A Comprehensive Method for Determination of Residual Protein in Rebaudioside M

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Article

A Comprehensive Method for Determination of Residual Protein in Rebaudioside M

Feng Li *, Wenjing Zhang, Xinjing Yang, Jiaxi Bai, Yanyan Huo and Yingjuan Qu

Xi'an Key Laboratory of Food Safety Testing and Risk Assessment, School of Chemical Engineering, Xi'an University, Xi'an 710065, China

* Correspondence: lifeng_sunny@163.com

Abstract: The sweetener Rebaudioside M (Reb M) has broad application prospects and can be produced on a large scale by enzymatic methods. However, detecting exogenous protein residues in Reb M products remains a major challenge, and methods for detecting protein residues in Reb M have rarely been reported. In this study, Reb M was dissolved in a sodium hydroxide solution to improve its solubility, and proteins were precipitated using deoxycholic acid sodium and trichloroacetic acid solutions. This precipitation method also eliminated the interference of Reb M in the detection results. After reconstituting the residual protein with water, the protein content in Reb M was measured using the bicinchoninic acid (BCA) method. The analytical method was internally validated according to the following parameters: range of linearity, limits of detection, limits of quantification, sensitivity, accuracy, and precision. The recoveries ranged from 100.90 to 109.40%, and the relative standard deviation (RSD) for precision was less than 4%. Results from 10 samples, including process products, final products, and products from different manufacturers, demonstrated that the method was suitable for the quantitative detection of residual proteins in enzyme-catalyzed Reb M, providing technical support for the quality control of Reb M.

Keywords: Rebaudioside M; protein residues; BCA method; enzymatic method; quality control

1. Introduction

In view of the growing global problem of obesity and diabetes, the World Health Organization (WHO) recommends limiting sugar consumption to less than 10% of total calorie intake, or less than 5% for additional health benefits [1–4]. As a result, the demand for low-sugar and low-calorie products that use sweeteners is steadily increasing. Sweeteners are significantly sweeter than sugar, with some being several hundred times sweeter than sucrose [5,6]. Therefore, only a small amount is needed to achieve the desired sweetness, allowing for a substantial reduction in sugar use, which in turn helps control both calorie intake and blood sugar levels. However, conventional synthetic options such as aspartame, acesulfame-K, sucralose, and neotame have raised health concerns regarding chronic consumption. In 2023, WHO classified aspartame as a potential carcinogen, intensifying global scrutiny over the food safety of sweeteners. Natural sweeteners, particularly the zero-calorie steviol glycosides (SGs) extracted from the leaves of *Stevia rebaudiana* Bertoni, represent the "third natural sweetening resource" after sugarcane and sugar beet [6,7]. With centuries of traditional use in South America, these compounds exhibit exceptional sweetening potency, negligible caloric content, and favorable sensory characteristics. Notably, they demonstrate anti-inflammatory and antimicrobial properties, and their non-metabolizable nature renders them biologically inert in humans, making them ideal sugar substitutes [8–10].

Steviol glycosides, classified as diterpenoid glycosides with multiple sugar chains at the C13 and C19 positions of ent-kaurene, comprise more than 40 identified compounds, including stevioside (ST), rebaudioside A (Reb A), rebaudioside B (Reb B), rebaudioside D (Reb D), and rebaudioside M (Reb M) [10,11]. Among these, Reb M is considered a next-generation sweetener due to its high-intensity sweetness, up to 350 times that of sucroseand its significantly reduced bitter aftertaste

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compared to ST and Reb A. However, the content of Reb M in *Stevia rebaudiana Bertoni* leaves is relatively low (0.4-0.5% of leaf dry weight), in contrast to ST (5-10%) and Reb A (2-4%). This low natural abundance hinders its large-scale production through traditional phytoextraction methods for commercial use [12–15].Consequently, the search for efficient and cost-effective synthesis methods has become a key research focus. The primary synthetic approaches for Reb M currently include chemical synthesis, fermentation, and enzymatic methods [16,17]. The elucidation of Reb M's enzymatic biosynthetic pathways has positioned biocatalytic synthesis as the predominant manufacturing paradigm, owing to its environmental sustainability, mild reaction conditions, operational simplicity, and enhanced yield efficiency [18,19]. Industry leaders such as Tate & Lyle (FDA GRAS No. 780) and INGIA-BIO (FDA GRAS No. 799) utilize sequential enzymatic modification processes to convert the precursor Reb A to Reb M via the intermediate Reb D through the coordinated action of UDP-dependent glucosyltransferases (UGTs) and sucrose synthases.

Notably, the incorporation of exogenous enzymatic proteins necessitates rigorous quality control, as residual proteins pose regulatory compliance challenges. Despite continuous improvements in enzymatic processes and purification technologies, proteins from the enzymatic reactions may still remain in the final products, potentially causing adverse reactions in humans. Therefore, controlling residual proteins in enzyme-catalyzed products is a critical aspect of process control in the production of fermentation-derived steviol glycosides [20]. While EU regulations mandate a protein residue limit of 5 mg/kg in steviol glycosides such as Reb A, Reb D, and Reb M, the FDA GRAS submissions by Tate & Lyle and INGIA-BIO report residual protein levels of no more than 5 ppm in Reb M products, as determined by the BCA assay method, although essential analytical validation data remain undisclosed [21–23]. Regulatory frameworks also differ across regions: the WHO has not issued specific guidance on Reb M protein residues, and China's GB1886.355-2022 standard for stevia extracts omits any specification for protein residues, highlighting the evolving nature of global food safety governance [24].

Current Reb M documentation reveals inconsistencies in the units used to report protein residues, with both ppm and mg/kg cited interchangeably. Conventionally, ppm may refer to weight-to-weight (w/w, equivalent to mg/kg) or weight-to-volume (w/v, equivalent to mg/L), leading to potential analytical discrepancies. Most FDA GRAS submissions ambiguously report protein residues as "ppm" without clarifying the measurement basis. An exception is Manus Bio (FDA GRAS No. 1010), which explicitly quantifies protein content on a w/v basis via the BCA assay. The detection limit of their method is 22.5 ppm (22.5 mg/L) at a Reb M solution concentration of 1 g/L, that translates to 22,500 mg/kg under w/w parameters, exposing significant challenges in unit conversion and cross-study comparison.This terminological ambiguity underscores the need for standardized metrological protocols in regulatory documentation. Current methodologies for analyzing residual proteins in Reb M also vary widely. For example, PureCircle's GRAS (No. 745) utilizes SDS-PAGE analysis with a detection threshold of 10,000 ppm—three orders of magnitude higher than Manus Bio's 22.5 ppm BCA-based protocol. This stark contrast exists alongside regulatory asymmetries: the EU mandates ≤5 mg/kg, while Tate & Lyle and INGIA-BIO claim compliance with ≤5 ppm without clear unit harmonization [25]. The increasing domestic demand for high-purity Reb M and the emergence of new enzymatic Reb M products in regulatory pipelines expose systemic quality control vulnerabilities rooted in unstandardized quantification frameworks. To ensure product integrity and consumer safety, regulatory modernization must mandate the following: explicit specification of protein residue limits in pharmacopeial standards; standardized unit declarations (*w*/*w* vs. *w*/*v*); and analytical method validation with sensitivity aligned to the claimed detection limits. Such harmonization is imperative to reconcile industrial practices with evolving food safety paradigms.

At present, protein analysis technologies are advancing rapidly, and several methods are commonly used for protein detection [26–28]. These include SDS-PAGE, the Lowry method, the Bradford method, and the BCA method. SDS-PAGE is a classic and widely accepted standard in protein analysis, particularly effective for studying large molecular substances. However, it is often

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time-consuming and labor-intensive. The Lowry and Bradford methods, while frequently employed, suffer from limitations such as interference from reagents and relatively low sensitivity.

In contrast, the BCA (bicinchoninic acid) method is widely used for quantifying soluble proteins in various matrices, including milk, plasma, tears, plant and animal tissues, food products, and urine. This method is based on the formation of a coordinated complex between Cu(II) ions and the nitrogen atoms in the peptide amide bonds, resulting in a color change from blue to violet. However, certain sample components—such as detergents, 2-mercaptoethanol, carbohydrates, ammonium, flavonoids, hemoglobin, and salts—can interfere with the reaction, leading to either overestimation or underestimation of protein content. Therefore, selecting an appropriate detection method is essential to minimize interference and obtain reliable results [29,30]. In the case of Rebaudioside M (Reb M), its inherent hydrophobicity and reactivity with assay reagents can cause spectral interference, systematically inflating absorbance values beyond actual protein content. To address this analytical challenge, Fan et al. introduced a pre-treatment protocol involving sodium deoxycholate-mediated protein stabilization followed by trichloroacetic acid precipitation [31,32]. This approach effectively isolates proteins from matrix interference prior to BCA quantification.

The aim of the present work was to develop an accurate BCA-based method for determining residual protein in Reb M. The units of protein residue were standardized as ppm (*w*/*w*). In addition, we optimized the pre-treatment protocol, selected appropriate solvents, and improved the solubility of Reb M. The finalized method was evaluated for its range of linearity, limits of detection, limits of quantification, sensitivity, accuracy, and precision. This validated workflow enables precise monitoring of residual protein levels throughout the manufacturing process, supporting both quality control standardization and purification process optimization. This systematic approach helps establish critical quality attributes for enzymatic Reb M production and informs the design of rational downstream processing strategies.

2. Materials and Methods

2.1. Samples and Reagents

Bovine Serum Albumin (BSA) (Solarbio Science & Technology Co., Ltd., Beijing; Batch 1229Z051, 98% purity). 2,2′-Biquinoline-4,4′-dicarboxylic acid disodium salt (Sigma-VETEC)Anhydrous sodium carbonate (Macklin Biochemical Co., Ltd., Shanghai). Sodium tartrate (Sinopharm Chemical Reagent Co., Ltd.). Sodium hydroxide (Sinopharm Chemical Reagent Co., Ltd.). Sodium bicarbonate (Titan Scientific Co., Ltd., Shanghai). Copper(II) sulfate pentahydrate (Sinopharm Chemical Reagent Co., Ltd.). Sodium deoxycholate (Macklin Biochemical Co., Ltd., Shanghai). Trichloroacetic acid (Titan Scientific Co., Ltd., Shanghai). All chemicals were of analytical grade. Experimental procedures utilized ultrapure water (18.2 MΩ·cm resistivity) generated by a Milli-Q water purification system (Millipore Corporation, USA).

Reb M test samples were obtained from a biotechnology enterprise and included the following batch identifiers: Reb M-Process1, Reb M-Process2, Reb M-2301, Reb M-2302, and Reb M-2303. Commercially available products of Reb A, Reb D, and Reb M were also sourced from five different manufacturers, with batch numbers: 20230327, 20240902, 02BRMGA0122014, Z046RP2306, and 240709006. These manufacturers have distributed their products in the European Union, the United States, and/or China.

2.2. Equipment and Instrument

Ultraviolet-Visible (UV-Vis) spectrophotometer (Model UV-1900i, Shimadzu Corporation (China) Management Co., Ltd.). Analytical balance (Model SQP; Sartorius Scientific Instruments Co., Ltd.). Compact high-speed centrifuge (Model 5425R; Eppendorf AG, Germany).

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2.3. Preparation of Standard Solutions

BCA Reagent A: Precisely weigh 1 g of 2,2′-biquinoline-4,4′-dicarboxylic acid disodium salt, 2 g anhydrous sodium carbonate, 0.16 g sodium tartrate, 0.4 g sodium hydroxide, and 0.95 g sodium bicarbonate. Dissolve in ultrapure water and adjust to a final volume of 100 mL. The resulting solution exhibits a pH of 11.2±0.1.

BCA Reagent B: Accurately weigh an appropriate quantity of copper(II) sulfate pentahydrate and dissolve in ultrapure water to prepare a 4% (w/v) aqueous copper sulfate solution.

BCA Reagent C: Mix BCA Reagent A and BCA Reagent B at a volumetric ratio of 50:1. This working solution must be freshly prepared prior to use.

Sodium Deoxycholate Test Solution: Dissolve sodium deoxycholate in ultrapure water to obtain an aqueous solution containing 15 mg per 10 mL.

Trichloroacetic Acid Test Solution: Prepare an aqueous solution by dissolving 7.2 g of trichloroacetic acid in 10 mL of ultrapure water.

Sodium Hydroxide Solution:Dissolve 4 g of sodium hydroxide in 100 mL of ultrapure water to prepare a 4% (w/v) aqueous solution.

Bovine Serum Albumin (BSA) Reference Stock Solution: Prepare a 1.0 mg/mL aqueous solution of BSA.

BSA Reference Working Solutions: Dilute aliquots of the BSA reference stock solution with the sodium hydroxide solution to obtain calibration standards at concentrations of 2, 2.5, 5, 7.5, and 10 mg/L.

Sample Solution**:** Accurately weigh an appropriate amount of Reb M, dissolve it in the sodium hydroxide solution, and dilute to a final concentration of 50 mg/mL. Prepare three independent replicates.

Spiked Sample Solutions: Dissolve Reb M in the sodium hydroxide solution, then add defined volumes of the BSA reference stock solution to prepare mixtures containing 50 mg/mL Reb M and 2, 5, or 10 mg/L BSA.

Blank Solution: Sodium hydroxide solution prepared as described above.

2.4. Sample Preparation

Interference Removal Protocol: To 1.0 mL of the test solution, add 0.1 mL of the sodium deoxycholate test solution. Vortex thoroughly and incubate at 25°C for 10 min. Then, add 0.3 mL of the trichloroacetic acid test solution, vortex again, and centrifuge at 12,000 rpm for 15 min. Carefully discard the supernatant. Resuspend the resulting protein pellet in 1.0 mL of sodium hydroxide solution, and repeat the vortexing, incubation, and centrifugation steps as described above. Reconstitute the final protein pellet with BCA Reagent C and incubate the mixture at 37 °C for 30 min. After incubation, allow the solution to cool to ambient temperature. Measure absorbance at 562 nm using a spectrophotometer. To ensure uniform reaction timing across all test samples and reference standards, implement a stopwatch-controlled protocol with 1-minute intervals between sequential reagent additions to each test tube.

3. Results and Discussion

The Kjeldahl method, Biuret assay, and UV-Vis spectrophotometry lack the necessary sensitivity for detecting protein residues at the mg/kg level. Although the Bradford assay is widely used, it operates under acidic conditions that are incompatible with the near-neutral pH of the Reb M matrix, thereby compromising the performance of the Coomassie Brilliant Blue G-250 reagent. In contrast, both the Lowry and BCA assays utilize alkaline detection reagents, rendering them more compatible with the physicochemical properties of Reb M solutions. Among these, the BCA assay offers superior sensitivity, operational simplicity, and a more streamlined workflow compared to the Lowry method, making it the most suitable choice for quantifying residual proteins in Reb M samples.

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3.1. Method Optimization

To optimize the detection of residual protein, we first evaluated the solubility of Reb M in various solvents. The experimental results indicated that Reb M exhibits limited solubility in water (<5 mg/mL), while significantly higher solubility was observed in 60% ethanol (v/v) and 60% DMSO (v/v) at 20 mg/mL. Notably, solubility in 1 M NaOH reached up to 60 mg/mL. To assess the impact of solvent choice on protein detection, Reb M was dissolved in 60% DMSO, 60% ethanol, and 1 M NaOH, and BSA recovery at a spiked concentration of 20 mg/L was evaluated using the protocol outlined in Section 2.4. As summarized in Table 1, recovery efficiencies in these matrices were 87.9, 90.3, and 93.2%, respectively, with 1 M NaOH demonstrating superior performance.

Given a constant protein concentration, increasing the Reb M concentration improved detection sensitivity. Accordingly, the Reb M concentration was raised from 20 to 50 mg/mL, reducing the relative protein concentration (20 mg/L BSA) from 0.10% (1000 mg/kg) to 0.04% (400 mg/kg), while maintaining high recovery (93.2–94.5%). However, further increasing the Reb M concentration to 60 mg/mL led to increased solution viscosity, which impaired protein sedimentation during centrifugation (12,000 rpm, 15 min), resulting in supernatant contamination and a reduced recovery rate (90.2%). Therefore, 50 mg/mL Reb M in 1 M NaOH was established as the optimal condition for subsequent analyses.

Table 1. Protein recovery from Reb M spiking with different solvents and Reb M concentration.

solvent	Reb M / (mg/mL)	spiked amount / (mg/L)	measured amount / (mg/L)	recovery rate /%
60% DMSO	20	20	17.58	87.9%
60% Ethanol	20	20	18.07	90.3%
1M NaOH	20	20	18.63	93.2%
1M NaOH	50	20	18.90	94.5%
1M NaOH	60	20	18.04	90.2%

To assess the anti-interference effect of the pre-treatment process on Reb M, we prepared a sample solution from the Reb M-2301 batch following the method outlined in Section 2.3. Different numbers of pre-treatment steps, as described in Section 2.4, were performed to evaluate the impact of varying processing times on interference reduction. As shown in Table 2, the absorbance of the untreated Reb M solution was 0.183, which was higher than that of the 7.5 mg/L BSA solution, indicating significant interference with the BCA detection reagents at a concentration of 50 mg/mL. After the first pre-treatment step, the background interference absorbance of Reb M decreased from 0.183 to 0.021, a reduction of 88%. However, a small amount of interference remained. Upon repeating the pre-treatment process, the absorbance of the solution was effectively reduced to near zero, indicating complete elimination of background interference. These results demonstrate that dual pre-treatment cycles are required to fully mitigate interference in the BCA assay, ensuring accurate protein quantification.

Table 2. Effect of pretreatment on the background absorbance of Reb M.

Sample	Processing mode	Absorbance / (AU)
50 mg/mL Reb M	Untreated	0.183
50 mg/mL Reb M	First pre-treatment	0.021
50 mg/mL Reb M	Second pre-treatment	0.001
7.5 mg/L BSA	Untreated	0.168

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3.2. Results of Method Validation

3.2.1. Linearity, Limit of Detection and Limit of Quantitation

The linearity of the BSA solution after pre-treatment was evaluated. The BSA reference solution, prepared as outlined in Section 2.3, was measured using sodium hydroxide solution as the blank control, following the method described in Section 2.4. The absorbance of the blank was subtracted from the measured absorbance to obtain the calibrated absorbance. A standard curve was plotted with the calibrated absorbance (y-axis) and the BSA reference solution concentration (x-axis, ranging from 2 to 10 mg/L). The resulting equation was $y = 0.0246x-0.0069$, with a slope (k) of 0.0246. The correlation coefficient (r) for this range was at least 0.994, confirming the robust linearity of the method for pretreated BSA quantification. To calculate the standard deviation (SD), the absorbance of the blank solution was measured, and the standard deviation was derived from the response values and the slope of the standard curve. The results are summarized in Table 3. Using the formula $LOD=3.3 \cdot SD/k$, the limit of detection (LOD) for this method was approximately 0.61 mg/L, which is equivalent to 12.2 mg/kg based on a Reb M solution concentration of 50 mg/mL. Similarly, using the formula $LOQ=10 \cdot SD/k$, the limit of quantification (LOQ) was calculated to be approximately 1.86 mg/L. As per methodological validation requirements, the LOQ of 1.86 mg/L was confirmed for accuracy and precision. This LOQ corresponds to 40 mg/kg (w/w), calculated based on a 50 mg/mL Reb M solution concentration. Given that the typical protein residue limit for food additives is around 100 mg/kg, the LOQ of this method is significantly lower than the established limit, ensuring that it can accurately quantify protein residues in Reb M products within the 100 mg/kg threshold.

Table 3. Methodological detection and quantification limits of Reb M protein residues.

No.	Absorbance /AU	Standard deviation	Limit of detection / (mg/L)	Limit of quantitation / (mg/L)
1	0.195			
2	0.201	0.00458	0.61	1.86
3	0.204			

3.2.2. Accuracy and Precision

The recovery rate of BSA in the Reb M solution was evaluated at three different spiked concentrations: low (40 ppm), medium (100 ppm), and high (200 ppm), prepared from Reb M batch Reb M-2301, as described in Sections 2.3 and 2.4. The results are presented in Table 4. The recovery rates for BSA across all spiked concentrations ranged from 100.90 to 109.40%. Additionally, the precision, measured as the relative standard deviation (RSD), was found to be less than 4% (n = 3). These results demonstrate that the method meets the quantitative detection requirements for residual proteins in Reb M matrices.

Table 4. Reb M protein residue methodological accuracy and precision.

No.	spiked amount / (mg/L)	measured amount / (mg/L)	recovery rate /%	average recovery rate /%	RSD /%
	2	2.09	104.4		
1	2	2.16	108.0	104.5	3.3%
	2	2.02	101.2		
	5	5.30	106.0		
2	5	5.47	109.4	106.3	2.7%
	5	5.18	103.6		

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	10	10.09	100.9		
3	10	10.15	101.5	101.5	0.5%
	10	10.20	102.0		

3.3. Sample Analysis

The protein residue levels of Reb M samples from different process batches and final product batches were examined. Reb M-Process 1 and Reb M-Process 2 represent crude products from different stages of the production process, while Reb M-2301, Reb M-2302, and Reb M-2303 are the final products. Residual protein levels in the samples were determined according to the method outlined in Section 2.4. As shown in Table 5, protein residues were detectable in the crude products from the Reb M process, with significant variation in the amount of residual protein. Notably, the protein residue in the Reb M-Process 2 batch was already below 100 mg/kg. In contrast, the final product batches showed protein levels below the limit of quantification (LOQ) of the method, indicating that the final processing stages effectively reduced protein residues in Reb M to levels below 40 mg/kg. These findings confirm that the protocol successfully quantifies protein contamination across a range of sample types, from crude intermediates to purified final products, validating its robustness for quality control throughout the Reb M manufacturing workflow. This analytical capability ensures reliable monitoring of process efficiency in eliminating proteinaceous impurities.

Table 5. Reb M sample test results.

No.	Batch number	Reb M concentration / (mg/mL)	Measured amount / (mg/L)	Residual Protein / (mg/kg)
		49.21	7.55	153.35
1	Reb M-Process1	48.68	7.46	153.20
		49.96	7.62	152.60
		49.62	4.37	88.07
2	Reb M-Process2	50.01	4.05	80.98
		49.83	4.11	82.48
		49.85	0.28	<LOQ
3	Reb M-2301	49.64	0.25	<LOQ
		49.81	0.19	<LOQ
		49.90	0.18	<LOQ
4	Reb M-2302	49.87	0.23	<LOQ
		49.81	0.23	<LOQ
		49.96	0.26	<LOQ
5	Reb M-2303	49.89	0.15	<LOQ
		50.06	0.20	<LOQ

The modified BCA method was also applied to detect residual proteins in other commercially available Reb A, Reb D, and Reb M products. As shown in Table 6, the residual protein in Reb A from Manufacturer 1 ranged from 82.79 to 89.56 mg/kg. Except for Reb M from Manufacturer 4, the Reb D and Reb M products from other manufacturers had residual protein levels exceeding 100 mg/kg. Comparative analysis reveals that Manufacturer 4's Reb M is the only formulation that meets protein

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residue compliance, with levels below 40 mg/kg, making it superior to the other Reb A, Reb D, and Reb M products from the other manufacturers.

Table 6. Commercial stevia sample testing.

No.	Sample	Manufacturers	Batch number	Residual Protein / (mg/kg)
1	RA	Manufacturer-1	20230327	82.79 89.56
2	RD	Manufacturer-2	20240902	109.92 111.57
3	Reb M	Manufacturer-3	02BRMGA0122014	121.66 117.48
4	Reb M	Manufacturer-4	Z046RP2306	<40 <40
5	Reb M	Manufacturer-5	240709006	141.39 139.73

4. Conclusions

In this study, we developed and validated a modified BCA method for determining residual proteins in Reb M samples, incorporating a protein precipitation pretreatment protocol. Compared to traditional BCA methods, this approach effectively addresses the matrix interference common in Reb M samples, offering notable advantages in analyzing complex and interference-prone matrices. The results demonstrated that trace amounts of BSA could be efficiently recovered after pretreatment, with satisfactory detection sensitivity and accuracy. The method showed excellent linearity, with correlation coefficient (r) values exceeding 0.994. Upon validation, the method's limit of quantification (LOQ) was determined to be 40 mg/kg. This methodology provides a versatile analytical platform for detecting trace residual protein in enzyme-catalyzed products, fulfilling a critical need for contamination control in precision manufacturing processes.

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Data Availability Statement: The data used to support the findings of this study are included in this article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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