

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Mustard in Foods

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ABSTRACT: Undeclared mustard residues in food products could trigger allergic reactions in mustard-allergic consumers. Our objective was to develop and validate a sandwich-type ELISA for the detection of mustard residues in foods. A mixture of yellow, brown, and oriental mustard seeds was used to immunize 3 rabbits and 1 sheep. Two mustard ELISAs were developed by utilizing the reciprocal combination of rabbit and sheep polyclonal antimustard sera as the capture and detector reagents. Binding was visualized by addition of rabbit antiship or goat antirabbit IgG antibody labeled with alkaline phosphatase and subsequent addition of substrate. The optimized ELISAs have limits of quantification (LOQ) of 1 and 3 ppm (μg of ground, whole mustard seeds/mL) for the sheep capture and rabbit capture formats, respectively. Only rapeseed cross-reacted in the rabbit and sheep capture mustard ELISAs at a level equivalent to 12300 and 16900 ppm of mustard. The mean percent recovery for cooked frankfurters spiked with 0 to 1000 ppm mustard flour was $95.3\% \pm 10.7\%$. A limited retail survey of 29 foods revealed that, of 15 samples having mustard declared on the ingredient list, 2 baked bean products contained no detectable mustard, possibly owing to a decrease in extractability and detectability of mustard proteins after subjecting to thermal processing. For the remaining 14 samples without mustard declared on the label, 3 samples contained detectable mustard, presumably due to the labeling of mustard as "spice" or inadvertent cross-contamination. This sandwich-type ELISA can serve as a powerful tool for food manufacturers and regulatory agencies to detect and quantify mustard residues in processed foods.

Keywords: allergen control, detection, ELISA, mustard allergy, validation

Introduction

Mustard belongs to the family of Brassicaceae (Cruciferae), which also includes other edible crops and oilseeds, including radish, rutabaga, cabbage, broccoli, turnip, watercress, horseradish, and rapeseed (Figueroa and others 2005). There are 3 types of mustard seeds produced worldwide: pale yellow or white mustard (*Brassica/Sinapis alba* or *B. hirta* Moench), brown and oriental mustard (*Brassica juncea* [L.] Cosson), and black or dark brown mustard (*Brassica nigra* [L.] Coch). Yellow, brown, and oriental mustard seeds are used predominantly in the United States or Europe, while black mustard seeds are not as popular due to difficulties in harvesting (Uhl 2000).

Mustard seed is used in a variety of food products to enhance flavor and nutritional values. Common foods containing mustard seeds include pickled products, processed meats, seasoning mixes, salad dressings, sauces, and condiments. Mustard oil is also widely used as an edible oil in India and as flavoring agent. Foods formulated with mustard seed are expected to increase in popularity in the future due to its sensory attributes, its high protein content, and its functional properties. However, the widespread use of mustard in foods has raised concerns as mustard can cause IgE-mediated allergic reactions in sensitive individuals, including occasionally severe reactions such as anaphylactic shock (Panconesi and others 1980; Winndström and Johansson 1986; Monreal and others 1992; Jorro and others 1995; Kanny and others 1995). The prevalence of mustard allergies appears to be on the rise, particularly among children (Rancé 2003). In France, mustard is ranked as the 4th most im-

portant food allergen for children (Rancé and others 1999). Mustard allergy is common only in France and Spain for unknown reasons (André and others 1994; Castillo and others 1996; Rancé and others 2000; Morisset and others 2003a; Figueroa and others 2005). Despite the high consumption of mustard elsewhere, allergy to mustard is relatively uncommon in other geographic regions. Currently, mustard is included in the list of 14 allergenic foods that must be declared on food labels of prepackaged foods in the EU (EC 2006).

No effective preventive treatments exist for mustard allergy at present (Schöll and Jensen-Jarolim 2004). Hence, susceptible individuals are required to strictly avoid mustard-containing foods within their diet (Rancé 2003; Sampson 2004). However, the presence of mustard allergens in food may not be discernable when residues are present due to processing errors such as unintentional cross-contamination during processing and product mislabeling issues (Taylor and Hefle 2005). Moreover, the presence of mustard is often designated only by the name "spices" on food labels in certain countries, a term that may or may not indicate the presence of mustard in the formulation. Developing an analytical method for the determination of mustard content in foods is therefore crucial for detecting inadvertent contamination during manufacturing processes.

ELISA is the method of choice for the detection of trace amounts of mustard in complex food matrices as it is simple, sensitive, specific, and reproducible (Taylor and Nordlee 1996). At present, only 1 ELISA method capable of detecting mustard has been published, but the use of this method was confined to the quantification of mustard protein in mustard seed oil (Koppelman and others 2007). In addition, a simple and rapid mustard ELISA test kit has recently been commercialized by ELISA Systems Pty. Ltd (Queensland, Australia). The test kit has a limit of quantification of 1 ppm mustard seed proteins, and it is capable of detecting mustard seed

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proteins from different species, including white or yellow, black, and brown mustard. The objective of this study was to develop a sensitive and specific sandwich-type ELISA for the detection of mustard residues in a wide range of foods. This assay is conceived to be practical for the food industry and regulatory agencies to monitor the safety of finished products and to ensure compliance with labeling regulations.

Materials and Methods

Polyclonal antisera production

Polyclonal antisera against mustard were developed at Lampire Biological Laboratories Inc. (Pipersville, Pa., U.S.A.). The mustard immunogen used to immunize 3 New Zealand rabbits and 1 sheep was composed of yellow (Minn-Dak Growers, Grand Forks, N.Dak., U.S.A.; Montana Specialty Mills, Great Falls, Mont., U.S.A.; Penzeys Spices, Brookfield Wis., U.S.A.; The Great American Spice Co., Fort Wayne, Ind., U.S.A.; Wisconsin Spice Inc., Berlin, Wis., U.S.A.), brown (Minn-Dak Growers; Montana Specialty Mills; Penzeys Spices; The Spice House, Evanston, Ill., U.S.A.; Wisconsin Spice Inc.), and oriental (Minn-Dak Growers; The Spice House; Wisconsin Spice Inc.) mustard seeds that were ground (6850 Freezer/Mill[®], SPEX CertiPrep, Metuchen, N.J., U.S.A.) and mixed in equal portions by weight. The mustard immunogen was reconstituted with PBS to 1 mg/mL at Lampire Biological Laboratories, Inc. For initial immunization, each rabbit was injected subcutaneously at 2 different sites with 25 μ g of protein emulsified with TiterMax Classic adjuvant (Sigma-Aldrich, St. Louis, Mo., U.S.A.) (200 μ L of 50:50 TiterMax to mustard immunogen solution at each site), whereas the sheep was injected intramuscularly at 4 different sites with 100 μ g of protein emulsified with TiterMax Classic adjuvant (250 μ L of 50:50 TiterMax to mustard immunogen solution at each site). For both the rabbits and the sheep, booster injections were administered at 28-d intervals after the initial immunization. The dose of immunogen–adjuvant mixture and the route of injection remained the same as the initial immunization with the exception that 2 boosts with Freund's Incomplete Adjuvant (FIA) were given between the TiterMax boosts. A total of 4 booster injections were given to each animal. Serum was collected at 10 and 20 d postbooster injections. The specificity and titer of the antisera were monitored throughout the immunization period using a noncompetitive ELISA as described by Hefle and others (2001), with the following modifications. Soluble proteins from the mustard immunogen were extracted and the protein content was determined by the Lowry method (Lowry and others 1951). Microtiter plates (NUNC-Immuno[™] MaxiSorp[™] 96-MicroWell[™] plates, Nalgel Nunc Intl., Rochester, N.Y., U.S.A.) were then coated with 1 μ g/mL of the soluble mustard proteins. All phosphate-buffered saline (PBS) solutions were adjusted to 0.025 M (0.005 M NaH₂PO₄, 0.02 M Na₂HPO₄, 0.85% NaCl, pH 7.4), and the incubation times after the addition of serially diluted antisera and the conjugate were changed to 2 h. In addition, the conjugate buffer, containing 0.025 M PBS, 0.5% bovine serum albumin (w/v) (RIA grade, Sigma Chemical Co.) and 0.2% Tween 20 (v/v), was used as the wash buffer throughout the assay. Titer was defined as the log reciprocal of the midlinear portion of the titration curve when 1 μ g/mL of soluble mustard proteins is coated onto the microtiter plate (Hefle and Lambrecht 2004). Antisera with titer values $\geq$ 10000 were pooled according to species and stored at -20°C for use in developing the mustard ELISA. The protein content of the pooled sera was determined by the Lowry method (Lowry and others 1951).

Standard and sample extractions

Commercial food samples used for the analysis were purchased from local grocery stores (Lincoln, Nebr., U.S.A.). Solid food samples were ground using an Osterizer[®] blender (Sunbeam Corp., Delray Beach, Fla., U.S.A.) or a commercial food processor (KPF600 Food Processor, KitchenAid, St. Joseph, Mo., U.S.A.), whichever was applicable. Acidic food samples were adjusted to a pH of 6.0 to 8.0 with 1.0 N NaOH prior to extraction.

Mustard immunogen, 0.01 M PBS, pH 7.4, containing either 1000 ppm mustard immunogen or mustard flour, cooked frankfurters containing 1000 ppm mustard flour, and food samples for the reference food standards and cross-reactivity study were extracted 1:10 (w/v) in 0.01 M PBS (0.002 M NaH₂PO₄, 0.008 M Na₂HPO₄, 0.85% NaCl, 0.02% NaN₃, pH 7.4) using gentle rocking (Labquake[™] Shaker, Barnstead/Thermolyne Corp., Dubuque, Iowa, U.S.A.), overnight at 25 $^{\circ}\text{C}$. On the other hand, frankfurter model foods and retail food samples were extracted 1:10 (w/v) in 0.01 M PBS, pH 7.4, at 140 rpm in a shaking water bath (Julabo SW22, Julabo USA Inc., Allentown, Pa., U.S.A.) at 60 $^{\circ}\text{C}$ for 2 h. Extracts were then centrifuged at 3612 $\times g$ (4100 rpm) in a tabletop centrifuge (Sorvall[®] Legend[™] RT, Kendro Laboratory Products, Newton, Conn., U.S.A.) at 10 $^{\circ}\text{C}$ for 30 min. The clarified solutions were divided into aliquots and stored at -20°C until analyzed. Protein content of the solutions was determined by the Lowry method (Lowry and others 1951).

Mustard standards

A series of standards with total soluble mustard proteins at levels ranging from 0 to 100 ppm were prepared by serially diluting the mustard immunogen extracts 1:3 (v/v) in 0.01 M PBS, pH 7.4. To construct a standard curve based on whole mustard, either 0.01 M PBS, pH 7.4 or cooked frankfurters were spiked with 1000 ppm mustard immunogen or mustard flour (w/w), and subsequently extracted and serially diluted in 0.01 M PBS, pH 7.4 or cooked frankfurter extracts at a volume ratio of 1:3 to achieve levels ranging from 0 to 1000 ppm of mustard immunogen or mustard flour. The standard curve in the cooked frankfurter extract was used only for the frankfurter model food experiments.

ELISA

Two sandwich-type ELISAs were developed utilizing capture antibodies from rabbit and sheep. The sandwich ELISA was developed according to a previously published protocol (Hefle and Lambrecht 2004) with minor modifications as described subsequently.

Rabbit capture mustard ELISA. Microtiter plates were coated with 100 μ L/well of rabbit antimustard total serum protein at 5 μ g/mL in coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, and 0.02% NaN₃, pH 9.6). The bound antigen was detected by adding 100 μ L/well of sheep antimustard total serum protein diluted to 10 μ g/mL in conjugate buffer (0.01 M PBS containing 0.1% bovine serum albumin [w/v], pH 7.4), followed by incubation with 100 μ L/well of rabbit antisheep IgG labeled with alkaline phosphatase (Immunopure[®], Pierce Biotechnology Inc., Rockford, Ill., U.S.A.) at 1:5000 (v/v) in conjugate buffer. The color produced after the addition of substrate was measured at 410 nm. Standard curves were generated using GraphPad Prism[®] Software (GraphPad Software Inc., San Diego, Calif., U.S.A.).

Sheep capture mustard ELISA. The sheep capture mustard ELISA was developed using a protocol similar to the rabbit capture mustard ELISA with the following modifications. Sheep antimustard total serum protein was diluted to 25 μ g/mL in coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, and 0.02% NaN₃, pH 9.6), and 100 μ L/well of this diluted solution was used to coat the microtiter

plates. The bound antigen was detected by adding 100 μ L/well of rabbit antimustard total serum protein diluted to 20 μ g/mL in conjugate buffer (0.01 M PBS containing 0.1% bovine serum albumin [w/v], pH 7.4). The bound rabbit antimustard sera was then detected by the addition of 100 μ L/well of goat antirabbit IgG labeled with alkaline phosphatase at 1:5000 (v/v) in conjugate buffer.

Reference food standards

Raw chicken breast was baked in a conventional oven at 350 °F for 45 min, and subsequently ground using a commercial food processor (KPF600 Food Processor) prior to extraction. Since meats and salad dressings are known to contain mustard and may be at risk for mustard contamination, baked chicken breast, raw hamburger, and French salad dressing were selected as reference food standards. These reference food standards were prepared by adding mustard immunogen extracts into extracts of the reference food standards to yield 100 ppm of soluble mustard proteins, followed by serial dilutions at 1:3 (v/v) with each of the corresponding control food extracts to obtain a set of standards containing 0 to 100 ppm mustard proteins. The food extracts spiked with mustard protein were then analyzed in the rabbit capture mustard ELISA.

Cross-reactivity studies

Foods that are botanically related to mustard and food ingredients that are present in the processed foods containing mustard were selected for the cross-reactivity studies. After extraction of the food samples, all extracts except rapeseed were diluted in 0.01 M PBS, pH 7.4, and then analyzed in both the rabbit capture mustard ELISA and sheep capture mustard ELISA on a protein content (10, 1, and 0.1 μ g/mL) and dilution (1:10, 1:100, 1:1000 [v/v]) basis. Protein content was determined by the Lowry method (Lowry and others 1951). Rapeseed was diluted from 1:10 to 1:1000000 (v/v) with 0.01 M PBS, pH 7.4, and then tested in both of the mustard ELISAs.

Frankfurter model foods preparations

The basic formulation of the frankfurters consisted of 8% water, 2% salt, 1% sugar, 0.0156% nitrite salt (93.75% sodium chloride with 6.25% sodium nitrite), and appropriate amounts of meat block (90% lean ground beef) to make up a 1.5-kg batch. Cure solution was prepared by mixing all dry ingredients with water listed previously. Subsequently, the cure solution was added evenly to the meat blocks that were placed in a food cutter (model 84181D, Hobart, Troy, Ohio, U.S.A.) and chopped under high speed for 3 min. The finished batter was immediately stuffed into 50-mL conical tubes (BD Falcon, Franklin Lakes, N.J., U.S.A.) and was cooked to an internal temperature of 71 °C in an 80 °C water bath (Julabo SW22). Frankfurters were weighed before and after cooking to determine the cooking yield. The cooked frankfurters were evaluated immediately, and any remainder was stored at -20 °C.

Mustard flour at 0, 1, 10, 50, 100, 500, and 1000 ppm (mg of mustard/kg of frankfurter) was introduced independently into the cure solution just prior to the addition to the uncooked meat block. Because the 1, 10, 50, and 100 ppm levels of mustard require minute amounts of mustard flour, a 10000 ppm mustard flour in salt mixture (1 g of mustard flour added to 99 g of salt) was prepared and then incorporated into the meat formulation to achieve the desired levels.

Frankfurter model foods and limited retail survey evaluations

Two frankfurter model foods and 2 retail foods from the same batch and lot number, respectively, were extracted. Each extract

was then analyzed in triplicate in 2 independent ELISA trials using the sheep capture mustard ELISA. The frankfurter model foods were also analyzed in a commercial mustard ELISA test kit (ELISA Systems Pty. Ltd.) by extracting the model foods in duplicate and testing each extract in duplicate in 2 separate trials according to the insert instructions.

Statistical analysis

The differences between the standard curves of reference food standards were performed using the *F* test (GraphPad Prism® Software). The percent recoveries of the frankfurter model food were compared for the sheep capture mustard ELISA and commercial test kit using Student's *t*-test with the Satterthwaite correction for unequal variance (SAS 9.1 software; SAS Inst. Inc., Cary, N.C., U.S.A.). A *P*-value of less than 0.05 was considered as significant.

Results and Discussion

Antibody production

The success in developing a specific and sensitive ELISA depends largely on the quality of the antibody produced; thus, the choice of the immunogen is particularly important. The reason for selecting the 3 varieties of mustard seeds was to acquire antisera capable of recognizing all varieties of mustard seeds that are commonly consumed and used in processed foods. No pre-existing antibodies were detectable in any of the animals before immunization (data not shown). Pooled rabbit and sheep antiserum with titers of 60300 ($10^{4.78}$) and 59000 ($10^{4.77}$), respectively, was used in developing the mustard ELISA.

ELISA

Lower limit of quantification (LOQ) is defined as the lowest level of mustard that the ELISA can detect with acceptable accuracy and precision. The ELISAs have limits of quantitation (LOQ) of 3 and 1 ppm (μ g of ground, whole mustard seeds/mL), respectively, for the rabbit capture and the sheep capture formats (Figure 1). These LOQs are reasonable when compared to the threshold dose for mustard determined in oral challenges of mustard-allergic subjects. Although a small number of mustard-allergic individuals have been subjected to low-dose challenge trials, the most sensitive individuals developed an allergic reaction to 1 mg of mustard seed, which equates to 20 ppm of mustard in a typical food serving size of 50 g (Taylor and others 2002). Furthermore, minimal eliciting doses for other allergenic foods, including peanuts, milk, and eggs, can be as low as 0.5 to 2 mg in the most sensitive individuals (Bindslev-Jensen and others 2002; Taylor and others 2002; Morisset and others 2003b).

Mustard proteins were effectively detected with the rabbit capture mustard ELISA in 3 different food matrices—hamburger, baked chicken, and French salad dressing (Figure 2). However, the slope of the standard curves was statistically different only for the PBS and hamburger curves ($P = 0.023$). Furthermore, the maximal absorbance of the hamburger standard curve was distinctly lower than for the PBS or other food standard curves. Therefore, with the possible exception of hamburger, this mustard ELISA was not subject to matrix interference that can occur with the analysis of some foods and food components (Hefle 1995; Keck-Gassenmeier and others 1999). The detection of mustard in hamburger is still clearly achieved so the matrix interference is modest.

Cross-reactivity

Ingredients commonly found in foods in association with mustard were analyzed to further explore possible matrix effects and

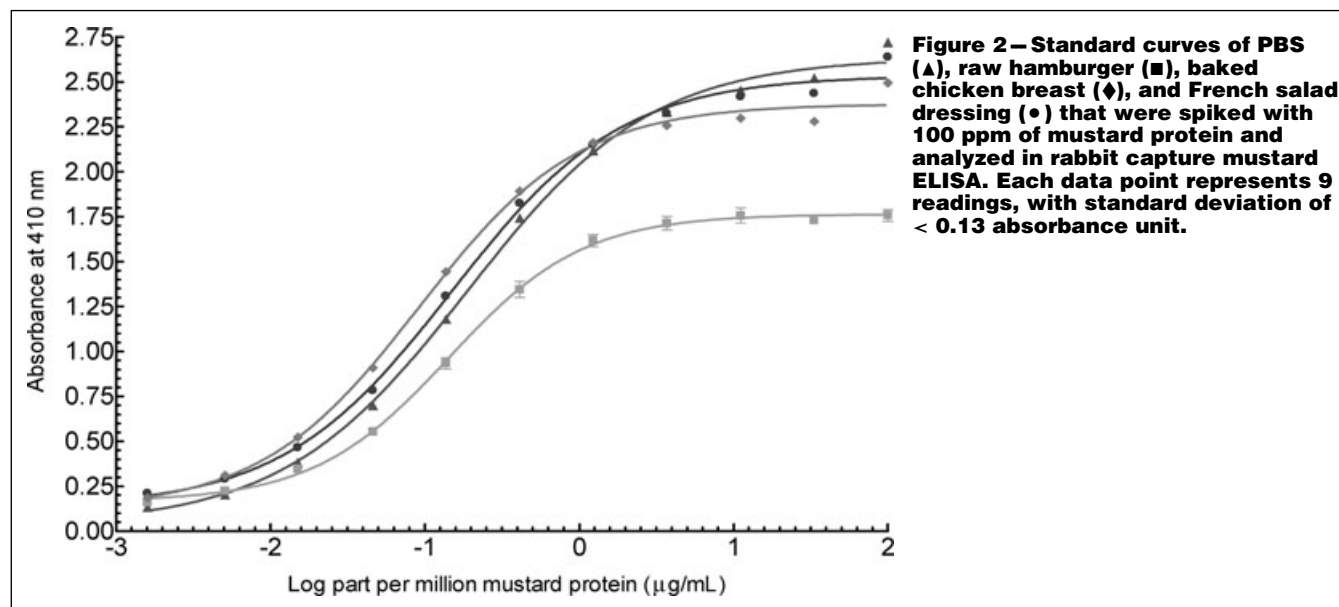
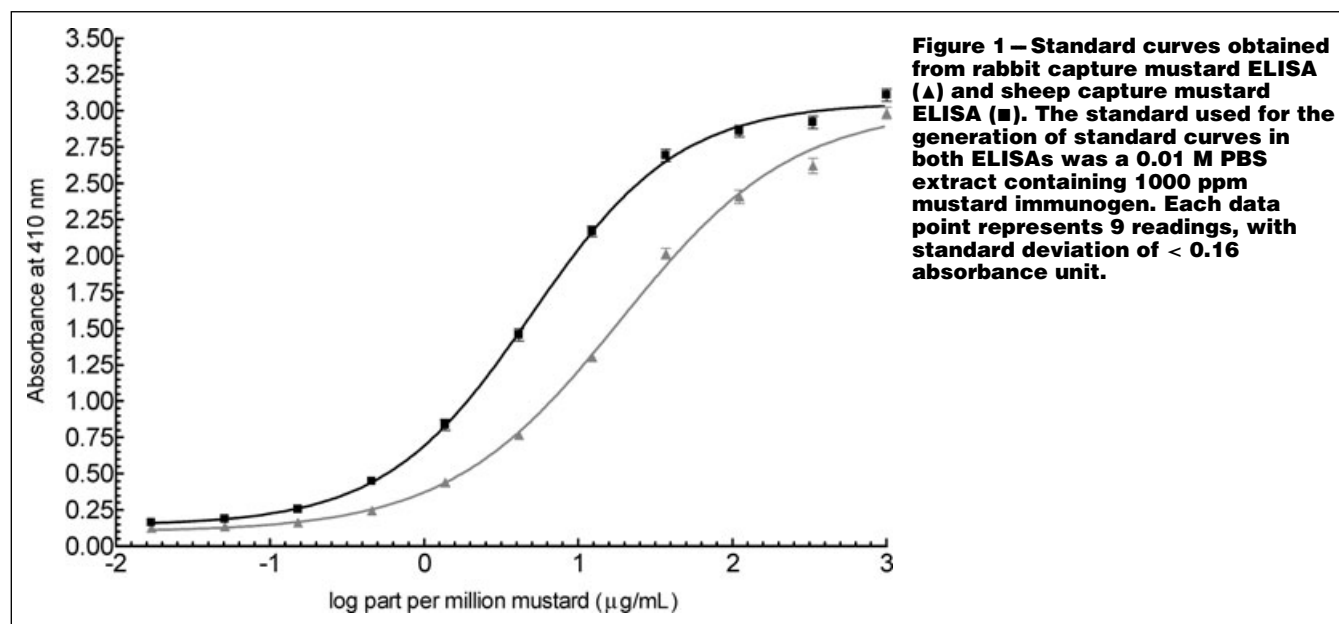
to evaluate the specificity of the antisera. These mustard ELISAs are specific for the detection of mustard residues with the exception of rapeseed. A total of 109 common foods and food ingredients, including foods derived from the Brassicacea family, were evaluated and, with the exception of rapeseed, showed no reactivity in these ELISAs, with responses equivalent to < 1 ppm of mustard (Table 1). A rapeseed sample containing 12 mg/mL of protein displayed strong cross-reactivity in both the rabbit capture (12300 ppm) and the sheep capture (16900 ppm) mustard ELISAs. The cross-reactivity with rapeseed was expected as it has been reported that the 2S albumin seed storage proteins in mustard seeds shared 94% sequence similarity with 2S albumins from rapeseed (Mon-salve and others 1997, 2001). Because rapeseed and mustard are closely related plants, similarities would also be expected between other proteins in the 2 species. The sequence similarity allows for binding of the mustard-specific antisera to the cross-reactive epitopes on these proteins (Koppelman 2006). This cross-reactivity between mustard and rapeseed will generally not affect mustard detection in foods because rapeseed is used mainly in the production

of canola oil or for animal feed; however, caution must be exercised when detecting mustard in foods that may have come into contact with rapeseed.

Frankfurter model food

Mustard is a common ingredient in processed meat products. Therefore, the ability of the sandwich ELISA to accurately quantify the levels of mustard in these foods is critically important. Thus, frankfurter was chosen as a model food and was produced under conditions that closely resemble the food industry manufacturing process to evaluate the performance of the assay in terms of extraction efficiency, matrix interference, and true recovery of the mustard residues after the mustard was incorporated in the initial formulation (as opposed to spiking) and subjected to processing conditions.

The mean percent recovery for mustard from the cooked frankfurters estimated using the sheep capture mustard ELISA was $95.3\% \pm 10.7\%$ (Table 2). The recovery was not corrected for the cooking loss as the weight loss was negligible (< 6%). The mean



percent recovery for mustard using the commercial mustard ELISA kit was $15.3\% \pm 2.8\%$ (Table 3). The discrepancy in the reported percent recoveries between the 2 ELISA detection methods could be explained by the differences in the measurements. The calculation in sheep capture mustard ELISA is based on whole mustard (mg of mustard/kg of frankfurters), whereas the commercial ELISA kit is based on soluble mustard proteins (mg of mustard protein/kg of frankfurters). If the percent recoveries in the commercial test kit

were multiplied by a correction factor of 5 as the soluble mustard proteins in mustard seed are estimated at 20%, the percent recoveries in the commercial test kit and the sheep capture mustard ELISA were not significantly different ($P = 0.348$).

Limited retail survey

To validate the performance and applicability of the sandwich-type mustard ELISA, a limited retail survey of 29 commercial

Table 1 – List of foods and food ingredients showing no cross-reactivity in the mustard ELISA.

Brassicaceae	Dairy products	Sugars and sweeteners
Bok choy	Buttermilk powder	Sugar (brown, light brown, white)
Broccoli	Casein	Corn syrup (high fructose, light, solids)
Brussel sprout	Nonfat dry milk	Maltodextrin
Cabbage	Skim milk powder	Molasses (light and dark)
Cauliflower	Whey powder	Sorbitol
Chinese kale		
Collard	Legumes	Preservatives
Horseradish powder	Peanut butter	Calcium disodium EDTA
Kale	Satay sauce	Citric acid
Kohlrabi	Soy sauce (acid hydrolyzed and naturally fermented)	Lactic acid
Mustard green		Nitrite (potassium and sodium)
Radish		Sodium benzoate
Rutabaga	Tree nuts	Sodium erythorbate
Turnips	Almond	
Wasabi paste	Cashew	Poultry and meat
Watercress	Hazelnut	Chicken (raw)
	Macadamia	Hamburger (raw)
Cereals	Pecan	Pork (raw)
Barley flour	Walnut	Turkey (raw)
Corn meal (yellow)		
Corn starch	Herbs and spices	Other food ingredients
Puffed rice	Allspice	Anchovies
Rye flour	Cinnamon	Chardonnay
Wheat flour (all-purpose)	Cloves	Honey
Wheat starch	Garlic (clove, powder, minced)	Ketchup
	Curry powder	Lemon juice
Color additives	Coriander seed	Maltol
Annatto	Cumin (ground and seed)	Merlot
Blue 1	Ginger powder	Monosodium glutamate
Caramel color	Onion (powder and minced)	Propyl gallate
Paprika	Parsley flakes	Tomato paste
Yellow 5	Pepper (black and white)	Spray-dried egg white
Yellow 6	Salt	Spray-dried whole egg
Oils	Tarragon leaves	Vinegar (apple cider, balsamic, distilled, red wine)
Canola oil	Tamarind	Yeast (active-dried and brewer)
Extra virgin olive oil	Turmeric	
Peanut oil		
Soybean oil		
Vegetable oil		

Table 2 – Recovery and percent recovery of mustard residues from the manufactured model cooked frankfurters, as determined by the sheep capture mustard ELISA.^a

Spike levels	Levels detected in ELISA (ppm ^b mustard)				Average ^c (ppm mustard)	% Recovery ^c
	Extract A		Extract B			
	Trial 1	Trial 2	Trial 1	Trial 2		
0 ppm	BLQ ^d	BLQ	BLQ	BLQ	NA ^e	NA
1 ppm	BLQ	0.8	0.8	0.8	0.8 ± 0	80.0 ± 0
10 ppm	10.3	12.2	10.1	10.5	10.8 ± 1.0	108 ± 10
50 ppm	46.1	45.2	45.0	43.4	44.9 ± 1.1	89.9 ± 2.2
100 ppm	91.6	91.9	88.5	95.2	91.8 ± 2.8	91.8 ± 2.8
500 ppm	496	469	455	475	474 ± 17	94.8 ± 3.4
1000 ppm	1050	1080	1020	1120	1068 ± 43	107 ± 4.3

^aStandard used for the generation of standard curve in ELISA was cooked frankfurter extracts containing 1000 ppm mustard flour.

^bppm = parts per million (mg of mustard per kg).

^cData are mean $\pm$ SD ($n = 12$ replicates).

^dBLQ = below limit of quantification, < 1.0 ppm.

^eNA = not applicable.

food samples were analyzed for the presence of mustard. Fifteen samples had mustard declared on the ingredient list, while 14 samples did not. Food samples were considered to be positive if the mustard levels detected were greater than the limit of quantification (LOQ), which is 1.0 ppm (μg mustard/g). Of the 15 samples containing mustard, 13 samples had detectable mustard content in the range between 2.3 and 59300 ppm (Table 4). The 2 exceptions were canned baked bean products where mustard proteins were not detected despite the fact that mustard was stated on the ingredient list. This is probably due to the heat treatment involved in the canning process that likely reduces the solubility and extractability of the mustard proteins. Thus, the sheep capture mustard ELISA is apparently not suitable for the analysis of mustard residues in food products that have been retorted. The 14 products without mustard declared on the label were selected because other related

products do contain mustard, so cross-contact is possible. Of the 14 samples without mustard listed, only 3 samples, 2 sausages and 1 salad dressing, contained detectable mustard. Two of 3 sausage samples contained detectable mustard. These products often include mustard under generic terms such as "spices" or "natural flavorings" on the food labels. Additionally, the Food Allergen Labeling and Consumer Protection Act (FALCPA) does not mandate the declaration of ingredients derived from mustard on food labels in the United States. One salad dressing sample (ranch dressing) showed some weak binding where the mustard level was quantified at 1.1 ppm.

Conclusions

The sandwich-type mustard ELISA developed in this study showed potential for use as an analytical tool for the food

Table 3 – Recovery and percent recovery of mustard proteins from the manufactured model cooked frankfurters, as determined by the mustard ELISA test kit from ELISA Systems.

Spike levels	Levels detected in ELISA (ppm ^a mustard protein)				Average ^b (ppm mustard protein)	% Recovery ^b
	Extract A		Extract B			
	Trial 1	Trial 2	Trial 1	Trial 2		
0 ppm	0.0	0.0	0.0	0.0	0.0	NA ^c
1 ppm	0.2	0.1	0.2	0.2	0.2 ± 0.05	20.0 ± 5.0
10 ppm	1.4	1.2	1.5	1.3	1.4 ± 0.1	14.0 ± 1.0
50 ppm	8.2	7.2	9.0	9.0	8.4 ± 0.9	16.8 ± 1.8
100 ppm	13.6	13.0	14.1	11.6	13.1 ± 1.1	13.1 ± 1.1
500 ppm	76.6	67.6	81.3	82.9	77.1 ± 6.9	15.4 ± 1.4
1000 ppm	123.2	120.8	130.9	129.6	126.1 ± 4.9	12.6 ± 0.5

^appm = parts per million (mg of soluble mustard protein per kg).

^bData are mean $\pm$ SD (n = 12 replicates).

^cNA = not applicable.

Table 4 – A limited retail survey of 5 categories of food items for mustard residues by sheep capture mustard ELISA.^a

Food product	Brand	Description	D ^b	Mustard (ppm ^c)
Baked beans	A	Chili beans	–	BLQ ^d
	B	Pork & beans	–	BLQ
	C	Country style baked beans	+	BLQ
	D	Original style baked beans	+	BLQ
Salad dressing	E	Sweet Honey Catalina	–	BLQ
	F	Italian	–	BLQ
	G	Ranch	–	1.1 $\pm$ 0.4
	E	Coleslaw	+	3507 $\pm$ 259
	E	Creamy poppy seed	+	328 $\pm$ 63
	H	Thousand island	+	5882 $\pm$ 950
	I	Honey dijon	+	317 $\pm$ 53
Sauce and marinade	J	Seafood cocktail sauce	–	BLQ
	E	Cocktail sauce	–	BLQ
	K	Pasta sauce	–	BLQ
	L	Italian sauce	–	BLQ
	M	Basting sauce	+	38.1 $\pm$ 8.4
	N	Honey & dijon marinade	+	29575 $\pm$ 1714
	E	Barbeque sauce	+	2.3 $\pm$ 0.3
	E	Tartar sauce	+	270 $\pm$ 53
Seasoning mix	N	Chili seasoning mix	–	BLQ
	O	Swiss steak mix	–	BLQ
	N	Meat loaf mix	+	48400 $\pm$ 12271
	O	Hollandaise sauce mix	+	59300 $\pm$ 10350
Sausage	P	Summer sausage	–	16100 $\pm$ 4529
	P	Lite smoked sausage	–	18000 $\pm$ 3932
	Q	Polish sausage	–	BLQ
	R	Summer sausage	+	30604 $\pm$ 10594
	R	Polish sausage	+	14400 $\pm$ 3000
	S	Smoked turkey sausage	+	145 $\pm$ 31

^aStandard used for the generation of standard curve in ELISA was 0.01 M PBS extracts containing 1000 ppm mustard flour.

^bD = declaration of mustard as an ingredient on food labels: –, nondeclared; +, declared.

^cppm = parts per million (mg of mustard per kg). Data are mean $\pm$ SD (n = 12 replicates).

^dBLQ = below limit of quantification, < 1.0 ppm.

industry and the regulatory agencies to verify the effectiveness of allergen control strategies, to investigate consumer complaints, and to ensure compliance with labeling guidelines. However, additional research is required to improve the detection of mustard in retorted products. Because different types of matrices and processing conditions have an effect on mustard detection, other foods should be evaluated before true applications can be ascertained. All efforts made in ensuring the safety of foods by using the mustard ELISA will ultimately lead to an improvement of the quality of life of mustard-allergic consumers.

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References

- André F, André C, Colin L, Cacaraci F, Cavagna S. 1994. Role of new allergens and of allergens consumption in the increased incidence of food sensitizations in France. *Toxicology* 93(1):77–83.
- Bindslev-Jensen C, Briggs D, Osterballe M. 2002. Can we determine a threshold level for allergenic foods by statistical analysis of published data in the literature? *Allergy* 57(8):741–6.
- Castillo R, Delgado J, Quirarte J, Blanco C, Carrillo T. 1996. Food hypersensitivity among adult patients: epidemiological and clinical aspects. *Allergol Immunopathol* 24(3):93–7.
- [EC] European Commission. 2006. Commission Directive 2006/142/EC of 22 December 2006 amending Annex IIIa of Directive 2003/13/EC of the European Parliament and of the council listing the ingredients which must under all circumstances appear on the labelling of foodstuffs. European Commission. Available from: <http://ec.europa.eu/>. Accessed Oct 22, 2007.
- Figuerola J, Blanco C, Dumpiérrez AG, Almeida L, Ortega N, Castillo R, Navarro L, Pérez E, Gallego MD, Carrillo LT. 2005. Mustard allergy confirmed by double-blind placebo-controlled food challenges: clinical features and cross-reactivity with mugwort pollen and plant-derived foods. *Allergy* 60(1):48–55.
- Hefle SL. 1995. Immunoassay fundamentals. *Food Tech* 49(2):102–7.
- Hefle SL, Lambrecht DM. 2004. Validated sandwich enzyme-linked immunosorbent assay for casein and its application to retail and milk-allergic complaint foods. *J Food Prot* 67(9):1933–8.
- Hefle SL, Jeanniton E, Taylor SL. 2001. Development of a sandwich enzyme-linked immunosorbent assay for the detection of egg residues in processed foods. *J Food Prot* 64(11):1812–6.
- Jorro G, Morales C, Brasó JV, Peláez A. 1995. Mustard allergy: three cases of systemic reaction to ingestion of mustard sauce. *J Invest Allergol Clin Immunol* 5(1):54–6.
- Kanny G, Fremont S, Talhouarne G, Nicolas JP, Moneret-Vautrin DA. 1995. Anaphylaxis to mustard as a masked allergen in “chicken dips.” *Ann Allergy Asthma Immunol* 75(4):340–2.
- Keck-Gassenmeier B, Bénét S, Rosa C, Hischenhuber C. 1999. Determination of peanut traces in food by a commercially-available ELISA test. *Food Agric Immunol* 11(3):243–50.
- Koppelman S. 2006. Detecting tree nuts and seeds in food. In: Koppelman SJ, Hefle SL, editors. *Detecting allergens in foods*. Boca Raton, Fla.: CRC Press. p 201–18.
- Koppelman SJ, Vlooswijk R, Bottger G, Van Duijn G, Van der Schaft P, Dekker J, Van Bergen H. 2007. Development of an enzyme-linked immunosorbent assay method to detect mustard protein in mustard seed oil. *J Food Prot* 70(1):179–83.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193(1):265–75.
- Monreal P, Botey J, Pena M, Marin A, Eseverri JL. 1992. Mustard allergy: two anaphylactic reactions to ingestion of mustard sauce. *Ann Allergy* 69(4):317–20.
- Monsalve RI, González De La Peña MA, López-Otín C, Fiandor A, Fernández C, Villalba M, Rodríguez R. 1997. Detection, isolation and complete amino acid sequence of an aeroallergenic protein from rapeseed flour. *Clin Exp Allergy* 27(7):833–41.
- Monsalve RI, Villalba M, Rodríguez R. 2001. Allergy to mustard seeds: the importance of 2S albumins as food allergens. *Internet Symp Food Allergens* 3(2):57–69.
- Morisset M, Moneret-Vautrin DA, Maadi F, Frémont S, Guénard L, Croizier A, Kanny G. 2003a. Prospective study of mustard allergy: first study with double-blind placebo-controlled food challenge trials (24 cases). *Allergy* 58(4):295–9.
- Morisset M, Moneret-Vautrin DA, Kanny G, Guénard L, Beaudouin E, Flabbée J, Hatahet R. 2003b. Thresholds of clinical reactivity to milk, egg, peanut and sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or single-blind placebo-controlled oral challenges. *Clin Exp Allergy* 33(8):1046–51.
- Panconesi E, Sertoli A, Fabbri P, Giorgini S, Spallanzani P. 1980. Anaphylactic shock from mustard after ingestion of pizza. *Contact Derm* 6(4):294–5.
- Rancé F. 2003. Mustard allergy as a new food allergy. *Allergy* 58(4):287–8.
- Rancé F, Kanny G, Dutau G, Moneret-Vautrin DA. 1999. Food hypersensitivity in children: clinical aspects and distribution of allergens. *Pediatr Allergy Immunol* 10(1):33–8.
- Rancé F, Dutau G, Abbal M. 2000. Mustard allergy in children. *Allergy* 55(5):496–500.
- Sampson HA. 2004. Update on food allergy. *J Allergy Clin Immunol* 113(5):805–19.
- Schöll I, Jensen-Jarolim E. 2004. Allergenic potency of spices: hot, medium hot, or very hot. *Int Arch Allergy Immunol* 135(3):247–61.
- Taylor SL, Nordlee JA. 1996. Detection of food allergens. *Food Tech* 50(5):231–8.
- Taylor SL, Hefle SL. 2005. Allergen control. *Food Tech* 59(2):40–3, 75.
- Taylor SL, Hefle SL, Bindslev-Jensen C, Bock SA, Burks AW, Christie L, Hill DJ, Host A, Hourihane JO, Lack G, Metcalfe DD, Moneret-Vautrin DA, Vadas PA, Rance F, Skrypec DJ, Trautman TA, Yman IM, Zeiger RS. 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *J Allergy Clin Immunol* 109(1):24–30.
- Uhl SR. 2000. *Handbook of spices, seasonings and flavorings*. 2nd ed. Lancaster, Pa.: Technomic Publishing Co. 352 p.
- Winndström L, Johansson SGO. 1986. IgE-mediated anaphylaxis to mustard. *Acta Derm Venereol* 66(1):70–1.