

Detection and Quantification of Soy Allergens in Food: Study of Two Commercial Enzyme-Linked Immunosorbent Assays

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ABSTRACT: The objective of this study was to evaluate the efficacy of 2 commercially available soy enzyme-linked immunosorbent assays (ELISA) and use them in detecting soy proteins in selected food commodities. Both ELISA kits exhibited high sensitivity. The determined limits of detection (LOD) (approximately 2 and <1 $\mu\text{g/mL}$ for Tepnel Biosystems and Elisa Systems kits, respectively) were lower than those claimed by the manufacturer. Quantification range for both kits was, however, narrower and in a lower concentration range than defined by the kit providers. Our examination revealed a positive cross-reactivity with chickpea proteins and matrix interferences for both kits. The immunoreactivity of soy proteins, when tested by the Tepnel Biosystems kit, was partially reduced by papain and bromelain hydrolysis; it was significantly decreased by protein glycation (>47%). Nondenatured and nonheated soy protein isolate (SPI) samples were also significantly less antigenic than the treated ones.

Keywords: antigenicity, cross-reactivity, ELISA kit, evaluation, immunoreactivity, matrix interference, soy allergens, soy proteins, validation

Introduction

The incidence of food allergy continues to be on the rise. An estimated 2% to 4% of the population and up to 8% of children suffer from food allergies (Bock 1987; Kanny and others 2001; Sampson 2005). Soy is one of the "big eight" foods that are believed to be responsible for 90% of all food allergies (Zarkadas and others 1999). Concerns about soybean allergy have become exacerbated due to increased use of soy ingredients in foods (for example, meat, bakery and dairy products, edible spreads). Although good manufacturing practices and accurate food labeling can enhance consumer safety, risks of contamination during shipping, storage, and processing, and from carryover due to inadequate cleaning of shared processing equipment (Huggett and Hischenhuber 1998) remain. Robust food allergen detection and quantification methods are, therefore, needed to minimize risks to highly sensitive consumers and ensure compliance with food labeling regulations. There is general agreement that the detection limits for different food products must be between 1 and 100 ppm (1 and 100 mg allergenic protein/kg food), depending on the respective food (Taylor and others 2002; Poms and others 2004). Food allergens are, however, often present in trace amounts and can be masked by the food matrix (Poms and others 2004). They are also subjected to a variety of processing techniques that can alter their immunogenic properties (Brandon and Friedman 2002; Koppelman and others 2004; Maleki and Hurlburt 2004; Sathe and others 2005).

To date enzyme-linked immunosorbent assays (ELISA) have been the preferred approach for allergen detection because of their high precision, simple handling, and good potential for standardization (Koppelman and others 2004; Poms and others 2004). Currently, 3 ELISA kits for soybean are available commercially. The Tepnel

Biosystems kit (Elisa Technologies, Gainesville, Fla., U.S.A.) is a competitive ELISA that targets renatured soy proteins with a detection limit of < 5000 mg/kg of food product. The 2nd, the Elisa Systems kit (Elisa Systems, Windsor, Australia) is based on a sandwich ELISA and determines the soy trypsin inhibitor in the food sample with a claimed sensitivity of 1 mg/kg. The 3rd, the Veratox for Soy Flour Allergen (Neogen Corporation, Mich., U.S.A.), which has recently come on the market, is a sandwich ELISA used for the quantitative analysis of minimally processed soy flour protein in food products such as cookies, crackers, chocolate bars, and cereals in the range of 2.5 to 25 mg/kg. These kits are generally designed to be easily applied by users. However, as with other ELISA tests, protein quantification could be strongly affected by antibody selectivity (cross-reactivity), matrix interference, and the manufacturing process (Hurst and others 2002; Koppelman and others 2004).

Unfortunately, none of the commercial kits above has been subjected to interlaboratory validation or qualification to confirm their sensitivity and specificity. In this context, the present paper evaluates the efficacy of Tepnel Biosystems and Elisa Systems soy testing ELISA kits in detecting soy proteins in various soy products and selected commercial food commodities. Their performance characteristics (accuracy, detection limit, precision, cross-reactivity, and influence of food matrix) were examined. The 2nd objective was to use the kits to monitor the effects of 2 processing modifications (hydrolysis and glycation) on the antigenicity and detectability of soy proteins.

Materials and Methods

Materials and chemicals

The Elisa Systems kit (Elisa Systems, Windsor, Australia) and Tepnel Biosystems kit (Tepnel Biosystems Ltd., Flintshire, U.K.) were purchased from Hallway Enterprises (Niagara-on-the-Lake, Ont., Canada) and ELISA Technologies (Gainesville, Fla., U.S.A.), respectively. Commercial soy flour (SF), soy protein concentrate (SPC), and

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soy protein isolate (SPI) were kindly provided by "Aliments Newly Weds" (Boucherville, Que., Canada). Commercial soy protein hydrolysate (SPH) was purchased from "Aliments UFL" (Boucherville, Que., Canada). Texturized soy protein (TSP) (Beef "Not!") was from Dixie Diners' Club. Chickpea protein isolate was prepared in our laboratory from locally purchased chickpeas. β -Lactoglobulin, ovalbumin, trypsin, chymotrypsin, papain, and bromelain were purchased from Sigma (St. Louis, Mo., U.S.A.). The commercial food commodities tested were all obtained from a local market. The 2% fat cow's milk (Natre) had no soy ingredients. Chicken sausages (no brand name) were labeled "may contain traces of soy." Tuna (Clover Leaf) and chicken flakes (no brand name) contained soy. The soup mix (cream of chicken, no brand name) contained hydrolyzed soy proteins, hydrogenated soy oil, and soy lecithin. The water used in all experiments was purified with a Milli-Q system (Millipore, Bedford, Mass., U.S.A.).

Preparation of in-house soy and chickpea protein isolates

Commercial defatted soy flour (100 g) and chickpea flour (ground whole chickpea beans; 100 g) were each dispersed in heated water (55 °C) at a ratio of 1:15. The pH values of the slurries were adjusted and maintained at 9.0 with 2N NaOH, and the slurries were stirred for 45 min at 55 °C. They were then allowed to stand for 15 min at room temperature and centrifuged at 4 °C for 30 min at 14300 $\times$ g. The supernatants were collected and the pH adjusted to 4.5 with 2N HCl and stirred for 45 min at 25 °C, followed by centrifugation at 2,830 g (4 °C) for 15 min. The precipitates obtained were washed twice with water and centrifuged each time at 2830 $\times$ g for 10 min. The washed precipitates were resuspended in a minimum amount of water and neutralized with 2N NaOH. Soy and chickpea protein isolates were then freeze-dried and stored at 4 °C until analyzed.

Hydrolysis of soy protein isolate

A 7.5% solution of the in-house soy protein isolate (SPI) in 0.1 M phosphate buffer (pH 7.6) was hydrolyzed with one of the following proteases: trypsin, papain, bromelain, or chymotrypsin. The enzyme: substrate ratio was 1:200 (by mass) for trypsin, papain, and bromelain, and 1:100 for chymotrypsin. The hydrolysis was done at the optimal pH and temperature of each protease as stated on the enzyme specification sheet. Hydrolysis was stopped after 60 min by heating at 75 °C for 15 min. The solutions were then cooled in an ice bath and centrifuged at 2830 $\times$ g for 15 min. Supernatants were collected and tested by the ELISA kits.

Glycation of soy protein isolate

Glycation of soy protein isolate was carried out according to the procedure of Yeboah and others (1999). Glucose was added to a 10% (w of protein/v) solution of in-house SPI. The amount of glucose was calculated based on the ratio of lysine residues to sugar carbonyl of 1:1. The solutions were adjusted to pH 7, stirred for 10 min, and freeze-dried. Aliquots of the freeze-dried protein-sugar mixtures were placed in the dark and incubated at 50 °C in separated sealed desiccators maintained at 65% relative humidity for 0, 3, 5, 10, and 14 d. The samples were then dialyzed for 48 h using Spectra/Por dialysis membrane (molecular weight cut-off 6000 to 8000) (Spectrum Laboratories Inc., Rancho Dominguez, Calif., U.S.A.). The samples were then freeze-dried and stored at -20 °C until analyzed.

Enzyme-linked immunosorbent assays

In-house soy proteins standards. In-house soy proteins standards covering a wide concentration range (0.5, 1.0, 3.5, 5.0, 15, 25, 35, 40, 70, 90 μ g/mL and 0.01, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 20 μ g/mL

for the Tepnel Biosystems kit and the Elisa Systems kit, respectively) were prepared using the in-house SPI. The in-house standards were treated and analyzed in the same way as the control samples provided with the kits.

Tepnel Biosystems kit. The Tepnel kit contained test strips consisting of 96 soy protein coated microwells, 5 soy protein standards of 3.5, 7, 15, 35, and 70 μ g/mL, and a soy protein control (soy protein isolate with no other additive). The soy protein standards (kit-provided and in-house) and all samples were prepared as per the manufacturer's recommendations. Briefly, samples were dispersed in Tris-HCl buffer (0.25 M, pH 8.6) and extracted with a preheated urea-DTT buffer at 100 °C for 60 min. The denatured protein extracts were then cooled to 50 °C and renatured with a solution of 0.35% NaCl and 0.18% L-cystine (pH 9.0; 50 °C). The final volume of the sample was adjusted by adding renaturation solution at room temperature. The renatured extract was then filtered and the filtrate was collected and analyzed according to the procedure described in the kit protocol at the time of the study. At least duplicate analyses were performed for each sample.

Elisa Systems kit. The Elisa Systems kit contains test strips (48 microwells coated with anti-soy trypsin inhibitor antibodies), a negative control (a buffered base), and 3 kit-provided standards of 1.0, 2.5 and 5.0 μ g/mL. All the samples were prepared as per the manufacturer's recommendations; they were extracted with a pre-warmed diluted extraction solution (provided with the kit) at 60 °C for 15 min with shaking. The extracts were then filtered and tested according to the manufacturer's instructions. At least duplicate analyses were performed.

Instrumentation

Optical densities (OD) were measured at 450 nm (Tepnel Biosystems kit) and 450 to 650 nm (Elisa Systems kit), using a Bench Mark Plus microplate reader (Bio-Rad Laboratories Ltd., Mississauga, Ont., Canada) controlled by a Microplate Manager Software (Bio-Rad Laboratories Ltd.).

Protein determination

The protein content of samples was determined according to the Dumas method (AOAC 1995) using an automated Leco system (Leco FP-428, Leco Corp., St-Joseph, Mich., U.S.A.). A nitrogen conversion factor of 6.25 was used.

Statistical analysis

Data were statistically evaluated by one-way analysis of variance (ANOVA) using the PRISM software, version 3.02 (Graph Pad Software, Inc. San Diego, Calif., U.S.A.). Significant differences between means were determined by Tukey's Multiple Comparison Test procedure at the 5% significance level.

Results and Discussion

Assay performance

Calibration curves. Figure 1A and 1B show the relationship between absorbance (OD) and analyte concentration for both ELISA kits. The relationship was nonlinear for both immunoassays. To fit the kit-provided and in-house standards, 2 mathematical models (Log-Log and Logistic models) were used whenever possible. The models functions were calculated by the BioRad Microplate Manager Data analysis software and the parameters are presented in Table 1.

For the Tepnel kit, the kit-provided and in-house standards could be fitted using both Log-Log and Logistic-Log models. For the Elisa Systems kit, however, the kit-provided standards could only be fitted

with a Log-Log equation due to insufficient data points; the in-house standards, which provided a larger data set, were adequately fitted with nonlinear Logistic-Log model, but badly fitted with the Log-Log model. As shown in Table 1, the slopes of the 3 Log-Log standard curves constructed with both the Tepnel and Elisa Systems kit-provided standards agreed well with each other with a coefficient of variation (CV) of 11.8% and 12.5%, respectively (below the acceptable limit of 15% [FDA 2001]). The slope-like parameter b for the 3 nonlinear logistic equations made with the Tepnel Biosystems kit-provided standards also agreed well with a CV of 10.0%.

Sensitivity and quantification range

The limit of detection (LOD) and the 50% B/B_0 value are the 2 major parameters generally measured to evaluate the sensitivity of immunoassays. LOD of the 2 soy ELISA test kits was determined according to 2 criteria. First, it was calculated as the concentration of soy protein corresponding to the mean OD of the negative control minus (competitive, Tepnel) or plus (direct, Elisa Systems) 3 times the standard deviation (European Commission 1986; Poms and others 2005) and second, as that yielding signals corresponding to 90% (10% inhibition) for the Tepnel kit and 10% B/B_0 (Midgley and others 1996) for the Elisa Systems kit.

The lower and upper limits of quantification (LLOQ and ULOQ) were estimated to be the concentrations that corresponded to quantification range generally represented by the 20% to 80% B/B_0 sig-

nals. The LLOQ was also estimated as the concentration corresponding to the absorbance of the mean negative control minus (competitive, Tepnel) or plus (direct, Elisa Systems) 5 times its standard deviation. As shown in Table 2, the ELISA kits specific quality parameters, such as sensitivity and quantification range, were very dependent on the criterion used for their calculation. The LOD and LOQ calculated as the mass equivalence of the negative control plus or minus 3 and 5 times, respectively, were generally found to be lower than those estimated by the other criteria [90% B/B_0 (Tepnel Biosystems) or 10% B/B_0 (Elisa Systems), and 20% B/B_0 for LOQ]. Certain determined LOD values for the Elisa Systems kit were unrealistically low. This could partly be explained by the fact that when LOD were calculated based on the criterion derived from the standard deviation of the mean blank values, only that standard deviation was taken in consideration. Therefore, low deviations will shift the LOD into very low concentration area. It is, thus, important to have a large number of blanks; unfortunately, this was not achievable in the present study due to the limited number of wells. Test kit sensitivity and quantification range are also very dependent on the calibration curve and on the curve-fitting model used for its construction. When these parameters were calculated using the Log-Log model, the Tepnel Biosystems kit was less sensitive (mean LOD = 2.10 $\mu\text{g/mL}$, and 50% B/B_0 = 6.36 $\mu\text{g/mL}$) compared to when determined with the Logistic function (mean LOD = 0.33 $\mu\text{g/mL}$ and 50% B/B_0 = 5.05 $\mu\text{g/mL}$). Accordingly, the quantification range was

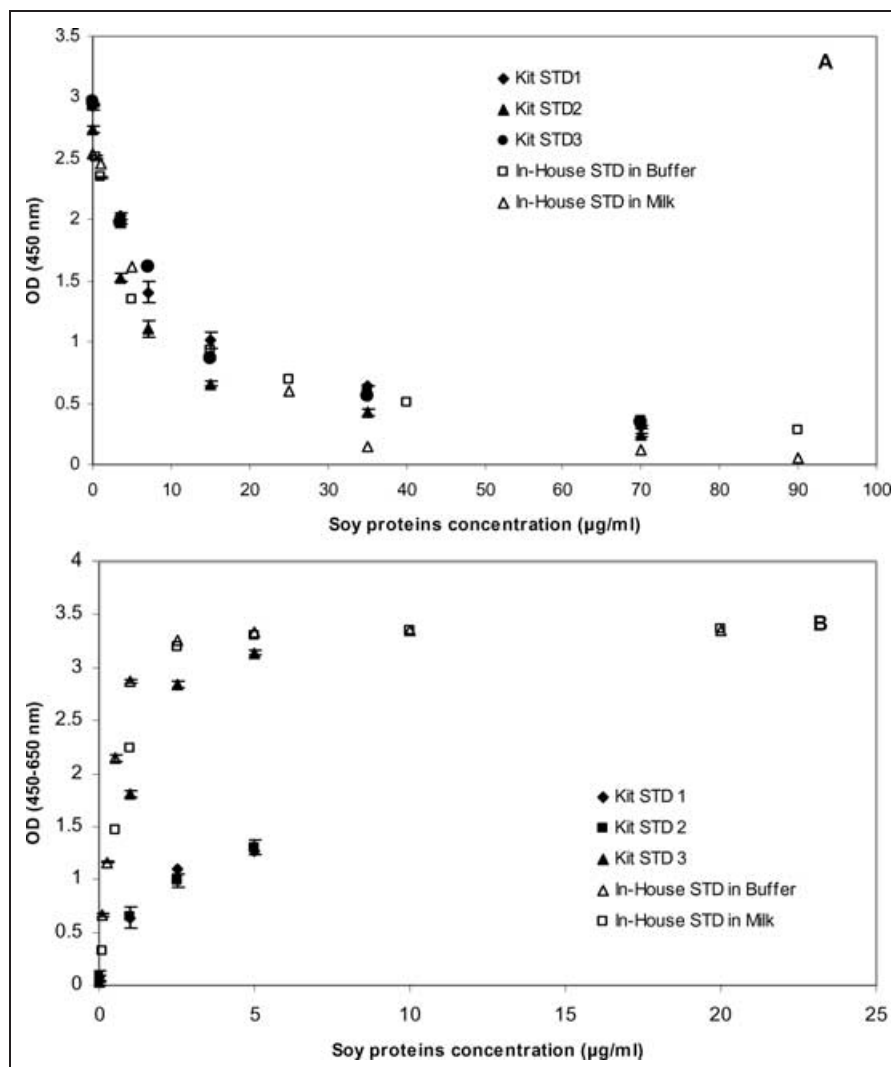


Figure 1 – Typical soy ELISA test kits dose-response data (A) Tepnel Biosystems kit; (B) Elisa Systems kit (Kit STD1 to 3 are replicates made with the kit-provided standards; in-house STD in buffer are made from in-house soy protein standards; in-house STD in milk were prepared by spiking 2% cow milk with known concentrations of soy proteins). Data are the average of triplicates.

narrower with the Log-Log model (2.78 to 12.50 $\mu\text{g/mL}$) as compared to the Logistic model (1.88 to 21.77 $\mu\text{g/mL}$). In both cases, however, the estimated sensitivity was found to be higher than that suggested by the kit manufacturer ($\text{LOD} < 5000 \text{ mg/kg}$). The latter also stated a wider dynamic range than was determined in the present study. The Elisa Systems kit was found to be more sensitive than the Tepnel Biosystems kit. The LOD was estimated to be less than 0.03 $\mu\text{g/mL}$ (using Log-Log function). However, because of the unacceptably high percentage of error obtained (CV of 86%), it can only be stated that it is lower than 0.1 $\mu\text{g/mL}$. The 50% B/B_0 was calculated to be 0.7 $\mu\text{g/mL}$ (CV = 7.54%). The kit's upper limit of quantification (2.5 $\mu\text{g/mL}$; CV = 6.0%) was also found to be much lower than that stated by the kit manufacturer (<5.0 $\mu\text{g/mL}$).

In-house standards in buffer and milk were also used to assess relevant performance parameters. The results showed (Table 1 and 2) that kit performance is strongly affected by the characteristics of the tested antigen (that is, if different from those against which the antibodies were raised) and those of the matrix in which they are tested (further discussions of these results are made below).

Precision

The precision of the ELISA kits was assessed by determining the intra-assay and inter-assay coefficients of variation (% CV), which measure the variability of the result for the same sample evaluated repeatedly in the same assay (run) and in separate assays (runs), respectively. Although there are no generally accepted minimal performance requirements for immunoassays, it is generally agreed that intra- and inter-assay variability of less than 10% to 15% is acceptable (FDA 2001).

In the present study, precision was assessed by running triplicates of each kit standard on 3 different days with independently prepared test solutions. The coefficient of variation (% CV) was determined

for each set of replicate within the same and between different days of analysis (Table 3). The data obtained with each standard curve were also compared. The intra-assay variations for the Tepnel and Elisa Systems kits ranged from 0.0% to 9.8%, and 0.1% to 15.9%, respectively, over the concentration range of the kit standards, demonstrating an acceptable precision for both test kits. Poor intra-assay repeatability (25.5%) was only found for the Elisa Systems kit negative blank (0.0 $\mu\text{g/mL}$), which made an accurate estimation of the kit LOD and LOQ difficult. Tepnel Biosystems kit inter-assay precision ranged from 5.6% to 28.7%; it was mostly in the high concentration range of the standard curve (>35 $\mu\text{g/mL}$) that unacceptable percentages of error (variations exceeding 20%) were observed. The poor inter-assay variability experienced with Elisa Systems (CV of 42.3% to 65.6%) could have been due to the fact that one of the 3 replicates runs was made with a kit having a different lot number. The inter-assay variation between the 2 sets of replicates made with the same Elisa Systems kit ranged only from 1.5% to 7.5%.

Accuracy

The accuracy of the 2 commercial soy testing kits was studied by a recovery test using the in-house standards. The accuracy (recovery) was expressed as the percentage of determined soy protein concentration over the expected concentration. Tepnel Biosystems kit showed (Table 4) good accuracy and precision for most concentrations, with both Logistic and Log-Log fittings. Coefficients of variations (CV) were below 8% and recovery values ranged from 81% to 116% (acceptable recovery rate ranging from 80 to 120% [FDA 2001]). Exceptionally, recovery rate for the concentration of 5.0 $\mu\text{g/mL}$ exceeded this range (recovery of 170%). Recovery rates for both kit-provided (40 $\mu\text{g/mL}$) and in-house controls (SPI, 40 $\mu\text{g/mL}$) were also acceptable with values superior to 94%. These results suggest that both Logistic and Log-Log models describe adequately and accurately the Tepnel Biosystems immunoassay.

Table 1 – Calibration curve parameters for Tepnel Biosystems and Elisa Systems soy test kits

Elisa kit	Standard curve fitting model						
	Log-Log ^a			Logistic-Log ^b			
	$\text{Log}(Y) = a \text{Log}(X) + b$			$Y = \frac{a - d}{1 + (X/c)^b} + d$			
	<i>a</i>	<i>b</i>	<i>r</i> ²	<i>a</i>	<i>d</i>	<i>c</i> ($\mu\text{g/mL}$)	<i>b</i>
Tepnel Biosystems kit							
Kit STD1	−0.593	0.655	0.994	2.96	0.265	6.14	1.27
Kit STD2	−0.482	0.651	0.984	3.29	0.399	6.62	1.09
Kit STD3 ^c	−0.598	0.664	0.985	2.97	0.175	6.33	1.06
In-house STD in buffer ^c	−0.424	0.395	0.970	2.91	0.169	5.03	0.93
In-house STD in milk ^c	−0.859	0.683	0.929	2.57	−0.097	9.01	1.25
% CV1 ^d	11.8	1.0	na ^h	6.1	na	3.8	10.0
% CV2 ^e	19.3	35.2	na	3.9	na	16.5	14.3
% CV3 ^f	30.1	2.8	na	12.6	na	24.3	6.5
% CV4 ^g	47.9	37.8	na	8.8	na	40.1	20.8
Elisa Systems kit							
Kit STD1	0.436	−0.187	0.999	Insufficient data			
Kit STD2	0.445	−0.182	0.972				
Kit STD3 ^c	0.352	0.274	0.962				
In-house STD in buffer ^c	Bad fit ($r^2 < 0.90$)			0.023	3.43	0.351	1.34
In-house STD in milk ^c				0.042	3.47	0.623	1.38
% CV1 ^d	12.5	na	na	na	na	na	na
% CV4 ^g	na	na	na	41.3	na	39.5	2.08

^aLog-Log equation parameters: *Y*, OD; *X*, concentration; *a*, y-intercept; *b*, slope.

^bLogistic-Log equation parameters: *Y*, OD; *X*, concentration; *a*, theoretical OD at zero concentration; *d*, theoretical OD at infinite concentration; *c*, concentration at 50% B/B_0 ; *b*, curvature parameter.

^cAssays were carried out within the same run.

^d% CV1 (coefficient of variation = (standard deviation/mean) $\times$ 100) between data obtained from kit STD1, kit STD2, and kit STD3

^e% CV2 between the mean data obtained from STD1, STD2 and STD3, and data obtained from In-House STD in buffer.

^f% CV3 between the mean data obtained from STD1, STD2 and STD3, and data obtained from In-House STD in milk.

^g% CV4 between data obtained from in-house STD in buffer and data obtained from In-House STD in milk.

^hna stands for not applicable.

Elisa Systems kit, on the contrary, exhibited poor and unacceptable recoveries for most studied concentrations, despite excellent precision ($CV < 3.3\%$). This could be explained by the fact that the Elisa Systems kit is specific to the soy allergen soybean trypsin inhibitor and therefore the kit response is very dependent on the level of this protein in the extracted sample, which in turn is very dependent on the source of the soy protein material (soybean variety) and processing history. It was, therefore, difficult to accurately study recoveries (accuracy) based on the calibration curve made with the kit controls. Elisa Systems immunoassay, as stated by the kit manufacturer, is best suited for screening purposes only. It should be mentioned that the present study challenged the 2 kits tested beyond what the test kit manufacturers have stated in their protocols. This must be taken into account in any interpretation of the results presented. Furthermore, recovery rates from defined sample buffer where soy proteins are fully solubilized cannot be translated to real

food matrices. Previous studies (Koppelman and others 2004) have shown that full recovery and, thus, true quantification of soy proteins in processed food matrices were difficult with the extraction methods that are used in the commercial soy protein assays.

Matrix interference

In the present study, cow's milk (2% fat) was selected as a model to test matrix interference. It was spiked with in-house SPI at different levels (1.0, 3.5, 5.0, 15, 25, 35, 70, 90 $\mu\text{g/mL}$ and 0.01, 0.1, 0.5, 1.0, 2.5, 5.0, 10, 20 $\mu\text{g/mL}$ for the Tepnel and Elisa Systems kits, respectively). The mathematical parameters of the calibration curves obtained in both the control matrix (buffered solution recommended by the kit provider) and the model matrix (milk) (Table 1 and 2) were compared as well as the percent recovery from the observed value over the known value of added soybean proteins in both the control and model matrices (Table 4). In the case of the Tepnel kit, the slope

Table 2 – Sensitivity and quantification range of Tepnel Biosystems and Elisa Systems soy test kits

STD curve fitting model	Standards/coefficient of variations	Tepnel Biosystems kit					
		LOD $3 \times SD$ $\mu\text{g/mL}$	LOD 10% B/B_0 $\mu\text{g/mL}$	LOD 50% B/B_0 $\mu\text{g/mL}$	LOQ $5 \times SD$ $\mu\text{g/mL}$	LLOQ 20% B/B_0 $\mu\text{g/mL}$	ULOQ 80% B/B_0 $\mu\text{g/mL}$
Log-Log	Kit STD1	2.113	2.336	4.683	2.166	2.711	10.564
	Kit STD2	1.959	2.328	5.642	2.097	2.812	16.233
	Kit STD3 ^a	2.231	2.444	4.838	2.298	2.829	10.712
	Kit Mean STD ^b	2.101	2.369	5.054	2.187	2.784	12.503
	In-House STD in Buffer ^a	0.720	0.838	2.576	0.750	1.06	10.896
	In-House STD in Milk ^a	2.226	2.307	3.586	2.307	2.540	5.872
	% CV1 ^c	6.5	2.7	10.2	4.7	2.3	25.8
	% CV2 ^d	69.2	67.5	45.9	69.2	63.4	9.7
	% CV3 ^e	4.1	1.9	24.0	3.8	6.5	51.0
	% CV4 ^f	72.3	66.1	23.2	72.0	58.1	42.4
Logistic-Log	Kit STD1	0.314	1.088	6.14	0.482	2.061	18.291
	Kit STD2	0.357	0.882	6.62	0.481	1.856	23.616
	Kit STD3 ^a	0.325	0.796	6.33	0.464	1.712	23.409
	Kit Mean STD						
	In-House STD in Buffer ^a	0.077	0.474	5.03	0.157	1.133	22.333
	In-House STD in Milk ^a	0.983	1.553	9.01	1.400	2.972	27.313
	% CV1	6.7	16.3	3.8	2.1	9.3	13.8
	% CV2	88.2	45.4	15.5	71.2	34.9	1.8
	% CV3	70.0	36.1	24.3	69.7	31.9	16.0
	% CV4	120.9	75.3	40.1	112.9	63.4	14.2
		Elisa Systems Kit					
		LOD $3 \times SD$ $\mu\text{g/mL}$	LOD 10% B/B_0 $\mu\text{g/mL}$	LOD 50% B/B_0 $\mu\text{g/mL}$	LOQ $5 \times SD$ $\mu\text{g/mL}$	LLOQ 20% B/B_0 $\mu\text{g/mL}$	ULOQ 80% B/B_0 $\mu\text{g/mL}$
Log-Log	Kit STD1	0.023	< 0.000	0.655	0.040	0.017	2.457
	Kit STD2	0.022	< 0.000	0.700	0.042	0.017	2.700
	Kit STD3 ^a	< 0.000	0.032	0.761	< 0.00	0.103	2.423
	Kit Mean STD	0.015	0.011	0.705	0.027	0.047	2.526
	In-House STD in Buffer ^a	na ^g	na	na	na	na	na
	In-House STD in Milk ^a	na	na	na	na	na	na
	% CV1	86.7	173.2	7.54	86.6	108.7	6.0
Logistic-Log	Kit STD1	na	na	na	na	na	na
	Kit STD2	na	na	na	na	na	na
	Kit STD3 ^a	na	na	na	na	na	na
	Kit Mean STD	na	na	na	na	na	na
	In-House STD in Buffer ^a	0.0046	0.068	0.351	0.005	0.125	0.987
	In-House STD in Milk ^a	0.0108	0.127	0.623	0.014	0.228	1.701
	% CV4	56.9	42.8	39.5	67.0	41.3	35.6

^aThe assays were carried out within the same plate.

^bAverage data from kit STD1, kit STD2, and kit STD3.

^c% CV1 (coefficient of variation = (standard deviation/mean) $\times$ 100) between data obtained from kit STD1, kit STD2, and kit STD3.

^d% CV2 between the Kit mean STD and in-house STD in buffer.

^e% CV3 between the Kit mean STD and in-house STD in milk.

^f% CV4 between data obtained from in-house STD in buffer and data obtained from in-house STD in milk.

^gna stands for not applicable.

^h B/B_0 : $B = Y - b$ and $B_0 = Y_0 - b$; Y is the sample OD; Y_0 is the maximum signal; b is the slope.

ⁱ B/B_0 : $B = Y - d$ and $B_0 = a - d$; Y is the sample OD; a , theoretical OD at zero concentration; d , theoretical OD at infinite concentration.

Table 3—Intra-assay and inter-assay precision and accuracy of Tepnel Biosystems and Elisa Systems soy test kits

Nominal concentration of kit standards ($\mu\text{g/mL}$)	Intra-assay range of % CV ^a	Inter-assay % CV
Tepnel Biosystems kit		
0.0	0.69–5.91	5.56
3.5	0.25–8.48	12.50
7.0	0.10–9.81	8.24
15	2.67–9.52	17.11
35	0.0–8.96	28.74
70	1.40–9.62	27.53
Elisa Systems kit ^b		
0.0	4.87–25.53	42.28
1.0	1.95–15.94	65.61
2.5	0.10–6.30	63.67
5.0	0.67–5.23	56.21

^a% CV : Coefficient of variance = (standard deviation/mean) $\times$ 100.

^bThe inter-assay % CV was determined from triplicate values (kit STD1, kit STD2, kit STD3) 2 of the replicates (kit STD 1 and kit STD 2) were from the same Elisa System kit and the 3rd one (kit STD 3) was from another kit having a different lot number.

of the calibration curve produced with the in-house standards in milk had a significantly lower value ($a = -0.859$) than that of the curve produced in buffer ($a = -0.424$), with a CV of 48%, demonstrating the presence of matrix interferences. Midgley and others (1996) have indicated that the slope of a standard calibration curve in matrix containing interferences is less steep than with the control system. The results of the spiking recovery in milk were interesting in that the recovery continuously increased as larger concentration of soy proteins was added to the sample, until it was rendered beyond the upper limit of quantification. One possible explanation is the presence in milk of interfering components that interact with

the target antigen (soy proteins) or the antibody and thereby modify the immunoassay response and sensitivity. Rozenfeld and others (2002) have isolated a 30-kD component from soy proteins that cross-reacted with cow milk caseins. Despite the observed matrix interference, the Tepnel Biosystems kit provided satisfactory intra-assay precision (CV < 6%) within the kit quantification range (3.5 to 25 $\mu\text{g/mL}$). Although similar trends were observed for the Elisa Systems kit (Table 1, 2, 4) (high variations in LOD and LOQ in buffer and milk), it is difficult to make any conclusive statement about matrix interference, due to insufficient accuracy of this immunoassay.

Cross-reactivity

The specificity of the 2 ELISA kits was evaluated by analyzing their cross-reactivity with selected food allergens (β -lactoglobulin, ovalbumin, and chickpea proteins) whose chemical structure or concurrent usage in food products might cause concern over potential interference in the assay. Cross-reactivity was established by testing the suspected cross-reactant at several concentrations (90, 180, 360 $\mu\text{g/mL}$) and was calculated as the percentage of the apparent mass equivalence determined from the kit-assembled standard curve (Log-Log function) over the added concentration of cross-reactant (Spiehler and others 1996).

Figure 2 depicts the profile of cross-reactivity of soybean proteins with the selected allergenic proteins. Results showed that there was a significant cross-reactivity with chickpea proteins for the Tepnel Biosystems kit (Figure 2A). Cross-reactivity of the Elisa Systems kit toward chickpea proteins was below the lower limit of quantification, but not negligible (Figure 2B). Cross-reactivity with chickpea proteins was not surprising due to the known structural homology between chickpea and soy proteins (Bernhisel-Broadbent and Sampson 1989). β -Lactoglobulin and ovalbumin showed no cross-reactivities when tested with both ELISA kits.

Table 4—Recovery of soy proteins in spiked buffer solutions and 2% cow milk

Added conc. of soy proteins ($\mu\text{g/mL}$)	Logistic-Log				Log-Log			
	Buffer		2% Milk		Buffer		2% Milk	
	Observed conc. (CV) $\mu\text{g/mL}$ (%)	Recovery (%)	Observed conc. (CV) $\mu\text{g/mL}$ (%)	Recovery (%)	Observed conc. ($\mu\text{g/mL}$)	Recovery (%)	Observed conc. ($\mu\text{g/mL}$)	Recovery (%)
Tepnel Biosystems kit								
0.5	1.34 (2.6)	268.6	nd ^c	na ^d	2.75	550.6	nd	na
1.0	1.94 (0.8)	194.1	1.54 (1.5)	153.7	3.09	309.0	2.86	286.0
3.5	3.55 (0.8)	101.4	3.57 (0.2)	102.1	4.08	116.7	4.10	117.1
5.0	8.52 (3.5)	170.3	5.98 (5.9)	119.6	7.75	155.1	5.78	115.6
5.0	15.85 (4.3)	105.7	17.36 (5.8)	115.7	14.24	94.9	15.67	104.5
25.0	25.39 (5.2)	101.6	31.49 (5.4)	125.9	23.57	94.3	29.71	118.8
35.0	30.31 (0.6)	86.6	>ULOQ ^f	na	28.52	81.5	332.07	948.7
40.0	42.14 (2.8)	105.3	nd	na	40.30	100.8	nd	na
70.0	77.14 (2.8)	110.2	>ULOQ	na	71.29	101.8	>ULOQ	na
90.0	133.67 (7.1)	148.5	>ULOQ	na	107.58	119.5	>ULOQ	na
40.0 (PC1) ^a	39.62 (0.9)	99.0	nd	na	37.83	94.6	nd	na
40.0 (PC2) ^b	36.26 (0.4)	98.1	nd	na	37.47	93.7	nd	na
Elisa Systems kit								
0.01					0.000 (14.8)	0.1	0.000 (7.3)	0.3
0.10					0.051 (0.2)	51.2	0.006 (5.4)	6.4
0.25					0.256 (1.3)	102.3	nd	na
0.50					1.466 (3.3)	293.2	0.494 (2.4)	98.8
1.00					3.353 (0.2)	335.3	1.660 (0.4)	166.0
2.50					4.814 (0.4)	192.6	4.504 (0.5)	180.2
5.00					5.089 (0.3)	101.8	5.006 (0.1)	100.2
10.00					5.170 (0.8)	51.7	5.175 (0.7)	51.7
20.0					5.163 (0.5)	25.8	5.249 (0.8)	26.2

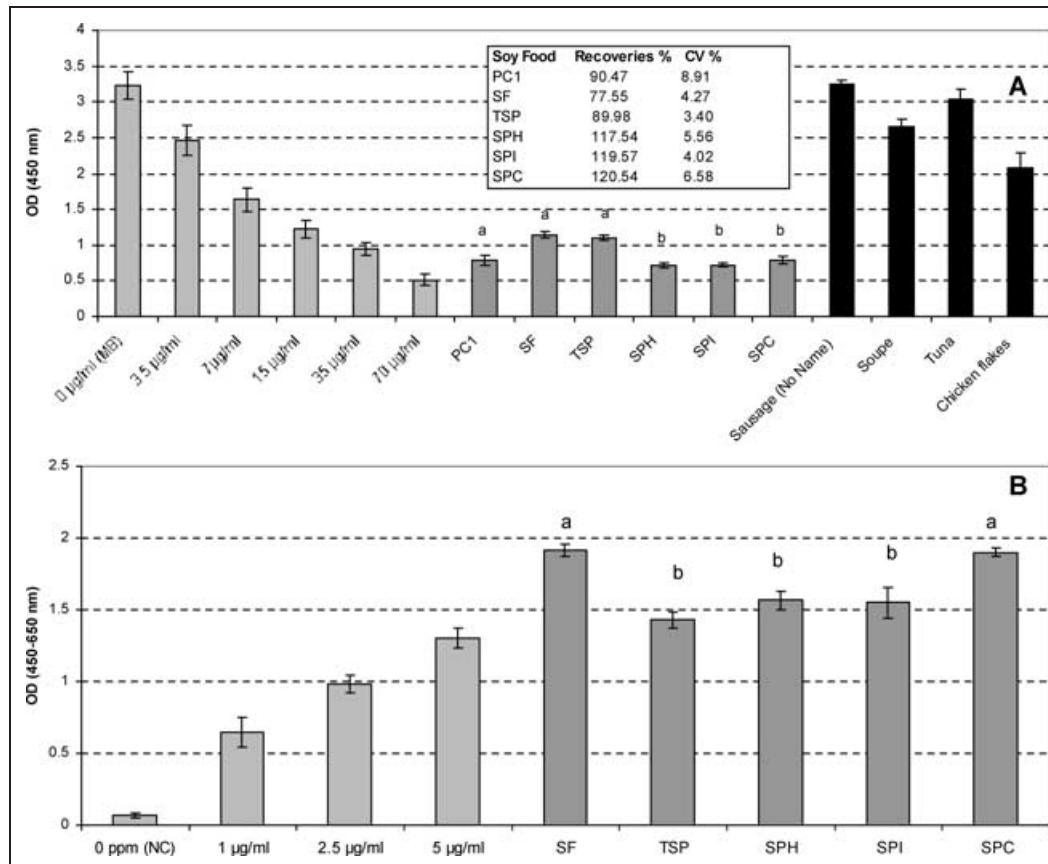
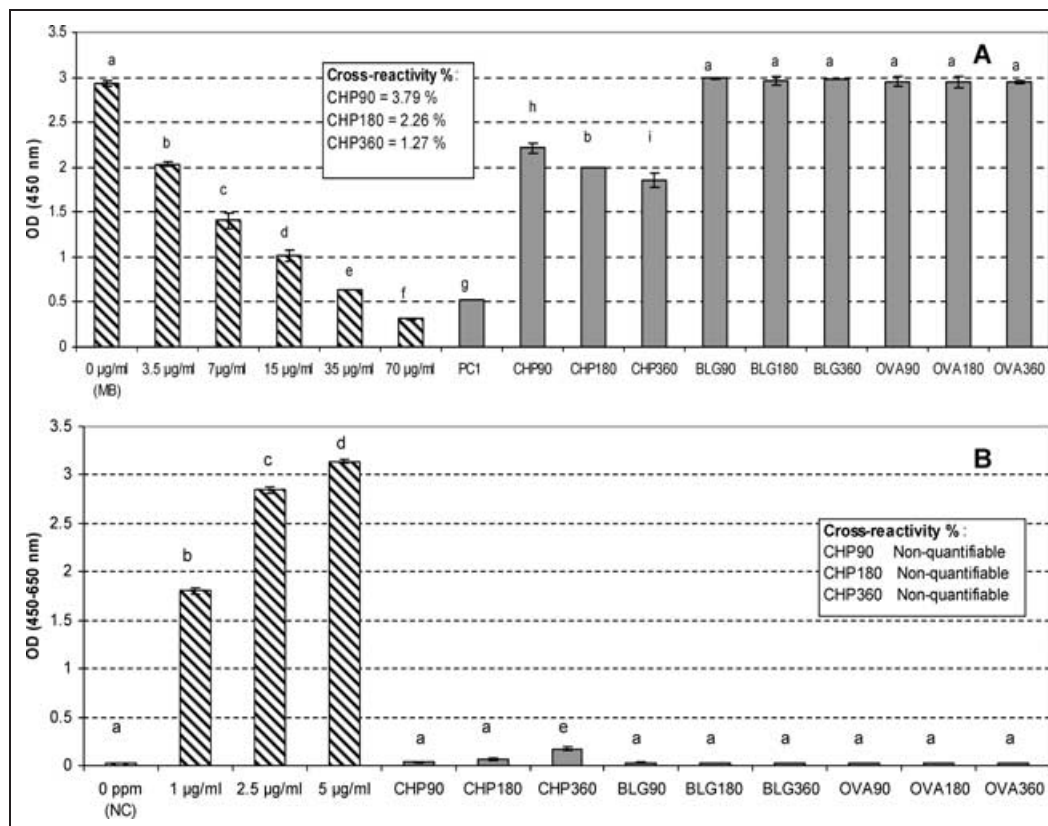
^aPC1 is the positive control provided with the Tepnel Biosystems kit.

^bPC2 is the in-house soybean protein positive control made from in-house SPI.

^cnd stands for not determined.

^dna stands for not applicable.

^f>ULOQ stands for beyond upper limit of quantification.



Performance of the two commercial ELISA kits on various soy and food products

To ascertain their effectiveness, both Tepnel Biosystems and Elisa Systems kits were tested on various soy products (flour, concentrate, isolate, hydrolysate, and texturized proteins). Other food products purchased from local stores, which may or may not contain soy proteins, were tested only by the Tepnel Biosystems kit due to limited availability of wells. Results are presented in Figure 3A and 3B. Acceptable levels of protein recoveries (77% to 120% based on the known protein content of the soy product) and coefficients of variation (0.89% to 14%) obtained for the various soy foods tested (Figure 3a) confirmed the good precision and accuracy of the Tepnel Biosystems kit. The results obtained for the other tested foods were in good agreement with the product labels (see materials and methods). Interestingly, when tested with the Elisa Systems kit, soy flour and soy protein concentrate exhibited higher absorbances compared to SPI and SPH, despite their lower protein content. This was probably due to their higher content in trypsin inhibitor. The difference in the level of detectable trypsin inhibitor in the various soy products could possibly result from differences in the product composition and manufacturing processes (Brandon and Friedman 2002; Koppelman and others 2004).

Effect of protein modification on ELISA kit response

In addition to interactions with compounds from the food matrix, proteins can be modified through various manufacturing processes; this may adversely affect their recognition in immunoassays, thereby resulting in altered test sensitivity and reliability. In this context, the effect of soy protein hydrolysis on the test kits response was assessed. The influence of protein glycation and alteration of recommended sample preparation procedure (denaturation-Urea-DTT compared with no denaturation, heating compared with no heating, renaturation compared with no renaturation, Table 5) were also investigated for the Tepnel Biosystems kit. Protein glycation could not be tested by the Elisa Systems kit due to the lack of solubility of the glycated proteins in the kit sample extraction buffer.

Table 5 shows that hydrolysis of in-house SPI with trypsin and chymotrypsin at the conditions used in this study did not significantly

modify their detectability by both test kits, indicating the presence of intact antigenic residues. Papain and bromelain resulted in significant reduction of SPI antigenicity only when tested with the Tepnel Biosystems kit (about 50% reduction; $P < 0.01$). Selective destruction of epitopes has been observed with a proportion of soybean allergens still maintaining their native configuration after certain hydrolytic treatments (Herian and others 1990, 1993; Wal 2002). For the Elisa Systems kit, it is difficult to make any statement on the effect of these 2 proteases on SPI antigenicity, since the data were statistically not different ($P > 0.05$) from that of nonhydrolyzed SPI control. Knowing that the Elisa Systems kit is designed with specific soy trypsin inhibitor antibodies, it might not detect changes in the antigenicity of other soybean proteins (such as glycinin and β -conglycinin).

Table 5 also shows that SPI immunoreactivity was significantly reduced by protein glycation ($>47\%$; $P < 0.01$). This decrease occurred rapidly after day 1 of glycation. Longer period of glycation did not further reduce SPI antigenicity. Oste and others (1990) have also found that antigenicity of glycated soybean trypsin inhibitor was reduced by up to 60% to 80% when heated at 120°C for 50 min compared to control samples lacking carbohydrates. Other workers have also shown that conjugation of soy protein with chitosan and galactomannan by means of the Maillard reaction greatly reduced soy allergenicity (Babiker and others 1998; Usui and others 2004). Significant alterations in protein structure do occur as a result of protein-carbohydrate interactions, such as the Maillard reaction, leading to epitope modification or masking, thereby affecting the antigen-antibody recognition (Babiker and others 1998; Davis and others 2001; Sathe and others 2005). Although the Maillard reaction has been reported to be a useful means of reducing allergenicity of some food proteins (Hansen and others 2003; Gruber and others 2004), it has been also shown to enhance the allergenicity of certain protein (that is, major peanut allergens Ara h1 and Ara h2) (Maleki and Hurlburt 2004).

The results further showed that the nondenatured and non-heated SPI samples were significantly ($P < 0.001$) less antigenic than the sample extracted as recommended in the kit instruction manual, demonstrating the importance of proper sample extraction and preparation procedures for the successful use (precision and accuracy) of the Tepnel Biosystems kit.

Table 5 – Detection of modified soybean proteins by Tepnel Biosystems and Elisa Systems soy test kits

Soy protein modification	Tepnel Biosystems kit		Elisa Systems kit	
	Observed ^a conc. (CV) μg/mL (%)	Deviation from control (%)	Observed conc. (CV) μg/mL (%)	Deviation from control (%)
Hydrolysis				
Nonhydrolyzed SPI (control)	12.77 (10.54) ^a ^b	—	8.78 (2.58) ^a	—
Trypsin (0.5% w/w; 60 min)	13.37 (6.01) ^a	+ 4.7	8.64 (5.23) ^a	−1.6
Chymotrypsin (1.0% w/w; 60 min)	11.74 (5.38) ^a	−8.1	10.42 (7.80) ^a	+ 19.1
Papain (0.5% w/w; 60 min)	7.26 (2.27) ^b	−43.2	10.73 (2.39) ^a	+ 22.7
Bromelain (0.5% w/w; 60 min)	6.20 (5.87) ^b	−51.5	7.54 (6.17) ^a	−14.3
Glycation				
Glycated SPI Day 01 (Control)	79.60 (13.02) ^a	—	Not applicable	
Glycated SPI Day 03	38.07 (10.4) ^b	−52.2		
Glycated SPI Day 05	40.01 (1.72) ^b	−49.7		
Glycated SPI Day 07	39.16 (1.13) ^b	−50.8		
Glycated SPI Day 10	41.64 (2.95) ^b	−47.7		
Glycated SPI Day 14	38.57 (0.56) ^b	−51.6		
Denaturation (urea-DTT) and heating (modified protein extraction procedure)				
Denatured, heated, and renatured (control) ^c	37.98 (1.16) ^a	—	Not applicable	
Denatured, heated, and nonrenatured	28.54 (4.31) ^b	−13.3		
Nondenatured and heated	5.37 (3.67) ^c	−84.5		
Nondenatured and nonheated	4.17 (0.66) ^d	−89.5		

^aConcentrations were calculated with the Log-Log function.

^bDifferent letters indicate statistically significant differences among samples within the same column ($P < 0.05$). Data are means $\pm$ standard deviation of duplicates.

^cSample was treated as recommended by the kit manufacturer for the soy protein positive control.

Conclusions

The present investigation evaluated the performance of Tepnel Biosystems and Elisa Systems kits in detecting and quantifying soy proteins. The results indicate that these 2 commercial ELISA kits could be useful tools for allergen monitoring. Both kits exhibited high sensitivity. The determined LODs (2 and $<1 \mu\text{g/mL}$ for Tepnel Biosystems and Elisa Systems kits, respectively) were found to be lower than those claimed by the manufacturer. The Tepnel Biosystems kit demonstrated good precision and accuracy at most concentrations studied. Our studies, however, revealed some drawbacks related to food matrix interferences and specificity (cross-reactivity with chickpeas proteins). The Elisa Systems kit showed poor accuracy but had high sensitivity, good precision, and was simple and rapid to use. Due to its specificity to the soy trypsin inhibitor, its ability to detect other soy proteins or soy protein materials that have been significantly treated or altered through processes such as high temperature and/or pressure, fermentation, or hydrolysis might be compromised. More work is clearly required in order to validate these 2 commercial soy test kits.

This study provided further evidence that protein modification that occurs during food processing, such as hydrolysis or glycation, may hamper their detectability and lead to inappropriate quantification by immunoassays. It also demonstrates the capability of immunoassays to detect chemical and structural modification of antigens. This information is useful and can contribute to the development of knowledge-based food processing strategies for controlling and manipulating food antigenicity.

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