

Commercial Milk Enzyme-Linked Immunosorbent Assay (ELISA) Kit Reactivities to Purified Milk Proteins and Milk-Derived Ingredients

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Abstract: Numerous commercial enzyme-linked immunosorbent assay (ELISA) kits exist to quantitatively detect bovine milk residues in foods. Milk contains many proteins that can serve as ELISA targets including caseins (α -, β -, or κ -casein) and whey proteins (α -lactalbumin or β -lactoglobulin). Nine commercially-available milk ELISA kits were selected to compare the specificity and sensitivity with 5 purified milk proteins and 3 milk-derived ingredients. All of the milk kits were capable of quantifying nonfat dry milk (NFDM), but did not necessarily detect all individual protein fractions. While milk-derived ingredients were detected by the kits, their quantitation may be inaccurate due to the use of different calibrators, reference materials, and antibodies in kit development. The establishment of a standard reference material for the calibration of milk ELISA kits is increasingly important. The appropriate selection and understanding of milk ELISA kits for food analysis is critical to accurate quantification of milk residues and informed risk management decisions.

Keywords: allergen, detection, ELISA, milk, milk protein

Practical Application: Commercial enzyme-linked immunosorbent assay (ELISA) kits are commonly used in the food industry to detect residues of allergenic foods. However, ELISA kits from different manufacturers designed to detect residues of the same food actually target different proteins and quantify the presence of residues of the allergenic food differently. As there is no standard reference material for calibration of ELISA kits, the appropriate selection of ELISA kits for quantitative measurement of allergenic residues in food can be challenging. This research examined the differences in the ability of commercial milk ELISA kits to accurately detect and quantify a variety of milk proteins and milk-derived ingredients. Results indicate that proper selection of an ELISA kit for detection of allergenic residues in foods is critical to protecting consumers, complying with regulatory requirements, and performing accurate risk assessments.

Introduction

Allergy to cow's milk affects many people in the U.S., and is especially common among children (Gupta and others 2011; McGowan and Keet 2013). Milk-allergic patients have variable reactivity to the different milk allergens and many patients are allergic to multiple milk proteins (Wal and others 1995, 2001). Cow's milk contains several major allergens that are classified as 2 major groups; casein and whey (Damodaran and Parkin 2008). The casein fraction of milk includes α -, β -, and κ -casein. The whey fraction contains 2 major allergenic proteins, α -lactalbumin (ALA) and β -lactoglobulin (BLG). Milk allergens exhibit substantial differences in sequence, functionality, reactivity, and allergenicity (Chatchatee and others, 2001). For a more in-depth analysis of cow's milk proteins and associated allergic response, the authors recommend the review article by Wal (2002).

The proportions of proteins in fluid milk remain relatively constant across bovine breeds and geographical changes (Wal and others 2001). Caseins comprise nearly 80% of milk proteins, of which, α -, β -, and κ -casein represent 50%, 37%, and 13% of total casein protein, respectively (Wal 2002). Whey contains the remaining 20% of milk protein, where BLG and ALA comprise roughly 50%

and 25%, respectively. Other milk proteins exist at low concentrations, but their allergenicity remains unproven (Wal 2002).

Fluid milk is modified in numerous ways to maximize its desirable characteristics for use in the food industry. Some milk-derived ingredients undergo processes to remove certain milk components. Furthermore, various methods of processing can alter the accessibility and reactivity of antibody-binding epitopes in foods (Poms and Anklam 2004). Due to widespread use of milk and derivative ingredients in the food industry and the comparative importance of milk allergy, accurate, and reliable methods of detecting small amounts of milk protein residues in products, equipment, and facilities must be available.

One of the most commonly used methods to quantitatively analyze target proteins in the food is the enzyme-linked immunosorbent assay (ELISA). Many commercialized ELISAs are available for use by industry and regulatory agencies. Variations among commercial ELISA kits include antigen sources, antibody production, kit calibrants, and reporting units. Antigens derived using different source species or breeds, processing conditions, and extraction conditions may be used to produce antibodies of varying epitope specificity and affinity (Cordle 2006; Abbott and others 2010). Kit manufacturers also use various materials to serve as kit calibrants or standards. Furthermore, the reporting units are often inconsistent among kits, making comparison of results difficult. The lack of widespread adoption of a standard reference material for the development of ELISA kits contributes to the complexity of kit comparison. The AOAC Task Force recommends the

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use of the National Institute of Standards and Technology (NIST) nonfat milk powder (NIST-RM-1549) as the standard reference material for calibration and quantification of commercial milk ELISAs (Abbott and others 2010). The NIST-RM-1549 nonfat milk powder; however, has been replaced by NIST with a whole milk powder standard reference material (NIST-RM-1549a). If a calibration material other than NIST NFDm is used to build the standard curve, it is suggested that the manufacturer provide an experimentally validated conversion factor between the calibration material and NIST milk powder.

Many ELISA kit users are unaware of the importance of kit specificities in accurate detection and quantification of allergen residues. In this research, the sensitivities of 9 milk-specific ELISA kits to 5 purified milk proteins and 4 milk-derived ingredients were determined. Kit selection and usage by the food industry as well as regulators must be driven by an appropriate understanding of the specificities, assumptions, and limitations of commercial milk ELISA kits. The importance of rapid, sensitive, robust, and specific methods for the detection of allergenic residues in packaged and processed foods is critical for the protection of food-allergic consumers, the validation of preventive allergen controls used on shared processing equipment, and compliance with labeling regulations.

Materials and Methods

Materials and ELISA kits

Milk protein fractions including α_s -casein ($\geq 70\%$ purity), β -casein ($\geq 98\%$ purity), κ -casein ($\geq 70\%$ purity), β -lactoglobulin ($\geq 90\%$ purity), and α -lactalbumin ($\geq 85\%$ purity) were purchased from Sigma-Aldrich Corporation (St. Louis, Mo., U.S.A.). Milk-derived ingredient samples of whey protein concentrate (WPC) at 2 concentrations, 34% protein (WPC34) and 80% protein (WPC80), and sodium caseinate (NaCas) were donated by Erie Foods International, Inc. (Erie, Ill., U.S.A.). Low-heat processed nonfat dry milk was obtained from Darigold® (Seattle, Wash., U.S.A.). C & H® Pure Granulated White Cane Sugar was purchased from a local retail store in Lincoln, Nebr. (distributed by Domino Foods, Yonkers, N.Y., U.S.A.).

Neogen® Veratox® Total Milk, Casein, and BioKits™ BLG ELISA kits were obtained from Neogen® Corporation (Lansing, Mich., U.S.A.). ELISA Systems™ Casein and β -lactoglobulin ELISA kits were obtained from ELISA Systems™ (Windsor, Qld., Australia). R-Biopharm RIDASCREEN® Fast Casein, Fast Milk, and Fast BLG ELISA kits were obtained from r-Biopharm, Inc. (Darmstadt, Germany). The Romer Labs® AgraQuant® BLG ELISA kit was obtained from Romer Labs (Union, Mo., U.S.A.). Kit information is listed in Table 1.

Spike preparation

Homogenous spikes were created in a neutral matrix of granulated cane sugar at levels that correlate with the levels detectable by milk ELISA kits. First, a 250 parts per million (ppm) concentrated spike of each purified protein or milk-derived ingredient was developed by adding 0.025 g of each purified protein or milk ingredient to 99.975 g of granulated sugar. The mixture was ground for 1 min in a Mr. Coffee® IDS77 Coffee Grinder (Jarden Consumer Solutions, Boca Raton, Fla., U.S.A.). After the sides of the bowl were scraped down with a rubber spatula, grinding was continued for another minute. Nine subsamples of each concentrated spike were evaluated for homogeneity using the Neogen Veratox® Total Milk kit.

The concentrated 250 ppm spike was used to create spikes of lower concentrations in granulated sugar. The concentrations were selected based on the standard solutions provided by each ELISA kit manufacturer. For example, a kit with standard solutions at 0, 2.5, 5, and 10 ppm NFDm would have spikes of 0, 2.5, 5.0, and 10 ppm of each purified protein or milk-derived ingredient. Spikes were produced using the same method of weighing and grinding, as described above. A total of 100 g of each concentration of spiked protein or milk-derived ingredient was produced.

Sample evaluation: protein concentration and electrophoresis

The protein content of each milk derivative was determined by LECO Dumas Protein analysis (Association of Official Analytical Chemists 1984). Proportions were applied to ELISA results to evaluate the capability of kits to quantify milk proteins in samples where the bovine milk micelle proportionality is not preserved.

Additionally, each purified protein and milk ingredient was evaluated using SDS-PAGE using the protocol of Laemmli (1970). Samples were solubilized in 0.01 M phosphate-buffered saline (PBS) and protein concentrations were estimated using the Lowry Protein Assay (Lowry and others, 1951). Proteins were prepared using reducing conditions by boiling 50 micrograms (μ g) of each sample in Laemmli sample buffer containing 5.4% dithiothreitol (percentage on a weight per volume basis (w/v)) for 5 min. Protein separation was performed using a Bio-Rad Mini-Protean® Tetra-cell electrophoresis unit (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). Five microliters of Precision Plus Protein™ Dual X-tra Standard was loaded in the first lane of each gel (Bio-Rad Laboratories). Multiple protein levels of each reduced sample were loaded on a 12% Mini-Protean® TGX™ Tris-HCl precast gel, 10 wells, with 30 μ L maximum volume per well (Bio-Rad Laboratories). Gels were run at a constant voltage of 200 V for approximately 30 min, or until the dye front reached the bottom of the gel.

Gels were fixed in a solution of 60% trichloroacetic acid (w/v) and 17.5% 5-sulfosalicylic acid (w/v) diluted 1:5 with deionized water (Sigma-Aldrich). Fixed gels were stained overnight using Coomassie Brilliant-Blue R-250 Staining Solution (Bio-Rad Laboratories). After a minimum of 8 h of staining, gels were destained using the Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad Laboratories). Gel images were captured using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, N.Y., U.S.A.) and evaluated using the Carestream Molecular Imaging software (v.5.02.30, Carestream Health, Rochester, N.Y., U.S.A.).

ELISA kit selection and performance

The 9 ELISA kits were selected based on prior history of acceptable performance based upon standard curves that exhibited an $r^2 \geq 0.98$ and a %CV $\leq 20\%$ among sample replicates. Each purified protein was analyzed using appropriate kits. Spiked proteins were weighed, extracted, and analyzed according to kit manufacturer's instructions for each kit. Sodium caseinate and whey protein concentrates were analyzed using only the total milk kits (Neogen Veratox® Total Milk and R-Biopharm RIDASCREEN® Fast Milk). NFDm was analyzed with all kits.

Briefly, 1 g of each spiked sample was weighed into a 50 mL polypropylene Falcon™ tube (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and the appropriate amount of prewarmed extraction buffer (60 °C) was added to the tube according to the manufacturers' instructions in each kit. The samples were vortexed and placed in a 60 °C shaking waterbath (Julabo™ SW22 shaking waterbath; Julabo U.S.A., Allentown, Va., U.S.A.) for the allotted extraction

Table 1—Commercial milk ELISA kit limits, calibrants, dynamic range, and reporting units.

Kit	LOD ^a	LOQ ^b	Dynamic range	Calibrant	Reporting units (ppm)
ELISA Systems™ Casein	0.5 ppm	1.0 ppm	1.0 to 10 ppm	a-Casein	Skim milk powder
Neogen Veratox® Casein	1.0 ppm	2.5 ppm	2.5 to 15 ppm	NFDM	NFDM
R-Biopharm RIDASCREEN® Fast Casein	0.12 ppm	0.5 ppm	0.5 to 13.5 ppm	Casein	Casein
ELISA Systems™ BLG	0.05 ppm	0.1 ppm	0.1 to 1 ppm	BLG	BLG
Neogen BioKits™ BLG	2.0 ppm	2.5 ppm	2.5 to 40 ppm	whole milk powder	BLG
R-Biopharm RIDASCREEN® Fast BLG	0.19 ppm	0.5 ppm	0.5 to 13.5 ppm	BLG	BLG
Romer Labs® AgraQuant® BLG	1.5 ppb	10 ppb	10 to 400 ppb	BLG	BLG ^c
Neogen Veratox® Total Milk	1.0 ppm	2.5 ppm	2.5 to 25 ppm	NFDM	NFDM
R-Biopharm RIDASCREEN® Fast Milk	0.7 ppm	2.5 ppm	2.5 to 67.5 ppm	Milk protein	Milk protein

^aLOD represents the kit limit of detection.
^bLOQ represents the kit limit of quantitation.
^cRomer Labs AgraQuant BLG kit reports in units of ppb BLG.

Table 2—Proportions for conversion of purified protein spikes to reporting units of ELISA kits.

Spike material	Proportion of casein	Proportion of MP	Proportion of NFDM
α-Casein	0.5	0.4	0.14
β-Casein	0.37	0.3	0.100
κ-Casein	0.13	0.1	0.036
β-Lactoglobulin (BLG)	n/a	0.1	0.035
α-Lactalbumin (ALA)	n/a	0.05	0.018

Eq. 1—Average Ratio of Detection. Proportions Represented in Table 2.

$$\sum \left[\left(\frac{\text{detected ppm with kit}}{\text{ppm spike level}} \right) \times (\text{proportion of respective milk derivative}) \right] \\ \text{total number of spike levels}^a \\ = \text{average ratio of detection}$$

^aApplicable only for spike levels > 0 ppm.

period. Tubes were then removed from the waterbath and cooled to room temperature. One milliter of each extracted sample was pipetted into a 1.5 mL Eppendorf™ microcentrifuge tube (Fisher Scientific), and centrifuged in a Thermo Scientific Legend Micro 17 centrifuge (ThermoScientific, Rockford, Ill., U.S.A.) for the recommended time and speed indicated in each kit manufacturer’s instructions. Three independent extractions were performed of each sample.

Extracted samples were analyzed with respective ELISA kits. Of the kits selected, 8 were sandwich ELISAs, and the Neogen® BioKits™ BLG was an indirect-competitive format. For the 8 sandwich ELISAs, antibody-coated wells, conjugate antibody, substrate, stop solution, wash buffer, and solutions of standard concentration were provided with each kit. For the indirect-competitive ELISA kit, antibody-coated wells, avidin peroxidase, conjugate antibody, substrate, and stop solution were provided. Prepared samples were applied to respective kits and evaluated according to manufacturer’s instructions. The optical density of each well was read at the appropriate wavelength recommended for each kit with a DYNEX Spectra MR™ Plate Reader (Dynex Technologies, Chantilly, Va., U.S.A.).

The optical density readings of each manufacturer-provided standard solution was used to create a standard curve. The optical density of each sample was then applied to the curve to establish the analyte concentration in the recommended reporting units.

Data analysis

Data was quantified and analyzed in Microsoft Excel, GraphPad Prism v.4.03, and any manufacturer-provided software (Neogen Veratox® v.3 and R-Biopharm RIDASCREEN® Ridasoft Win v.1.42). Curve fits were applied with software as recommended per manufacturer’s instructions. If no curve was recommended, a 4-parameter sigmoidal dose-response curve was used for quantitation. Statistical analyses were performed in Microsoft Excel and GraphPad Prism v.4.03.

Kit comparison approaches

ELISA kit results must be converted into consistent units for comparison purposes. Estimates for the proportion of individual protein fractions to the overall milk protein concentration were obtained (Wal and others 2001). Estimates for the concentration of milk protein in NFDM were obtained from the USDA National Nutrient Database for Standard Reference and through LECO Dumas Analysis.

Using estimates, several equations were constructed and used to convert between concentrations of individual proteins and the equivalent concentrations of milk or milk derivatives (Eqs S.1 to S.4). These equations were used to calculate the proportions displayed in Table 2. An additional equation was constructed to calculate the efficiency of each kit. The resulting ratio of detection is a measure of detected milk residues observed in each kit relative to the actual concentration of milk residues in each spiked sample, and is calculated by application of the proportions listed in Table 2 to Eq. 1. Additional supporting equations and information are included in the Supplementary Materials.

Results and Discussion

SDS-PAGE

All purified proteins and milk ingredients were analyzed with SDS-PAGE (data not shown) to determine their relative purity. Visual inspection of protein profiles of all purified proteins (α-, β-, and κ-casein, β-lactoglobulin, and α-lactalbumin) confirmed their relative purity with the value listed on the Certificate of Analysis. α-, β-, and κ-casein exhibited purity levels of 90%, 98%, and 89% purity, respectively. β-Lactoglobulin and α-lactalbumin were described to be 97% and 99% pure, respectively. The SDS-PAGE profiles of sodium caseinate, WPC34, and WPC80 were as expected (data not shown). Thus, the purified proteins and milk-derived ingredients were determined to be of sufficient quality to use for these ELISA comparisons.

Casein kits and detection of α -, β -, and κ -casein

Spiked samples of α -, β -, and κ -casein were analyzed with Neogen Veratox[®] Casein, ELISA Systems[™] Casein, and R-Biopharm RIDASCREEN[®] Fast Casein ELISA kits. Using the Neogen Veratox[®] Casein kit, spikes of both α - and β -casein were detected with a ratio of approximately 1.0, corresponding to 100% detection efficiency of the spike level. However, κ -casein was not detected at any of the spike levels evaluated with the Neogen Veratox[®] Casein kit (Figure 1A). Evaluation of casein spikes with the ELISA Systems[™] Casein kit revealed that the kit primarily detects α -casein, exhibiting a ratio of detection of 0.9

(Table 3). Spikes of β - and κ -casein were not detected at any spike level evaluated (Figure 1B). The R-Biopharm RIDASCREEN[®] Fast Casein kit detected κ -casein at a ratio of 1.0, β -casein at a ratio of 0.1, and α -casein at a ratio of 0.01 (Figure 1C and Table 3). The varying reactivities of the kits to different milk protein fractions create challenges for comparison of results between kits.

BLG kits and detection of whey proteins

BLG kits evaluated in this study included the Neogen BioKits[™] BLG, ELISA Systems[™] BLG, R-Biopharm RIDASCREEN[®] Fast BLG, and Romer Labs[®] AgraQuant[®] BLG. Although a

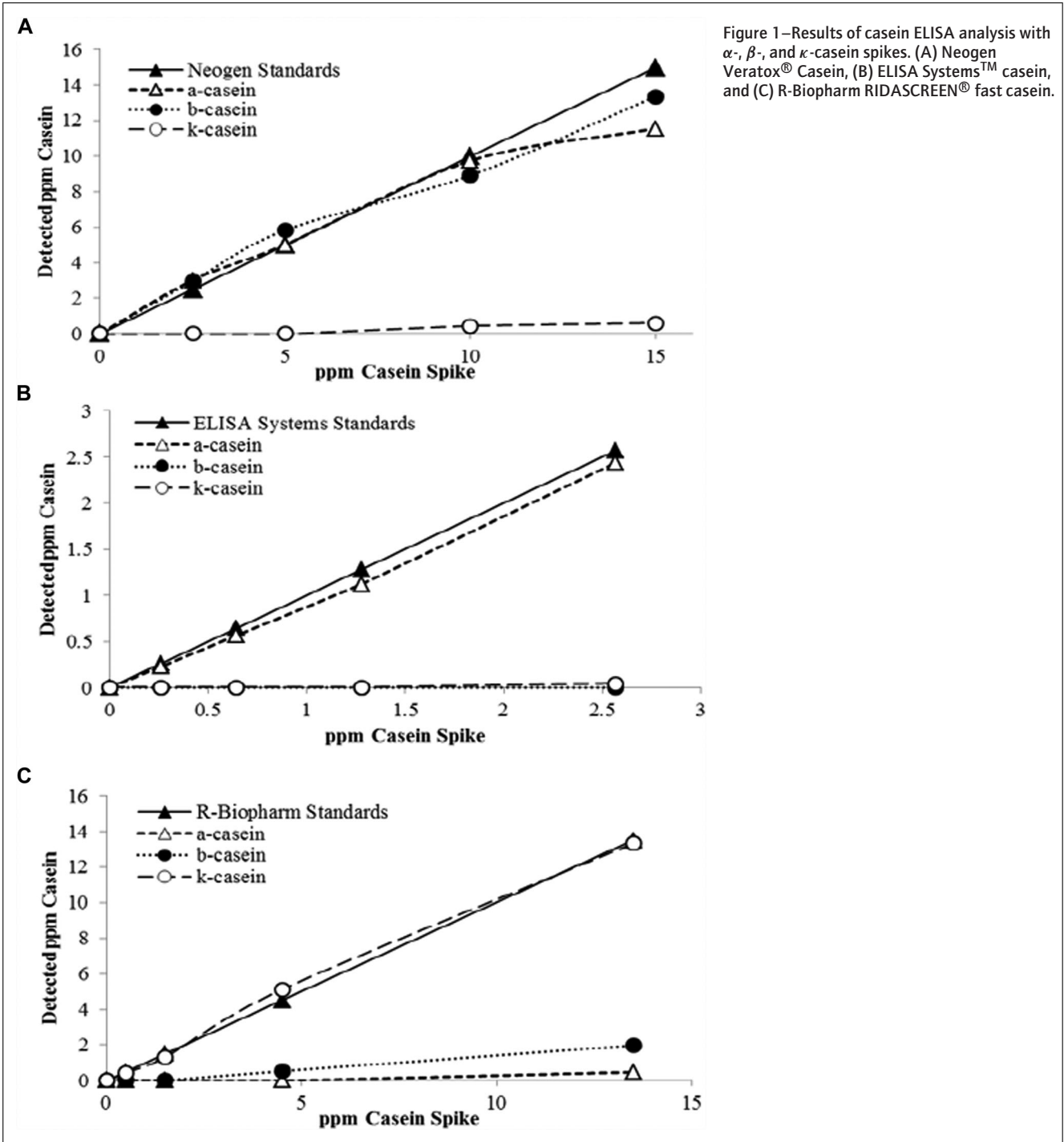


Figure 1—Results of casein ELISA analysis with α -, β -, and κ -casein spikes. (A) Neogen Veratox[®] Casein, (B) ELISA Systems[™] casein, and (C) R-Biopharm RIDASCREEN[®] fast casein.

major whey protein and an important allergen, ALA is not detected by any milk kit at any spike concentration evaluated (data not shown). All BLG kits are capable of detecting BLG in spiked samples, albeit at various levels (Figure 2). Results are expressed in Figure 2 as percent detection of BLG in the spikes. This was done to more clearly illustrate the differences among the kits. None of the kits evaluated detected BLG with a ratio of 1.0. Two kits, the Romer Labs® AgraQuant® BLG and ELISA Systems™ BLG kits detect BLG at ratios higher than the level in the spikes (ratios of 1.73 and 1.20, respectively) (Table 3). The R-Biopharm Fast Milk and the Neogen BioKits™ BLG kits detect BLG at ratios of 0.73 and 0.68, respectively (Table 3).

BLG and ALA spikes were also analyzed using Neogen Veratox® Total Milk and R-Biopharm Fast Milk kits. The Neogen Veratox® Total Milk kit did not detect BLG at any of the spike concentrations evaluated. The R-Biopharm Fast Milk kit exhibited a ratio of detection of 0.42 (Table 3).

Less variation exists among BLG kits than among casein kits; all BLG kits are capable of detecting BLG, while not all casein kits are capable of detecting all fractions of casein (Figure 1 and 2).

Total milk kits and detection of milk proteins

Two total milk kits were assessed in this study, Neogen Veratox® Total Milk and R-Biopharm RIDASCREEN® Fast Milk. Neither kit was capable of detecting ALA at any concentration evaluated (data not shown). While the Neogen Veratox® Total Milk kit detected α -, β -, and κ -casein, it fails to detect purified BLG and ALA at any spike concentration evaluated (Figure 3A). The detection of milk residues with the Neogen Veratox® Total Milk primarily depends on sensitivity to κ -casein (ratio of 0.48) and less on sensitivity to α - and β -casein (ratios of 0.11 and 0.13, respectively, Table 3). Upon evaluation with the purified milk proteins, the R-Biopharm Fast Milk kit did not detect α -casein, κ -casein, and ALA (Figure 3B). However, the kit detects β -casein and BLG at ratios of approximately 20% and 40%, respectively (Table 3).

Detection of milk-derived ingredients with ELISA kits

Spikes of milk-derived ingredients were evaluated with the Neogen Veratox® Total Milk and the R-Biopharm RIDASCREEN® Fast Milk kits. The concentrations of milk protein present in NFDM, WPC34, WPC80, and NaCas were determined using LECO Dumas Analysis. The protein concentration estimates were used to determine equivalent or expected

concentrations of spikes created from each derivative. Both total milk kits are capable of detecting residues in all milk-derived ingredients evaluated.

WPC80 contains approximately 78.5% protein, while sodium caseinate contains 90% protein. WPC34 contains 32.4% protein. LECO Dumas Analysis indicated the NFDM used in spikes contained approximately 36% protein. The USDA National Nutrient Database for Standard Reference indicates that NFDM has an average concentration of 35% protein. Using conversion factors (Table 2) to normalize the protein concentrations in the milk-derived ingredients, detection can be compared among samples (Eqs S.8 and S.9, Supporting Information). The Neogen Veratox® Total Milk kit most accurately quantified milk residues in sodium caseinate and NFDM, exhibiting ratios of detection of 0.71 and 0.70, respectively (Table 3 and Figure 4). Similar detection of milk residues is observed when the Neogen Veratox® Total Milk kit is used to analyze WPC34 and WPC80. Ratios of 0.27 and 0.24 were recorded for spiked samples of WPC34 and WPC80, respectively (Table 3 and Figure 4). The detection of WPC34 and WPC80 with this kit is surprising given the lack of detection observed with spikes of BLG and ALA, but may reflect the presence of κ -casein in these commercial whey products. Despite its lack of reactivity with ALA and BLG, the Neogen Veratox® Total Milk kit appears to function reasonably well with the major milk ingredients, casein and whey.

Purified protein analysis reveals that R-Biopharm RIDASCREEN® Fast Milk kit detects 1 protein each from the casein and whey fractions: β -casein and BLG. However, the Neogen Veratox® Total Milk kit only detects proteins from the casein fraction of milk. It is known that κ -casein is more sensitive to proteolysis than other caseins and will interact with other milk proteins upon heat treatment (Wal 2002). Due to the ability of casein to interact with other milk proteins during heat treatment, however, κ -casein peptides may be found in a variety of heat-treated milk products thus making κ -casein an acceptable target protein for the detection of milk residue derived from both the casein and whey fractions.

After the application of conversion factors (Table 2) to normalize the protein concentrations of the milk-derived ingredients in the units used to express kit results, the R-Biopharm RIDASCREEN® Fast Milk kit was determined to be the most sensitive to NFDM, exhibiting a ratio of 1.14 (Table 3 and Figure 4). The kit's sensitivity was similar for samples of WPC34, WPC80, and NaCas, with ratios of detection at 0.54, 0.45, and 0.48, respectively (Table 3 and Figure 4). According to previous results, the R-Biopharm Fast Milk kit exhibits primary sensitivity to BLG and a low level of sensitivity to β -casein. Because of the accuracy and sensitivity exhibited with the detection of NFDM, it is possible that NFDM or a closely related milk derivative was used for kit calibration.

The R-Biopharm RIDASCREEN® Fast Milk kit detected WPC and NFDM at higher levels than the Neogen Veratox® Total Milk kit. However, NaCas was detected more efficiently using the Neogen Veratox® Total Milk kit than when the R-Biopharm RIDASCREEN® Fast Milk kit was used (Figure 4). Given the increased sensitivity of the Neogen Veratox® Total Milk kit to κ -casein and the primary sensitivity of the R-Biopharm RIDASCREEN® Fast Milk kit to BLG, these results are expected. NFDM and NaCas exhibited similar levels of reactivity in the Neogen Veratox® Total Milk Kit, while NFDM exhibited the highest level of reactivity for the R-Biopharm

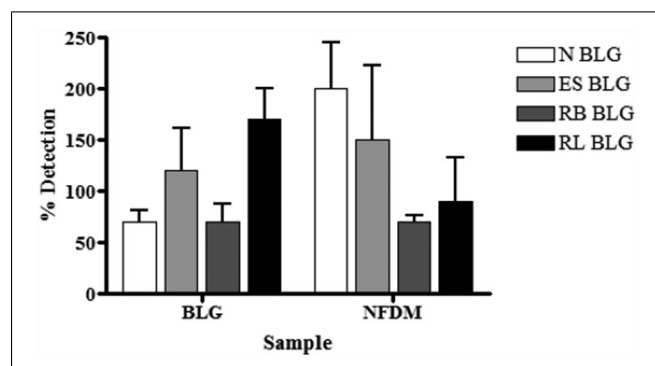


Figure 2—Observed detection (%) of spiked samples containing BLG and NFDM with BLG ELISA kits (Neogen BioKits™ BLG, ELISA Systems™ BLG, R-Biopharm RIDASCREEN® Fast BLG, Romer Labs® AgraQuant® BLG).

RIDASCREEN® Fast Milk kit among milk-derived ingredients (Figure 4).

Detection of NFDM using ELISA kits

NFDM was evaluated using all 9 commercial milk ELISA kits mentioned above. All kits evaluated were capable of detecting milk residues in samples of NFDM. Variation in quantitation of milk residue may be a result of kit development. Because kits are not developed using the same materials, antibody sensitivities, or calibrated to a standard reference material, the testing of the same sample with multiple kits would not be expected to provide consistent results. Results indicate that the Neogen BioKits™ BLG

kit detects NFDM at the highest ratio of all kits evaluated (2.0) (Figure 5). However, the Neogen BioKits™ BLG kit is not capable of detecting trace residues of NFDM at concentrations lower than 100 ppm NFDM. The Romer Labs® AgraQuant® BLG kit is most sensitive to NFDM, as it is able to detect milk residues in spikes containing 1 ppm NFDM. Kit limits of detection, limits of quantitation, and other vital kit information are listed in Table 1.

Of the 2 total milk kits evaluated, the Neogen Veratox® Total Milk kit is slightly more sensitive to low levels of milk residues in NFDM than the R-Biopharm RIDASCREEN® Fast Milk kit (Figure 5). Both kits detect milk residues in the range of 10 to

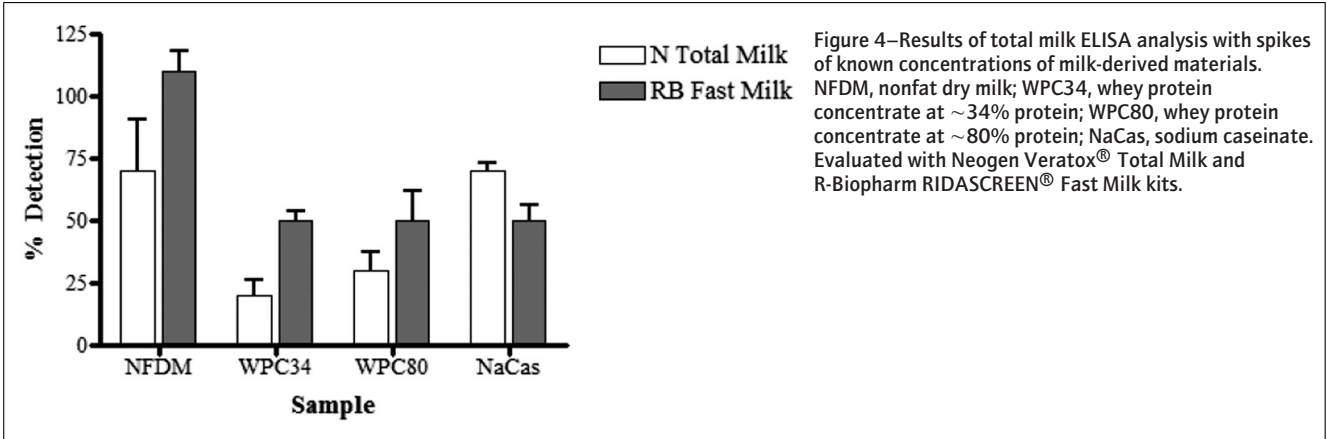
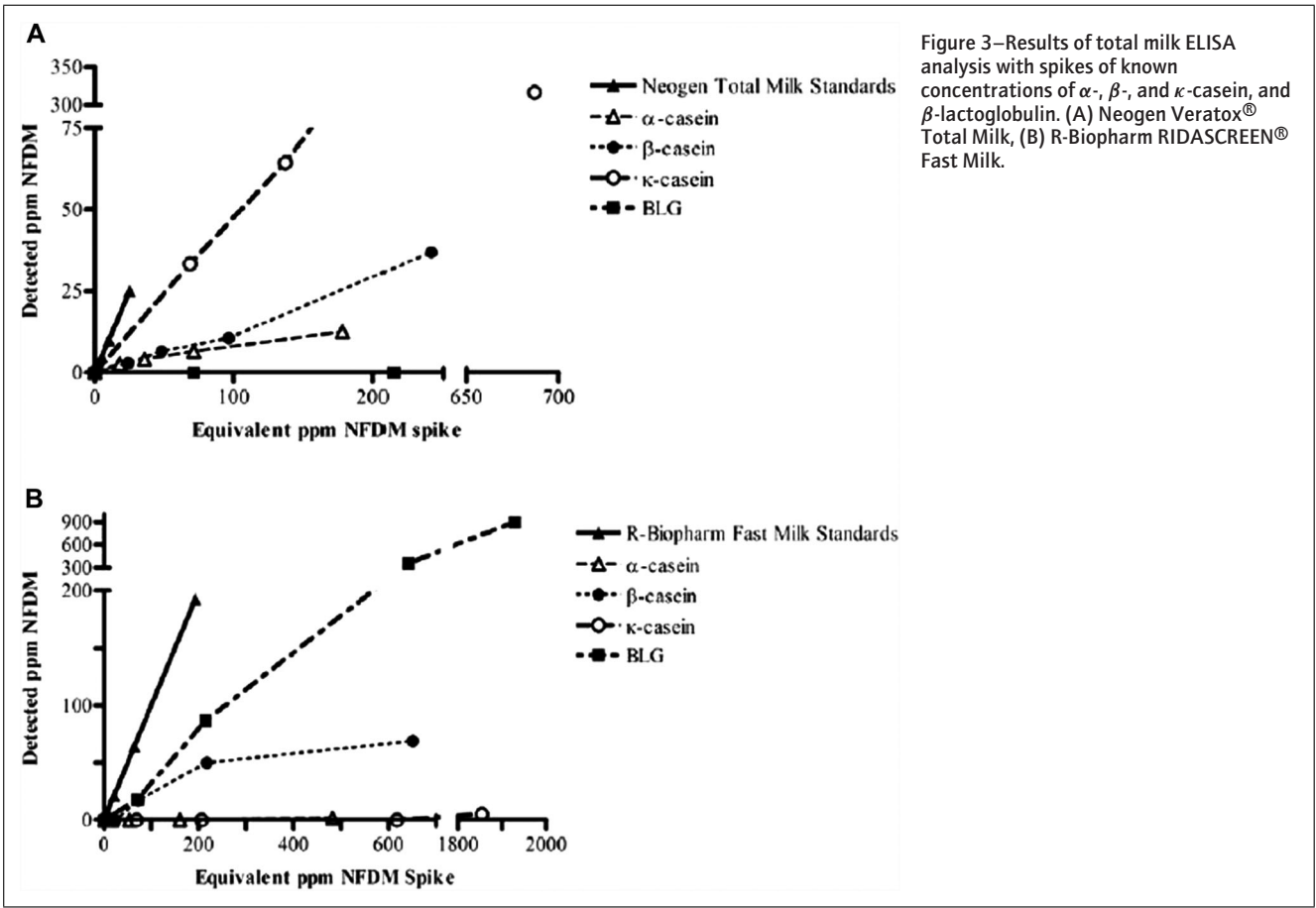


Table 3—Percent detection of milk proteins and ingredients by commercial milk ELISA kits.^{a,b}

Kit	α -Casein	β -Casein	κ -Casein	BLG	WPC34	WPC80	NaCas	NFDM
Neogen Veratox Casein	100 $\pm$ 18	100 $\pm$ 17	BLQ					50 $\pm$ 6.0
ELISA Systems Casein	90 $\pm$ 3.5	BLQ	BLQ					120 $\pm$ 30
R-Biopharm Fast Casein	BLQ	10 $\pm$ 2.0	100 $\pm$ 13					70 $\pm$ 13
Neogen Veratox Total Milk	10 $\pm$ 3.6	10 $\pm$ 1.8	50 $\pm$ 2.6	BLQ	20 $\pm$ 3.6	30 $\pm$ 7.4	70 $\pm$ 3.5	70 $\pm$ 21
R-Biopharm Fast Milk	BLQ	20 $\pm$ 7.4	BLQ	40 $\pm$ 13	50 $\pm$ 4.0	50 $\pm$ 12	50 $\pm$ 6.2	110 $\pm$ 8.2
Neogen BioKits BLG				70 $\pm$ 11				200 $\pm$ 45
ELISA Systems BLG				120 $\pm$ 41				150 $\pm$ 73
R-Biopharm Fast BLG				70 $\pm$ 18				70 $\pm$ 6.5
Romer Labs BLG				170 $\pm$ 30				90 $\pm$ 43

^aBLQ indicates a reading below the level of quantitation for the ELISA kit.

^b α -lactalbumin (ALA) data not included; Results were BLQ for all kits evaluated.

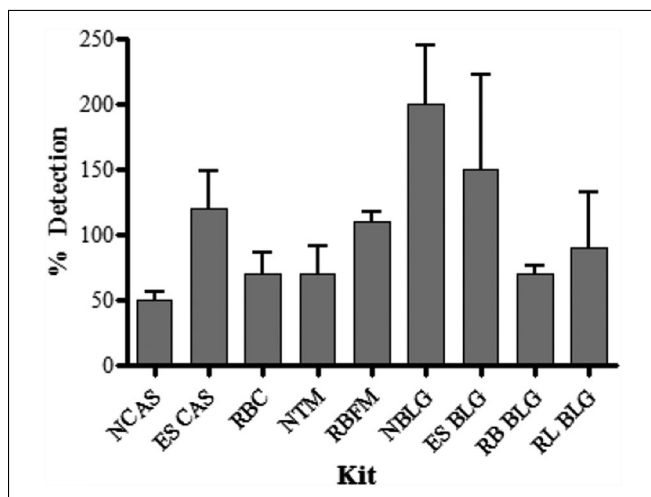


Figure 5—Detection of spikes of known concentration of NFDM with commercial milk ELISA kits. NCAS, Neogen Veratox[®] Casein; ESCAS, ELISA Systems[™] Casein; RBC, R-Biopharm RIDASCREEN[®] Fast Casein; NTM, Neogen Veratox[®] Total Milk; RBFM, R-Biopharm RIDASCREEN[®] Fast Milk; NBLG, Neogen BioKits[™] BLG; ES BLG, ELISA Systems[™] BLG; RB BLG, R-Biopharm RIDASCREEN[®] Fast BLG; RL BLG, Romer Labs[®] AgraQuant[®] BLG.

25 ppm NFDM, with neither kit able to detect residues at concentrations near their published limits of quantitation, 2.5 ppm NFDM for the Neogen Veratox[®] Total Milk kit and 2.5 ppm milk protein for R-Biopharm RIDASCREEN[®] Fast Milk kit in this matrix. In terms of quantitative accuracy at all levels of NFDM evaluated, the R-Biopharm Fast Milk kit somewhat more accurately represents the concentration of milk residues present in the sample than does the Neogen Veratox[®] Total Milk kit (ratios of 1.1 ± 0.082 and 0.7 ± 0.21 , respectively), although the difference is not statistically significant. While dependent on application, both the Neogen Veratox[®] Total Milk kit and the R-Biopharm RIDASCREEN[®] Fast Milk kit are suitable ELISA kits for the detection of trace levels of milk residues in foods.

Detecting milk residues in foods is a challenge due to the variety of milk-based ingredients and the availability of ELISA kits with differing targets. The selection of an appropriate kit for any given application is an additional challenge. As no 2 kits equally detected the same ingredient, this research highlights that each kit manufacturer uses different antibodies in the development of commercial milk ELISAs. While most ELISA manufacturers do not reveal the nature of the antibodies used in the detection of milk residues, some information can be inferred from these

data. In addition to varying antibody specificities, kits recommend or provide extraction buffers comprised of various chemical components that affect the extraction of proteins from a food matrix and solubility in the buffer system, in turn affecting the results.

Unless a standard reference material is widely adopted for the calibration of milk ELISA kits, the quantitative accuracy of the kits is questionable. The previous and current NIST milk reference materials (NIST-RM-1549 and NIST-RM-1549a, respectively) have not been widely adopted. While no comparison was made between the NIST milk reference standards and NFDM in this study, NFDM may serve as a reasonable reference standard and kit calibrator. As a mildly processed material with high consistency and accuracy of detection across all milk ELISA kits evaluated, NFDM appears to be a good choice for a standard reference material. Proprietary differences in kit development can be acceptable provided that each kit is calibrated to a standard reference material and conversion factors are provided by the kit manufacturer to convert from the units provided by the kit to relevant reference units such as ppm milk protein or ppm NFDM. A critical need exists to implement consistent criteria for ELISA kit validation and adopt a standard reference material for milk as previously recommended (Abbott and others 2010).

Conclusions

The research performed further supports the establishment and widespread adoption of a standard reference material for the development, calibration, and quantification commercial allergen ELISA kits. Currently, not all milk-specific ELISA kits were capable of detecting the individual milk proteins or modified milk-derived ingredients. Using a standard reference material to build a standard curve would allow comparison among ELISA kits and would assist in the protection of allergic individuals, as well as allow for more informed industry risk management decisions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Detailed Eqs S.1 to S.14 are listed online for conversion between concentrations of milk proteins and other milk ingredients and their equivalent concentration in relevant reference units.

Supplementary Materials

Equation S.1. Converting ppm casein to ppm milk protein

$$\text{ppm casein} \times \frac{1 \text{ ppm milk protein}}{0.8 \text{ ppm casein}} = 1.25 \text{ ppm milk protein}$$

Equation S.2. Converting ppm BLG to ppm milk protein

$$\text{ppm BLG} \times \frac{1 \text{ ppm milk protein}}{0.1 \text{ ppm BLG}} = 10 \text{ ppm milk protein}$$

Equation S.3. Converting ppm milk protein to ppm NFDM

$$\text{ppm milk protein} \times \frac{1 \text{ ppm NFDM}}{0.35 \text{ ppm milk protein}} = 2.86 \text{ ppm NFDM}$$

Equation S.4. Converting ppm NFDM to ppm milk protein

$$\text{ppm NFDM} \times \frac{0.35 \text{ ppm milk protein}}{1 \text{ ppm NFDM}} = 0.35 \text{ ppm milk protein}$$

Equation S.5. Applying conversion factors to spike concentrations for equivalent casein estimate

Protein	Conversion Factor
α -casein	2
β -casein	2.7
κ -casein	7.7

$$\text{ppm spike } (\alpha-, \beta-, \kappa - \text{casein}) \times \text{conversion factor} = \text{equivalent ppm casein}$$

Equation S.6. Applying conversion factors to kit results for whey protein spikes

Protein	Conversion Factor
β -lactoglobulin (BLG)	10
α -lactalbumin (ALA)	20

$$\text{ppm spike (BLG or ALA)} \times \text{conversion factor} = \text{equivalent ppm milk protein}$$

Equation S.7. Calculating conversion factors: WPC34 to ppm milk protein (MP).

$$\frac{0.3255 \text{ g protein}}{1 \text{ g WPC34}} \times \frac{100\% \text{ WP}}{\text{WPC34 protein}} \times \frac{1 \text{ unit MP}}{0.2 \text{ units WP}} = 1.62$$

Equation S.8. Calculating conversion factors: WPC34 to ppm NFDM

$$\frac{0.3255 \text{ g protein}}{1 \text{ g WPC34}} \times \frac{100\% \text{ WP}}{\text{WPC34 protein}} \times \frac{1 \text{ unit MP}}{0.2 \text{ units WP}} \times \frac{1 \text{ unit NFDM}}{0.35 \text{ units MP}} = 4.63$$

Equation S.9. Calculating conversion factors: WPC80 to ppm milk protein (MP)

$$\frac{0.7847 \text{ g protein}}{1 \text{ g WPC80}} \times \frac{100\% \text{ WP}}{\text{WPC80 protein}} \times \frac{1 \text{ unit MP}}{0.2 \text{ units WP}} = 3.92$$

Equation S.10. Calculating conversion factors: WPC80 to ppm NFDM

$$\frac{0.7847 \text{ g protein}}{1 \text{ g WPC80}} \times \frac{100\% \text{ WP}}{\text{WPC80 protein}} \times \frac{1 \text{ unit MP}}{0.2 \text{ units WP}} \times \frac{1 \text{ unit NFDM}}{0.35 \text{ units MP}} = 11.21$$

Equation S.11. Calculating conversion factors: Sodium Caseinate to ppm milk protein (MP)

$$\frac{0.8988 \text{ g protein}}{1 \text{ g NaCas}} \times \frac{100\% \text{ casein}}{\text{NaCas protein}} \times \frac{1 \text{ unit MP}}{0.8 \text{ units casein}} = 1.12$$

Equation S.12. Calculating conversion factors: Sodium Caseinate to ppm NFDM

$$\frac{0.8988 \text{ g protein}}{1 \text{ g NaCas}} \times \frac{100\% \text{ casein}}{\text{NaCas protein}} \times \frac{1 \text{ unit MP}}{0.8 \text{ units casein}} \times \frac{1 \text{ unit NFDM}}{0.35 \text{ units MP}} = 3.21$$

Equations S.7-S.12 were developed using values for percent protein contained in each milk-derived ingredient as determined from the LECO Dumas Analysis. Although the LECO Analysis indicated the NFDM used in this research as containing approximately 36% protein, the USDA National Nutrient Database for Standard Reference suggests that commercially-produced

NFDM contains an average of 35% protein. Equations were built using the 35% estimation. The proteins present in WPC34 and WPC80 were assumed to be 100% whey protein. The proteins present in sodium caseinate were assumed to be 100% caseins. Additional terms are added into the equation in case different estimations of the source of protein are obtained in the future.

Table S.1. Conversion Factors for ppm spike to ppm reporting unit, calculated using equations S.7-S12.

Spike Ingredient	CF ₁ - equivalent MP	CF ₂ - equivalent NFDM
WPC34	1.62	4.63
WPC80	3.92	11.21
NaCas	1.12	3.21

Equation S.13. Converting spike concentration to ppm milk protein

$$ppm\ spike \times CF_1^a = equivalent\ ppm\ MP$$

^aConversion factors taken from Table S.1. Detailed equations shown in Equations S.7, S.9, and S.11.

Equation S.14. Converting spike concentrations to ppm NFDM

$$ppm\ spike \times CF_2^a = equivalent\ ppm\ NFDM$$

^aConversion factors listed in Table S.1. Detailed equations for the calculation of conversion factors are shown in Equations S.8, S.10, and S.12.