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Analysis of Bovine Lactoferrin in Infant Formula and Adult Nutritional Products by Optical Biosensor Immunoassay: Collaborative Study, Final Action 2021.07

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Abstract

Background: Bovine lactoferrin is increasingly being used as an ingredient in infant formula manufacture to enhance nutritional efficacy through the provision of growth, immunoprotective, and antimicrobial factors to the neonate.

Objective: To evaluate method reproducibility of AOAC First Action Official Method 2021.07 for compliance with the performance requirements described in Standard Method Performance Requirement (SMPR) 2020.005.

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Method: Eight laboratories participated in the analysis of blind-duplicate samples of seven nutritional products. Samples were diluted in buffer, and an optical biosensor immunoassay was used in a direct-assay format to quantitate bovine lactoferrin by its interaction with an immobilized anti-lactoferrin antibody. Quantitation was accomplished by the external standard technique with interpolation from a four-parameter calibration regression.

Results: After outliers were removed, precision as reproducibility was found to be within limits set in SMPR 2020.005 (≤ 9%) for six out of seven samples and all had acceptable Horwitz Ratio ($HorRat_R$) values ranging from 1.0 to 2.1. Additionally, comparison with an alternative independent Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) First Action method (heparin cleanup LC-UV), showed negligible difference between results.

Conclusions: The method described is suitable for the quantification of intact, undenatured bovine lactoferrin in powdered infant formulas. The SPIFAN Expert Review Panel evaluated the method and accompanying validation data from this multi-laboratory testing (MLT) study in July 2023 and recommended Official Method 2021.07 for adoption as a Final Action Official Method.

Highlights: A multi-laboratory validation study of an automated optical biosensor immunoassay for the determination of intact, undenatured bovine lactoferrin is described.

Introduction

Lactoferrin (approximately 80 kDa, pI: approximately 9.0) is a secretory glycoprotein characterized by carbonate-anion dependent, high-affinity reversible binding of two Fe^{3+} ions per molecule. As a minor milk protein, lactoferrin exhibits a range of physiological properties that provide growth, immunoprotective, and antimicrobial factors to the neonate. The lactoferrin content of milk is species-dependent, with significantly higher levels in human milk and colostrum than in the bovine equivalents. For these reasons, bovine lactoferrin is increasingly used as an ingredient in infant formula manufacture to enhance the nutritional efficacy of this food. In addition to its antimicrobial activity, lactoferrin may function in the regulation of intestinal iron uptake, immune response, growth factor activity, bone growth, and antioxidant activity (1).

Rapid, high-throughput analytical methods for the determination of lactoferrin content in infant and adult nutritionals are needed for routine testing to meet product specifications, and reference methods utilizing contemporary techniques are needed to demonstrate product compliance with strict global regulations. A variety of analytical techniques have been used in the analysis of lactoferrin in dairy products, including capillary electrophoresis (2), reversed-phase HPLC–UV (3, 4) gel permeation fast protein liquid chromatography (FPLC) (5), UHPLC–MS (6), and ELISA (7). A comprehensive survey of current analytical methods for the quantitation of lactoferrin in a range of clinical and food matrixes has recently been

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reported (8). An HPLC–UV method utilizing a heparin column cleanup is currently undergoing a collaborative study to evaluate method reproducibility for Final Action review by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) expert review panel (ERP (9)).

A label-free, real-time, automated optical surface plasmon resonance (SPR)-based biosensor immunoassay for the quantitation of intact, undenatured lactoferrin in milk, colostrum, and lactoferrin-supplemented infant formulas has been routinely implemented within the New Zealand dairy industry for approximately 15 years (10). The method recently underwent a comprehensive single-laboratory validation (SLV) study using the SPIFAN kit (a set of infant formula and adult nutritional products that were selected as a representative sub-sample of the wide range of commercially available products) manually fortified with bovine lactoferrin as part of the study. Performance parameters evaluated complied with Standard Method Performance Requirements (SMPR), thereby demonstrating the method to be suitable for the analysis of bovine lactoferrin in a wide range of infant formulas and nutritional products (11, 12). In December 2021, this SLV study was reviewed by the SPIFAN ERP: it was approved for First Action status as AOAC Method 2021.07 (12, 13) and was further recommended to advance to a multi-laboratory testing (MLT) study for determination of method reproducibility.

Multi-Laboratory Testing Study

The MLT was undertaken to evaluate the analytical performance of an optical biosensor immunoassay method for compliance with SMPR 2020.005 (11) and for consideration for Final Action status.

Despite the ubiquitous use of SPR-based biosensors in drug discovery and life science laboratories, recruiting sufficient laboratories to participate in the MLT was a challenge, given the lack of SPR instrumentation in most food testing laboratories. Therefore, many of the participants were recruited from academic laboratories familiar with this platform for kinetic evaluation of molecular interactions but generally unfamiliar with its application to concentration analysis. Prior to the commencement of the MLT study, each collaborator received a detailed study protocol to allow familiarization with the technique and an opportunity to communicate any difficulties.

Samples used in this MLT reproducibility study were limited to bovine milk-based infant nutritional powders manufactured in New Zealand due to the challenge of compliance with restrictions placed upon the importation of samples into many of the participant laboratories. While a limiting factor in establishing the scope of the assay under reproducibility conditions, the applicability of the assay to other infant and adult nutritional powders such as soy-based, amino acid-based, and hydrolyzed protein-based was demonstrated during the SLV study previously completed prior to the awarding of First Action Official Method status.

A practice sample was run by participants and, when acceptable results had been obtained, approval to proceed to the analysis of the test samples was given. The test samples were analyzed as blind-coded duplicate pairs in a single analytical run.

All data were statistically analyzed using the harmonized guidelines for collaborative studies to establish overall mean, intra-laboratory repeatability (S_r), repeatability relative standard deviation (RSD_r), inter-laboratory reproducibility (S_R), reproducibility relative standard deviation (RSD_R), and Horwitz ratio ($HorRat^R$; 10). Cochran (P = 0.025, one-tail) and Grubbs (single and double, P = 0.025, two-tail) tests were utilized to determine outliers.

AOAC Official Method 2021.07

Analysis of Bovine Lactoferrin in Infant Formula and Adult Nutritionals by

Optical Biosensor Immunoassay

First Action 2021

Final Action 2023

[Applicable to the determination of bovine lactoferrin in bovine-milk protein-based infant formula.]

Caution: Refer to the material safety data sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

A. Principle

The bovine lactoferrin content in infant, adult, and/orpediatric formulas is determined by an automated biosensor-SPR- based immunoassay using immobilized goat anti-bovine lactoferrin antibody as the detecting molecule. The method is configured as a direct and non-labelled immunoassay, with quantitation against an authentic bovine lactoferrin calibrant. The sample is prepared for analysis by simple dilution into buffer.

B. Apparatus

(a) Automated biosensor instrument.—Biacore T200 or Biacore Q (GE Healthcare, Uppsala, Sweden) or equivalent SPR- based biosensor.

(b) Sensor chip.—CM5 (GE Healthcare) or equivalent.

(c) Micropipettes.—10–100 mL, 100–1000 mL, 1–10 mL.

(d) Centrifuge tubes.—15 mL, polypropylene.

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(e) Volumetric flasks.—5 mL, 10 mL, 50 mL, 100 mL.

(f) Microtiter plates.—96-well, polystyrene.

(g) Microcentrifuge tubes.—1.5 mL, polystyrene.

(h) Balance.—Accurate to 4 decimal places.

C. Reagents

(a) Antibody.—Affinity-purified, polyclonal goat anti-bovinelactoferrin, 1 mg/mL (A10-126A, Bethyl Laboratories, Montgomery, TX or equivalent).

(b) Bovine lactoferrin.—Approximately 50 mg (CertifiedReference Material, Cerilliant, Round Rock, TX or equivalent).

(c) Amine coupling kit.—GE Healthcare or equivalent individual commercially available reagents.

(1) EDC.—0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl.

(2) NHS.—0.1 M N-hydroxysuccinimide.

(3) Ethanolamine.—1 M, pH = 8.5.

(d) Immobilization buffer.—Sodium acetate, 10 mM, pH = 5.0 (GE Healthcare/Cytiva or equivalent).

(e) Running buffer (HPS-EP).—10 mM HEPES, 0.15 M NaCl, 3.0 mM EDTA, 0.005% surfactant P20, pH = 7.4 (GE Healthcare or equivalent).

(f) Glycine-HCl.—10 mM, pH = 1.5 and pH = 2.0 (GE Healthcare or equivalent).

(g) Hydrochloric acid.—0.1 M (Sigma, St. Louis, MO or equivalent).

(h) Sodium chloride.—AR grade (Sigma, St. Louis, MO or equivalent).

(i) Water.—Purified with resistivity ≥ 18 MΩ.

D. Reagent Preparation

(a) Solution buffer.—Dissolve 2.044 g sodium chloride in 100 mL running buffer. Store in a refrigerator at 4 °C. Expiry: 3 months.

(b) Regeneration reagent (10 mM glycine-HCl, pH = 1.75).—Prepare a fresh mixture of 10 mM glycine, pH = 1.5 and 10 mM glycine, pH = 2.0 (36 + 64, v/v).

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E. Immobilization

(a) Immobilization of anti-bovine lactoferrin antibody to a CM5 sensor surface is via amine coupling.

(b) A designated flow cell is activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC, 0.4 M) and N-hydroxysuccinimide (NHS, 0.1 M) reagents (1:1, v/v, 10 mL/min, 7 min), followed sequentially with the injection(10 mL/min, 7 min) of goat anti-bovine lactoferrin (50 mg/mL in 10 mM sodium acetate, pH = 5.0).

(c) Unreacted ester functionalities are deactivated with ethanolamine (1 M, pH = 8.5, 10 mL/min, 7 min).

(d) Final immobilization levels in resonance units (RU, where 1 RU = 1 pg/mm^2) are determined from the sensorgram and are typically approximately 10–15 kRU. The chip may be stored between analyses over desiccant at 4 °C in a sealed container.

F. Standard Preparation

(a) Stock standard (SS).—Approximately 5 mg/mL corrected for purity from the certificate of analysis (mass balance, chromatographic purity, moisture and LC-MS analysis).
- (1) Dissolve accurately approximately 50 mg lactoferrin in 10 mL water.
- (2) Store in a freezer under nitrogen at less than −10 °C until required.

(b) Intermediate standard (IS).—100 µg/mL.
- (1) Dilute SS to exactly 100 µg/mL by appropriate dilution inrunning buffer (HPS-EP).
- (2) Sub-aliquots may be stored in a freezer under nitrogen at less than −10 °C until required.

(c) Working standards (WS).—
- (1) To 990 µL solution buffer in a plastic vial, add 10 µL IS.This is WS1 (1000 ng/mL). Prepare fresh each run.
- (2) Prepare a single set of calibration standards by 2-fold serial dilution. Label five plastic vials WS2–WS6. Pipette 500 µL solution buffer into each vial. Prepare fresh each run.
  - (a) WS2 = 500 ng/mL, pipette 500 µL WS1 into vial 2; cap and vortex mix.
  - (b) WS3 = 250 ng/mL, pipette 500 µL WS2 into vial 3; cap and vortex mix
  - (c) WS4 = 125 ng/mL, pipette 500 µL WS3 into vial 4; cap and vortex mix.
  - (d) WS5 = 62.5 ng/mL, pipette 500 µL WS4 into vial 5; cap and vortex mix.
  - (e) WS6 = 0 ng/mL, pipette 500 µL solution buffer only into vial 6; cap and vortex mix.

G. Sample Preparation

(a) Infant formula powder or milk powder (diluted 1:2000, w/v).—

(1) Accurately weigh approximately 0.50 g powder into a 15 mL centrifuge tube; record the weight.

(2) Dissolve in 8 mL running buffer and vortex mix. Make up to 10 mL with running buffer. Store in the dark for 15–20 min.

(3) Pipette 990 µL solution buffer into a microcentrifuge tube; add 10 µL diluted sample. Cap and vortex mix.

(b) Powder reconstituted ready-to-feed basis (diluted 1:2000, w/v).—

(1) Accurately weigh approximately 25 g powder into acontainer; record the weight.

(2) Tare the balance and accurately weigh approximately 200 g water into the same container; record the weight.

(3) Warm to 25 °C for 10 min; mix the sample solution thoroughly to ensure complete dissolution.

(4) Weigh a 4.5 g aliquot of sample solution in a centrifuge tube; make up to 10 mL with running buffer; cap and vortex mix.

(5) Pipette 990 µL solution buffer into a microcentrifuge tube; add 10 µL diluted sample; cap and vortex mix.

H. SPR Analysis

(a) Calibration standards and sample extracts (200 mL) are dispensed (in duplicate) into the appropriate wells of a 96-well microtiter plate and sealed with adhesive foil.

(b) Include in each run:

(1) sample blank;

(2) repeat check standard (WS2);

(3) duplicate in-house infant formula QC sample.

(c) The instrument system and docked sensor chip is equilibrated and analysis is initiated under optimized conditions. Note: Automated analysis with the Biacore T200 maybe performed via the concentration assay wizard or method builder options. The former is simpler, where as the latter gives more flexibility with respect to flow path, reference subtraction, and assignment of report points. Ifan alternative vendor SPR system is used, follow the manufacturer's instructions for use of the software system for quantitative analysis.

(d) Running buffer.—Flow rate: 10 mL/min; contact time: 300 s.

(e) Regeneration.—10 mM glycine-HCl, pH = 1.75; flow rate:50 mL/min, contact time: 32 s.

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(f) The response at 10 s after commencement of the dissociation phase, relative to the baseline sampled 10 s before sample injection, is used for quantitation.

(g) Each injection cycle requires approximately 15 min, with a complete 96-well microtiter plate completed in approximately 24 h, including system equilibration and duplicate multi-level calibration.

I. System Suitability

(a) Perform a surface performance test to stabilize the chip. The system suitability test is performed: (i) when a sensorchip has been freshly immobilized with an antibody, and (ii) at the beginning of every analytical run.

(1) Run: 3 × lactoferrin standard WS1, 1000 ng/mL.

(2) Repeatability of binding response should be < 5%.

J. Characterization of a New Antibody

The performance of an alternative commercially available candidate affinity-purified and non-labelled polyclonal antibody should be compared relative to the prescribed antibody (Bethyl Laboratories, affinity-purified, polyclonal goat anti-bovine lactoferrin), under direct assay conditions.

(a) Using the concentration of the antibody provided by the vendor, dilute the antibody in 10 mM sodium acetate,pH = 5.0 prior to coupling to the sensor chip surface to achieve comparable immobilization levels (10–15 kRU).

(b) Multiple sensor chip flow cells may be covalently immobilized with different candidate antibodies using standard amine-coupling chemistry, and with lactoferrin calibrant subsequently injected over all surfaces under direct assay conditions to compare antibody performance (additionally, the assay may be reversed, with lactoferrin immobilized and multiple candidate antibodies injected sequentially as analyte).

(c) Binding responses with the top-level lactoferrin calibration standard should be confirmed to be comparable (± 10%) under recommended direct assay operating conditions. In addition, a four-parameter regression curve over the prescribed calibrant range should yield comparable (±10%) values for curve parameters and detection limits.

(d) Signal-to-noise and dynamic range should be comparable (± 10%) under recommended direct-assay operating conditions.

(e) Confirm specificity and selectivity of the immobilized antibody by estimating minimal or negligible cross-reactivity(B_{50}) to other major milk proteins relative to bovine lactoferrin.

(f) A competitive inhibition experiment may also be performed to confirm the specificity of the tethered antibody for bovine lactoferrin. This is accomplished by evaluating the extent of binding inhibition of lactoferrin to the immobilized antibody after incubating with excess antibody in solution.

(g) Confirm that the sensor surface regeneration protocol is effective for the alternative antibody.

(h) Confirm the stability of the immobilized alternative antibody surface over replicate cycles.

(i) Confirm comparable precision estimates (repeatability and intermediate precision) as described in the System Suitability section.

K. Calculations

Calibration standards are run at the beginning and end of each sequence of samples to ensure minimal response drift across the analytical run. The calibration trend feature can be implemented to account for minimal drift across a sequence. The calibration curve is constructed using the four-parameter polynomial regression given in Equation (1).

$$y = R_{hi} - \left(\frac{(R_{hi} - R_{lo})}{1 + \left(\frac{Lf_{conc}}{A1}\right)^{A2}} \right) \quad (1)$$

where:

y = instrument response (RU); R_{hi} = response at infinite concentration; R_{lo} = response at zero concentration; A_1 = B_{50} (concentration at 50% binding saturation); A_2 = slope factor; and Lf_{conc} = concentration of lactoferrin (ng/mL).

For long sample sequences, a further set of calibration standards can be included within the sequence. The result file is processed within the T200 Evaluation Software (or alternative SPR system), where both the calibration curve and raw data interpolation are automated as part of instrument operation.

L. Data Handling

Report data to the nearest µg/mL (liquids) and µg/g (powders).

Results and Discussion

Optical biosensors utilizing SPR detection are an important technique for the label-free and real-time monitoring of biomolecular interactions. Although predominantly used for evaluating kinetic and

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thermodynamic characteristics in drug discovery and life science environments, they have also found application within the food sector to facilitate concentration analysis. An automated biosensor platform, incorporating SPR optics, directly detects the interaction between the immobilized lactoferrin-specific recognition antibody and bovine lactoferrin in solution. The method is rapid, sensitive, precise, and accurate, and provides specific analytical information of the undenatured physiologically active protein content. Further, due to the inherent specificity of the antibody-based detection strategy, the technique facilitates a simple sample preparation involving direct dilution in buffer.

Optimization of antibody selection, immobilization chemistry, immobilization level, regeneration protocol, buffer selection and assay run conditions, and the evaluation of critical method performance parameters, such as specificity, sensitivity, range, repeatability, intermediate precision, recovery, and accuracy were previously reported (10, 12). The purpose of the present multi- laboratory study was therefore to estimate reproducibility precision of the method across several independent laboratories.

A total of 10 laboratories agreed to participate as part of this study, of which 8 laboratories were able to submit data for evaluation prior to the submission deadline. Different models of SPR instruments were used (Biacore T200, 8K+, 3000). It should be noted that, although Biacore instruments are the most widely used biosensor to characterize molecular interactions, any SPR- based instrument from alternative vendors may be used to implement this analytical method.

All laboratories returned comparable and expected values for both antibody ligand immobilization levels and relative binding responses for calibration standards. This confirms the robustness and reliability of amine coupling immobilization chemistry and instrument performance across all laboratories and multiple models of SPR instruments from a single manufacturer.

The initial phase of method evaluation within the participating laboratories involved in the MLT required the analysis of a practice sample, which was an extra pair of blind duplicates of one of the test samples.

Upon completion of the analysis of all test samples, each participating laboratory reported measured results, as well as information such as sample identification, weights, volumes, dilution level, antibody immobilization level, binding responses and calibration curve parameters. Participants were also asked to document any deviation from the method and any other pertinent comments based on their experiences in adapting the method into their laboratory. The results received from participants were tabulated and are summarized in Table 1. One laboratory (Laboratory 5) reported problems with the stability of the calibration standard between analyzing the practice samples and test samples, with results obtained typically lower in comparison with other laboratories results; therefore, the complete data set from Laboratory 5 was removed prior to statistical analysis. The remaining raw data were subjected to Cochran and Grubb tests and no outliers were identified.

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Precision as repeatability was 3.8–6.8% RSD_r with a reproducibility of 5.7–11.4% RSD_R (Table 2), and $HorRat_R$ values for the method ranging from 1.0 to 2.1 (expected range 0.5 to 2.0; 14, 15). Despite the ubiquitous use of ELISA in the food sector for concentration analysis, biosensors such as those utilizing SPR optics that exploit the same analytical principles, are less accepted in regulated food testing environments. This is despite their many advantages such as specificity, sensitivity, and a more facile sample preparation compared to alternative separation-based HPLC platforms. Although the precision of generic immunoassays is often reported to be higher compared with separation-based platforms (16), the reproducibility precision reported in this MLT study is comparable with HPLC-based methods.

A comparison between AOAC Method 2021.07 and the heparin cleanup LC-UV AOAC First Action method was undertaken that involved testing the same set of MLT samples by both analytical techniques. Despite the fundamentally different analytical principles used (biosensor immunoassay versus LC-UV), both techniques yielded comparable estimates of lactoferrin with negligible difference between results ($\bar{d}$ = 4.4 mg/hg, $P = 0.01$, $\alpha = 0.05$) confirming that both methods provide a reliable estimate of the lactoferrin content in nutritional products (Figure 1).

A summary of each laboratory's performance was sent to participants, along with an invitation to make comments on the performance of the method in their laboratory. In general, comments were positive with respect to the ease of use of the method. Safety concerns with this method were evaluated and there were no major hazards beyond those typically found in chemistry laboratories. Users of the method are directed to use appropriate safety equipment when handling acids, bases, and solvents, and to refer to material safety data sheets for detailed safety instructions for each chemical used.

Conclusions

The results of the MLT study have been demonstrated to meet the performance requirements defined in SMPR 2020.005 providing evidence of its suitability for the quantitation of intact, undenatured bovine lactoferrin in bovine milk-based infant formula and adult nutritional powders.

Recommendation

A study report summarizing the outcomes of this multi- laboratory collaborative study was submitted for evaluation by the SPIFAN ERP. The SPIFAN ERP evaluated the method and accompanying validation data from this MLT study in July 2023 and recommended Official Method 2021.07 for adoption as a Final Action Official Method (approved by the Official Methods Board February 2024).

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CRediT Author Statement

Brendon Gill: Writing—original draft, Conceptualization, Data curation, Writing—review & editing. Harvey Indyk: Methodology, Writing—original draft, Writing—review & editing. Tadashi Kobayashi: Formal analysis. Jackie Wood: Writing—review & editing. Fiona Clow: Formal analysis, Writing—review & editing. Olan Dolezal: Formal analysis, Writing—review & editing. Lauren Hartley-Tassell: Formal analysis, Writing—review & editing. Martina Jones: Formal analysis, Writing—review & editing. William Kelton: Formal analysis, Writing—review & editing. Robyn Stoller: Formal analysis, Writing—review & editing. Lorna Wilkinson-White: Formal analysis, Writing—review & editing.

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Conflict of Interest

All authors declare no conflict of interest.

References

1. Iglesias-Figueroa, B.F., Espinoza-Sánchez, E.A., Siqueiros-Cendón, T.S., & Rascón-Cruz, Q. (2019) *Int. Dairy J.* 89, 37–41. doi: 10.1016/j.idairyj.2018.09.004

2. Li, J., Ding, X., Chen, Y., Song, B., Zhao, S., & Wang, Z. (2012) *J.Chromatogr. A* 1244, 178–183. doi: 10.1016/j.chroma.2012.05.004

3. Dračková, M., Borkovcová, I., Janštová, B., Naiserová, M.,Přidalová, H., Navrátilová, P., & Vorlová, L. (2009) *Czech. J. Food Sci.* 27, S102–S104. doi: 10.17221/944-CJFS

4. Palman, K.P., & Elgar, D.F. (2002) *J. Chromatogr. A* 947, 307–311. doi: 10.1016/S0021-9673(01)01563-1

5. Law, A.J.R., Leaver, J., Banks, J.M., & Horne, D.S. (1993) *Milchwissenschaft* 48, 663–666

6. Zhang, J.S., Lai, S.Y., Cai, Z.X., Chen, Q., Huang, B.F., & Ren, Y.P. (2014) *Anal. Chim. Acta* 829, 33–39. doi: 10.1016/j.aca.2014.04.025

7. Chen, P.W., & Mao, F.C. (2004) *J. Food Drug Anal.* 12, 9. doi: 10.38212/2224-6614.2653

8. Zhang, Y., Lu, C., & Zhang, J. (2021) *Nutrients* 13, 2492. doi: 10.3390/nu13082492

9. Official Methods of Analysis (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, **AOAC 2021.10**. doi: 10.1093/9780197610145.001.0001

10. Indyk, H.E., & Filonzi, E.L. (2005) *Int. Dairy J.* 15, 429–438. doi: 10.1016/j.idairyj.2004.09.003

11. Official Methods of Analysis (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, **SMPR 2020.005**. doi: 10.1093/9780197610145.003.005

12. Gill, B.D., Kobayashi, T., Wood, J.E., & Indyk, H.E. (2022) *J. AOAC Int*. 105, 1605–1610 doi: 10.1093/jaoacint/qsac066

13. Official Methods of Analysis (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, **AOAC 2021.07**. doi: 10.1093/9780197610145.001.0001

14. International Council for Harmonisation (ICH) (2005) Validation of Analytical Procedures: Text and Methodology Q2 (R1), https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf (accessed June 12, 2024)

15. Horwitz, W., & Albert, R. (2006) *J. AOAC Int.* 89, 1095–1109. doi: 10.1093/jaoac/89.4.1095

16. Cross, T.G., & Hornshaw, M.P. (2016) *J. Appl. Bioanal.* 2, 108–116. doi: 10.17145/jab.16.015

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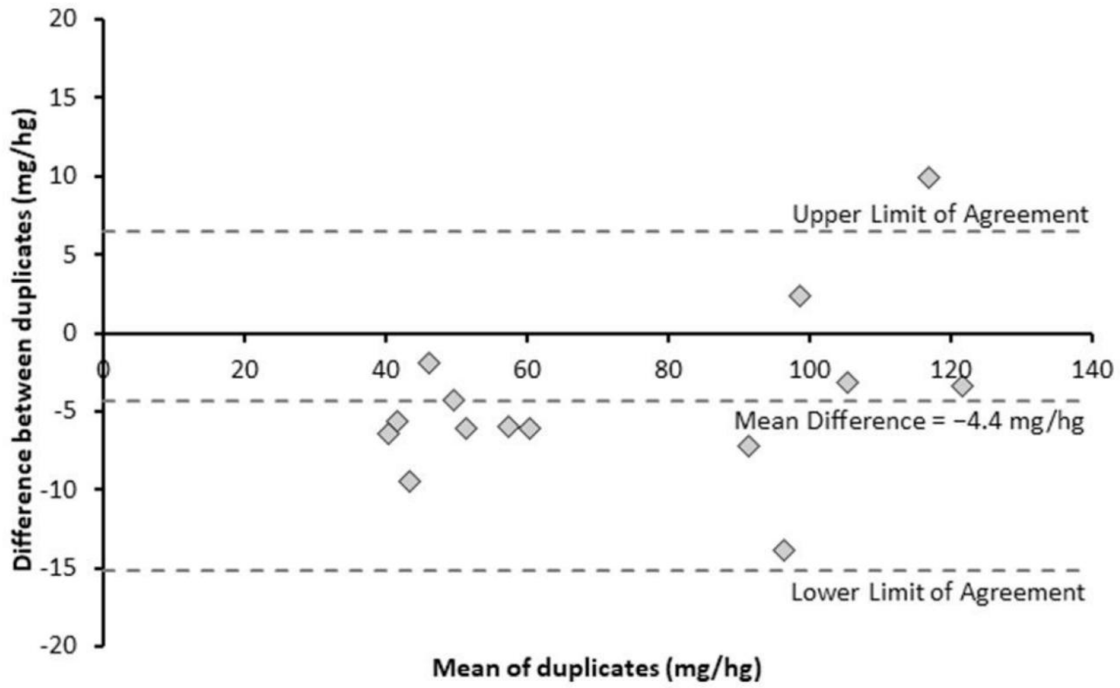

Figure 1. Bland-Altman plot comparing lactoferrin results using the AOAC Method 2021.07 SPR method and the heparin LC-UV method.

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Table 1. Raw data for replicate analyses of powdered nutritional products

	Lactoferrin, mg/hg[a]													
Lab No.	**Follow-on formula**		**Infant formula**		**Toddler formula**		**Toddler formula**		**Infant formula**		**Follow-on formula**		**Nutritional powder**	
1	123.4	112.1	103.4	95.1	107.1	97.6	48.1	47.2	51.9	54.5	63.6	60.4	44.6	43.7
2	120.4	133.9	127.6	122.2	128.2	123.7	59.6	57.7	59.1	64.9	72.6	71.7	47.3	50.3
3	124.8	124.0	117.9	116.1	148.8	130.8	56.3	47.2	70.1	60.3	77.6	70.1	43.8	47.3
4	132.5	126.2	118.9	111.6	120.4	114.8	53.3	48.0	59.3	65.8	65.6	68.3	42.3	41.4
5[b]	112.4	98.2	100.6	88.8	90.7	91.2	38.8	44.7	45.3	50.9	54.3	48.0	36.1	41.6
6	126.9	128.0	109.4	109.1	110.4	113.0	51.3	50.8	63.3	61.8	66.2	68.8	46.3	43.1
7	122.6	124.7	108.9	119.2	113.2	104.2	47.2	46.3	58.0	66.0	61.1	70.0	45.4	43.0
8	122.6	141.9	101.9	116.4	122.0	128.7	49.1	47.2	67.5	64.2	64.2	66.3	39.6	40.4

[a] Results reported on a 'dry-weight' basis

[b] Data were removed prior to statistical analysis due to user concerns over the stability of the calibration standard between the practice samples and test samples

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Table 2. Method precision for samples

Lab No.	Lactoferrin, mg/hg a						
	Follow-on formula	**Infant formula**	**Toddler formula**	**Toddler formula**	**Infant formula**	**Follow-on formula**	**Nutritional powder**
Total number of laboratories, L	7	7	7	7	7	7	7
Total number of replicates, na	14	14	14	14	14	14	14
Grand mean, x̅ mg/hg	126.0	112.7	116.6	50.7	61.9	67.6	44.2
Repeatability standard deviation (SDr), mg/hg	7.2	5.8	6.5	2.9	4.3	3.4	1.7
Reproducibility standard deviation (SDR), mg/hg	7.2	9.0	13.3	4.5	5.1	4.8	3.0
SMPR repeatability limit (RSDr,%)	6	6	6	6	6	6	6
Repeatability relative standard deviation (RSDr, %)	5.7	5.1	5.6	5.8	6.8	5.1	3.8
SMPR reproducibility limit (RSDR, %)	9	9	9	9	9	9	9
Reproducibility relative standard deviation (RSDR, %)	5.7	8.0	11.4	8.9	8.3	7.1	6.9
Horwitz ratio (HorRatR)	1.0	1.4	2.1	1.4	1.4	1.2	1.1

[a] Cochran and Grubb tests identified no statistical outliers