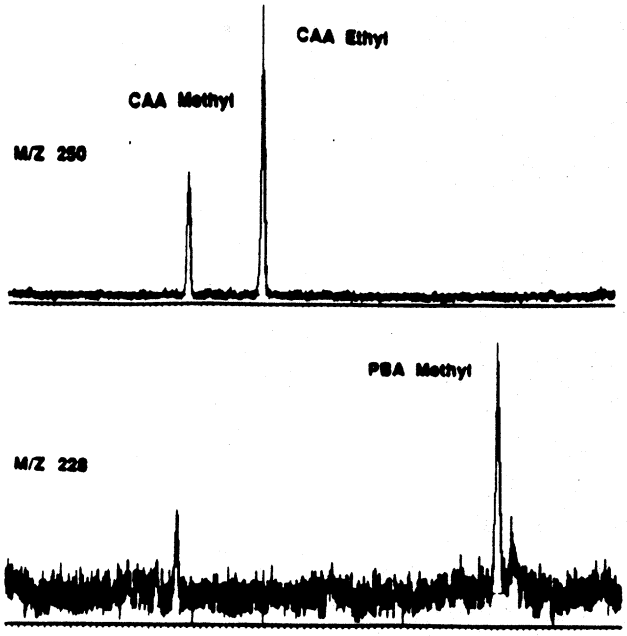

Figure 2. GC/MS chromatograms for a cottonseed sample spiked with CAA and PBA at 0.05 ppm. The lower trace shows m/z 228 detection of the PBA methyl ester. The upper trace shows m/z 250 detection of the CAA methyl and ethyl esters.

drying, and extract evaporation.

Figure 2 shows the reconstructed and single-ion chromatograms from the analysis of a cottonseed sample. The method detection limit is estimated at 0.02 ppm for CAA and 0.05 ppm for PBA. The high detection limits are due to the presence of interfering compounds in many of the matrices tested and to variable GC/MS sensitivities.

CONCLUSION

The method described herein allows reliable determination of trace quantities of the fluvalinate metabolites CAA and PBA in a variety of matrices. The methods have been used in these laboratories to analyze samples of crops that have been field treated with fluvalinate. The sensitivity of fluvalinate to hydrolysis and the difficult analytical characteristics of CAA and its methyl ester require a careful observance of several method details.

Registry No. 2, 3739-38-6; 3, 74971-63-4.

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Extraction and Quantitation of Soy Protein in Sausages by ELISA

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An indirect ELISA procedure was applied to detect and measure soy protein in sodium carbonate buffer extracts (pH 9.8) of cooked and uncooked sausages without prior delipidation or protein isolation. The method was evaluated on frankfurters prepared in our laboratory containing 0, 1, 2, 3, 4, and 5% soy isolate. With food-grade soy isolate as standard, results showed that experimental values were 94% in agreement with all levels of added soy isolate and had 95% confidence limits of 80–107%. When the extraction and ELISA procedures were applied to 23 commercial samples, results were negative on 18 products with no soy additive label while four lots of one product showed <2.5% soy protein and another product had 2.6% (±0.4, $p < 0.05$). This procedure provides a more simple, rapid, and direct analysis of soy additives suitable for monitoring adherence to the legal restrictions regarding use of soy protein additives in processed meat products.

Regulatory agencies in the United States, Canada, and EEC (European Economic Community) member countries are concerned about illegal addition of vegetable protein in meat products. Soy protein isolate, concentrate, and flour are commonly used as meat additives. In the United States, up to 2% soy protein isolate and up to 3.5% soy flour or concentrate can be added to sausage products, loaves, stews, or soups (*Code of Federal Regulations*, 1987).

To date, there is no satisfactory routine method used to measure the quantity of soy protein in meat products. Nonimmunochemical methods for detection of soy and vegetable proteins, reviewed by Llewellyn (1979, 1982), Olsman and Hitchcock (1980), Eldridge and Wolfe (1980), and Eldridge (1981), lack sensitivity and specificity. However, immunochemical methods that offer high sensitivity, specificity, and large sample throughput merit further investigation. Immunochemical methods devel-

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Table I. Apparent Accuracy of Quantitative Immunochemical Methods for Soy Protein Detection in Meat Products

reference	method	% soy added	heat treatment	estd % of actual amt
Koh (1978)	immunodiffusion rocket immunoelectrophoresis	5	Δ to 71 °C[a]	95–106
		25 (in beef patties)		96–104
Poll et al. (1978)	crossover, immunoelectrophoresis	1	90 °C, 60 min	+–[b]
		5	125 °C, 25 min	+–
		10 (in beef patties)	125 °C, 25 min	+–
Griffiths et al. (1984)	ELISA[c]	2–4	100 °C, 30 min	80–115
		8–32	100 °C, 30 min	86–102
		45–55 (in beef patties)	121 °C, 30 min	
Olsman et al. (1985)	ELISA[c]	1.5–2.81 (meat emulsion)	80 °C, 60 min	115
Ravestein and Driedonks (1986)	ELISA	1	90 °C, 120 °C	nd[d]
		3.25, 4.25 (in beef patties)	90 °C 120 °C, 30 min	80, 120
		1.5–2.81 (in luncheon meat)	90 °C	51–73
			120 °C, 30 min	90, 120
Rittenburg et al. (1987)	ELISA	1.2–2.4 (in sausages)	commerical processing temperature	47–106
		3.7–5.8 (in loaf, mince, luncheon meat)	commerical processing temperature	71–107
this paper	ELISA	1.3–3.15 (in burger, sausage, loaf)	121 °C, 120 min	71–86
		0, 1, 2, 3, 4, 5 (in frankfurter)	Δ to 71 °C[a]	94 ($p < 0.05$)

[a] (Δ) heated to temperature indicated. [b] Not quantifiable results. [c] Based on ELISA procedure of Hitchcock et al. (1981). [d] Not detectable.

oped for detection and quantitation of soy proteins in heated, pasteurized or sterilized beef patties, luncheon meats, and emulsions are summarized in Table I. Crimes et al. (1984) reported that results of a 22-laboratory collaborative study using the ELISA method developed by Hitchcock et al. (1981) had 30% and 60% repeatability values when soy flours and textured vegetable soy proteins were used as additives in sausage-type products, respectively. This method utilized antibodies made against urea-denatured and renatured soy protein. The preparation for this ELISA analysis is tedious, and analysis of 10 samples is completed in 1 week. Olsman et al. (1985), using the same procedure, reported a 115% mean estimate of soy proteins added to meat emulsions in the range of 1.5–2.81% and subsequently heated for 1 h at 80 °C. Ravestein and Driedonks (1986) also develop an ELISA procedure using antibodies to SDS-denatured soy protein. Analysis of beef patties and luncheon meats fortified with 1.5–4.25% soy protein resulted in 80–120% prediction of actual values, and the pasteurized luncheon meats fortified with 1.5–2.81% soy protein were 51–73% in agreement with added amounts. Sample preparations prior to ELISA analysis reported in these papers are tedious and can be simplified. Menzel and Hagemeister (1982) reported a solid-phase RIA for detection of native and sterilized soy protein. Immunoblotting technique with detectability of 100 ng to 2 μg (an equivalent of 0.02–0.4% soy protein isolate) was also reported by Janssen et al. (1986). These RIA and immunoblotting methods have not been applied to food products.

This research describes a simple and rapid sample preparation technique that does not compromise the antigenicity of the soy proteins in the meat samples and an ELISA procedure that can measure amounts of soy protein (as protein additives in sausage products) within the range allowed by United States food standards.

MATERIALS AND METHODS

Reagents and Equipment. Soy protein isolate (SPI 620) was a gift from Ralston Purina. Soy protein fractions (7S, 11S, basic, and acidic) were gifts from M. Groves of USDA–ARS, ERRC. Rabbit antibody to whole soy protein and goat anti-rabbit IgG peroxidase were purchased from Calbiochem (San Diego, CA); 2,2′-azinobis[3-ethylbenzthiazolinesulfonic acid] (ABTS) and Tween 20 were from Sigma Chemical Co. (St. Louis, MO); 96-well Nunc Immunoplate was from Vanguard International (Neptune, NJ); blood serum separator and filter were from Sarstedt (Princeton, NJ); microdispenser (with eight-needle manifold) was from Drummond (Broomall, PA); Polytron PT N/35 with PTA 20 generator was from Brinkman (Westbury, NY); sonicator was from Heat Systems-Ultrasonics (Plainview, NY); ELISA plate reader was from Dynatech (Alexandria, VA).

Meat Products. Frankfurters containing 49% lean meat, 25.2% beef fat, 21.2% water, 4.6% spice, sugar, and salt were prepared in our laboratory according to industrial standards, i.e. chopped until an emulsion was formed at 18 °C, stuffed into casing, smoked, and cooled to 71 °C internal temperature. Beef proteins were substituted with 1, 2, 3, 4, or 5% SPI 620 based on total weight of sausage mixture. Commercial fresh and cooked sausage products were obtained from local markets.

Characterization of Immunoreagents. The antibody to soy protein was screened for its avidity with 11S and 7S fractions, basic and acidic subfractions of 11S, soy isolate, and soy meal. Concentrations of antigen and first and second antibodies used in the assay, time and temperature of incubations of antigen–solid phase, antigen–antibody, and first antibody–antiglobulin interactions, and pH of buffers used in extraction and adsorption of antigens to polystyrene titer plates for antigen–antibody interaction were optimized. The cross-reactivity of anti-soy protein with other species of proteins was also determined.

Preparation of Standards. Soy protein isolate (1–2 mg) was sonicated in carbonate buffer (3.2 mM Na_2CO_3, 6.8 mM $NaHCO_3$, 0.1% thimersol, 0.05% Tween 20, pH 9.8) for 2 min with use of a macrotip (power 6, 50% pulse) at 0.1 μg/μL concentration and further diluted to 0.01 μg/μL. This standard solution was prepared 3–4 h prior to ELISA determination, and 5, 10, 20, 40, 60, or 80 μL was applied onto microtiter plates.

Sample Preparation. Samples were prepared as shown in Figure 1. To enhance sample homogeneity, 50 g (or one piece) of sausage product was macerated in a Waring Blendor for 30 s. Proteins were extracted in aliquots of 1-g sample by homogenizing with the Polytron for 1 min

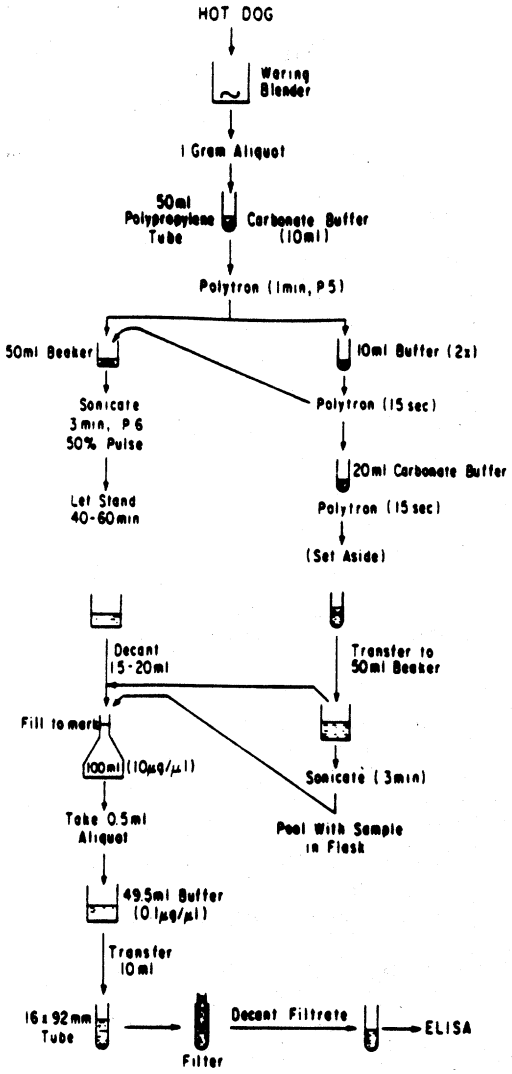

Figure 1. Extraction of proteins in cooked sausages by sodium carbonate buffer (pH 9.8) for direct analysis by ELISA technique. P5 and P6 mean power levels 5 and 6, respectively.

at power 5 and sonicated for 3 min at 50% pulsed power (level 6) with a macrotip. Samples were diluted to a final protein concentration of 0.1 $\mu g/\mu L$ and subsequently filtered through a serum separator to remove particulates. Aliquots of 10 mL were transferred into 16 × 92 mm polypropylene tubes, and an inner tube with a filter attached at the bottom was pushed down, allowing the clarified extract to transfer into the inner tube. Of the clarified extract, 50 μL was taken for ELISA analysis.

Enzyme-Linked Immunosorbent Assay. Standards (0.01 $\mu g/\mu L$ soy protein isolate) containing 0, 0.05, 0.10, 0.2, 0.4, 0.6, and 0.8 μg of protein were applied to the wells of microtiter plates in triplicate. Three blanks containing only reagent buffer and antibody enzyme–conjugate–antiglobulin were also analyzed to determine nonspecific binding to solid phase. Sample aliquots of 50 μL were applied to microtiter plates. Carbonate buffer was added to all standards and samples to make up 100-μL volume. The plates were incubated at room temperature overnight (16–18 h) to allow binding of antigen to solid phase, and the ELISA procedure was followed as shown in Figure 2. The plates were blocked with 100 μL of PBS-G (1% gelatin in PBS: 0.041 M $NaH_2PO_4 \cdot H_2O$, 0.061] M Na_2HPO_4, 0.01% thimersol, 0.9% NaCl, pH 7.0). The bound antigens were then incubated with 100 μL of anti-soy (1:5000) and subsequently allowed to react with goat anti-rabbit IgG peroxidase (1:750) for 90 min each at 37 °C. Excess

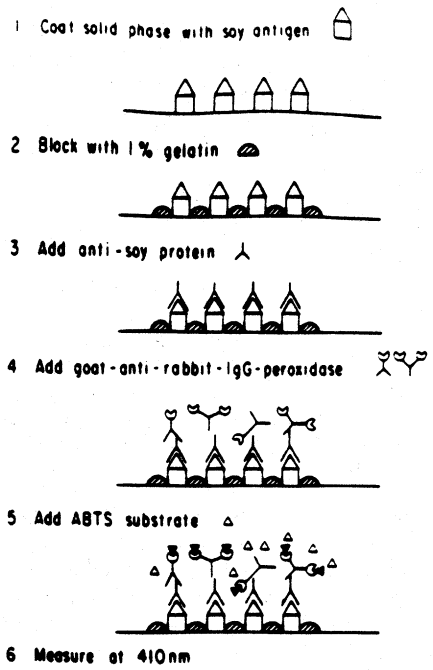

Figure 2. Indirect ELISA procedure for detection of soy proteins in carbonate buffer extracts of cooked sausages. Excess reagents were discarded and washed 4× with buffer after steps 1–4.

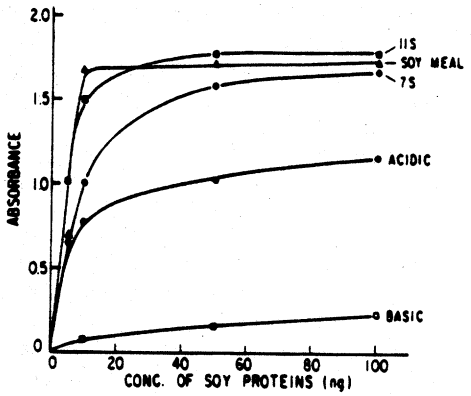

Figure 3. Binding of antibody to whole soy protein with soy meal, glycinin (11S), and conglycinin (7S) fractions and acidic and basic subfractions of 11S.

reagents were rinsed off 4× with 200 μL of PBS-T (0.05% Tween in PBS) after each step. Substrate (100 μL) containing 0.022% w/v ABTS and 30 μL of H_2O_2 (30% v/v) in citrate buffer (0.23 M sodium citrate monohydrate, 0.36 M citric acid, pH 4.0) were added onto microtiter plates. The chromogens were allowed to develop for 15 min at room temperature. The reaction was stopped by addition of 50 μL of 1.25% aqueous KF, and absorbance was measured at 410 nm.

RESULTS AND DISCUSSION

The Calbiochem antibody to soy protein showed varying degrees of binding (Figure 3) with soy meal protein and other protein fractions, glycinin (11S), conglycinin (7S), and basic and acidic subfractions of 11S. Antisoy showed no cross-reactivity or detectable binding with spices used in sausages and other protein species (beef, pork, chicken, whole milk powder, whey, casein, surimi) when tested at 10× or 100× concentration of standards. At concentrations of 0–100 ng, the 11S fraction had similar binding response with an equivalent amount of proteins in soy meal containing 50% protein. Soy proteins were isolated and fractionated from soy meal according to procedures of

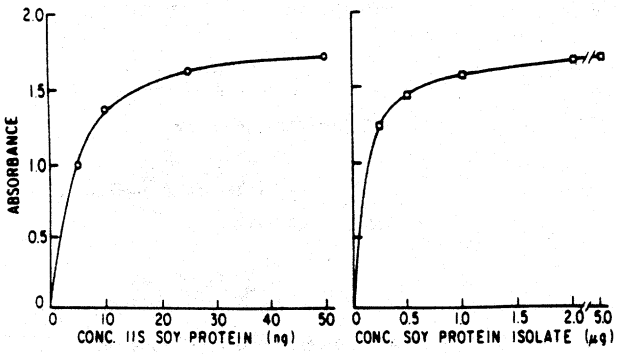

Figure 4. Binding of glycinin (11S) soy protein and soy isolate (SPI 620) to anti-soy. 11S soy protein was analyzed at 0–50-ng concentration, and soy isolate was analyzed at 0–5-μg concentration.

German and co-workers (1982). Slightly less binding activity was observed with the 7S fraction and much less with the acidic subfraction. Minimal binding was observed with the basic subfraction. The latter was least soluble in carbonate buffer among the fractions tested and resulted in lack of binding ability as the epitopes were perhaps mostly in the intact 11S or 7S segments. When the 11S fraction was compared with food-grade SPI 620, results showed that 11S had 50× greater binding ability than SPI 620 (Figure 4). Soy protein contains 40–50% glycinin and 16–20% conglycinin while the glycinin fraction consists of acidic and basic subfractions (Brooks, and Morr, 1985). Results of these binding studies indicate that the antibody to whole soy had lower binding affinity to smaller protein subunits of the basic and acidic subfractions while the intact protein in soy meal and the glycinin major fraction had equivalent binding affinity to this antibody, suggesting that epitopes lie in the glycinin region. There is also no indication of an additive effect on the binding avidity of glycinin and conglycinin fractions. Perhaps, some intact proteins in soy meal apparently are not available for binding wherein its epitopes may be bound to cellular constituents or are buried within the protein structure. Use of reducing agents in our laboratory to isolate the glycinin and conglycinin fractions unfolded the proteins, thus exposing their epitopes. Another reason for the discrepancy in binding abilities of various proteins is that the laboratory-isolated soy proteins have significantly different conformations (due to use of reducing agents) than the antigen used for commercial antibody production. Soy protein additives in meat products perhaps have similar conformations to that of the protein used as antigen for antibody production and were prepared without reducing agents. This may account for the discrepancy between SPI and glycinin.

Selecting which antigen standard to use for measuring soy protein additives, acetone powder samples of laboratory-prepared frackfurters fortified with 0.5–2.0% soy proteins (Promax 70) were analyzed by ELISA procedure. A more accurate measurement was obtained (90% of theoretical amount) with SPI 620 standard than with glycinin fraction as protein standard. Calculated results were underestimated by 50× with glycinin, and therefore it was practical to use the food-grade (SPI 620) as standard antigen. Textured vegetable soy proteins (TVP) are highly denatured protein ingredients and had varying degrees of antigenic response depending upon its processing treatment, also shown by differences in solubility and color. Processing conditions vary in alkali solubilization/acid precipitation cycles and roasting temperature used in forming solid product. Two samples of TVP had approximately 90% and 25% activities of the SPI 620

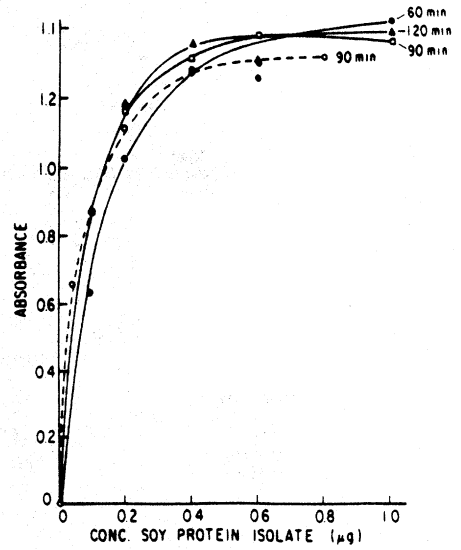

Figure 5. Optimum incubation periods for antigen–antibody binding indicated by solid lines. Dashed line shows repeated assay at 90-min incubation.

standard, suggesting that measurement of TVP will yield varying results.

Studies on optimum conditions showed that 1:5000 antibody dilution in PBS (pH 7.0) and incubation at 60, 90, or 120 min showed maximum absorbance of enzyme substrate (Figure 5). One-way analysis of variance of the three incubation periods showed no significant differences ($p < 0.01$). Later studies also showed no significant difference ($p < 0.01$) between two separate assays at 90-min incubation. Anti-globulin–peroxidase conjugate was used at 1:750 or 1:1000 dilutions. Optimum binding with the first antibody was demonstrated after 75–90-min incubation at 37 °C. Incubation periods of less than 1 h resulted in variability of the height of standard curves while slopes did not change. Longer incubation period allowed the proteins to saturate binding sites in the solid phase, antigen, or the antibodies. When such a state is achieved, there is a dynamic exchange of the excess and the bound reagent. For example, a bound antigen will be exchanged with the excess free antigen without the concentration of the free antigen in solution or bound antigen changing. When the ligand exchange is allowed to proceed to equilibrium, better interassay precision results and a more reliable quantitative measurement are obtained. In shorter incubation periods of rapid assays where equilibrium of ligand exchange is not achieved, results tend to be qualitative and such an assay can be used for screening purposes to determine the presence or absence of the compound investigated.

ELISA protocols were designed with three blanks as quality control checks to determine nonspecific color formation of enzyme substrate attributed to nonspecific binding of first or second antibodies adsorbed directly onto the solid phase. Sufficient blocking of unbound sites in the solid phase by gelatin or another appropriate protein such as bovine serum albumin can eliminate this problem. Maximum binding of antigens or antibody to plates was obtained at highly basic pH (9.8) in carbonate buffer compared to lower binding achieved with Tris (pH 8.0) or phosphate (pH 7.0) buffers. The maximum amount of soy protein isolate applied to wells of titer plates was in the 0.5–0.6-μg range when incubated at static conditions (no shaking). Assay sensitivity can be increased by limiting amounts of antibodies, i.e. decreasing amounts or increasing dilutions of the first and second antibodies. The

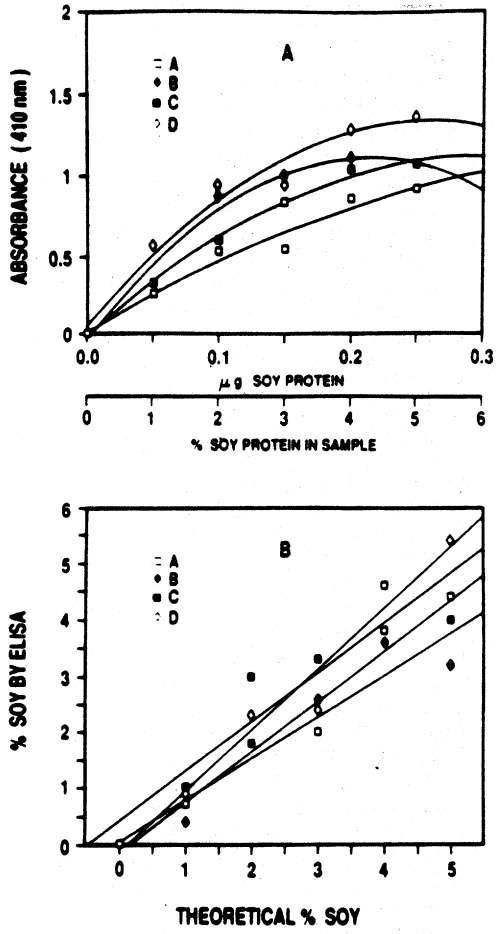

Figure 6. (A) Plot of amounts of soy protein in model frankfurters measured by ELISA techniques in four separate assays. Mean absorbance values are plotted against estimated micrograms of soy protein derived from standard plots. Each point is a mean of triplicate analysis. The regression equations and correlations of the four assays are as follows: (A) $y = 0.010 + 5.229x - 6.357X^2$, $R = 0.99$; (B) $y = -0.041 + 10.893x - 25.857X^2$, $R = 0.99$; (C) $y = -0.0118 + 7.651x - 13.071X^2$, $R = 1.0$; (D) $y = 0.050 + 9.906x - 19.214X^2$, $R = 0.99$. An expanded scale of microgram concentration (0–0.30) can give a direct estimate of 0–6% from absorbance readings of samples following protein extraction and ELISA procedures. (B) Plot of ELISA-measured soy protein in model frankfurters containing 0–5% soy protein vs theoretical amounts added to frankfurters. Each point is a mean of triplicate analysis and is derived from absorbance values in Figure 6A. The regression equations and correlations are as follows for each trail: (A) $y = 0.848 + 0.908x$, $R = 0.98$; (B) $y = -0.707 + 0.754x$, $R = 0.95$; (C) $y = 0.46 + 0.889x$, $R = 0.93$; (D) $y = -1.22 + 1.091x$, $R = 0.98$. Mean experimental percent soy protein in four trials has a regression correlation of $R = 0.98$, $y = 0.848 + 0.098x$.

most linear part of the standard curve when 0–0.8 μg of proteins was applied to the plates was in 0–0.4-μg range, the region of maximum accuracy for soy protein measurement (Figures 5 and 6). Samples analyzed containing >0.4 μg of soy protein must be analyzed again at higher dilution. Solid-phase adsorption of antigens was carried out overnight for convenience because 3–4 h was needed for preparation of 10–20 samples for ELISA. Control samples containing known amounts of soy protein in low, medium, or high range must be analyzed in every assay to monitor the precision and accuracy of the assay.

Since legal limits of protein additives are based on total weight of sausage product, it was practical to analyze and measure protein additives on an "as is" or a "wet" basis vs analysis of acetone powders. Direct analysis of wet samples eliminated the need for determination of total protein or total nitrogen from which the amount of soy proteins is extrapolated. Furthermore, when small amounts (1–2 mg) of acetone powders are sampled, there is some problem of homogeneity of soy protein distribution. In this study, great variability of results was observed when acetone powders were used.

Table II. Soy Protein Content of Commerical Cooked Sausages Measured by Indirect ELISA Technique

A. Sausages with Soy Flour Additive

	sample	sets of analysis I[a]	II	III
1.	PM-1	1.85 (1)[a]		
2.	PM-3	<2.5 (5)	1.0[b]	0.2[c]
3.	PM-4		1.0	0.3[c]
4.	PM-5			1.0
5.	RH	2.6 ± 0.4 (8)	1.6[b]	1.7[d]

B. Sausages without Soy Protein Additive
18 brands of cooked/fresh sausages; results, negative

[a] Mean soy protein content from various numbers of analyses indicated in parentheses.[b–d] Samples analyzed after storage at −10 to −20 °C for periods of 4 (*b*), 2 (*c*), and 6 months (*d*). Each value in II and III indicates the mean of triplicate analyses.

This study shows that total soy protein in sausage product can be determined directly by reading off the standard curves (Figure 6A) or derived from the equation of the line derived from plotting soy protein concentration vs absorbance. Analysis of model frankfurters fortified with soy proteins showed that mean recovery was 93.7% with confidence intervals (95%) of 80–107%. The absorbance values and estimated soy protein (μg) in these four trials (Figure 6A) were not significantly different. The F value ($p < 0.05$) was 2.05 (estimated) vs 3.24 (tabular) when analyzed by one-way analysis of variance. In Figure 6B, the ELISA-determined percent soy protein vs theoretical percent soy protein values were in close agreement. Individual trials had regression correlations of 0.98, 0.95, 0.93, and 0.98 for A–D, respectively. The mean values in four trials had a regression correlation of 0.98 ($y = 1.22 + 1.0914x$).

False positives resulting from cross-reactivities and nonspecific binding of immunochemicals were not observed in this assay but had occurred only from washing contamination of the wells, which can be eliminated by blotting dry the plate surfaces after each wash cycle. The extra step of blocking the plates with gelatin was necessary for the elimination of false positive results as such results can pose problems in monitoring additives in meat procuts.

Analysis of commercial products (Table II) showed negative results on 18 brands of cooked and uncooked sausages which had no soy protein additives in labels. Four lots of one brand of frankfurter containing soy flour additive showed an equivalent of <2.5% soy protein isolate. In the earlier stage of these studies, the lower limit of standard concentration was 0.25 μg of soy protein, an equivalent of 2.5% soy in the product. In later studies, the lower limit was 0.05 μg, which gave a more accurate measurement of <2% soy protein. Soy protein values measured in fresh PM-3 and PM-4 in the amount of 1% were within the limits of legal standards of 1.75% soy protein when soy flour was used as additive in sausages. Soy flour contains approximately 50% protein. Another product was shown to contain 2.6 (±.4)% soy protein equivalent. Only one batch was analyzed due to the unavailability of this brand in later studies. Results in column I are average values derived from various experiments. Samples in columns II and III were simultaneously analyzed with the model frankfurters. Storage of sausage products from 2 to 6 months at −20 °C showed a decrease

in immunochemical response. The decrease is apparently due to freeze denaturation of proteins or their interactions with other components in the sausage, thereby blocking or destroying antigenic sites. The results of these assays suggest that analysis must be made on fresh products and, perhaps, should be stored in ultralow-temperature freezer or as acetone powder for later analysis. These storage conditions are important to consider should samples be stored for longer periods for reanalysis by immunoassays.

In conclusion, the procedure described offers a simple and rapid extraction of proteins in cooked sausages for ELISA to quantitate soy protein content (0–5%) in model frankfurters and commercially obtained cooked and uncooked sausages. Since the soy antibody has no cross-reactivity with pork or chicken, this assay can be used to detect soy proteins in these mixtures. Preparation of 10 samples was achieved in 4 h, and the ELISA assay can be completed in 21 or 6 h when adsorption of antigens to plates was carried out at room temperature overnight or 2 h at 37 °C, respectively. In contrast, analyses of 10 samples were completed for 1 week by the procedure of Hitchcock and co-workers (1981). In laboratories requiring high sample throughput, both sample preparation and ELISA procedures can be automated by using standard robotics, thus speeding analysis of larger number of samples.

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