

THE ASSESSMENT OF PROTEIN CONCENTRATION AND TYPE IN NUTRITIONAL PROTEIN SHAKES

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Abstract

Pre-made protein shakes are a popular, accessible, and convenient way to increase protein intake. They are used as nutritional supplements and often substituted for meals to provide a protein boost. The convenience of these ready-to-drink shakes also allows people with specific dietary needs to meet their protein nutrient needs. This study assessed the protein content of 12 nutritional protein shakes to determine total protein and whey protein content. Three experimental methodologies were used in tandem. These included the Bradford assay (to measure the total protein content in the shakes). Reverse phase high-performance liquid chromatography (RP-HPLC) was utilized to quantify the concentrations of Bovine serum albumin (BSA), α -Lactalbumin (α -LA), β -Lactoglobulin A (β -LGA), and β -Lactoglobulin B (β -LGB); the whey proteins in each protein shake. SDS-PAGE was used to assess the number of distinct proteins in the shakes and to estimate their molecular weights for identification. The animal-based protein shakes contain casein as the primary protein, with some also containing whey protein. The plant-based protein shakes did not contain any casein or whey protein, as expected.

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Introduction

Protein supplements are the most used dietary supplements among athletes, recreationally active adults, and soldiers¹. Primary consumption of protein supplements occurs before or after workouts to help with muscle recovery, growth, and development. While pre-made shakes and protein powders have many benefits, they are not FDA-regulated or pre-approved, as they are classified as dietary supplements². Most users obtain information about these supplements from family, friends, and coaches who rely on marketing materials. Without third-party confirmation of these marketing materials, there is no way to verify the veracity of these marketing claims. In recent times, multiple manufacturers have been hit with false-claims lawsuits, including Premier Protein and Owyn^{3,4}. It is important to conduct third-party testing to ensure that the protein amounts manufacturers claim in the shakes are accurate. Doing so allows consumers to know whether they are getting the protein content they expect. The shakes can contain additives including sugars, vitamins, minerals, thickeners, artificial flavoring, and sweeteners. Additionally, these shakes can be animal-based or plant-based, depending on their source. It is also important to note that some protein supplements may pose health risks due to the presence of harmful heavy metals, including arsenic, cadmium, lead, and mercury⁵.

The goal of our research lab is to determine the protein content of protein products such as protein powders and protein shakes^{6,7}. We have prior experience with protein powders and were eager to determine whether there were any differences in the protein content of milkshakes. Milkshakes present several advantages over protein powders. These advantages include a ready-to-use formulation, as these shakes are premade and deliver protein in liquid form, which may also contribute to improved hydration status⁸. We used our established protocols to determine total protein content by the Bradford Assay, followed by High-Performance Liquid Chromatography (HPLC) analysis of the 4 major whey proteins, in tandem with SDS-PAGE for protein visualization.

The Bradford assay is a protein detection method that is fast, simple, and sensitive enough to detect even low protein concentrations^{9,10}. It uses a Coomassie Brilliant Blue G-25 dye to bind to

proteins, resulting in a color change that is measured with a spectrophotometer. A more accurate and precise method is to determine the concentration of each protein type present in the protein product using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). RP-HPLC identifies, separates, and quantifies the components in chemical mixtures¹¹. Since we did not know which proteins might be present in our protein milkshake, we used the four major whey proteins as our HPLC standards. These four major proteins were Bovine serum albumin (BSA), α -lactalbumin (α -LA), β -lactoglobulin A (β -LGA), and β -lactoglobulin B (β -LGB). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a method used to separate and visualize proteins by molecular weight^{12,13}, was used in our final step, as discussed in our prior paper⁷. SDS-PAGE can help us determine which other protein types may be present in protein shakes.

Materials and Methods

Twelve protein shakes were purchased from Walmart. The Bradford reagent was acquired from Bio-Rad and the Thermo Scientific Genesys 30 was used as the spectrophotometer. Bovine serum albumin (BSA), α -Lactalbumin (α -LA), β -Lactoglobulin A (β -LGA), and β -Lactoglobulin B (β -LGB) standards were acquired from Sigma-Aldrich. A Poroshell StableBond 300 C8, 2.1 x 75 mm, 5 μ m was used as our reversed-phase C8 HPLC column with superficially porous particles. Trifluoroacetic acid (TFA) and Acetonitrile (ACN) were acquired from Sigma-Aldrich. Agilent Technologies' 1260 Infinity HPLC was used. The Mini-Protean Tetra Cell System and Comparative Proteomics I kit were obtained from BIORAD and contained Kaleidoscope™ Prestained Protein Standard, 10x Tris-Glycine-SDS electrophoresis buffer (TGS buffer), Laemmli sample buffer, 4–20% Mini-PROTEAN® TGX™ Precast Gel, and Bio-Safe™ Coomassie stain.

Bradford Assay Method

32 μ L of each protein shake was added to 968 μ L of 0.1 M Pi Buffer and mixed thoroughly. 20 μ L of this solution was pipetted into a cuvette, followed by the addition of 3000 μ L of Bradford reagent, and the mixture was then mixed. A spectrophotometer was used to measure the absorbance of the cuvettes at 595 nm

after 5 minutes of incubation at room temperature. Each protein shake was measured three times to assess statistical significance and guarantee accuracy and repeatability of the data.

Preparation of Bradford Assay Standards

A stock solution of BSA at a concentration of 2 mg/mL (in 0.1 M Phosphate buffer) was created. The stock was diluted to concentrations ranging from 200 µg/mL to 700 µg/mL. A graph of absorbance versus concentrations was constructed to obtain the calibration curve. The calibration curve was used to calculate the amount of protein present in the milkshake. The amount of protein calculated using the calibration curve was compared to the actual amount of protein expected (found on the label of the milk shakes) using the formula:

$$\% = \frac{(\text{actual concentration of protein})}{(\text{expected concentration of protein})} * 100$$

RP-HPLC Method

0.1% TFA and 5.0% ACN in water was used as solution A, whereas 0.1% TFA in ACN was solution B. The two solutions, A and B, were combined to perform the gradient elution for HPLC. The gradient started at 5% solution B, reached 15% after 0.5 minutes, and proceeded as follows: 0.5 – 1 min, 15% – 18% B; 1 – 2 min, 18% – 27.5% B; 2 – 3 min, 27.5% – 30.5% B; 3–3.25 min, 30.5% – 31% B; 3.25 – 4 min, 31% – 32% B; 4 – 4.58 min, 32% – 33.8% B; 4.58–7.50 min, 33.8% – 37% B; 7.50 – 7.51 min, 37% – 50% B; 7.51 – 8.50 min, 50% – 50% B; 8.50 – 8.51 min, 50% – 5% B; 8.51 – 10.0 min, 5% – 5% B. Each sample took ten minutes to analyze in total. The injection volume was 2 µL, the column temperature was 70 °C, the flow rate was 2 mL/min, and the detection wavelength was 280 nm.

Preparation of RP-HPLC Standards

A 5 mg/ml stock solution of BSA was created and diluted to concentrations ranging from 0.25 mg/mL to 2.00 mg/mL. The stock solution of α-LA, β-LGB and β-LGA were prepared using the same process. To ensure the precision and repeatability of the outcomes, each standard was performed in triplicate.

Preparations of protein shakes for RP-HPLC

A 1:5 dilution was used to prepare each protein shake by adding 200 µL of protein shake to 800 µL of 0.1 M Pi Buffer, then vortexing until completely dissolved.

SDS-PAGE standard and sample preparation

An approximate 2mg/mL solution of protein shakes was made by diluting 32 µL of each milkshake to a final volume of 1000 µL with phosphate buffer. 2mg/mL protein standard solutions were also made. All standard and sample protein solutions were stored at 4 °C between assays.

Sample Laemmli Dye Buffer Preparation

The Bio-Rad Mini-Protean Tetra System kit with a compatible Bio-Rad power supply box was used throughout the experiment. 50 µL of 2-mercaptoethanol was added to 950 µL of the 2x Laemmli sample buffer. The 2x concentration of Laemmli buffer stock with mercaptoethanol solution provided was diluted to 1x using the 2mg/mL protein shake sample and standard solutions in the microcentrifuge tube, then heated at 95 °C for 5 minutes.

Running SDS-PAGE Procedure

A 1X Tris/Glycine/SDS Buffer was prepared and used as the running buffer for SDS-PAGE. 10 µL of each standard and sample protein stock solution was pipetted into each gel well, and 5 µL of the protein ladder was pipetted into a gel well. The gels were run at 200 volts for 35 minutes. Then, the gels were removed from the gel cassette by gently separating and breaking the arrows and inverting them into a water bath.

Coomassie Staining and Imaging Procedure

The gels were washed 3 times for 5 minutes each in 200 mL of DI water to remove SDS and prevent interference with staining. All the water was poured out of the staining container, and 50 mL of Coomassie stain was added until it covered the gel. This was gently shaken for an hour and a half, and the Coomassie stain was poured out. The gel was rinsed in 200 mL of DI water with gentle shaking. The gel was destained for 30 minutes. The water from the staining container was poured out, and the gels were gently placed on a lightbox. Photos of the gels were taken, and the gels were disposed of in biological waste.

SDS-PAGE Image Analyzing Procedure

The ImageJ Image Processing and Analysis in Java program was used to analyze the SDS-PAGE images. A scatter plot of R_f against the log of molecular weight of the protein ladder was created to obtain a calibration curve. The calibration curve was used to determine the molecular weight of each standard and sample protein.

$$R_f = \frac{\text{distance from the top of the gel to the protein}}{\text{Distance from top of the gel to the solvent front}}$$

Results and Discussion

Our lab has established that using a single method to analyze protein content in protein powders is insufficient to capture the full picture of the protein present in the sample, given the limitations of the available instruments. Three protocols have been established to identify the proteins present, including the *Bradford Assay*, *HPLC with UV detection*, and *SDS-PAGE*. The goal is to understand the amount of protein present in protein products on the market. We start by quantifying total protein using Bradford, then analyze 4 proteins (major proteins found in whey protein) by HPLC, and finally use SDS-PAGE to assess whether any other proteins have been missed. Using the Bradford assay, the absorbances of the protein shakes were measured and used to calculate the actual concentration. None of the proteins had the amount of proteins expected; they all had less than the protein amounts listed. The data showed that 3 of the protein shakes contained 60% (or greater) of the amount of proteins that we were expecting to be present (Figure 1).

Using the HPLC-UV method, data were collected for the protein standards (α-Lactalbumin (α-LA), β-Lactoglobulin A (β-LGA), β-Lactoglobulin B (β-LGB), and Bovine serum albumin (BSA) to make the calibration curves. The calibration curves all had R^2 values greater than 0.96, indicating a strong correlation between average area and concentration. For the R^2 values, the absolute minimum standard value was 0.95. Using this data, the amounts of each of these four proteins were calculated (based on the retention times and peak area) in the different milkshakes. Our previous work, however, indicated that HPLC-UV detection data alone were insufficient to determine whether these proteins were

present. There was always a need to pair the retention data with SDS-PAGE to obtain a true measure of the four proteins present. Based on our initial SDS-PAGE runs (triplicate; single data point shown in Figure 2), the Bovine Serum Albumin (BSA) was not present in any of the protein shake samples. This was also the case in the protein powders that were tested. The only two milkshakes that did not have any of the four major whey proteins were Owyn chocolate and Owyn vanilla, which are plant-based milkshakes, so this data is expected (Table 1). Four of the 12 milkshakes contained about 10% of the major whey proteins. LA was the most common whey protein found in all the milkshakes; it was only absent only in the plant-based milkshakes and Equate Milkshake. Equate, however, contained a significant amount of LGA and LGB combined. (Table 1). Atkins contained three of the major whey proteins, accounting for about 15% of the expected protein amount on its label (Table 1 and Figure 3).

In the animal-based milkshakes (all the milkshakes except Owyn Chocolate and Vanilla), another protein was found in significant amounts. Using the molecular weights on the Bio-Rad protein ladder standards (Figure 4), the proteins' molecular weight

(Mwt) were determined (Table 2). To assess the integrity of our dataset, we also performed calculations on our original protein standards. For BSA, LGA, and LA, the calculated molecular weights were within 1 kDa of the expected values. The unknown protein had a molecular weight of 36 kDa, which meant that the unknown protein had a molecular weight between 35 and 37 kDa. Another piece of information that helped us was the characteristic two-line pattern we observed for this unknown protein. The most likely protein was casein, based on our literature search^{14,15}. We added casein as one of our protein standards to another batch of SDS-PAGE runs and confirmed that the protein observed in our animal-based milkshakes was casein (Figures 5 and 6). The plant-based milk (Owyn chocolate and Owyn vanilla) did not contain casein, as expected.

Conclusion

The pre-made protein shakes we tested do not contain significant amounts of the four major proteins typically found in whey protein; however, they contained at least 40% of the proteins they claimed to contain. Of the 12 milkshakes tested, at most 3 of the 4 major whey proteins were found in the animal-based milkshakes; none of the milkshakes contained BSA. The animal-based milkshakes also contained significant amounts of casein. The plant-based ones contained neither casein nor the major proteins found in whey, as expected. Using the Bradford Assay, there was variation in protein content among the different milkshakes. Core Power and Premier Protein had the highest total protein content among

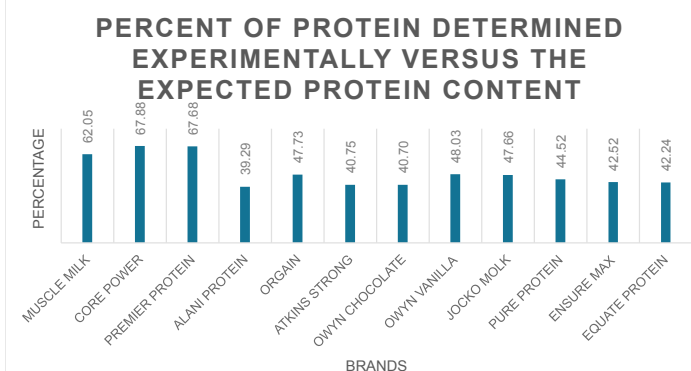


Figure 1. Percentages of proteins present in the milkshakes. This was calculated using the formula $\% = (\text{actual concentration of protein}) / (\text{expected concentration of proteins}) * 100$

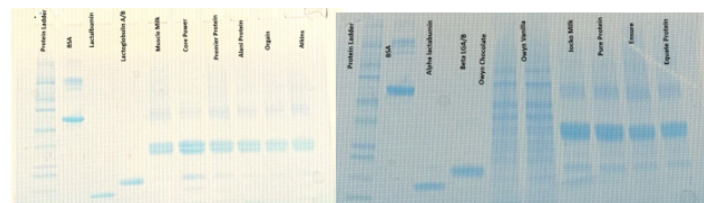


Figure 2. Single run for SDS-PAGE showing the protein standards and the 12 protein milkshakes tested. From left to right: Protein Ladder, BSA, LA, LGA, Muscle Milk, Core Power, Premier Protein, Alani protein, Orgain, Atkins Strong, Protein ladder, BSA, LA, LGA, Owyn Chocolate, Owyn Vanilla, Jocko Milk, Pure Protein, Ensure Max Protein and Equate Protein

Table 1. A table showing the results of analyzing both the SDS-PAGE and HPLC-UV data for the four major whey proteins. In red, the four milkshakes that contained the major whey proteins in amounts greater than or equal to 10%.

Shakes at 280 nm	Expected Amount of Proteins (mg/mL)	LA (mg/mL)	BSA (mg/mL)	LGA (mg/mL)	LGB (mg/mL)	Total whey proteins (mg/mL)	%
Muscle Milk	60.4	2.04				2.04	3.37
Core Power	101.4	4.71				4.71	4.64
Premier Protein	88.2	1.11				1.11	1.26
Alani Protein	84.5	0.66		6.66	5.51	12.83	15.19
Orgain	90.9	1.03		5.25		6.27	6.90
Atkins	92.3	0.71		10.07	3.24	14.02	15.19
Owyn Chocolate	97.0						
Owyn Vanilla	97.0						
Jocko Milk	90.9	1.05				1.05	1.16
Pure Protein	92.3	1.10		7.99		9.09	9.84
Ensure	90.9	1.17		2.77		3.94	4.34
Equate Protein	92.3			7.04	4.20	11.24	12.17

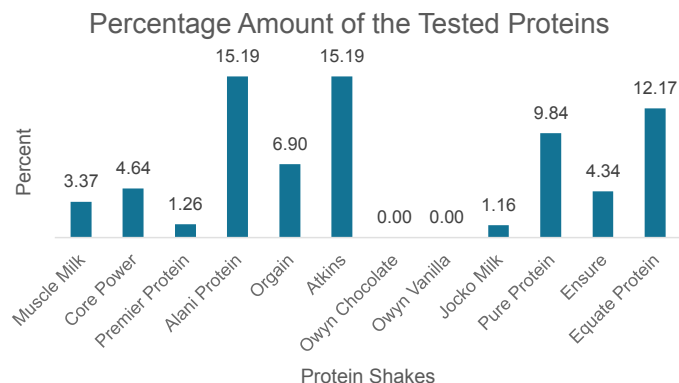


Figure 3. Percentage of whey proteins in milkshakes. This was calculated based on the amount of protein on the label, not the total from the Bradford Assay.

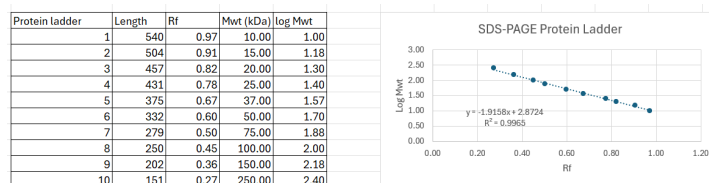


Figure 4. A graph of log molecular weight (y-axis) and R_f values (x-axis) obtained using the Bio-Rad precision plus protein standards on SDS-Page.

Table 2. Comparing the calculated molecular weight for the protein standards and comparing it to their literature values.

Proteins	Calculated Mwt (kDa)	Expected Mwt (kDa)
BSA	66.8	66.5
LA	13.6	14.2
LGA/B	17.5	18.4
Unknown Protein	36.6	**

the twelve milkshakes tested. A future direction for this work is to determine the amount of casein in animal-based milkshakes and to extend the analysis to examine the protein composition of plant-based milkshakes. We also found that the marketing of these shakes fails to mention which proteins or amino acids are included, so the goal is to determine the amino acid content to assess their contribution.

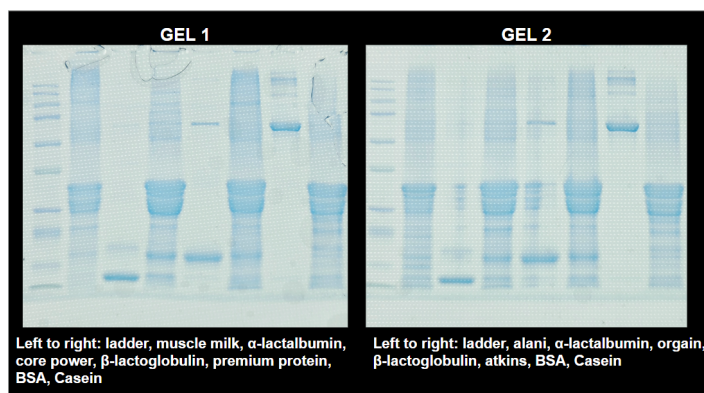


Figure 5. SDS-PAGE on the twelve protein powders, including the major whey proteins and Casein.

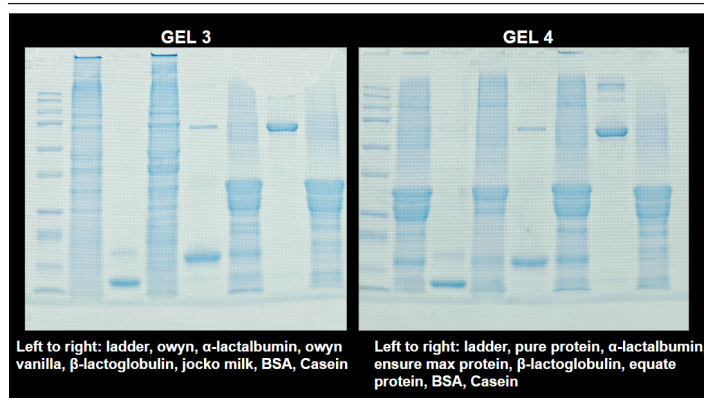


Figure 6. SDS-PAGE on the twelve protein powders, including the major whey proteins and Casein.

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