

Detection of hazelnuts and almonds using commercial ELISA test kits

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Abstract Three commercial sandwich enzyme-linked immunosorbent assay (ELISA) test kits for the detection of hazelnuts and almonds were evaluated. Limits of detection and dynamic ranges were determined for hazelnuts and almonds spiked into cooked oatmeal, dipping chocolate, and muffins (baked). The limit of detection values varied from 1 to 38 $\mu\text{g/g}$, depending on the food matrix and ELISA test kit. Percent recoveries based on the standards supplied with the test kits varied from 10% to 170%. It is impossible to ascertain whether the percent recoveries reflect the performance of the ELISAs or differences between the protein content of the nuts used to spike the samples and the test kit standards. Unfortunately, reference materials do not exist that can be used to compare the results from different test kits and standardize the test kit standards. Also, insufficient knowledge regarding the epitope specificity of the antibodies used in the ELISAs further hinders interpretation of the results generated by the different test kits.

Keywords Almond · Hazelnut · ELISA · Food allergen · Foods/beverages · Immunoassays/ELISA · Bioanalytical methods

Introduction

Tree nuts account for one of the major allergenic food groups in the USA. Peanut, shellfish, and tree nut allergies

have a high rate of anaphylaxis, and only an estimated 10% of children allergic to tree nuts outgrow their allergies while an estimated 68% of children allergic to eggs acquire tolerance [1–3]. Surveys conducted in 1997 and 2002 in the USA estimated the incidence of tree nut allergic adults at 0.7% and children at 0.2% [4, 5].

Several major and minor hazelnut and almond food allergens have been identified [6, 7]. Questions have been raised regarding the allergenicity of the glycan forms of some of the proteins following posttranslational glycosylation [8]. Regional differences in the sensitivity of different nationalities to specific allergens have been observed [9]. The primary allergen of almonds is the storage protein known as almond major protein (AMP) or amandin. AMP accounts for approximately 65% of the aqueous extractable proteins and is detectable by IgG and IgE binding after various forms of heating, including blanching and roasting [10–12].

Patients sensitive to one tree nut have a high probability of being allergic to other tree nuts and plant products, including peanut proteins [4, 13, 14]. Immunoblot analysis of IgE subpopulations isolated from a patient who suffered anaphylaxis due to walnut exposure displayed cross-reactivity with hazelnut and Brazil nut proteins but not almond [15]. Further, a patient who suffered anaphylaxis due to coconut exposure displayed IgEs that cross-reacted with hazelnut [16]. In contrast, IgE from patients allergic to the legumes peanut and lupine cross-reacted with almond [17, 18].

Numerous methods have been employed to detect hazelnut and almonds including DNA-based technologies such as real-time polymerase chain reaction (PCR), array technology to detect PCR-amplified DNA, high-resolution melting single-nucleotide polymorphism analysis [19–24], and immunology-based methods such as enzyme-linked immunosorbent assays (ELISAs) [10, 25–27].

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Currently, avoidance is the only approach by which tree-nut-allergic individuals can avoid adverse reactions to the consumption of tree nuts. Acknowledging the importance of accurate food labels, the US Congress passed in 2004 the Food Allergen Labeling Consumer Protection Act. To meet the requirements of FALPCA and help insure the accuracy of food labels, reliable analytical methods are required. To facilitate such, the limits of detection (LODs), dynamic range, and percent recoveries for the detection of hazelnut and almonds using three commercial ELISA test kits were determined and compared to manufacturer's claims for food representative of different forms of processing (e.g., baking) and known to be problematic (i.e., chocolate).

Materials and methods

Almonds, hazelnuts, and food samples were purchased locally. Thimerosal, BSA, carboxymethyl cellulose (CMC) sodium salt, phosphate-buffered saline (PBS), and other chemical reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Food samples

Hazelnut and almond spiking solutions were prepared as previously described for peanuts [28] with the following modifications: The hazelnuts and almonds were ground to a mesh size of 20 (0.84 mm) and suspended in 2% CMC at either 200 mg/50 g mixture or 800 mg/50 g mixture. The CMC suspension was then diluted in PBS to final concentrations of 100, 406, 643, and 2,600 µg/ml. One- or 5-g food samples were spiked with a maximum volume of 100 µl of spiking solution to achieve the desired concentrations of hazelnut or almond. Food samples were typically spiked at six concentrations designed to encompass the detection limits reported by the test kit manufacturer and the standards supplied with the test kit. The maximum level was typically chosen to represent twice the upper limit of the assay and thereby provide an indication of the response associated with saturation. Further, use of a sample spiked with a high level of tree nut guaranteed that a food displaying a low percent recovery would generate a signal within the dynamic range of the assay.

The foods chosen for evaluation in this study included oatmeal (uncooked and cooked), chocolate, and muffins. Baked muffins provided a test of the methods' ability to detect the target antigen after exposure to heat. Chocolate is a food shown to commonly have undeclared tree nuts present and considered a problem matrix due to reduced levels of recovery. Uncooked oatmeal represented a control for the cooked oatmeal; however, elevated backgrounds and

erratic results, possibly due to variable hydration during sample preparation, limited its utility.

Preparation of the uncooked oatmeal samples entailed spiking 1 or 5 g samples of ground oatmeal. Cooked oatmeal samples were prepared by mixing 40 g of ground oatmeal with 175 ml of boiling water for 2–3 min. While still hot, 1- or 5-g portions of cooked oatmeal were transferred to individual vessels, fortified with spiking solution, stirred, and heated an additional 1 or 2 min, depending whether the sample contained 1 or 5 g.

Muffins were prepared using Jiffy Corn Muffin Mix following the instructions on the package. One- or 5-g samples were placed in a small muffin pan and fortified with the spiking solution prior to baking for 14 min at 204°C.

Chocolate samples were prepared by melting Baker's Dipping Chocolate (ingredients: milk chocolate) in a beaker immersed in a boiling water bath and adding the spiking solution to the melted chocolate.

ELISAs

ELISA test kits were purchased from the manufacturer. Specifically, Veratox® for Hazelnut Allergen (catalog # 8420) and Veratox® for Almond Allergen (catalog # 8440) were purchased from Neogen Corporation (Lansing, MI, USA). RIDASCREEN®FAST Hazelnut (R6802) and RIDASCREEN®FAST Mandel/Almond (R6901) were purchased from R-Biopharm Inc. (Marshall, MI, USA). ELISA Systems Hazelnut (catalog # 95204 ESHRD-48) and ELISA Systems Almond (catalog # 95200 ESARD-48) ELISA test kits were purchased from bioMerieux Industry (Hazelwood, MO, USA).

The spiked samples were extracted according to the protocols supplied with the ELISA test kits and immediately analyzed according to the manufacturer's instructions for the ELISA. The evaluations typically entailed the analysis of samples prepared in triplicate.

Veratox® for Hazelnut and Veratox® for Almond are sandwich ELISAs that employ 0-, 2.5-, 5-, 10-, and 25-ppm standards. The sample is diluted 25-fold and extracted at 60°C for 15 min with shaking at 150 rpm followed by either filtration using Whatman #4 filter paper or centrifugation at 14,000×g for 5 min. The sample, conjugate, and substrate incubation steps are each 10 min with the presence of hazelnut and almond indicated by the generation of color as read at 650 nm. The ELISAs are described as having a range of quantitation of 2.5–25 ppm.

RIDASCREEN®FAST Hazelnut and RIDASCREEN®FAST Mandel/Almond are sandwich ELISAs that employ 0-, 2.5-, 5-, 10-, and 20-ppm standards. The food sample is diluted 20-fold and extracted for 10 min at 60°C followed by either filtration or centrifugation. The sample, conjugate, and substrate incubation steps are each 10 min with the presence of hazelnut or almond indicated by the generation of color as

read at 450 nm and the concentration determined by interpolation. The hazelnut assay is described as having a limit of detection of 1.5 ppm and a limit of quantitation of 2.5 ppm. Roasting has been shown to reduce the recovery of the hazelnut ELISA by 30%. The RIDASCREEN®FAST Hazelnut ELISA has been validated for dark chocolate by the German BVL (§35 method LMBG (German Federal Laboratory for Consumer Protection and Veterinary Medicine)) and the results published [29]. The Mandel/Almond assay is described as having a limit of detection of 1.7 ppm and a limit of quantitation of 2.5 ppm. The ELISA does not cross react with hazelnut, walnut, coconut, cashew nut, brazil nut, peca nut, macadamia nut, chestnut, sunflower seeds, sesame, and lima beans. Cross reaction of >100% with apricot stone was reported by R-Biopharm.

ELISA Systems Hazelnut Test Kit and Almond Test Kit are sandwich ELISAs that employ 0-, 0.5-, 1-, 2.5-, and 5-ppm protein as standards. For the Hazelnut ELISA, the standards are equivalent to 0-, 3.6-, 7.1-, 18-, and 36-ppm hazelnut, while for the Almond ELISA, the standards are equivalent to 0-, 2.4-, 4.8-, 12-, and 24-ppm almond. Analysis of food samples entails tenfold dilution, 15 min of extraction at 60°C with shaking for one out of every 5 min, followed by removal of insoluble matter by either filtration or centrifugation prior to ELISA analysis. Each of the ELISA incubation steps (sample, conjugate, and substrate) are 10 min with the presence of hazelnut indicated by the generation of color as read at 450 nm (preferably relative to 650 nm). The assays are described as each having a limit of quantitation of 0.5-ppm protein (3.6-ppm hazelnut, 2.4-ppm almond). The standards supplied with the ELISA Systems Hazelnut ELISA span a wider range than those supplied with other test kits. To accommodate this wider range, the food samples were spiked with 0-, 3-, 6-, 22-, 40-, and 105-ppm hazelnut.

Data analysis

The detection and quantitation of hazelnuts and almonds entailed ascertaining the amount of spiked material necessary to generate a signal three times the standard deviation above the background and by comparing the response to standard curves generated using the test kit standards. The standard curves were based on linear, third-order polynomial, and log-logit fitting of the responses generated by the test kit standards. Inasmuch as the RIDASCREEN and ELISA Systems ELISAs did not prescribe a maximum response, the data generated using these assays could not be analyzed using log-logit analysis, which requires a defined maximum (limiting) response. Further, only those fits displaying average R^2 values (square of the Pearson product-moment correlation coefficients) of 0.9 or greater were used, though R^2 values <0.98 were considered questionable.

Detection limits were determined using both the absorbance cutoffs of the standards defined by the manufacturer as the limits of quantitation and the onset of a saturation response as evidenced by a deviation from a linear (DfL) correlation between analyte concentration and response. The onset of saturation was determined using multiple DfL cutoffs, specifically 20%, 25%, and 50% and the level observed for the test kit standard defined by the manufacturer as the upper limit of the assay. The onset of saturation is a more reliable indicator of the upper limit inasmuch as it avoids the assumption that the food or processing has no effect on either the background response or the detection of the target analyte as represented by the titration curve.

All analyses were done in triplicate unless noted otherwise and the results presented as the average $\pm$ the standard deviation. When comparing two results (i.e., the percent recoveries calculated using two trend line equations), the results are presented as the average $\pm$ the range divided by two. Outliers greater than four times the standard deviation from the mean were omitted from the analyses.

Results and discussion

The results obtained analyzing oatmeal (cooked and uncooked), chocolate, and muffins (baked) for the presence of spiked hazelnuts and almonds using the various test kits are presented in Tables 1, 2, and 3.

The level of the response generated by a food lacking the target analyte versus the response of the 0-ppm standard supplied with the test kit is one measure of the suitability of the assay. An elevated background response can limit the sensitivity of the assay and under extreme conditions result in false-positive responses, making the assay unsuitable for the food being analyzed. Once it is established that the food does not generate a false-positive response, the focus is on whether the assay can detect the target analyte in the food and what is the dynamic range. This involves determining the LOD and the upper limit of the assay as described in the “Materials and methods” section.

Uncooked oatmeal samples gave elevated background absorbance (responses) with four of the six test kits examined ranging from 0.32 ± 0.15 , $n=7$ ($OD_{650\text{ nm}}$) for the Veratox® for Almond to 0.78 ± 0.18 , $n=4$ ($OD_{450\text{ nm}}$) for the ELISA Systems Almond Test Kit. Further indicative of a matrix unsuitable for ELISA analysis was the observation of an inability to detect changes in analyte concentration as illustrated by both the RIDASCREEN ELISAs and the ELISA Systems Hazelnut Test Kit, an assay that did not display an elevated background. Only the Veratox® for Hazelnut test kit displayed an ability to reliably detect the analyte in uncooked oatmeal. The Veratox® for Hazelnut displayed a lower limit of $1.4 \pm$

Table 1 Veratox® test kits for hazelnut and almond detection

	Background (OD)	Lower limit (µg/g tree nut) ^a	Upper limit	
			SD (µg/g tree nut) ^a	5% DfL (µg/g tree nut) ^b
Hazelnut				
0 ppm standard	0.13±0.06 (n=7)			
Cooked oatmeal	0.18±0.15 (n=6)	1.4 ± 0.7 (n=6)	18 ± 9 (n=6)	19 ± 1 (n=6)
Uncooked oatmeal	0.19±0.08 (n=5)	1.4 ± 0.7 (n=4)	55 ± 15 (n=4)	19 ± 2 (n=4)
Chocolate	0.12 ± 0.02 (n=3)	1.1 ± 1.8 (n=5)	22 ± 8 (n=5)	22 ± 2 (n=5)
Muffins	0.15±0.02 (n=6)	5.6 ± 2.1 (n=4)	46 ± 7 (n=4)	33 ± 11 (n=4)
Almond				
0 ppm standard	0.08±0.01 (n=6)			
Cooked oatmeal	0.07±0.02 (n=4)	4 ± 1 (n=4)	64 ± 20 (n=4)	22 ± 3 (n=4)
Uncooked oatmeal	0.32 ± 0.15 (n=7)	3 ± 3 (n=6)	— ^c	— ^c
Chocolate	0.065±0.006 (n=4)	4.2 ± 0.4 (n=4)	34 ± 5 (n=6)	28 ± 11 (n=6)
Muffins	0.070±0.005 (n=6)	9 ± 2 (n=6)	114 ± 50 (n=6)	19 ± 2 (n=6)

Spiked food samples analyzed according to the test kit's protocol. Data presented as the average ± standard deviation

^a Concentration that generates a response comparable to either the lower limit standard of 2.5 ppm (hazelnut 0.43±0.11, *n*=7; almond 0.40±0.05, *n*=6) or upper limit standard of 25 ppm (hazelnut 2.1±0.3, *n*=7; almond 1.9±0.3, *n*=7)

^b Concentration that generates a 25% deviation between the responses calculated using a third-order polynomial describing all spiked samples versus linear analysis of samples spiked with ≤11 $\mu\text{g/g}$

^c Unable to ascribe an upper limit due to erratic data that could not be interpreted

Table 2 RIDASCREEN®FAST test kits for hazelnut and almond detection

	Background (OD)	Lower limit (µg/g tree nut) ^a	Upper LIMIT	
			SD (µg/g tree nut) ^a	25% DfL (µg/g tree nut) ^b
Hazelnut				
0 ppm standard	0.06±0.03 (n=6)			
Cooked oatmeal	0.07±0.03 (n=3)	2±1 (n=2)	26±8 (n=2)	28±0 (n=2)
Uncooked oatmeal	0.35±0.05 (n=2)	— ^c	— ^c	— ^c
Chocolate	0.09±0.03 (n=7)	1±1 (n=6)	20±8 (n=6)	>54 ^d
Muffins	0.07±0.03 (n=3)	2±0 (n=3)	38±6 (n=3)	>54
Almond				
0 ppm standard	0.12±0.02 (n=7)			
Cooked oatmeal	0.14±0.05 (n=5)	2.5±1.3 (n=4)	16±4 (n=4)	27±3.6 (n=4)
Uncooked oatmeal	0.92±0.04 (n=2)	— ^c	— ^c	— ^c
Chocolate	0.17±0.05 (n=5)	3±1 (n=5)	11±4 (n=5)	39±9 (n=5)
Muffins	0.13±0.02 (n=9)	7±3 (n=9)	58±33 (n=9)	>54

Spiked food samples analyzed according to the test kit's protocol. Data presented as the average±standard deviation

^a Concentration that generates an absorbance comparable to either the lower limit standard of 2.5 ppm (hazelnut 0.17±0.08, *n*=6; almond 0.49±0.04, *n*=7) or upper limit standard of 20 ppm (hazelnut 1.17±0.08, *n*=6; almond 2.3±0.2, *n*=7)

^b Concentration that generates a 25%, deviation between the responses calculated using a third-order polynomial describing all spiked samples versus linear analysis of samples spiked with ≤11 $\mu\text{g/g}$

^c Impossible to calculate due to elevated background and lack of correlation between response and spike level

^d Wide linear range prevented detection of a 25% deviation from linearity at <54 $\mu\text{g/g}$

Table 3 ELISA systems test kits for hazelnut and almond detection

	Background (OD)	Lower limit (μg/g tree nut) ^a	Upper limit	
			SD (μg/g tree nut) ^a	25% DfL (μg/g tree nut) ^b
Hazelnut				
0 ppm standard	0.054±0.007 (n=5)			
Cooked oatmeal	0.07±0.01 (n=3)	5.8±0.7 (n=3)	46.7±1.6 (n=3)	>54 ^c
Uncooked oatmeal	0.049±0.002 (n=3)	16±8 (n=4)	— ^d	>54
Chocolate	0.057±0.006 (n=5)	8±3 (n=5)	87±19 (n=5)	41±3 (n=5)
Muffins	0.055±0.006 (n=6)	38±16 (n=3)	— ^e	>54
Almond				
0 ppm standard	0.039±0.010 (n=7)			
Cooked oatmeal	0.047±0.012 (n=6)	4.3±1.2 (n=6)	23±7 (n=6)	18±3 (n=6)
Uncooked oatmeal	0.78±0.18 (n=4)	— ^d	— ^d	— ^d
Chocolate	0.047±0.005 (n=3)	5±0 (n=4)	38±5 (n=4)	21±1 (n=4)
Muffins	0.033±0.006 (n=6)	7±3 (n=6)	57±13 (n=6)	24±11 (n=6)

Spiked food samples analyzed according to the test kit's protocol. Data presented as the average $\pm$ standard deviation

^a Concentration that generates an absorbance comparable to either the lower limit standard of 2.5 ppm (hazelnut 0.45 $\pm$ 0.06, $n=5$; almond 0.852 $\pm$ 0.085, $n=7$) or upper limit standard of 20 ppm (hazelnut 2.6 $\pm$ 0.1, $n=5$; almond 2.1 $\pm$ 0.2, $n=7$)

^b Concentration that generates a 25% deviation between the response calculated using a third order polynomial describing all spiked samples versus linear analysis of samples spiked with ≤ 22 $\mu\text{g/g}$ hazelnut or ≤ 11 $\mu\text{g/g}$ almond

^c Wide linear range prevented detection of a 25% deviation from linearity at <54 $\mu\text{g/g}$

^d Impossible to calculate due to elevated background and lack of correlation between response and spike level

^e Unable to assign an upper limit due to low recovery (10%)

0.7 $\mu\text{g/g}$ ($n=4$) and based on generating a response comparable to the upper limit standard and a 25% deviation from linearity displayed upper limits of 55 $\pm$ 15 $\mu\text{g/g}$ ($n=4$) and 19 $\pm$ 2 $\mu\text{g/g}$ ($n=4$), respectively. The average recovery was 133% within the dynamic range of the Veratox[®] for Hazelnut assay.

Veratox[®] ELISAs

The Veratox[®] for Hazelnut and the Veratox[®] for Almond ELISAs displayed responses (OD_{650 nm}) for the cooked oatmeal, chocolate, and baked muffins comparable to the zero standard (see Table 1). The observation of comparable responses between the zero standard and the food samples indicates the unlikelihood of false positives with these foods for the two ELISAs.

The instructions supplied with the Veratox[®] assays describe the ELISAs as having range of quantitation of 2.5–25 ppm. The lower limits of quantitation calculated by fitting the data generated with the spiked samples to third-order polynomials and interpolating the amount of analyte necessary to generate an absorbance comparable to the 2.5-ppm standard (hazelnut 0.43 $\pm$ 0.11, $n=7$; almond 0.040 $\pm$ 0.05, $n=6$) ranged from 1 to 6 $\mu\text{g/g}$ hazelnut and 4 to 9 $\mu\text{g/g}$ almond for the two assays (Table 1). The upper limit for quantitation based on the concentration necessary to generate

an absorbance comparable to the 25-ppm test kit standard (hazelnut 2.1 $\pm$ 0.3, $n=7$; almond 1.9 $\pm$ 0.3, $n=7$) did not always agree with those based on DfL calculations. Though the hazelnut ELISA displayed comparable upper limits for cooked oatmeal and chocolate, the Veratox[®] for Almond did not (see Table 1). The occurrence of substantial differences between the two approaches used to calculate upper limits is dependent on whether the presence of the food or processing during preparation affects the titration profile or the maximum response. A food whose partial volume is small and does not entail processing that could affect the binding of antibodies to the antigenic epitope should generate comparable upper limits irrespective of whether calculated based upon either the response by the upper limit standard or its level of DfL.

The recovery data for cooked oatmeal, chocolate, and baked muffins spiked with hazelnut and almond were determined based on linear regression, third-order polynomial, and log-logit (maximum OD=3) fitting of the responses generated by the test kit standards. Linear analysis displayed the greatest variability with R^2 values as low as 0.85 and 0.90 for the hazelnut and almond assays, respectively. In contrast, the third-order polynomial and log-logit fits were comparable with R^2 values typically ≥ 0.98 and ≥ 0.95 , respectively, for the hazelnut ELISAs and ≥ 0.99 and ≥ 0.96 , respectively, for the almond Veratox[®] ELISA. The average percent recovery from cooked oat-

meal, chocolate, and muffins for hazelnuts/almonds were 190%/60%, 137%/65%, and 62%/37%, respectively, when limited to those samples within the dynamic range of the assay. Despite the lower recovery for hazelnut from baked muffins, the reproducibility of the results was acceptable (hazelnut average %CV=20±9%, $n=15$).

RIDASCREEN®FAST ELISAs

The RIDASCREEN®FAST Hazelnut ELISA and RIDASCREEN®FAST Mandel/Almond generated background responses ($OD_{450\text{ nm}}$) comparable to the zero standards (hazelnut 0.06 ± 0.03 , $n=6$; almond 0.12 ± 0.02 , $n=7$) and indicate that false positives should not be a problem with these foods (Table 2).

The RIDASCREEN®FAST Hazelnut ELISA is described by the manufacturer as having a limit of detection of 1.5 ppm and a limit of quantitation of 2.5 ppm while the limits for the RIDASCREEN®FAST Mandel/Almond ELISA are 1.7 and 2.5 µg/g. The concentration of hazelnut necessary to generate absorbance values comparable to the 2.5-ppm standard (hazelnut 0.17 ± 0.08 , $n=6$; almond 0.49 ± 0.4 , $n=7$) ranged from 1 to 2 µg/g hazelnut and 3 to 7 µg/g almond (see Table 2). Since the test kit manufacturer recommends a limit for interpolation of 20 ppm, upper limits based on the absorbance generated by the 20-ppm standard (hazelnut 1.17 ± 0.08 , $n=6$; almond 2.3 ± 0.2 , $n=7$) were compared to those calculated based on a 25% DfL. The upper limits calculated based on the concentration of analyte necessary to generate a response comparable to the 20-ppm standard yielded limits considerably less from those based on a 25% DfL. Such differences are consistent with the RIDASCREEN®FAST ELISAs displaying exceptionally wide ranges of linearity such that the upper limit exceeded the range suitable for calculation (>54 µg/g) based on the concentrations of the spiked samples and the test kit standards.

The recovery of hazelnut and almond was calculated using linear and third-order polynomial analysis ($R^2\geq0.99$).

The average percent recoveries for hazelnut/almond in cooked oatmeal, chocolate, and baked muffins were 89/140%, 108/148%, and 61/52% percent (average %CV 28%/18%), respectively.

ELISA system test kits

The background responses ($OD_{450\text{ nm}}$) observed with the ELISA Systems Hazelnut and ELISA Systems Almond test kits generated comparable responses among the 0-ppm standards (hazelnut 0.054 ± 0.007 , $n=5$; almond 0.039 ± 0.010 , $n=7$) and cooked oatmeal, chocolate, and baked muffins samples not spiked with either hazelnut or almonds (Table 3). Using the responses generated by the 0.5- and 5.0-ppm protein standards along with 25% DfL to define the lower and upper limits generated considerably different results for the two assays. The hazelnut ELISA yielded elevated lower limits and upper limits consistent with the low recoveries observed for cooked oatmeal (59%, %CV=22%), chocolate (46%, %CV=34%), and baked muffins (10%, %CV=30%). In contrast, the almond ELISA generated lower limits of detection ranging from 4 to 7 µg/g and upper limits ranging from 18 to 57 µg/g depending on the food and whether calculated based on the response generated by the 5-ppm protein standard or 25% DfL. A correlation between recovery (cooked oatmeal 98%, chocolate 69%, baked muffins 44%, average %CV=20%) and the upper limit was observed, but not as extensive as observed with the hazelnut ELISA.

Conclusion

All three ELISA test kits detected the presence of hazelnut and almonds in cooked oatmeal, chocolate, and baked muffins. The limits of detection for the Veratox® and RIDASCREEN® Fast Hazelnut ELISAs were comparable with the ELISA Systems assays, displaying a slightly greater lower limit as summarized below.

	Cooked oatmeal		Chocolate		Baked muffins	
	Dynamic range (µg/g)	Recovery (%)	Dynamic range (µg/g)	Recovery (%)	Dynamic range (µg/g)	Recovery (%)
Hazelnut						
Veratox®	1.4–19	169	1–22	128	6–33	60
RIDASCREEN®	2–28	89	1 to >54	108	2 to >54	61
ELISA systems	6 to >54	59	8–41	46	38 to >54	10
Almond						
Veratox®	4–22	60	4–28	80	9–19	65
RIDASCREEN®	3–27	140	3–39	148	7 to >54	52
ELISA Systems	4–18	98	5–21	69	7–24	44

The dynamic ranges displayed variability primarily due to differences in recovery and the degree of linearity, which affected the ability to assign an upper limit based on the observation of a saturation response.

Differences in the percent recoveries for the three test kits may reflect performance and inherent properties associated with recognizing different epitopes. The lack of hazelnut and almond reference materials and methods to quantify the presence of the antigenic epitopes makes interpretation of recovery values difficult. Variability in protein content between cultivars and crops may make the standards supplied with the test kit inappropriate for the food samples with apparent recoveries of less than or greater than 100% reflecting these variables rather than recovery efficiency. Also, food processing and preparation can modify or affect the accessibility of critical epitopes. This latter problem is amplified by different epitopes undergoing different levels of modification or changes in accessibility affecting the binding of antibodies differently. As a result, the recoveries measured for the different foods or the same foods using different ELISA test kits can only be used as an indication of assay performance and should not be relied on for accuracy. Quantitation may require identification of the antigenic epitopes and research on the effects of food processing and preparation on the antigenic properties of the epitopes. The establishment of reference materials may facilitate this process but is unlikely to eliminate the problem nor would the availability of a reference material provide an understanding of the affects of processing on allergenicity or the detection of allergenic food. Such an understanding is dependent on identifying the allergenic epitopes and clinical studies.

Despite these potential sources of quantitative variability, specific problems were observed that limit the usefulness of the test kits. Low percent recoveries for baked muffins made the detection at levels approaching the limits of detection problematic and can increase the variance. A reduction in the recovery of food allergens from baked foods has been observed with other ELISA test kits [30]. Possible reasons for the reduction in recovery upon baking includes heat-induced denaturation or chemical modification resulting in either reduced extraction efficiency of the antigenic protein or reduced accessibility of the epitope. The observation that ELISAs based on the extraction and detection of the SDS-denatured/ β -mercaptoethanol-reduced proteins, using antibodies stable under these conditions, display superior recoveries [31] is consistent with these explanations.

The detection in uncooked oatmeal using the RIDASCREEN® Fast and ELISA Systems ELISAs and the Veratox® for Almonds was erratic. The Veratox® Hazelnut ELISA did not display such problems with uncooked oatmeal possibly due to its use of the greatest level of dilution (25-fold) and continuous shaking during extraction at 60°C, which would facilitate hydration.

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