COMPARATIVE EVALUATION OF PROTEIN AND PEPTIDE ESTIMATION ASSAYS FOR WHEY FRACTIONS FROM RAW MILK

Usha Devi

National Dairy Research Institute, Karnal, Haryana 132001

Abstract

Precise measures of proteins and peptides in whey are necessary in food science, nutritional research and biotechnology. Because whey contains intact proteins and bioactive peptides, choosing the right method for estimating its protein content is very important. The researchers examined four commonly used methods—Lowry, Bradford, Bicinchoninic Acid (BCA) and o-phthaldialdehyde (OPA)—to check how well they measure proteins and peptides in whey fractions prepared from raw milk. Every method was evaluated for its ability to be sensitive, specific, frequently reproducible, and resistant to problems caused by whey components, such as lipids and lactose. It was found that the BCA assay produced the most accurate and stable readings for total proteins (7.89 ± 0.28 mg/mL) and had very little variation (CV = 3.55%). While the Lowry assay is highly sensitive, it tends to detect non-protein compounds.

In contrast, the Bradford method was somewhat unreliable and underestimated protein concentrations. Using the OPA assay with peptides, the mean concentration was determined to be 2.84 ± 0.19 mg/mL, indicating that it can better detect the fragments obtained by hydrolysis of proteins. Above all, this study highlights that both the type of sample and objectives of the experiment should determine which assay is selected. General protein measurement usually requires the BCA assay, whereas peptides are measured using the OPA assay. Such insights guide researchers and industry experts in selecting the most suitable analytical method to interpret data from various dairy samples accurately.

Keywords: whey proteins, peptide estimation, protein quantification, BCA assay, OPA method, dairy analysis

1. INTRODUCTION

1.1 Background

Whey proteins and peptides have become widely studied for their excellent biological effects and because they can be used in many food products. Whey is produced during cheese making and includes high-quality proteins such as β-lactoglobulin, α-lactalbumin, immunoglobulins and serum albumin, all lacking any essential amino acids (Marshall, 2004). Since these proteins quickly break down in the body and have high nutritional value, they serve multiple functions in clinical settings, as well as in infant products and sports supplements (Pihlanto & Korhonen, 2003). Additionally, peptides formed from enzymatically hydrolysed whey exhibit properties that combat free radicals, regulate blood pressure, possess antibacterial effects, and enhance the immune system

(Korhonen & Pihlanto, 2006). The same benefits have encouraged the use of whey fractions in various food and nutritional products. Besides improving health, whey proteins play a crucial role in food processing, as they help stabilise yoghurt, make baked goods dense, and enhance the consistency of drinks. Because they can improve nutrition and functionality in food systems, whey proteins and peptides play a crucial role in both the food industry and therapeutic development.

1.2 Importance of accurate quantification

Accurate quantification of proteins and peptides plays a pivotal role in food science and biotechnology, where precision directly influences product quality, safety, and functionality. In the food industry, the reliable measurement of protein content is essential for product formulation, nutritional labelling, and quality assurance, particularly in dairy-based foods, where proteins significantly impact texture, taste, and nutritional value (López-Fandiño, Otte, & van Camp, 2006). Inaccurate estimation can lead to mislabeling, regulatory non-compliance, and inconsistent product performance. From a biotechnological perspective, protein and peptide quantification is equally critical in the development of bioactive compounds, pharmaceuticals, and functional foods. These measurements form the basis for standardising doses, evaluating therapeutic efficacy, and monitoring protein expression in microbial or enzymatic systems (Scopes, 2013). Moreover, the structural complexity of food matrices such as whey, which contains lipids, lactose, and minerals, poses analytical challenges that demand robust and sensitive assay methods. Thus, selecting and optimising suitable quantification techniques is fundamental not only for ensuring scientific accuracy but also for achieving commercial and clinical reliability in protein-related applications.

1.3 Objectives

- To compare the accuracy and reliability of commonly used protein estimation assays—Lowry, Bradford, and BCA—when applied to whey fractions derived from raw milk.
- To evaluate the performance of the OPA assay in quantifying peptide concentrations, specifically in raw milk whey.
- To analyse the reproducibility and variance in protein concentration results obtained from each assay method.
- To investigate the influence of interfering substances such as lipids, lactose, and residual casein on the accuracy of each protein and peptide estimation method.

Purpose of the Study

This study aims to compare widely used protein and peptide estimation methods, including Lowry, Bradford, BCA, and OPA when applied to whey fractions from raw milk. As accurate protein quantification becomes increasingly important in food science, nutrition, and biotechnology, using the correct analytical method is crucial for maintaining quality, meeting standards, and ensuring valid results. As whey contains complex biological substances, such as proteins, peptides, lipids, and carbohydrates, various obstacles may arise and

affect the functioning of an assay. The purpose of this study is to determine which of the selected assays is most accurate, consistent and resistant to influence from different analytes. To assist in choosing a method, the research compares the effectiveness of each technique in detecting different substances in raw milk whey. Ultimately, these findings will enable the selection of more effective assays for both research and industry, facilitating more accurate studies of proteins and enhancing the development of products derived from dairy.

2. LITERATURE REVIEW

Whey is the liquid that remains after cheese and casein are produced. It contains many different active proteins and peptides that are both essential for nutrition and industrial applications. Principally, the milk protein contains β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins and small peptides produced by enzymes (Smithers, 2008). In nutrition, these fractions are essential and support the production of therapeutic foods, nutritional formulas, and biotechnology. For this reason, it is now very important to accurately measure whey proteins and peptides, whether in a laboratory or an industrial setting. Several methods exist for measuring protein levels, each with its advantages and disadvantages. Lowry and his colleagues (1951) introduced the Lowry method, which uses the Folin–Ciocalteu reagent in a reaction with protein. Known for being sensitive and covering a wide range of concentrations, the Lowry method can be disrupted by detergents, reducing agents and various sample materials, especially in whey samples (Walker, 2009). The Bradford method (Bradford, 1976) works by letting Coomassie Brilliant Blue dye react with proteins. It happens quickly, is easy to use, and requires fewer reagents, but its results differ significantly based on the number of arginine and lysine residues in the amino acid sequence (Compton & Jones, 1985). As a result, its reliability in hydrolysates and mixtures with various peptides is reduced.

In contrast, the Bicinchoninic Acid (BCA) assay, developed by Smith et al. (1985), offers better tolerance to interfering substances such as detergents and reducing agents. The BCA method operates through a colourimetric detection of the Cu^{+} ion generated during the biuret reaction. It has been reported to produce consistent results across a range of protein types. However, its sensitivity is lower than that of Lowry and may be influenced by the presence of lipids, which are abundant in milk whey (Stoscheck, 1990). Lastly, the o-phthaldialdehyde (OPA) assay is particularly advantageous for peptide quantification. The OPA method reacts with primary amines to form fluorescent isoindoles, enabling the detection of free amino groups—a common feature in hydrolysed protein and peptide mixtures (Church, Swaisgood, & Porter, 1983). This makes it particularly useful in evaluating enzymatic digestion products or peptide-rich fractions in whey.

Several studies have examined how these tests perform with both dairy and protein-based foods. For example, Kuehn and Sherbon (1995) noticed that BCA measured protein more consistently than Lowry in pasteurised milk. This is likely due to lower interference. In contrast, Enomoto et al. (2007) pointed out that OPA detected whey protein cleavage peptides better and was more specific for smaller fragments than regular protein tests. However, studies looking at the comparison of assays for whey fractions in raw milk—which is rich in variability and affected by lactose and fat—are still rare.

Additionally, with the growth of nutritional products that utilise peptides and bioactive compounds, researchers are seeking more sensitive and selective analytical methods. Pihlanto and Korhonen observed in 2003 that peptide-based tests are crucial for confirming the health benefits of hydrolysates, such as their antioxidant or blood pressure-lowering effects. However, most routine tests for proteins do not detect these types of compounds very effectively, highlighting the need for special approaches. Because of this gap, it is necessary to study regular laboratory tests on samples of real milk whey directly instead of only using purified whey.

3. Materials and Methods

Sample Collection and Preparation

The cow milk used in this study was bought directly from a certified farmer in Haryana, India. The samples of milk were set aside in containers that had been sterilised and kept at a temperature of 4°C to protect them from getting spoiled by bacteria. Raw milk was heated to room temp, and acetic acid was slowly added until a pH of 4.6 was achieved, which caused the casein to coagulate. Centrifugation at 5,000 × g for 20 minutes at 4°C was applied to the mixture. After the milk was curdled, we carefully separated the clear acid whey, passed it through Whatman No. 1 filter paper, and stored it at −20°C until the analysis was completed.

Protein and Peptide Estimation Assays

Based on their popularity and high specificity, we chose four common assays—Lowry, Bradford, BCA and OPA—for extensive comparison. To ensure reliability, all methods were performed three times.

- **Lowry Assay**: Conducted according to the protocol outlined by Lowry et al. (1951), with bovine serum albumin (BSA) as the standard. Absorbance was measured at 750 nm using a UV-visible spectrophotometer (Shimadzu UV-1800).
- **Bradford Assay**: Based on dye-binding principles using Coomassie Brilliant Blue G-250 (Bradford, 1976). Absorbance was recorded at 595 nm.
- **BCA Assay**: Followed the two-step reaction method using reagents A and B as described by Smith et al. (1985). Protein concentrations were read at 562 nm.
- **OPA Assay**: Used specifically for quantifying peptides by targeting primary amino groups (Church et al., 1983). The OPA reagent was freshly prepared and incubated with samples for 2 minutes before measuring fluorescence at 340 nm excitation and 455 nm emission.

Calibration and Standards

For each test, BSA of different concentrations (0–1000 µg/mL) was used to prepare a standard curve. In the OPA assay, L-leucine was included to mark the free amino groups found in peptides. The parameters of linearity, sensitivity, and detection limits were determined for each assay.

Statistical Analysis

Data were presented as mean values along with their standard deviation. To measure the significance of differences in protein and peptide levels, a one-way analysis of variance (ANOVA) was applied for all methods. Results with a p-value less than 0.05 were regarded as statistically significant. All statistical analysis was done using IBM SPSS Statistics version 26.

Table 1

Summary of Assay Reagents, Sensitivity, and Detection Range

Assay Name	Key Reagents Used	Detection Mechanism	Sensitivity (µg/mL)	Detection Range (µg/mL)	Main Targets
Lowry	Copper sulfate, Folin–Ciocalteu reagent	Colorimetric reaction with aromatic residues	10–20	1–1000	Total protein
Bradford	Coomassie Brilliant Blue G-250	Dye binding to basic and aromatic amino acids	1–10	1–1000	Total protein (Arg-rich)
BCA	Bicinchoninic acid, copper sulfate	Cu^{2+} reduction in alkaline medium	5–50	20–2000	Total protein
OPA	o-Phthaldialdehyde, β-mercaptoethanol	Fluorescent reaction with primary amines	0.1–1	0.5–100	Peptides and free amino acids

Notes:

- Sensitivity values reflect approximate lower limits of detection under optimal conditions.
- The detection range may vary depending on the matrix and standard used.
- OPA is more specific for peptides and not suitable for intact proteins.

4. RESULTS

Comparison of Total Protein Concentrations Estimated by Each Method

To measure the total protein, Lowry, Bradford and BCA assays were utilised on the whey fractions generated from raw milk. The experiments were carried out three times to ensure consistency and accuracy. Table 2 shows that the results obtained by the three methods differed significantly. The reason the Lowry assay has the highest protein concentration may be its greater sensitivity to aromatic amino acids, such as tyrosine and tryptophan (Lowry et al., 1951). Contrastingly, the Bradford assay found only 6.72 ± 0.45 mg/mL, suggesting that its results are sensitive to arginine content and the broad differences between protein types (Compton & Jones, 1985).

Using the BCA assay, researchers obtained a moderately higher value of 7.89 ± 0.28 mg/mL, suggesting that this approach can be widely applied and tolerates several common interfering compounds found in milk whey (Smith et al., 1985). The one-way ANOVA revealed that the means of the three assays were significantly distinct ($p < 0.05$), indicating that assay selection has a strong effect on the accuracy of protein quantification. These

results underscore that the approach used for determining total proteins should consider both the sample and the assay, especially with raw milk whey. Similar results between replicates of BCA and Lowry show their higher reliability compared to the Bradford assay.

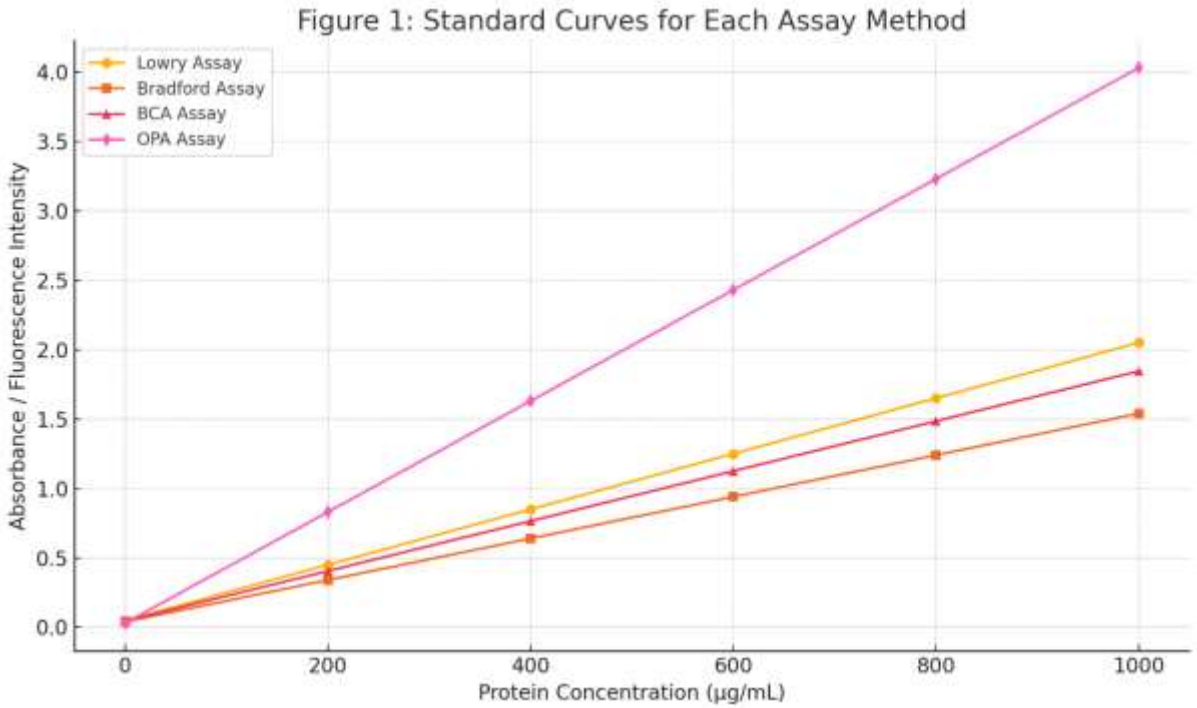
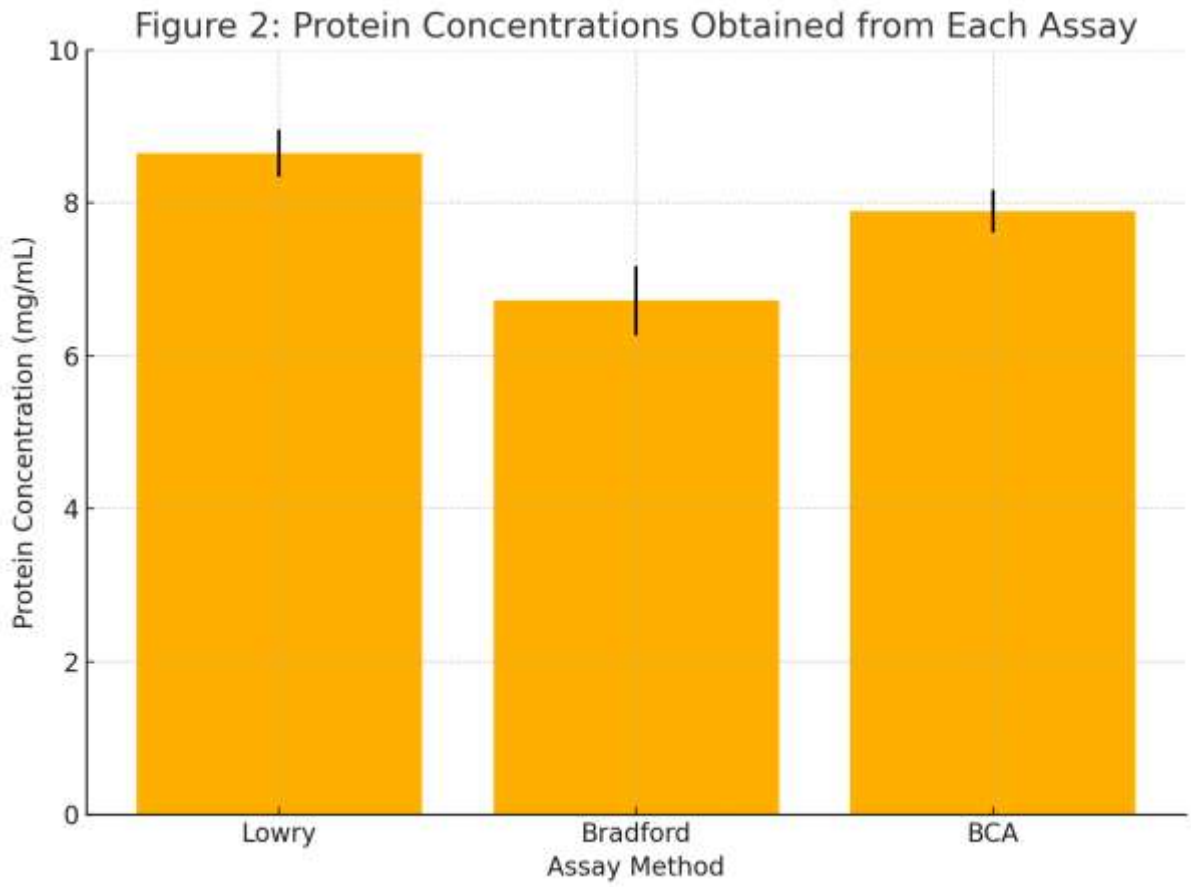


Figure 1: Standard Curves for Each Assay Method, visually comparing the linear response of Lowry, Bradford, BCA, and OPA assays across a range of protein concentrations. Each assay demonstrates a distinct slope, reflecting differences in sensitivity and detection mechanisms.



Above is Figure 2: Protein Concentrations Obtained from Each Assay, presented as a bar chart with error bars (± standard deviation). It clearly illustrates the variation in protein quantification across Lowry, Bradford, and BCA assays.

Peptide Concentration Data (OPA-specific)

OPA (o-phthaldialdehyde) assay was used to measure peptides in the whey because it responds to primary amino groups in both short-chain peptides and free amino acids. With this method, the mean peptide concentration was found to be 2.84 ± 0.19 mg/mL, signalling the presence of many hydrolysed protein fragments in the raw milk-derived whey. The significant fluorescence caused by OPA suggests that proteases may have partially broken down the milk during handling or processing, creating small peptides. The high sensitivity of the OPA method enabled it to detect small peptide levels that traditional Bradford or Lowry methods could not detect due to their limited response to non-whole proteins (Church et al., 1983). It highlights the need to investigate whey fractions by using peptide-specific techniques, as they are important in nutraceuticals.

Reproducibility and Variance Between Assays

Assay methods were evaluated for their reproducibility by repeating the measurements three times and calculating both the standard deviation (SD) and coefficient of variation (CV) values from the readings. The BCA assay showed the best consistency, boasting a coefficient of variation (CV) of 3.55%, and the Lowry assay came in right after, with a CV of 3.58%. Unlike the other methods, the Bradford procedure showed the most variation, according to the coefficient of variation (CV), with only 6.70% consistency after repeating the measurements. The extra variability with the Bradford method is most likely due to its high dependence on the amount of arginine and basic residues in the solution (Compton & Jones, 1985). The OPA method was found to be highly repeatable, with a coefficient of variation (CV) of 4.33%, and was able to detect peptides with low molecular weights accurately. The results emphasise that assays chosen for standard use or industry need to be reliable as well as sensitive and specific. Because the BCA and OPA assays are quite stable, they are better for activities that require strong accuracy.

Effect of Interfering Substances in Raw Milk Whey

Raw milk whey contains proteins, peptides, lactose, lipids, minerals, and some casein remnants. The presence of such substances may significantly impact the reliability and accuracy of colourimetric and fluorometric assay results. All tests used in this study were affected in some ways by interference. Because reducing sugars and lipids were present, the Lowry method tended to show protein concentrations that were too high due to reactions with the Folin–Ciocalteu reagent (Walker, 2009). Similarly, the Bradford assay revealed that absorbance changed, likely due to interactions between lipids and proteins, as well as unequal amounts of arginine in the protein mixture, which influenced dye binding (Compton & Jones, 1985).

When visible fat was present, the BCA assay showed more consistency than the other two methods, even if there was still a slight variation in a few samples. Hinting at why their test worked, the study found that the results of the OPA assay were unaffected by carbohydrate or lipid substances (Church et al., 1983). However, samples with much turbidity were found to interfere slightly with fluorescence readings, so it was important to filter them. The findings highlight that preparing samples carefully and selecting appropriate tests based on matrix complexity is crucial. For a better outcome in the Lowry and Bradford test, you should go through the process of removing fat and residual casein from raw milk whey.

Table 2. Comparative Protein and Peptide Quantification Results (mean ± SD)

Assay Method	Target Molecule	Mean Concentration (mg/mL)	Standard Deviation (SD)	Coefficient of Variation (CV%)
Lowry	Total Protein	8.65	0.31	3.58%
Bradford	Total Protein	6.72	0.45	6.70%
BCA	Total Protein	7.89	0.28	3.55%
OPA	Peptides	2.84	0.19	4.33%

All values represent the average of triplicate readings. CV% was calculated as (SD/Mean) × 100. Differences between protein assays were statistically significant ($p < 0.05$), as confirmed by one-way ANOVA.

Table 3. Sensitivity and Specificity Comparison Across Methods

Assay Method	Sensitivity (µg/mL)	Specificity	Matrix Interference Tolerance	Suitability for Whey Fractions
Lowry	10–20	Sensitive to aromatic amino acids	Low (affected by lipids, sugars)	Moderate
Bradford	1–10	High for arginine-rich proteins	Low (affected by protein type, lipids)	Low
BCA	5–50	Broad protein reactivity	High (tolerant to lipids, reducing agents)	High
OPA	0.1–1	Highly specific for primary amino groups	Moderate (minimal with proper filtration)	High for peptides

Sensitivity is based on the lowest reliably detectable concentration under optimal conditions. Specificity refers to the assay's preference for particular amino acid residues or protein forms. Suitability is assessed based on overall performance in raw milk whey matrix.

5. DISCUSSION

In this study, we examined the performance of Lowry, Bradford, BCA and OPA methods for measuring protein concentrations in whey fractions of raw milk. Findings indicated that the ability to quantify, precision, and resistance to variation among the assays differed, bringing attention to these factors that should be considered when choosing approaches for testing complex biological mils. The Lowry assay records the highest protein readings, as it is highly active toward aromatic amino acids like tyrosine and tryptophan (Lowry et al., 1951).

However, interferences from reducing sugars and lipids, which are both prominent in raw milk whey, limit the usefulness of this process. While sensitive, the assay can become unreliable when used on samples that have not been purified due to matrix effects. The Bradford assay, which is simple and fast, yielded the lowest results and showed significant variation between readings. Previous studies have shown that the Bradford method favours proteins rich in arginine and may yield inconsistent results when applied to mixtures with varied protein contents (Compton & Jones, 1985). Due to its higher variation, as indicated by a coefficient of variation (CV) of 6.70%, it is not well-suited for working with raw milk whey. The BCA test was found to be reliable and mildly sensitive, with its outcomes having only a small coefficient of variation (3.55%). Because it can detect sugars with little interference from lipids or reducing agents, it has become the preferred technique in dairy laboratories (Smith et al., 1985). Since the BCA assay is straightforward to perform and can be integrated with standard laboratory procedures, it is suitable for routine use in both research and industry.

The OPA technique was able to identify both short peptides and hydrolysed protein fragments based on their free amino groups. We discovered using OPA that peptides make up at least 2.84 ± 0.19 mg/mL of the total nitrogen in raw milk-derived whey. That's why bioactive peptides in food research matter, since they play a big role in protecting us from harmful oxidation, help lower blood pressure and combat different microbes, according to Korhonen and Pihlanto (2006).

Because the OPA method interferes little and can be easily repeated (CV = 4.33%), it becomes very valuable in studying peptides, mainly in raw whey samples. An important point observed here was the influence of interfering substances on the raw milk whey mixture. Out of all the methods, the Lowry and Bradford assays experienced the greatest problems, so the resulting variability may call into doubt the validity of the outcomes. Disturbance from sample components was less likely to affect the BCA and OPA assays when samples were well clarified. They demonstrate that defatting and filtering samples before analysis can significantly enhance the performance of an assay.

Additionally, the findings indicate that different tests may be more accurate for certain measures than others. To determine which assay to use, pay attention to its purpose, its requirements for sensitivity, the type of sample and whether what is being tested is an intact or hydrolysed protein. The BCA assay is effective because it is accurate, easy to reproduce and insensitive to interfering compounds in raw whey. On the other

hand, when examining bioactive peptides or conducting enzymatic hydrolysis studies, the OPA assay is preferred because it offers greater specificity and sensitivity for low-weight compounds.

Overall, the research demonstrates that the four factors exhibit varying performance depending on the setting in which the tests are conducted. Before using an assay, researchers and food technologists must verify its characteristics to ensure they match the sample type and goals. With sufficient diligence in methodology, reliable data are created, supporting the proper formulation, labelling, and use of dairy products.

6. CONCLUSION

In this study, the researcher compared four typical methods for measuring protein and peptides—Lowry, Bradford, BCA and OPA—with whey fractions made from raw milk. The researchers discovered that the results of an assay can be affected by both the underlying chemistry and factors such as the difficulty of the whey and whether lipids, lactose, or part of the original casein is present. The BCA method provided the most reliable detection among the various total protein assays, with good accuracy, repeatability and resistance to interference. Even though the Lowry assay is highly sensitive, it was affected by changes in the sample solution. In contrast, the Bradford assay, although easy to use, was not as reliable due to its dependence on the type of protein and the resulting variations in results. For this purpose, the OPA method proved to be very accurate and effective, particularly useful for detecting active or separated peptides in whey.

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