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QUANTIFYING AMOUNT OF WHEY PROTEIN IN VARIOUS PROTEIN POWDERS

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Abstract

Protein powders are a prevalent way to add protein to a diet, especially before workouts. The main protein in these powders is whey proteins, a product of the cheese-making process. This study aims to quantify the amount of whey protein in 10 protein powders in the market to verify the number of proteins present versus the amount on their nutrition label. Bradford's Assay was used to estimate the total protein concentration in each powder. Reverse phase high-performance liquid chromatography (RP-HPLC) was used to determine the concentrations of α-Lactalbumin (α-LA), β-Lactoglobulin A (β-LG_A), β-Lactoglobulin B (β-LG_B), and Bovine serum albumin (BSA) in each protein sample. The Bradford assay reveals that, on average, the ten powders only contained about 36% of the protein they claimed. The RP-HPLC determined that the majority of the proteins in the powders are β-Lactoglobulin A and β-Lactoglobulin B.

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Introduction

Many people use protein powders as a source of extra protein in their diet. However, the FDA does not regulate protein powders[1], which means that manufacturers can use protein quantification methods, such as the Dumas and Kjeldahl methods, that over-inflate the value of total protein concentration in their powders. The Dumas and Kjaldahl methods work by detecting the nitrogen concentration in the sample; however, they detect all nitrogen in the proteins as well as any amino acids, flavoring and preservatives[2]. This can over-inflate the amount of protein in the powder and make people think it is a better source of protein than it is. Over-inflated protein values highlight the need for more accurate third-party detection methods that verify whether or not manufacturer-reported values are correct. This can give customers a better idea of what they are consuming. Bradford's Assay uses the Coomassie Brilliant Blue G-250 reagent dye, which changes color from brown to blue as it binds to proteins[3]. This assay is a great candidate for this work due to its good protein sensitivity and cheap and quick method of analyzing proteins. Reverse Phase – High-Performance Liquid Chromatography (RP-HPLC) is the better method to find the amount of each type of protein present. RP-HPLC is a good candidate due to its high sensitivity and ability to separate compounds, allowing us to determine the exact amount of each protein within the powder. We chose the four protein standards: α-Lactalbumin (α-LA), β-Lactoglobulin A (α-LA), β-Lactoglobulin B (β-LG_B), and Bovine serum albumin (BSA). These standards were chosen because they are the most abundant protein in whey proteins[5]. Using both methods, we can create separate calibration curves to measure the total protein concentration and the individual protein in the ten marketed protein powders.

Materials/Methods

The ten protein powders were ordered from Amazon. Bradford reagent was obtained from Bio-Rad. The spectrophotometer used was Genesys 30 from Thermo Scientific. We obtained α-Lactalbumin (α-LA), β-Lactoglobulin A (β-LG_A), β-Lactoglobulin B (β-LG_B), and Bovine serum albumin (BSA) standards from Sigma-Aldrich. A Poroshell StableBond 300 C8, 2.1 x 75 mm, 5 µm. A reversed-phase C8 HPLC column with superficially porous particles was obtained from Agilent. Acetonitrile (ACN) and Trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich. The HPLC used was an Agilent Technologies 1260 Infinity.

Bradford Assay Method

100 uL of standards and samples were transferred to a cuvette, followed by 3000 uL of Bradford reagent, and mixed with a pipette. The cuvettes were incubated at room temperature for 5 minutes, and then their absorbances were measured with a spectrophotometer at 595 nm. Triplicates of each standard and sample were measured to ensure the accuracy and reproducibility of results and to calculate statistical significance.

Preparation of Bradford Assay Standards

A 2 mg/mL stock solution of bovine serum albumin (BSA) was made by adding 2 mg of BSA, followed by 1 mL of a 0.1M Pi Buffer. The stock was diluted to concentrations ranging from 100 ug/mL to 650 ug/mL in 50 ug/mL intervals for 11 data points. A calibration curve was created by plotting the absorbance readings against their respective concentrations.

Protein Powders Analysis using Bradford Assay

A 2 mg/mL stock solution of each protein powder was made by adding 2 mg of powder followed by 1 ml of a 0.1 M Pi Buffer. Three dilutions of concentration 600 ug/mL were made by pipetting 60 uL of stock into three separate microcentrigue tubes, followed by 140 uL of Pi buffer. Calculations were made to determine the amount of expected protein concentration in each powder. The detected protein is calculated using the absorbance readings and the calibration curve of the BSA.

RP-HPLC Method

The gradient elution was carried out with a mixture of 2 solutions, A and B. Solution A contained 0.1% TFA and 5.0% ACN in water, and solution B contained 0.1% TFA in ACN. The gradient began with 5% solution B; after 0.5 min, the gradient was 15% and continued 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. The total analysis time per sample

was 10 min. The flow rate was 2 mL/min, the column temperature was 70°C, the injection volume was 2 uL, and the detection was at a wavelength of 214 nm.

Preparation of RP-HPLC Standards

A 5 mg/ml stock solution of BSA was made by adding 15 mg of BSA to 3 mL of Milli-Q water. The stock was diluted to concentrations ranging from 0.25 mg/mL to 2.00 mg/mL in 0.25 mg/mL intervals. The same procedure was performed to prepare the stock solution of α-LA and was diluted to concentrations ranging from 0.25 mg/mL to 1.50 mg/mL in 0.25 mg/mL intervals. A 5 mg/ml stock solution of β-LG_B was made by adding 10 mg of β-LG_B in 2 mL of Milli-Q water. The stock was diluted to concentrations ranging from 0.3 mg/mL to 1.00 mg/mL in 0.1 mg/mL intervals. The same procedure was performed to prepare the stock solution of β-LG_A. Each standard was performed in triplicates to ensure the accuracy and reproducibility of results.

Preparations of protein powders for RP-HPLC

15 mL of a 5 mg/mL solution was prepared for each protein powder sample analyzed in triplicates by adding 75 mg of protein into 15 mL of Milli-Q water. Calculations were made to determine how many mg of powder is required to obtain 75 mg of protein based on the reported serving size and nutrition label. The summary of the calculations is included in **Table 1.** Each of the resulting masses was added to 15 mL Milli-Q water. Each protein powder sample was vortexed for 30 seconds and then left for 5 min to let the undissolved flavoring settle to the bottom.

Results and Discussion

Bradford assay

Table 2 Shows the data obtained from the BSA standards for the Bradford assay. **Figure 1** shows our calibration curve using the data form **Table 2**. The R-squared value was 0.9868, indicating a linear fit between our concentration and the absorbance values obtained from the spectrometer. **Table 3** shows the absorbances for each protein powder and the detected amount of protein. The data allowed us to find the actual protein amount per serving size of the powder and the percent difference for each of the protein powders. The percent difference reveals that powder 6 (Vitron) had the lowest percent, with only 7% of the proteins stated on the label. The highest percent difference was for Ultimate Nutrition, which contained 47% of the protein stated on the label. **Figure 2** compares the reported protein value per serving and the experimental amount for each of the ten powders. On average, the protein amount per serving is only 36% of the labeled value. It is important to note that Bradford's Assay binds to arginine, lysine, and histidine residues. All proteins are assumed to have the same relative amounts of these residues based on their size. If the protein does not have these residues, Bradford's Assay will not detect it, which results in an undervalued protein concentration. Bradford's Assay is also impacted by the presence of any basic buffering agents or strong detergents that could be in the protein powder[3]. Despite these limitations, Bradford's Assay is still a cost-effective and fast protein quantification method that gives relatively accurate data.

Table 1: Data used to create 15 mL of a 5 mg/mL stock solution of each Protein Powder.

Protein Powder	Brand	Serving Size	Grams/Serving	Ratio	Powder (mg) for 75 mg Protein
1	ProCareHealth	30	26	1.15	86.5
2	MUSCLE FX	35	26	1.35	101.0
3	Ultimate Nutrition	34	20	1.70	127.5
4	Nutrisite Restore	29	20	1.45	108.8
5	Natreve MOOLESS	28	20	1.40	105.0
6	Vitron	40	23	1.74	130.5
7	BULKSUPPLEMENTS	30	23	1.30	97.8
8	Animal Clear Whey	25	20	1.25	93.8
9	Icon Muscle	35	25	1.40	105.0
10	Myprotein	24.7	20	1.24	92.6

Table 2: Data used to create a Calibration curve for BSA using the Bradford assay.

Concentration of BSA (ug/ml)	100	150	200	250	300	350	400	450	500	550	600	650
Absorbance	0.121	0.307	0.410	0.496	0.564	0.645	0.713	0.796	0.874	0.942	1.015	1.100

RP-HPLC calibration curve

The retention time of four major proteins in whey was used to determine the amounts of each protein in 10 whey protein samples. These proteins eluted in a consistent order: α-LA eluted

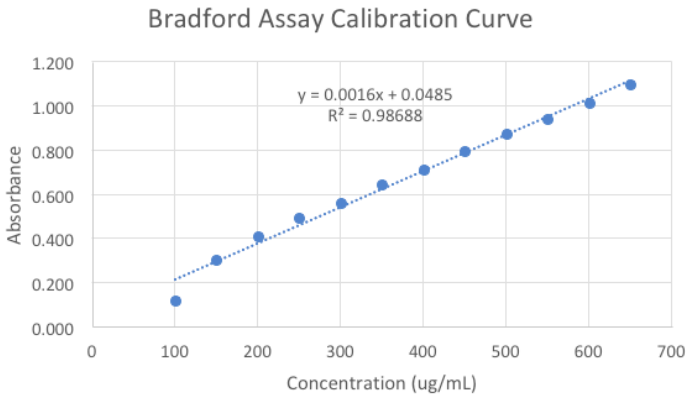

Figure 1: Calibration curve for BSA using the Bradford assay.

Table 3: Data showing the absorbance of each protein powder along with the calculated concentration

Protein Powder	Brand	Protein per serving (g)	Serving Size (g)	Ratio	Expected Amount of Protein in 600 ug of powder (ug)	Absorbance			Average absorbance	Protein detected (ug) from 600 ug of powder
1	ProCareHealth	26	30	0.87	520.0	0.360	0.390	0.335	0.362	195.9
2	MUSCLE FX	26	35	0.74	445.7	0.292	0.287	0.284	0.288	149.7
3	Ultimate Nutrition	20	34	0.59	352.9	0.311	0.316	0.314	0.314	165.9
4	Nutrisite Restore	20	29	0.69	413.8	0.257	0.258	0.260	0.258	130.9
5	Natreve MOOLESS	20	28	0.71	428.6	0.252	0.237	0.236	0.242	120.9
6	Vitron	23	40	0.58	345.0	0.085	0.085	0.091	0.087	24.1
7	BULKSUPPLEMENTS	23	30	0.77	460.0	0.349	0.347	0.349	0.348	187.2
8	Animal Clear Whey	20	25	0.8	480.0	0.320	0.326	0.319	0.322	170.9
9	Icon Muscle	25	35	0.71	428.6	0.271	0.284	0.284	0.280	144.7
10	Myprotein	20	24.7	0.81	485.8	0.199	0.195	0.175	0.190	88.4

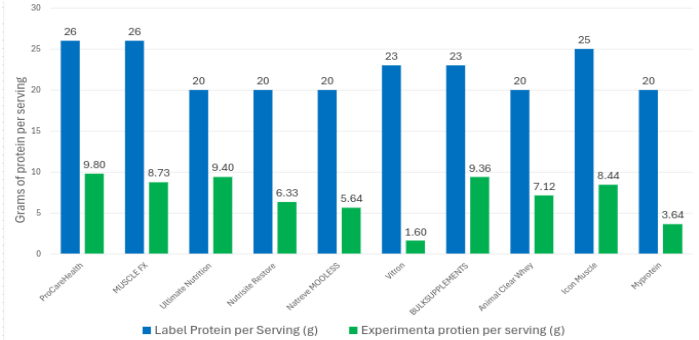

Figure 2: Chart comparing the expected and observed grams of protein per serving using the Bradfords Assay.

first, followed by BSA, β-LG_B, and β-LG_A (**Figure 3**). Calibration curves were constructed from chromatography data using standard protein solutions analyzed in triplicate (**Tables 4-7)**. Among the four protein standards, BSA contained the highest R^2 value of 0.9989 (**Figure 4**). However, BSA could not be detected at concentrations equal to and lower than 0.25 mg/ mL. α-LA contained an R^2 value of 0.9971 (**Figure 5**). β-LG_B contained an R^2 value of 0.997 (**Figure 6**). β-LG_A contained the lowest R^2 value of 0.9743 (**Figure 7**). All of the standards did have an R^2 higher than .97, which indicates that they all had a good linear fit.

RP-HPLC results

All of the protein powders except MUSCLE FX, Ultimate Nutrition, Natreve MOOLESS, and Icon Muscle were able to dissolve completely. The four mentioned powders were chocolate-flavored, and all left undissolved brown powder at the bottom of the test tube. After decreasing the concentration of the four powders, they still had problems dissolving completely. In addition, vortexing them for longer proved unsuccessful. The assumption was then made that the undissolved brown powder collected at the bottom of the test tube was the chocolate flavoring and that all of the protein was fully dissolved in the solution as the proteins we were testing were all soluble in the solvent system we were using.

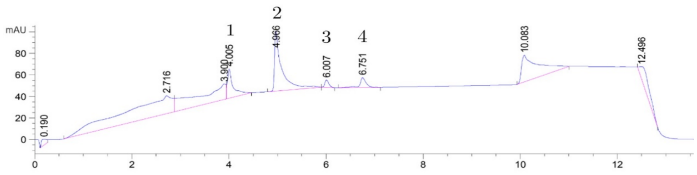

Figure 3: Displays relative retention times of the whey protein fractions: α-LA (1), BSA (2), β-LG_B (3), and β-LG_A (4).

Table 4: Data for calibration curve of BSA for RP-HPLC.

The average peak area of BSA		
Concentration (mg/mL)	Area	Standard Deviation
0.25	n/a	n/a
0.50	201	16.3
0.75	351	36.7
1.00	509	28.6
1.25	662	34.8
1.50	837	24.7
1.75	977	28.1
2.00	1169	55.6

Table 5: Data for calibration curve of α-LA obtained from RP-HPLC

The average peak area of alpha-LA		
Concentration (mg/mL)	Area	Standard Deviation
0.25	362	12.6
0.50	542	10.1
0.75	699	10.1
1.00	898	1.6
1.25	1108	13.3
1.50	2036	520.8
1.75	2328	32.1
2.00	2523	7.1

Table 6: Data for calibration curve of β-LG_B obtained from RP-HPLC

The average peak area of Beta-LG-B		
Concentration (mg/mL)	Area	Standard Deviation
0.30	152	24.4
0.40	205	11.3
0.50	263	20.6
0.60	294	1.8
0.70	341	8.6
0.80	390	0.28
0.90	438	3.6
1.00	496	3.1

Table 7: Data for calibration curve of β-LG_A obtained from RP-HPLC

The average peak area of Beta-LG-A		
Concentration (mg/mL)	Area	Standard Deviation
0.30	204	35.8
0.40	254	35.8
0.50	268	32.9
0.70	362	28.6
0.80	457	58.8
0.90	513	62.8
1.00	584	63.8

Table 8 shows the area and standard deviations of α-LA, β-LG_A, β-LG_B, and BSA in the ten protein powders. BSA was not detected in ProCareHealth, MUSCLE FX, Ultimate Nutrition, Nutrisite Restore, Vitron, BULKSUPPLIMENTS, Animal Clear Whey or Icon Muscle. Some possible explanations for these results are that the concentration of BSA present was too small to be quantified for each powder or there was no BSA present in the powders. Natreve MOOLESS and Myprotein had detectable amounts of BSA (**Table 8**). In **Table 9,** the calculated value of the powders that detected BSA was negative. This could indicate that the amount of BSA in all of the protein powders is less than our limit of detection (less than 0.25mg/mL), which makes it an unreliable detection. After

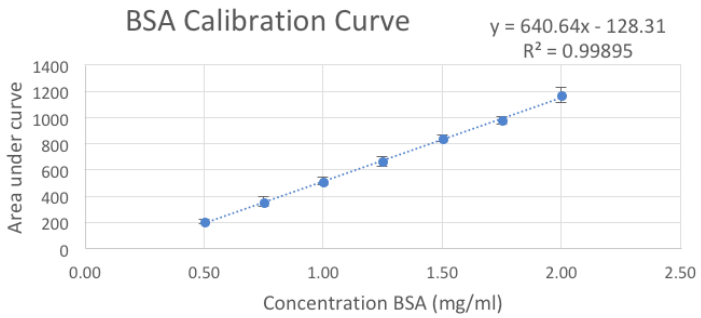

Figure 4: Calibration curve of BSA obtained from RP-HPLC

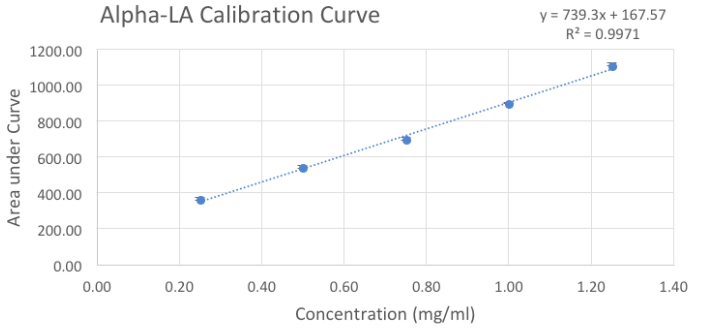

Figure 5: Calibration curve of α-LA obtained from RP-HPLC

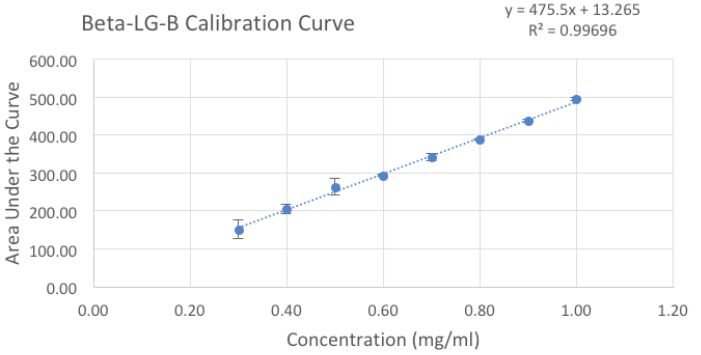

Figure 6: Calibration curve of β-LG_B obtained from RP-HPLC

calculations, Ultimate Nutrition, Vitron, and Icon Muscle also had negative α-LA values (**Table 9**). This could indicate that there is a small amount of α-LA in those powders that are near our limit of detection. The α-LA was not detected in Natreve MOOLESS, implying that it is absent from the powder or present in quantities lower than the limited detection. The most abundant proteins in the powders were β-LG_A and β-LG_B, which were detected in every sample except Natreve Mooless and Myprotein and had higher calculated concentrations.

ProCareHealth, Muscle FX, and Nutrisite Restore powders had total concentrations of proteins close to or greater than 1 mg/ml, meaning they had more protein than the rest of the protein powders. All these protein powders are made from whey protein isolates, making them purer for the four proteins analyzed than whey protein concentrates. Ultimate Nutrition and Vitron had the lowest total detected protein at 0.05 mg/mL, potentially indicating they were not good protein sources for the four analyzed proteins. My Protein and Natreve Mooless had a total detected concentration of 0.00 mg/ml; these proteins had labels showing that they were animal-free whey protein powders. Further research needs to be done to understand the composition of these animal-free whey protein powders.

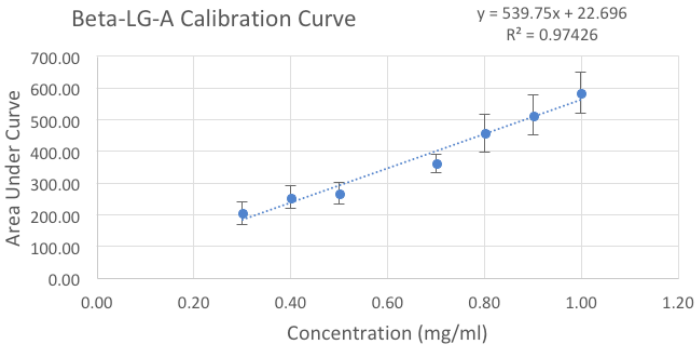

Figure 7: Calibration curve of β-LG_A obtained from RP-HPLC

Table 8: Table displaying the statistics (mean and standard deviation) of the peaks for ten protein powders

	Alpha-LA		BSA		Beta-LG-B		Beta-LG-A	
Proteins Powders	Area	Standard deviation	Area	Standard deviation	Area	Standard deviation	Area	Standard deviation
ProCareHealth	329	35.7	-	-	168	23.1	334	24.5
MUSCLE FX	393	1.9	-	-	189	7.7	385	34.6
Ultimate Nutrition	54	0.6	-	-	22	17.6	39	1.7
Nutrisite Restore	235	28.6	-	-	149	6.0	293	7.8
Natreve MOOLESS	-	-	65	5.5	-	-	-	-
Vitron	49	1.9	-	-	21	0.5	38	4.0
BULKSUPPLEMENTS	184	2.2	-	-	59	8.3	111	29.0
Animal Clear Whey	482	2.0	-	-	69	14.1	-	-
Icon Muscle	125	32.3	-	-	51	1.3	96	12.4
Myprotein	121	4.4	114	8.4	-	-	-	-

Table 9: The table displaying the amount of each protein in 10 protein powders. Negative values were included on the table to indicate where the calculation from our calibration curve equation gave a negative value. However, the negative values were not used in our total calculations

Average Protein Detected (mg/ml)					
Protein	Alpha-LA	BSA	Beta-LG-B	Beta-LG-A	Total
ProCareHealth	0.22	-	0.41	0.58	1.21
MUSCLE FX	0.31	-	0.47	0.67	1.44
Ultimate Nutrition	-0.15	-	0.02	0.03	0.05
Nutrisite Restore	0.09	-	0.36	0.50	0.95
Natreve MOOLESS	-	-0.10	-	-	0.00
Vitron	-0.16	-	0.02	0.03	0.05
BULKSUPPLEMENTS	0.02	-	0.12	0.16	0.31
Animal Clear Whey	0.43	-	0.15	-0.04	0.57
Icon Muscle	-0.06	-	0.10	0.14	0.24
Myprotein	-0.06	-0.02	-	-	0.00

Comparing the Bradford and RP-HPLC results

On the whole, the Bradford assay detected more proteins than the RP-HPLC. This is expected as the Bradford assay analyzes the total protein concentration while our RP-HPLC method only analyzes the four major proteins expected to be present in whey proteins. Our protein powders fall into three categories: Whey Protein Concentrate, Whey Protein Isolate, and Animal Free Whey Proteins **(Table 10)**.

The Whey Protein Concentrate had greater variability with the Bradford assay, ranging from 7% to 47% in terms of the proteins detected versus the amount of protein claimed on the label. With the RP-HPLC method, the whey protein concentrates consistently had low amounts of the four proteins analyzed, indicating that they included other proteins that were not measured. The Animal Free Whey Proteins had low amounts of proteins with the Bradford Assay with My Protein (18%) and Natreve MOOLESS (28%) and did not have any or low amount (less than the limit of detection) of the four proteins analyzed with RP-HPLC. All the whey protein isolates detected proteins within a close range (33% to 38%) using the Bradford. With the exception of ICON Muscle protein powder, all the whey protein isolates had more than 10% of the protein they claimed to contain on the label with the RP-HPLC method.

Conclusion

The data from this study show that most protein powders may not be as good of a protein source as they claim. The amount of protein detected in the powders varied considerably. ProCareHealth and Muscle FX were the best protein sources out of the ten powders tested. Arguably, these protein powders may contain other proteins besides the four proteins we tested, as indicated by the results of the Bradford Assay. As such, some future work includes using Gel Electrophoresis to determine how many different proteins are present in each powder and estimating their relative sizes to determine their identity. This can help identify other proteins that manufacturers may add to inflate the amount of whey protein.

Table 10: The table compares the amount of protein detected in 10 protein powders by the Bradford and HPLC methods, respectively. The values are lower in the HPLC method than in the Bradford, indicating that other proteins are present in these protein powders besides the four analyzed with HPLC.

Protein Powder	Brand		Chocolate Flavor	Bradford (% difference)	HPLC (% difference)
1	ProCareHealth	Whey Protein Isolate		37.67	24.20
2	MUSCLE FX	Whey Protein Isolate	Yes	33.59	28.80
3	Ultimate Nutrition	Whey Protein Concentrate	Yes	47.01	1.00
4	Nutrisite Restore	Whey Protein Isolate		31.63	19.00
5	Natreve MOOLESS	Animal Free Whey Protein	Yes	28.21	0.00
6	Vitron Nutripure	Whey Protein Concentrate		6.99	1.00
7	BULKSUPPLEMENTS	Whey Protein Concentrate		40.70	6.20
8	Animal Clear Whey	Whey Protein Isolate		35.60	11.40
9	Icon Muscle	Whey Protein Isolate	Yes	33.76	4.80
10	Myprotein	Non-Animal Whey Protein		18.20	0.00

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