

FOOD COMPOSITION AND ADDITIVES

Immunochemical-Based Method for Detection of Hazelnut Proteins in Processed Foods

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A competitive enzyme-linked immunosorbent assay (ELISA) was developed to detect hazelnut by using polyclonal antibodies generated against a protein extract of roasted hazelnut. No cross-reactivity was observed in tests against 39 commodities, including many common allergens, tree nuts, and legumes. Hazelnut protein standard solutions at 0.45 ng/mL [inhibition concentration (IC₈₀) of the competitive test] were clearly identified by the ELISA. An extraction and quantification method was developed and optimized for chocolate, cookies, breakfast cereals, and ice cream, major food commodities likely to be cross-contaminated with undeclared hazelnut during food processing. No sample cleanup was required when extracts were diluted 10-fold. Recovery results were generated with blank matrixes spiked at 4 levels from 1 to 10 µg/g hazelnut protein. With the developed extraction and sample handling procedure, hazelnut proteins were recovered at 64–83% from chocolate and at 78–97% from other matrixes. A confirmatory technique was developed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western transfer. The developed methods were applied to a small market survey of chocolate products and allowed the identification of undeclared hazelnut in these products.

0.1–0.5% of hazelnut allergy among Europeans. Holzhauser and Vieths (5) reviewed several reported cases of severe allergic reactions caused by hazelnut. One piece of chocolate containing 0.2% of undeclared hazelnut caused an asthmatic attack in an allergic patient, and severe laryngeal edema was caused by a small fragment of hazelnut in a teaspoon of breakfast Muesli.

Hazelnuts are used in a wide variety of confectionery food products because of their nutritive value and taste. The amount of hazelnut included in a recipe is even considered a mark of quality. Several products containing hazelnut, such as cookies, Muesli bars, and chocolate spreads are manufactured using production lines on which other products that do not contain hazelnut are also formulated. Cross-contact may therefore occur, leading to undeclared amounts of hazelnut in commercial products.

Analytical methods are required to monitor these products to minimize the risk to highly sensitive consumers and to enforce labeling regulations concerning allergens in food products. Of the various analytical techniques developed to detect and quantify hazelnut in foods, some were meant to be quality control systems to check for product adulteration in hazelnut-containing products. Immuno-electrodiffusion allowed the quantification of 1–15% hazelnut in food products (6). A sandwich immunoassay recently detected hazelnut protein between 0.001 and 10% (5) and was applied with the corresponding extraction procedures to complex food matrixes, leading to recoveries of 67–132%. Koppelman et al. (4) reported a similar method that detected as little as 5 ng/mL hazelnut in solution, corresponding to 1 µg/g in food products.

This study describes a competitive enzyme-linked immunosorbent assay (ELISA) developed to detect and quantify undeclared hazelnut in processed food products. Newly generated hazelnut-specific polyclonal antibodies were characterized for their specificity to particular hazelnut proteins and for their selectivity to this commodity in comparison to other tree nuts and common allergenic proteins. An extraction procedure developed to be applied on major commodities of interest led to optimized recoveries in chocolate, cookies, ice cream, and breakfast cereals. The assay was later applied to

Food allergies are the cause of many health problems among an increasing number of individuals. Although peanut allergy is reportedly the most prevalent in the Western population (1–3), affecting 1–2% of all individuals, tree nuts such as hazelnuts constitute another important source of allergies and affect a substantial part of the population. Koppelman et al. (4) reported an estimated prevalence of

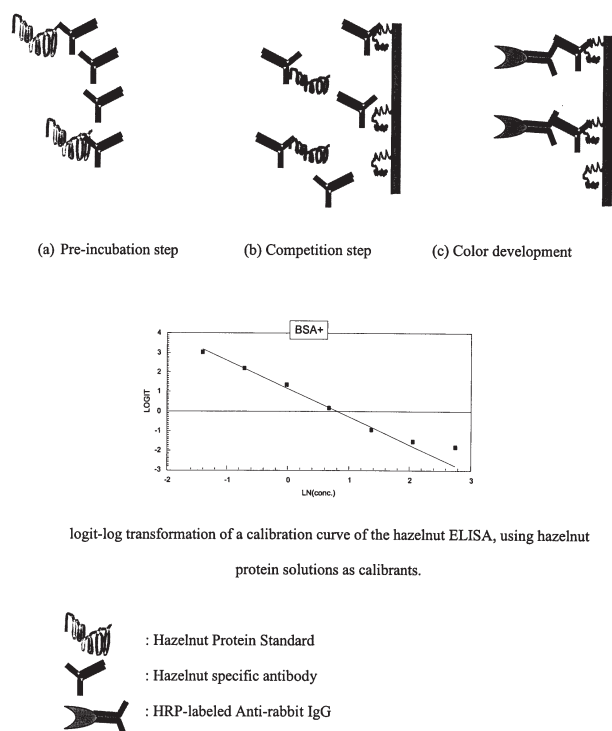


Figure 1. Assay format, conditions of use, and example of calibration curve.

chocolate samples collected as part of a small survey of commercial products and helped to identify undeclared hazelnut in foods. With a confirmation method based on electrophoretic techniques and Western transfer, hazelnut proteins were identified in samples screened positive by the ELISA. An interlaboratory validation of the ELISA methodology is under way in collaboration with the laboratories of the Canadian Food Inspection Agency prior to its implementation as an official testing methodology for monitoring undeclared hazelnut proteins in foods sold on the Canadian market.

Experimental

Reagents and Supplies

(a) *Bovine serum albumin (BSA; RIA grade), goat anti-rabbit IgG peroxidase conjugate (second antibody), Tween 20, Triton X-100, polyethylene glycol (PEG) with average molecular weight of 8000, and glycerol.*—Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

(b) *O-phenylenediamine dihydrochloride (OPD).*—Pierce Chemical Co. (Rockford, IL).

(c) *Freund's complete and incomplete adjuvants.*—Gibco (Grand Island, NY).

(d) *Immunolon 2 microtiter plates and different salt buffers.*—VWR Canlab (Mississauga, Ontario, Canada).

(e) *Dialysis tubing (25 mm diameter with 12 000–14 000 molecular weight cut-off).*—Fisher Scientific (Pittsburgh, PA).

(f) *Food samples.*—For cross-reactivity studies; either purchased locally or obtained as food composites used for a

total diet study on pesticides, and extracted in phosphate buffered saline (PBS).

(g) *Minigels for electrophoresis (1.0 mm by 10 wells).*—Novex (San Diego, CA).

(h) *Gel Code Blue stain reagent.*—Pierce.

(i) *Buffers.*—PBS, pH 7.4, contained 20mM NaH_2PO_4 and 140mM NaCl. Extraction buffer consisted of 0.1% (v/v) Tween 20, and 0.5% (v/v) Triton X-100 in PBS. Antibody diluent (BSA^+) consisted of 0.1% (w/v) BSA, 0.1% (v/v) Tween 20 and 3% (v/v) PEG in PBS. Washing buffer consisted of 0.1% (v/v) Tween 20 in PBS. Plate coating buffer consisted of 13mM Na_2CO_3 and 35mM NaHCO_3 at pH 9.6. Citrate buffer consisted of 51mM Na_2HPO_4 and 24mM citric acid at pH 5.0. The substrate consisted of 17.5 mg OPD in 25 mL citrate buffer with 10 μL 30% H_2O_2 added.

Instrumentation

(a) *Microplate washer.*—Bio-Rad (Hercules, CA). Microtiter plates were washed with washing buffer in microplate washer with 5 wash and soak cycles of 8 s each. A 12-channel pipettor was used to dispense solutions into wells on microtiter plates.

(b) *Microplate reader.*—Bio-Tek (Winooski, VT) ELx800 with KC3 software. Used to measure optical densities of wells. A 490 nm sample filter and 630 nm reference filter were used. Data were transferred to a spreadsheet program for further analysis.

(c) *Test equipment.*—Bio-Tek Universal Calibration Test Plate. Used to periodically check the plate reader to ensure that the reader was functioning within factory specifications.

(d) *ThermoFlow Mini Cell.*—Cell X Cell II ThermoFlow Mini Cell (Novex, San Diego, CA), with a programmable power supply Model 3540. Used to perform the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of Hazelnut Protein Solutions

Roasted hazelnuts were obtained from a local store and chopped in a blender. The hazelnut was then defatted by washing several times with hexane, filtered, and air-dried at room temperature for 2 days. The defatted hazelnut was homogenized and extracted in PBS (10 g in 100 mL buffer). This extract was further dialyzed against PBS for 36 h, using dialysis tubing with 12 000–14 000 MW cut-off. The dialyzed extract was then centrifuged, and the supernatant was checked for protein concentration with the Bradford test. Hazelnut protein solutions set at 1 mg/mL were then combined with Freund's complete and incomplete adjuvant and used for immunization of animals (7). The dialyzed extracts were also used to generate standard hazelnut protein solutions. The Bradford test was performed in order to calibrate stock solutions of hazelnut proteins at 5 and 10 mg/mL. Aliquots of these solutions were stored at -20°C if not used immediately.

Immunization

Three rabbits were immunized against defatted roasted hazelnut protein extracts. Trial bleeds were regularly monitored

Table 1. Food commodities that tested negative for cross-reactivity with the hazelnut competitive ELISA

Nuts ^a	Other allergens and food ingredients		
Almond	Beef ^b	Buckwheat ^a	Bread (whole wheat) ^b
Brazil nut	Chocolate ^b	Carob ^b	Chick pea ^b
Cashew	Egg ^a	Cocoa ^b	Corn ^b
Peanut	Milk ^a	Cod ^a	Fish ^b
Pinenut	Mustard seed ^a	Cream cheese ^b	Kidney bean ^b
Pistachio	Poppy seed ^a	Emmental cheese ^b	Latex ^a
Walnut	Rice ^b	Gluten flour ^a	Lentil bean ^b
	Sesame seed ^a	Ice cream ^b	Navy bean ^b
	Soy ^a	Poultry ^b	Pinto bean ^b
	Veal ^b	Prawn shrimp ^a	White bean ^b
	Wheat ^a	Tuna ^a	

^a No inhibition at 1 mg/mL protein.^b No inhibition when extracted with 10 g/100 mL PBS.

by ELISA for their titer and the lowest inhibition concentration corresponding to antibody–antigen binding [inhibition concentration 50 (IC₅₀)]. The ex-sanguination bleeds of Rabbit C82 gave the best results with a working antibody concentration of 1:35 000 allowing a final reading of the optical density (OD) of 1, after the substrate development step. The IgG fraction was obtained using affinity chromatography purification of the anti-serum according to a previously described protocol (8). The obtained antibodies were also conjugated to horseradish peroxidase (HRP; 9).

Sample Preparation

Sample amounts of 10 g were weighed into 250 mL screw-top centrifuge tubes. Chocolate, cookies, and cereals were broken up into small pieces and ice cream was added as is. Extraction buffer was added to each sample at 100 mL/10 g. The extraction buffer consisted of a high salt (1M NaCl) buffer, incorporating 0.1M PBS, 0.1% (v/v) Tween and 0.5% (v/v) of Triton X-100. The cookie and cereal samples were homogenized in a polytron before the next step. The samples were shaken vigorously for 1 h in a heated water bath at 45°C. Chocolate and ice cream were allowed to melt and did not require sample homogenization. The rest of the extraction procedure was kept unchanged for all types of matrixes. Each sample was centrifuged at 3000 rpm for 5 min; the supernatant was then removed and centrifuged for 30 min at 20 000 × *g* in a refrigerated centrifuge at 4°C. Finally, the extract was filtered through Whatman No. 1 filter paper, and refrigerated. Extracts remained stable for 7 days at 4°C or could be stored at –20°C for longer periods.

Immunoassay

The ELISA test is based on an indirect competitive configuration (Figure 1). A 100 µL volume of each standard or diluted sample was added to 100 µL hazelnut antiserum diluted at 1:3500 (10-fold more concentrated than the final antibody

concentration on the plates) in BSA⁺ buffer, in a glass culture tube. The tubes were capped and left incubating at 4°C for 1 h. An 800 µL volume of BSA⁺ buffer was added to each solution, and mixed. The content of each tube was then transferred onto a 96-well plate (3 wells were each filled with 200 µL solution), already sensitized with water-dialyzed hazelnut protein extract, coated according to a previously described procedure (10). The concentration of the coating solution was set at 0.5 µg/mL hazelnut protein. The plate was then shaken for 10 s, and the first incubation was maintained for 30 min at 4°C. Washing was done on a plate washer (5 cycles, 300 µL rinse, 8 s soak time) and 200 µL of 1/5000 diluted HRP-labeled second antibody was added to each well.

The second incubation was also maintained for 30 min at room temperature. Subsequently, the substrate (OPD) and H₂O₂ were added. The color development was stopped after 30 min by adding 50 µL 2.5M H₂SO₄, and the ODs were read 10 min later at 492 nm with reference at 630 nm. The calibration curve consisted of the logit–log transformation of the relative OD response given by a blank and 7 concentrations (15.6, 7.8, 3.9, 1.9, 0.9, 0.5, and 0.25 ng/mL) hazelnut protein standard.

Sample extracts were diluted a minimum of 1/10 in BSA⁺ buffer to eliminate matrix effects. If samples contained higher concentrations of hazelnut, then larger dilutions were used. For unknown samples, a series of dilutions were run.

According to the test conditions, a sample containing 1 µg/g hazelnut would give a response equivalent to that of a hazelnut protein solution set at 1 ng/mL, as a final concentration on the plate.

The recovery study was performed by spiking blank matrixes with previously prepared solutions of standard hazelnut protein. The blank matrixes were chosen among marketed products with no nut warning and were checked for the absence of hazelnut by the developed ELISA (OD > IC₈₀ of the assay with no dilution of extract). Bittersweet dark chocolate,

Table 2. Recovery of hazelnut protein from target matrixes, using developed extraction procedure, and quantitative determination of undeclared hazelnut in commercial samples

Fortification with hazelnut protein, µg/g	Recovery from dark chocolate, %			
	Day 1 (n = 3)	Day 2 (n = 3)	Day 3 (n = 3)	Average
1	67.2	57.6	66.7	63.8
2.5	67.7	80.7	62.7	70.4
5	85.4	80.2	68.3	78.0
10	85.5	88.7	76.1	83.4
	Recovery from breakfast cereals, %			
	Day 1 (n = 3)	Day 2 (n = 3)	Day 3 (n = 3)	Average
1	75.8	106.2	82.0	88.0
2.5	75.6	85.2	80.4	80.4
5	105.8	88.9	74.3	89.7
10	113.1	95.4	71.5	93.3
	Recovery from cookies, %			
	Day 1 (n = 3)	Day 2 (n = 3)	Day 3 (n = 3)	Average
1	114.4	76.9	101.0	97.4
2.5	97.3	82.5	94.1	91.3
5	93.9	77.6	94.7	88.7
10	106.7	81.7	89.1	92.5
	Recovery from chocolate ice cream, %			
	Day 1 (n = 3)	Day 2 (n = 3)	Day 3 (n = 3)	Average
1	80.3	79.9	74.5	78.2
2.5	93.0	78.4	72.8	81.4
5	93.2	92.9	77.1	87.7
10	96.5	82.9	70.1	83.2
Commercial sample No.	Amount of hazelnut protein determined in µg/g, %			
	Day 1 (n = 3)	Day 2 (n = 3)	Day 3 (n = 3)	Average, µg/g
HZ-01	69.8	72.3	66.0	69.4
HZ-02	9.2	11.5	9.6	10.1

milk chocolate, cookies, cereals, and chocolate ice cream were chosen as the matrixes of interest and were spiked at 4 levels of hazelnut protein (1, 2.5, 5, and 10 µg/g). These spiked samples were fortified at the solid state with a solution of hazelnut protein standard prepared from the previously calibrated stock hazelnut protein extract. After spiking, samples were left to soak for 5 min.

SDS-PAGE and Confirmation of Positive Chocolate Samples

A 1:1 ratio of standard protein or sample extract in Tris-glycine running buffer (25mM Tris base, 192mM glycine, 0.1% SDS) were mixed, and 20 µL of each solution was added to the minigel. The solutions to be added to 4–20% Tris-glycine minigel consisted of 5 µg PBS dialyzed hazelnut protein standard, a blank chocolate extract, and spiked chocolate samples (at a level corresponding to 10 µg/g hazelnut protein). A solution of 5 µg peanut protein extract was also tested under similar conditions.

After addition of the samples and standards to the gel, 125 V were applied for 2.5 h. The gel was removed from the cassette and washed 3 times (10 min each) in deionized water. It was then placed in Gel Code Blue stain reagent for 1.5 h and destained in deionized water overnight and scanned for permanent record.

For each confirmation experiment, 2 gels were run simultaneously, one intended for a protein stain and the other for Western transfer. The second gel was blotted onto a nitrocellulose membrane under saturating conditions in a transfer buffer (12mM Tris base, 96mM glycine). Thirty volts were applied for 2 h; the membrane was then removed from the cell and placed in a blocking solution of 3% (w/v) skimmed milk and PBS. The blot was then placed in a solution of an HRP-labeled hazelnut antibody (diluted 1/10 000 with blocking solution) and incubated for 1 h at room temperature. The membrane was subsequently washed 3 times (10 min each) with 0.1% Tween 20 in PBS. The transfer was visualized with tetramethylbenzidine (TMB) membrane peroxidase sub-

Table 3. Survey of commercial chocolate for presence of declared and undeclared hazelnut proteins

Sample	Description	Nut declared/allergen warning	Result ^a and hazelnut content, ppm	
Dark chocolate				
SV-11	Bittersweet chocolate	No	(+)	3352
SV-04	Bittersweet chocolate	No	(+)	0.4 ^a
SV-13	Bitter dark chocolate	Yes	(−)	/
SV-34	Chocolate orange sticks	Yes	(+)	10
SV-38	Bittersweet chocolate	No	(+)	4
SV-43	Bittersweet chocolate	No	(+)	23
SV-44	Bittersweet chocolate	No	(+)	7
HZ-03	Dark chocolate	No	(-)	/
HZ-05	Dark chocolate with orange peel	Yes	(+)	10
HZ-06	Dark chocolate with hazelnut paste	Yes	(+)	3750
Milk chocolate				
SV-05	Rich milk chocolate bar	No	(−)	/
SV-24	Milk chocolate	No	(−)	/
SV-25	Milk chocolate	No	(+)	38
SV-27	Milk chocolate	No	(+)	55
SV-29	Whole milk chocolate	No	(+)	33
HZ-01	Milk chocolate	No	(+)	69
HZ-02	Coffee-flavored milk chocolate (contains white chocolate)	No	(+)	10
HZ-04	Milk chocolate	Yes	(+)	59
HZ-07	Milk chocolate with nuts	Yes	(+)	3800
HZ-08	Milk chocolate with nuts	Yes	(+)	10100
JQ-01	Milk orange chocolate	Yes	(+)	15

^a Close to quantitation limit of assay when 1/100 diluted extract was tested.

strate until the bands of proteins appeared (ca 30 min). The staining was stopped by placing the blot in deionized water for 15 min; it was then dried and scanned for permanent record.

Results and Discussion

The sensitivity of the indirect immunoassay was optimized by varying the nature and concentration of the coated hazelnut protein and altering the assay conditions at the first preincubation stage (Figure 1). Results were better when antibodies with free analytes, in standard or sample extracts, were preincubated in small volumes of more concentrated antibody solutions before transfer to the plate. Lowering the concentration of the coating hazelnut extract resulted in an improved sensitivity of the assay, as reported previously (7, 11). The optimum sensitivity was reached with a water-dialyzed hazelnut extract set at 0.5 µg/mL in coating buffer. Under optimum conditions of use, the IC₅₀ of the ELISA was 2.2 ng/mL (concentration of hazelnut inhibiting 50% of the antibody binding on the plate). Calibration curves performed with standard hazelnut protein solutions gave a linear range of 0.49–7.8 ng/mL (Figure 1). The IC₈₀ and IC₉₀, considered respectively as the

limit of quantitation (LOQ) and limit of detection (LOD) of the assay could be evaluated at 0.45 ng/mL (equivalent to 0.45 µg/g hazelnut protein in the solid matrix) and 0.25 ng/mL (equivalent to 0.25 µg/g), respectively.

Thirty-nine commodities, including common legumes, tree nuts, and other food ingredients, were tested for cross-reactivity by preparing protein extracts in PBS. All commodities (Table 1) showed no cross-reactivity at ≤1 mg/mL of their buffer-soluble fraction versus the Bradford test, thus demonstrating the selectivity of the hazelnut-specific antibody.

Chocolate is the major food commodity of concern for possible inadvertent cross-contamination, because of the wide variety of nut-containing formulations. A high salt (1M NaCl) buffer, incorporating 0.1M PBS and 0.1% Tween 20, was used to extract hazelnut protein from foodstuff. A blank chocolate matrix was necessary to generate the recovery data and to test the efficiency of the extraction procedure. A 100 mL volume of extraction buffer was used to extract a 10 g sample; 0.5% Triton X-100 in the extraction buffer and shaking the samples in a heated water bath (45°C) for 1 h helped improve reproducibility and consistency of recovery results.

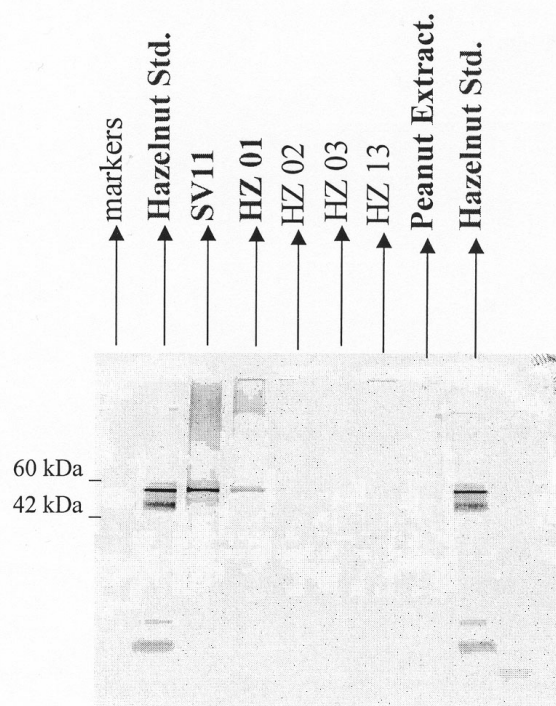


Figure 2. Results of electrophoresis and Western transfer for hazelnut standards and chocolate extracts. Confirmation of hazelnut proteins were achieved clearly for HZ-01, HZ-02, and SV-11.

Some blank chocolate extracts showed nonspecific inhibition of the maximum OD with no evidence of hazelnut, particularly in some brands of milk chocolate. A dilution of the extract 1/10 in BSA⁺ buffer overcame this matrix effect. Spikes realized on blank chocolate (1, 2.5, 5, and 10 µg/g) and extracted with optimized buffer, gave recoveries between 63.8 and 83.4% with coefficients of variation of 6–11% (Table 2). There was no distinct difference between the behavior of milk or dark chocolate with the 1/10 dilution of extracts in BSA⁺ buffer. Recoveries obtained with both dark and milk chocolate at the spiking levels investigated averaged 73%. The LOD of the assay was statistically determined for chocolate by a Student's *t*-test. The results of 5 spiked chocolate at 0.25 and 0.5 µg/g were compared with those of blank chocolate extracts. The 0.5 µg/g (ppm) spike was clearly distinguished from the blank with 99% confidence, whereas the 0.25 µg/g spike gave a 90% confidence. The LOQ was therefore considered equal to 0.5 µg/g hazelnut protein in chocolate, determined by this assay with no prior treatment of the extract.

The efficiency of the extraction procedure and the applicability of the assay was also checked on other matrixes of interest such as cookies, chocolate ice cream, and breakfast cereals. The optimized extraction buffer and sample handling procedure were kept unchanged and allowed to reach satisfactory results for all these matrixes. Average recoveries of hazelnut protein from cookies, chocolate ice cream, and breakfast cereals (Table 2) varied from 78 to 97% for spiking levels

between 1 and 10 µg/g. An excellent day-to-day reproducibility was demonstrated when these spiked samples were run for 3 days. The result of each day corresponded to the average recovery obtained for 3 extracts performed on the same day for each spiked sample.

The results of a limited survey of some commercial chocolates are shown in Table 3. Undeclared hazelnut was quantified by the same extraction and dilution procedures and led to identification of a range of levels of cross-contact, varying from 4 µg/g to about 0.3% of undeclared hazelnut protein in chocolate (Table 3).

One of the major issues of the detection and quantification of potential allergens in foods is reliability and reduction of false positives caused by matrix effects. Confirmation methodologies are therefore required to complement ELISA results. In this study, SDS-PAGE coupled with Western transfer was used for confirmation purposes. HRP-labeled hazelnut antibodies were used for recognition of separated protein fragments blotted on nitrocellulose.

Hirschwehr et al. (12) reported that the main allergens to be encountered in hazelnut include an 18 kDa allergen, related to Cor a1, the major hazel pollen allergen. Koppelman et al. (4) identified several hazelnut proteins, giving a high binding to immunoglobulin E (IgE) from hazelnut-sensitive individuals: 25 kDa (doublet), 37 kDa (doublet), and 55 kDa.

Our hazelnut-specific antibody featured a strong binding to 2 particular groups of hazelnut proteins, which are clearly identified between 42 and 60 kDa. These proteins were also clearly visible in extracts of chocolate samples HZ-01 and SV-11, the most contaminated samples quantified by ELISA (Figure 2). Sample HZ-02 showed a smaller indication of these 2 protein markers. This technique demonstrated positives and confirmed the specificity of the ELISA. However, the development of an independent technique from immunochemical-based methodologies giving a higher sensitivity will be necessary for confirmation of trace amounts. Capillary electrophoresis coupled to mass spectrometry offers a perspective for this purpose and may complement the ELISA for determining and quantifying potential allergens in foods.

The methodology described in this report and, in particular, the competitive ELISA method, is currently being validated under an interlaboratory trial prior to its use for testing of products on the Canadian market that are likely to contain undeclared hazelnut proteins.

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