

Immunoassay Platform for Casein Antigens in Hydrolyzed Casein-Based Infant Formula

Christopher Butler G¹, Michael B Miklus¹, Cynthia M Barber², Steven M Tennyson¹, Pedro A Prieto^{1,2*}

¹Perrigo Nutritionals R&D, 147 Industrial Park Road, Georgia Vermont, 05468, USA; ²Perrigo Nutritionals R&D, 652 Peter Jefferson Parkway, Charlottesville Virginia, 22911, USA

ABSTRACT

Hypoallergenic formulas are the sole source of nutrition for infants that are either allergic to milk proteins or at risk of developing allergies. Strategies to provide nutritional sustenance, while preventing allergic reactions, include designing formulas based on extensively hydrolyzed casein which is presumably devoid of antigenic epitopes. Assays devoted to the assessment of antigenic protein motifs are crucial to verify the absence of relevant antigens in formulas and the raw materials used in their preparation. Evaluation of commercial immunoassay kits intended for the detection of milk proteins in foods led to the conclusion that a specific assay for extensively hydrolyzed casein-based formulas was necessary to improve allergen recoveries and assay consistency. The purpose of this investigation was to develop a reproducible path, from the generation of antibodies to the pre-validation of immunoassays optimized for the analysis of hydrolyzed casein-based infant formula. We prepared purified antisera from sheep immunized with bovine acid-precipitated casein to establish a platform consisting of a slot blot immunoassay and an enzyme-linked immunosorbent assay. Results indicate that sheep can reliably produce antibodies against epitopes in the casein fraction of bovine milk, thus providing a quantitative reagent that binds to immobilized casein in different formats. The limits of detection and quantitation for standard solutions for the enzyme-linked immunoassay were 0.8 and 2.5 ppm, respectively. The limit of detection in the extensively hydrolyzed casein-based formula was 0.5 ppm and the limit of quantitation 1.4 ppm. This account describes two reproducible immunoassays that are accessible to any laboratory or manufacturing setting and do not require proprietary ingredients or undisclosed extraction solutions. While these tests were developed to quantitate casein in hypoallergenic formula matrices, an application of the slot blot immunoassay to assess residual casein on manufacturing surfaces is also described in the present account.

Keywords: Infant formula; Infant nutrition; Milk allergies; Hydrolyzed casein; Hydrolyzed milk; Hydrolyzed formulas; Casein ELISA; Food analysis

INTRODUCTION

Infant formulas based on extensively hydrolyzed milk proteins emerged to satisfy the nutritional needs of infants who have been diagnosed with milk allergies or who are at risk of developing them [1-3]. Several risk factors have been identified for cow's milk allergy (CMA) being family history one of most salient [4,5] although diagnosed CMA cases are increasing in certain communities which also points to environmental factors [4,6]. Diagnosis of CMA range from skin prick tests and response to oral challenges, to detection of relevant immunoglobulins [7-9]; once a diagnosis has been established, or high-risk has been determined, the health care professional has to determine a course of action. Strategies to provide nutritional sustenance, while preventing

allergic reactions, include feeding formulas based on extensively hydrolyzed casein (EHC) or extensively hydrolyzed whey (EHW) which are presumably devoid of antigenic epitopes. The final demonstration of the immunological suitability of a hypoallergenic formula is ascertained through clinical trials, which are expensive and difficult to conduct due to the limited availability of allergy-prone infants as study subjects [3,10-12]. In addition to, or as part of clinical experiences, biochemical analyses can be used to provide molecular-based evidence of reactivity towards food antigens by assessing the ability of immunoglobulins from human sera to bind antigenic proteins blotted in nitrocellulose [13,14]. These experiments illustrate the versatility of immunological methods which can reveal links between the composition of foods and immunoglobulin repertoires in humans while also

Correspondence to: Pedro Antonio Prieto, Ph.D, VP Nutrition R&D, Global Scientific Affairs, 652 Peter Jefferson Parkway, Charlottesville, Virginia 22911, USA, Tel: (434) 297 1104, (434) 446 9229, 36-1104; E-mail: pedro.prieto@perrigo.com

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confirming allergy diagnoses. The study by Hochwallner [14], used sera from several subjects to determine the reactivity of their IgEs towards different hydrolyzed protein-based infant formulas and explored the relationship of the degree of protein hydrolysis with allergenicity. These studies exemplify the sensitivity and accuracy of immunoassays based on reagents that recognize antigenic determinants immobilized in inert matrices. The same principles used to design and refine immunoassays in the medical field have been used for decades to assure food safety regarding the presence of antigens in hypoallergenic and other products.

Scientists have developed and tested different immunological methods to detect and characterize allergens in food products and raw materials. Bardran et al. [15] and other researchers have developed immunoassays to determine the presence and quantity of antigenic proteins in different food matrices; casein and whey from bovine milk were amongst the targets of these studies. Analyses developed by Planque et al. [16] were also designed to test simultaneously for a spectrum of known antigens including those from peanut, milk, and egg. Assays are needed to detect food antigens even when the composition of a food product or its manufacturing process theoretically preclude the presence of antigenic epitopes. These tests act as safeguards that are especially relevant when the foods in question are intended for consumption by a vulnerable population as in the case of hypoallergenic infant formula.

The analysis of sensitive products frequently requires ad hoc matrix treatments and reagents that may or may not be suitable for general food applications. For example, Ivens et al. [17] compared several commercially available ELISA kits regarding their performance on nonfat dry milk (NFDM), purified caseins (α , β , and κ), β -lactoglobulin, different whey protein concentrates, and sodium caseinate. This study noted that in many cases different commercial kits designed to detect the same allergen, target different proteins. Since the kits use antibodies raised against various milk proteins or fractions, it is not surprising that they perform differently. If an antibody is raised against purified β -lactoglobulin, it may not cross react with casein. Furthermore, some anti-casein antibodies do not recognize κ -casein and others have a low affinity for α - or β -casein. When these assays are used to quantify NFDM, their performance is not consistent, precisely due to the binding predilections of different antibodies. While it is true that calibration may support validation of particular immunoassay applications, Ivens and coworkers [17] concluded that a consensus adoption of a standard reference would allow for a fair comparison of different kits. To our knowledge, none of the commercially available kits were designed specifically for the detection and quantitation of residual antigenic determinants in infant formulas based on extensively hydrolyzed casein.

Isolated bovine β -lactoglobulin has also been studied as an allergen targeted by immunoassays due to its allergenicity in humans. He et al. [18] raised polyclonal rabbit antibodies versus tandem-expressed lactoglobulin epitopes recognized by Human IgE and used them to detect and quantify this bovine milk protein [19]. The elegant experiments of He et al. provide the fundamentals for tests that are aimed at the detection of residual antigens from a single bovine milk protein and could be applied to different matrices. While this test could be useful for products that contain whole milk or whey-based proteins (such as infant formulas containing EHW), it has

limited application for products based on EHC or for products that can be contaminated with casein rather than whole milk. A broader test described by Weiss et al. [20] addressed this limitation; they devised a Sandwich ELISA that uses immobilized antibodies raised against casein and β -lactoglobulin and defined the scope of the method to encompass the determination of milk proteins in cookies, infant formula, ice cream, and other foods. A sentence in their publication illustrates the delicate balance that exists between aiming at broad versus specific immunoassay applications using the same reagents: "If the milk protein composition of the sample is not known, and the milk protein ratio is different than the natural ratio in milk (e.g., a sample primarily consisting of whey), the results may be underestimated." An underlying assumption in the design of milk protein immunoassays is that milk antigens from different species –such as bovine, ovine and caprine casein– are similarly allergenic. However, we can find disagreement amongst publications on the subject. For example, David [21] states: "The marked similarities between the proteins in the milk of cows, goats, sheep, and horses means that almost all subjects who are allergic to cow's milk protein are allergic to milk of these other animals." On the other hand, Viñas et al. [22] demonstrated that some adult and pediatric subjects with allergies to goat and sheep cheese are tolerant of cow's milk. It should be pointed out that physical-chemical methods such as ultrahigh-performance liquid chromatography and mass spectrometry (LC-MS) were used by Heick et al. [23] to analyze different foods after protein extraction and trypsin digestion. Their method provided useful data on the presence of proteins in processed foods by calibrating quantitation versus *in silico* peptides with distinctive transitions.

Our purpose was to establish immunoassays devoted to the detection and quantitation of antigenic determinants in the acid casein fraction of bovine milk to assess infant formula prepared with EHC, and the raw materials used in its manufacture. Sheep were immunized to obtain anti-casein antibodies due to the evidence suggesting that ovine and bovine casein are immunologically different as stated above [22]. The immunization protocol generated liters of pooled antisera thus allowing us to deep freeze antisera that will be available for a long period. Availability of this key reagent supports the aim to set a reproducible process, from the generation of antibodies to the conditions of immunoassays that would not depend on the use of proprietary reagents. The design strategy for the immunoassay platform was based on direct immobilization of the target antigenic determinants used as controls or present in tested materials.

In comparison with competitive or sandwich ELISA, indirect immunoassays do not rely on competition between true antigens and test article. A slot blot version of the ELISA was co-developed to provide an alternative to antigen binding to plastic surfaces since SBIA rely on the retention of material to nitrocellulose filters. In this format protein antigens or particles containing them are trapped and immobilized while the solution or suspension in which they are present is forced through the filter by a vacuum. Thus, antigens are presented on the surface of the filter to antibodies, independently of their proclivity to bind plastic. The present account consists of evidence that supports the use of a particular immunoassay platform for a narrow application in hypoallergenic infant formulas that are based on extensively hydrolyzed casein.

MATERIALS AND METHODS

Materials

Reagents: Unless otherwise indicated under Methods, reagents and other materials were from the suppliers listed in this section. Polypropylene centrifuge tubes (50 ml), and COSTAR™ 96-well flat-bottom polystyrene microtitration plates were both from Corning, (Corning NY, USA), and Protein LoBind (5 ml) polypropylene centrifuge tubes and Protein LoBind 2 ml microcentrifuge tubes were both from Eppendorf® (Hamburg, Germany). Water used for all the experiments was HPLC grade (Resistivity > 16 M Ω.cm). Sodium bicarbonate, Tris-buffered saline 10 X solution, Tween 20, ELISA blocker buffer, Pierce rabbit anti-sheep IgG (H+L) conjugated with horse radish peroxidase, sodium hydroxide (50% w/w), ammonium bicarbonate, acetonitrile, hydrochloric acid, formic acid, trifluoroacetic acid, sodium chloride, phosphate buffered saline 10X solution, NP-40 Surfact-Amps detergent solution (10% w/v solution), sodium deoxycholate (SDC), normal rabbit serum, 1-Step™ 3,3',5,5'-Tetramethylbenzidine (Ultra TMB-ELISA) and Stop Solution were purchased from Fisher Scientific (Pittsburgh, PA). Opti-4CN Substrate Kit was purchased from Bio-Rad (Hercules, CA). Tris(hydroxymethyl)aminomethane and dodecyl sulfate sodium salt were from Acros Organics (Geel, Belgium).

Casein reference standards: For the Slot Blot experiments, NFDM from Agri-Mark, Inc. (West Springfield, MA) was used as a source of casein. Standards prepared from this material were used to emulate potential antigen contamination in a manufacturing setting. Protein concentration in the NFDM was determined using a Leco Corporation Nitrogen Analyzer and a conversion factor (Nitrogen to Protein) of 6.38. Casein concentration was assumed to be 80% of the protein concentration as determined for NFDM. Stock Standard containing 320 µg/ml of casein was prepared by dissolving 27 mg of NFDM (equivalent to 8 mg casein) in diluent (25.0 ml of 25 mM sodium bicarbonate, pH 9.5 with 0.1% w/v Tween 20). Dissolution was expedited by ultrasonication for 10 minutes followed by incubation at 37°C for 10 minutes. Working standards were prepared from the stock standard using the diluent to attain casein concentrations of 0.4 µg/ml, 0.8 µg/ml, 1.2 µg/ml, and 1.6 µg/ml (equivalent to 10, 20, 30, and 40 parts per million, respectively).

Extensively hydrolyzed casein-based formulas: Two HEC-based infant formula formulas were produced by Perrigo® Product Development (Georgia, Vermont). One version was formulated with the incurrence of 20 ppm casein, using NFDM as the source of casein, and the other was produced as intended for product release. Positive control samples (PCS) were prepared from the incurred EHC formula while negative control samples (NCS) were prepared from the EHC-based formula as intended for product release.

Antisera production: Acid casein was dispersed in PBS by pH adjustment, filtered and used to immunize three sheep (400 µg primary injection with complete Freund's adjuvant, boosts were identical but with incomplete Freund's adjuvant). A 49-day immunization and test bleed protocol was followed including primary injection, regular boost injections, and production bleeds. Anti- β -casein antibody titer was determined in pre-bleed and test

bleed samples using a direct binding assay. Protein A column chromatography purification was performed on the dilute antisera once the antibody titer was confirmed (Protein A IgG Purification Kit, Thermo Scientific). Protein concentration in the purified antisera was determined by BCA (Pierce BCA Protein Assay Kit). A β -casein competitive ELISA was used to determine the relative affinity, specificity, and the cross-reactivity of the Protein A purified anti-casein polyclonal antibody with α -casein and κ -casein. This assay used immobilized β -casein and either β -, α -, κ -casein to compete with immobilized caseins for binding by the sheep anti-casein polyclonal antibody. The biotinylated anti-casein antibody was then added followed by streptavidin-peroxidase (SA-HRP) followed by peroxidase substrate (TMB) for color generation.

Methods

Western blots: Nitrocellulose sheets blotted with electrophoresed proteins were probed with antisera according to Towbin et al. [24] and Tharin and Yang [25]. Samples were prepared by adding approximately 4 g of infant formula to a 50 ml flat bottom polypropylene centrifuge tube containing 4.0 ml Extraction Buffer (25mM Sodium Bicarbonate, pH 9.5 w/ 0.1% w/v Tween 20). Dissolution and extraction were expedited by ultrasonication for 10 minutes followed by incubation at 37°C for 25 minutes. An aliquot was transferred to a microcentrifuge tube and centrifuged at 16,000 g for 20 minutes. The supernatant was mixed (1:1) with Sample Buffer (Tricine sample buffer and 125 mM Dithiothreitol), incubated in a water bath at 95°C for 5 minutes, and centrifuged at 16,000 g for 20 minutes. The blue layers from samples and standards and Precision Plus molecular weight standards were loaded into designated wells of 10-20% Acrylamide Mini-PROTEAN Tris-tricine pre-cast gels assembled in a Mini-PROTEAN Tetra System (Bio-Rad,) and electrophoretically separated using Tris/Tricine/SDS running buffer and a PowerPac HC Power Supply (Bio-Rad) with parameters of 125 volts, 3.00 amperes, and 300 watts for 60 minutes. The resulting electrophoretograms were removed from the cassette and proteins were transferred to a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Bio-Rad) and a Trans-Blot Turbo Transfer Pack. Blotted membranes were transferred to trays for processing and washed for 5 minutes in Tris Buffered Saline, 1X with 0.1% (w/v) Tween 20 (wash solution). The membrane was blocked at room temperature in ELISA Blocking Buffer for 90 minutes followed by washing twice (five minutes each) in the wash solution. Next, the membrane was incubated with working primary antibody (Antisera diluted 1:500 in wash solution) for 90 minutes, followed by two washings (five minutes each) in Wash Solution, incubated with working secondary antibody (Reconstituted Secondary antibody conjugate diluted 1:2000 in wash solution) for 50 minutes, and followed by two washings (five minutes each) in wash solution and two washings (5 minutes each) in TBS, 1X. Color development was accomplished by incubating in substrate working solution composed of Opti-4CN diluent, water and Opti-4CN substrate (according to manufacturing directions) for at least 11 minutes. Finally, the membrane was washed two times for 5 minutes in water, dried on a paper towel, and imaged using a Gel Doc XR + Imager (Bio-Rad, Hercules, CA).

SBIA: Slot blot immunoassays have been used to compare performance with ELISA; the test described in this section was based on a method developed by Stone et al. [26]. Samples were prepared by adding 660 mg of infant formula to a 50 ml flat bottom

polypropylene centrifuge tube containing 15.0 ml of the diluent used for standard preparation. Dissolution and extraction were expedited by ultrasonication for 10 minutes followed by incubation at 37°C for 25 minutes. An aliquot was then centrifuged at 16,000 g for 20 minutes. The supernatant was then filtered using a 0.22 µm polyethersulfone syringe filter. A Bio-Dot SF Microfiltration Slot Blot apparatus (Bio-Rad, Hercules, CA) was assembled with three sheets of pre-wet filter paper and a nitrocellulose membrane from the same company. The apparatus was operated using a vacuum. Wells were rehydrated using 100 µl of 25mM Tris (hydroxymethyl) aminomethane, pH 7.4 with 0.13 M NaCl and 0.0027 M KCl (Tris Buffered Saline, 1X). Samples and working standard solution (200 µl) were loaded into the wells, drained using a mild vacuum, and washed with 200 µl of Tris Buffered Saline, 1X. The resulting membrane was removed from the apparatus, placed in a Midigel incubation tray (Fisher Scientific, Pittsburgh, PA) for processing using a MiniRocker Rocking Platform (Bio-Rad). The membrane was then washed for 5 minutes in Tris Buffered Saline, 1X with 0.1% (w/v) Tween 20 (wash solution). The membrane was then soaked in ELISA blocker buffer for 90 minutes and washed twice (five minutes each) in the wash solution. Next, the membrane was incubated with working primary antibody (antisera diluted 1:500 in wash solution) for 90 minutes, and was washed twice (five minutes each) in the wash solution, incubated with working secondary antibody (reconstituted secondary antibody conjugate diluted 1:2000 in wash solution) for 50 minutes, and washed twice again (five minutes each) in wash solution. Color development is accomplished by incubating in substrate working solution (Opti-4CN Diluent: Water: Opti-4CN Substrate (4.5:40.5:0.9) for at least 11 minutes. Finally, the membrane is washed for 5 minutes in water, moved to a new Midigel incubation tray, washed for 5 minutes in water, and imaged using a Gel Doc XR + Imager (also from Bio-Rad).

SBIA applied to swabs from manufacturing surfaces: The slot immunoassay was evaluated for the detection of residual antigens on manufacturing equipment surfaces. A total of nine Texwipe® Clean Tips Swabs (TX716, Texwipe, Kernersville, NC) were individually placed in separate 50 ml plastic flat-bottom centrifuge tubes containing 10.0 ml of 25 mM Sodium Bicarbonate, pH 9.5 with 0.1% (w/v) Tween 20. Each swab was removed from its corresponding tube and used once to wipe 77.4 cm² (12 in²) surface of pilot plant equipment. Three locations were selected for swabbing; the swab was then placed back in the designated tube. Same surface locations were swabbed three times; the first time after cleaning in place (CIP) but before production of non-hypoallergenic milk-based infant formula, the second after production of non-hypoallergenic milk-based infant formula, and the third after the manufacturing surfaces were cleaned (CIP) after the production of non-hypoallergenic milk-based infant formula. Tubes with swabs and diluent were sonicated for 10 minutes and filtered through 0.22 µm polyethersulfone syringe filters before applying to the wells of the apparatus.

ELISA: Sample preparation was performed using an Eppendorf® 5418 centrifuge (Eppendorf®, Hamburg, Germany), a Vortex-Genie 2® mixer (Scientific Industries, Bohemia, NY, USA), a Branson 8510® Ultrasonic Cleaner (purchased from Fisher Scientific, Pittsburgh, PA) and a MaxQ™ 2506 Reciprocating shaker (Thermo Scientific, Waltham, MA, USA). Assays were performed using an

ELx508 Microplate Strip Washer and an ELx808 Absorbance Microplate Reader; both purchased from BioTek® Instruments, Inc. (Winooski, VT, USA). The volumes of reagents (primary antibody, secondary antibody conjugate, TMB substrate and stop solution) and incubation times were established such that the absorbance values at 450 nm (OD 450) of a casein concentration of 40 ppm were from 0.9 to 1.0. For each incubation, plates were sealed with Parafilm to minimize evaporation. Plates were washed five times with 0.5% Tween 20® in PBS using an ELx508 Microplate Strip Washer (BioTek® Instruments, Inc., Winooski, VT, USA). Flat-bottomed 96-well polystyrene microplates (Corning, Corning NY) were coated with 100 µl of either standards or sample extract solutions in three consecutive wells and incubated at 4°C overnight. Following incubation, the plate was washed, the wells were blocked with 250 µl of blocking buffer (2% Normal Rabbit Serum in PBS) and incubated for 60 minutes at ambient temperature. The plate was then washed and the target proteins immobilized in the plate (either from samples or from casein standards) were probed with primary antibody (Sheep Protein A- purified antisera, diluted 1:1200 in Extraction Buffer) and incubated for 90 minutes at ambient temperature. The plate was then washed, and 100 µl of the secondary antibody conjugated with HRP (reconstituted rabbit anti-sheep IgG-horseradish peroxidase conjugate diluted to 1:3200 in Extraction Buffer) was added to each well to bind the primary antibody, and the plate was incubated for 75 minutes. After a final wash, 100 µl of TMB substrate was added to each well to react with the immobilized peroxidase to develop color, and the plate was covered with Parafilm followed by aluminum foil and incubated at ambient temperature for 15 minutes. Color development was stopped by adding 100 µl of stop solution (0.16M sulfuric acid) to each well and absorbance of each well was measured at 450 nm using an ELx808 ELISA plate reader (BioTek®, Winooski, VT). Gen5 Secure software (BioTek Instruments, Winooski, VT) was used to collect the absorbance at 450 nm (OD 450) data for each well, construct a least-squares regression linear calibration curve from the standard calibration solutions, calculate its coefficient of determination (r²), and the casein concentration for each well. The sample extract well concentrations were corrected for sample weight and averaged for each sample using an Excel spreadsheet.

RESULTS

Antisera reactivity and characterization

Three immunized sheep consistently produced antibodies against epitopes of the major protein components of acid-precipitated casein (α , β , and κ -casein). A competitive immunoassay format using β -casein as a capture reagent showed that α and κ -casein were able to inhibit the binding of biotinylated, protein A-purified sheep antibody against acid casein from each animal in the study. This result confirmed the specificity and cross-reactivity of the purified antibodies for the major casein proteins. Results presented in Figure 1 show that the addition of higher concentrations of either α , β , and κ -casein inhibited the binding of biotinylated anti-casein polyclonal antibody available to recognize the β -casein bound to the well.

Specificity of the purified antisera was further investigated by using it as a primary reagent to probe for EHC-formulas after separation by electrophoresis. Western Blots were prepared as described

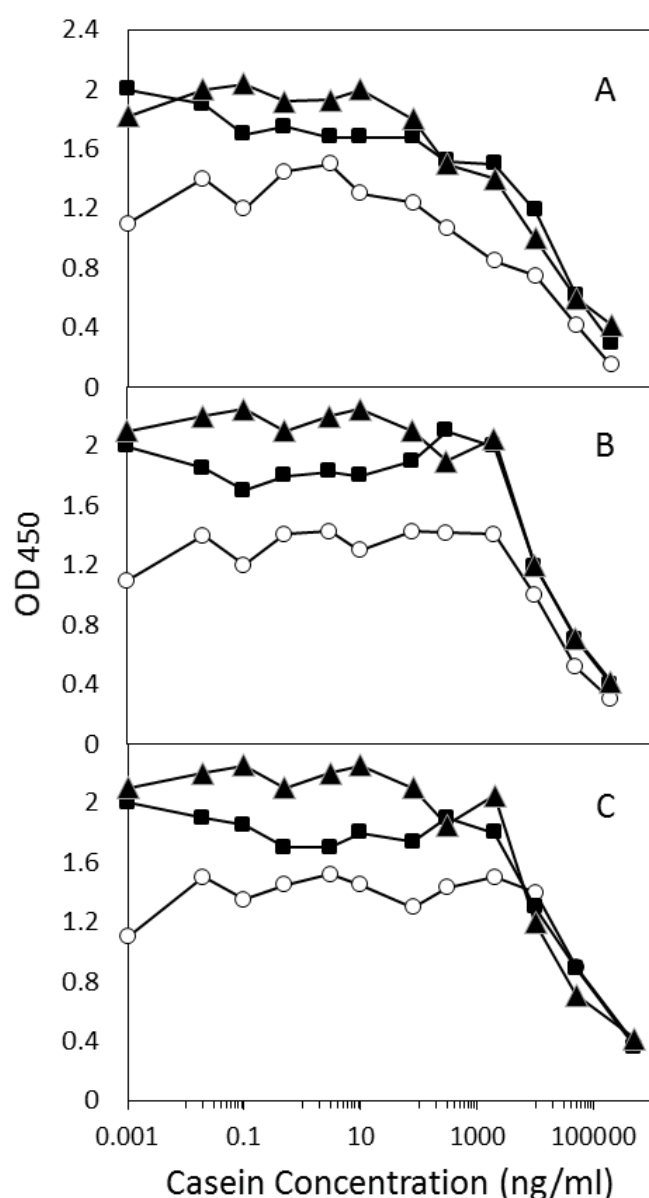


Figure 1: Reactivity of the anti-casein polyclonal antibody: Pooled, Protein A-purified antisera from different animals (Sheep 1 -▲-, Sheep 2 -■-, Sheep 3 -○-) was tested for reactivity with either β - (Panel A), α - (Panel B) or κ -casein (Panel C). Each casein fraction inhibited the binding of the purified immunoglobulin to b-casein indicating the Protein A purified immunoglobulins contained reactivity to all three forms of casein.

under Methods, transferring electrophoresed proteins from SDS gels to nitrocellulose membranes. The EHC-formula incurred with 20 ppm of casein was used as a positive control; the results of this experiment are depicted in Figure 2. This experiment had the additional purpose of determining if the purified pooled anti-sera were suitable for the development of matrix-specific (EHC-based infant formulas) immunoassays.

Slot blot

Working standards were prepared as described above, loaded into the Slot Blot apparatus (loading volume, 200 μ l), and processed for each sample analysis. Band intensity was measured using the Gel Doc XR+. A plot of band intensity versus casein concentration (μ g/ml) was generated and least squares linear regression was used to calculate the best linear fit along with correlation coefficient (R). The correlation coefficient was ≥ 0.95 for each sample analysis. Six

to eight replicates of the 20 ppm casein-incurred EHC-based infant formula were prepared and analyzed by two different analysts on multiple days. Band intensity of samples was measured as above. Casein concentrations, Mean, and percent relative standard deviation (%RSD) are shown in Table 1. To assess intermediate precision, the pooled Mean and pooled %RSD were calculated for Analyst 1 (Day 1, Day 2, and Day 3) From Table 1. The pooled Mean casein concentration was 25.2 ppm and the pooled %RSD was 17.5%. To assess reproducibility, the pooled Mean and pooled %RSD were calculated for Analyst 1, Day 3 and Analyst 2 from Table 1. Pooled Mean casein concentration was 23.7 ppm and pooled %RSD 10.7%.

For a preliminary assessment of accuracy, the casein concentration (ppm) from Table 1 was converted to percent of casein recovered versus the 20 ppm target. Mean percent casein recovered ranged from 113.9% to 141.9%. The limit of detection (LOD) was estimated based on the band for a 0.4 μ g/ml working standard

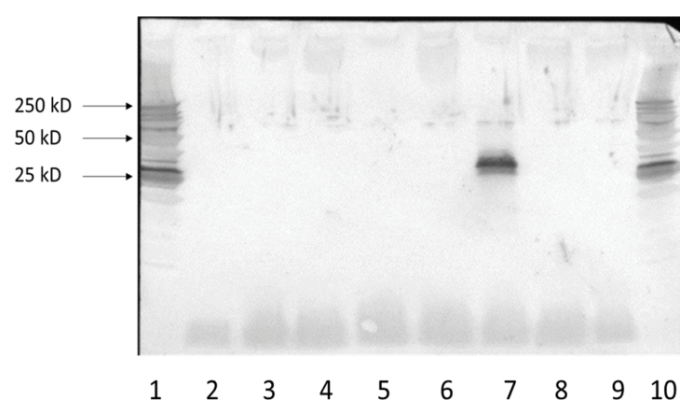


Figure 2: Western blot of hypoallergenic formulas: Proteins from infant formulas were prepared as described under Methods and subjected to SDS gel electrophoresis. Western blots were probed with the anti-casein sheep Protein-A purified antisera used to base the SBIA and ELISA described above. Lanes were as follows: 1. Molecular weight Standards, 2. Alimentum®, 3. Nutramigen®, 4. Nutramigen®, 5. New Hypoallergenic Formula (G19A Pilot Plant Batch, 6. New formula G19 prepared to support a clinical trial, 7. G19A positive control (incurred with 20 ppm of casein), 8. G19A formula used as negative control, 9. Same as in 8, 10. Molecular weight standards. Arrows indicate the position of three MW standards.

Table 1: Repeatability for Slot Blot Immunoassay of Results of analyses of casein (20 ppm) in a hypoallergenic matrix by two analysts were conducted in different days. The tests were carried out in ways that would reflect the assessment of casein content in a EHC-based formula in an industrial setting.

Experiment	Analyst 1 Day 1	Analyst 1 Day 2	Analyst 1 Day 3	Analyst 2 Single day
Observations (ppm)	23.7	29.5	27.8	22.8
	20.1	24.2	19.2	24.8
	19.7	21.8	20.1	22.6
	23.6	25.9	24.9	23.8
	25.3	29.0	26.9	22.5
	25.3	37.9	25.6	23.2
	22.6	26.7		
	22.0	32.0		
Mean	22.8	28.4	24.1	23.3
%RSD	9.2	17.7	14.9	3.8

and the corresponding Signal-to-noise ratio (S/N) in the z-axis. The casein concentration corresponding to S/N=3:1 was calculated to be 0.09 µg/ml or 2 ppm, which was the estimated LOD.

SBIA applied to swabs from manufacturing surfaces

A slot blot format is less dependent on solubility or efficiency of antigen extraction because proteins are trapped by the nitrocellulose filter even if they are not fully solubilized or in suspension. For this reason, the SBIA was applied to the analysis of swabs from manufacturing surfaces. This application of the immunoassay platform allows for the use of the same reagents and extraction solutions used for sample testing (0, 2, 10 and 20 ppm of casein). While the assay was not validated for this application, results suggest that SBIA may be a useful tool that relies on the same antibody preparation used for sample analysis either in slot blot or ELISA formats. Figure 3, shows results for casein detected after extraction from swabs of manufacturing surfaces. The test was linear for standards as expected and was sensitive enough to distinguish cleaned surfaces before exposure to non-hypoallergenic formula (U1 to U3, Figure 3), areas that had not been cleaned after manufacturing (U4 to U6, Figure 3) and areas that had been cleaned (U7 to U9, Figure 3). It can also be observed that U8 yielded a low but detectable response which indicates that the test is sensitive enough to detect areas that contain residual casein even after CIP. The bands of Figure 3 were also quantitated using the same protocol for a regular SBIA experiment (Table 2).

ELISA

Cross-reactivity studies were performed to determine the specificity of the method by analyzing samples presumably devoid of casein (Non-Casein Samples or NCS). Since infant formulas have many ingredients other than the protein source it is imperative to determine if any of its components are detected by the primary

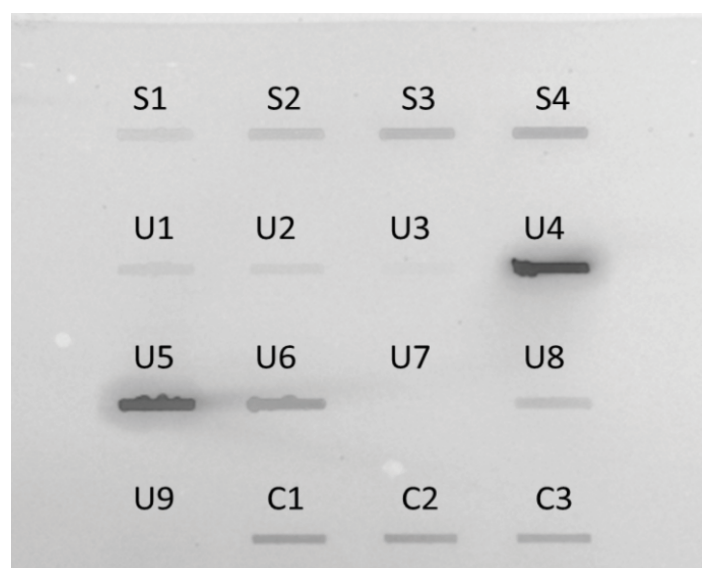


Figure 3: SBIA membrane image from swabs of manufacturing surfaces: The SBIA was used to test for casein content from swabs after extraction as described under Methods. Bands are: S1-S4 working standards, U1-U3 swab samples collected after the cleaning procedure, but prior to running milk-based product, U4-U6 swab samples collected after the manufacturing of milk-based product, U7-U9 swab samples collected after a milk-based product and a subsequent cleaning procedure, C1-C3 are samples of the hypoallergenic formula G19A incurred with 20 ppm of casein.

Table 2: Results for Swabs from Manufacturing Surfaces Using the Slot Blot Immunoassay: Swabs of manufacturing surfaces were extracted as described under Materials and Methods. The resulting solutions were filtered through nitrocellulose and imaged after incubation with primary and secondary antibodies. Cleaning-in-place (CIP) was conducted according to Standard Operating Procedures. Casein was not detected (ND) in two instances after CIP.

ID	Description	Location	Casein recovered (µg/in ²)
U1	After CIP but before production of MBF	1	0.31
U2	After CIP but before production of MBF	2	0.26
U3	After CIP but before production of MBF	3	0.11
U4	After production of MBF	1	6.54
U5	After production of MBF	2	4.59
U6	After production of MBF	3	1.77
U7	After production of MBF and subsequent CIP	1	ND
U8	After production of MBF and subsequent CIP	2	0.55
U9	After production of MBF and subsequent CIP	3	ND

antibody (sheep protein A-purified antisera) and thereby interfere with the detection or determination of casein concentration. One NCS sample extract was prepared and assayed in each of nine tests. For eight of the assays, no casein was detected, while in the ninth, casein was detected at 2.7 ppm. Two pure whey proteins, alpha-lactalbumin (α -LAC) and beta-lactoglobulin (β -LAC), which are known to be present in NFDM were also assayed; no casein was detected in these samples. To assess linearity or best fit for the response of the ELISA, three measurements of a blank (extraction buffer) and six working standard solutions across the calibration range (2 to 40 ppm) were made and, the OD 450 of each (y-axis) was plotted against the corresponding casein concentration in ppm (x-axis). The least squares linear regression line was fitted to the data (Figure 4) The OD 450 of the medium, i.e., extraction buffer (y-intercept) was 0.1031, the response factor (i.e., slope) was 0.020 OD 450/ppm, and the coefficient of determination (r^2) was 0.99.

To determine limit parameters of the ELISA for casein standard solutions, samples ranging in concentration from 0.2 to 10 ppm were prepared by diluting the casein stock standard solution described above in extraction buffer. The LOD and LOQ were determined from six measurements of a solution blank (extraction buffer only) and for three measurements of each casein standard solution using the Linear Regression Method [27]. The LOD and LOQ for the ELISA were 0.8 and 2.5 ppm, respectively. To assess functional sensitivity, working sample solutions at casein concentrations from 0.2 to 10 ppm were prepared by combining PCS, NCS, and extraction buffer in different volume combinations. Six measurements were made of a sample blank (NCS) and each working sample solution; LOD and LOQ were determined using the method described above for standard solutions. The LOD and LOQ of working casein samples (casein in finished product matrix) were 0.5 and 1.4 ppm, respectively. Accuracy was determined from three sets of positive sample extracts prepared from three pairs of PCS and NCS stock solutions (NCS and PCS diluted to 10 mg/ml in extraction buffer) and combined with extraction buffer in five different volume combinations for a total of 15 separate solutions

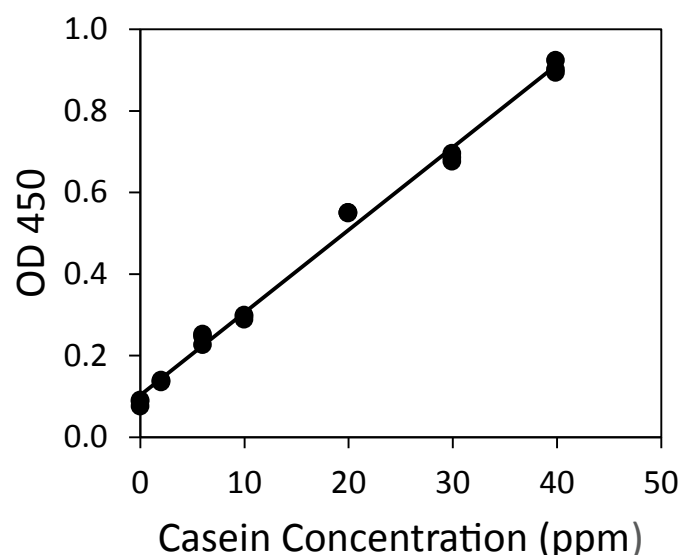


Figure 4: Linear fit of ELISA response: Response of the ELISA is expressed as optical density (OD 450) versus casein concentration (in parts per million) of NFDM diluted in extraction buffer. Selected calibration range was from 2 to 40 ppm of casein. Linear fit required a particular set of components for sample extraction buffer.

with a final matrix concentration of 2.5 mg/ml and casein concentrations from 2 to 20 ppm. Recovery (%) of casein from each solution was calculated by dividing the Mean of the concentrations of recovered casein by the theoretical casein concentration and multiplying by 100. Results are summarized in Table 3, and OD 450 versus theoretical concentration of casein are plotted in Figure 5. The plot of residual errors Figure 6 shows that there are no trends in recovery by Theoretical Casein Concentration.

To determine intermediary precision, two analysts prepared replicates of PCS. One analyzed replicates on seven different days while the other analyzed replicates on a single day. The mean percent casein recovered from Positive Control Sample for each analyst, the pooled mean, and %RSD are shown in Table 4. We adopted the definition of false positive results as the weight-adjusted recovered casein from the NCS when the OD was greater than the OD generated by the lowest calibration concentration (2 ppm). Only one false positive result out of 11 assays was observed. Similarly, false negative results were defined as observations when the weight-adjusted recovered casein from the PCS was less than the lowest calibration concentration (2 ppm). No false negative results were observed out of 24 samples. To verify the performance of the ELISA, we tested several commercial products for residual casein and compared the results with those obtained for our PCS and NCS (Table 5).

DISCUSSION

Hypoallergenic formulas are the sole source of nutrition for infants; they constitute a unique case amongst foods designed to prevent the contact of allergens with the final consumer. These formulas are thus in the toolbox of the health care professionals to provide sustenance, promote the growth of infants and prevent adverse immunological reactions at a critical developmental stage. Inconsistent results were obtained with one commercial assay, and the company that produced a promising second test abandoned the marketplace. These circumstances prompted us to develop a

Table 3: Recovery of Incurred Casein from Working Sample Solutions: Casein recoveries at concentrations of 20 ppm and lower were determined using the ELISA to assess recoveries at low concentrations. Each value was obtained from three observations.

Casein Target (ppm)	n	Mean recovery (%)	%RSD	Comments
2	3	95%	N/A	Two of the three samples had a mean OD 450 less than that of the lowest concentration Standard (2 ppm)
3	3	138%	52%	One of the three samples had a mean OD 450 less than that of the lowest concentration Standard Solution
4	3	94%	6%	
10	3	105%	5%	
20	3	97%	5%	

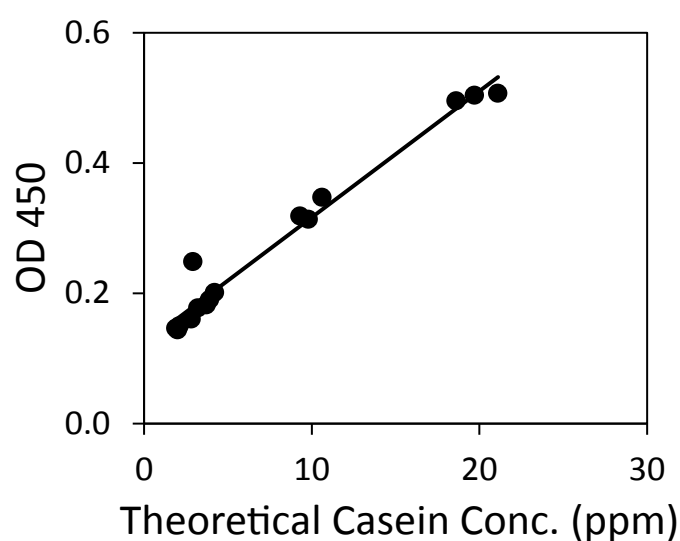


Figure 5: ELISA accuracy: The accuracy for the ELISA was determined from three sets of positive samples prepared from pairs of PCS and NCS sample stock solutions diluted with Extraction Buffer in five different volume combinations for a total of 15 separate observations. OD450 was plotted versus the theoretical concentration of casein in the samples.

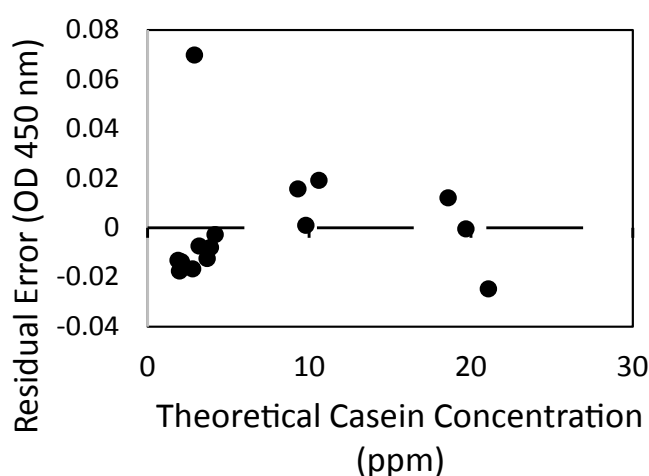


Figure 6: Plot of residuals by theoretical casein concentration: Errors were estimated for each observation from the linear fit results shown in Figure 1. These errors were plotted versus casein concentrations. The dotted line represents a theoretical no-error reference.

Table 4: Intermediate precision: results for the ELISA test for a multi-day two-analyst precision study are shown for triplicate observations. The shadowed area corresponds to repeated experiments by one analyst, and the clear lane is the result for a second analyst on a single day of experiments.

Analyst	Assay	n samples	Mean recovery assay	%RSD Assay	Mean recovery pooled	%RSD pooled
1	1	3	117%	4%	107%	7%
	2	3	114%	3%		
	3	3	114%	7%		
	4	3	105%	5%		
	5	3	94%	4%		
	6	3	106%	9%		
	7	3	105%	1%		
2	1	3	105%	4%		

Table 5: Recovery of Casein from Hypoallergenic product: Samples of two commercial hypoallergenic formulas and two batches of a new EHC-based formula were analyzed for the casein content alongside a hypoallergenic formula incurred with 20 ppm of casein as a positive control. ¹ <2.0 was assigned when calculated concentration is less than that of the lowest concentration standard used to quantitate this sample, i.e., 2.0 ppm.

Sample	Mean recovered casein concentration (ppm)
Negative Control Sample	<2.0 ¹
Positive Control Sample	20.8
G19A, Lot # E12LTXV	<2.0 ¹
G19A, Lot # PP17-015	<2.0 ¹
Nutramigen®	<2.0 ¹
Alimentum®	<2.0 ¹

reliable, repeatable immunoassay devoted to assessing the presence of residual casein antigens in EHC-based formulas. Also, while attempting to improve recoveries using extraction solutions from commercial kits, it was found that these were proprietary thus preventing optimization of their components for an EHC-based formula. Sheep were immunized using the target proteins in the acid bovine casein fraction as immunogens, similarly to what other groups have done for general food applications [28]. Curiously, most of the efforts carried out at the onset of monoclonal antibody technology were aimed at detecting contamination of ovine and caprine milk cheeses with less expensive bovine milk as opposed to allergy prevention [29]. This supported the notion that it was feasible to obtain sheep antibodies capable of binding bovine casein antigens. A well-managed program of bleeds and immunization boosters also yields relatively large quantities of antisera. Our assumptions were confirmed by data shown in Figure 1 and by the volumes of unprocessed antisera (liters). Each casein fraction inhibited the binding of the purified immunoglobulin indicating that protein-A purified immunoglobulins contained reactivity to all three forms of casein. To further verify the suitability of the pooled purified antisera to develop immunoassays, Western Blots of hypoallergenic formulas and a positive control incurred with bovine casein were probed with the purified antisera (Figure 2). No binding was detected to the EHC-based formulas, and an intense band with significant microheterogeneity was detected close to the 25,000 Kd molecular weight marker in casein incurred formula. This was expected since there are isoforms of all the caseins due to co- and posttranslational modification.

Once the specificity of pooled purified antisera from sheep was determined, different immunoassay formats including a

competitive ELISA were explored. During these investigations, it was found that casein binds quantitatively to ELISA plates and that it can be retained quantitatively in nitrocellulose membrane/filters thus facilitating binding by the antisera. These antibodies also bound quantitatively to the immobilized casein, thus providing us with the ability to amplify its binding signal by using a labeled secondary antibody in a classic indirect ELISA format.

The use of immunoassays is predicated on their simplicity of use and the inherent specificity conferred by antibodies, on the other hand, several authors describe that ELISA tests are prone to errors such as false positives [30], and sensitivity to the type and brand of the multi-well plate used [31]. Some of the false positives termed “hot wells” by users of ELISA normally result in the repetition of the test. While conducting the experiments described above, it became apparent that every step and material used in the ELISA must be carefully controlled; in our hands, results varied when extraction solutions and, particularly, detergents were exchanged for chemically equivalent reagents from different manufacturers. For example, the sodium deoxycholate (SDC) from Fisher Scientific as part of the extraction buffer, generated data with a linear fit. When SDC from five additional suppliers was tested, it became clear that these alternate forms of the same detergent shifted the response from linear to quadratic. This susceptibility of ELISA tests to seemingly minor changes of reagents have been observed by others [31,32].

The SBIA that was co-developed with the ELISA was originally intended to assess the performance of the sheep antisera in an assay that does not depend on antigen binding to plastic polymers. However, the data presented in Figure 3 indicates that it is well suited for swab test analysis precisely because larger amounts of fluid can be filtered through the nitrocellulose membrane. The ELISA proved to be sensitive, precise and accurate for the quantitation of casein in hypoallergenic formula matrices (Figures 4-6) including commercially available infant formulas (Table 5). These data prompted the full validation of the method for internal use and the desire to share it with the community of food scientists.

It should be mentioned that the pooled purified sheep antisera was tested in a sandwich format, but the results were poor (data not shown). We speculated that relatively large concentrations of non-antigenic peptides present in casein hydrolysate-based formulas cause an apparent reduction of antibody affinity perhaps by non-specific inhibition of the secondary antibody.

We conclude that the ELISA and SBIA methods are suitable to test residual casein in infant formula, but it is important to point

out some of their limitations; even though the method performed as expected when applied to commercially available hypoallergenic formulas, we believe that it should be validated for each matrix or formula to optimize linearity and recoveries. It is likely that different lipid blends, amounts or proportions of hydrolysate and concentrations of other ingredients will affect the ELISA performance. The lack of consistency that was observed for general food allergen tests when applied to hypoallergenic infant formulas is likely to occur when the tests described in the present account are applied to other food matrices. Our contribution to the field resides in the design and development of a non-competitive, non-sandwich assay platform that is reproducible and does not depend on proprietary reagents. This may be particularly important for hypoallergenic infant formulas. To foster attempts to reproduce the experiments described herein, we would be glad to share aliquots of the sheep anti-casein protein-A purified sera with other manufacturers of infant formula or academic institutions. Hypoallergenic formulas are tools for the health care professionals that face the dilemma of providing proper nutrition to infants while treating or addressing the risk of allergies to cow's milk. However, not all formulas made of protein hydrolysates are truly hypoallergenic since some contain partially hydrolyzed proteins which may contain antigenic epitopes [33]. The importance of tests that detect minute amounts of antigens is to provide assurances on the hypoallergenic nature of infant formulas thus supporting the efforts of the medical community to prevent allergen exposure at a critical developmental stage in human life.

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