HUMAN NUTRIENT METHODS

Determination of Casein Allergens in Extensively Hydrolyzed Casein Infant Formula by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Background: The use of hypoallergenic infant formulas and the need for reliable tests to determine the presence of residual antigens have increased in parallel.

Objective: An LC-MS method for quantitation of casein was validated using incurred samples and a matrix-matched external standard curve.

Method: Powdered infant formula samples were extracted in a buffer of sodium deoxycholate and ammonium bicarbonate at 60°C and filtered through 7 kDa desalting columns. Samples were digested overnight with trypsin and precipitated with acid prior to analysis of marker peptides by tandem mass spectrometry.

Results: Based on three marker peptides, the linear range for casein was 1.8–42 μg/g of powdered infant formula with an LOQ of 1.8 μg/g. The determination coefficients (R2) for each curve were ≥0.99 for casein peptides. Method repeatability was ≤22% RSD and intermediate precision was ≤23% RSD; recovery of casein from incurred material (2–20 μg/g) ranged from 78% to 118%.

Conclusions: An LC-MS/MS method was developed and validated for confirmation of casein allergens in hypoallergenic infant formula.

Highlights: A method was developed to accurately and reliably quantify casein allergens in extensively hydrolyzed casein infant formula by LC-MS without the need for custom peptide standards.

While breast milk is considered the optimal source of nutrition for infants (1, 2), infant formula is commonly used as an alternative when feeding with mother’s milk is not possible or practical. Infant formulas made with extensively hydrolyzed protein or amino acids that meet hypoallergenic standards are indicated for infants with cow’s milk allergy to prevent immune reactions to intact protein epitopes (3). Although there is no legal definition for extensively hydrolyzed infant formula, it is normally manufactured with peptides less than 3 kDa, while amino acid-based formulas are free of peptides and contain only amino acids (4).

Because hypoallergenic infant formula may be produced in facilities that also use milk ingredients, it is necessary to test for allergens to ensure that no cross-contamination occurs. In the

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case of formulas made with extensively hydrolyzed proteins such as casein, allergen testing also provides assurance that no intact protein contaminates either ingredients or the final product. Several analytical methods have been used to measure allergens in extensively hydrolyzed casein infant formula, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA), and immunoblotting (5-7). Of these, SDS-PAGE and immunoblotting are more time-consuming and are primarily used for research and validation purposes, while ELISA is more often used for quality control. Although an SDS-capillary gel electrophoresis method for whey protein in infant formula was validated, it does not measure casein and is not applicable to hydrolyzed products (8).

Despite its widespread use for allergen testing, ELISA has several limitations when used for extensively hydrolyzed infant formula. For example, the use of polyclonal antibodies may overestimate allergens because of their different affinities for proteins and peptides (5). Antibodies raised against protein epitopes are also prone to cross reactivity with proteins of similar structure and, in changes in protein conformation during food processing may influence results (9, 10). Due to its specificity, LC-MS/MS has been proposed as a confirmatory test for allergens in a number of processed foods, including pasta, baked goods, baby food, breakfast cereal, and infant formula (9-13). Although two of these LC-MS/MS methods measured allergens in infant formula, neither were evaluated with hypoallergenic infant formula, which requires validation to ensure that hydrolyzed protein fragments do not interfere with the analysis.

To address the lack of specific confirmatory tests for routine use with hypoallergenic infant formula, we developed and validated extraction and LC-MS/MS procedures for determination of intact protein contaminates either ingredients or the final product (9-13). This is enough solution for 18 samples.

METHOD
Apparatus

(a) LC system.—Agilent 1290 UHPLC system consisting of binary pump, autosampler, thermostatted column compartment, and UV-Vis detector (Agilent Technologies, Santa Clara, CA), or equivalent.

(b) Mass spectrometer.—Agilent 6460 triple quadrupole mass spectrometer with MassHunter data acquisition software (Agilent Technologies), or equivalent.

(c) Analytical column.—BioZen peptide XB-C18 column, 1.7 μm, 2.1 x 150 mm (part no. 00F-4774-AN; Phenomenex, Torrance, CA), or equivalent.

(d) Guard column.—C18 Guard cartridge, 2.1 x 2 mm (part no. AJ0-8948; Phenomenex), or equivalent.

(e) Guard cartridge holder.—Part no. AJ0-9000 (Phenomenex), or equivalent.

(f) Protein desalting columns.—Polypropylene or polycrylamide resin, 7K MWCO (part no. 89882 or 89849; Thermo Scientific, Rockford, IL), or equivalent.

(g) Top loading balance.—Capable of weighing to 0.1 g.

(h) Analytical balance.—Capable of weighing to 0.01 mg.

(i) Ultrasonic water bath.—40 kHz.

(j) Water bath.—Capable of heating to 60°C.

(k) Microcentrifuge.—For 2 mL tubes; capable of 16 000 x g.

(l) Centrifuge tubes.—1.5 mL, plastic.

(m) Centrifuge tubes.—2 mL, protein low-bind (part no. 022431102; Eppendorf, Hamburg, Germany), or equivalent.

(n) Kohlrusche flasks.—100 mL.

(o) HPLC vials.—Clear, with 300 μL inserts.

(p) Class A volumetric pipets.—Various sizes.

(q) Adjustable pipets.—2-20 μL, 20-200 μL, and 100-1000 μL.

(r) Syringe filters.—Polyethersulfone (PES), 0.2 μm, 4 mm diameter.

Reagents
Note: Reagent volumes may be scaled up or down provided good laboratory practices are followed.

(a) Laboratory water.—>18 megaohm-cm.

(b) Ammonium bicarbonate (NH₄HCO₃).—99%.

(c) Sodium deoxycholate (SDC).—99% (part no. BP349-100; Fisher Scientific, Fair Lawn, NJ), or equivalent.

(d) Trypsin.—From bovine pancreas, treated with tosylphenylalanyl chloromethyl ketone (TPCK) (part no. T1426; Sigma-Aldrich, St. Louis, MO), or equivalent.

(e) Formic acid.—LC/MS grade.

(f) Hydrochloric acid (HCl).—12.1 N, American Chemical Society (ACS) grade.

(g) Trifluoroacetic acid (TFA).—LC/MS grade.

(h) Acetonitrile (ACN).—LC/MS grade.

(i) Extraction buffer (50 mM NH₄HCO₃, 1% SDC).—Add 3.6 g NH₄HCO₃ and 900 mL water to a 2 L beaker and mix on a spin plate to dissolve. Add 9 g SDC and allow to slowly dissolve (approximately 30 min). When dissolved, mix briefly on a spin plate and transfer to a media bottle. This is enough solution for eight calibration solutions or samples.

(j) Trypsin storage solution (1 mM HCl).—Add 8.26 μL HCL to 100 mL water.

(k) Trypsin buffer solution (100 mM NH₄HCO₃).—Dissolve 395 mg NH₄HCO₃ in 50 mL water.

(l) Trypsin stock solution (1 mg/mL in trypsin storage solution).—Transfer 10 mg trypsin to a 10 mL volumetric flask and dilute to volume with trypsin storage solution. Vortex to completely dissolve. Aliquot 1 mL to separate LC vials and store at -20°C.

(m) Trypsin working solution (200 μg/mL in trypsin buffer solution).—Make immediately before use. Combine 200 μL trypsin stock solution and 800 μL trypsin buffer solution. This is enough solution for 18 samples.

(n) 0.5% TFA.—Add 50 μL TFA to 10 mL water.

(o) Mobile phase for LC system.—(1) Phase A: 0.1% formic acid aqueous. Combine 500 mL water and 500 μL formic acid and mix to dissolve. (2) Phase B: ACN.

Standards and In-House Reference Materials
The assay was tested using extensively hydrolyzed casein infant formula (Perrigo Nutritional; Georgia VT, USA). This formula was manufactured by adding externally sourced
extensively hydrolyzed casein to liquid ingredients prior to heat treatment and drying, and incurred sample was produced by adding nonfat dry milk (NFDM) to the same liquid ingredients during the manufacturing process. NFDM was used for the incurred sample because it is an ingredient for other formulas and is therefore a potential contaminant.

Because of the known ion suppression of the sample matrix in LC-MS/MS methods, the untreated extensively hydrolyzed casein infant formula was used as the blank matrix. NFDM from Agri-Mark, Inc. (West Springfield, MA, USA) was used to make the in-house reference material for casein allergen and as a standard to quantify casein allergen in samples by spiking the blank matrix. The nitrogen concentration in the NFDM was determined by combustion using a LECO analyzer (LECO Corp., St. Joseph, MI, USA), and protein in the sample was calculated to be 35.3% using a nitrogen conversion factor of 6.38 (14). To calculate the casein concentrations in the standards and samples, it was assumed that casein accounted for 80% of protein (15). The incurred sample contained 20 mg/g casein (70.8 mg/g NFDM) on a powder basis, which is below the lowest observed adverse effect level (LOAEL) for milk allergens according to the U.S. Food and Drug Administration (16) based on a 100 mL dose for infant formula. We estimated that a level of casein \( \leq 10 \) ppm is clinically relevant based on LOAELs for whole milk protein in children (17) and results from a clinical trial confirming the hypoallergenicity of an extensively hydrolyzed casein formula (18). However, the method has been validated near the LOQ since clinically relevant targets may change as new information becomes available. For incurred samples at 2 and 10 mg/g, the in-house reference material was diluted with the blank matrix at the time of sample preparation.

### Preparation of Standard Solutions

Stock solution of NFDM (1 mg/mL) in water was prepared fresh daily. NFDM intermediate solution (100 mg/mL) was made by diluting NFDM stock solution with extraction buffer (50 mM \( \text{NH}_4\text{HCO}_3 \), 1% SDC).

### Calibration Solutions

Protein extraction and trypsin digestion were done according to Lin et al. (19) and Lutter et al. (12) with modifications. A matrix-matched calibration curve was used to account for ion suppression in the sample matrix, a common challenge with processed foods (9, 12, 20). The calibration solutions were prepared alongside the test samples as described in the Sample Preparation section.

### Sample Preparation

(a) Preheat water bath to 60°C.
(b) Warm extraction buffer to 40°C.
(c) Weigh approximately 4 g of extensively hydrolyzed casein infant formula sample into five separate Kohlausch flasks and label them M1–M5. These will be used for the NFDM calibration solutions.
(d) Weigh approximately 4 g of each test sample into a Kohlausch flask.
(e) Add approximately 80 mL extraction buffer to each flask and swirl to mix.
(f) Add NFDM intermediate solution to flasks M1–M4 according to Table 1.
(g) Dilute all standard and sample flasks to volume with extraction buffer and shake vigorously to mix.
(h) Extract in a 60°C water bath for 30 min.
(i) Shake each flask for a few seconds and cool in a room-temperature water bath for 10 min.
(j) Desalt 120 \( \mu \)L standard or sample suspension in order to remove extensively hydrolyzed casein peptides, which interfere with the analysis.

1. Remove the bottom closure and loosen the cap on each desalting column.
2. Place each column in a 1.5 mL centrifuge tube and spin at 1500 \( \times g \) for 1 min.
3. Blot the tip of each column dry, mark on the side where the resin is highest, and place in a clean 2 mL low-bind tube.
4. Load 120 \( \mu \)L standard or sample suspension onto each column and cap loosely.
5. Place columns in the centrifuge so that the marks are facing outward and spin at 1500 \( \times g \) for 2 min.
6. Discard desalting columns. Add 50 \( \mu \)L of trypsin working solution to each desalted standard or sample and vortex briefly.

(k) Incubate standards and samples at 37°C for 16 h.
(l) Vortex tubes for a few seconds and centrifuge at 1000 \( \times g \) for 1 min.
(m) Add 170 \( \mu \)L of 0.5% TFA to each tube and vortex for a few seconds.
(n) Centrifuge at 16 000 \( \times g \) for 10 min.
(o) Filter approximately 200 \( \mu \)L of supernatant from each tube through a 0.2 \( \mu \)m PES syringe filter into a 300 \( \mu \)L LC vial.

### LC-MS Analysis

Quantitation by LC-MS/MS was done with an Agilent 1290 UHPLC system and Agilent 6460 triple quadrupole mass spectrometer using an ESI Jet Stream source and MassHunter software (Santa Clara, CA, USA). LC gradients were optimized with fused core columns using acidified water and acetonitrile mobile phases (11). A fused core XB-C18 analytical column (1.7 \( \mu \)m, 2.1 \times 150 mm) was connected to a matching guard column (Phenomenex, Torrance, CA) and the injection volume was 5 \( \mu \)L. The column temperature was set to 60°C and the flow rate to 0.3 mL/min with the mobile phases 0.1% v/v formic acid.

### Table 1. Composition and nominal concentrations of NFDM calibration solutions

|       | EH casein infant formula, g | Intermediate solution, mL | Total vol, mL | NFDM conc, mg/mL | Casein concn, mg/mL |
|-------|-----------------------------|---------------------------|---------------|------------------|--------------------|
| M1    | 4                           | 6.0                       | 100           | 6.0              | 1.69               |
| M2    | 4                           | 3.0                       | 100           | 3.0              | 0.85               |
| M3    | 4                           | 1.0                       | 100           | 1.0              | 0.28               |
| M4    | 4                           | 0.25                      | 100           | 0.25             | 0.07               |
| M5    | 4                           | —                         | 100           | 0.0              | 0.00               |

*Assuming NFDM is 35.3% protein and protein is 80% casein.
Table 2. MRM parameters for marker peptides from bovine casein analyzed by LC-MS/MS

| Protein | Peptide     | Retention time, min | Precursor, m/z | Product, m/z | Fragmentation, V | CE°, V |
|---------|-------------|---------------------|----------------|--------------|------------------|--------|
| αS2 Casein | NAVPITPTLNR | 5.4                 | 598.3          | 285.1\(^a\)  | 75               | 10     |
|          |             |                     | 598.3          | 911.5\(^b\)  | 75               | 10     |
|          |             |                     | 598.3          | 456.3\(^b\)  | 75               | 10     |
| αS1 Casein | YLGYLEQLLR | 8.2                 | 634.4          | 249.2\(^a\)  | 140              | 15     |
|          |             |                     | 634.4          | 991.3\(^b\)  | 140              | 15     |
|          |             |                     | 634.4          | 771.4\(^b\)  | 140              | 15     |
| αS1 Casein | FFVAPFPEGK | 8.9                 | 692.9          | 295.2\(^a\)  | 125              | 20     |
|          |             |                     | 692.9          | 991.5\(^b\)  | 125              | 15     |
|          |             |                     | 692.9          | 920.5\(^b\)  | 125              | 15     |

\(^a\) Quantifying ion.
\(^b\) Qualifying ion.

° CE – collision energy.

Table 3. Relative ion ratios from marker peptides used for casein analysis. The average ion ratio from calibration solutions was compared with three incurred samples (20 µg/g casein) prepared on each of three days

| Protein | Peptide     | Precursor ion | Quantifier ion | Qualifier ion | Day 1       | Day 2       | Day 3       |
|---------|-------------|---------------|----------------|---------------|-------------|-------------|-------------|
| αS2 Casein | NAVPITPTLNR | 598.3         | 285.1          | 911.5         | ≤27.8       | ≤21.4       | ≤21.4       |
|          |             | 598.3         | 285.1          | 456.3         | ≤13.2       | ≤9.5        | ≤12.4       |
| αS1 Casein | YLGYLEQLLR | 634.4         | 249.2          | 991.3         | ≤21.6       | ≤6.0        | ≤15.0       |
|          |             | 634.4         | 249.2          | 771.4         | ≤14.2       | ≤11.8       | ≤24.0       |
| αS1 Casein | FFVAPFPEGK | 692.9         | 295.2          | 991.5         | ≤15.5       | ≤11.3       | ≤8.8        |
|          |             | 692.9         | 295.2          | 920.5         | ≤9.9        | ≤7.2        | ≤5.9        |

(a) Calculate the concentration of casein, in µg/mL, in each calibration solution:

\[ C_{Cas} = \frac{W_{NFDM} \times 1000 \times (5/50) \times 0.353 \times 0.80 \times (V_{NI}/V_{Total})}{V_{NI}} \]

where \( W_{NFDM} \) = the weight of nonfat dry milk used to make the stock solution, in mg; 50 = stock solution volume, in mL; 1000 = the conversion from mg to µg; (5/50) = the dilution of stock solution to NFDM intermediate solution; 0.353 = the percentage of protein in NFDM, expressed as a decimal; 0.80 = the percentage of casein in milk protein, expressed as a decimal; \( V_{NI} \) = the volume of NFDM intermediate solution, used; \( V_{Total} \) = dilution volume.

(b) Build a 5-point calibration curve for each peptide analyte by plotting peptide peak areas against concentrations, with casein concentration on the x-axis. The coefficient of determination (R²) should be > 0.95 for each curve. The calibration and calculation may be done using the instrument software or off-line.

(c) Calculate the contents of casein in the test samples based on each marker peptide, e.g., NAVPITPTLNR. For peak identification, use the MS transitions specified in Table 3.

\[ \text{Cas}_{NAVP} = \left( \frac{A_{NAVP} - I_{NAVP}}{RF_{NAV}} \times 100 \right) \times \frac{M_{S}}{V_{NI}} \]

where \( \text{Cas}_{NAVP} \) = the casein in the sample (µg/g powder), based on the marker peptide NAVPITPTLNR; \( A_{NAVP} \) = the area of the quantifier ion of the marker peptide in the sample chromatogram, in arbitrary units (AU); \( I_{NAVP} \) = the y-intercept of the calibration curve for the quantifier ion of the marker peptide; \( RF_{NAV} \) = the slope of the calibration curve for the quantifier ion of the marker peptide; \( M_{S} \) = the sample weight, in g.

Method Validation

A single-laboratory validation was performed in accordance with ICH guidelines (23). Quantifying ions were used for calibration and quantitation, and specificity of the method was confirmed with qualifying ions for each marker peptide. To measure linearity and range, a 5-point standard curve for casein was made according to the method, and each standard was injected at the beginning and end of each chromatographic sequence. LOD was calculated as (3.3 x) slope and LOQ was...
calculated as \((10 \times s)/\text{slope}\), where \(s\) is the standard deviation of the response of a blank sample. Precision experiments were performed using the incurred infant formula samples. All samples were prepared in triplicate on each of four different days. Intermediate precision (RSD\(_i\)) was calculated as the relative standard deviation of all replicates. Repeatability (RSD\(_r\)) for triplicate samples was calculated from the within-day variance after a one-way analysis of variance (ANOVA) of all replicates. Recovery was calculated as a percentage of the measured protein in an incurred sample relative to the amount of protein added to the sample. To test the robustness of the casein allergen method with different types of desalting (size exclusion) columns, three different size exclusion materials were tested.

Selectivity
Because the allergen contamination could potentially occur at any point in the production process, it was necessary to demonstrate the ability to quantify intact proteins in a relevant incurred matrix. Extensively hydrolyzed casein infant formula incurred with NFDM was passed through 7 kDa MWCO desalting columns to ensure that only intact casein submitted to analysis. This proved effective, as marker peptides for casein were easily detected in incurred formula (Figure 1) and no signal was seen in the blank sample matrix at the same retention time. Several marker peptides reported in the literature were initially tested, and those that showed the best selectivity were chosen for further method validation. Of the three eventually chosen to quantify intact casein in the samples, FFVAPFPFEVFGK and YLGYLEQLLR from sS2-casein were reportedly found in IgE binding epitopes identified in sera of allergic patients (24). Thus, in the case of these two peptides the test quantified potential contamination that is relevant from an immunological standpoint.

For LC-MS/MS analysis, the product ion with the greatest signal was used as the quantifying ion and the other product ions were used as qualifying ions. A total of four casein peptides were originally tested. Although measuring FALPQYLK from sS2-casein resulted in multiple peaks, the remaining three peptides showed good selectivity (Figure 1) and were chosen for further validation. Relative intensity of qualifying ions in calibration solutions and samples is often used to confirm the presence of analytes by LC-MS/MS. Although criteria are not specified for relative ion intensity for peptide analysis, the requirements for identification of pesticide residues in foods based on relative ion intensity is outlined by the European Commission (25). To determine relative ion intensities (ion ratios), first ion ratios (quantifier ion peak area: qualifier ion peak area) were calculated for each calibration solution and incurred sample. The ratios of the samples were then compared with the average of the ion ratios of the calibration solutions in the same sequence. According to SANTE/11813/2017, the ion ratio of the samples should be within 30% (relative) of the average ion ratio of the calibration standards (25). All three casein peptides met these criteria (Table 3).

Linearity and range
Data for linearity and range from the 5-point calibration curve for casein are summarized in Table 4. The determination coefficients (R\(^2\)) for each curve were >0.99 for casein peptides. The range for casein in infant formula (approximately 1.8–42 µg/g) corresponds to approximately 6–150 µg/g NFDM in powdered infant formula and is a suitable range for hypoallergenic formula. This is similar to the range of 20–150 µg/g milk powder in soy-based infant formula reported by Lutter et al., who used FALPQYLK from sS2-casein as a marker peptide (12). It is smaller than the range of 1–1000 µg/g whole milk in infant formula reported by New et al., who used YLGYLEQLLR from sS1-casein as a marker peptide (13).

LOD and LOQ
The calculated LOD and LOQ varied with the marker peptide used for measurement (Table 4). Although these calculated values are lower than the range of the standard curve, it is recommended that the low end of the calibration range be used as the LOQ because the use of qualifier ions provides confidence in the results. The low LOQ with this method can likely be attributed to the desalting (size exclusion) step in the sample preparation. Size exclusion has been used in sample preparation for allergen detection in order to reduce ion suppression (9, 13, 26), but ideally smaller peptides would be included in the analysis. Casein fragments in the 3–6 kDa range have been shown to bind to human IgG antibodies (27), suggesting that our method cannot be expected to detect all immunogenic peptides.

Recovery and precision
Recovery of casein from the incurred infant formula samples ranged from 78 to 118%, varying with the peptide used for quantitation and the concentration of casein in the formula (Table 5). Previous studies have given various results. Lutter et al. were not able to recover β-, κ-, or sS2-casein from soy-based infant formula spiked with 5–15 µg/g NFDM (12). New et al. spiked 10, 100, and 1000 µg/g whole milk powder into an infant formula and recovered 71–83% (13). AOAC INTERNATIONAL has published Standard Method Performance Requirements (SMPRs) for allergens by LC-MS (SMPR 2016.002) (28), and our method meets the requirement of 60–120% recovery. The NFDM used for the calibration curve in this study came from a local supplier, but it has nearly the same protein concentration as the MoniQA reference material for skim milk powder (35.3% versus 35.4%) (29) and would presumably produce similar results for quantifying milk in this assay. Repeatability and intermediate precision data are also shown in Table 5. With the exception of one peptide measured at 2 µg/g, the method meets the repeatability limit of 20% RSD specified in SMPR 2016.002. Although the SMFR does not specify criteria for intermediate precision, the maximum value for the method was 23% RSD\(_i\).

Robustness
As stated above, we evaluated three different resins to desalt samples during the preparation process: polypropylene, polyacrylamide, and Sephadex G25 (dextran/epichlorohydrin). Samples prepared with the Sephadex G-25 columns showed the highest recovery with all three marker peptides. The peptide FFVAPFPFEVFGK gave the most variable results of the three peptides used. The polypropylene resin columns provided the most consistent recovery regardless of the peptide used for measurement. All of the columns tested met the recovery requirements of SMPR 2016.002.

Conclusions
Many LC-MS/MS methods use isotope-labeled peptides as internal standards to allow quantification in multiple matrices, but we chose to use a matrix-matched external calibration curve.
because it provided acceptable accuracy in our samples as it accounted for losses in signal due to sample preparation and ion suppression. It was also more cost effective than using isotope-labeled internal standards, which must be custom made. Although internal standard addition and matrix matching can be used together (11, 30), we found that matrix matching

Table 4. Slopes, y-intercepts, coefficients of determination ($R^2$), LOD, LOQ, and linear ranges of marker peptides used for quantitation of casein

| Protein | Peptide   | Slope  | y-intercept | $R^2$ | Calculated LOD, µg/g | Calculated LOQ, µg/g | Linear range, µg/g |
|---------|-----------|--------|-------------|-------|-----------------------|----------------------|-------------------|
| αs2 Casein | NAVTPTLNR | 3438   | 100.7       | 0.997 | 0.4                   | 1.3                  | 1.8–42            |
| αs1 Casein | YLGYLEQLLR | 9656   | -9.8        | 0.998 | 0.5                   | 1.6                  | 1.8–42            |
| αs1 Casein | FFVAPFPEVFGK | 5033  | 40.9        | 0.995 | 0.6                   | 1.8                  | 1.8–42            |
alone provided good results. The method is intended to be used in a manufacturing setting in addition to food analysis laboratories; using matrix matching simplifies the implementation and use of the method on a day-to-day basis. We also found that overnight trypsin digestion without prior reduction and alkylation of samples provided good recovery, possibly because the marker peptides lack cysteine residues (31). The LC-MS method for casein allergens presented here can accurately and reliably quantify intact casein in infant formula made with extensively hydrolyzed casein. All three marker peptides for casein provided acceptable accuracy and precision under specific experimental conditions, even when different desalting columns were used. One limitation of the method is its inability to detect whey protein allergens, but by measuring casein allergens, the method can detect intact casein from milk as well as any peptide fragments above 7 kDa that might be in the extensively hydrolyzed casein ingredient. Although it is possible that smaller peptides can cause an allergic reaction, most antigenicity in hypoallergenic formulas is associated with residual that smaller peptides can cause an allergic reaction, most anti-

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Conflict of interest statement
All authors are current or former employees of Perrigo Nutritionals, an infant formula manufacturer.

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| Protein                  | Peptide          | Casein concn, µg/g | Avg. recovery, % | RSD, % | RSD\(_{lab}\), % |
|-------------------------|------------------|--------------------|-----------------|--------|-----------------|
| αS2 Casein              | NAVPITPTLNR      | 2                  | 118             | 22     | 22              |
|                         |                  | 10                 | 111             | 8.1    | 14              |
|                         |                  | 20                 | 94              | 8.4    | 8.8             |
| αS1 Casein              | YLGYLEQLLR       | 2                  | 102             | 9.2    | 21              |
|                         |                  | 10                 | 81              | 6.0    | 10              |
|                         |                  | 20                 | 84              | 6.1    | 5.6             |
| αS1 Casein              | FFVAPFPEVFGK     | 2                  | 78              | 13     | 23              |
|                         |                  | 10                 | 113             | 18     | 20              |
|                         |                  | 20                 | 99              | 13     | 21              |

Table 5. Recovery of allergens from extensively hydrolyzed casein infant formula incurred with nonfat dry milk. Each data point is the average of 12 samples (3 replicates × 4 days)
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