Journal of Food Protection, Vol. 79, No. 9, 2016, Pages 1572–1582
doi:10.4315/0362-028X.JFP-15-493

A Novel Immunoassay Test System for Detection of Modified Allergen Residues Present in Almond-, Cashew-, Coconut-, Hazelnut-, and Soy-Based Nondairy Beverages

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MS 15-493: Received 28 October 2015/Accepted 5 May 2016

ABSTRACT

A growing number of plant-based milk substitutes have become commercially available, providing an array of options for consumers with dietary restrictions. Though several of these products rival cow's milk in terms of their nutritional profiles, beverages prepared with soy and tree nuts can be a significant concern to consumers because of potential contamination with food allergens. Adding to this concern is the fact that allergen residues from plant-based beverages are modified during manufacturing, thereby decreasing the sensitivity of antibody-based detection methods. Consequently, many commercially available allergen detection kits are less effective for allergens derived from nondairy milk substitutes. To address this limitation, we developed a panel of polyclonal antibodies directed against the modified proteins present in almond, cashew, coconut, hazelnut, and soy milks and incorporated them into rapid lateral flow immunoassay tests configured in both sandwich and competitive format. The tests had robust detection capabilities when used with a panel of various brand-name products, with a sensitivity of 1 ppm and selectivity values of 3 to 5 ppm in nondairy beverages. Minimal cross-reactivity to extracts prepared from common commodities was observed. The development of a highly sensitive and rapid test specifically designed to detect trace quantities of highly modified allergen residues in plant-based, dairy-free beverages will aid food manufacturers and regulatory agencies in monitoring products for these modified allergens when testing environmental and food samples.

Key words: Allergens; Almond milk; Cashew, coconut, hazelnut, and soy milk; Lateral flow device; Nondairy beverage; Polyclonal antibodies

An increasing number of plant-based milk substitutes have become commercially available, providing suitable alternatives for consumers with lactose intolerance, galactosemia, phenylketonuria, milk allergy, and vegan dietary habits. Many of these nondairy beverages are manufactured in such a manner as to enhance the overall organoleptic properties and nutritional profiles *(8)*. However, soy and tree nut beverages pose a risk for certain consumers because food products made with equipment also used with allergen-containing ingredients are at increased risk for contamination with allergens through cross-contact. Inadvertent contamination of foods and incorrect food labeling is estimated to account for 47% of adverse reactions to food allergens *(13)*. Tree nuts are one of the most common triggers of acute food allergies and are frequently associated with severe anaphylaxis *(9)*. Though typically not life threatening, immunotoxic reactions to soy proteins, including immunoglobulin (Ig) E-dependent and IgE-independent mechanisms, are relatively common in children *(10, 14)* and typically present as chronic symptoms, including intestinal and dermatological manifestations. Consequently, federal food labeling laws mandate that labels for foods containing soy and/or tree nuts declare in plain language the presence of these foods or their derivatives *(16)*.

Risk of allergen contamination is addressed through implementation of allergen control measures, which include frequent monitoring of manufacturing surfaces and foods (commodities and finished product) for allergen residues. Allergen residue detection is based largely on antibodies that recognize specific epitopes present on the allergen residues, allowing for rapid detection and quantitation using such platforms as enzyme-linked immunosorbent assays (ELISAs), bead arrays, and lateral flow immunochromatographic assays. Antibody-based detection of target analytes is dependent on the precise form of analyte present in a sample. Analytes that have undergone extensive processing, including fermentation, enzymatic hydrolysis, heat, or high pressure, can undergo fragmentation, chemical alterations, and conformational changes that affect the ability of diagnostic antibodies to recognize cognate epitopes *(2, 11)*.

Although numerous methods are employed in preparation of plant-based beverages, commercially dominant products rely on conventional sterilization processes such

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as ultrahigh temperature processing (UHT; 142°C for 6 s) and ultrahigh pressure homogenization (UHPH) at 200 and 300 MPa in combination with elevated temperatures (55 to 75°C) *(8, 12, 17)* to address microbial concerns for low-acid foods. Additional pH extremes and thermal treatments may be implemented to enhance protein levels, remove off-flavors, and inactivate plant-based protease inhibitors and lectins, which are antinutritives known to disrupt digestive health *(4)*. The consequence of such processing has been well described for dairy milk, in which UHT and UHPH treatments denature milk proteins, particularly whey proteins *(3, 6)*. Corresponding analyses in plant-based nondairy beverages is much more limited, although effects of processing have been reported for soy milk proteins and to a lesser extent almond milk proteins; the effects on more recently developed beverages have yet to be reported. In soy milk, the 2S albumin and the 7S (beta-conglycinin) and 11S (glycinin) globulins are the principal soy proteins present *(18)*. Both heat and high pressure treatments can cause aggregate formation and increase the display of hydrophobic regions to solvent-exposed surfaces, thereby imposing major structural changes starting at 70°C and between 300 and 400 MPa for both the 7S and 11S globulins *(5, 7, 15, 19)*. This effect has been empirically demonstrated with a monoclonal antibody assay of thermally treated soybean proteins, which revealed that heat-induced loss of enzyme function is linearly correlated with loss of antigenicity *(1)*. Changes associated with formation of aggregates and novel solvent-exposed surfaces result in a loss of native epitope structures and the formation of neoepitopes, considerably altering the antigenicity or antibody-binding capability relative to native proteins. Similar but cruder effects have been reported for almond milk proteins, where processing has ablated antigenicity in ELISAs *(7)*. In plant-based, nondairy beverages, both UHT and UHPH treatments induce protein aggregate formation *(5, 7, 12, 15, 19),* indicating that these processes greatly reduce the occurrence of native protein conformation in finished product. A potential and concerning consequence of this processing is the reduced effectiveness of standard immunodiagnostic tools for detecting allergens present in these plant-based, nondairy beverages. To address this diagnostic concern, we developed a panel of rapid immunochromatographic tests for almond milk, cashew milk, coconut milk, hazelnut milk, and soy milk proteins, in the form of a lateral flow device (LFD), which when paired with a calibrated electronic strip reader can be applied to the semiquantitative screening of foods and environmental samples in under 25 min, with a limit of detection (LOD) of 1.0 ppm in food. Application of this novel test system should improve allergen control procedures and critical control point measures and assist food manufacturers and regulatory agencies in monitoring for allergens derived from plant-based, nondairy beverages in environmental and food test samples.

MATERIALS AND METHODS

Reagents and assay buffers. Nondairy, plant-based beverages, soybeans, and nuts were obtained from various grocery stores in the United States and Europe. Newpro soy protein concentrate (New Asia Ingredients, Singapore) and Profam soy protein isolate and defatted soy flour (ADM Foods and Wellness, Decatur, IL) were used for making specific extracts of known protein concentrations with Tris-buffered saline. Igs were obtained from Pi Bioscientific (Seattle, WA). Vaccine material (800 μg) was prepared from Silk brand almond milk and soy milk, So Delicious coconut milk and cashew milk, and Pacific Foods hazelnut milk (relying on 60 to 75% ammonium sulfate–based precipitation to concentrate the proteins) and emulsified with complete and incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). For basic cross-reactivity analysis, proteins were extracted from commodities using extraction LFD running buffer (Pi Bioscientific). Protein concentrations for reference materials used in the LOD and dynamic range testing were determined by combustion analysis using a Dumas FP-328 instrument (Leco Corporation, St. Joseph, MI) and the coefficients 5.28 for nuts and 5.50 for soy to convert nitrogen values to calculated protein values. In other instances, protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Wilmington, DE), with bovine serum albumin (BSA) as a reference standard. Affinity-purified polyclonal antibodies (pAbs) against the five targets were obtained from Pi Bioscientific. The pAbs against the individual targets were raised separately in goats, purified from serum on protein G columns, and then subsequently purified on columns (AKTA Prime FPLC, GE Healthcare Life Sciences, Pittsburgh, PA) with agarose beads conjugated with almond milk protein, cashew milk protein, coconut milk protein, hazelnut milk protein, or soy milk protein (Agarose Bead Technologies, Tampa, FL). To make protein-agarose columns for affinity purification, agarose beads were glyoxalated, periodate oxidized, and then conjugated to primary amines present on target proteins. The ensuing matrices were rinsed and packed into the chromatographic columns.

Preparation of gold conjugates. Citrate-capped 40-nm gold nanoparticles were obtained from Pi Bioscientific. The affinity-purified pAbs were individually diluted in borate buffer to a final concentration of 0.1 mg/ml, and then 7.5 ml was added to 250 ml of gold nanoparticles ($A_{530} = 1$) in a dropwise fashion while stirring for 30 min. To block, 2.5 ml of 10% BSA (in borate buffer) was added, and the colloid was pelleted by centrifugation at 3,000 $\times g$ for 1.5 h. Spectral analysis was performed on the resuspended soft pellet, and the absorbance was adjusted to a final reading of $A = 20$ (at the absorption maxima) using 1% BSA plus 10% sucrose in 8 mM borate buffer.

Preparation of LFDs. Nitrocellulose membrane (Sartorius, Goettingen, Germany) was lined with affinity-purified antibodies for each of the targets to prepare the sandwich format test (T1) line; almond proteins, cashew proteins, coconut proteins, hazelnut proteins, and soy proteins for the competitive format test (T2) line; and chicken anti-goat antibodies (Pi Bioscientific) for the procedural control (PC) line using an IsoFlow Reagent Dispenser (Imagene Technology, Hanover, NH). To prepare the conjugate pad, the gold conjugates were sprayed on strips of glass fiber conjugate pad material (Ahlstrom, Mt. Holly Springs, PA) using the IsoFlow Dispenser. To assemble the test strips, the nitrocellulose membrane, conjugate pad, sample pad (Ahlstrom, Mt. Holly Springs, PA), and absorbent pad (Advanced Micro Devices, Mumbai, India) were adhered to the adhesive laminate of the backing card (Lohmann, Precision Die Cutting, San Jose, CA) with overlapping surfaces to ensure continuous capillary transfer. The assembled cards were then cut into 5-mm-wide strips using a Matrix 2360 programmable shear (Kinematic Automation, Sonora, CA), housed in plastic cassettes (Advanced Micro Devices), and

stored with desiccant in sealed foil bags at room temperature until used. The LFD was configured such that the sample first encountered the T1 line, then the T2 line, and then the PC line.

Sample preparation. To prepare full-strength extracts, samples were mixed and homogenized and then diluted as follows: 1 ml in 9 ml of extraction buffer for liquids and 1 g in 10 ml of extraction buffer for solids. Samples were then extracted at 95°C using Allergen LFD buffer B (Pi Bioscientific) in a water bath for 1 min, the resulting extracts were cooled to room temperature and centrifuged ($\sim$2,500 $\times$ *g*) for 15 min to promote phase separation, and then 100 μl of the aqueous phase was collected and directly applied to the sample port of the LFD. For sensitivity and selectivity testing, serial dilutions of known protein concentrations or beverage volumes were made in buffer or a separate matrix (as indicated), extracted, and then tested.

Assay procedure. Before starting the assay, extraction buffer and LFDs were equilibrated to room temperature. Cooled sample extract (100 μl) was applied to the sample port of the LFD and allowed to hydrate the gold conjugate and wick across the nitrocellulose membrane. The sample was run for 15 min, and the results were read using an ESE-Quant Gold strip reader (Qiagen, Stockach, Germany).

Interpretation of results. Unless otherwise mentioned, the results reported are the mean (standard deviation) for three replicates performed by a single analyst. The measure of parts per million of protein can be used interchangeably with protein concentration in milligrams or micrograms per liter. The results of the assay were interpreted as follows. The T1 line (sandwich assay) will not appear in the absence of analyte, but the T2 line (competitive assay) will appear. When the analyte concentration is at or just above the LOD (1 ppm of protein), a clearly visible T1 line appears along with the T2 line. As the concentration of analyte increases (from 1 to 10 ppm), the T1 line also increases in intensity and the T2 line will begin to decrease in intensity. In general, above 10 ppm of analyte concentration, both T1 and T2 lines will decrease in intensity, with the T2 line disappearing at high analyte concentrations (generally >100 ppm). Thus, the T2 line indicates analyte detection when the target concentration is sufficiently high that prozone effects become problematic for the T1 line. Although not tested, the T2 test, which is a competitive format, allows for detection of target analyte that has undergone significant processing such that only single epitopes remain, addressing a limitation of the sandwich T1 line, which requires plural tandem epitopes to remain fully operational.

RANN scoring is a universal visual scoring system based on colorimetric intensity determined with a score card consisting of five lines of defined intensity, ranging from very faint to very intense. For basic assay parameter analyses, a strip reader value of 60 units (RANN 4) was used for determining the threshold for the T1 sandwich line, and strip reader values of less than 100 units were used to denote the threshold for the T2 competitive line. Strip reader values of 35 to 59 units indicated weak positive results to allow comparison with the Romer Labs (Getzersdorf, Austria) kit values, which rely on visual interpretation of RANN scoring, where RANN 2 is the threshold for a positive result at the test line.

RESULTS AND DISCUSSION

Sensitivity and dynamic range testing. The sandwich T1 line of all five LFD tests was assessed for the analytical LOD using known protein concentrations from each beverage diluted at log-fold serial dilutions in LFD extraction buffer. In each instance, 100 μl of each sample "extract" was applied to the sample port of the LFD, the test ran for 15 min, and the results were read using an electronic strip reader. For these experiments, the threshold of positivity was set at 60 units, which is when the test line starts becoming visible to the naked eye. At 0 ppm of the target analyte, none of the five LFD tests registered any signal at the T1 sandwich test line (Tables 1 through 5). Although spiked samples at 0.01 ppm gave varying results for the five LFD tests, those spiked at 0.1 ppm consistently registered signals >60 units at the T1 sandwich test line. To confirm this value, 0.01 and 0.1 ppm of target analyte (protein) concentrations were tested 10 times each by two independent analysts: signals $\geq$60 units were observed for 20 of 20 tests for the sandwich test line for each of the five LFD kits (data not shown). Therefore, the overall analytical LOD at the T1 sandwich test line for all five tests was set at 0.1 ppm of protein, which translates to 1 ppm in foods to account for the 10-fold dilution during sample preparation. Another key consideration was that the rate of disappearance of the T2 line (competitive assay) was faster than the increased density observed for the T1 line (sandwich assay) for each of the fives test kits across the dynamic range tested. This feature enables the operator to correctly distinguish very low target analyte concentrations from very high concentrations. However, the T2 competitive line was more variable in repeat testing than was the T1 line, as reflected in the standard deviations in Tables 1 through 5. Consequently, unlike the T1 line, which can be used for semiquantitative analysis when the kit is operated with the standard protein prep, the T2 line is more useful for confirming prozone effects at high target analyte concentrations, confirming the absence of hydrolyzed or heavily modified targets and potential lectin-mediated reactions, which cause false-positive results at the T1 line. The T1

TABLE 1. *Analytical sensitivity and dynamic range for almond milk LFD*[a]

Milk protein (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0	798 (61)	Negative
0.01	0	735 (34)	Negative
0.1	102 (6)	758 (50)	Positive
1	315 (15)	623 (25)	Positive
10	605 (40)	361 (7)	Positive
100	589 (82)	70 (1)	Positive[b]
1,000	334 (42)	0	Positive[b]

[a] Almond milk (Silk brand, unsweetened original) was diluted at indicated protein concentrations in extraction buffer, and then 100 μl was applied to the LFD. Results were read at 15 min using a strip reader. Means were calculated from single tests performed by two independent analysts. Threshold for determining positivity at the T1 sandwich line was set to 60 units.

[b] High concentrations of almond milk protein resulted in attenuation (strip reader value, <100) of the competitive test line 2.

TABLE 2. *Analytical sensitivity and dynamic range for cashew milk LFD*[a]

Milk protein (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0	587 (278)	Negative
0.01	42 (9)	603 (215)	Weak positive
0.1	160 (94)	567 (205)	Positive
1	324 (75)	494 (55)	Positive
10	428 (88)	253 (104)	Positive
100	291 (155)	49 (14)	Positive[b]
1,000	86 (7)	0	Positive[b]

[a] Cashew milk (So Delicious brand, unsweetened) was diluted at indicated protein concentrations in extraction buffer, and then 100 μl was applied to the LFD. Results were read at 15 min using a strip reader. Means were calculated from single tests performed by two independent analysts. Threshold for determining positivity at the T1 sandwich line was set to 60 units.

[b] High concentrations of cashew milk protein resulted in attenuation (strip reader value, <100) of the competitive test line 2.

TABLE 4. *Analytical sensitivity and dynamic range for hazelnut milk LFD*[a]

Milk protein (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0	373 (35)	Negative
0.01	123 (53)	420 (28)	Positive
0.1	259 (98)	394 (189)	Positive
1	286 (94)	214 (66)	Positive
10	212 (35)	62 (2)	Positive[b]
100	91 (11)	0	Positive[b]
1,000	0	0	Positive[b]

[a] Hazelnut milk (Pacific Foods brand, original) was diluted at indicated protein concentrations in extraction buffer, and then 100 μl was applied to the LFD. Results were read at 15 min using a strip reader. Means were calculated from single tests performed by two independent analysts. Threshold for determining positivity at the T1 sandwich line was set to 60 units.

[b] High concentrations of hazelnut milk protein resulted in attenuation (strip reader value, <100) of the competitive test line 2.

versus T2 values were plotted as a function of target analyte concentration to demonstrate how the combined electronic values can be used in semiquantitative analysis (Fig. 1).

Cross-reactivity analysis. To determine the specificity of each assay, full-strength extracts were prepared from a panel of select commodities using extraction buffer. Limited cross-reactivity was detected, with an occasional weak signal (<40 units) inconsistently reported by the analysts as reflected by the variability of the separate tests (Table 6). Significant and reportable (above threshold) cross-reactivity was observed for kidney bean extract with the almond milk, cashew milk, coconut milk, and soy milk LFDs at the T1 line (sandwich assay) only, most likely reflecting lectin-mediated bridging of the antibody printed on the membrane and the antibody conjugated to the gold nanoparticles. Significant cross-reactivity was observed for sesame extract with the hazelnut milk LFD. These cross-reactivities fully disappeared when the kidney bean or sesame extracts were diluted 1/100 before testing (data not shown). Very low level cross-reactivity was observed for egg and mung bean full-strength extracts with the soy milk LFD. With the exception of the almond milk LFD, the other LFDs were fully overloaded by samples derived from native cognate targets; raw cashew, raw coconut, raw hazelnut, and raw soy extracts extinguished the competitive T2 line (data not shown), with

TABLE 3. *Analytical sensitivity and dynamic range for coconut milk LFD*[a]

Milk protein (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0	748 (109)	Negative
0.01	15 (21)	752 (76)	Negative
0.1	114 (22)	722 (43)	Positive
1	152 (18)	571 (58)	Positive
10	141 (21)	294 (81)	Positive
100	77 (6)	65 (2)	Positive[b]
1,000	30 (42)	0	Positive[b]

[a] Coconut milk (So Delicious brand, original) was diluted at indicated protein concentrations in extraction buffer, and then 100 μl was applied to the LFD. Results were read at 15 min using a strip reader. Means were calculated from single tests performed by two independent analysts. Threshold for determining positivity at the T1 sandwich line was set to 60 units.

[b] High concentrations of coconut milk protein resulted in attenuation (strip reader value, <100) of the competitive test line 2.

TABLE 5. *Analytical sensitivity and dynamic range for soy milk LFD*[a]

Milk protein (ppm)	Mean (SD) strip reader value: Test line 1 (sandwich)	Mean (SD) strip reader value: Test line 2 (competitive)	Result
Blank	0	743 (22)	Negative
0.01	50 (7)	746 (14)	Weak positive
0.1	124 (3)	675 (22)	Positive
1	365 (50)	554 (16)	Positive
10	374 (41)	250 (7)	Positive
100	214 (53)	48 (1)	Positive[b]
1,000	65 (13)	0	Positive[b]

[a] Soy milk (Silk brand, original) was diluted at indicated protein concentrations in extraction buffer, and then 100 μl was applied to the LFD. Results were read at 15 min using a strip reader. Means were calculated from single tests performed by two independent analysts. Threshold for determining positivity at the T1 sandwich line was set to 60 units.

[b] High concentrations of soy milk protein resulted in attenuation (strip reader value, <100) of the competitive test line 2.

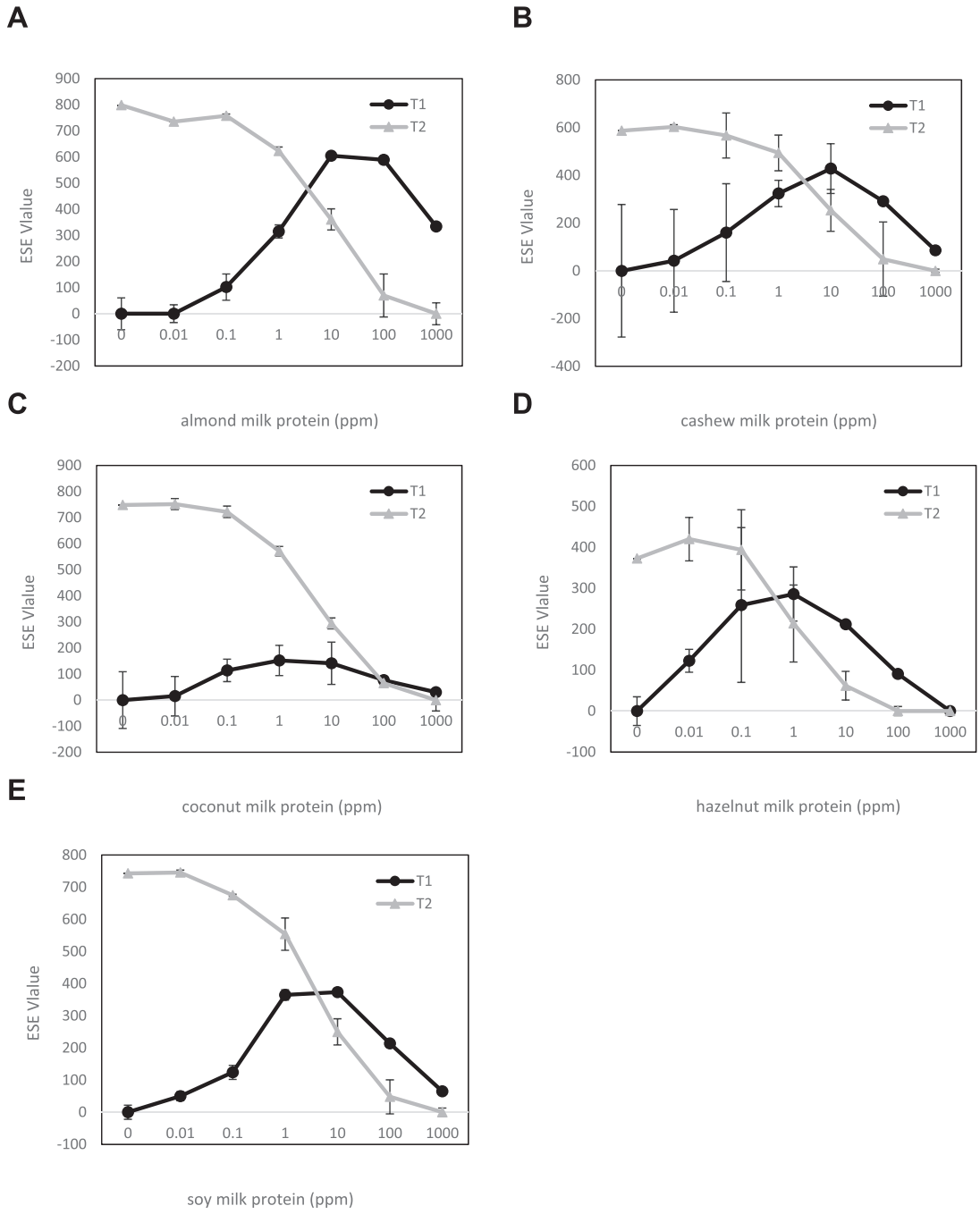

FIGURE 1. *T1 and T2 ESE values as a function of target analyte concentration for (A) almond milk, (B) cashew milk, (C) coconut milk, (D) hazelnut milk, and (E) soy milk LFDs.*

near total loss of signal at the sandwich T1 line. In contrast, the almond milk LFD produced a significant signal (121 [4] units) at the sandwich T1 line for raw almond extract. Overall, the dynamic range testing for all five tests was suitable.

Spiking and matrix effects. The selectivity of each LFD assay was assessed by spiking each nondairy beverage into soy milk and by spiking soy milk into almond milk. Serial dilutions were made and extracted at desired dilutions 1/9 in LFD extraction buffer for 1 min at 95°C. To broadly assess different products, brands, and processing treatments, as many products as were available were tested using cognate LFD assays. Assessment of protein parts per million for the spiked samples was calculated based on the protein estimate indicated on the product label; BCA values were also reported but were not considered in our analyses because these values were generally higher than the manufacturers' reported protein concentrations. This increase is likely due to the presence of complex polysaccharides such as starch and additives such as locust gum, gellan gum, xanthan gum, guar gum, and carrageenan, which are known to interfere with most protein quantification assays. With the LFDs, 24 of the 25

TABLE 6. *Specificity analysis of full-strength extracts*[a]

Food	Mean (SD) strip reader value at T1 sandwich line				
	Almond milk	Cashew milk	Coconut milk	Hazelnut milk	Soy milk
Lima bean	0	0	0	10 (17)	0
Mung bean	0	0	0	0	15 (13)
Green pea	0	10 (17)	0	0	0
Lupin	0	0	0	0	0
Kidney bean	369 (52)	380 (31)	245 (56)	0	433 (33)
Adzuki bean	0	0	0	0	0
Coconut	0	0	Overloaded[b]	0	0
Chick pea	0	0	0	0	0
Poppy seed	0	0	0	0	0
Banana	0	0	0	7 (11)	0
Apple	0	0	0	0	0
Raw chicken	0	0	0	0	0
Raw beef	0	0	0	0	0
Sesame seed	0	0	0	249 (45)	0
Peanut	0	0	0	0	6 (10)
Almond	Overloaded	0	0	0	0
Brazil nut	0	10 (17)	0	0	0
Macadamia nut	0	0	5 (9)	0	17 (24)
Pine nut	0	0	8 (14)	0	0
Walnut	0	0	0	8 (14)	0
Hazelnut	0	0	22 (31)	Overloaded	17 (24)
Cashew nut	0	Overloaded	0	0	0
Pistachio nut	0	0	0	0	0
Soybean	0	0	0	5 (9)	Overloaded
Celery seed	0	0	0	0	0
Mustard	0	0	0	0	0
Milk	0	0	0	0	0
Egg	0	0	0	0	35 (<1)

[a] Extracts were prepared from a panel of common commodities using the Pi Bioscientific extraction buffer (1 g in 10 ml of buffer, 1 min at 95°C) and then applied directly to the LFD. Results were read at 15 min using a strip reader. Means were calculated from triplicate tests performed by a single analyst. Threshold for determining positivity at the T1 sandwich line was set to 60 units except where the T2 competitive line was overloaded. Results for only T1 are reported.

[b] Overloaded indicates that full-strength extract resulted in complete overload of T2 competitive test line (data not shown); T1 sandwich test line value was zero or close to zero due to the presence of extremely high concentrations of target analyte.

almond milk products tested (all except the 365 Organic original, with no protein listed on the label) were detected at a 1/1,000 dilution in soy milk, where estimated almond concentrations (based on food labels) were 4.2 to 20.8 ppm (Table 7). Blue Diamond almond milk in refrigerated (non-UHT) and shelf-stable (UHT) preparations were further tested at 1/10,000 dilutions (estimated 0.4 ppm of protein); both products produced low positive results at this low concentration, indicating no significant differences between shelf-stable and non-UHT product allergens in terms of detectability.

The cashew milk LFDs were tested using two products, So Delicious and Silk cashew milks, at 1/1,000 dilution with estimated 0 to 4.2 ppm based on label information (Table 8). In both instances, the LFD was able to detect cashew contamination in the soy milk background.

The coconut milk LFD was tested using nine coconut milk products containing vastly different amounts of protein based on label information (Table 9). Of these products, five tested positive and four tested negative at a 1/10 dilution, independent of estimated protein concentration, suggesting the following possibilities: (i) inconsistent labeling information, (ii) vastly different processing methods, and (iii) significant influence of thickening agents such as carrageenan and guar gum, which are routinely added to coconut milk and thus impact the fluidics of the assay. Three of the coconut milk products (Pacific Organic brand) listed 0% protein on the labels but tested positive for coconut milk proteins at 1/10 dilution, one product (365 Organic Light) tested positive at 1/100 dilution.

The hazelnut milk LFD was tested using two brands, Pacific Organic and Alpro, with strong signals obtained at 1/1,000 sample dilution (estimated 4 to 8.3 ppm of protein based on labels) (Table 10). The soy milk LFD was tested using 19 products at a 1/5,000 dilution, equaling 0 to 8.3 ppm of protein based on the label information (Table 11). Four of these products failed to register a positive test signal at the T1 sandwich line: Silk formulated with dark chocolate (high polyphenol content), a culinary kitchen product

TABLE 7. *Selectivity analysis for various almond milks spiked into soy milk*[a]

Almond milk brand (dilution)	Estimated protein from label (ppm)	BCA "almond" protein (ppm)	Mean (SD) strip reader value		Result
			Test line 1 (sandwich)	Test line 2 (competitive)	
Blank (soy milk alone)	0	NT[b]	0	757 (16)	Negative
Simple Truth, unsweetened, UHT (1/1,000)	4.2	7.8	145 (12)	500 (18)	Positive
Simple Truth, chocolate (1/1,000)	4.2	7.3	73 (5)	502 (22)	Positive
Silk, unsweetened, original (1/1,000)	4.2	8.5	66 (4)	478 (25)	Positive
Silk, vanilla (1/1,000)	4.2	9.3	116 (7)	499 (43)	Positive
Silk, original (1/1,000)	4.2	8.4	135 (11)	492 (34)	Positive
Silk, dark chocolate (1/1,000)	4.2	8.1	45 (2)	496 (23)	Weak positive
Silk, unsweetened, vanilla (1/1,000)	4.2	8.5	86 (4)	529 (36)	Positive
Silk, original, almond-coconut blend (1/1,000)	<4.2	NT	98 (10)	565 (37)	Positive
Pacific Organic, original, UHT (1/1,000)	4.2	8.4	68 (8)	528 (33)	Positive
Pacific Organic, almond-coconut blend (1/1,000)	4.2	NT	119 (17)	569 (36)	Positive
Pacific Organic, original, unsweetened UHT (1/1,000)	4.2	7.3	50 (3)	484 (27)	Weak positive
Pacific Organic, vanilla UHT (1/1,000)	4.2	5.6	49 (4)	546 (48)	Weak positive
Pacific Organic, original, unsweetened UHT (1/1,000)	4.2	5.2	45 (6)	597 (33)	Weak positive
So Delicious, 5× protein, original (1/1,000)	20.8	23.6	75 (5)	606 (44)	Positive
So Delicious, 5× protein, unsweetened UHT (1/1,000)	20.8	24.4	68 (6)	585 (38)	Positive
So Delicious, 5× protein, vanilla, UHT (1/1,000)	20.8	20.4	64 (5)	591 (48)	Positive
Califia, creamy, original (1/1,000)	4.2	10.3	98 (4)	527 (23)	Positive
Califia, original, almond-coconut blend (1/1,000)	4.2	NT	110 (19)	560 (25)	Positive
Alpro, original (1/1,000)	5	8.1	85 (10)	527 (33)	Positive
Alpro, dark chocolate (1/1,000)	8	6.8	43 (6)	521 (33)	Weak positive
Blue Diamond, original non-UHT (1/1,000)	4.2	8.1	137 (10)	532 (42)	Positive
Blue Diamond, original, non-UHT (1/10,000)	0.41	0.8	50 (2)	473 (43)	Weak Positive
Blue Diamond, original, UHT (1/10,000)	0.41	1.0	47 (2)	500 (33)	Weak positive
Blue Diamond, almond-coconut blend, UHT (1/1,000)	4.2	NT	115 (7)	543 (27)	Positive
365 Organic, original (1/1,000)	0	NT	0	544 (50)	Negative

[a] Almond milks were serially log-fold diluted in soy milk (Silk brand, original), extracted in LFD buffer (1 ml in 9 ml of buffer, 1 min at 95°C), and then tested with the almond milk LFD. Means were calculated from triplicate tests performed by a single analyst. Threshold for determining positivity at the T1 sandwich line was set to 35 units (RANN 2).
[b] NT, not tested.

(thickening agent), a red fruit soy product (low pH), and Pacific select soy original (which contains carrageenan).

Despite these few limitations, overall each of the five LFD assays generally resisted the effects of the matrix (other nondairy beverages). Sensitivity of the LFDs was affected by dark chocolate, acidic pH, and thickening agents. None of the system subcomponents cross-reacted with nontarget nondairy beverages at full strength except in one instance, where contamination of soy milk with almond was established by PCR (data not shown). This analysis included a broad array of products, subjected to various processing treatments and with commodities from different sources. The findings suggest that these LFDs are suitable for use in testing different types of plant-based, nondairy products, both finished products and residues that might be present on manufacturing surfaces.

TABLE 8. *Selectivity analysis for various cashew milks spiked into soy milk*[a]

Cashew milk brand (dilution)	Estimated protein from label (ppm)	BCA "cashew" protein (ppm)	Mean (SD) strip reader value		Result
			Test line 1 (sandwich)	Test line 2 (competitive)	
Blank (soy milk alone)	0	NT[b]	0	493 (32)	Negative
So Delicious, vanilla, unsweetened (1/1,000)	0	3.3	92 (10)	251 (25)	Positive
Silk, original (1/1,000)	<4.2	3.1	109 (11)	274 (23)	Positive

[a] Cashew milks were serially log-fold diluted in soy milk (Silk brand, original), extracted in LFD buffer (1 ml in 9 ml of buffer, 1 min at 95°C), and then tested with the cashew milk LFD. Means were calculated from triplicate tests performed by a single analyst. Threshold for determining positivity at the T1 sandwich line was set to 35 units (RANN 2).
[b] NT, not tested.

TABLE 9. *Selectivity analysis for various coconut milks spiked into soy milk*[a]

Coconut milk brand (dilution)	Estimated protein from label (ppm)	BCA "coconut" protein (ppm)	Mean (SD) strip reader value		Result
			Test line 1 (sandwich)	Test line 2 (competitive)	
Blank (soy milk alone)	0	NT[b]	0	479 (22)	Negative
Pacific Organic, original, unsweetened, UHT (1/10)	0	0.6	45 (4)	302 (19)	Weak positive
Pacific Organic, vanilla, unsweetened, UHT (1/10)	0	0.6	41 (3)	306 (18)	Weak positive
Pacific Organic, original, UHT (1/10)	0	0.8	21 (18)	297 (49)	Negative
Simple Truth, unsweetened (1/10)	416	0.9	0	451 (43)	Negative
Alpro, original (1/10)	100	0.7	73 (5)	332 (20)	Positive
Alpro, dark chocolate (1/10)	400	0.6	0	465 (21)	Negative
So Delicious, original (1/10)	0	NT	0	401 (14)	Negative
365 Organic, light (1/100)	0	0.9	51 (5)	350 (23)	Weak positive
365 Organic, original (1/100)	155	0.9	48 (6)	274 (22)	Weak positive

[a] Coconut milks were serially log-fold diluted in soy milk (Silk brand, original), extracted in LFD buffer (1 ml in 9 ml of buffer, 1 min at 95°C), and then tested with the coconut milk LFD. Means were calculated from triplicate tests performed by a single analyst. Threshold for determining positivity at the T1 sandwich line was set to 35 units (RANN 2).
[b] NT, not tested.

Method concordance. Method concordance was assessed using two separate approaches. In the first approach, the performance of a soy LFD assay developed against soy flour protein (Pi Bioscientific) was compared with that of a soy milk LFD using highly processed soy residues derived from soy protein concentrate, soy protein isolate, and soy milk with soy flour extract used as a control (Table 12). Soy protein concentrate (SPC) is frequently incorporated into baked goods, breakfast cereal, some meat products, and pet foods. Soy protein isolate (SPI) is commonly used to improve the texture of meat products, as an emulsifying agent, and as a protein supplement in health foods. The rationale for testing these foods in this manner is based on the fact that the processing of soy concentrate and isolate involves several key steps that are shared with soy milk processing, including exposure to extreme pH. Consequently, soy residues derived from concentrate and isolate are not as easily detected with most immunodiagnostic kits as are soy residues derived from soy milk. Both kits had an LOD of 1 ppm for soy protein derived from soy flour. The sensitivity of the soy LFD was ~500 ppm for SPI and ~100 ppm for SPC. In comparison, the sensitivity of the soy milk LFD was ~50 ppm for SPI and ~10 ppm for SPC, a roughly 10-fold improvement in detection of each target. Likewise, the soy milk LFD was more reliable than the soy LFD for detecting soy milk, although both tests were comparable for detection of soy flour protein. Although the soy milk LFD was not ideal for detection of SPC and SPI, it was a much better diagnostic tool for detection of SPI- and SPC-modified soy residues than the soy LFD that was developed using pAbs raised against soy flour proteins.

The second method concordance analysis involved comparison of the soy milk LFD with a commercial soy LFD test purchased from Romer Labs (Table 13). Soy milk formulations (Alpro brand original, dark chocolate, red fruit punch, and culinary) were serially diluted in buffer and then extracted 1/10 in respective extraction buffers and tested with the soy milk and Romer soy LFD strips. Tests were performed in triplicate, with visual qualitative scores assigned to the Romer soy LFDs and electronic values measured for the soy milk LFD. Visual scoring for the Romer strips was necessary because of the generation of false-positive signals that evolve at the T1 line shortly after 5 min and because this assay does not incorporate a competitive assay (T2) line. Although the Romer soy LFD is supposed to have a 2.0-ppm LOD for soy protein, the test

TABLE 10. *Selectivity analysis for various hazelnut milks spiked into soy milk*[a]

Hazelnut milk brand (dilution)	Estimated protein from label (ppm)	BCA "hazelnut" protein (ppm)	Mean (SD) strip reader value		Result
			Test line 1 (sandwich)	Test line 2 (competitive)	
Blank (soy milk alone)	0	NT[b]	10 (17)	402 (20)	Negative
Pacific Organic, original (1/1,000)	8.3	14.6	215 (18)	278 (19)	Positive
Alpro, original (1/1,000)	4	9.2	127 (7)	274 (23)	Positive

[a] Hazelnut milks were serially log-fold diluted in soy milk (Silk brand, original), extracted in LFD buffer (1 ml in 9 ml of buffer, 1 min at 95°C), and then tested with the hazelnut milk LFD. Means were calculated from triplicate tests performed by a single analyst. Threshold for determining positivity at the T1 sandwich line was set to 35 units (RANN 2).
[b] NT, not tested.

TABLE 11. *Selectivity analysis for various soy milks spiked into almond milk*[a]

Soy milk brand (dilution)	Estimated protein from label (ppm)	BCA "soy" protein (ppm)	Mean (SD) strip reader value		Result
			Test line 1 (sandwich)	Test line 2 (competitive)	
Blank (almond milk alone)	0	NT[b]	0	593 (33)	Negative
Simple Truth, unsweetened, UHT (1/5,000)	5.8	4.8	65 (6)	570 (21)	Positive
Simple Truth, chocolate (1/5,000)	4.2	6.0	43 (4)	573 (24)	Weak positive
Simple Truth, vanilla (1/5,000)	5	4.7	51 (5)	568 (24)	Weak positive
Pacific, original (1/5,000)	8.3	4.5	48 (4)	620 (18)	Weak positive
Pacific, ultra soy, original (1/5,000)	8.3	6.6	47 (3)	568 (31)	Weak positive
Pacific, select soy, original (1/5,000)	4.2	4.0	0	521 (20)	Negative
Westsoy, vanilla, unsweetened, UHT (1/5,000)	7.5	6.5	48 (3)	588 (32)	Weak positive
Westsoy, plus, vanilla, UHT (1/5,000)	6.7	5.3	41 (3)	585 (16)	Weak positive
Westsoy, original, unsweetened, UHT (1/5,000)	7.5	6.0	73 (5)	581 (16)	Positive
Silk, original (1/5,000)	6.7	5.5	89 (7)	606 (15)	Positive
Silk, vanilla (1/5,000)	5	4.9	44 (10)	550 (31)	Weak positive
Silk, light, original (1/5,000)	5	4.8	50 (5)	560 (26)	Weak positive
Silk, light, chocolate (1/5,000)	2.5	4.8	22 (19)	619 (30)	Negative
Alpro, original (1/5,000)	6	4.6	46 (4)	602 (19)	Weak positive
Alpro, chocolate (1/5,000)	6.2	9.1	73 (11)	636 (19)	Positive
Alpro, red fruit (1/5,000)	5.2	4.4	0	586 (20)	Negative
Alpro, culinary kitchen (1/5,000)	4	6.5	8 (14)	611 (31)	Negative
365 Organic, original, UHT (1/5,000)	5	6.3	69 (8)	555 (23)	Positive
365 Organic, vanilla, UHT (1/5,000)	5	3.7	50 (5)	621 (18)	Weak positive

[a] Soy milks were serially log-fold diluted in almond milk (Silk brand, unsweetened original), extracted in LFD buffer (1 ml in 9 ml of buffer, 1 min at 95°C), and then tested with the soy milk LFD. Means were calculated from triplicate tests performed by a single analyst. Threshold for determining positivity at the T1 sandwich line was set to 35 units (RANN 2).
[b] NT, not tested.

TABLE 12. *Method concordance for soy milk LFD versus soy LFD for highly processed soy protein residues derived from soy protein isolate, soy protein concentrate, and soy milk*[a]

Commodity	Concn (ppm)	Mean (SD) soy LFD[b]			Mean (SD) soy milk LFD[c]		
		Test line 1 (sandwich)	Test line 2 (competitive)	Result	Test line 1 (sandwich)	Test line 2 (competitive)	Result
Soy protein isolate	10	0	280 (22)	Negative	0	535 (24)	Negative
	50	0	262 (13)	Negative	115 (9)	481 (19)	Positive
	100	0	254 (16)	Negative	117 (13)	398 (27)	Positive
	500	51 (5)	249 (46)	Weak positive	190 (10)	183 (14)	Positive
	1,000	58 (5)	211 (21)	Weak positive	156 (8)	127 (16)	Positive
Soy protein concentrate	10	0	305 (19)	Negative	44 (8)	564 (9)	Weak positive
	50	0	244 (7)	Negative	106 (16)	476 (22)	Positive
	100	47 (5)	281 (18)	Weak positive	176 (11)	414 (8)	Positive
	500	69 (7)	208 (28)	Positive	176 (22)	212 (14)	Positive
	1,000	71 (12)	198 (10)	Positive	136 (25)	118 (15)	Positive
Soy milk	1	0	215 (10)	Negative	0	593 (26)	Negative
	5	19 (17)	334 (12)	Negative	46 (4)	566 (39)	Weak positive
	10	55 (10)	309 (21)	Weak positive	103 (12)	600 (12)	Positive
	20	59 (6)	280 (17)	Weak positive	128 (6)	552 (34)	Positive
Soy flour	1	60 (4)	349 (13)	Weak positive	70 (5)	557 (49)	Positive
	5	113 (11)	201 (32)	Positive	77 (2)	472 (42)	Positive
	10	128 (8)	244 (18)	Positive	111 (10)	528 (24)	Positive
	20	156 (9)	205 (40)	Positive	120 (4)	435 (8)	Positive
Blank	0	0	299 (23)	Negative	0	573 (23)	Negative

[a] Proteins (1 ml of 1,000 ppm in 9 ml of buffer, 1 min at 95°C) were prepared in extraction buffer and then serially diluted in the same buffer and used for the soy LFD and soy milk LFDs. Means were calculated from triplicate tests performed by a single analyst. Threshold for determining positivity at the T1 sandwich line was set to 35 units (RANN 2).
[b] Analytical LOD (limit of detection) is 1 ppm of soy protein in food.
[c] Analytical LOD is 1 ppm of soy milk protein in food.

TABLE 13. *Method concordance for the soy milk LFD versus a commercial soy LFD kit (Romer Labs) using various Alpro brand soy milks diluted into the respective kit extraction buffers, extracted, and then applied and read as per the instructions supplied with each kit*[a]

Commodity	Dilution	Estimated protein from label (ppm)	Romer soy kit[b]			Soy milk kit[c]			
Soy Drink Original	1/10	3,000	NT	NT	NT	392, 0	285, 39	408, 0	Pos
	1/100	300	NT	NT	NT	294, 268	356, 282	418, 259	Pos
	1/1,000	30	Pos	Pos	Pos	218, 539	211, 549	203, 425	Pos
	1/5,000	6	Neg	Neg	Neg	83, 527	83, 557	73, 574	Pos
	1/10,000	3	NT	NT	NT	45, 640	59, 596	43, 596	Weak pos
Soy Drink Chocolate	1/10	3,100	NT	NT	NT	225, 40	210, 0	205, 38	Pos
	1/100	310	NT	NT	NT	402, 235	336, 232	330, 249	Pos
	1/1,000	31	Pos	Pos	pos	227, 534	269, 501	228, 512	Pos
	1/10,000	3	Neg	Neg	Neg	83, 498	118, 556	84, 570	Pos
Soy Drink Red Fruit	1/10	2,600	NT	NT	NT	385, 316	383, 435	319, 353	Pos
	1/100	260	NT	NT	NT	211, 537	193, 548	207, 523	Pos
	1/1,000	26	Neg	Neg	Neg	120, 647	112, 689	130, 696	Pos
	1/4,000	6.5	Neg	Neg	Neg	65, 657	65, 621	70, 621	Pos
	1/10,000	2.6	NT	NT	NT	0, 643	0, 612	0, 686	Neg
Soy Drink Culinary Kitchen	1/10	2,000	NT	NT	NT	266, 0	323, 41	348, 44	Pos
	1/100	200	NT	NT	NT	283, 268	361, 261	298, 279	Pos
	1/1,000	20	Neg	Neg	Neg	226, 567	127, 531	130, 543	Pos
	1/5,000	4	Neg	Neg	Neg	68, 602	59, 585	61, 495	Pos
	1/10,000	2	NT	NT	NT	47, 589	46, 645	42, 620	Weak pos

[a] For the soy milk LFD, triplicate tests were performed by a single analyst, and results were read using a strip reader. The threshold for determining positivity at the T1 sandwich line was set to 35 units (RANN 2). For the Romer soy LFD, triplicate tests were performed by a single analyst, and results were read visually using a RANN score of 2 for the threshold of positivity; electronic reads were not feasible because of the formation of a false-positive signal after 5 min at the test line. NT, not tested; Pos, positive result; Neg, negative result.
[b] Analytical LOD (limit of detection) is 1 ppm of soy protein in food.
[c] Analytical LOD is 1 ppm of soy milk protein in food. Replicate test results for T1 sandwich and T2 competitive test lines are shown.

could only weakly detect soy milk proteins in the range of 20 to 30 ppm (RANN 2, which equals approximately 35 to 40 units on the electronic strip reader). In comparison, the soy milk LFD detected soy residues down to 2 to 3 ppm, with the exception of the soy red fruit drink, in which residues were detected down to ~6.5 ppm. Thus, the soy milk LFD sensitivity was higher than that of the commercial Romer test kit by ≥10-fold, with the added benefit of an improved design, i.e., inclusion of a competitive test element, housing cassettes, and resistance to false-positive results, which can complicate interpretation.

ACKNOWLEDGMENTS

We thank Drs. Walter Hill and Steven Gendel for assistance in manuscript preparation.

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