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UNDERSTANDING THE PROTEIN CONTENT IN TEN COMMERCIALLY AVAILABLE PROTEIN POWDERS

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Abstract

Previously, ten commercially available protein powders were found to contain only 36% of the protein that was claimed on their nutrition labels. The process used the Bradford Assay to determine protein content and High-Performance Liquid Chromatography with UV-VIS at 214 nm for detection (HPLC-UV). HPLC tested four proteins (α-Lactalbumin (α-LA), β-Lactoglobulin A (β-LGA), β-Lactoglobulin B (β-LGB), and Bovine serum albumin (BSA)) that have been shown to be present in protein powders. To further understand the discrepancy between the calculated protein content and the expected, three protein methods: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), UV-VIS, and HPLC-UV at 280nm wavelength were used to further explore if other proteins were present. No new proteins were found in significant amounts, but we were able to ascertain that 280 nm is a better wavelength for the detection of proteins with HPLC-UV detection.

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Introduction

Many people use protein powders as a source of extra protein in their diet. This is due to the increasing trend of healthy living in society. Athletes, for example, use protein to increase body mass, muscular strength, and size[1]. Protein is currently the most valuable component of whey. The major proteins that contribute to the protein fraction of the whey stream are α-Lactalbumin (α-LA), β-Lactoglobulin (β-LG), serum albumin, immunoglobulins, and protease peptone [2,3]. About 80% of the total whey protein is composed of β-LG and α-LA [2]. However, the FDA does not regulate protein powders because it is classified as special foods instead of a drug[4]. Due to this lack of oversight, 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 [5]. In our previous study, we found out that ten commercially available protein powders tested had less than 36% of the protein that was promoted on the nutrition label [5]. The study used High Performance Liquid Chromatography (HPLC) and Bradford Assay to come to this conclusion. The Bradford Assay analyzed total protein content, while our HPLC process analyzed four proteins that have been tested to be very common in protein powders. With the protein content so low, we decided to follow up on this work to understand if there are any other proteins present aside from the four proteins that we tested. We deemed it important to use different techniques to understand the protein composition and content of the protein powders.

We had speculated in our original paper that Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of our protein content would be essential to visualizing if there are any other proteins present aside from the four proteins we had chosen to analyze with HPLC using UV detection at a wavelength of 214 nm[5]. High-Performance Liquid Chromatography (HPLC) is a technique that separates proteins based on their chemical properties. In protein powder analysis, reversed-phase HPLC was used to separate individual proteins in a mixture based on their retention times. This method allowed us to identify and quantify specific proteins in the powder. However, with UV detection, there were no guarantees that the proteins were actually the four that we had chosen to analyze. We had tried to mitigate that by using the commercially available proteins as our standards and ensuring that the retention times were close to what we obtained for the standards. Further analysis using SDS-PAGE added another layer of guarantee. SDS-PAGE uses sodium dodecyl sulfate and beta-mercaptoethanol to break the protein-protein disulfide bonds, linearize the proteins, and add negative charges from their native folded state. From there, the linear protein runs through a gel matrix, which uses only the protein's molecular weight to determine the migration distance. The heavier the protein, the slower it moves and stays closer to the top of the gel, and vice versa. Using these migration distances and a set of known protein molecular weights, we can visualize the number of proteins present and estimate the molecular weight of the unknown proteins if there were any[6,7,8].

Experimental Methods

The ten protein powders were ordered from Amazon. Bradford reagent was obtained from Bio-Rad. The spectrophotometers used were Genesys 30 from ThermoScientific and Agilent G1103B Cary 8454 UV-Vis Spectrophotometer. We obtained α-Lactalbumin(α-LA), β-Lactoglobulin A (β-LGA), β-Lactoglobulin B (β-LGB), and 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 Agilent Technologies 1260 Infinity. The materials from SDS-PAGE were from Bio-Rad. This included the Mini-Protean Tetra Cell System and Comparative Proteomics I kit, which contained Kaleidoscope™ Prestained Protein Standards, 10x Tris-Glycine-SDS electrophoresis buffer (TGS buffer), Laemmli sample buffer, 4–20% Mini-PROTEAN® TGX™ Precast Gel, and Bio-SafeTM Coomassie stain

SDS-PAGE standard and sample preparation

10 ml samples each of 20mg/mL stock solution of protein were made using the protein amounts listed on the nutrition labels

on each protein powder to determine what amount of the powder needed to be weighed (**Table 1**). The 20 mg/mL stock solutions were vortexed to self-homogenize, then centrifuged until pellet formation occurred. Using dilution, 2 mg/mL solutions for each protein powder were created and then pipetted into separate microfuge tubes. 2mg/mL protein standard solutions were 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. This became our stock solution for the sample buffer, which was used for our sample preparation. The 2x concentration of Laemmli buffer stock solution provided in the kit was diluted to 1x for use in the assay with the 2mg/mL protein powder sample and standard solutions. It is important to note that this procedure was done under a fume hood due to the scent of 2-mercaptoethanol. Anything that touched the 2-mercaptoethanol was placed in a water and bleach solution for at least 30 minutes to remove the smell.

Dyeing Protein Powder Samples with Sample Laemmli Dye Buffer Solution

10 µL of standard and sample protein powder stock solutions were individually pipetted into PCR tubes. For each PCR tube, 10 µL of Laemmli dye buffer was added. The PCR tubes were heated in the thermocycler at 95 °C for 5 minutes.

Running SDS-PAGE Procedure

The Bio-Rad Mini-Protean Tetra System kit provided a 10x buffer solution, which was used to create a 1X Tris/Glycine/SDS Buffer by mixing 100 mL of 10x buffer solution with 900 mL of DI water. 700 mL of the 1X Tris/Glycine/SDS Buffer was needed for running 2 gels. 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 Bio-Rad Mini-Protean Tetra System box with a compatible Bio-Rad power supply box was set up mainly based on the procedure outlined in the Bio-Rad instruction manual. The proper orientation of the power supply box was indicated by the red marking on the right and the black marking on the left. The comb and green tape at the bottom of the Ready Gel Cassette were removed. To run 1-2 gels, the mini buffer dam and only the Banana plugs electrode assembly should be used. It is important to note that the companion running module should not be used. Set and clamp gels in the back of the power supply box. The gels ran at 200 volts for 35 minutes. Then, the gels were removed from the gel cassette by gently separating and breaking the arrows. To remove the gel from the plate, invert the plate over a water bath so that the gel can float.

Table 1. Amount of protein powder weighed to create a 20 mg/mL protein solution

Commercially Available Protein Powder	Amount weighed
Powder 1 - Pro	232 mg
Powder 2 - Muscle	270.3 mg
Powder 3 - Ultimate	339.5 mg
Powder 4 - Nutrisite	290.4 mg
Powder 5 - NATREVE	280.9 mg
Powder 6 – Nutripure/Vitron	348.0 mg
Powder 7 - BULK	260. 8 mg
Powder 8 - Animal	252.0 mg
Powder 9 - ICON	280.7 mg
Powder 10 - MYPROTEIN	247.3 mg

Coomassie Staining and Imaging Procedure

The gels were washed 3 times for 5 minutes each in 200 mL of DI water; this was done to remove SDS so that it does not interfere with the staining. All the water was poured out from the staining container, and 50 mL Coomassie stain was added until it was enough to cover the gel. The staining container was taped to an orbital shaker and gently shaken for an hour and a half. The Coomassie stain was poured out, and the gel was rinsed in 200 mL of DI water. Enough DI water was added to cover the gels, and the staining container was re-taped to the orbital shaker. 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. First, the full length of each of the 6 SDS-PAGE images was measured. Then, for each standard and sample protein, measuring was done until the bottom of each of their bands. The distance obtained by taking these measurements was used to find the Rf values and average triplicate values of the Rf values in Excel. A scatter plot against the log of molecular weight of the ladder was created to obtain a calibration curve. The calibration curve was used to find the molecular weight of each of the standard and sample proteins.

UV-Vis standard and sample preparation

5 mg of BSA, Lactalbumin, beta-lactoglobulin A, and beta-lactoglobulin B were weighed and then separately dissolved in 5 mL of deionized water. The 20 mg/mL stock solution made in the SDS-PAGE standard and sample preparation step was diluted using deionized water to reach a concentration of 1 mg/mL.

BSA Calibration Curve Creation

A 5mg/mL BSA stock solution was prepared by measuring 20 mg of BSA and mixing it with 4 mL of DI water. Then, this solution was used to create dilutions of 5.00 mg/mL, 2.50 mg/mL, 1.250 mg/mL, 0.625 mg/mL, 0.313 mg/mL, 0.156 mg/mL, 0.078 mg/mL, and 0.039 mg/mL in addition to 0 mg/mL. UV-Vis was run for each of these dilutions at 280 nm. The absorbance of each dilution was used to create a calibration curve of absorbance versus concentration using Excel.

UV-Vis Method and Analysis

Deionized water was used as a blank. All samples and blanks were measured using the Agilent spectrophotometer between the range 250 nm and 400 nm[9]. Each sample was measured in tripli-

cate from different aliquots of the stock solution. The triplicate measurements of protein were averaged to determine the absence and presence of proteins. An attempt was made to use the BSA calibration curve equation to determine the concentration of protein in each protein powder.

RP-HPLC Method

Two solutions, A and B, were combined to perform the gradient elution. 0.1% TFA and 5.0% ACN in water were present in solution A, whereas 0.1% TFA in ACN was present in solution B. 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 wavelength for detection was 280 nm.

Results and Discussion

The SDS-PAGE gels were run in triplicate, and proteins were visualized using Coomassie Blue dye (**Figure 1**).

The images were analyzed using Image J to obtain the distance of migration (Rf), and the average of the Rf values was calculated. The Rf values of the standards were used to make a calibration curve (Figure 2), which was used to calculate the molecular weight of the proteins present in the gels.

The molecular weights obtained from the calculation were compared with literature values. The approximate molecular weight for BSA is 66 kDa[1], for α-Lactalbumin is 14 kDa[4], and for β-Lactoglobulin (A and B) is 18 kDa[2]. Based on our calculation

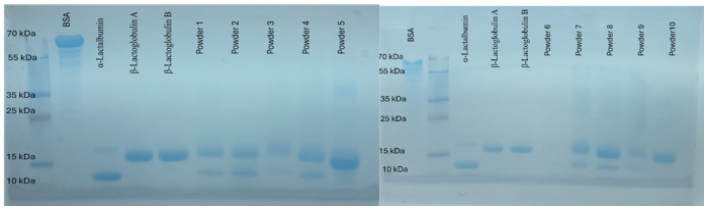

Figure 1.SDS-PAGE with powders 1-10 compared to the standard protein powders

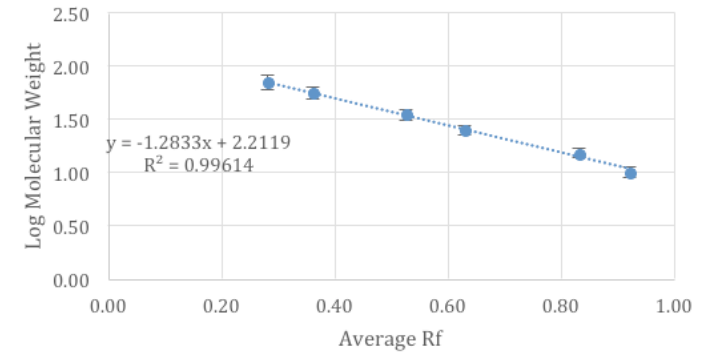

Figure 2. A graph of log molecular weight against Rf value of the proteins present on the protein ladder.

from the data from SDS-PAGE, we had about 63 kDa for BSA, 12 kDa for α-LA, and 15 kDa for β-LGA and β-LGB. As these values were consistently within 2-3 kDa of the actual, we understood that these differences were introduced by our experimental run, as we had taken steps to ensure the accuracy of our data. These measures included running pure protein samples alongside the protein powders in each run. The bands from our protein powders aligned with the pure protein standard.

The data that was obtained from the SDS-PAGE have been summarized in **Table 2**. Assessment of the data indicated that none of the powders contained BSA, or the amount of BSA present was too small to be visualized using SDS PAGE. The migration distances for beta-lactoglobulin A and B were the same, so we could not distinguish between the two using SDS-PAGE. All protein powders contained β-Lactoglobulin (β-LG), except for Powder 6 (Nutripure, which used to be Vitron), which had a very faint protein line in one trial. α-Lactalbumin (α-LA) was also present in all powders except for Powders 5, 6, and 10.

This aligns with data we had obtained with HPLC-UV (214 nm) in that none of the protein powders contained BSA. Because of the strictness of retention time, to ensure the integrity of our data, we had originally indicated that Powder 5 (Natreve Mooless) and Powder 10 (Myprotein) did not contain any β-Lactoglobulin (β-LG)[5]. These animal-free protein powders, which had shown different retention times in HPLC for LGA and LGA, were shown to have the same Rf value with SDS-PAGE, indicating that the proteins had similar molecular weights but interacted with the HPLC column differently.

The biggest surprise for us, however, was the fact that we did not detect any other protein in significant amounts aside from some of the four protein standards that we had originally tested. Some protein powders showed faint bands in some of our runs, but there was not enough consistency across multiple runs for us to have conclusive data that there were other proteins present. One significant data point, which had been consistent irrespective of

Table 2. Table showing the protein content of 10 commercially available protein powders. When a protein was absent, it was marked as an X, and when present, it was marked as a Y. There were instances of inconclusive data (Inconclusive indicated that 2 out of the 3 runs per protein did not yield any data)

Powder Number	Powder Name	BSA (63 kDa)	α-LA (12 kDa)	β-LG (15 kDa)
1	ProCareHealth	X	Y	Y
2	MuscleFX	X	Y	Y
3	Ultimate Nutrition	X	Y	Y
4	Nutrisite Restore	X	Y	Y
5	MOOLESS	X	X	Y
6	Nutripure	X	*I	*I
7	BULKSUPPLEMENTS	X	Y	Y
8	Animal Clear Whey	X	Y	Y
9	Icon Muscle	X	Y	Y
10	Myprotein	X	X	Y

the protein analysis method used, was that Nutripure protein had either no protein or the amount of protein present was too low to be accurately quantified by any of our protein methods.

With this information from SDS-PAGE, we revisited our data from HPLC-UV (214 nm) analysis (**Table 3**). All the values obtained for Natreve Mooless, Myprotein, and Animal Clear Whey far overshadow the amount of protein that we are expecting.

Using UV spectrophotometry at 280 nm wavelength, the absorbance of the protein powders and the standards was measured at this wavelength in triplicate. UV-VIS spectrophotometry measures the absorbance at 280 nm [10], as an indication of protein concentration. This works best if the protein samples are purified. In our case, however, our proteins were not purified (**Figure 3**).

We attempted to see whether we could quantify this data in any meaningful way. We created a calibration curve (**Figure 4**) of BSA at 280 nm. The calibration curve gave us a good R^2 value of 0.991. We used the equation of the line to attempt to calculate the total protein ocncentrations of our protein powders.

Although we tried to use BSA as a measure of total protein content, we quickly found that aside from indicating that proteins were absent, it was not useful in quantifying total protein content (**Table 4**). Our values were consistently higher than 1mg/mL, which was greater than 100% the proteins that we had dissolved in solution. For example, Bulk protein had a 4.3 mg/mL concentration, Nutrisite had a 1.6 mg/mL concentration, and the protein standards (LGA and LGB) had concentration values of 1.5 mg/mL and 1.4 mg/mL, respectively.

Table 3. Reanalysis of the data from HPLC-UV (214 nm), accounting for the presence of Lactoglobulin

	Protien detected (mg/ml)				
Protien	Alpha-LA	BSA	Beta-LG-B	Beta-LG-A	Total detected
ProCareHealth	0.2176	0.0000	0.4120	0.5764	1.2061
MUSCLE FX	0.3053	0.0000	0.4673	0.6720	1.4447
Ultimate Nutrition	-0.1536	0.0000	0.0237	0.0303	0.0540
Nutrisite Restore	0.0912	0.0000	0.3607	0.5011	0.9530
Natreve MOOLESS	0.0000	-0.4976	1.5598	1.1877	2.7475
Vitron	-0.1606	0.0000	0.0198	0.0277	0.0476
BULKSUPPLEMENTS	0.0225	0.0000	0.1212	0.1636	0.3074
Animal Clear Whey	0.4257	0.0000	2.0170	2.7807	5.2235
Icon Muscle	-0.0582	0.0000	0.1006	0.1359	0.2365
Myprotein	0.0000	-0.0228	4.6319	2.9379	7.5698

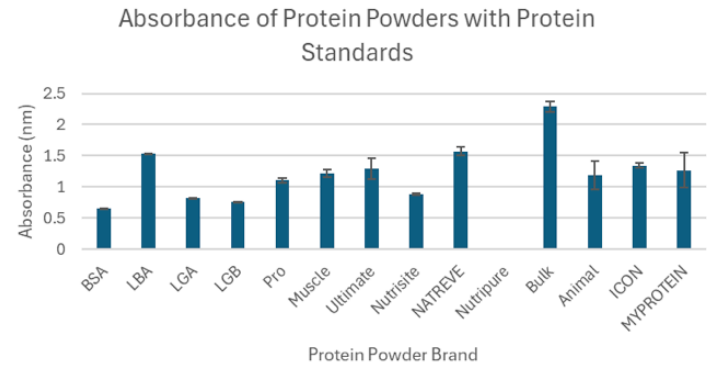

Figure 3.Using UV-Vis Spectrophotometry to measure absorbance at 280 nm.

We therefore went back to analyzing the 4 protein standards with HPLC-UV (280 nm). HPLC can separate the proteins for analysis, making them easier to quantify. Analysis of the protein powders at 280 nm using HPLC gave the following data (**Table 5**):

Pairing the HPLC-UV (280 nm) data with the data obtained using SDS-PAGE, we eliminated all numbers that were not associated with visualized proteins and recalculated the protein content (**Table 6**). This step was necessary because we understood that multiple compounds could have the same retention time on HPLC.

We then calculated the amount of protein that was obtained as a percentage of the amount of protein we were expecting in solution. For each of our protein powders, we were expecting 5 mg of protein to be present in solution. The data (**Figure 5**) indicates that Myprotein and Animal Clear Whey had about 50% of the expected proteins. The Icon Muscle protein powder had the least amount of protein.

Conclusions

This study confirms the findings of the previous research conducted, which is that the commercially available protein powders

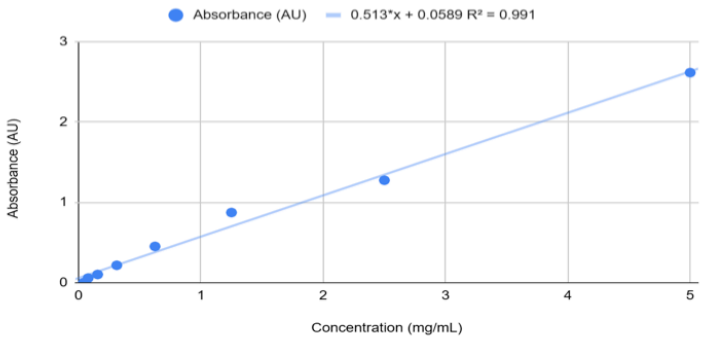

Figure 4. Calibration Curve of BSA obtained using UV-spectrophotometry at 280 nm

Table 4. Using a BSA calibration curve to calculate the concentration of each powder

Sample	Concentration (mg/mL)	Standard Deviation
BSA	**1.158**	0
LBA	**2.881**	0
LGA	**1.475**	0.003
LGB	**1.362**	0
Pro	2.033	0.035
Muscle	2.26	0.065
Ultimate	2.393	0.165

Sample	Concentration (mg/mL)	Standard Deviation
Nutrisite	1.602	0.011
NATREVE	2.948	0.064
Nutripure	0	
Bulk	4.347	0.089
Animal	2.196	0.224
ICON	2.506	0.032
MYPROTEIN	2.355	0.276

Table 5. Average Peak Area and Concentration of the 4 proteins investigated using HPLC with detector wavelength of 280 nm. Retention times were calculated within +/- 0.1 of the retention times obtained from the pure protein standards.

		LA		BSA		LGA		LGB		PROTEIN CONTENT
Powder Number	Powder at 280 nm	Avg. Area	Concentration	Avg. Area	Concentration	Avg. Area	Concentration	Avg. Area	Concentration	(mg/ml)
1	ProCareHealth	29.50	0.36	-	0.00	-	0.00	25.34	0.82	1.18
2	MuscleFX	40.26	0.47	-	0.00	-	0.00	-	0.00	0.47
3	Ultimate Nutrition	50.41	0.57	-	0.00	-	0.00	-	0.00	0.57
4	Nutrisite Restore	41.16	0.48	-	0.00	-	0.00	23.28	0.78	1.26
5	MOOLESS	-	0.00	18.05	0.98	-	0.00	44.93	1.22	2.21
7	BULKSUPPLEMENTS	41.45	0.48	-	0.00	-	0.00	9.76	0.50	0.98
8	Animal Clear Whey	23.26	0.30	51.46	2.00	-	0.00	99.95	2.36	4.65
9	Icon Muscle	13.57	0.20	-	0.00	-	0.00	-	0.00	0.20
10	Myprotein	-	0.00	94.14	3.29	-	0.00	89.12	2.13	5.43

do not have as high a protein content as they claim to contain. The top two proteins in terms of protein content were Animal Clear Whey at 53% and Myprotein at 43%. We also concluded that when analyzing the HPLC with UV detection, the 280 nm wavelength is a better wavelength for the analysis of protein powders and also works much better when paired with SDS-PAGE. Further studies on other protein products, such as milkshakes, are being conducted to determine if there are any discrepancies between the amount of protein advertised and what they contain.

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Table 6. Protein Content of Protein Powders obtained by analyzing 4 proteins at 280 nm HPLC-UV detection and pairing it with SDS-PAGE detection

Powder Number	Powder	LA	BSA	LGA	LGB	Total Protein (mg/mL)	%
1	ProCare Health	0.36	0.00	0.00	0.82	1.18	23.64
2	Muscle FX	0.47	0.00	0.00	0.00	0.47	9.37
3	Ultimate Nutrition	0.57	0.00	0.00	0.00	0.57	11.41
4	Nutrisite Restore	0.48	0.00	0.00	0.78	1.26	25.13
5	MOOLESS	0.00	0.00	0.00	1.22	1.22	24.49
7	BULKSUPPLEMENTS	0.48	0.00	0.00	0.50	0.98	19.63
8	Animal Clear Whey	0.30	0.00	0.00	2.36	2.65	53.09
9	Icon Muscle	0.20	0.00	0.00	0.00	0.20	4.02
10	MyProtein	0.00	0.00	0.00	2.13	2.13	42.67

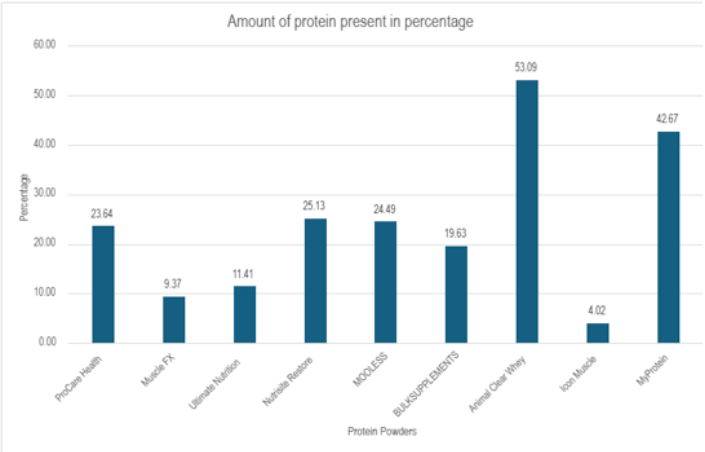

Figure 5. A graph showing the percentage amount of proteins present. This was obtained by taking the amount of protein calculated experimental/5mg and multiplying the value by 100

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