2002). In early 2004, the USDA-FSIS declared that specified risk materials, such as the skull, brain, eyes, vertebral column, and spinal cord, of cattle 30 months of age or older and the small intestine of all cattle were forbidden for human consumption (USDA-FSIS 2004).

A number of analytical techniques have been developed for the inspection and detection of these illegal meat and feed products. Overall, these methods can be classified into five types: chromatography, electrophoresis, spectroscopy, deoxyribonucleic acid (DNA) based techniques and immunochemical techniques.

Chromatography is used to separate molecules based on differential absorption and elution, which involves the flow of a fluid carrier over an immobile absorbing phase. Several substances, such as fatty acid profiles, myoglobin and hemoglobin, dipeptides, and myofibrillar proteins, have been utilized to identify meat species and detect animal tissues in feedstuffs through their characteristic component patterns. However, this method is less efficient when identifying adulterated meat species because of the increased complication of

the chromatographic patterns in meat mixtures. In addition, the requirements of expensive instruments and laborious sample preparation procedures have restricted its use in this area.

Electrophoresis, such as isoelectric focusing (IEF) and capillary electrophoresis, is another commonly used technique for differentiation of meat species. The basic principle of separation is the differential movement of protein molecules based on electric charges or molecular weights in an electric field. Although electrophoresis can rapidly separate the protein samples and even automate the system of analysis, it still suffers the same disadvantage as chromatography, as it is difficult to interpret the results of the meat mixture samples unambiguously.

Spectroscopy is a technique based on the unique absorbance profiles at specific wavelengths of the electromagnetic spectrum of the sample components. Near-infrared reflectance spectroscopy (NIRS) is the most important spectroscopic method for analysis of meat and feed samples. Several studies have utilized mid-infrared spectroscopy (MIRS) to identify offal adulteration in meat (Fontaine and others 2001, 2002 and 2004). The advantages of the NIRS techniques include its rapidity, use of innocuous reagents, minimal sample preparation, noninvasive analysis, and its good and repeatable signal intensity. However, the main drawback of NIRS is that the method is indirect and therefore requires a reference database. The detection limit of spectroscopy is also relatively high, so other techniques are required to confirm the results.

DNA-based techniques, including DNA hybridization and polymerase chain reaction (PCR) provide a sensitive and powerful technique for the inspection of meat and feed products. Tartaglia and others (1998) were the first to design a PCR test enabling the detection of ruminant tissues in feedstuffs. The multiplex PCR (MPCR) was able to detect 0.02% bovine meat and bone meal (MBM) in feedstuffs (Gao and others 2003), while the detection limit of the restriction fragment length polymorphism PCR (RFLP/PCR) was even better, at 0.01% (Tajima and others 2002). More recently, real-time PCR (RT/PCR) has been found to detect 0.001% bovine MBM in feedstuffs (Lahiff and others 2002). Although PCR techniques have the advantage of achieving a very low detection limit, they suffer from serious drawbacks. Besides the requirement of expensive instrument and reagents, PCR methods are prone to contamination and are unable to distinguish between DNA from prohibited animal protein and DNA from allowed animal proteins of the same species, such as milk, fat and blood.

Immunochemical techniques are based on the specific immunoreactions between a species-specific antibody and target antigen. The most extensively employed technique in this

area is enzyme-linked immunosorbant assay (ELISA). ELISA methods, such as indirect ELISA, competitive ELISA and sandwich ELISA, have been widely applied to analyze meat and feed products. There are many advantages to ELISA. For example, no major instrument is required; it is easy to operate and employs minimal reagents. Furthermore, ELISA not only has a large-scale screening and field test capability but is also a rapid, specific, sensitive and reliable technique.

Several commercial ELISA kits for the inspection of meat and feed products have been developed and are marketed worldwide (Table 4). Sandwich ELISA is the dominant format of assays. Briefly, one species-specific antibody (the capture antibody) is bound to a solid phase. The sample is then added, upon which the target protein binds to the capture antibody. Unbound molecules are removed with a washing step, and a labeled second antibody (the detection antibody) is added that binds to the target protein, thus completing the "sandwich." The major advantages of sandwich ELISA are that the sample does not need to be purified prior to use, and the assay is highly sensitive. Furthermore, the capture antibody is already bound to the microplate, eliminating the time and effort required for an overnight incubation step. If the assay format is in the form of a rapid lateral-flow immunoassay device, an inexpensive test strip that incorporates antibodies onto a paper strip that reacts with the sample to form a color band, the entire time for analysis can be reduced to less than an hour.

Recently, Chen and others (2004) developed a sandwich ELISA for the detection of ruminant proteins, namely skeletal muscle protein troponin I (sTnI), in feedstuffs. Because of the specificity, high immunoreactivity and complementary epitopes of two monoclonal antibodies (MAbs), the detection limit of the assay for the detection of bovine and ovine sTnI, can be as low as 5.0 and 4.0 ng/ml, respectively, in feed samples. Chen and Hsieh (2002) reported that sTnI was a 24 kD thermo-stable species marker protein (TSMP), which could maintain the stability of the epitopes even after undergoing severe heat treatment (132ºC/2.0 bar, 120 min). In addition, sTnI has specific antigenicity and has an even distribution in skeletal muscles. Therefore, sTnI appears to be an ideal biomarker for which antibodies can be developed for the detection of animal tissues in severely heat-treated samples.

Currently, there is no rapid, specific and reliable assay that is able to detect all types of mammalian tissues in highly processed meat and feed samples; hence it is imperative to develop an effective, rapid analytical method to do so. In the light of the excellent properties of ELISA and sTnI, in this project we attempted to develop a rapid MAb-based sandwich

ELISA based on the detection of mammalian sTnI as the target protein in meat and feed products.

OBJECTIVES

The overall goal of this research was to develop a sandwich ELISA for the detection of mammalian meat in raw, cooked and autoclaved meat and feed products.

The specific objectives were: a) to select a pair of suitable MAbs (sTnI specific) for the construction of a sandwich ELISA; b) to optimize the sandwich assay conditions; and c) to determine the detection limit of mammalian tissue in laboratory-adulterated raw, cooked and autoclaved samples.

REVIEW OF THE LITERATURE

Problems of Meat Species Adulteration

Meat products sold for human consumption should be accurately labeled as to which meat species they contain. However, fraudulent or unintentional mislabeling still exists that may not be visually detectable. Meat adulteration occurs not only at importation but also at the restaurant and retail level, where the substitution is easier to conceal. The problem of meat species adulteration has been reported in several countries (Barai and others 1992; Kang'ethe and others 1982). Species adulteration of 22.5% was found in cooked meat products and 15.9% in fresh ground meat sold in Florida (Hsieh and others 1995). In Alabama meat markets, 90% of market-made ground pork samples contained undeclared beef and/or poultry, while 54% of the pork sausage samples contained undeclared meat species. In adulterated pork sausage samples, 62% were contaminated with a single species, 36% with two species, and 2% with three species (Hsieh and others 1996). Surveys conducted by the USDA-FSIS in 1996 and 1999 showed that more than 40% of food companies were unwilling to voluntarily provide label nutrition information for their single-ingredient, raw meat and poultry products (USDA-FSIS 2001). A survey by the Health Department of Western Australia reported that 11% of cooked meat samples and 13% of raw meat samples were inaccurately labeled (Chemistry Centre of Western Australia 1999). In the UK, 14.6% of meat samples were identified as having labels that did not declare a species of meat detected within them (MAFF 1999). The rate of suspicious ground meat samples was even higher in Ontario, at up to 32.5% (Odumeru 2003).

Meat species adulteration not only constitutes economic fraud, thereby violating consumers' trust in the meat industry, but there are also concerns for those with religious taboos, such as kosher food for Jews and Muslim prohibitions, moral aversions, or individuals allergic to particular meat species. A study diagnosed beef allergy among 11 (3.28%) of 335 atopic children (Werfel and others 1997). The incidence of beef allergy may be as high as 0.3% in the general population (Fiocchi and others 2000). In addition, once a meat product has been adulterated by unknown meat, the ability to trace the sources of pathogen contamination is lost. This could be a serious issue for food safety if the contaminated meat is not cooked to the minimum internal cooking temperature required by the contaminating species to destroy pathogens (Hsieh and others 1999). Furthermore, if meat

that is contaminated by PrP^{sc}, the BSE disease causing agent, has been used to adulterate other meats, it could lead to a serious risk of consumers developing fatal TSEs.

Problems of Animal Feedstuffs

Bovine spongiform encephalopathy. The TSEs are chronic, progressive, and fatal neurodegenerative disorders of both animals and humans (Wilesmith and others 1988; Prusiner 1994). More than 20 countries have reported cases of BSE, a type of TSE found in cattle, since this disease was first identified in the UK around 1987 (Anon 2000). By 2003, approximately 183,000 cases of BSE had been confirmed worldwide (OIE 2003). However, the actual numbers that have been affected are more likely to be around one million, with perhaps 750,000 BSE-infected cattle entering the human food chain in the 1980s and early to mid-1990s (Anderson and others 1996). To date, the US has detected a sheep version of BSE in imported sheep (Enserink 2001) and on December 25, 2003, the first case of BSE in a dairy cow in Washington State was confirmed by the USDA (CDC 2004b).

BSE is a fatal degenerative disease affecting the central nervous system (CNS) of cattle through an abnormal conformation form of a normal prion protein (PrP) (Trevitt and Singh 2003). The normal PrP, found predominantly on the surface of neurons attached by a glycoinositol phospholipid anchor, is a normal host protein encoded by a single exon of a single copy gene and may be involved in synaptic function and is protease sensitive. It begins with signs of anxiety, restlessness, and aggressive behavior, thus leading to the name "mad cow disease". Death usually occurs between two weeks and six months after the onset of clinical symptoms. Currently, no test to detect the disease in live cattle has been validated. The only identifiable common factor in BSE-infected cows is the consumption of cattle feed manufactured from animal by-products containing ruminant-derived protein. BSE can be experimentally transmitted to goats and sheep by the oral route through feeding them as little as 0.5 g of brain tissue from BSE-infected cattle (Foster and others 1993).

New variant Creutzfeldt-Jakob disease. Creutzfelt-Jakob disease (CJD) is a rare, fatal, human TSE with a current worldwide incidence of about one case per million per year. In 1995, a novel form of CJD, new variant CJD (nvCJD), was discovered in the UK (Will and others 1996). Around 70% of those infected die within six months (Trevitt and Singh 2003). The disease was not considered a public health threat until March 1996, when the UK Government announced that there might be a positive association between BSE and human nvCJD (Brown 1996; Butler 1997). Up to December 2003, a total of 153 cases of nvCJD had

been reported in the world (CDC 2004a). In early 2002, the first nvCJD patient in the US was confirmed in Florida (Wiersma and others 2002).

Laboratory studies provided strong evidence that the causative agents of BSE in cattle and nvCJD in humans shared a common origin (Irani and Johnson 2003). Furthermore, experimental data showed that nvCJD resulted from exposure to the BSE agent (Collinge and others 1996; Bruce and others 1997; Cousens and others 1997; Taylor 2002). Although the mechanism of transmission of the BSE agent to human beings has not been established, it was thought to be through consumption of bovine meat products contaminated with BSE agent. However, current slaughtering processes could not absolutely exclude the CNS contamination of beef meat (Lucker and others 2002). Frighteningly, epidemiologic models have suggested that the number of future nvCJD cases could vary from fewer than 100 to upwards of hundreds of thousands (Ghani and others 2000; Valleron and others 2001). In the latest study of the potential effects of nvCJD in the UK, scientists predicted that up to 4,000 people might already be infected with the fatal prion disease (Hawkes 2004).

Due to consumers' awareness of health issues and the lethality of BSE, the consumption of beef and lamb has been decreasing while that of poultry has shown a progressive rise. For instance, in the UK, poultry consumption in 1999 was up 22% compared to 1979 (MAFF 2000). The consumption of beef and lamb dropped more than 11% in France in 1996 − 1997 (Sorenson and others 2003), while poultry consumption increased 23% (Klipstein-Grobusch and others 1998). In 1996, beef consumption in Slovenia dropped by 16% (Curk 1999). In Geneva, the percentage of female meat consumers who did not eat pork or lamb had increased to 26.4% by late 1996. However, chicken consumption per week for female meat consumers increased markedly to 19% over the baseline (January 1993 − April 1996) level in 2000 (Sorenson and others 2003). In the US, per capita beef consumption peaked around 1976. In 2001, it was 29% down compared with that in 1976. In contrast, primary poultry consumption had risen by 86% in 2001 compared with 1976 levels (USDA 2004).

Regulation of Meat and Feed Products

To prevent adulterated or misbranded products from being sold as food, and to ensure meat products are slaughtered and processed under sanitary conditions, many countries have enacted very strict laws. In the US, the Federal Meat Inspection Act of 1906 requires the USDA to inspect all cattle, sheep, swine, goats, and horses brought into any plant to be slaughtered and processed into products for human consumption (FDA 1906). The original

Meat Inspection Act did not include the poultry industry, which at the time was mainly small production by independent farmers. The Poultry Products Inspection Act, passed in 1957, made poultry inspection mandatory (FDA 1957).

The Nutrition Labeling and Education Act of 1990 requires nutrition labeling of most foods regulated by the FDA. In 1993, the FDA published final regulations requiring comparable nutrition labeling requirements for multi-ingredient and heat processed meat and poultry products (FDA 1993).

Since the consumption of infected feedstuffs by ruminants has been indubitably recognized as the main BSE transmission channel, legislation has been enacted to avoid certain or all animal by-products such as MBM entering the ruminant feed chain throughout the world and prevent the establishment and amplification of BSE through feed, thereby minimizing any risk to animals and humans. The EU in 1994 and the US in 1997 banned the use of proteins originating from mammalian tissues for feeding ruminants (EC 1994; FDA 1997). The World Health Organization (WHO) has recommended that all countries should ban the use of ruminant tissues in ruminant feed since 1996 (WHO 1996). Furthermore, the EU decided in 2000 that processed animal proteins should be banned for all farmed animals kept, fattened or bred for the production of food (EC 2000; EC 2002).

The recent discovery of a single cow with BSE in a Washington State dairy herd illustrates the stringent need for an accurate, rapid, national livestock identification system to trace infected cattle in the US. The US Animal Identification Plan (USAIP), an efficient and effective animal identification program, has been implemented through the use of radio frequency identification (RFID) technology (Troyk 1999). The USAIP recommends that all cattle, swine, and small ruminants possess individual or group/lot identification for interstate movement by July 2005 (National Identification Development Team 2003). In early 2004, the USDA-FSIS declared that skull, brain, trigeminal ganglia, eyes, vertebral column, spinal cord and dorsal root ganglia of cattle 30 months of age or older and the small intestine of all cattle were specified risk materials and prohibited their use in human food (USDA-FSIS 2004).

Techniques for Meat Species Identification and Detection of Animal Tissues in Feeds

Chromatography. Chromatographic techniques for species identification and detection animal tissues in feeds can be classified as gas chromatography (GC), liquid chromatography (LC), or high performance liquid chromatography (HPLC), which itself can be further categorized as cation exchange HPLC, anion exchange HPLC, size exclusion HPLC or reverse-phase HPLC based on the different separation theories utilized.

Several different substances have been used as the basis of speciation in conjunction with the chromatographic techniques in meat and feed products. Unique fatty acid profiles of different meat species can be obtained from GC analysis, thus differentiation of meat species in meat products can be conducted through an examination of the animal fat present (Araujo de Vizcarrondo and others 1998). A study of triglycerides using LC reported that the ratio of triglyceride containing saturated fatty acids vs. triglyceride containing unsaturated fatty acids at the same (C-2) position in a sample could be compared with those of pure meats, and pork fat had larger amounts of triglyceride containing saturated fatty acid at the C-2 position than that found in other kinds of meat. The presence of pork in the sample would thus cause the ratio to increase compared with the ratios for pure beef or mutton, which could then be used to detect pork and lard adulterants in beef or mutton (Saeed and others 1989). However, the sensitivity of the technique varied with the types of samples; 1% pork in beef significantly increases the ratio, while up to 3% pork is needed to cause a noticeable change in mutton.

Hemoglobin also has been used as a biomarker to detect meat adulteration. A biomarker is a specific biochemical substance that has a particular molecular feature that makes it useful for measuring the qualities of meat or feed samples. As different meat species produce different peak patterns for hemoglobin with HPLC separation of the meat extracts, as little as 0 to 1% hemoglobin in experimentally contaminated tissue could be detected in poultry muscle tissue through cation exchange HPLC (Lyon and others 1986). In another study, Oellingrath and others (1990) showed that 2% of myoglobin and hemoglobin could be detected in ground beef by HPLC. Using these characteristic peak patterns, Wissiack and others (2003) also screened samples to detect meat adulteration through the determination of hemoglobin by cation exchange HPLC with a diode array detector. However, the detection limit of the assay was poor, being limited to approximately 10% to 30% of different species adulteration levels.

Carnosine and related dipeptides (balenine and anserine) belong to another class of biomarkers that have been used to identify different meat species. They are naturally-occurring histidine-containing compounds and are present in the fraction of the total water-soluble nitrogen-containing substances. They are found in animal tissue exclusively, including in heart muscle, kidney, and liver, but are present in extremely high concentrations in muscle tissue (Quinn and others 1992). It had been reported that the ratios of anserine and carnosine were sufficiently different between sheep, cattle, horse and kangaroo that the detection of species adulteration could be achieved by HPLC (Carnegie and others 1985). Similarly, Huang and Kuo (2000) found that the ratios of carnosine and anserine were also

very specific in the chicken, duck and turkey meat extracts (breast and thigh). Schonherr (2002) detected animal tissue in feeds by the determination of carnosine and related dipeptides using HPLC, because these dipeptides do not present in either plants or bacteria. A detection limit of about 0.5% can be reached. Toorop and others (1997a, 1997b) extracted myofibrillar proteins with an extraction buffer (0.4 M NaCl at pH 6.0) in raw meat, from which they also obtained the typical chromatograms of raw beef, lamb, veal, pork and turkey through size exclusion HPLC. Subsequently, they were able to detected raw and cooked pork meat which had been adulterated in veal meat using the method they had developed and analyzed the results using a multiple regression to form prediction equations. However, these equations had a 3% variation that could not be accounted for. Overall, chromatographic methods are limited to identifying the species in raw and cooked pure meat; they are not satisfactory for meat mixtures.

Electrophoresis. The process of electrophoresis is defined as the differential movement of ions by attraction or repulsion in an electric field. In practical terms, a positive (anode) and a negative (cathode) electrode are placed in a solution containing ions. Then, when a voltage is applied across the electrodes, solute molecules of different charges will move through the solution towards the electrode of opposite charge. It is a powerful technique for the separation of proteins. IEF and capillary electrophoresis are the two methods most often used to identify meat species.

IEF is a method of determining the isoelectric point of a protein by carrying out electrophoresis in a microchannel or a piece of gel containing a pH gradient. Slattery and Sinclair (1983) demonstrated that beef and buffalo meat, and meat from red and grey kangaroos could be clearly distinguished by IEF on polyacrylamide or agarose gel in the pH range 5.5 to 8.5. Skarpeid and others (1998) also identified samples containing various amounts of beef, pork and turkey meat though IEF. However, the prediction errors were close to 10%.

Capillary electrophoresis is the technique of performing electrophoresis in buffer-filled, narrow-bore capillaries. Cota-Rivas and Vallejo-Cordoba (1997, 1998) developed a sodium dodecyl sulfate (SDS) polymer-filled capillary gel electrophoresis (CE-SDS) method for meat species differentiation. They then utilized linear discriminant analysis (LDA) in the interpretation of CE-SDS meat protein profiles to identify 42 samples of raw beef, pork and turkey meats.

Although electrophoresis can rapidly separate the samples and even automatically analyze them, it is still difficult to interpret the results when more than one species coexist in a meat sample.

Spectroscopy. This technique usually involves three essential factors: (1) a source of light, (2) an element to separate the light into its component wavelengths, and (3) a detector to sense the presence of light after separation of wavelengths. The most widely used spectroscopic analytical technique used for monitoring the quality of meat and feed products is NIRS, Near Infrared Reflectance Spectroscopy, which is based on the absorption of the electromagnetic spectrum at wavelengths in the range 780 − 2500 nm by specific molecules in the analyzed samples (Dyer and Feng 1997).

Fontaine and others (2001, 2002 and 2004) published the results of the NIRS amino acid calibrations of protein-rich feedstuffs and cereals including brans or middlings. With a few exceptions, corroboration showed that 70 − 98% and 85 − 98% of the amino acid variance in the cereal samples and in the feed samples, respectively, could be revealed using NIRS. This technique has been used not only for feed composition determination, but also for meat authentication (Davies and Grant 1987; Downey 1996). Ding and others (1999) indicated that NIRS could be used to identify broiler meat or carcasses from those of local chickens. Overall identification accuracies of 100%, 92%, 96% and 92% in chickens were achieved for minced thigh meat, minced breast meat, breast cut without skin and breast cut with skin, respectively. In another study (Ding and Xu 1999), differentiation accuracy of beef and kangaroo meat was 83% to 100%, respectively, and no kangaroo meat was misclassified. Ding and Xu (2000) developed NIRS to detect beef hamburgers adulterated with 5 − 25% mutton, pork, skim milk power, or wheat flour with an accuracy up to 92.7%, while the accuracy of detection increased with the increase of adulteration level. NIRS has also been applied to differentiate refrozen and thawed beef from unfrozen beef with accuracies between 90 and 100% (Thyholt and Isaksson 1997).

Mid-Infra Red Spectroscopy (MIRS) is another useful spectroscopic technique for the authentication of meat products. Al-Jowder and others (1997) utilized MIRS for species identification and semi-quantitative control of turkey, chicken and pork mince between fresh and thawed samples. They also showed that MIRS, combined with appropriate chemometric methods, could discriminate between pure beef and beef mixed with 10 − 100% (w/w) ox kidney or liver (Al-Jowder and others 1999). They went on to apply the same method to other similar problems and ascertained that it could be used to discriminate between pure cooked

beef and adulterated cooked beef containing 20% (w/w) of each of four ox adulterants: heart, tripe, kidney and liver (Al-Jowder and others 2002).

Although spectroscopy is an attractive option for quality screening because it is rapid, and noninvasive, it still requires sophisticated instrumentation and the development of a database. The detection limit of NIRS can be down to 1%; however, it is not as sensitive as other confirmation methods.

DNA-based techniques. Identification of animal species through an examination of the nucleic acids in meat products was first achieved by simple DNA hybridizations, generally in a type of dot blot design on filters, using labeled DNA probes (Chikuni and others 1990; Winterø and others 1990; Ebbehøj and Thomsen 1991a, 1991b; Buntjer and others 1995). Although the heat treatment of meat samples in some studies can be up to 120ºC for 30 min, the detection limit fluctuated around 1%. With the development of effective genetic amplification methods, mainly PCR, a technique for amplifying a specific region of DNA defined by a set of two primers, the sensitivity of DNA-based meat or feed component analysis increased dramatically.

Gao and others (2003) amplified a highly conserved eucaryotic DNA region of the 18S ribosomal gene using multiplex PCR (MPCR), which employs different primer pairs in the same amplification reaction, and were able to detect levels as low as 0.02% bovine MBM in feedstuffs. Bellagamba and others (2003) detected 0.25% ruminant or pig adulterants in fish meal by MPCR.

Lahiff and others (2002) detected the presence of bovine DNA in MBM samples using real-time PCR (RT/PCR). RT/PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection employed by conventional quantitative PCR methods. A 271-bp region of the mitochondrial ATPase 8-ATPase 6 gene was amplified with a detection limit of 0.001%.

Lahiff and others (2001) also identified the presence of ovine, porcine and chicken species in MBM samples through the use of restriction fragment length polymorphism PCR (RFLP/PCR). RFLP is a method that allows familial relationships to be established by comparing the characteristic polymorphic patterns that are obtained when certain regions of genomic DNA are amplified and cut with specific restriction enzymes. The detection limits reported for the bovine, porcine, ovine and poultry specific PCR assays were 1%, 1%, 5% and 5% (w/v), respectively. Tartaglia and others (1998), as well as other authors (Wang and others 2000; Tajima and others 2002; Bottero and others 2003; Frezza and others 2003), have

chosen mitochondrial gene targets for the design of RFLP/PCR assays. These authors reported different detection limits with their PCR methods. Tartaglia and others (1998) and Wang and others (2000) reported a sensitivity of 0.125% using the bovine primers L8129 and H8357, whereas the detection limits were even lower in the studies of Bottero and others (2003) and Tajima and others (2002), which were 0.0625% and 0.01%, respectively.

RFLP/PCR technique has been widely applied to meat species identification (Meyer and others 1994; Guglich and others 1994; Calvo and others 2001; Bellis and others 2003; Rodriguez and others 2004). In a study reported by Montiel-Sosa and others (2000), highly species-specific primers of pork mtDNA D-loop region fragments were amplified. This method was able to detect 5% (w/w) of raw pork in beef. In another study, Calvo and others (2002a, 2002b) detected 1% raw and heated (30 min at 50ºC, 80ºC and 120ºC) pork in beef, and pork in duck, and 0.01% raw beef in pork and 1% cooked (30 min at 80ºC and 120ºC) beef in pork. Seyboldt and others (2003) utilized a 168-bp glial fibrillary acidic protein (GFAP) mRNA of bovine CNS as a specific marker to detect bovine CNS tissue in minced meat products through RFLP/PCR assays. 0.5% (w/w) of bovine brain homogenate was successfully detected in raw and cooked meat products.

By using taxon and/or species-specific DNA as the animal markers, the detection limit of PCR techniques can be as low as 0.001%. However, the equipment and reagents are costly. It is also vulnerable to contamination and interference due to the presence of DNA from allowed animal proteins of the sample species in ruminant nutrient (i.e. milk), or other animal materials (fat and blood, etc.).

Immunochemical techniques. Based on the primary interaction of an antibody with an antigen, immunochemical techniques have been adapted to analyze trace amounts of target components in complex mixtures. These techniques have been widely applied to meat species identification for some time (Hitchcock and Crimes 1985) and several commercial kits have been developed for the analysis of meat and feed products (Table 4).

Immunodiffusion is a common immunochemical techniques. This method, when employed for meat species identification, involves the diffusion of the antigen and antibody through a semisolid medium, usually agar or agarose gel, resulting in a precipitin reaction. Precipitin lines or bands are formed on the gel wherever the concentrations of the antigen and antibody are equivalent (Cutrufelli and others 1988, 1993). Martin and others (1998) quantitatively evaluated raw pork adulterated in ground beef by agar gel radial immunodiffusion and found the detection limit to be 3 to 5% (w/w).

ELISA is one of the most powerful immunochemical techniques available and has been extensively employed in meat species detection because of its rapidity, specificity, sensitivity, reliability and low cost. Based on the assay format, ELISA can be classified into three types, namely (1) antibody capture assays, (2) antigen capture assays, and (3) two-antibody sandwich assays (Harlow and Lane 1988).

Dot-ELISA is an antibody capture assay where the antibody binds to the immobilized antigen. In a study of hamburger meat identification by Macedo-Silva and others (2000), this method detected adulteration of the meat extract of the homologous species at a concentration of 0.6%.

Indirect ELISA is an extensively applied antibody capture assay often used in meat authentication (Kang'ethe and others 1982; Martin and others 1989; Pickering and others 1995; Sheu and Hsieh 1998; Asensio and others 2003). Hsieh and others (1998) reported that an indirect ELISA assay using monoclonal antibody (MAb) specific to cooked mammalian meats could detect 0.5% (w/w) of cooked pork, beef, lamb, and horse meats in a chicken-based mixture.

In recent years, several studies have reported the use of detection of biomarkers by immunochemical methods to identify species adulteration in processed-meat or feed. Chen and others (1998) developed MAbs (5H9, 5H8, 2F2 and 8A4) specific to porcine thermal-stable muscle proteins (TSMPs), which could detect at least 1% (w/w) pork in raw and cooked heterogeneous meat mixtures. In another research study (Chen and Hsieh 2000), the detection limit of MAb 5H9 was improved to 0.5% (w/w) by indirect ELISA. Subsequently, Chen and Hsieh (2002) confirmed that the porcine TSMP involved was skeletal troponin I (sTnI). The main function of sTnI in the body is the inhibition of actomyosin ATPase (Leavis and Gergely 1984). It is a thermo-stable species marker protein whose epitope could be recognized by MAb 5H9 even after severe heat treatment (132ºC/2.0 bar, 120 min). The authors used sTnI specific MAbs (7A12, 8A12 and 2A8) for the detection of the rendered muscle tissues in animal feedstuffs by indirect ELISA. The detection limits of the mammalian and ruminant assays were between 0.3 and 2% (Chen and others 2002). In addition, Hsieh and others (2002) developed an indirect ELISA using MAb 2F8 to evaluate the endpoint heating temperature (EPT) in ground pork and beef.

The sensitivity could be greatly enhanced if a sandwich ELISA could be applied. Sandwich ELISA utilizes a pair of MAbs as the capture and detection antibodies (Martin and others 1988, 1991; Ansfield and others 2000a, 2000b). More recently, Chen and others (2004) developed a sandwich ELISA for the detection of ruminant proteins in feedstuffs.

Their assay used MAb 5G9 (specific to bovine and ovine sTnI) as the capture antibody and the biotin-conjugated MAb 2G3 (reacting to all animal sTnI) as the detection antibody and the assay achieved detection limits for bovine and ovine sTnI as low as 5.0 and 4.0 ng/ml, respectively.

The causative agent of BSE, PrP^{sc}, is found in Central Nervous System (CNS) tissues. Because the contamination of CNS tissue cannot be effectively controlled, two kinds of biomarkers of the CNS have recently been utilized to detect CNS tissue in meat and feed products. Lucker and others (1999) reported that neuron-specific enolase (NSE), the most abundant form of the glycolytic enzyme enolase found in mature neurons, could be a useful marker protein in a study using Western blotting. Western blotting is an electroblotting method in which proteins are transferred from a gel to a thin, rigid support (nitrocellulose) and detected by binding to a labeled antibody. Through using the MAb-based Western blotting, the detection limit of brain tissue could be reduced to 0.25% (w/w) in both raw and heat-treated meat products.

GFAP, which is restricted to the CNS, is another useful biomarker of CNS tissue. Schmidt and others (1999, 2001) developed a sandwich ELISA, which could detect about 0.037 ng/mg of GFAP in beef. However, severe heat treatment (115ºC, 100 min) eliminated the detectability of GFAP. In two comparative evaluations of the performance of these two techniques (Agazzi and others 2002; Hughson and others 2003), very little difference in the detection limits between the two methods was observed, from 0.5% to 1% (w/w) in both raw and cooked (80ºC, 20 min) meat samples. However, when the samples were cooked at 120ºC for 20 min, the detection limit was raised to 2% (w/w) CNS for the NSE test kit, while the detection limit of the GFAP test kit remained at the same level.

Comparing the GFAP test kit and the NSE test kit, the methods based on the detection of sTnI appear to offer more advantages due to their applicability in severely heat-treated samples, their better sensitivity, and their more ubiquitous target molecules (i.e. muscle vs. brain) in MBM samples.

METHODOLOGY

Materials

Sodium chloride, sodium phosphate (monobasic anhydrous), sodium phosphate (dibasic anhydrous), sodium bicarbonate, sodium carbonate (anhydrous), Tween-20, citric acid monohydrate and bovine serum albumin (BSA) were purchased from Fisher Scientific (Fair Lawn, NJ). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG, ethylenediaminetetraacetic acid (EDTA), 2,2' -azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals and reagents were of analytical grade. Glassware was washed using Versa-Clean® detergent (Fisher Scientific, Fair Lawn, NJ) and rinsed with pure water from the NANOpure DIamond® ultrapure water system (Barnstead International, Dubuque, IA). The pure water was used to prepare all solutions used in this study. Soy Best, a soy-based feed that contained high bypass soybean meal, was obtained from Crain States Soya Inc. (West Point, NE).

MAbs (2G3, 6G1, 8A12 and 8F10), specific to sTnI, were previously prepared in Dr. Hsieh's laboratory. MAbs 2G3 and 6G1 react with all mammalian and poultry meat samples, while MAbs 8A12 and 8F10 recognize mammalian meat samples. HRP-conjugated MAb 8F10 was provided by Neogen Co. (Lansing, MI).

Preparation of Meat Samples

Beef chuck, lamb thigh, pork shoulder, turkey thigh, whole chicken and duck meats were purchased from local supermarkets in Tallahassee, FL. Deer and elk steak meat samples were provided by the Fats and Proteins Research Foundation (Bloomington, IL). Horse meat was obtained from the College of Veterinary Medicine, Auburn University (Auburn, AL). All meats were intact pieces and stored at -80ºC until use.

Extraction of Meat Soluble Proteins

All lean meats were cut into small pieces and finely ground separately with a Hamilton Beach® grinder (Proctor-Silex, Inc., Washington, NC). The particle size of the ground meats was less than 50 mm. A portion of 20 g of ground lean meat from each species was transferred to a beaker and heated the beaker in boiling water (100ºC) for 30 min.

Another 20 g of ground lean meat from each species was autoclaved at 121ºC/1.2 bar for 30 min using a NAPCO® 8000-DSE Benchtop Autoclave (Jouan Inc., Winchester, VA). Raw meat extracts were prepared by mixing 10 g of ground lean meat from each species (1:5) (w/v) with the antigen extract solution (10 mM phosphate-buffered saline (PBS) buffer containing 0.5 M NaCl (pH 7.0)) in a sterile plastic bag (Fisher, Pittsburgh, PA) and homogenized by a stomacher (Tekmar Co., Cincinnati, OH) for 30 seconds. Two-fold (w/v) of the antigen extract solution was added to the cooked and the autoclaved meat samples and homogenized well by ULTRA-TURRAX® T 25 basic homogenizer (IKA Works, Inc., Wilmington, NC) for two min at 11,000 rpm. All sample homogenates were held at 4ºC for 120 min before the mixture was centrifuged at 3,220 × g for 30 min at 4ºC. Supernatants were filtered through Whatman No. 4 filter paper (Fisher Scientific, Fair Lawn, NJ) at 4ºC and stored at -20ºC until use. Protein concentration of the extracts was determined by Protein Assay kit II (Bio-Rad, Hercules, CA) following the manufacture's instructions. BSA was used as the protein standard (0.05 to 0.50 mg/ml).

Preparation of Laboratory-Adulterated Meat Samples

Two methods were used to prepare the adulterated meat extracts. The first was to mix extracts of raw, cooked and autoclaved 4.00% of beef in turkey and 4.00% of pork in chicken (w/w) with pure raw, cooked and autoclaved turkey and chicken extracts, to a series of final concentrations of adulterated beef in turkey or pork in chicken of 0.05, 0.10, 0.50, 1.00 and 2.00% (v/v). The other method was to mix ground pork in chicken at each adulteration level (0.00, 0.05, 0.10, 0.50, 1.00 and 5.00% on a weight basis) and then follow the method described previously to prepare cooked and autoclaved meat protein extracts.

Preparation of Laboratory-Adulterated Feed Samples

Dry bovine meat meal was prepared according to Chen and others (2004). Two percent (w/w) dry beef meat meal in Soy Best and pure Soy Best were separately extracted by the antigen extract solution (1:5, w/v) and held at room temperature for 120 min. After centrifugation of the mixture at 3,220 × g for 30 min at 20ºC, the supernatants were filtered through Whatman No. 4 filter paper in room temperature. The extract of dry bovine meat meal was used as the adulterant to Soy Best. Different percentages (0.01, 0.05, 0.10, 0.50 and 1.00%, v/v) of bovine meat meal extracts were mixed well with pure Soy Best extracts and stored at -20ºC until use.

Indirect ELISA

To demonstrate the specificities of the MAbs, two micrograms of protein from each extract, properly diluted in 100 µl of 0.06 M carbonate buffer (3.806 g of sodium bicarbonate and 1.93 g of sodium carbonate in 1000 ml pure water) containing 10 mM EDTA (pH 9.6), was coated to each well of a microplate (polyvinyl chloride plates) (Costar Co., Cambridge, MA) and incubated at 37˚C for 60 min. The microplate was washed 3 times with 250 µl/well of the washing buffer (10 mM PBS containing 0.05% Tween-20 (pH 7.2)) using a microplate washer (Bio-Rad Model 1575) (Bio-Rad, Hercules, CA), and then the remaining binding sites were blocked by adding 200 µl/well of blocking buffer (1% BSA in 10 mM PBS (pH 7.2)). The microplate was incubated for 60 min at 37˚C and washed two times with 250 µl/well of the washing buffer. A portion of 100 µl/well of properly diluted MAb in the antibody buffer (1% BSA in 10 mM PBS containing 0.05% Tween-20 (pH 7.2)) was added to the microplate, and incubated at 37˚C for 60 min. After washing the microplate three times with the washing buffer, 100 µl of HRP-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) 1:3,000 (v/v) diluted in the antibody buffer was added to each well and the plate was incubated at 37˚C for 60 min. The microplate was then washed five times with the washing buffer before the addition of 100 µl/well of ABTS substrate solution (22 mg of ABTS and 15 µl of 30% hydrogen peroxide in 100 ml of 0.1 M phosphate-citrate buffer, pH 4.0). Color was developed at room temperature for 30 min and the reaction was stopped by adding 100 µl/well of 0.2 M citric acid. Absorbance was read using a microplate reader (Bio-Rad Model 450) (Bio-Rad, Hercules, CA) at 415 nm. The indirect ELISA model is shown in Figure 20.

Epitope Comparison

A competitive immunoassay was used to test for potential steric competition of MAbs 2G3, 6G1 and 8A12 with MAb 8F10. The wells of a microplate were coated with 100 µl of cooked beef extract diluted in 0.06 M carbonate buffer containing 10 mM EDTA (pH 9.6); the protein concentration was 0.25 µg/well. After incubation at 37ºC for 60 min, the microplate was washed three times with the washing buffer, and then blocked with 200 µl/well of the blocking buffer and incubated at 37ºC for 60 min followed by washing two times.

Experiment 1: To determine the epitope competition between three MAbs and MAb 8F10, sequential dilutions of purified MAbs (6G1, 8A12 and 8F10) (1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:16,000, 1:32,000, and 1:64,000 (v/v)) or MAb 2G3 supernatant (1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1,280 (v/v)) were prepared with the antibody buffer

containing 1:9,000 (v/v) HRP-conjugated MAb 8F10 in a low-binding reagent reservoir (polystyrene reagent reservoir) (Corning Inc., Corning, NY) and then 100 µl/well of each diluted MAb reagent was transferred to the coated and blocked microplate. After the microplate was incubated at 37ºC for 120 min and washed five times, 100 µl ABTS substrate solution was added to each well and incubated for 25 min at room temperature for color development, followed by adding 100 µl of 0.2 M citric acid to each well to stop the reaction. Absorbance was measured by the microplate reader at 415 nm. The assay color only revealed the binding between MAb 8F10 and the coated antigen in the sample extract. Since the concentration of HRP-conjugated MAb 8F10 in each well was constant, the absorbance was expected to remain constant at each dilution level of the other MAbs tested.

Experiment 2: To identify the binding between the MAbs and the coated antigen, the same MAbs at the same sequential dilutions as Experiment 1 were diluted in the antibody buffer without HRP-conjugated MAb 8F10 and transferred 100 µl/well to the coated and blocked microplate. After incubation at 37ºC for 120 min and washing three times with the washing buffer, the wells were loaded with 100 µl HRP-conjugated goat anti-mouse IgG 1:3,000 (v/v) diluted in the antibody buffer and the microplate was incubated at 37ºC for 60 min. After washing five times, 100 µl ABTS substrate solution was added to each well and incubated for 25 min at room temperature for color development, followed by adding 100 µl of 0.2 M citric acid to each well to stop the reaction. Absorbance was determined at 415 nm. The assay color revealed the binding between the tested MAb and the coated antigen. The lower the dilution, the higher the color development anticipated.

Experiment 3: To further identify the results from Experiment 1, the same MAbs at the identical sequential dilutions as above were diluted in the same dilution buffer (the antibody buffer containing 1:9,000 (v/v) HRP-conjugated MAb 8F10) and transferred to the coated and blocked microplate at 100 µl/well. After the microplate was incubated at 37ºC for 120 min and washed three times, 100 µl/well HRP-conjugated goat anti-mouse IgG 1:3,000 (v/v) diluted in the antibody buffer was added to the microplate and incubated at 37ºC for 60 min. After washing five times, 100 µl ABTS substrate solution was added to each well and incubated for 25 min at room temperature for color development. Absorbance was measured at 415 nm after adding 100 µl of 0.2 M citric acid to each well to stop the reaction. The assay color revealed the binding between both MAb 8F10 and the other competing MAb and the coated antigen. If there was no competition between two MAbs, the absorbance was greater than that obtained from either Experiment 1 or Experiment 2.

The overall results are shown in Figures 2, 3, 4 and 5. MAb 6G1 was selected as the capture antibody and MAb 8F10 as the detection antibody for the sandwich ELISA.

Sandwich ELISA

The basic sandwich ELISA (Figure 21) was performed according to the following procedure. The microplate was coated with the capture MAb 6G1 1:1,000 (v/v) diluted in 10 mM PBS (pH 7.2) and then incubated at 37ºC for 60 min. The plate was washed three times with the washing buffer and blocked with 200 µl/well of the blocking buffer, and then incubated at 37ºC for 60 min. After blocking, the plate was washed two times, and then 100 µl/well of sample extract was added. For cooked and autoclaved samples, non-diluted extracts were used, while for raw samples, meat extracts were further diluted 1:10 (v/v) with antibody buffer containing 10 mM EDTA. The plate was incubated at 37ºC for 120 min. After washing three times, 100 µl of HRP-conjugated MAb 8F10 1:1,000 (v/v) diluted in the antibody buffer was added to each well and the plate was incubated at 37ºC for 60 min. After washing five times, 100 µl ABTS substrate solution was added to each well and incubated for 30 min at room temperature for color development, followed by adding 100 µl of 0.2 M citric acid to each well to stop the reaction. The color developed was measured by the microplate reader at 415 nm.

To optimize the conditions of the sandwich ELISA, the best values for the dilutions of the capture and detection antibodies, selection of antigen buffer, incubation time for antigen-antibody binding and dilutions of meat and feed samples were determined.

Selection of dilution of the capture antibody: To determine the optimal dilution of the capture antibody, MAb 6G1, the microplate was coated with MAb 6G1 1:1,000, 1:2,000, 1:4,000 and 1:8,000 (v/v) diluted in 10 mM PBS and diluted autoclaved meat extracts (beef, deer, elk, horse, lamb, pork, chicken, duck and turkey) (1:2 (v/v) in the antibody buffer) were added. The HRP-conjugated MAb 8F10 was 1:3,000 (v/v) diluted in the antibody buffer. The color development time was 10 min. The optimum dilution of the capture antibody, MAb 6G1, was found to be 1:1,000 (v/v) (Figure 6).

Selection of dilution of the detection antibody: To determine the optimal dilution factor of detection antibody, HRP-conjugated MAb 8F10, four different dilutions of MAb 8F10 (1:1,000, 1:2,000, 1:3,000 and 1:6,000 (v/v)) were evaluated. MAb 6G1 was diluted 1:1,000 (v/v). Non-diluted autoclaved adulterated meat extracts (0.50% beef in turkey and 0.50% pork in chicken) (w/w) and pure autoclaved turkey and chicken meat extracts were used. The lengths of the incubation of antigen and color development were selected to be 120

min and 30 min, respectively. The optimum dilution of the detection antibody was found to be 1:1,000 (v/v) (Figure 7).

Selection of antigen buffer: To select the antigen buffer, each of the beef or pork (raw, cooked and autoclaved) meat extracts was 1:2 (v/v) diluted in either a) an antibody buffer containing 10 mM EDTA, or b) an antibody buffer without EDTA. Both MAb 6G1 and HRP-conjugated MAb 8F10 were diluted 1:1,000 (v/v). The color development time was 20 min. The antigen buffer selected for raw meat samples was dilution in the antibody buffer containing 10 mM EDTA, while cooked and autoclaved meat extracts were diluted in the antibody buffer without EDTA (Figure 8).

Selection of incubation time for antigen-antibody binding: To determine the optional incubation time of antigen and antibody binding, three incubation times, 30 min, 60 min and 120 min, were evaluated. Raw (dear, elk, lamb and chicken) meat extracts were 1:10 (v/v) diluted in the antibody buffer containing 10 mM EDTA, and cooked meat extracts (dear, elk and lamb) were 1:2 (v/v) diluted in the antibody buffer. MAb 6G1 and HRP-conjugated MAb 8F10 were diluted 1:1,000 and 1:3,000 (v/v), respectively. The color development time was 30 min. The incubation time selected for antigen-antibody binding was 120 min (Figure 9).

Selection of dilution of laboratory-adulterated meat samples: To determine the optimal dilution of adulterated meat samples, the non-diluted and diluted (1:2 (v/v) in the antibody buffer) adulterated cook and autoclaved meat extracts (beef in turkey) were evaluated, as well as adulterated raw meat extracts (pork in chicken) which were diluted (1:5, 1:10, 1:20, 1:30, 1:50 and 1:100 (v/v)) in the antibody buffer containing 10 mM EDTA. The incubation time for antigen-antibody binding was 120 min. Both MAb 6G1 and MAb 8F10 were diluted 1:1,000 (v/v). After the color substrate was added the microplate for 30 min, the absorbance was read.

The dilution selected for adulterated meat samples was that raw meat samples were 1:10 (v/v) diluted in the antibody buffer containing 10 mM EDTA (Figure 10), while cooked and autoclaved meat extracts were non-diluted (Figures 11 and 12).

Selection of dilution of laboratory-adulterated feed samples: To optimize the dilution of adulterated feed samples, both non-diluted and diluted (1:2 (v/v) in the antibody buffer) adulterated feed extracts were used. Both MAb 6G1 and MAb 8F10 were diluted 1:1,000 (v/v). The incubation time for antigen-antibody binding was 120 min and the color was developed for 30 min. The dilution selected for adulterated feed samples was non-diluted (Figure 13).

Effect of Different Fat Content of Meat Samples

To determine if different fat content of the meat samples would interfere with the performance of the sandwich ELISA developed, beef and pork samples containing different fat contents (0, 10, 20 and 30%, w/w) were prepared and extracted as described previously, and the meat extracts were analyzed using the sandwich ELISA (Figure 15).

Three equal portions of each sample were treated as raw, cooked and autoclaved and then extracted according to the method described previously. However, the raw samples were diluted 1:4 (w/v), and the cooked and the autoclaved samples were diluted 1:1 (w/v) with the antigen extract solution. All meat extracts were stored at -20ºC until use. For the sandwich ELISA, the raw meat extracts were 1:2 (v/v) diluted in the antibody buffer containing 10 mM EDTA, while the cooked and autoclaved meat samples were 1:2 (v/v) diluted in the antibody buffer without EDTA. Other steps followed the procedure described previously.

Effect of Different Heat-Treatment of Meat Samples

To study the effects of heat-processing conditions on the assay signals, six equal portions of pure ground lean beef or pure ground lean pork samples were treated with six different conditions (a) 121°C/1.2 bar, 30 min; b) 128°C/1.6 bar, 30 min; c) 132°C/2.0 bar, 30 min; d) 132°C/2.0 bar, 60 min; e) 132°C/2.0 bar, 90 min; and f) 132°C/2.0 bar, 120 min), and the meat extract from each condition was analyzed using the sandwich ELISA developed (Figure 16).

Statistical Analysis

Data were analyzed using One-Way ANOVA to compare the means for the differences among the treatment groups. The sample size of each group was larger than four. Each sample was measured at least twice. Post hoc analyses were performed using Tukey HSD. Significance was accepted at $P \leq 0.05$. Statistical analysis was performed with SPSS software (11.0 for Windows) (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Species Specificity of Monoclonal Antibodies

The species specificities of the four MAbs, 6G1, 8A12, 2G3 and 8F10 determined by indirect ELISA are shown in Figure 1. The characteristics of these MAbs are summarized in Table 1. MAb 6G1 displayed a broad reactivity to mammalian (beef, deer, elk, horse, lamb and pork) and poultry (chicken, duck and turkey) meats. However, the reaction signals of cooked and autoclaved poultry were not as strong as those of mammalian species. MAb 2G3 reacted strongly with almost all mammalian and poultry species except horse meat samples. Chen and others (2002) reported that MAb 2G3 could react with all species including horse. The possible explanation for this disagreement of results between Chen and others and the present study was that the different methods for the sample preparation could affect the concentration of target protein in horse meat. Chen and others (2002) used purified horse sTnI for their assay, while horse meat protein extract was used in the present study. Both MAbs 8A12 and 8F10 showed similar reaction patterns with the mammalian species samples, whereas the immunoreactivity of MAb 8F10 was stronger in heat-treated samples than those of MAb 8A12. Neither MAbs 8A12 nor 8F10 presented a cross-reaction with heat-treated poultry meat samples, although both slightly cross-reacted with raw poultry samples. The results for MAb 8A12 agreed with those of Chen and others (2002).

Epitope Comparison

To develop a sandwich ELISA for the detection of mammalian tissue in meat and feed products, the initial task was to select a pair of suitable MAbs in which the capture antibody and the detection antibody would be able to bind to different epitopes on the surface of the antigen molecule and be able to efficiently distinguish mammalian species. From a comparison of the species specificities of MAbs (Figure 1), MAb 8F10 was selected as the detection antibody in the light of its higher affinity to mammalian species and no or weak cross reactivity to poultry samples than other MAbs tested. MAb 8F10 was conjugated to the enzyme, HRP, with a concentration of 1.000 mg antibody/ml.

The selection of a capture MAb to pair with MAb 8F10 was determined by comparing epitopes using competitive ELISA. The competitive ELISA method for epitope comparison between MAbs is based on the study of Vilim and others (2003). Briefly, the basic principle is that a pair of MAbs competes for binding to the limited numbers of epitopes at the same

time. If these two MAbs have the same epitope, they will inhibit each other from binding to the same antigen. On the other hand, if they have different epitopes, then the reactivity should be additive.

In the experiments as a whole, the assay conditions (incubation times and temperature) and the concentrations of the coated antigen, HRP-conjugated MAb 8F10 and HRP-conjugated goat anti-mouse IgG were kept constant except for the concentrations of the MAbs tested. The results are presented in Figures 2, 3, 4 and 5.

The purpose of using HRP-conjugated goat anti-mouse IgG in the ELISA system containing no HRP-conjugated MAb 8F10 was to reveal any binding between the MAbs tested and the coated antigen. In the MAb 6G1 experiment, the results showed that the readings of the assay were increased when the concentration of MAb 6G1 was increased (see the bottom curve in Figure 2). Similar trends were also observed with MAbs 2G3, 8A12 and 8F10 (see the bottom curves in Figures 3, 4 and 5). This indicates that the MAbs had bound to the antigen epitopes as anticipated, and increased antibody concentrations exhibited higher readings.

Due to the fact that HRP-conjugated MAb 8F10 could be inhibited by itself, when a limited amount of HRP-conjugated MAb 8F10 was added to the wells containing MAb 8F10, the absorbencies of HRP-conjugated MAb 8F10 were proportionally decreased as the concentration of MAb 8F10 was increased (Figure 5). In contrast, the readings remained fairly constant when HRP-conjugated MAb 8F10 was added together with other MAbs (2G3, 6G1 and 8A12), even with an increased concentration of the competing MAbs (see the middle curves in Figures 2, 3 and 4). These results indicate that there was no competition for binding sites between MAb 8F10 and the other MAbs tested.

This observation was further confirmed by another experiment, in which HRP-conjugated goat anti-mouse IgG was added to the system after HRP-conjugated MAb 8F10 and another MAb tested had bound to the coated antigen. The reading was an additive result, revealing both the bound MAb with the antigen and the bound HRP-conjugated MAb 8F10 with the same antigen. The absorbencies of the MAbs, 2G3, 6G1 and 8A12, slowly increased as the concentrations of the MAbs tested increased (see the top curves in Figures 2, 3 and 4), while those of MAb 8F10 remained at the same level (see the top curve in Figure 5) due to the competition between its own molecules.

The overall results showed that any of the three MAbs (2G3, 6G1 and 8A12) could form a pair with MAb 8F10 as the capture and detection antibodies in a sandwich ELISA for the detection of mammalian tissue in meat and feed products. However, based on the species

specificity, MAb 6G1 was selected as the capturing antibody paired with HRP-conjugated MAb 8F10 as the detection antibody for the mammalian sandwich ELISA. The concentration of MAb 6G1 was 1.374 mg antibody/ml.

Optimization of Mammalian Sandwich ELISA Conditions

To maximize the sensitivity of the mammalian sandwich ELISA using MAbs 6G1 and 8F10, the conditions of the assay, including dilutions of capture and detection antibodies, selection of antigen buffer, incubation time for antigen-antibody binding and dilutions of meat and feed samples, were optimized. Through a series of experiments described in the previous section, Methodology, the dilution selected for MAbs 6G1 and 8F10 was 1:1,000 (v/v), which gave the highest readings of the dilutions tested (Figures 6 and 7). To determine the optimal dilution of MAb 6G1, the autoclaved meat samples were used. As the readings shown in Figure 6 demonstrate, there was no significant difference among the four dilutions of MAb 6G1 in autoclaved poultry samples ($P \geq 0.05$). However, the reaction signals increased sharply in autoclave mammalian meat extracts when the dilution was decreased from 1:8,000 to 1:1,000 (v/v). The selection of optimal dilution for the detecting MAb 8F10 was made in autoclaved laboratory-adulterated samples. From Figure 7, there was little variation in the absorbencies of the pure autoclaved chicken and turkey samples when the dilution of HRP-conjugated MAb 8F10 was changed from 1:6,000 to 1:1,000 (v/v) due to the lack of reactivity with poultry meats, while the absorbance increased as the dilution decreased from 1:6,000 to 1:1,000 (v/v) in 0.50% pork adulterated chicken samples. A similar pattern was also observed in the autoclaved 0.50% beef in turkey samples. Therefore, 1:1,000 (v/v) was selected as the optimal dilution for HRP-conjugated MAb 8F10.

Adequate incubation time of the assay ensures the primary antigen-antibody binding, which relies on the forces between antibody and antigen molecules. The binding involves non-covalent binding, such as electrostatic, hydrogen bonding, hydrophobic and Van der Waals forces. Raw and cooked meat extracts from four species were used to study the effect of incubation time on the assay response. The assay signals of raw and cooked mammalian meat species (deer, elk and lamb) were significantly increased ($P \leq 0.05$) when the incubation time was prolonged from 30 min to 120 min, while no significant difference ($P \geq 0.05$) was shown in raw chicken samples (Figure 8). Therefore, to increase the sensitivity of the assay, the incubation time for antigen and antibody binding was selected to be 120 min.

To maximize the specific immunoreactivity, the selection of antigen buffer was studied with or without the presence of EDTA. It could be clearly seen that raw meat extracts

diluted in the antibody buffer containing 10 mM EDTA had a significantly higher reading than the samples without EDTA. However, the opposite was observed in heat-treated samples (Figure 9). The results indicated that raw beef and pork meat samples should be diluted in an antibody buffer containing 10 mM EDTA, while cooked and autoclaved beef and pork meat extracts should be diluted in an antibody buffer without EDTA.

EDTA is a commonly used chelator of divalent cations which can tightly bind calcium (Ca^{2+}) with an affinity constant (K_a) of $10^{7.27}$ M^{-1} at pH 7.0 (Bryan and others 1992; Ribou and others 1999). In raw meat extracts, troponin C (TnC) is the Ca^{2+} binding component and is one of the subunits (TnC, TnI and TnT) of the troponin complex. The three subunits are associated together in the native form of troponin (Filatov and others 1999). The K_a between Ca^{2+} and TnC in raw samples is in the range from 10^5 M^{-1} to 10^7 M^{-1}, which is lower than that of either Ca^{2+} or EDTA (Li and others 1997). Ca^{2+} in raw samples can greatly reduce the immunoreactivity of the assay based on the detection of sTnI in meat extracts. The presence of EDTA in the raw meat extract appears to effectively modulate the interference of Ca^{2+} due to the higher K_a between EDTA and Ca^{2+}. Nielsen and others (1994) indicated that the addition of EDTA to the serum diluent resulted in an increase in specificity from 96.0% to 99.4% in an indirect ELISA for detection of bovine antibody to Brucella abortus. From Figure 9, the readings of the raw beef and pork samples diluted in an antibody buffer containing 10 mM EDTA were increased by about 700% and 210%, respectively, compared with those diluted in the antibody buffer without EDTA. However, the absorbencies of cooked and autoclaved beef samples diluted in the antibody buffer containing 10 mM EDTA were decreased by about 30% and 40%, respectively. These similar phenomena were also seen in the cooked and autoclaved pork extracts, although to a lesser degree. A possible explanation is that the heat treatment leads to the separation of the Ca^{2+} binding protein, TnC, from TnI, which greatly reduces the interference of Ca^{2+}. However, it is possible that unchelated EDTA might contribute negatively, slightly decreasing the reaction signal in heat-treated samples (pH 7.0). The mechanism remains uncertain.

The optimal dilution for laboratory-adulterated meat samples was also studied. In raw meat extracts, the extractable proteins are primarily sarcoplasmic proteins and myofibrillar proteins, which are soluble in water and salt solution, respectively. The total protein concentration of the raw samples is a hundred times higher than in heat-treated meat samples, in which most soluble proteins are denatured and degraded by the heat treatment. Excessive amounts of non-specific protein molecules may result in hindering the binding of the target protein, sTnI, to the capture MAb 6G1. To minimize non-specific binding signals, a series of

raw adulterated meat samples (pork in chicken) were prepared with dilutions of 1:5, 1:10, 1:20, 1:30, 1:50 and 1:100 (v/v) in the antibody buffer containing 10 mM EDTA. The selection criterion was that at the selected dilution there should be a significant difference between the baseline (pure chicken extract) reading and the reading for the 0.50% pork in chicken sample ($P \leq 0.05$). From Figure 10 (c, d, e and f), the data for the raw samples diluted to 1:20, 1:30, 1:50 and 1:100 (v/v) showed that no significant difference between these two adulteration levels could be observed ($P \geq 0.05$). However, the readings for the dilutions of 1:5 and 1:10 (v/v) were acceptable ($P \leq 0.05$) (Figure 10 a and b). Further comparing the absorbencies of these two dilutions, the reading for the pure raw chicken sample diluted to 1:5 (v/v) was slightly higher than that diluted to 1:10 (v/v). Therefore, to moderately decrease the cross-reaction of pure raw chicken extract to the assay, 1:10 (v/v) was selected as the dilution factor for raw adulterated samples.

The selection of the dilutions for heat-treated meat samples was made in laboratory-adulterated beef in turkey samples between 1:2 (v/v) diluted and non-diluted meat extracts. From Figures 11 and 12, a detection limit at 0.50% adulteration level was achieved in the non-diluted cooked and autoclaved samples. However, in the 1:2 (v/v) diluted samples, no significant difference among the adulteration levels could be observed ($P \geq 0.05$) as the concentration of the target protein, sTnI, was too low to be seen. In addition, no cross-reactions of the assay were observed in undiluted heat-treated samples. Therefore, 1:10 (v/v) dilution in antibody buffer containing 10 mM EDTA was recommended for raw adulterated sample preparation, while no dilution was needed for heat-treated adulterated samples.

Tests of the laboratory-adulterated feed samples were also conducted in undiluted or 1:2 (v/v) diluted adulterated bovine meat meal in soy-based feed samples. The results showed a detection limit of 0.50% could be achieved in undiluted samples ($P \leq 0.05$), while no significant difference could be observed between adulteration levels of 0.00 and 2.00% ($P \geq 0.05$) (Figure 13). The most likely explanation is the same as for the heat-treated laboratory-adulterated meat samples, where the concentration of sTnI was insufficient to differentiate between the samples.

The experimental conditions selected for the sandwich ELISA are summarized in Table 2. The species specificity of the optimized sandwich ELISA is shown in Figure 14. This assay provides a strong and specific immunoreactivity to raw, cooked and autoclaved mammalian meat extracts (beef, deer, elk, horse, lamb and pork) and does not show any non-specific binding with heat-treated poultry meat extracts (chicken, duck and turkey), although a weak cross-reactions with raw poultry samples was observed.

Effect of Different Fat Content of Meat Samples

Fat is an important component of animal meat products and cannot be completely eliminated in meat samples. The fat content of different cuts and in different products varies. For example, regular ground beef contains 30% fat, while in extra lean ground beef, the fat content is limited to 10% or less (Hillbilly Housewife 2004). It is thus important to study the potential interference of the fat content of meat on the reaction signals of the mammalian sandwich ELISA developed by investigating the extraction method used for preparation of meat samples containing fat to see whether enough target antigens (sTnI) can be extracted to react with the MAbs for the sandwich ELISA. In this experiment, lab-made beef and pork samples (raw, cooked and autoclaved) containing different fat contents (0%, 10%, 20% and 30%, w/w) were prepared and tested by the optimized mammalian sandwich ELISA. The results showed that there were no significant differences in assay signals among the raw beef or pork samples with different fat contents which were 1:2 (v/v) diluted in the antibody buffer containing 10 mM EDTA ($P \geq 0.05$) (Figure 15 a and d). The heat-treated beef and pork samples produced the same results as the raw samples ($P \geq 0.05$) (Figure 15 b, c, e and f).

These results showed that a fat content of up to 30% in the meat samples did not significantly affect the reaction signal of the mammalian sandwich assay, indicating that the method used for sample preparation was able to extract similar amounts of sTnIs from the high fat content meat samples sufficient to react with the MAbs of the sandwich assay developed.

Effect of Different Heat-Treatment of Meat Samples

In 1996, the EC took the decision to alter the heat treatment for processing animal waste to 133ºC/3 bar for 20 min in order to inactivate PrP^{sc} (EC 1996). To ensure compliance, effective analytical methods are required. However, there are few methods that can meet these analytical requirements in the currently available commercial ELISA kits for meat speciation (Table 4). To assess the performance of the new mammalian sandwich ELISA in evaluating the effectiveness of the assay in heat-processed samples, six different heat-treated autoclaved meat extracts were prepared in addition to raw and cooked samples. In both beef and pork samples, as the heat treatment became more severe, the reaction signals increased regularly, reaching a peak immunoreactivity at the condition of 128°C/1.6 bar for 30 min then decreasing slightly (Figure 16).

The overall reactivity of pork was stronger than beef. However, the absorbance of the most severe treatment (132°C/2.0 bar, 120 min) in beef samples remained as high as 0.9,

indicating that the sandwich ELISA developed was adequate to analyze samples subject to even the most severe heat treatment in this study. From this experiment, the result also confirmed that sTnI is a highly heat-resistant biomarker and suitable for the detection of target muscle tissue in meat and feed products regardless of the extent of heat processing.

Detection Limits for Laboratory-Adulterated Meat and Feed Products

Detection of mammalian tissue in non-mammalian meat or feed products is important for enforcement of food-labeling laws and prevention of the spread of TSEs. For this purpose, raw, cooked and autoclaved laboratory-adulterated meat and feed samples were prepared to determine the detection limit of the assay developed. The detection limit of the assay was defined as the lowest adulteration level that produced a significant difference between the baseline (unadulterated poultry extract) reading and the reading of the selected adulteration level ($P \leq 0.05$).

As shown in Figure 17 (a, b, and c), the absorbencies of the samples of 0.50% raw, cooked and autoclaved pork in chicken samples prepared by the volume to volume dilution method displayed a significantly higher reading than those of the unadulterated chicken samples ($P \leq 0.05$). An identical detection limit was also seen in heat-treated pork adulterated chicken samples that were prepared by mixing meats on a weight basis (Figure 18) ($P \leq 0.05$). This result illustrates that the detection limit was the same in spite of the two distinctly different methods used to prepare the meat samples.

In another experiment, the detection limit of raw beef adulterated in a turkey sample was as low as 0.05% of adulterated level ($P \leq 0.05$), while the detection limits of cooked and autoclaved beef in turkey samples were 0.50% and 1.00%, respectively ($P \leq 0.05$) (Figure 17 d, e and f). It must be emphasized that there were no cross-reactions observed between heat-treated unadulterated poultry extracts and the MAbs used in the mammalian sandwich ELISA.

The detection limit for the lab-made samples of bovine meat meal mixed with soy-based feed was also tested. This assay was able to detect 0.50% bovine tissues in a soy-based feed sample ($P \leq 0.05$) (Figure 19).

CONCLUSIONS

Based on the epitope compatibility and species specificity, MAb 6G1, specific to mammalian and poultry sTnI, was selected as the capture antibody and MAb 8F10, specific to mammalian sTnI, was selected as the detection antibody to construct a mammalian sandwich ELISA in this study.

After optimizing the dilutions of the capture and detection antibodies and the adulterated meat and feed samples, and selecting the most effective antigen buffer and incubation time for antigen-antibody binding, this sandwich assay was capable of detecting mammalian tissue in meat and feed products at levels as low as 0.05% of adulteration. This MAb-based sandwich ELISA had no cross-reactions with heat-treated poultry samples. Although it had slight cross-reactions with raw adulterated poultry samples and soy-based feed extracts, it was still able to achieve a detection limit of less than 0.50% of the adulteration level.

Different fat contents (0 − 30%, w/w) of raw, cooked and autoclaved meat samples did not significantly affect the quantitative measure of the assay. This mammalian sandwich assay can be utilized to effectively analyze even severely heat-treated (132°C/2.0 bar for 120 min) meat and feed samples.

This is the first assay that can be used for rapid, sensitive and reliable detection of undeclared mammalian proteins in meat and feed products.

APPENDIX

Figures and Tables

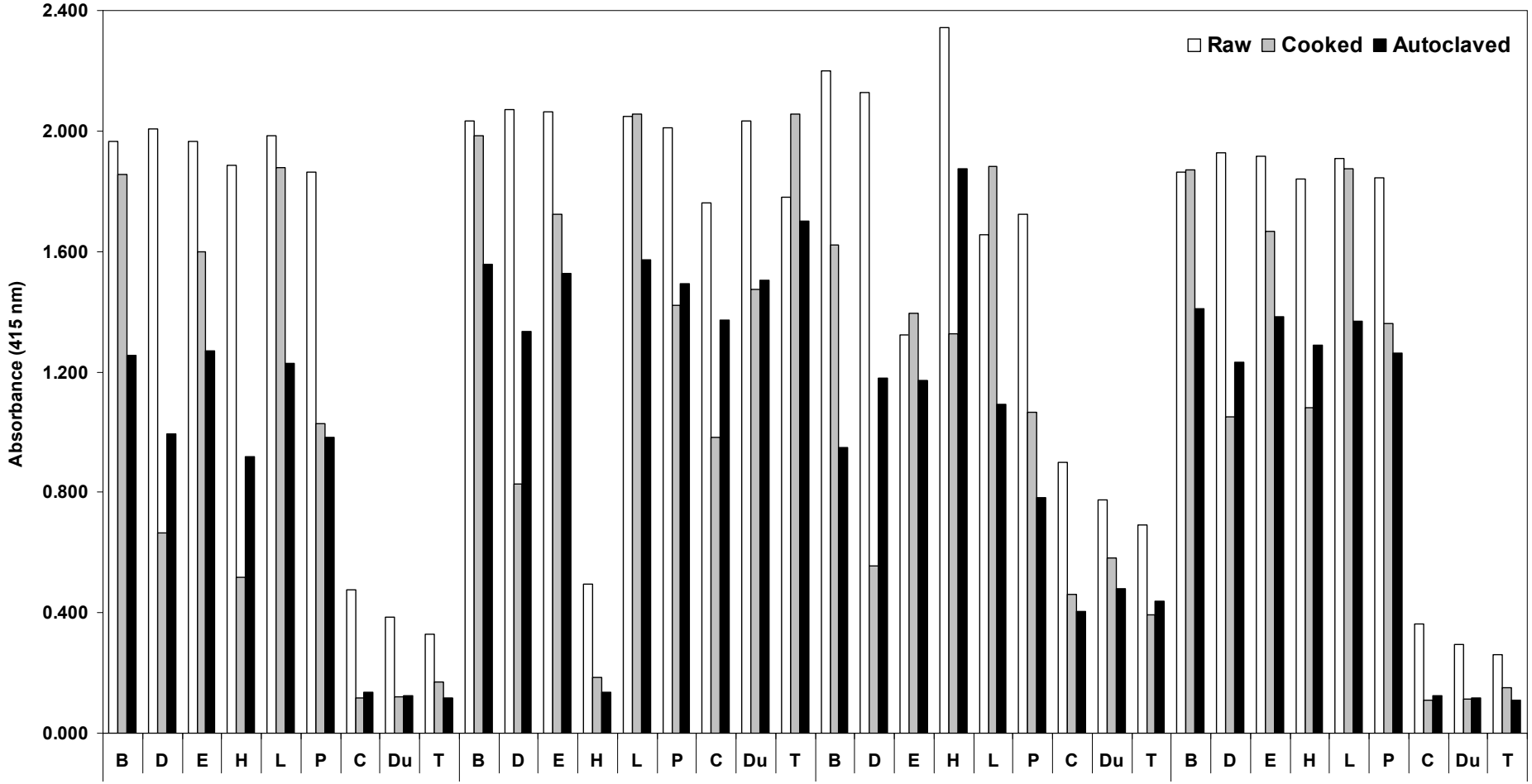

Figure 1—Species specificity of MAbs (8A12, 2G3, 6G1 and 8F10) using indirect ELISA.
* B: Beef; D: Deer; E: Elk; H: Horse; L: Lamb; P: Pork; C: Chicken; Du: Duck; T: Turkey.

Table 1—Species specificity of MAbs using indirect ELISA

MAbs	Class	Species Specificity
2G3	IgG	B D E L P C Du T *
6G1	IgG	B D E H L P C Du T
8A12	IgG	B D E H L P
8F10	IgG	B D E H L P

* B: Beef; D: Deer; E: Elk; H: Horse; L: Lamb; P: Pork; C: Chicken; Du: Duck; T: Turkey.

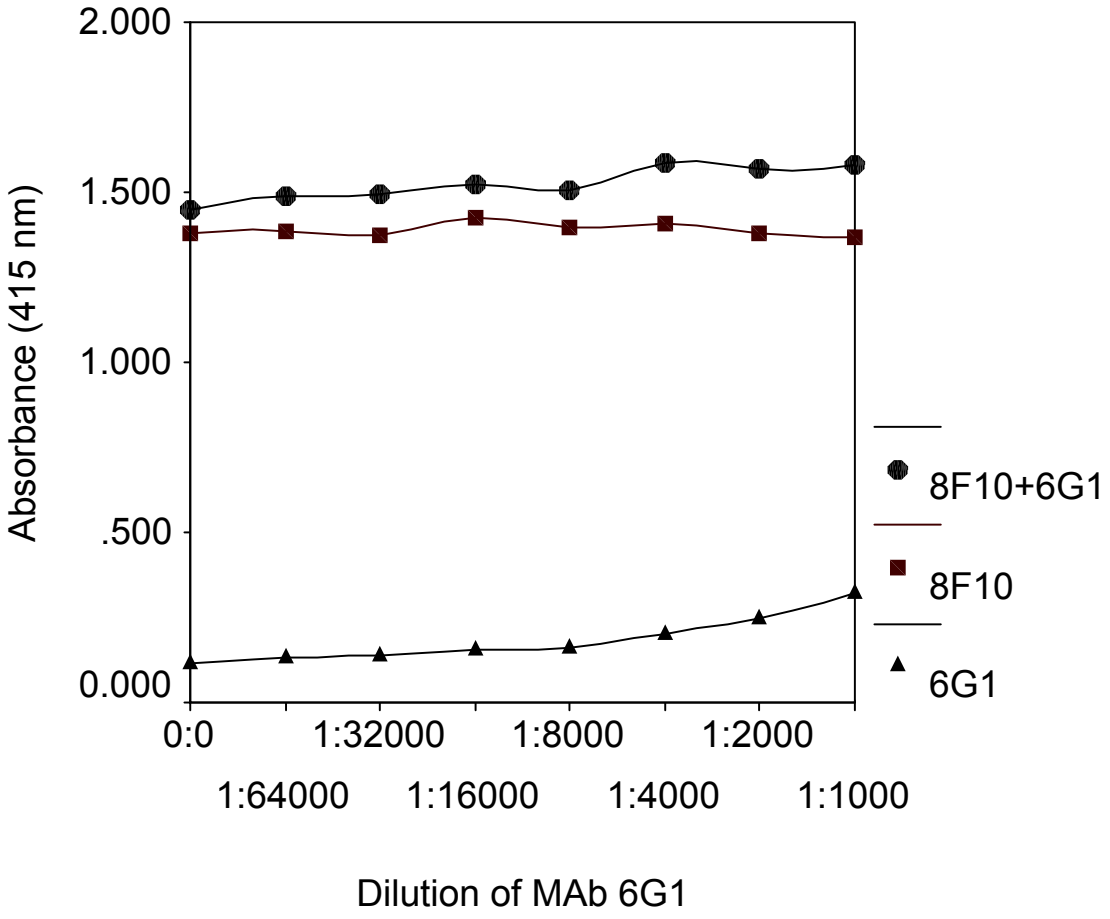

Figure 2— Epitope comparison of MAb 6G1 and MAb 8F10 using competitive ELISA. 0.25 µg protein/well cooked beef extract was coated. HRP-conjugated MAb 8F10 and HRP-conjugated goat anti-mouse IgG were 1:9,000 and 1:3,000 (v/v) diluted in the antibody buffer, respectively.

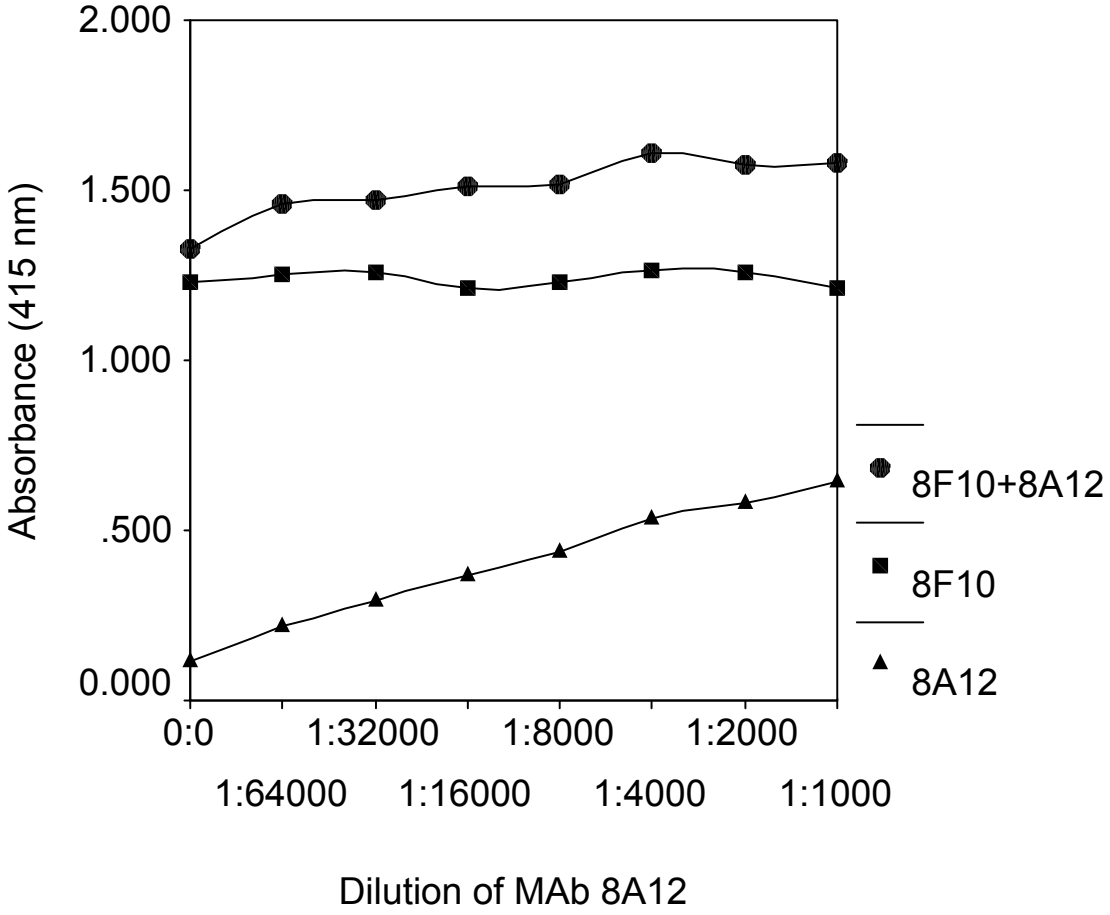

Figure 3— Epitope comparison of MAb 8A12 and MAb 8F10 using competitive ELISA. 0.25 µg protein/well cooked beef extract was coated. HRP-conjugated MAb 8F10 and HRP-conjugated goat anti-mouse IgG were 1:9,000 and 1:3,000 (v/v) diluted in the antibody buffer, respectively.

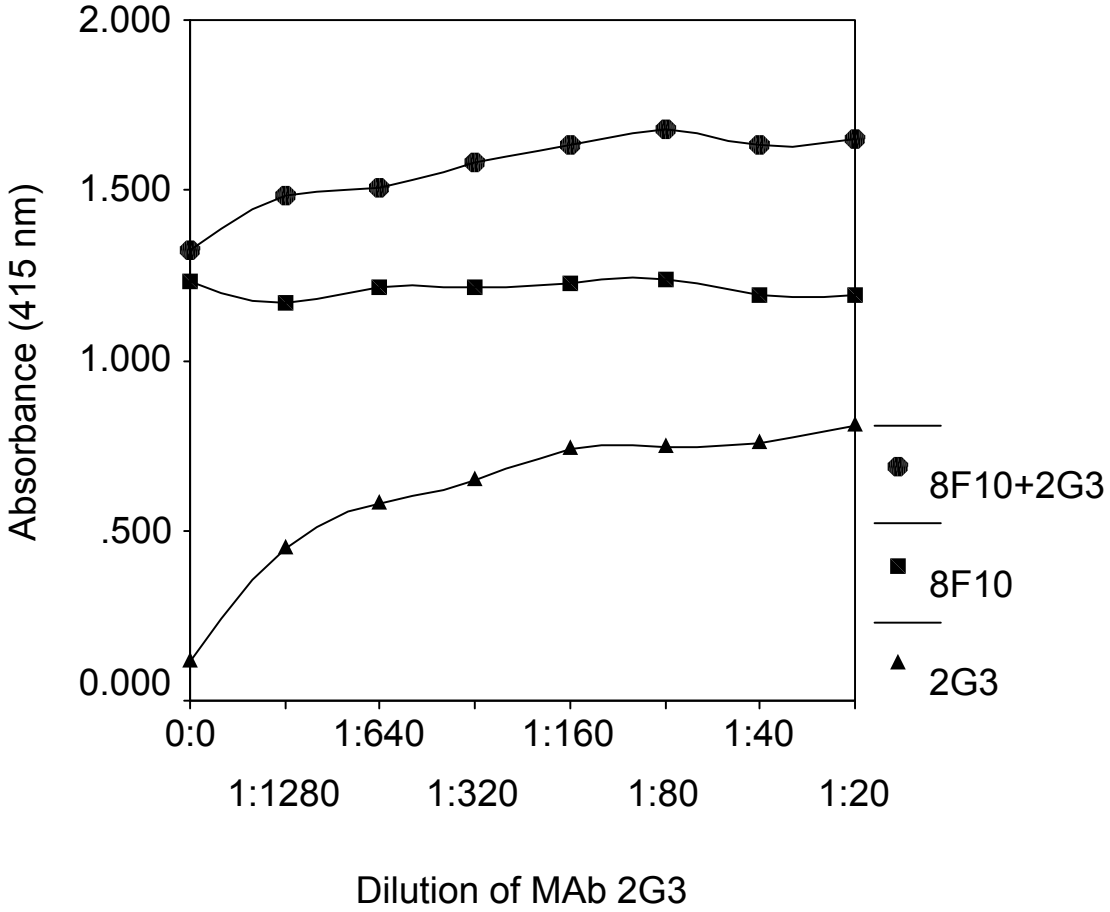

Figure 4— Epitope comparison of MAb 2G3 and MAb 8F10 using competitive ELISA. 0.25 µg protein/well cooked beef extract was coated. HRP-conjugated MAb 8F10 and HRP-conjugated goat anti-mouse IgG were 1:9,000 and 1:3,000 (v/v) diluted in the antibody buffer, respectively.

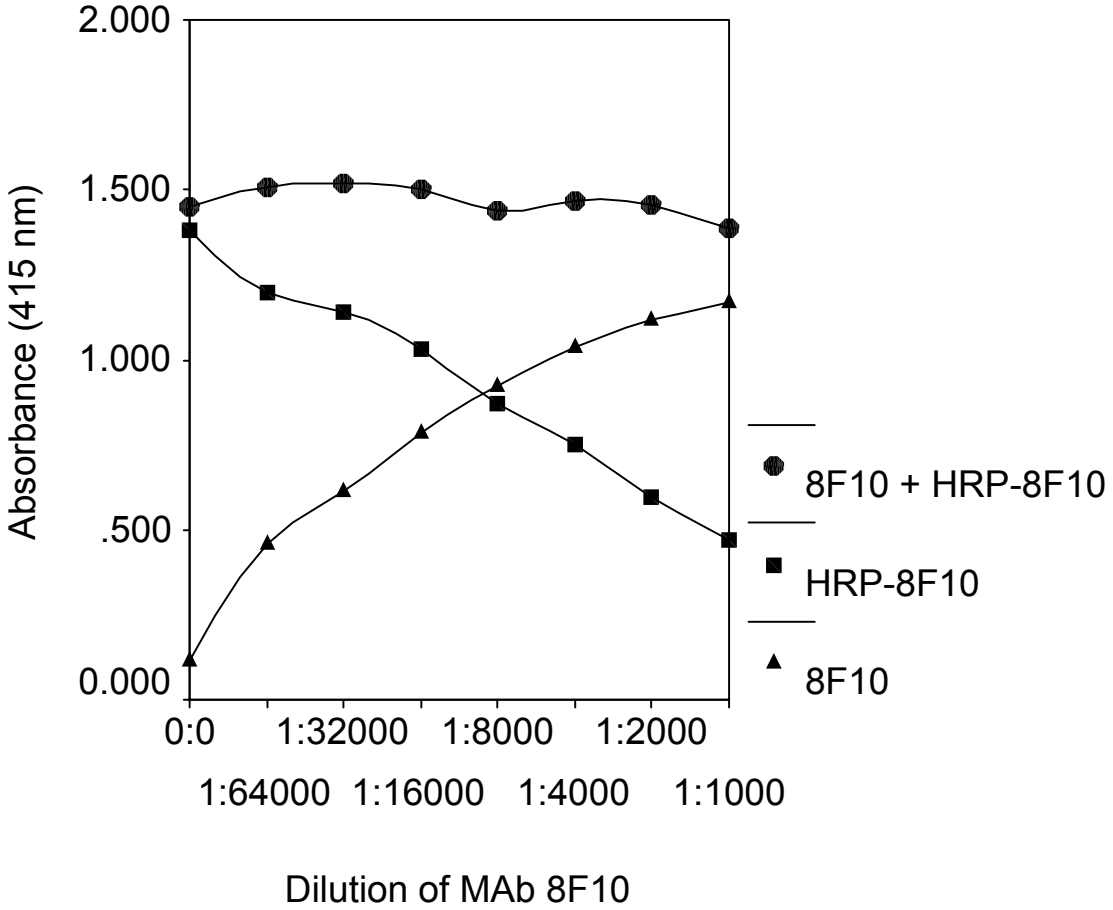

Figure 5— Inhibitory binding between MAb 8F10 and MAb 8F10 using competitive ELISA. 0.25 μg protein/well cooked beef extract was coated. HRP-conjugated MAb 8F10 and HRP-conjugated goat anti-mouse IgG were 1:9,000 and 1:3,000 (v/v) diluted in the antibody buffer, respectively.

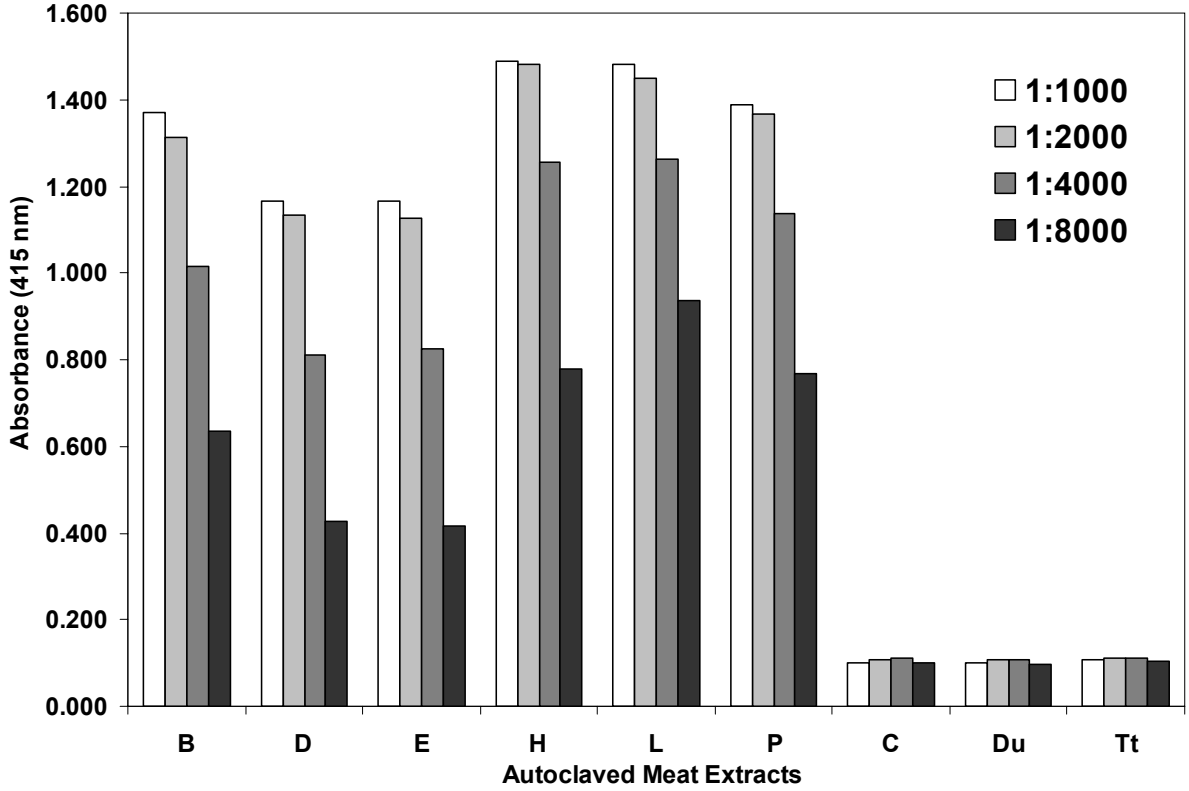

Figure 6—Selection of dilution of the capture antibody using sandwich ELISA. The microplate was coated with MAb 6G1 diluted (1:1,000, 1:2,000, 1:4,000 and 1:8,000 (v/v)) in 10 mM PBS and diluted autoclaved meat extracts (1:2 (v/v) in the antibody buffer) were added. The selection of dilution of the capture antibody, MAb 6G1, was 1:1,000 (v/v).
* B: Beef; D: Deer; E: Elk; H: Horse; L: Lamb; P: Pork; C: Chicken; Du: Duck; T: Turkey.

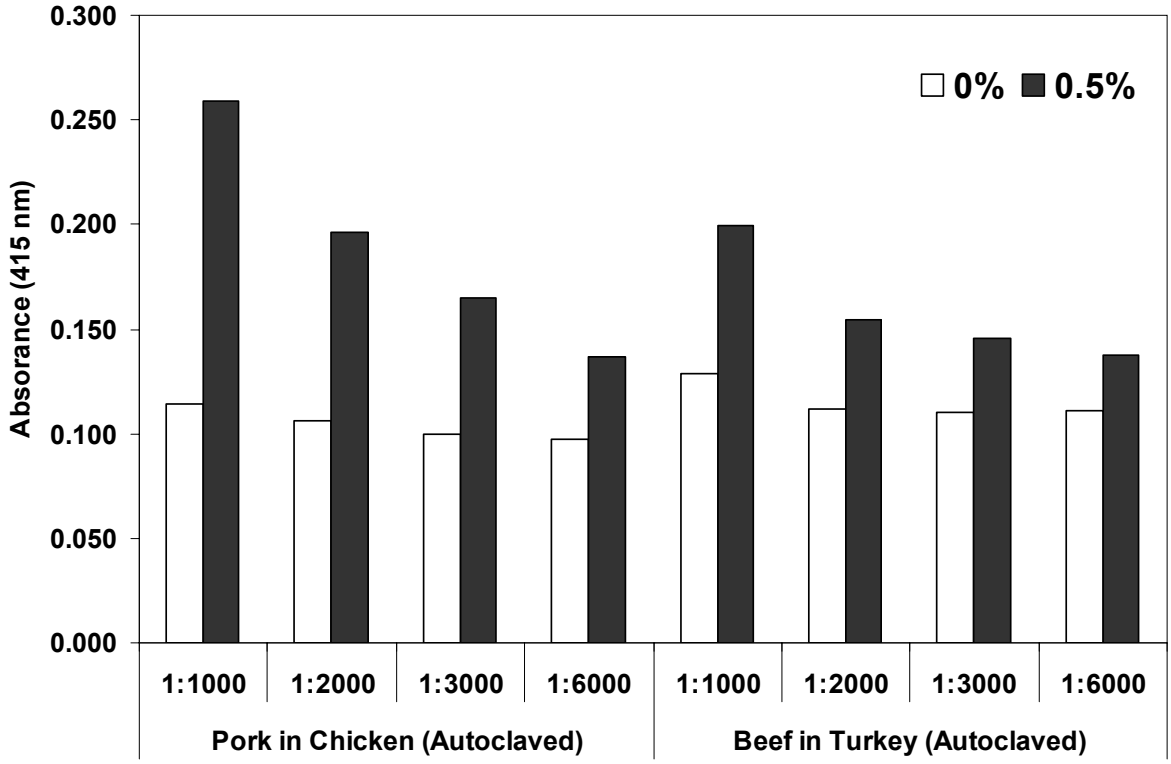

Figure 7—Seletion of dilution of the detection antibody using sandwich ELISA. Four different dilutions of MAb 8F10 (1:1,000, 1:2,000, 1:3,000 and 1:6,000 (v/v)) were evaluated. MAb 6G1 was diluted 1:1,000 (v/v). Non-diluted autoclaved adulterated meat extracts (0.50% beef in turkey and 0.50% pork in chicken, w/w) and pure autoclaved turkey and chicken meat extracts were used. The selection of dilution of the capture antibody, MAb 8F10, was 1:1,000 (v/v).

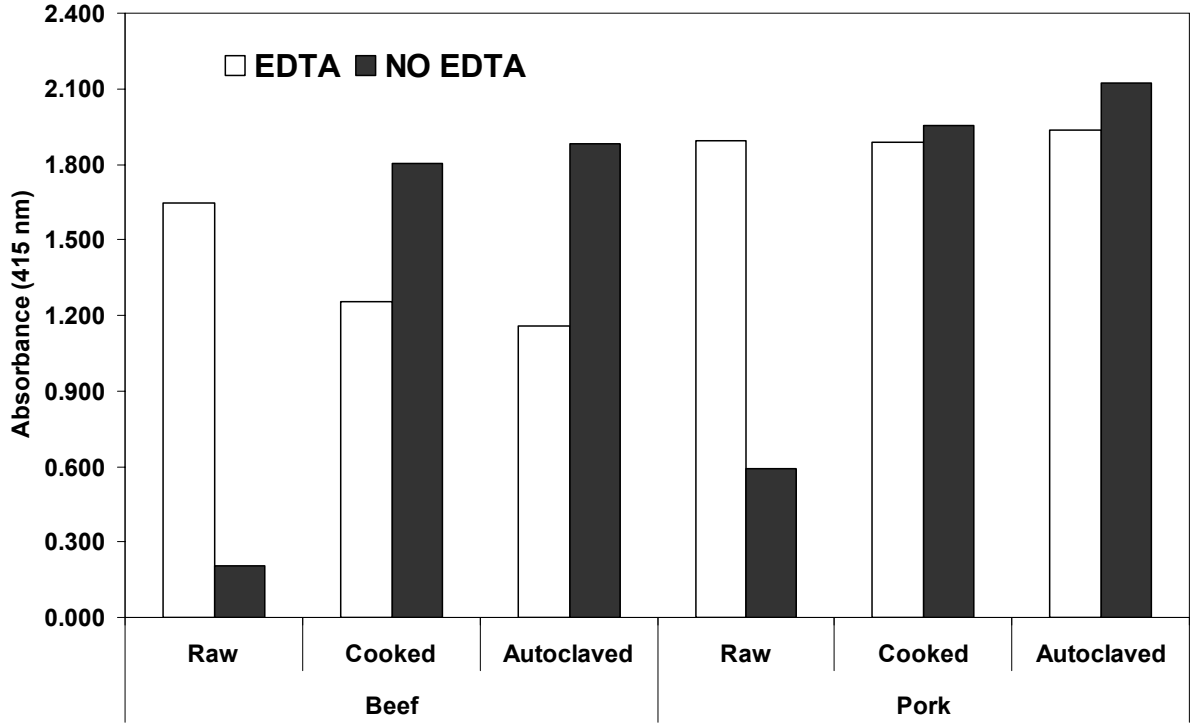

Figure 8—Selection of antigen buffer using sandwich ELISA. Each beef and pork (raw, cooked and autoclaved) meat extract was 1:2 (v/v) diluted in the antibody buffer containing 10 mM EDTA, or in the antibody buffer without EDTA. The selection of the antigen buffer was that raw meat samples were diluted in the antibody buffer containing 10 mM EDTA, while cooked and autoclaved meat extracts were diluted in the antibody buffer without EDTA.

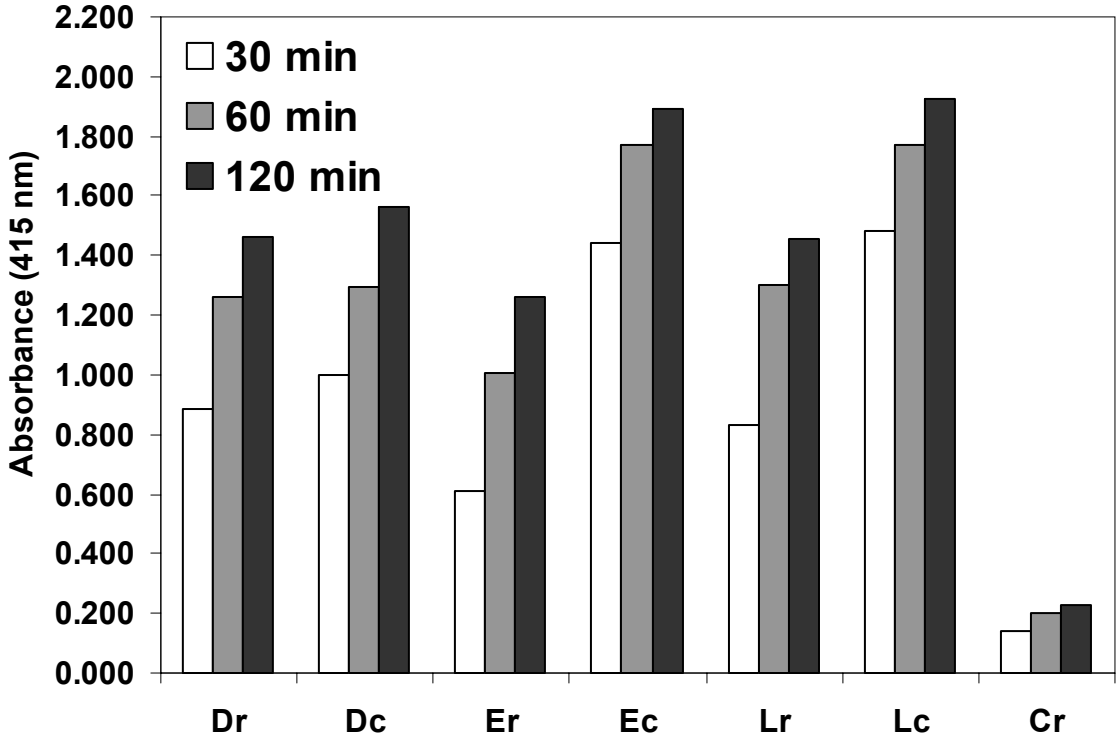

Figure 9—Selection of incubation time for antigen-antibody binding using sandwich ELISA. Raw (dear, elk, lamb and chicken) meat extracts were 1:10 (v/v) diluted in the antibody buffer containing 10 mM EDTA, and cooked meat extracts (dear, elk and lamb) were 1:2 (v/v) diluted in the antibody buffer. MAb 6G1 and HRP-conjugated MAb 8F10 were diluted 1:1,000 and 1:3,000 (v/v), respectively. The selection of incubation time for antigen-antibody binding was 120 min.

* D: Deer; E: Elk; L: Lamb; C: Chicken; r: Raw; c: Cooked.

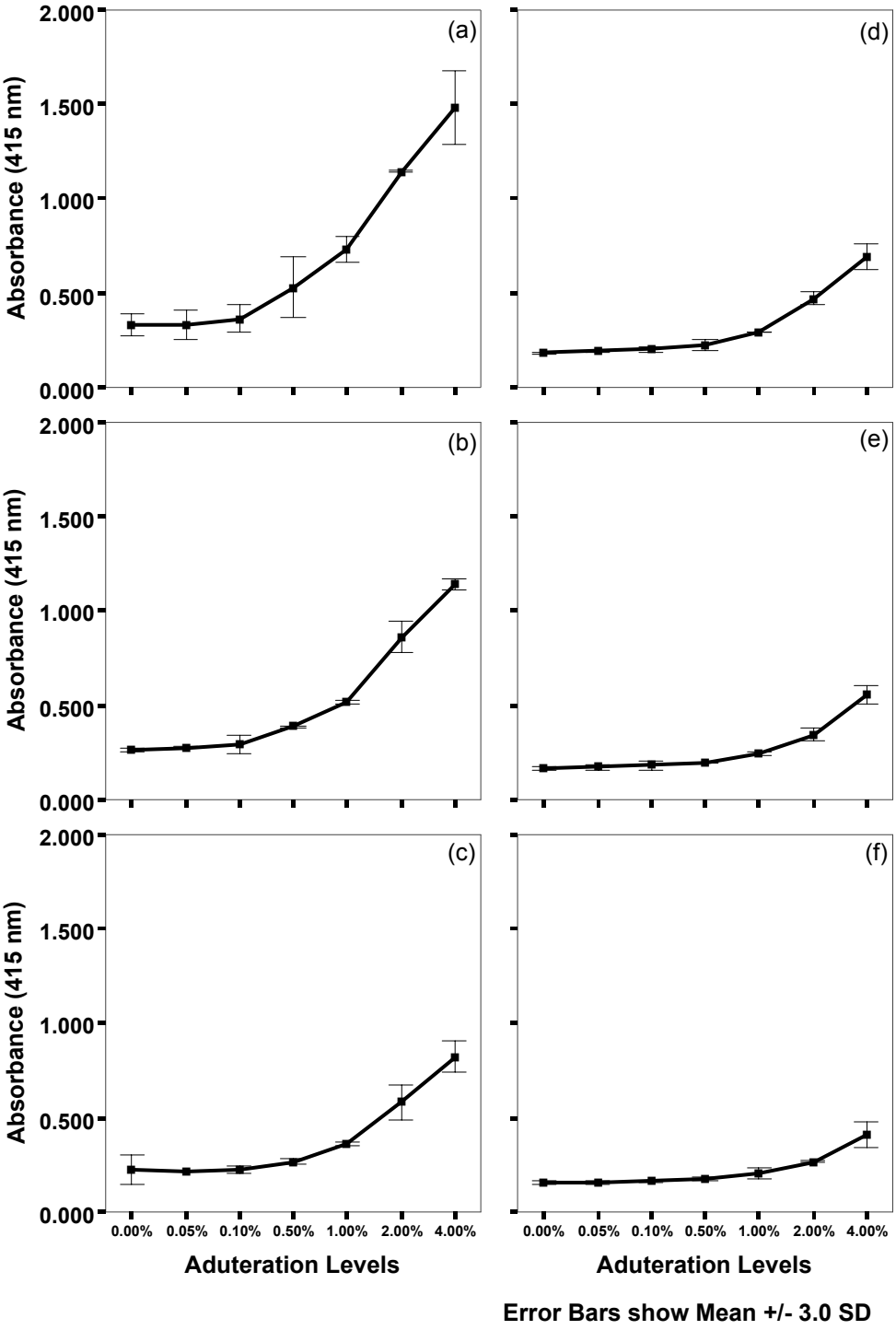

Figure 10—Selection of dilution of laboratory-adulterated raw meat samples using sandwich ELISA. The raw samples (pork in chicken) were diluted in the antibody buffer containing 10 mM EDTA. The dilutions were: (a) 1:5; (b) 1:10; (c) 1:20; (d) 1:30; (e) 1:50; (f) 1:100 (v/v). Both MAb 6G1 and MAb 8F10 were diluted 1:1,000 (v/v). The selection of dilution of adulterated raw meat samples was 1:10 (v/v) diluted in the antibody buffer containing 10 mM EDTA ($P \leq 0.05$).

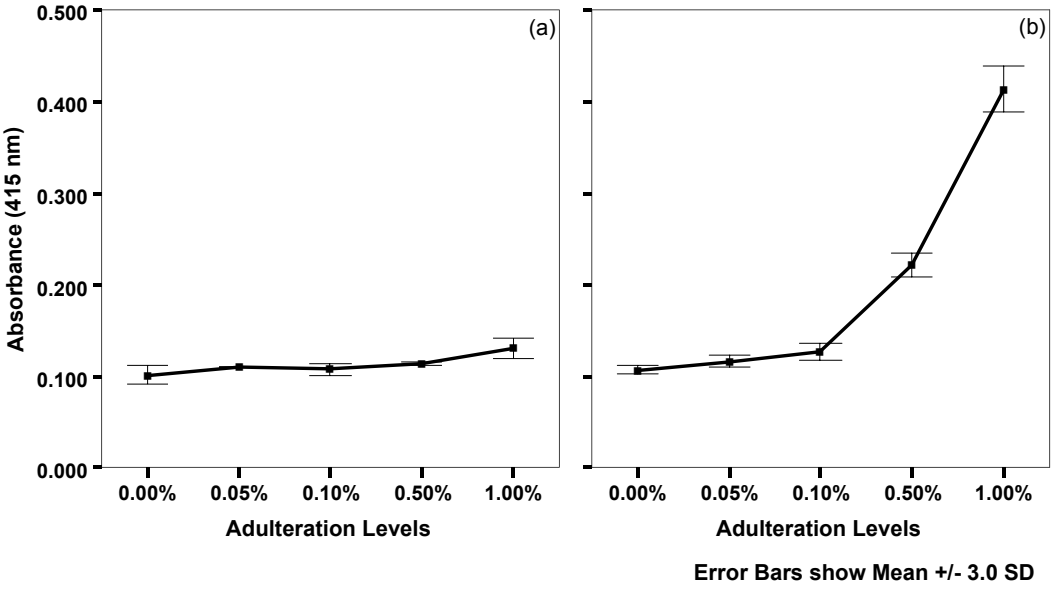

Figure 11—Selection of dilution of laboratory-adulterated cooked meat samples using sandwich ELISA. The cooked samples (beef in turkey) were (a) 1:2 (v/v) diluted in the antibody buffer, and (b) non-diluted. Both MAb 6G1 and MAb 8F10 were diluted 1:1,000 (v/v). The selection of dilution of adulterated cooked meat samples was non-diluted ($P \leq 0.05$).

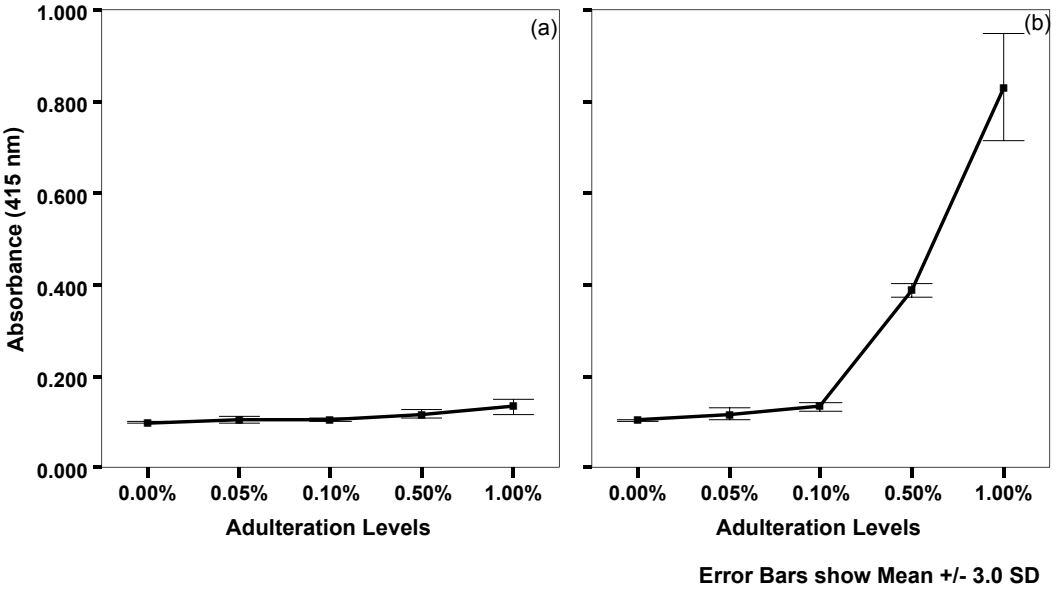

Figure 12—Selection of dilution of laboratory-adulterated autoclaved meat samples using sandwich ELISA. The autoclaved samples (beef in turkey) were (a) 1:2 (v/v) diluted in the antibody buffer, and (b) non-diluted. Both MAb 6G1 and MAb 8F10 were diluted 1:1,000 (v/v). The selection of dilution of adulterated autoclaved meat samples was non-diluted ($P \leq 0.05$).

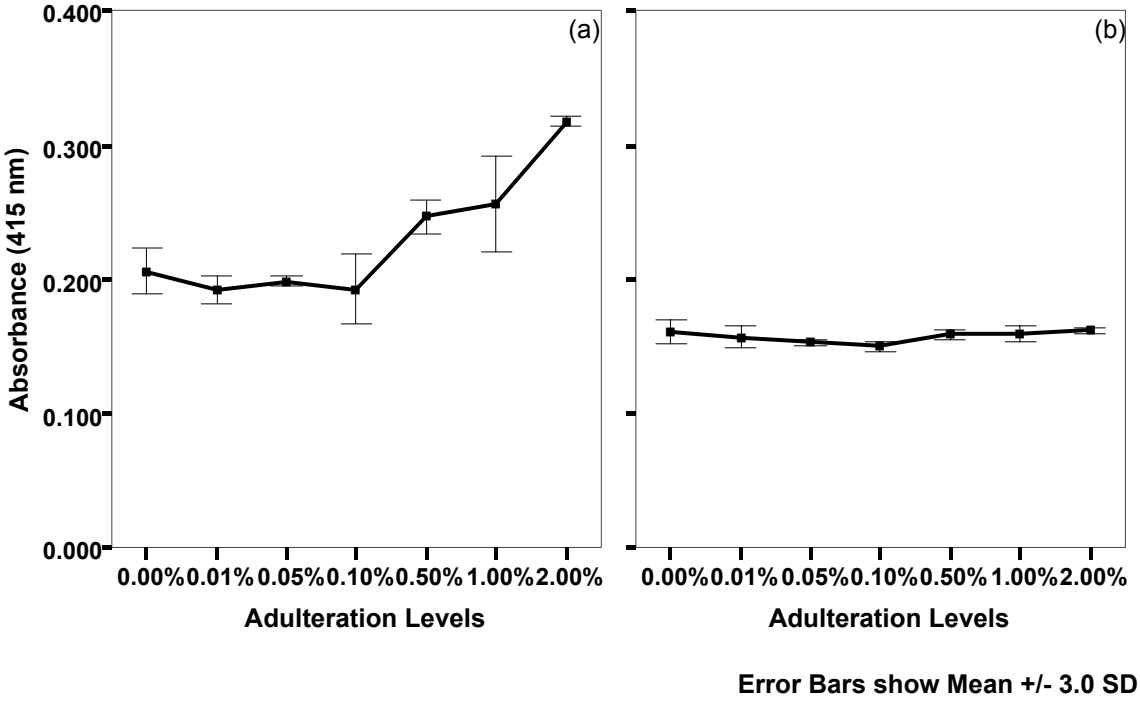

Figure 13—Selection of dilution of laboratory-adulterated feed samples using sandwich ELISA. The (a) non-diluted, and (b) diluted (1:2 (v/v) in the antibody buffer) adulterated bovine meat meal in soy-based feed extracts were used. Both MAb 6G1 and MAb 8F10 were diluted 1:1,000 (v/v). The selection of dilution of adulterated feed samples was non-diluted ($P \leq 0.05$).

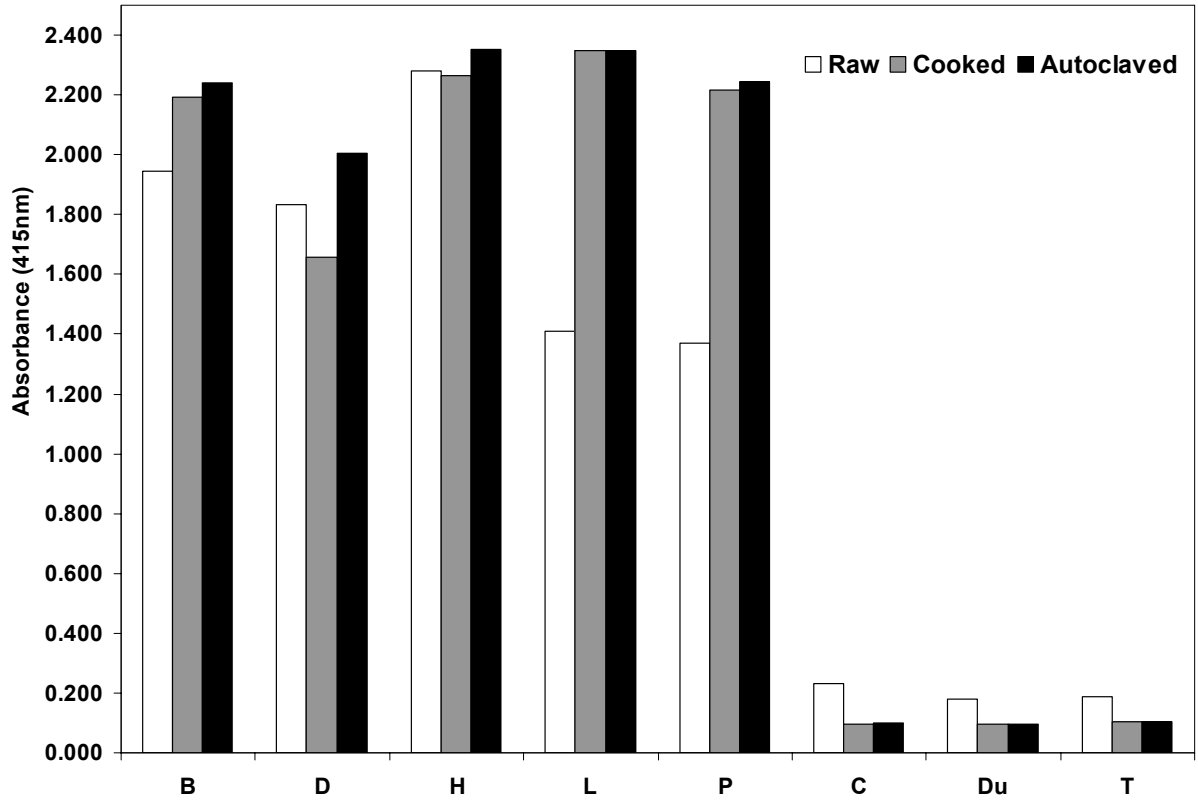

Figure 14—Species specificity of the optimized sandwich ELISA using MAb 6G1 (capture) and MAb 8F10 (detection). The assay is specific to mammalian meat (B, D, H, L and P).
* B: Beef; D: Deer; H: Horse; L: Lamb; P: Pork; C: Chicken; Du: Duck; T: Turkey.

Table 2—Optimized conditions of the mammalian sandwich ELISA using MAb 6G1 (capture) and MAb 8F10 (detection)

<table>
<tr><td rowspan="4">Preparation of Meat Sample Extracts</td><td>Type</td><td>Weight</td><td>Extract Solution</td><td>Dilution (w/v)</td><td>Homogenization</td><td>Extract Time</td><td>Centrifuge</td><td>Storage</td></tr>
<tr><td>Raw</td><td rowspan="3">10 − 50 g</td><td rowspan="3">10 mM PBS
0.5 M NaCl
(pH 7.0)</td><td>1:5</td><td>Stir Bar/2 min</td><td rowspan="3">4°C
120 min</td><td rowspan="3">4°C
3,220 × g
30 min</td><td rowspan="3">-20°C</td></tr>
<tr><td>Cooked
(100 °C/30 min)</td><td rowspan="2">1:2</td><td rowspan="2">11,000 rpm
2 min</td></tr>
<tr><td>Autoclaved
(121°C/1.2 bar/30 min)</td></tr>
<tr><td rowspan="12">Conditions of Sandwich ELISA</td><td rowspan="2">Type</td><td colspan="2">MAbs</td><td colspan="3">Antigen</td></tr>
<tr><td>6G1</td><td>HRP-8F10</td><td>Raw</td><td>Cooked</td><td>Autoclaved</td></tr>
<tr><td>Incubate Time</td><td colspan="2">37°C/60 min</td><td colspan="3">37°C/120 min</td></tr>
<tr><td>Dilution Buffer
(pH 7.2)</td><td>10 mM PBS</td><td>1% BSA
10 mM PBS
0.05% Tween-20</td><td>1% BSA
10 mM PBS
0.05% Tween-20
10 mM EDTA</td><td colspan="2">NO</td></tr>
<tr><td>Dilution (v/v)</td><td colspan="2">1:1,000</td><td>1:10</td><td colspan="2">NO</td></tr>
<tr><td>Coating Buffer</td><td colspan="5">10 mM PBS (pH 7.2)</td></tr>
<tr><td>Blocking Buffer</td><td colspan="5">1% BSA/10 mM PBS (pH 7.2)</td></tr>
<tr><td>Blocking Time</td><td colspan="5">37°C/ 60 min</td></tr>
<tr><td>Washing Buffer</td><td colspan="5">10 mM PBS/0.05% Tween-20 (pH 7.2)</td></tr>
<tr><td>Substrate Solution</td><td colspan="5">22 mg of ABTS & 15 µl of 30% hydrogen peroxide
in 100 ml of 0.1 M phosphate-citrate buffer (pH 4.0)</td></tr>
<tr><td>Stop Solution</td><td colspan="5">0.2 M citric acid</td></tr>
<tr><td>Color Development Time</td><td colspan="5">Room temperature/30 min</td></tr>
<tr><td>Absorbance Wavelength</td><td colspan="5">415 nm</td></tr>
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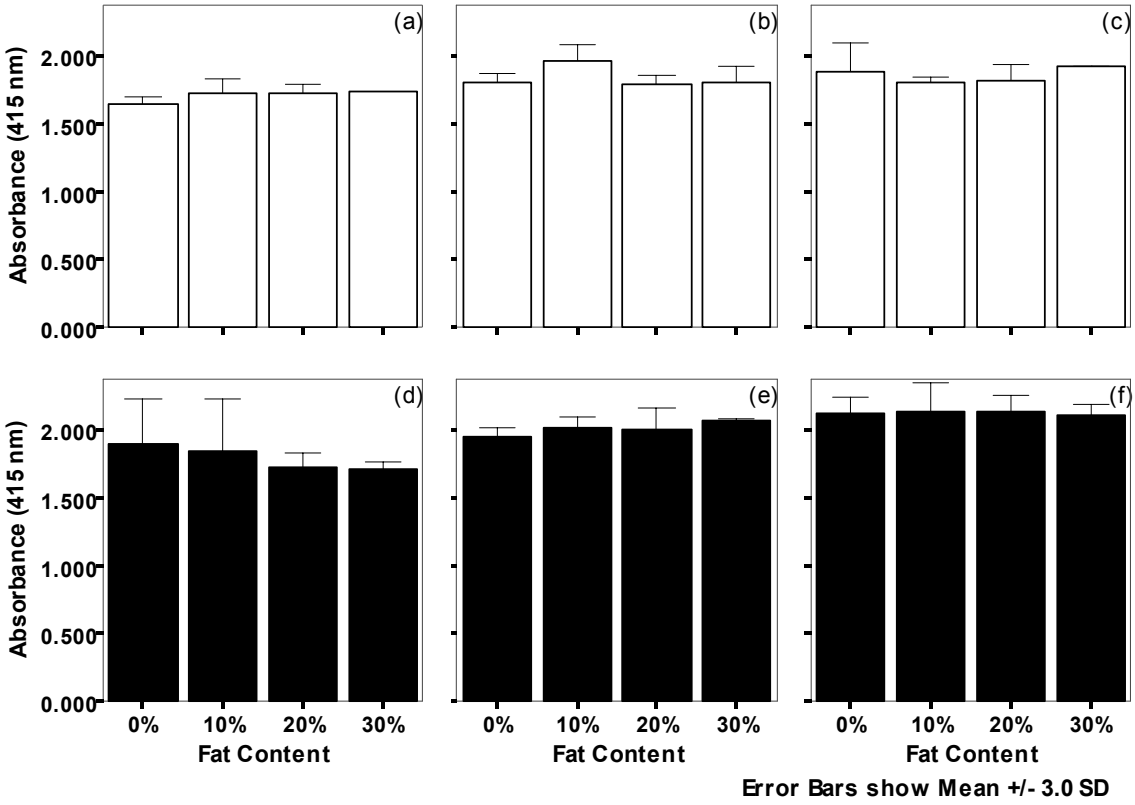

Figure 15— Effect of different fat contents (0, 10, 20, and 30% of fat, w/w) in beef and pork meat samples on reactivity of the optimized sandwich ELISA using MAb 6G1 (capture) and MAb 8F10 (detection). The raw meat samples were 1:2 (v/v) diluted in the antibody buffer with 10 mM EDTA, and the cooked and autoclaved meat samples were 1:2 (v/v) diluted in the antibody buffer without 10 mM EDTA. (a) Raw beef; (b) Cooked beef; (c) Autoclaved beef; (d) Raw pork; (e) Cooked pork; (f) Autoclaved pork. The effects of meat samples with different fat contents on the sandwich ELISA produced no significant differences among raw, cooked and autoclaved meat samples ($P \geq 0.05$). Fat content did not significantly affect the reaction signal of the sandwich ELISA developed.

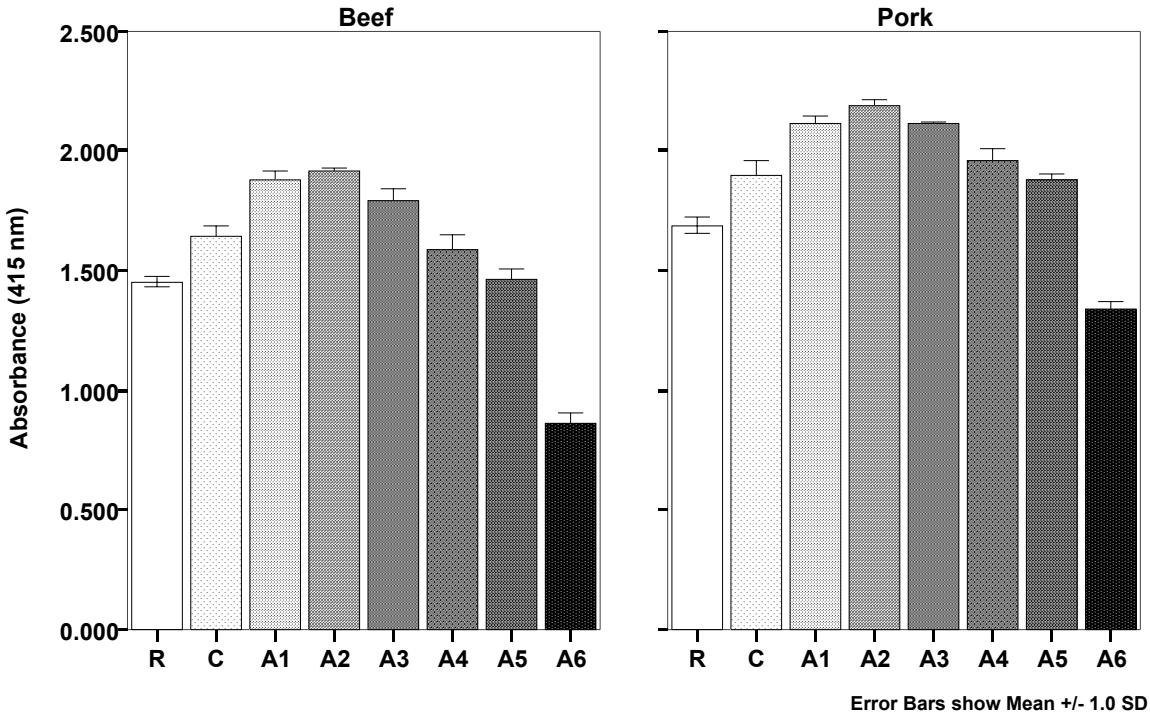

Figure 16—Effect of different heat treatments of beef and pork samples on the reactivity of the optimized sandwich ELISA using MAb 6G1 (capture) and MAb 8F10 (detection). The raw meat samples were 1:2 (v/v) diluted in the antibody buffer with 10 mM EDTA, and the cooked and autoclaved meat samples were 1:2 (v/v) diluted in the antibody buffer. The overall reactivity of pork was stronger than beef. The data for A2 showed the highest immunoreactivity to both beef and pork. As the reactivity of heat treatment increased, the reactivity decreased gradually. However, the assay was still adequate to analyze samples subject to the most severe heat treatment.

* R: no heat treatment; C: 100°C, 30 min; A1: 121°C/1.2 bar, 30 min; A2: 128°C/1.6 bar, 30 min; A3: 132°C/2.0 bar, 30 min; A4: 132°C/2.0 bar, 60 min; A5: 132°C/2.0 bar, 90 min; and A6: 132°C/2.0 bar, 120 min.

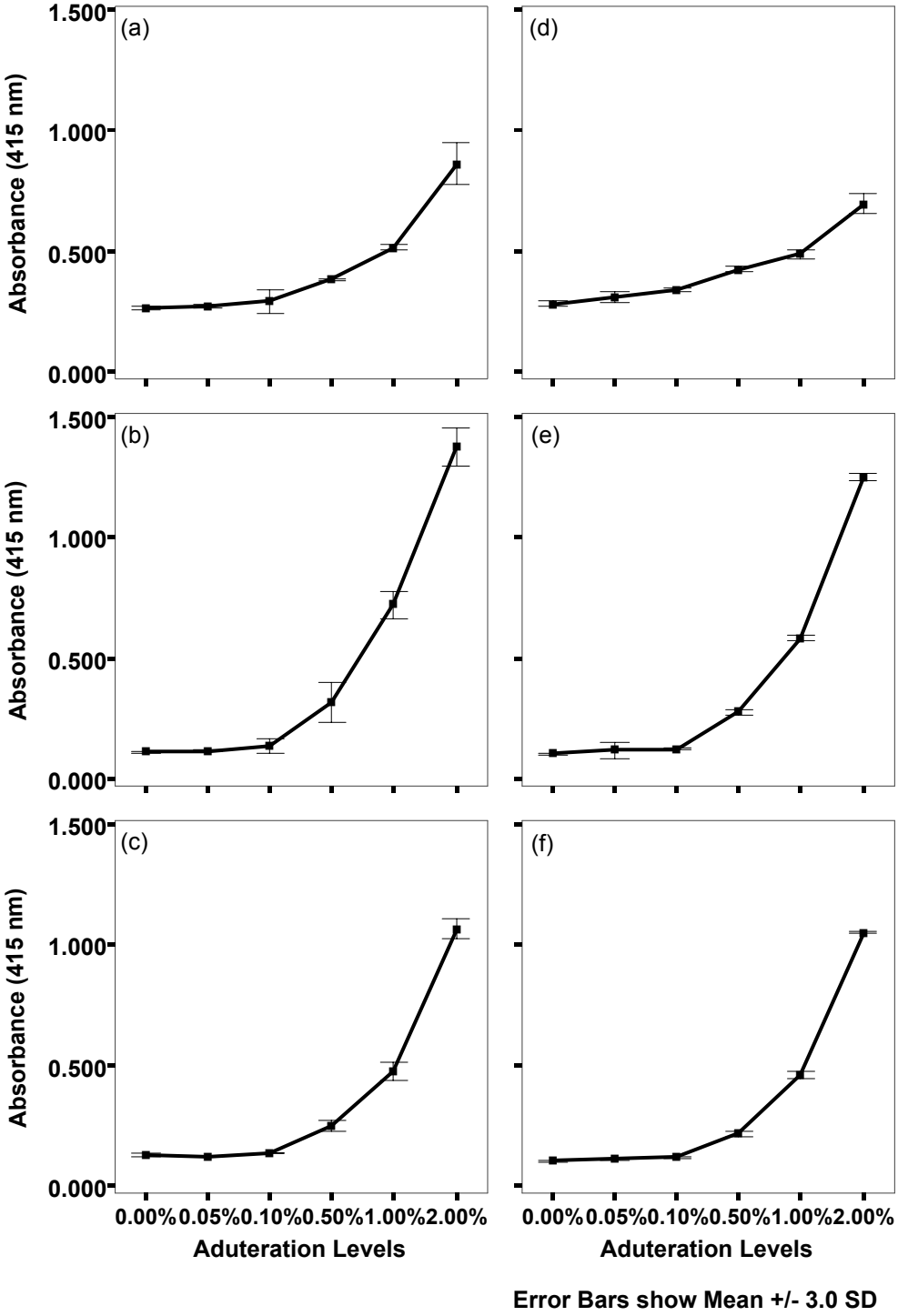

Figure 17—Detection limits of the optimized sandwich ELISA using MAb 6G1 (capture) and MAb 8F10 (detection) for detecting laboratory-adulterated meat samples (v/v). The raw adulterated meat samples diluted 1:10 (v/v) in the antibody buffer containing 10 mM EDTA, and the undiluted cooked and autoclaved adulterated meat samples were analyzed using the sandwich ELISA. (a) Raw pork in chicken; (b) Cooked pork in chicken; (c) Autoclaved pork in chicken; (d) Raw beef in turkey; (e) Cooked beef in turkey; (f) Autoclaved beef in turkey.

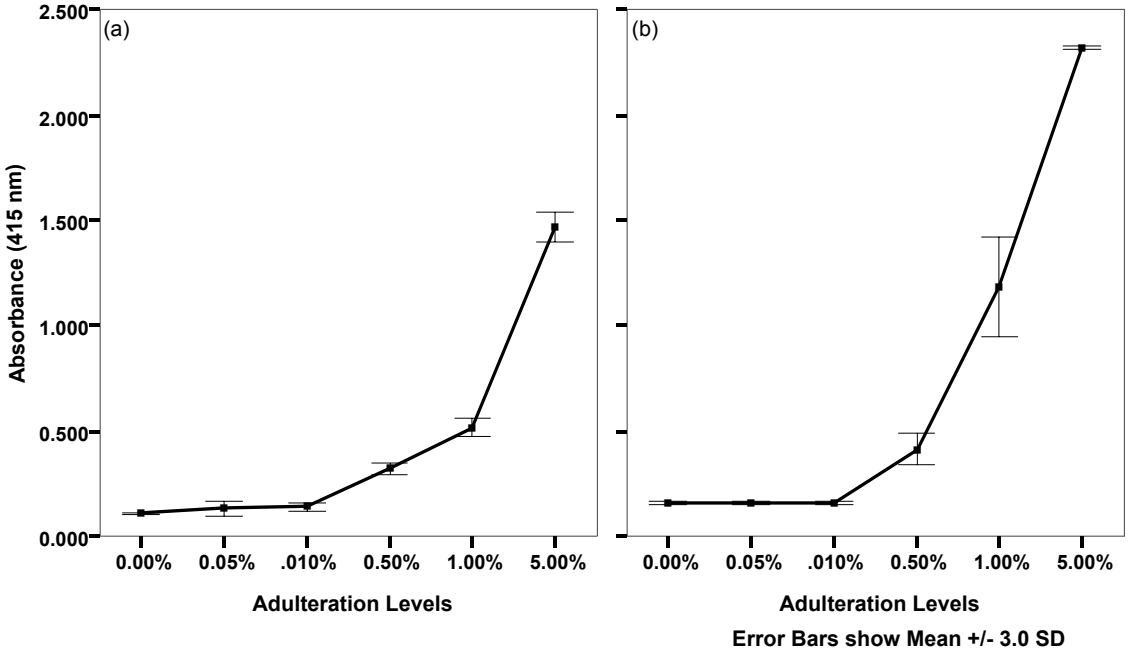

Figure 18—Detection limits of the optimized sandwich ELISA using MAb 6G1 (capture) and MAb 8F10 (detection) for detecting cooked and autoclaved pork adulterated in chicken (w/w). Undiluted adulterated meat samples were used. (a) Cooked pork in chicken; (b) Autoclaved pork in chicken.

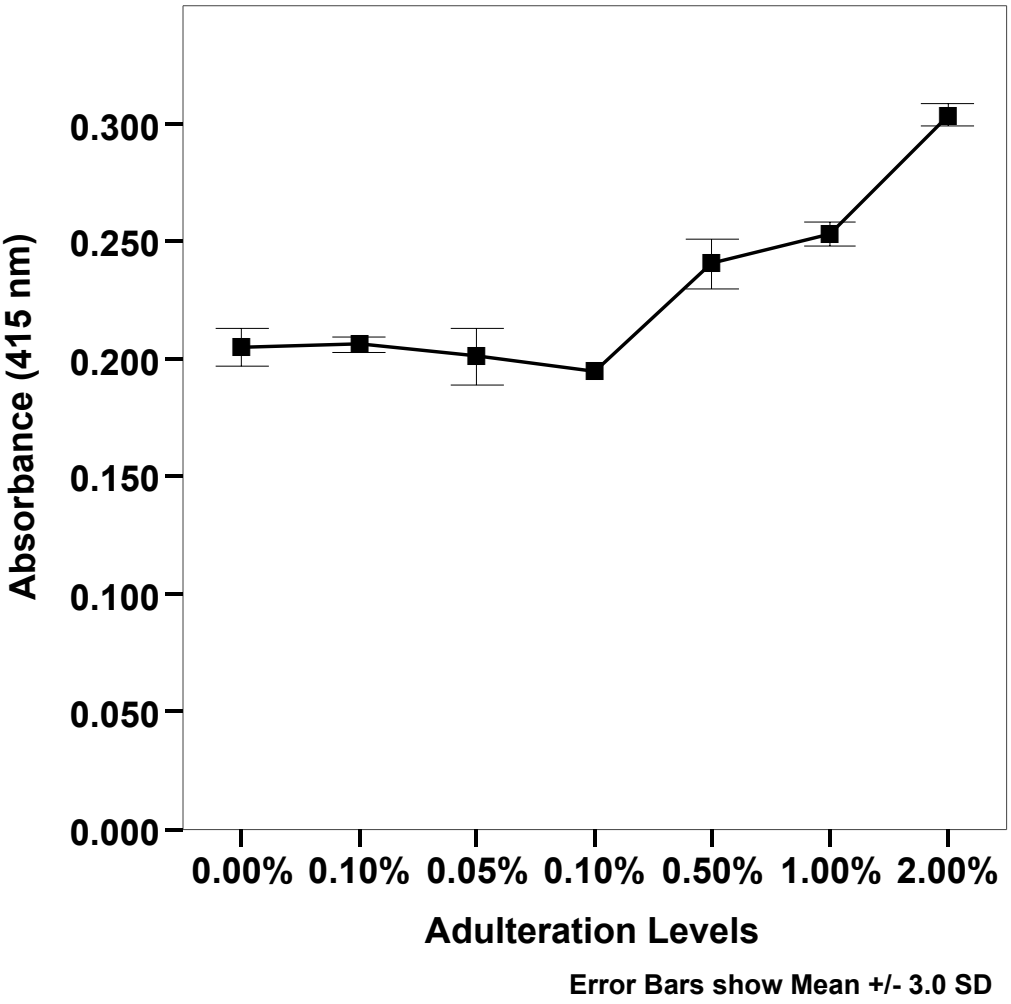

Figure 19— Detection limit of the optimized sandwich ELISA using MAb 6G1 (capture) and MAb 8F10 (detection) for detecting bovine meat meal adulterated in soy-based feed samples (v/v). Undiluted adulterated feed samples were used.

Table 3—Summary of the characteristics of the optimized sandwich ELISA for detection of mammalian tissue in meat and feed products

Assay Type			Sandwich ELISA
Biomarker			sTnI
Antigen Type	**Meat Species**	**Raw**	B, D, H, L, P
		Cooked	
		Autoclaved	
	Soy-based Feedstuffs		Bovine meat meal
Hands-on-time	**Preparation & Extraction (min)**		180
	Immunoassay (min)		240
Fat content in meat samples (0 − 30%, w/w)			No significant effect ($P \leq 0.05$)
Heat treatment of meat samples			Valid up to 132°C/2.0 bar, 120 min
Detection Limit ($P \leq 0.05$)	**Pork in chicken (w/w)**	**Raw**	0.50%
		Cooked	0.50%
		Autoclaved	0.50%
	Beef in turkey (w/w)	**Raw**	0.05%
		Cooked	0.50%
		Autoclaved	1.00%
	Bovine meat meal in soy-based feed samples		0.50%

* B: Beef; D: Deer; H: Horse; L: Lamb; P: Pork.

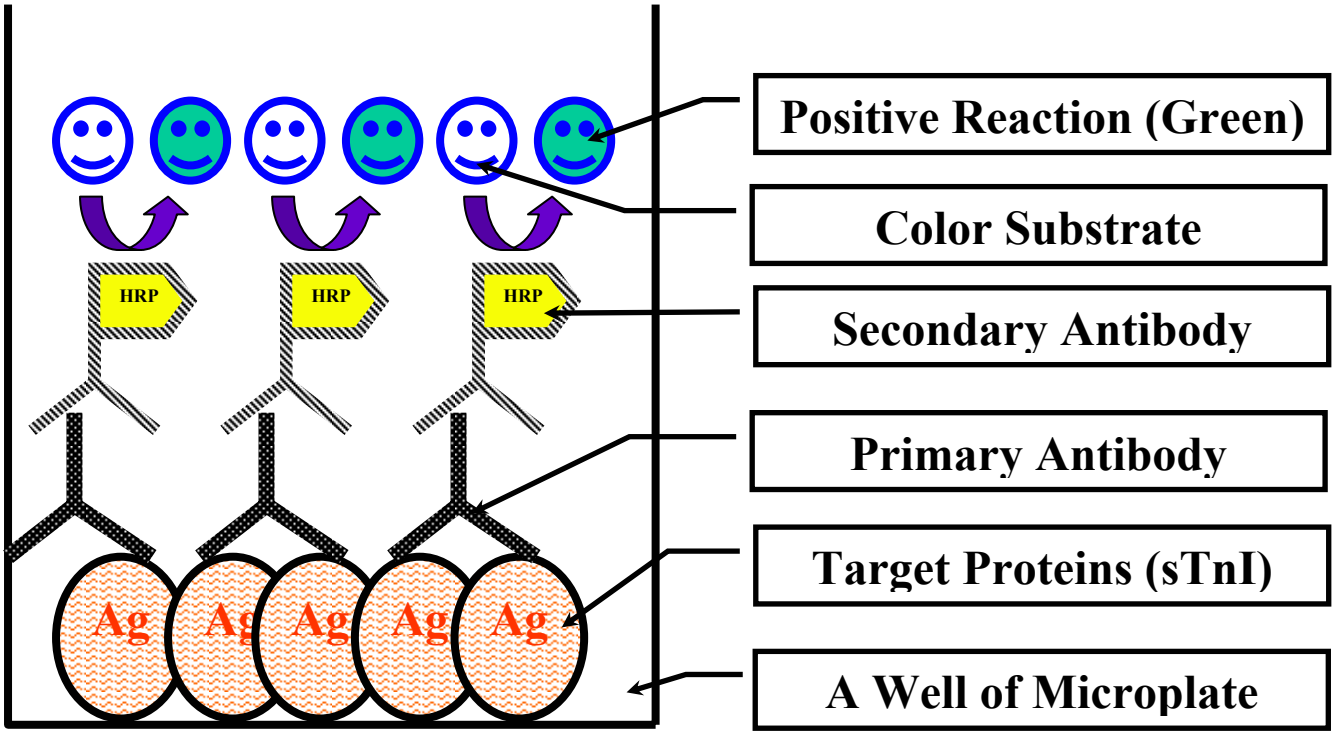

Figure 20—Model of indirect ELISA

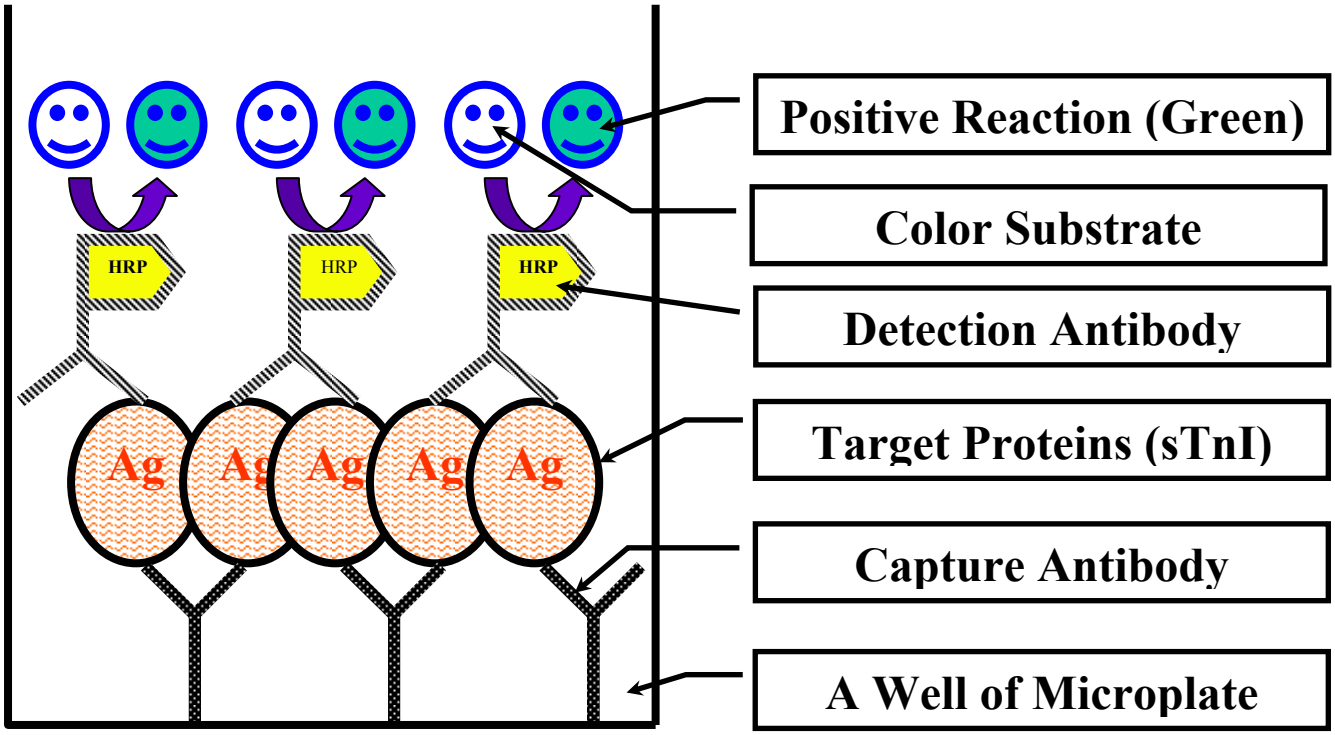

Figure 21—Model of sandwich ELISA

Table 4—Commercial ELISA kits for meat speciation

Brand Name	Assay Type	Biomarker	Antigen Type				Hands-on-time		Detection Limit
			Meat Species			Feedstuffs	Preparation & Extraction (min)	Immunoassay (min)	
			Raw	Cooked	Autoclaved				
MELISA-TEK® Meat Species Kit	Sandwich ELISA	sTnI*	P	P		MBM & animal feeds	30 (five samples)	90	<1.0% in feed samples <0.1% in MBM samples <75 ppm in 138°C tissue samples
ELISA-TEK® Cooked Meat Species Kit	Sandwich ELISA	TSMG		B, D, H, P, Po, S			30 (five samples)	180	1.0% in canned, cooked, or processed foods
ELISA-TEK® Raw Meat Species Kit	Sandwich ELISA	Serum albumin	B, H, P, Po, S				10	60	<1.0% for meat and Po (all species) <5.0% for milk (relevant species only)
FeedChek® MBM Test Kit	Lateral flow	Processed animal proteins				Mammalian, avian, & fish MBM	15 sec	10	≥ 0.1% MBM
Reveal for Ruminant in MBM (Neogen Co.)	Lateral flow	TSMP				Ruminant MBM	10	10	2% MBM
Reveal for Ruminant in Feed (Neogen Co.)	Lateral flow	TSMP				Ruminant by-products	10	10	1% in feed and feed supplements
BioKits® (Cooked) Species Identification Kits	Sandwich ELISA	TSMP		B, H, P, Po, S			60 (five samples)	210	<1% B, P, Po <2% S
BioKits® (Raw) Species Identification Kit	Sandwich ELISA	Species albumin	B, P, Po, S				60 (five samples)	30	<1% B, P, Po, S
ABC Research Corporation Meat Species ELISA	Sandwich ELISA	GFAP	B, P, Po, S	B, P, Po, S			N/A	N/A	N/A
RIDASCREEN® Risk Material 10/5 Test Kits	Sandwich ELISA	GFAP	Raw meat, meat products				< 1 min/sample	15	≤ 0.1% CNS tissue
RIDASCREEN® Risk Material Test Kits	Sandwich ELISA	GFAP		Processed & cooked meat products			60 (ten samples)	60	≤ 0.2% CNS tissue

* Abbreviations: sTnI: skeletal muscle protein troponin I; TSMP: thermal-stable muscle proteins; TSMG: thermo-stable muscle-related glycoproteins; GFAP: glial fibrillary acidic protein; CNS: central nervous system; B: beef; D: deer; H: horse; P: pork; Po: poultry; S: sheep; MBM: meat and bone meal.

REFERENCES

Agazzi ME, Barrero-Moreno J, Lucker E, von Holst C, Anklam E. 2002. Performance comparison of two analytical methods for the detection of tissues of the central nervous system in sausages: Results of an interlaboratory study. Euro Food Res Tech 215:334-339.

Al-Jowder O, Kemsley EK, Wilson RH. 1997. Mid-infrared spectroscopy and authenticity problems in selected meats: A feasibility study. Food Chem 59:195-201.

Al-Jowder O, Defernez M, Kemsley EK, Wilson RH. 1999. Mid-infrared spectroscopy and chemometrics for the authentication of meat products. J Agric Food Chem 47(8):3210-3218.

Al-Jowder O, Kemsley EK, Wilson RH. 2002. Detection of adulteration in cooked meat products by mid-infrared spectroscopy. J Agric Food Chem 50(6):1325-1329.

Anderson RM, Donnelly CA, Ferguson NM, Woolhouse ME, Watt CJ, Udy HJ, MaWhinney S, Dunstan SP, Southwood TR, Wilesmith JW, Ryan JB, Hoinville LJ, Hillerton JE, Austin AR, Wells GA. 1996. Transmission dynamics and epidemiology of BSE in British cattle. Nature 382:779-788.

Anon. 2000. Identification of a new disease in cattle: The report of the bovine spongiform encephalopathy (BSE) Inquiry. 3:33-34. Available from MAFF (http://www.bseinquiry.gov.uk). Posted 03 01 04.

Ansfield M, Reaney SD, Jackman R. 2000a. Production of a sensitive immunoassay for detection of ruminant and porcine proteins, heated to > 130°C at 2.7 bar, in compound animal feedstuffs. Food Agri Immuno 12:273-284.

Ansfield M, Reaney SD, Jackman R. 2000b. Performance assessment and validation of a sensitive immunoassay for detection of ruminant and porcine heat stable proteins in compound animal feedstuffs. Food Agri Immuno 12:285-297.

Appel T, Wolff M, von Rheinbaben F, Heinzel M, Riesner D. 2001. Heat stability of prion rods and recombinant prion protein in water, lipid and lipid-water mixtures. J Gen Virol 82:465-473.

Araujo de Vizcarrondo C, Carrillo de Padilla F, Martin E. 1998. Fatty acid composition of beef, pork, and poultry fresh cuts, and some of their processed products. Arch Latinoam Nutr 48:354-358.

Asensio L, Gonzalez I, Rodriguez MA, Mayoral B, Lopez-Calleja I, Hernandez PE, Garcia T, Martin R. 2003. Development of a specific monoclonal antibody for grouper (Epinephelus guaza) identification by an indirect enzyme-linked immunosorbent assay. J Food Prot 66(5):886-889.

Barai BK, Nayak RR, Singhal RS, Kulkarni PR. 1992. Approaches to the detection of meat adulteration. Trends in Food Sci & Technol 4:69-72.

Bellagamba F, Valfre F, Panseri S, Moretti VM. 2003. Polymerase chain reaction-based analysis to detect terrestrial animal protein in fish meal. J Food Prot 66(4):682-685.

Bellis C, Ashton KJ, Freney L, Blair B, Griffiths LR. 2003. A molecular genetic approach for forensic animal species identification. Forensic Sci Int 134(2-3):99-108.

Bottero MT, Dalmasso IA, Nucera D, Turi RM, Rosati S, Squadrone S, Goria M, Civera T. 2003. Development of a PCR assay for the detection of animal tissues in ruminant feeds. J Food Prot 66(12):2307-12.

Brown P. 1996. Bovine spongiform encephalopathy [corrected] Creutzfeldt-Jakob disease. Br Med J 312:790-791.

Brown P, Rohwer RG, Green EM, Gajdusek DC. 1982. Effect of chemicals, heat, and histopathologic processing on high-infectivity hamster-adapted scrapie virus. J Infect Dis 145(5):683-687.

Brown P, Liberski PP, Wolff A, Gajdusek DC. 1990. Resistance of scrapie infectivity to steam autoclaving after formaldehyde fixation and limited survival after ashing at 360 degrees C: Practical and theoretical implications. J Infect Dis 161(3):467-472.

Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. 1997. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. Nature 389:498-501.

Bryan P, Alexander P, Strausberg S, Schwarz F, Lan W, Gilliland G, Gallagher DT. 1992. Energetics of folding subtilisin BPN'. Biochemistry 31(21):4937-4945.

Buntjer JB, Lenstra JA, Haagsma N. 1995. Rapid species identification in meat by using satellite DNA probes. Z Lebensm Unters Forsch 201(6):577-582.

Butler D. 1997. How BSE crisis forced Europe out of its complacency. Nature 385:6-7.

Calvo JH, Zaragoza P, Osta R. 2001. Technical note: A quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. J Anim Sci 79(8):2108-2112.

Calvo JH, Rodellar C, Zaragoza P, Osta R. 2002a. Beef- and bovine-derived material identification in processed and unprocessed food and feed by PCR amplification. J Agric Food Chem 50(19):5262-5264.

Calvo JH, Osta R, Zaragoza P. 2002b. Quantitative PCR detection of pork in raw and heated ground beef and pate. J Agric Food Chem 50(19):5265-5267.

Carnegie PR, Ilic MZ, Etheridge MO, Stuart S. 1985. Use of histidine dipeptides and myoglobin to monitor adulteration of cooked beef with meat from other species. Aust Vet J 62:272-276.

CDC. 2004a. Fact Sheet: New Variant Creutzfeldt-Jakob Disease. Available from CDC (http://www.cdc.gov/ncidod/diseases/cjd/cjd_fact_sheet.htm). Posted 03 01 04.

CDC. 2004b. Bovine spongiform encephalopathy in a dairy cow--Washington State, 2003. Morb Mortal Wkly Rep 52:1280-1285.

Chemistry Centre of Western Australia. 1999. Western Australian Food Monitoring Program. Available from Department of Health of Western Australia (http://www.population.health.wa.gov.au/Environmental/Resources/Whats%20the%20beef%20with%20sausages.pdf). Posted 03 01 04.

Chen FC, Hsieh Y-HP. 2000. Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. J AOAC Int 83(1):79-85.

Chen FC, Hsieh Y-HP. 2002. Porcine troponin I: A thermostable species marker protein. Meat Sci 61:55-60.

Chen FC, Hsieh Y-HP, Bridgman RC. 1998. Monoclonal antibodies to porcine thermal-stable muscle protein for detection of pork in raw and cooked meats. J Food Sci 63(2):201-205.

Chen FC, Hsieh Y-HP, Bridgman RC. 2002. Monoclonal antibodies against troponin I for the detection of rendered muscle tissues in animal feedstuffs. Meat Sci 62:405-412.

Chen FC, Hsieh Y-HP, Bridgman RC. 2004. Monoclonal antibody-based sandwich enzyme-linked immunosorbent assay for sensitive detection of prohibited ruminant proteins in feedstuffs. J Food Prot 67(3):544-549.

Chikuni K, Ozutsumi K, Koishikawa T, Kato S. 1990. Species identification of cooked meats by DNA hybridization assay. Meat Sci 27:119-128.

Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. 1996. Molecular analysis of prion strain variation and the etiology of "new-variant" CJD. Nature 383:685-690.

Cota-Rivas M, Vallejo-Cordoba BV. 1997. Capillary electrophoresis for meat species differentiation. J Capillary Electrophor 4:195-199.

Cousens SN, Zeidler M, Esmonde TF, De Silva R, Wilesmith JW, Smith PG, Will RG. 1997. Sporadic Creutzfeldt-Jakob disease in the United Kingdom: analysis of epidemiological surveillance data from 1970-1996. British Medical J 315:389-395.

Cutrufelli ME, Mageau RP, Schwab B, Johnston RW. 1988. Development of porcine rapid identification method (PRIME) by modified agar-gel immunodiffusion. J Assoc Off Anal Chem 71:444-445.

Cutrufelli ME, Mageau RP, Schwab B, Johnston RW. 1993. Development of a multispecies identification field test by modified agar-gel immunodiffusion. J AOAC Int 76(5):1022-1026.

Curk A. 1999. Bovine spongiform encephalopathy crisis in Europe and its impact on beef consumption in Slovenia. Rev Sci Tech 18:758-763.

Davies A, Grant A. 1987. Near infrared analysis of food. Int J Food Sci Technol 22:191-207.

Ding HB, Xu RJ, Chan DKO. 1999. Identification of broiler chicken meat using a visible/near-infrared spectroscopic technique. J Sci Food Agric 79:1382-1388.

Ding HB, Xu RJ. 1999. Differentiation of Beef and Kangaroo Meat by Visible/Near-Infrared Reflectance Spectroscopy. J Food Sci 64(5):814-817.

Ding HB, Xu RJ. 2000. Near-infrared spectroscopic technique for detection of beef hamburger adulteration. J Agric Food Chem 48(6):2193-2198.

Downey G. 1996. Authentication of food and food ingredients by near infrared spectroscopy. J Near Infrared Spectrosc 4:47–61.

Dyer DJ, Feng P. 1997. NIR destined to be major analytical influence. Feedstuffs 10:16-25.

Ebbehoj KF, Thomsen PD. 1991a. Species differentiation of heated meat products by DNA hybridization. Meat Sci 30:221-234.

Ebbehoj KF, Thomsen PD. 1991b. Differentiation of closely related species by DNA hybridization. Meat Sci 30:359-366.

EC. 1994. Commission Decision 94/381/EC of 27 June 1994 concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of mammalian derived protein. Off J Eur Communities L172:23-24.

EC. 1996. Commission Decision 96/449/EC of 18 July 1996 on the approval of alternative heat treatment systems for processing animal waste with a view to the inactivation of spongiform encephalopathy agents. Off J Eur Communities L184:43-46.

EC. 2000. Council Decision 2000/766/EC of 4 December 2000 on concerning certain protection measures with regard to transmissible spongiform encephalopathies and the feeding of animal protein. Off J Eur Communities L306:32-33.

EC. 2002. Commission Decision 2002/248/EC of 27 March 2002 on amending Council Decision 2000/766/EC and Commission Decision 2001/9/EC with regard to transmissible spongiform encephalopathies and the feeding of animal proteins. Off J Eur Communities L84:71-72.

Enserink M. 2001. Is the U.S. doing enough to prevent mad cow disease? Science 292:1639-1641.

FDA. 1906. Federal Meat Inspection Act. 21 chapter 12. Available from FDA (http://www.fda.gov/opacom/laws/meat.htm). Posted 03 01 04.

FDA. 1957. Federal Meat Inspection Act. 21 chapter 10. Available from FDA (http://www.fda.gov/opacom/laws/pltryact.htm). Posted 03 01 04.

FDA. 1993. Food labeling: Mandatory status of nutrition labeling and nutrient content revision, format for nutrition label; Final rule. 21 Code of Federal Regulations. Parts 1 and 101.

FDA. 1997. Substances prohibited from use in animal food or feed; Animal proteins prohibited in ruminant feed; Final rule. 21 Code of Federal Regulations. Part 589.2000.

Filatov VL, Katrukha AG, Bulargina TV, Gusev NB. 1999. REVIEW: Troponin: Structure, properties, and mechanism of functioning. Available from BioProt Network (http://www.protein.bio.msu.ru/biokhimiya/contents/v64/full/64091155.htm). Posted 02 14 04.

Fiocchi A, Restani P, Riva E. 2000. Beef allergy in children. Nutrition 16:454-457.

Fontaine J, Horr J, Schirmer B. 2001. Near-infrared reflectance spectroscopy enables the fast and accurate prediction of the essential amino acid contents in soy, rapeseed meal, sunflower meal, peas, fishmeal, meat meal products, and poultry meal. J Agric Food Chem 49:57-66.

Fontaine J, Schirmer B, Horr J. 2002. Near-infrared reflectance spectroscopy (NIRS) enables the fast and accurate prediction of essential amino acid contents. 2. Results for wheat, barley, corn, triticale, wheat bran/middlings, rice bran, and sorghum. J Agric Food Chem 50:3902-3911.

Fontaine J, Horr J, Schirmer B. 2004. Amino Acid contents in raw materials can be precisely analyzed in a global network of near-infrared spectrometers: Collaborative trials prove the positive effects of instrument standardization and repeatability files. J Agric Food Chem 52:701-708.

Foster JD, Hope J, Fraser H. 1993. Transmission of bovine spongiform encephalopathy to sheep and goats. Vet Rec 133:339-341.

Frezza D, Favaro M, Vaccari G, von-Holst C, Giambra V, Anklam E, Bove D, Battaglia PA, Agrimi U, Brambilla G, Ajmone-Marsan P, Tartaglia M. 2003. A competitive polymerase chain reaction-based approach for the identification and semiquantification of mitochondrial DNA in differently heat-treated bovine meat and bone meal. J Food Prot 66(1):103-9.

Gao HW, Zhang DB, Pan AH, Liang WQ, Liang CZ. 2003. Multiplex polymerase chain reaction method for detection of bovine materials in foodstuffs. J AOAC Int 86(4):764-767.

Ghani AC, Ferguson NM, Donnelly CA, Anderson RM. 2000. Predicting vCJD mortality in Great Britain. Nature 406:583-584.

Grummer RR, Luck ML, Barmore JA. 1994. Lactational performance of dairy cows fed raw soybeans, with or without animal by-product proteins, or roasted soybeans. J Dairy Sci 77(5):1354-1359.

Guglich EA, Wilson PJ, White BN. 1994. Forensic application of repetitive DNA markers to the species identification of animal tissues. J Forensic Sci 39(2):353-361.

Harlow E, Lane, D. 1988. Antibodies: A laboratory manual. New York: Cold Spring Harbor Laboratory. 555p.

Hawkes N. 2004. Thousands carrying hidden CJD timebomb. Available from the Times (http://avantgo.thetimes.co.uk/services/avantgo/article/0,,1117957,00.html). Posted 05 24 04.

Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, Doey LJ, Lantos P. 1997. The same prion strain causes vCJD and BSE. Nature 389(6650):448-450, 526.

Hillbilly Housewife. 2004. Ground Beef. Available from Hillbilly Housewife (http://www.hillbillyhousewife.com/groundbeefbargain.htm). Posted 02 14 04.

Hitchcock CHS, Crimes AA. 1985. Methodology for meat species identification: A review. Meat Sci 15:215-224.

Hughson E, Reece P, Dennis MJ, Oehlschlager S. 2003. Comparative evaluation of the performance of two commercial kits for the detection of central nervous system tissue in meat. Food Addit Contam 20(11):1034-1043.

Hsieh, Y-HP. 1999. Current development for the detection of meat species adulteration. 53rd Annual Reciprocal Meat Conference 72-73.

Hsieh Y-HP, Chen FC, Djurdjevic N. 1999. Monoclonal antibodies against heat-treated muscle protein for species identification and end-point temperature determination of cooked meats. In: Xiong YL, Ho CT, Shahidi F, editors. Quality attributes of muscle foods. New York: Plenum Publishing. p 287-307.

Hsieh Y-HP, Sheu SC, Bridgman RC. 1998. Development of a monoclonal antibody specific to cooked mammalian meats. J Food Prot 61(4):476-481.

Hsieh Y-HP, Wetzstein CJ Green NR. 1996. Retail pork products often contain meats other than pork. Highlights Agri Res 43.

Hsieh Y-HP, Woodward BB and Ho SH. 1995. Detection of species substitution in raw and cooked meats using immunoassays. J Food Protection 58:555-559.

Hsieh Y-HP, Zhang S, Chen FC, Sheu SC. 2002. Monoclonal Antibody-based ELISA for Assessment of Endpoint Heating Temperature of Ground Pork and Beef. J Food Sci 67(3):1149-1154.

Huang SC, Kuo JC. 2000. Concentrations and antioxidative activity of anserine and carnosine in poultry meat extracts treated with demineralization and papain. Proc Natl Sci Counc Repub China B 24:193-201.

Irani DN, Johnson RT. 2003. Diagnosis and prevention of bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease. Annu Rev Med 54:305-319.

Kang'ethe EK, Jones SJ, Patterson RLS. 1982. Identification of the species origin of fresh meat using an enzyme-linked immunosorbent assay procedure. Meat Sci 7:229-240.

Klipstein-Grobusch K, Kroke A, Voss S, Boeing H. 1998. [Influence of lifestyle on the use of supplements in the Brandenburg nutrition and cancer study]. Z Ernahrungswiss 37:38-46.

Lahiff S, Glennon M, Lyng J, Smith T, Shilton N, Maher M. 2002. Real-time polymerase chain reaction detection of bovine DNA in meat and bone meal samples. J Food Prot 65(7):1158-1165.

Lahiff S, Glennon M, O'Brien L, Lyng J, Smith T, Maher M, Shilton N. 2001. Species-specific PCR for the identification of ovine, porcine and chicken species in meat and bone meal (MBM). Mol Cell Probes 15(1):27-35.

Leavis PC, Gergely J. 1984. Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction. CRC Crit Rev Biochem 16(3):235-305.

Li HC, Hideg K, Fajer PG. 1997. The mobility of troponin C and troponin I in muscle. J Mol Recognit 10(4):194-201.

Lucker E, Eigenbrodt E, Wenisch S, Failing K, Leiser R, Bulte M. 1999. Development of an integrated procedure for the detection of central nervous tissue in meat products using cholesterol and neuron-specific enolase as markers. J Food Prot 62(3):268-276.

Lucker E, Schlottermuller B, Martin A. 2002. Studies on contamination of beef with tissues of the central nervous system (CNS) as pertaining to slaughtering technology and human BSE-exposure risk. Berl Munch Tierarztl Wochenschr 115:118-121.

Lyon BG, Davis CE, Lyon CE. 1986. Detection of blood (hemoglobin) contamination in poultry muscle tissue. Poult Sci 65:1919-1924.

Macedo-Silva A, Barbosa SFC, Alkmin MGA, Vaz AJ, Shimokomaki M, Tenuta-Filho A. 2000. Hamburger meat identification by dot-ELISA. Meat Sci 56:189-192.

MAFF. 1999. MAFF UK - Meat Speciation Survey. No. 176. Available from Food Standards Agency (http://archive.food.gov.uk/maff/archive/food/infsheet/1999/no176/176meat.htm). Posted 03 03 04.

MAFF. 2000. National Food Survey 1999. Available from British Nutrition Foundation (http://www.nutrition.org.uk/medianews/pressinformation/nfs.htm). Posted 03 01 04.

Martin DR, Chan J, Chiu JY. 1998. Quantitative evaluation of pork adulteration in raw ground beef by radial immunodiffusion and enzyme-linked immunosorbent assay. J Food Prot 61(12):1686-1690.

Martin R, Azcona JI, Garcia T, Hernandez PE, Sanz B. 1988. Sandwich ELISA for detection of horse meat in raw meat mixtures using antisera to muscle soluble proteins. Meat Sci 22:143-153.

Martin R, Wardale RJ, Jones SJ, Hernandez PE, Patterson RLS. 1989. Production and characterization of monoclonal antibodies specific to chicken muscle soluble proteins. Meat Sci 25:199-207.

Martin R, Wardale RJ, Jones SJ, Hernandez PE, Patterson RLS. 1991. Monoclonal antibody sandwich ELISA for the potential detection of chicken meat in mixtures of raw beef and pork. Meat Sci 30:23-31.

Meyer R, Candrian U, Luthy J. 1994. Detection of pork in heated meat products by the polymerase chain reaction. J AOAC Int 77(3):617-622.

Montiel-Sosa JF, Ruiz-Pesini E, Montoya J, Roncales P, Lopez-Perez MJ, Perez-Martos A. 2000. Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitochondrial DNA. J Agric Food Chem 48(7):2829-2832.

National Identification Development Team. 2003. United States Animal Identification Plan - "Work in Progress". 1:1. Available from USAIP (http://usaip.info/USAIP4.1.pdf). Posted 03 01 04.

Nielsen K, Kelly L, Gall D, Smith P, Bosse J, Nicoletti P, Kelly W. 1994. The use of divalent cation chelating agents (EDTA/EGTA) to reduce non-specific serum protein interaction in enzyme immunoassay. Vet Res Commun18(6):433-437.

Odumeru J. 2003. EF5070: Field application of protein fingerprinting technology for assessment of meat adulteration incidence in meat processing plants and retail stores in Ontario. Ontario Ministry of Agriculture and Food. Enhanced Food Quality and Safety Research Program.

Oellingrath IM, Iversen A, Skrede G. 1990. Quantitative determination of myoglobin and haemoglobin in beef by high-performance liquid chromatography. Meat Sci 28:313-320.

Office International des Epizooties (OIE). 2003. Number of reported cases of bovine spongiform encephalopathy (BSE) worldwide. Available from OIE (http://www.oie.int/ eng/info/en_ esbmonde.htm). Posted 03 01 04.

Pickering K, Griffin M, Smethurst P, Hargin KD, Stewart CA. 1995. Investigation of methods to detect mechanically recovered meat in meat products — IV: Immunology. Meat Sci 40:327-336.

Prusiner SB. 1994. Biology and genetics of prion diseases. Annu Rev Microbiol 48:655-686.

Quinn PJ, Boldyrev AA, Formazuyk VE. 1992. Carnosine: Its properties, functions and potential therapeutic applications. Mol Aspects Med 13:379-444.

Ribou AC, Vigo J, Viallet P, Salmon JM. 1999. Interaction of a protein, BSA, and a fluorescent probe, Mag-Indo-1, influence of EDTA and calcium on the equilibrium. Biophys Chem 81(3):179-189.

Rodriguez MA, Garcia T, Gonzalez I, Asensio L, Hernandez PE, Martin R. 2004. PCR identification of beef, sheep, goat, and pork in raw and heat-treated meat mixtures. J Food Prot 67(1):172-177.

Saeed T, Ali SG, Rahman HA, Sawaya WN. 1989. Detection of pork and lard as adulterants in processed meat: Liquid chromatographic analysis of derivatized triglycerides. J Assoc Off Anal Chem 72:921-925.

Schmidt GR, Hossner KL, Yemm RS, Gould DH, O'Callaghan JP. 1999. An enzyme-linked immunosorbent assay for glial fibrillary acidic protein as an indicator of the presence of brain or spinal cord in meat. J Food Prot 62(4):394-397.

Schmidt GR, Yemm RS, Childs KD, O'Callaghan JP, Hossner KL. 2001. The detection of central nervous system tissue on beef carcasses and in comminuted beef. J Food Prot 64(12):2047-2052.

Schonherr J. 2002. Analysis of products of animal origin in feeds by determination of carnosine and related dipeptides by high-performance liquid chromatography. J Agric Food Chem 50:1945-1950.

Seyboldt C, John A, von Mueffling T, Nowak B, Wenzel S. 2003. Reverse transcription-polymerase chain reaction assay for species-specific detection of bovine central nervous system tissue in meat and meat products. J Food Prot 66(4):644-651.

Sheu S-C, Hsieh Y-HP. 1998. Production and partial characterization of monoclonal antibodies specific to cooked poultry meat. Meat Sci 50:315-326.

Skarpeid HJ, Kvaal K, Hildrum KI. 1998. Identification of animal species in ground meat mixtures by multivariate analysis of isoelectric focusing protein profiles. Electrophoresis 19:3103-3109.

Slattery WJ, Sinclair AJ. 1983. Differentiation of meat according to species by the electrophoretic separation of muscle lactate dehydrogenase and esterase isoenzymes and isoelectric focusing of soluble muscle proteins. Aust Vet J 60:47-51.

Sorenson AW, Delhumeau C, Bernstein MS, Costanza MC, Morabia A. 2003. Impact of 'Mad Cow Disease' publicity on trends in meat and total vitamin A consumption in Geneva between 1993 and 2000. Eur J Clin Nutr 57:177-185.

Tajima K, Enishi O, Amari M, Mitsumori M, Kajikawa H, Kurihara M, Yanai S, Matsui H, Yasue H, Mitsuhashi T, Kawashima T, Matsumoto M. 2002. PCR detection of DNAs of animal origin in feed by primers based on sequences of short and long interspersed repetitive elements. Biosci Biotechnol Biochem 66(10):2247-50.

Tartaglia M, Saulle E, Pestalozza S, Morelli L, Antonucci G, Battaglia PA. 1998. Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived materials. J Food Prot 61(5):513-8.

Taylor DM. 2002. Current perspectives on bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease. Clin Microbiol Infect 8:332-339.

Taylor DM, Fraser H, McConnell I, Brown DA, Brown KL, Lamza KA, Smith GR. 1994. Decontamination studies with the agents of bovine spongiform encephalopathy and scrapie. Arch Virol 139(3-4):313-326.

Thyholt K, Isaksson T. 1997. Differentiation of frozen and unfrozen beef using near-infrared spectroscopy. J Sci Food Agric 73:525-532.

Toorop MR, Murch SJ, Ball, RO. 1997a. Development of a rapid and accurate method for separation and quantification of myofibrillar proteins in meat. Food Res Int 30:619-627.

Toorop MR, Murch SJ, Ball, RO. 1997b. Methodology and development of prediction equations for the determination of pork substitution in veal. Food Res Int 30:629-636.

Trevitt CR, Singh PN. 2003. Variant Creutzfeldt-Jakob disease: Pathology, epidemiology, and public health implications. Am J Clin Nutr 78:651S-656S.

Troyk PR. 1999. Injectable electronic identification, monitoring, and stimulation systems. Annu Rev Biomed Eng 1:177-209.

USDA. 2004. Red meat, poultry, and fish (boneless, trimmed equivalent): Per capita consumption. Available from USDA (http://www.ers.usda.gov/Data/FoodConsumption/Spreadsheets/Boneless!A1). Posted 06 02 04.

USDA-FSIS. 2001 Nutrition labeling proposed for raw meat and poultry products. Available from USDA-FSIS (http://www.fsis.usda.gov/ OA/background /nutrlabel.htm). Posted 05 03 04.

USDA-FSIS. 2003. Nutrition labeling: Nutrient content claims on multi-serve, meal-type meat and poultry products. Available from USDA-FSIS (http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/00-046P.htm). Posted 05 03 04.

USDA-FSIS. 2004. Prohibition of the use of specified risk materials for human food and requirements for the disposition of non-ambulatory disabled cattle. Federal Register 69:1862.

Vallejo-Cordoba B, Cota-Rivas M. 1998. Meat species identification by linear discriminant analysis of capillary electrophoresis protein profiles. J Capillary Electrophor 5:171-175.

Valleron AJ, Boelle PY, Will R, and Cesbron JY. 2001. Estimation of epidemic size and incubation time based on age characteristics of vCJD in the United Kingdom. Science 294:1726-1728.

Vilim V, Voburka Z, Vytasek R, Senolt L, Tchetverikov I, Kraus VB, Pavelka K. 2003. Monoclonal antibodies to human cartilage oligomeric matrix protein: Epitope mapping and characterization of sandwich ELISA. Clin Chim Acta 328(1-2):59-69.

Wang RF, Myers MJ, Campbell W, Cao WW, Paine D, Cerniglia CE. 2000. A rapid method for PCR detection of bovine materials in animal feedstuffs. Mol Cell Probes 14(1):1-5.

Werfel SJ, Cooke SK, Sampson HA. 1997. Clinical reactivity to beef in children allergic to cow's milk. J Allergy Clin Immunol 99 (3):293-300.

WHO. 1996. Report of a WHO consultation on public health issues related to human and animal transmissible spongiform encephalopathies. Available from WHO (http://www.who.int/emc-documents/tse/docs/whoemcdis96147.html). Posted 03 04 04.

Wiersma S, Cooper S, Knight R, Kennedy AM, Joiner S, Belay E, Schonberger LB. 2002. Probable variant Creutzfeldt-Jakob disease in a U.S. resident—Florida. Morb Mortal Wkly Rep 51:927-929.

Wilesmith JW, Wells GA, Cranwell MP, Ryan JB. 1988. Bovine spongiform encephalopathy: epidemiological studies. Vet Rec 123:638-644.

Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG. 1996. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 347:921-925.

Winterø AK, Thomsen PD, Davies W. 1990. A comparison of DNA-hybridization, immunodiffusion, countercurrent immunoelectrophoresis and isoelectric focusing for detecting the admixture of pork to beef. Meat Sci 27:75-85.

Wissiack R, de la Calle B, Bordin G, Rodriguez AR. 2003. Screening test to detect meat adulteration through the determination of hemoglobin by cation exchange chromatography with diode array detection. Meat Sci 64:427-432.

BIOGRAPHICAL SKETCH

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