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Quantification of Allergenic Bovine Milk α_{S1}-Casein in Baked Goods Using an Intact ^{15}N-Labeled Protein Internal Standard

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Supporting Information

ABSTRACT: Intact bovine ^{15}N-α_{S1}-casein was used as an internal standard in a selected reaction monitoring (SRM) assay for milk protein in baked food samples containing fats, sugar, and gums. Effects on SRM results of sample matrix composition in two biscuit recipes containing nonfat dry milk (NFDM) were studied, including samples from a milk allergen ELISA proficiency trial. Following extraction of defatted samples with carbohydrate-degrading enzymes and acid precipitation of casein, the SRM assay exhibited an LOQ of <3 ppm NFDM with 60−80% recovery. NFDM levels measured by the SRM assay were 1.7−2.5 times greater than median levels determined by ELISA. Differences were observed in the α_{S1}-casein interpeptide SRM ion abundance profile between recipes and after baking. ^{15}N-α_{S1}-Casein increases SRM analysis accuracy by correcting for extraction recovery but does not eliminate underestimation of allergen concentrations due to baking-related milk protein transformation (modifications).

KEYWORDS: *baked, casein, food allergen, isotope dilution, mass spectrometry, milk, nonenzymatic glycation, quantification, regulatory enforcement, selected reaction monitoring*

INTRODUCTION

Three percent of adults and 6% of children in the United States exhibit allergies to milk, peanuts, tree nuts, eggs, seafood, soy, or wheat, and 200 deaths per year are attributed to allergen exposure.[1,2] To protect susceptible populations, the Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) requires that food containing major allergens declare them on package labels. However, FALCPA did not establish threshold values for labeling because of considerable uncertainty about analytical detection limits and exposure thresholds.[3] Industry uses advisory labeling to indicate products may contain allergens but does not provide quantitative information. Consumers make purchasing decisions in a landscape where market surveys have detected milk allergens (17−222 ppm) in foods that bore advisory labels or declared no allergen content.[4,5] Development of improved detection assays and use of their performance metrics have been suggested as a strategy to help establish allergen labeling thresholds and enforce FALCPA.[6] Commercial ELISA kits are the principal allergen detection tools, but mass spectrometry (MS) is increasingly being used.[7−10]

Bovine milk is a source of calcium and protein for many children and adults. It contains more than 25 proteins, many of which are allergenic.[11−13] α_{S1}-Casein is the most abundant milk protein, comprising 29% of the total protein weight.[14] α_{S1}-Casein epitopes recognized by human IgEs from allergic individuals have been characterized.[15,16] Peptide sequences YLGYLEQLLR and FFVAPFPEVFGK are present in immune-dominant epitopes that potently stimulate human T cells in vitro.[17−19] Milk fractions (e.g., casein, whey, and carbohydrates) are used to produce infant formulas, dietary supplements, and many other products.[20] Foods can unexpectedly contain milk protein due to contamination of milk-derived components (e.g., lactose), cross-contamination from multiuse industrial processing equipment, or product mislabeling.[21]

The low cost, ease of use, and extensive experience with milk allergen ELISA kits have made them widely accepted. However, due to its greater molecular specificity, MS is a complementary tool for confirming litigated ELISA results in industrial and regulatory arenas. It can be used as a sensitive screening tool or to perform multiplexed quantitative analyses. Weber et al. demonstrated the LC-MS/MS detection of α_{S1}-casein in foods spiked with 1.25 ppm nonfat dry milk (NFDM).[22] Heick et al. performed simultaneous detection of seven allergens in sugar-free, nonfat bread using selected reaction monitoring (SRM).[23] α_{S1}-Casein and α_{S2}-casein surrogate peptides were used to detect added NFDM (10 ppm), and SRM was shown to be more sensitive than ELISA.[24] In flour and bread containing added NFDM, baking decreased SRM signal abundance by 55% and in one case eliminated the casein ELISA signal. Lutter et al. measured spiked milk proteins in infant foods by SRM using ^{13}C^{15}N-labeled β-casein, α_{S2}-casein, κ-casein, and β-lactoglobulin peptides.[25] The limit of detection and recovery estimates ranged from 1 to 50 ppm and from 16 to 66%, respectively. The study by Lutter et al. is the most extensive SRM report of measuring milk protein concentrations in unbaked food. Although α_{S1}-casein was not measured, the concentration of individual milk proteins in three primary milk standards was reported, including the National Institutes of Standards and Technology (NIST) NFDM standard 1549.[25,26] These studies

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represent best-case scenarios because they were performed in model systems that minimize the possibility of protein modifications, for example, by Maillard reactions. More difficult analytical problems are expected in complex baked foods containing sugars, fats, and thickeners, where nonenzymatic protein glycation of milk proteins occurs.

ELISA and SRM methods do not account for losses during sample preparation and thereby underestimate allergen concentration. Protein standard absolute quantification (PSAQ) is an SRM-based technique for managing this problem to acquire the most rigorously quantitative data possible.[27] PSAQ is performed by spiking intact isotopically labeled protein into samples at the earliest stage of processing.[28] Samples are extracted and digested, and natural isotopic and labeled peptide abundances are measured. PSAQ comprehensively corrects for extraction and digestion inefficiencies. It has been used to analyze staphylococcal enterotoxins, ubiquitin, DNA repair enzymes, and serum biomarkers but has not been applied to allergens.[29−33]

α_{S1}-Casein was chosen to explore the utility of an intact ^{15}N-labeled protein as an internal standard to increase the accuracy of an SRM assay for milk protein in baked samples containing fats, sugar, and gums. The use of an intact protein standard was compared to stable isotope-labeled peptide standards in the quantification of α_{S1}-casein in the NIST NFDM standard 1549 and samples containing added NFDM. Two recipes were employed to study sample matrix composition effects on assay performance. A nonionic surfactant carrier was used to increase peptide recovery for measurement of internal standards in the absence of the baked sample matrix. New sample-handling protocols were developed that included enzymatic treatment to degrade carbohydrates and increase sample protein extraction efficiency. SRM results are compared to previous ELISA results of the same samples from a multilaboratory milk allergen proficiency study.[34]

■ EXPERIMENTAL PROCEDURES

Materials. NIST NFDM standard 1549 was purchased from the National Institutes of Standards and Technology (Gaithersburg, MD, USA). Natural isotopic abundance and ^{13}C^{15}N-labeled α_{S1}-casein peptide sets (HQGLPQEVLNENLL**R**, YLGYLEQLL**R**, and FFVAPFPEV**F**GK) were purchased from 21st Century Biochemicals (Marlboro, MA, USA). Peptide chemical purities were determined by HPLC with UV (215 nm) detection, and concentrations were determined by amino acid analysis by the vendor. Peptides were supplied in polyethylene vials as an equal mass mixture (10 μg each) of three natural isotopic abundance peptides or the corresponding ^{13}C^{15}N-labeled peptides. The bovine α_{S1}-casein expression vector was synthesized by GeneScript Corp. (Piscataway, NJ, USA), and the corresponding ^{15}N-labeled protein was produced by M-Fold Biotechnology (Tübingen, Germany). Sequencing grade trypsin (V511) was purchased from Promega (Madison, WI, USA). Heat stable α-amylase (A3306), nonionic surfactant Pluronic F-127 (poloxamer), and angiotensin II were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pectinase (P062L) and mannanase (PDN X4/1) were supplied as a gift by Biocatalysts Ltd. (Parc Nantgarw, Wales, U.K.). Protein extraction from solid samples was performed in 15 mL polypropylene centrifuge tubes, and all other sample procedures were performed in 0.5 or 2 mL LoBind Eppendorf (Hauppauge, NY, USA) tubes. Co-Star Spin-X 0.22 μm nylon membrane filters were purchased from Corning (Corning, NY, USA). Glass LC injector vials (2 mL, maximum recovery) were purchased from Waters Corp. (Milford, MA, USA). Amicon Ultra-4 molecular weight cutoff (30 kDa MWCO) filters were purchased from Millipore (Billerica, MA, USA).

Instrumentation. SRM was performed using an Acquity UPLC system (Waters, Manchester, U.K.) connected to a hybrid triple-quadrupole 4000 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a Turbospray V ESI source. Sample vials were maintained at 10 °C, and injections were made using a full loop (10 μL) injection mode. Peptides were separated at 100 μL/min and 35 °C on a BEH C18 column (1.7 μm particles, 1 × 150 mm) equipped with a matching Vanguard precolumn (2.1 × 5 mm). Mobile phases A and B respectively consisted of 0.1% HCOOH and 1% CH_3CN in water and 0.1% HCOOH and 1% water in CH_3CN. The mobile phase consisted of 15% B from 0 to 1.5 min and was linearly changed to 40% B at 10 min and 15% B at 10.5 min. The column was re-equilibrated for 9.5 min to complete the 20 min analysis. The injection needle was washed with mobile phase B (200 μL) followed by mobile phase A (600 μL) between samples. Analyst 1.5 (AB Sciex) was used to acquire and analyze data.

Methods. *Preparation of Recombinant ^{15}N-Labeled α_{S1}-Casein.* An rTEV protease cleavable His-tag was fused to the N terminus of the mature coding sequence (UniProtKB, accession no. P02662) of bovine α_{S1}-casein and subcloned into pET32a vector (GeneScript Corp., Piscataway, NJ, USA). Plasmid was transfected into *Escherichia coli* strain Bl21(DE) and expressed in M9 medium with ^{15}N-ammonium sulfate (>99% isotopic purity, M-fold Biotechnology. Fusion protein was purified via Ni-NTA agarose, treated with acTEV protease, and repurified with Ni-NTA agarose. The concentration, purity, and molecular weight of repurified protein were measured by OD280 corrected to 0.95 mg/mL by Vector NTI and SDS-PAGE by the vendor. Positive ionization ESI FT-MS spectra (Exactive, ThermoScientific, Waltham, MA, USA) were acquired at a resolving power of 100,000 from the intact protein via loop injection. The spectrum of the multiple charge state envelope was deconvoluted using the ProMass algorithm (ThermoScientific). Recombinant protein was digested with trypsin and characterized by UPLC-MS (AB Sciex 4000 QTRAP) and nano-LC-MS/MS (LTQ, Thermo).

Titration of NIST NFDM Trypsin Digest Samples with Poloxamer. DTT was added to NIST NFDM aliquots to a final concentration of 5 mM in 25 mM NH_4HCO_3. Diluted ^{13}C^{15}N-labeled peptide standard and various concentrations of poloxamer in 10 μL of water were added, and the digestion vessel was heated (60 °C × 20 min) and rapidly cooled on wet ice. Trypsin (0.5 μg/μL in 50 mM CH_3COOH) was added at 1:50 (w/w) and incubated for 1 h at 37 °C. Digestions were quenched by addition of 10% HCOOH to yield a final 0.2% HCOOH (v/v). SRM assay parameters are described below.

Measurement of ^{13}C^{15}N-Peptide, ^{15}N-Labeled Recombinant Protein, and α_{S1}-Casein Concentration in NIST NFDM Standard 1549. Unlabeled synthetic peptide concentrations determined by amino acid analysis were used as datum points to measure ^{13}C^{15}N-labeled peptide and intact ^{15}N-α_{S1}-casein standard concentrations. The ^{13}C^{15}N-labeled peptide and intact ^{15}N-α_{S1}-casein standards were then independently used to measure the concentration of α_{S1}-casein in the NIST NFDM standard. Poloxamer concentrations >20 μM, or at least 100-fold greater than the unlabeled peptide concentration, were used in all standardization solutions and digests. After spiking with an internal standard, proteins were heated (60 °C × 20 min) in 50 mM NH_4HCO_3 containing 5 mM DTT and rapidly cooled on wet ice. Trypsin was added to substrate protein at a ratio of 1:50 (w/w), and samples were digested overnight at 37 °C. Digestions were quenched by the addition of 10% HCOOH to yield a final 0.2% HCOOH (v/v). SRM assay parameters are described below.

Preparation of Baked Samples. NIST NFDM standard 1549 (0, 1, 3, 10, 30, and 100 ppm) was added to cookie dough and baked at the Center for Food Safety and Nutrition (CFSAN) for 16 min at 180 °C using a modification of the recipe described by Scaravelli.[35] Cookie disks were approximately 3 cm in diameter and 1 cm thick after cooking. Biscuits containing milk were obtained from a U.S. FDA-sponsored ELISA comparison study organized and managed in October 2010 by the Food Analysis Performance Assessment Scheme (FAPAS) organization (Sand Hutton, York, U.K.).[34] Briefly, FAPAS biscuits (A, B, and C, respectively containing gravimetrically determined concentrations of 0, 20, and 50 ppm NFDM milk) were baked for 45 min at 180 °C. Baked samples (5−18 g) were crushed, weighed, and extracted with 4 volumes (25 mL) of $CHCl_3$. Defatted

solids were air-dried and weighed. Cookie and biscuit recipes are provided in Supplemental Tables 1 and 2 of the Supporting Information.

Dilution of FAPAS Biscuit C with FAPAS Matrix Blank. Defatted biscuit A (0 ppm NFDM) and C (50 ppm) solids were blended to yield virtually incurred samples (0, 3, 10, 25, and 50 ppm NFDM).

Spiking Defatted Samples with Intact ^{15}N-α_{S1}-Casein and/or NIST NFDM Standard 1549. Defatted cookie and biscuit samples (0.5 g) were spiked with ^{15}N-α_{S1}-casein (3 μM) to simulate an equivalent 25 ppm NFDM level. The method of standard additions was implemented by spiking FAPAS biscuit samples in triplicate with NIST NFDM standard 1549 (0.5 mg/mL 15% CH_3CN, 50 mM NH_4HCO_3) to yield 0, 10, and 25 ppm NFDM.

Extraction of α_{S1}-Casein from Baked FAPAS Biscuit. Dry, defatted 0.5 g samples spiked with ^{15}N-α_{S1}-casein and/or NIST NFDM standard additions were mixed with α-amylase (390 IU), pectinase (22 IU), and mannanase (4 IU) in 3 mL of buffer (15% CH_3CN, 50 mM NH_4HCO_3, pH 9). Samples were extracted at 40 °C with shaking for 1 h and clarified by centrifugation (6000g × 20 min at 4 °C), and the supernatant was transferred to a clean LoBind centrifuge tube.

Acid Precipitation of Caseins from Extract. The clarified supernatant pH was adjusted to 4.6 by the addition of 20% acetic acid (HOAc) in water and verified using narrow-range (4−5) pH paper (ColorpHast, EMD). Samples were left at room temperature for 30 min and centrifuged (18000g × 10 min at 20 °C). The supernatant was transferred to a separate tube. The pellet was resuspended in 15% CH_3CN, 12.5 mM NH_4HCO_3 by aggressive vortex mixing and modest sonication at 20 °C.

Measurement of Total Protein Concentration in Extract. Protein concentrations in the initial extract, HOAc supernatant, and resuspended pellet were measured in a BCA assay (Pierce) microplate format using BSA as the calibration standard.

^{13}C^{15}N-Peptide Internal Standards. A stock solution (5 μM) of three ^{13}C^{15}N-labeled peptides (HQGLPQEVLNENLL[**R-^{13}C^{15}N**], YLGYLEQLL[**R-^{13}C^{15}N**], and FFVAPFPEV[**F-^{13}C^{15}N**]GK) in 50% CH_3CN, 2% HCOOH was prepared in the polyethylene vial provided by the vendor. The stock solution was diluted to 100 nM with 15% CH_3CN, 0.1% HCOOH, and water in a LoBind centrifuge tube for spiking of trypsin digests.

Reduction, Thermal Denaturation, and Trypsin Digestion of Extracted Protein. DTT was added to the reconstituted pellet to a final concentration of 5 mM. Diluted ^{13}C^{15}N-peptide standard was added, and the digestion vessel was heated to 60 °C for 20 min and rapidly cooled on wet ice. Trypsin (0.5 μg/μL 50 mM HOAc) was added at an enzyme to a substrate ratio of 1:50 (w/w) and incubated for 16 h at 37 °C. Digestions were quenched by the addition of 10% HCOOH to yield a final 0.2% HCOOH (v/v). A 40 μL aliquot of 50 nM angiotensin II was added to each sample as an injection standard. Each sample had a final labeled peptide concentration of 3 nM.

Filtration of Acidified Trypsin Digests. Quenched digests were centrifuged (15000g for 10 min, 20 °C), and the supernatant was filtered (0.22 μm) before transfer to glass LC vials. A subset of samples, including some extracted without enzymes, were further processed with a 30 kDa MWCO filter.

Standard Curve Preparation. Separate matrix-matched standard curves were prepared containing 0 ppm NFDM CFSAN cookie or FAPAS biscuit samples with or without 50 μM poloxamer. Unlabeled peptide solution was diluted in baked good matrix to yield 0, 0.13, 0.49, 1.5, 4.9, or 15 nM (CFSAN) and 0, 0.14, 0.63, 1.6, 3.9, or 8.4 nM (FAPAS). Labeled peptides in standard curve samples were diluted to a constant concentration of 1.8 nM (CFSAN) and 2.8 nM (FAPAS). Standard curves were generated by injecting the same solution three times.

NFDM SRM Assay Parameters. FAPAS biscuits were assayed by extraction in triplicate, acid precipitation and digestion of three aliquots per extract, and triplicate injection of each digested sample. Milk concentration in unknowns was calculated from the SRM-measured α_{S1}-casein concentration in NIST NFDM standard 1549. CFSAN cookie, dilution series, and NFDM standard addition sample assays were performed with one extraction per sample. Extraction recovery was calculated as the percentage of ^{15}N-α_{S1}-casein protein spiked into the sample recovered in the extracted sample, as measured using the ^{13}C^{15}N-labeled synthetic peptides. Total assay uncertainty was calculated by propagating the experimental error from analyte and internal standard SRM area ratios with systematic error from measurements of internal standard stock concentrations, SRM channel crosstalk, and α_{S1}-casein concentration in NIST NFDM.

Parent ion/product pairs and instrument parameters were selected on the basis of infusing peptide standards at the flow rate and mobile phase composition used for quantitative analysis. The most abundant product ions of doubly charged parent ions were selected: HQGLPQEVLNENLLR y_{11} and y_{13}; YLGYLEQLLR y_6 and y_8; and FFVAPFPEVFGK y_8, y_9, and y_{10}. An SRM transition table indicating the specific m/z employed is provided in Supplemental Table 3 of the Supporting Information. HQGLPQEVLNENLLR ion sets were detected using a 130 V declustering potential (DP) and 48 V collision energy (CE); 75 V DP and 30 V CE were used for YLGYLEQLLR sets; and 70 V DP and 25 V CE were used for FFVAPFPEVFGK sets. SRM transitions from [angiotensin II]$^{2+}$, b_6 and y_2, used a 50 V DP and a 35 V CE. A 65 ms dwell time was used for each SRM transition. FFVAPFPEVFGK y_8 and YLGYLEQLLR y_6 and y_8 SRMs were routinely used for quantification. The grouping of unlabeled analyte and ^{15}N-labeled and ^{13}C^{15}N-labeled peptide SRM areas is referred to as an SRM set.

■ RESULTS AND DISCUSSION

The FDA Allergen Threshold Working Group proposed the development of new assays as a policy to guide the establishment of labeling thresholds and more effectively enforce FALCPA.[6] Improved food allergen assays should address current analytical limitations; for example, ELISA and SRM assays systematically underestimate allergen concentration by not correcting for sample extraction recovery. A new PSAQ approach utilizing ^{15}N-labeled intact α_{S1}-casein protein was developed to increase the quantitative accuracy of an α_{S1}-casein SRM assay applied to baked cookies and biscuits containing NFDM. The present work extends the studies of Heick and Lutter by using more complex food models containing sugar, fat, and gum thickeners.[23−25] These components affect Maillard reactions with the added allergen proteins, factors that were minimized by recipes used in earlier studies. Analyses of samples prepared with different recipes can provide insight into effects of the food matrix and processing on SRM results. This study yielded several additional findings that are significant for food allergen detection. First, during assay development a strong carrier effect was discovered and managed to more accurately characterize α_{S1}-casein peptide and intact protein standards. Second, extraction efficiency was increased by using carbohydrate-degrading enzymes. Third, differences in the interpeptide SRM profile between recipes indicate α_{S1}-casein undergoes recipe- and baking-dependent changes.

Poloxamer Minimized Carrier Effects during Standardization of Synthetic Peptide, ^{15}N-α_{S1}-Casein, and NIST NFDM Standard 1549 Concentrations. Unlabeled synthetic α_{S1}-casein peptides were used as primary reference standards to measure the corresponding concentration of ^{13}C^{15}N-labeled peptides, ^{15}N-labeled recombinant protein, and the NIST NFDM standard. These were subsequently used as internal standards. A strong carrier effect was observed when 5% CH_3CN/0.1% HCOOH/94.9% H_2O (v/v/v) solvent was used for synthetic peptides, even at milk digest total protein concentrations >30 ng/μL. Low recoveries were similarly observed by Lutter et al. in the SRM analysis of NFDM-fortified water.[25] Techniques for minimizing peptide loss to

sample vials have been studied.[36−39] Nonionic surfactant poloxamer was added to synthetic peptide solutions, NIST NFDM standard, and ^{15}N-α_{S1}-casein digests to determine its efficacy as a carrier. The addition of poloxamer to 20 μM and increasing the solvent CH_3CN concentration to 15% minimized losses without interfering with chromatographic separation or MS system performance. Peptide responses in NFDM digests sharply increased with poloxamer concentration and plateaued at 5-fold higher levels than digests without poloxamer (Figure 1a). Normalized peptide response factors in

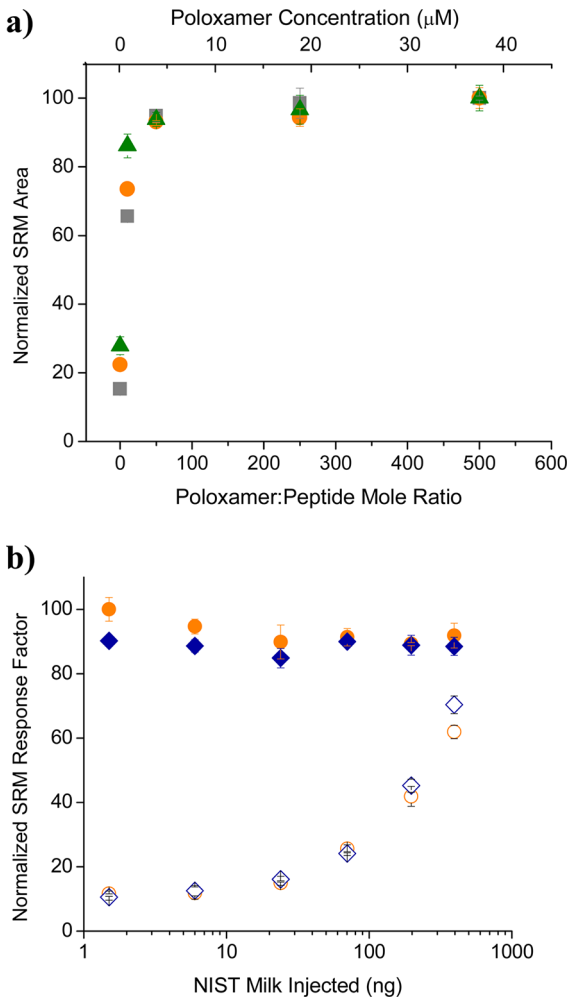

Figure 1. Titration of α_{S1}-casein assay peptides with poloxamer: (a) response of FFVAPFPEVFGK y_8 (gray squares), YLGYLEQLLR y_8 (orange circles), and HQGLPQEVLNENLLR y_{11} (green triangles) from injection of a fixed amount (60 ng protein) of NIST NFDM standard 1549 trypsin digest titrated with poloxamer (average (n = 3) ± propagated error); (b) response factors of YLGYLEQLLR y_8 ^{13}C^{15}N-labeled peptide internal standard (blue diamonds) and analyte peptide (orange circles) from injection of NIST NFDM standard digests with (solid) or without (open) poloxamer present at a 100-fold mole excess relative to the NIST α_{S1}-casein concentration (average (n = 3) ± propagated error).

NFDM digests without poloxamer decreased, relative to digests with poloxamer, as lower NFDM digest concentrations were injected (Figure 1b). Glucagon and angiotensin I gave similar results in carrier titration experiments (data not shown), but the much lower cost of poloxamer favored its use. Although carrier was needed for the analysis of neat peptides, ^{15}N-α_{S1}-casein, and the NIST NFDM standard, it was not necessary for baked samples in which total digested protein concentrations were ≥1 μg/μL and analyte losses were corrected by the labeled internal standard peptides. Although the direct injection of surfactants is generally avoided in MS applications, poloxamer was a very effective carrier.[37] Poloxamer may be a generalizable carrier for other SRM assays, such as samples with low total protein concentration from extensive sample enrichment protocols (e.g., SISCAPA).[33,40] LC columns should be reserved for applications using poloxamer to avoid surfactant carry-over.

α_{S1}-Casein Standards. His-tag-free recombinant ^{15}N-α_{S1}-casein protein was obtained at >85% chemical purity determined by Coomassie stain SDS-PAGE analysis. The deconvoluted intact mass was 23293.0 Da (Supporting Information, Supplemental Table 3). Enhanced resolution Q1 scans of three SRM target peptides determined the isotopic purity to be >99% (data not shown). The average (n = 12) concentration of the stock ^{15}N-α_{S1}-casein standard was determined to be 40 ± 4 pg/mL (mean ± σ_{n-1}) on the basis of SRM analyses of separate trypsin digestions using three unlabeled, amino acid analysis-quantified, synthetic α_{S1}-casein peptide standards.

Various amounts of SRM signal crosstalk from ^{13}C^{15}N-labeled synthetic peptides into ^{15}N-peptide channels were observed when labeled peptides were injected alone (Supporting Information, Supplemental Table 3). Corrections to ^{15}N-SRM areas were made to account for overlapping precursor−fragment ion transition cross-talk in assay calculations. Quantification was based on SRMs with <1% crosstalk. Unlabeled and ^{15}N-labeled peptides did not contribute signal to other SRM channels.

The average concentration of α_{S1}-casein in NIST NFDM standard 1549 determined using labeled synthetic peptides or the ^{15}N-labeled intact protein standard was 80 mg/g dry weight (Table 1). Determination of the same α_{S1}-casein concentration using different labeled synthetic peptides and ^{15}N-α_{S1}-casein peptides is evidence that trypsin digestion efficiencies at the corresponding sites in the NIST NFDM standard and the ^{15}N-α_{S1}-casein were equivalent. No increase in SRM signal intensity was observed at longer incubation times or higher trypsin concentrations to suggest that extensive or essentially complete digestion occurred. The data collectively demonstrate an operational equivalency of the ^{15}N-α_{S1}-casein and NIST NFDM 1549 standard as α_{S1}-casein standards.

Sample Defatting, Protein Extraction, and Postdigestion Filtration. CFSAN cookies and FAPAS biscuits have different recipes, and the latter contain guar and xanthan gum (Supporting Information, Supplemental Tables 1 and 2). Cookie and biscuit samples respectively lost 15 ± 1% (n = 6) and 20 ± 2% (n = 3) of their initial weight after defatting.

CFSAN cookies were processed and injected without incident. In contrast, FAPAS biscuits produced rapid, progressive increases in column back pressure that resulted in the irreversible loss of column performance. Particle filtration (0.22 μm) and C_{18} solid-phase extraction of trypsin digests did not solve the problem. SDS-PAGE analysis of HOAc-precipitated biscuit protein revealed a high molecular weight (>150 kDa) smeared band that resisted Coomassie staining and disrupted background gel staining (data not shown). MWCO (30 kDa) filtration of enzyme-free extracts or extraction of FAPAS samples using α-amylase, pectinase, and mannanase eliminated the back pressure problem, ostensibly by removing

Table 1. α_{S1}-Casein Concentration (Milligrams per Gram) Measured in NIST Nonfat Dry Milk Standard 1549 Using Intact ^{15}N-α_{S1}-Casein or ^{13}C^{15}N-Labeled Peptide Standards[a]

	FFVAPFPEVFGK			HQGLPQEVLNENLLR		YLGYLEQLLR	
internal standard	y_{10}	y_8	y_9	y_{11}	y_{13}	y_6	y_8
^{15}N-α_{S1}-casein	77 ± 9	81 ± 10	80 ± 11	88 ± 22	88 ± 30	85 ± 10	82 ± 11
^{13}C^{15}N-peptides	76 ± 11	80 ± 11	79 ± 11	73 ± 18	78 ± 28	88 ± 14	86 ± 13

[a]Average (n = 15) ± propagated error.

or degrading gum thickeners. Centrifugation of enzyme-treated FAPAS biscuit extracts produced distinct layers of solid and transparent liquid in the pellet, but extraction without enzymes rendered a more translucent, gelatinous third layer. Samples extracted with enzymes produced larger and more uniform pellets that contained 4-fold more protein. The 6-fold higher extraction efficiency of the enzyme extraction technique resulted in its routine use with all samples. ^{13}C^{15}N-Labeled peptide response relative to the angiotensin II internal standard was 4-fold lower than in samples extracted without enzymes. Twenty-five percent of total protein was recovered in the pellet and 75% in the HOAc supernatant in biscuit extracts containing carbohydrate-degrading enzymes. In extracts prepared without carbohydrate-degrading enzymes 18% was recovered in the pellet and 82% in the supernatant. CFSAN cookie digests extracted with enzymes performed identically to those prepared without enzymes. The use of MWCO filtration and enzymatic extraction may be advantageous when details about the sample composition are not known. Care should be exercised to determine that carbohydrate-degrading enzyme preparations do not contain proteases that could potentially eliminate target protein signals.

Measurement of α_{S1}-Casein and Calculation of Milk Concentration. CFSAN cookie and FAPAS biscuit digests were routinely injected (11−13 μg total digested protein) without increasing column back pressure, changing retention times by more than ±0.1 min, or altering chromatographic peak shapes.

CFSAN Cookies. The matrix-matched standard curve was unaffected by poloxamer, and measurements with and without carrier were averaged. The blank cookie matrix made no contribution to SRMs used to measure α_{S1}-casein. Peptide responses were linear from 1.3 to 150 fmol (Figure 2a). The limit of quantitation (LOQ) was 1.3 fmol, at which YLGYLEQLLR and FFVAPFPEVFGK SRMs exhibited signal-to-noise ratios >5. The limit of the blank (LOB) and limit of detection (LOD), respectively, were 0.4 and 0.6 fmol.[41] HQGLPQEVLNENLLR was detected but not used for quantification because it exhibits a much less abundant signal.

In baked cookies, NIST NFDM incursions calculated using ^{15}N-α_{S1}-casein were linearly correlated (R^2 > 0.996) with experimentally added levels (Figure 2b). Extraction recovery of ^{15}N-peptides was 66%, measured relative to ^{13}C^{15}N-labeled peptides (Supporting Information, Supplemental Table 4). However, the α_{S1}-casein peptide SRM profile in baked cookies was different from the unbaked spiked NIST NFDM and ^{15}N-α_{S1}-casein profiles, which were superposable (Figure 3). FFVAPFPEVFGK SRM peak areas were proportionately smaller relative to YLGYLEQLLR and HQGLPQEVLNENLLR and indicate a preferential loss during baking. NFDM concentrations calculated using YLGYLEQLLR and FFVAPFPEVFGK, respectively, were ∼60 and ∼80% below the

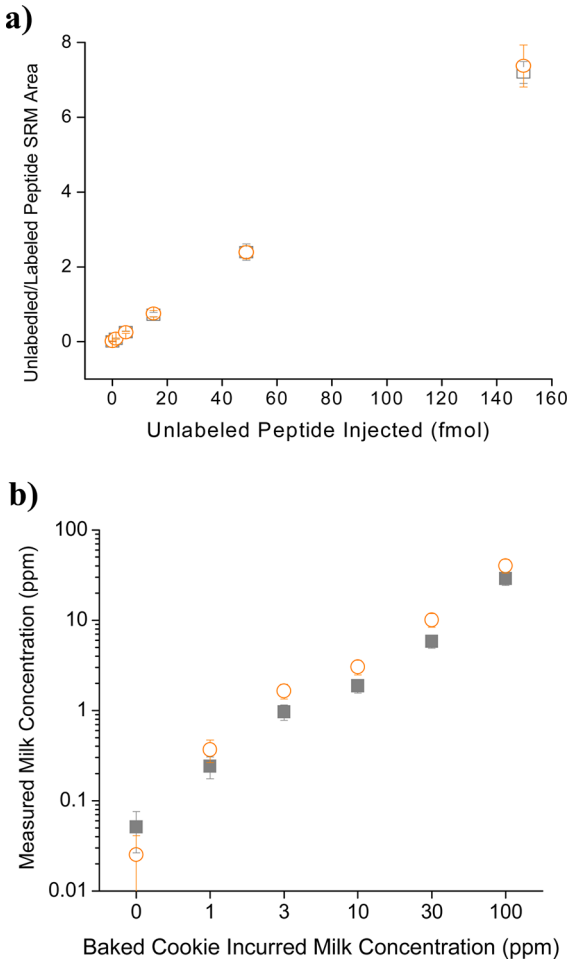

Figure 2. CFSAN baked cookie α_{S1}-casein SRM data. (a) Matrix-matched standard curve (0 ppm NFDM); ratio of unlabeled to ^{13}C^{15}N-labeled α_{S1}-casein peptide SRM areas: FFVAPFPEVFGK y_8 (black squares) and YLGYLEQLLR y_8 (orange circles). Average (n = 3) ± propagated error. CVs = 8%. (b) Calculated NFDM-incursion level from peptide transitions FFVAPFPEVFGK y_8 (gray squares) and YLGYLEQLLR y_6 (orange circles). Average (n = 3) ± propagated error. CV = 19% at 3 ppm.

experimentally added NFDM levels (Figure 2b; Supplemental Table 4 in the Supporting Information).

SRM analysis assumes protein digestion efficiency is equivalent at the respective trypsin sites and all peptide recoveries are equal.[42] The mole ratios of different peptides measured from a single protein are expected to equal 1, and therefore the peptide relative ion abundances should be constant across different samples if the source protein does not change. The assumptions are inconsistent with the baked cookie FFVAPFPEVFGK SRM data, although equimolar peptide ratios were observed in unbaked cookies spiked with NIST NFDM standard and ^{15}N-labeled α_{S1}-casein. Complex

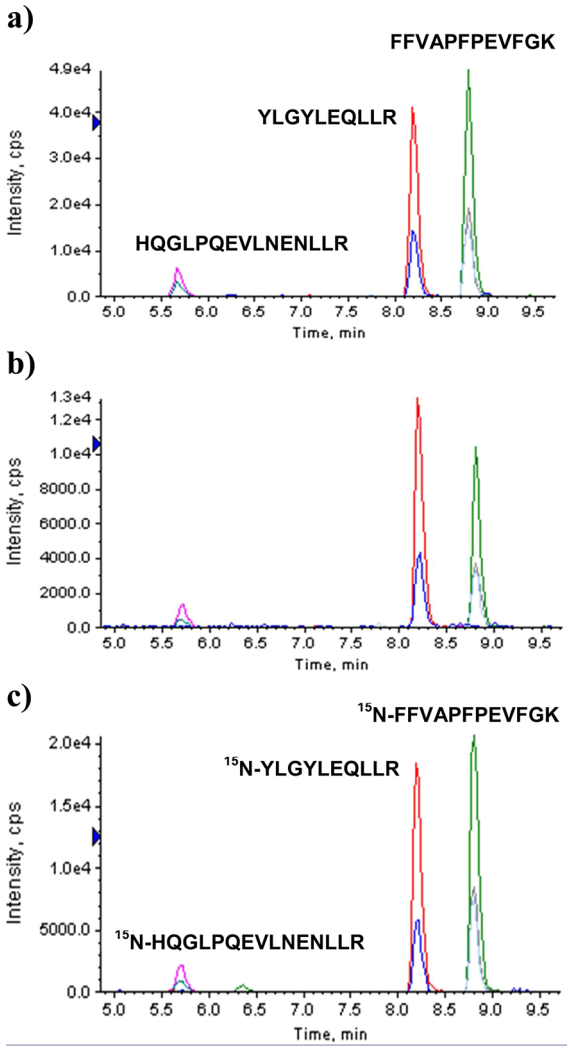

Figure 3. Extracted SRM ion chromatograms of unlabeled peptides in (a) unbaked NIST NFDM standard 1549 digest and (b) baked CFSAN cookie (30 ppm) and of ^{15}N-labeled peptides spiked in (c) baked CFSAN cookie (30 ppm).

Maillard reactions are important in the development of browning, aroma, and flavor during baking and affect the protein quality of heat-treated milk. Although milk protein nonenzymatic glycation has been extensively studied, more subtle effects on the quantitative protein analysis by SRM have not been documented.[43] The experimental biscuits and cookies contained reducing (e.g., lactose) and nonreducing (e.g., sucrose) sugars. Sucrose inversion during baking is known to yield glucose and fructose (both reducing sugars), and carmelization (at 180 °C) yields autoxidation products (e.g., dicarbonyls) that produce protein glycation.[43−46] Casein incubation with glucose and fructose was reported to yield glycation products and to decrease lysine content and casein solubility and was used to model Maillard reactions.[47−50] Lactosylation of milk protein during heating inhibits protein digestion by trypsin and decreases lysine bioavailability.[51,52]

The peptide SRM profile difference produced by baking is proposed to arise from nonenzymatic glycation of α_{S1}-casein. An unspecified covalent modification (or perhaps modifications) interferes with FFVAPFPEVFGK detection either by changing its mass or by inhibiting trypsin digestion at the C-terminal lysine (K34). HQGLPQEVLNENLLR and FFVAPFPEVFGK are contiguous, share an arginine-dependent tryptic site (R22), and are flanked by lysine-dependent sites at K7 and K34 (Scheme 1). This is different from YLGYLEQLLR, which contains arginine-dependent tryptic sites at R90 and R100. Maillard reactions preferentially occur at the ε-amino position of lysine upon heating with reducing sugars at neutral pH.[43,53] Nonenzymatic glycation of K34 should inhibit digestion with trypsin and decrease the peptide amount released for detection without affecting HQGLPQEVLNENLLR and YLGYLEQLLR, as shown in Scheme 1. The inhibition of α_{S1}-casein digestion by trypsin was previously mapped to lactosylation at K34, but arginine-dependent cleavage sites at R90 and R100 were unaffected in heated milk.[54,55] Maillard reactions can yield many different protein glycation products. Identification of α_{S1}-casein modifications that interfere with quantification in baked goods requires more detailed investigation to more fully complete the protein mass balance.

The peptide SRM profile in baked CFSAN cookies may have implications for selection of peptide sequences to detect other food allergens. Because Maillard reactions preferentially occur at the ε-amino position of lysine, peptides with arginine-dependent trypsin sites may be more resistant to nonenzymatic glycation and could yield more robust quantitative assays than lysine-dependent tryptic peptides that exhibit higher response factors. As illustrated by FFVAPFPEVFGK, a peptide that produces the strongest SRM signal does not necessarily confer the greatest quantitative accuracy.

FAPAS Biscuits. The matrix-matched standard curve was unaffected by poloxamer, and measurements with and without carrier were averaged. The biscuit matrix produced an interfering signal in the ^{15}N-YLGYLEQLLR y_8 channel that eluted 0.1 min later. The next most abundant y_6 product ion was instead used for quantification. Unlabeled peptide responses were linear from 6 to 85 fmol (Figure 4a). The LOQ was 6 fmol, at which SRMs exhibited signal-to-noise ratios >8. The LOB and LOD, respectively, were 0.5 and 0.7 fmol.

Baked biscuit α_{S1}-casein peptide SRM profiles were superposable on unbaked NIST NFDM and ^{15}N-α_{S1}-casein-spiked biscuit profiles. NFDM concentrations calculated using ^{15}N-α_{S1}-casein from the two SRM sets were equal within 0.6−6% (Table 2). Extraction recovery was 65−80%, measured relative to ^{13}C^{15}N-labeled peptides. Calculated NFDM concentrations were ∼66% lower than the initial experimentally added values

Scheme 1. Annotated Amino Acid Sequence of Bovine α_{S1}-Casein (UniProt P02662)[a]

RPKHPIK^{7}**HQGLPQEVLNENLLR**^{22}**FFVAPFPEVFGK**^{34}EKVNELSKDIGSESTEDQA
MEDIKQMEAESISSSEEIVPNSVEQKHIQKEDVPSER^{90}**YLGYLEQLLR**^{100}LKKYKVPQ
LEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWY
YVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW

[a]Lactosylation was previously identified at underlined lysines in heated milk.[54,55]

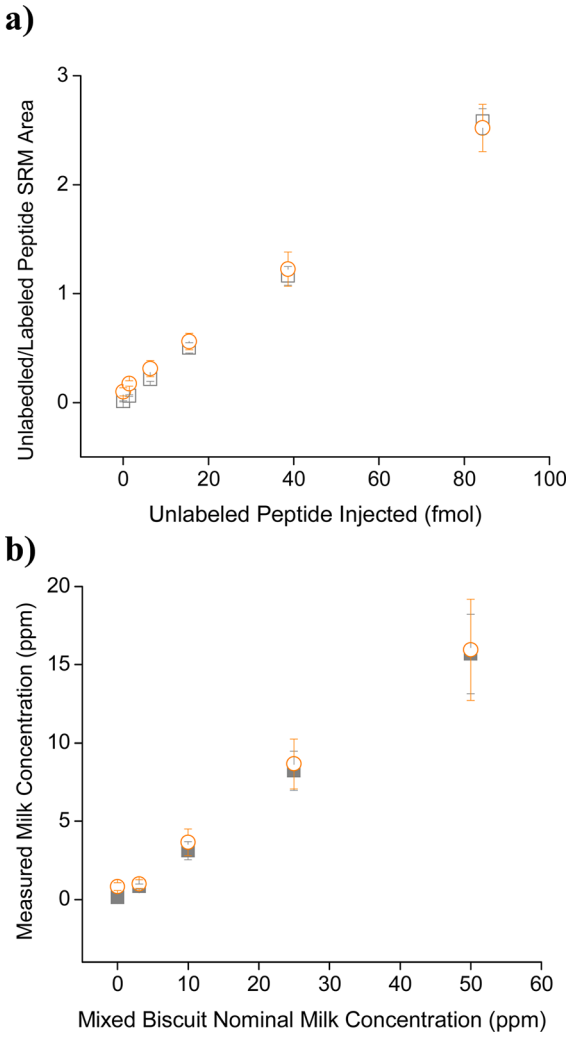

Figure 4. FAPAS baked biscuit α_{S1}-casein SRM data. (a) Matrix-matched standard curve (0 ppm NFDM, biscuit A); ratio of unlabeled to $^{13}C^{15}N$-labeled synthetic peptide area: FFVAPFPEVFGK y_8 (black squares) and YLGYLEQLLR y_6 (orange circles) ± propagated error. (b) Calculated NFDM concentration in samples prepared by dilution of biscuit C (50 ppm NFDM) in biscuit A (0 ppm): FFVAPFPEVFGK y_8 (gray squares) and YLGYLEQLLR y_6 (orange circles) ± propagated error.

(Table 2). Unbaked biscuit samples were not available for analysis.

FAPAS biscuit C (50 ppm NFDM) was diluted with biscuit A (0 ppm) to provide samples virtually incurred at lower NFDM levels. Calculated incursions were 66% lower and linearly correlated (R^2 > 0.997) with the experimentally added value (Figure 4b). Added NFDM concentrations determined from the two SRM sets were equal within 16% at 3 ppm. The noise (or background) signal level in analyte SRM channels from biscuit A was <0.9 ppm, an amount less than the error in measurement of the 3 ppm sample. Analysis of the biscuit dilution series (Figure 4) demonstrated that the assay is linear to the lowest concentration analyzed (3 ppm), at which the signal-to-noise ratio was 6 at a 12% CV.

NIST NFDM standard 1549 was used in the method of standard additions to measure NFDM concentrations in FAPAS samples (Table 3). Calculated concentrations were 40−60% lower than experimentally added levels. Agreement between NFDM concentrations determined by the method of standard additions and from using ^{15}N-α_{S1}-casein (Table 2; Figure 4b) demonstrated the internal consistency of SRM measurements in the experimental model.

Table 3. NFDM Levels in FAPAS Milk Allergen ELISA Proficiency Samples Measured Using ^{15}N-α_{S1}-Casein and NIST NFDM Standard 1549 by the Method of Standard Additions[a]

SRM set	"A" NFDM (ppm)	"B" NFDM (ppm)	"C" NFDM (ppm)
FFVAPFPEVFGK y_8	−0.08 ± 0.02	9.6 ± 0.9	23 ± 4
YLGYLEQLLR y_6	0.5 ± 0.1	9 ± 2	17 ± 3

[a]Average (n = 3) ± propagated error.

SRM-calculated incursions were 1.7−2.5 times greater than median levels determined in a multilaboratory milk allergen ELISA proficiency study (Tables 2 and 3).[34] Expected SRM results would be only ∼1.3 times greater than ELISA if the only difference was due to adjustment of SRM data for extraction efficiency (∼75%). However, the recovery-corrected biscuit and cookie SRM results respectively accounted for only 34 and 30% of added NFDM. α_{S1}-casein modifications that made it insoluble or resistant to acid precipitation or digestion or that altered the target peptide mass were not determined because spiked protein does not compensate for transformation and loss of milk protein due to baking. Baking-associated losses of α_{S1}-casein and milk proteins in other SRM and ELISA studies range from 25 to 91%.[24,56,57]

SRM profiles of FAPAS biscuits and CFSAN cookies were different; CFSAN cookies exhibited a preferential decrease of FFVAPFPEVFGK after baking. To determine if this phenomenon was unique to CFSAN cookies, analysis was performed on 10 different baked cookie products purchased at a local market. The range of differences in the FFVAPFPEVFGK SRM profiles

Table 2. Average (n = 3) Nonfat Dry Milk (NFDM) Concentration and ^{15}N-α_{S1}-Casein Recovery in FAPAS Milk Allergen ELISA Proficiency Biscuit Samples ± Propagated Error

	A (0 ppm NFDM)		B (20 ppm NFDM)		C (50 ppm NFDM)	
SRM set	recov (%)	calcd (ppm)	recov (%)	calcd (ppm)	recov (%)	calcd (ppm)
FFVAPFPEVFGK y_8	69 ± 14	0.2 ± 0.1	80 ± 11	7 ± 1	76 ± 14	18 ± 3
	64 ± 17	0.1 ± 0.1	76 ± 11	7 ± 1	82 ± 19	17 ± 3
	70 ± 13	0.3 ± 0.1	76 ± 16	7 ± 1	80 ± 13	17 ± 3
YLGYLEQLLR y_6	67 ± 24	0.6 ± 0.3	77 ± 23	7 ± 2	71 ± 13	17 ± 3
	61 ± 26	0.5 ± 0.3	74 ± 27	7 ± 2	78 ± 13	16 ± 3
	67 ± 19	0.7 ± 0.3	74 ± 25	7 ± 1	75 ± 12	16 ± 3

was similar to that between CFSAN cookies and FAPAS biscuits (data not shown). This demonstrates recipes can affect α_{S1}-casein quantification in products made using a wide range of ingredients and processing conditions. Previously reported effects of ingredients on nonenzymatic protein glycation can be used to rationalize why different results were obtained. Ammonium-based leavening agents can increase nonenzymatic glycation relative to $NaHCO_3$ in baked cookies.[58] Use of ammonium-based leavening in CFSAN cookies may have contributed to the decreased FFVAPFPEVFGK SRM relative to FAPAS biscuits made with $NaHCO_3$. Higher moisture baking conditions decrease the rates of Maillard reactions.[59] Hydrophilic gum thickeners and chickpea flour are expected to have produced higher moisture conditions during baking of FAPAS biscuits relative to CFSAN cookies lacking gums and containing only wheat flour.[60] The composition of commercial baked goods is extremely diverse and may affect the degree to which allergens are modified during food processing.

The PSAQ technique increased the accuracy of the α_{S1}-casein concentration measured in baked goods by correcting for extraction recovery. However, the SRM assay underestimated concentrations because the allergen protein is transformed by food processing in ways that interfered with peptide detection. When possible, the preferential use of arginine-dependent instead of lysine-dependent tryptic peptides could minimize SRM interferences caused by Maillard and other modifying reactions. Because the ingredients and processing history of foods encountered in regulatory analyses are likely to be unknown, high internal standard extraction recovery does not guarantee accurate quantification at concentrations demonstrated to be within assay range in validation studies. The influence of protein modifications encountered in processed food on the SRM quantification of allergens needs to be considered in future MS-based studies to more realistically examine and address practical analytical challenges. If chemical modifications of allergen proteins due to food processing are sufficiently abundant and limited to a particular type (e.g., deamidation, oxidation, and nonenzymatic glycation yielding furosine), the inclusion of modified peptides to make more complete SRM lists may account for some currently missing allergen protein, minimize false-negative results, and help asymptotically approach the elusive goal of performing absolute quantitation. To expand on the results reported here, our next studies will include the addition of ^{15}N-α_{S1}-casein in a cookie/biscuit recipe prior to baking. These analyses should yield further insights into the value of a labeled protein internal standard for quantitation as well as for the determination of the chemical fates of such food ingredients, for which this work provides provocative data.

■ ASSOCIATED CONTENT

Supporting Information

Positive ESI spectrum and deconvoluted zero charge-state mass of intact ^{15}N-α_{S1}-casein; SRM cross-talk of $^{13}C^{15}N$-labeled α_{S1}-casein peptides into ^{15}N-α_{S1}-casein peptides; CFSAN cookie and FAPAS biscuit recipes; and NFDM concentration and ^{15}N-α_{S1}-casein recovery measured in baked CFSAN cookie samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HOAc, acetic acid; BCA, bicinchoninic acid; CFSAN, Center for Food Safety and Nutrition; DP, declustering potential; FAPAS, Food Analysis Performance Assessment Strategy; NIST, National Institute of Standards and Technology; NFDM, nonfat dry milk; SRM, selected reaction monitoring.

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List of Supplemental Material

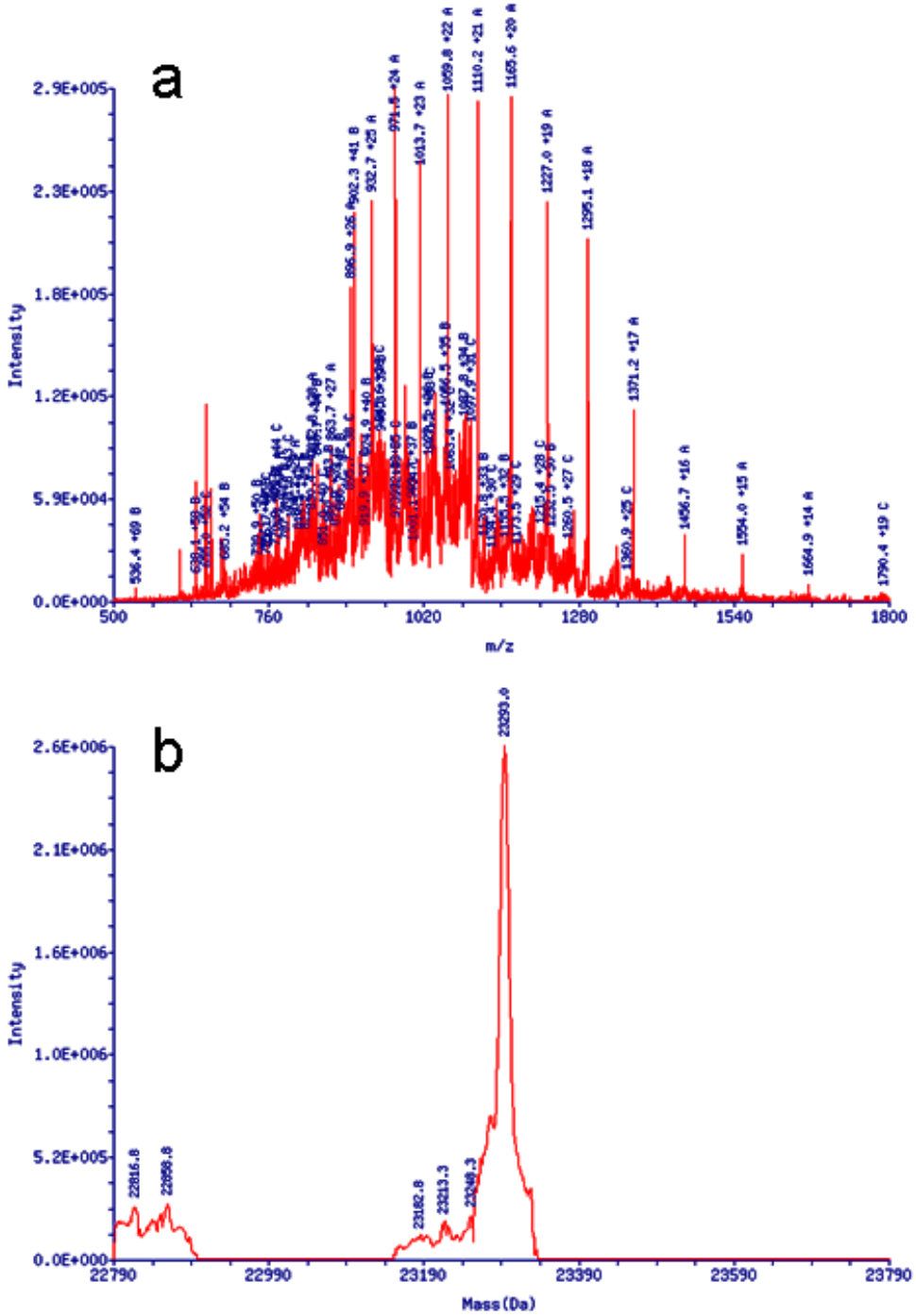
Supplemental Figure 1. Positive ion ESI mass spectrum of ^{15}N-labeled recombinant bovine α-S1-casein (a) and the corresponding deconvoluted zero-charge state average mass (M^0+Na+2(H_2O)) (b).

Supplemental Table 1. Ingredients in CFSAN cookies.

Supplemental Table 2. Ingredients in FAPAS biscuits.(*34*)

Supplemental Table 3. SRM cross-talk of $^{13}C^{15}N$-labeled α-S1-casein peptides into ^{15}N-labeled α-S1-casein peptide SRM channels, average (n=12) ± propagated error.

Supplemental Table 4. Non-fat dry milk (NFDM) concentration and ^{15}N-α-S1-casein recovery measured in CFSAN baked cookie samples ± propagated error.



Supplemental Figure S1. Positive ion ESI mass spectrum of ^{15}N-labeled recombinant bovine α-S1-casein **(a)** and the corresponding deconvoluted zero-charge state average mass 23,293.0 Da (M^0+Na+2(H_2O)) **(b)**.

Supplemental Table 1. CFSAN cookie ingredients.(*61*)

Component	Weight (grams)
olive oil	300
wheat flour	1180
powdered sugar	380
water	130
NaCl	6
$NaHCO_3$	2
$(NH_4)_2SO_4$	2
Sum	2000

Supplemental Table 2. FAPAS biscuits ingredients.(*34*)

Component	Weight (grams)
corn flour, rice flour, chickpea flour, starch, sugar, guar gum, xanthan gum, $Na_2H_2P_2O_7$, $NaHCO_3$, NaCl[a]	1429
vegetable fat	643
water	428
Sum	**2500**

a) FAPAS could not provide more detailed information regarding the individual amounts of these components.

Supplemental Table 3. SRM cross-talk of $^{13}C^{15}N$-labeled α-S1-casein peptides into ^{15}N-labeled α-S1-casein peptide SRM channels. Average (n = 12) ± propagated error.

SRM set	Product Ion	Unlabeled	$^{13}C^{15}N$	^{15}N	Crosstalk (%)
FFVAPFPEVFGK	y_8	692.9>920.5	697.9>930.5	699.4>929.5	0.4 ± 0.1
	y_9	692.9>991.5	697.9>1001.6	699.4>1001.5	9 ± 2
	y_{10}	692.9>1090.6	697.9>1100.6	699.4>1101.6	3.5 ± 0.8
HQGLPQEVLNENLLR	y_{11}	880.5>1324.7	885.5>1334.7	892.5>1341.7	0 ± 0
	y_{13}	880.1>1494.8	855.5>1504.8	892.5>1513.8	0 ± 0
YLGYLEQLLR	y_6	634.4>771.5	639.4>781.5	641.4>781.5	5 ± 1
	y_8	634.4>991.6	639.4>1001.6	641.4>1003.6	0.6 ± 0.2

Supplemental Table 4. Non-fat dry milk (NFDM) concentration and ^{15}N-α-S1-casein recovery measured in CFSAN baked cookie samples ± propagated error.

Incurred NFDM Concentration (ppm)	FFVAPFPEVFGK y_8		YLGYLEQLLR y_8		HQGLPQEVLNENLLR y_{11}	
	Recovery (%)	Calculated (ppm)	Recovery (%)	Calculated (ppm)	Recovery (%)	Calculated (ppm)
0	65 ± 10	0.05 ± 0.02	64 ± 12	0.02 ± 0.02	57 ± 12	0.09 ± 0.06
1	65 ± 9	0.24 ± 0.07	67 ± 10	0.4 ± 0.1	59 ± 15	0.4 ± 0.3
3	70 ± 10	1.0 ± 0.2	69 ± 10	1.7 ± 0.3	57 ± 11	2.1 ± 0.7
10	70 ± 9	1.9 ± 0.3	67 ± 11	3.0 ± 0.6	64 ± 17	3.3 ± 0.9
30	69 ± 9	5.9 ± 0.9	65 ± 11	10 ± 2	60 ± 18	9 ± 2
100	64 ± 9	29 ± 5	62 ± 9	40 ± 7	64 ± 14	45 ± 12