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Initial Investigation on Protein Extracts of Gamma-Irradiated Robusta, Excelsa and Liberica Civet Coffee Beans:Bradford Assay and SDS-PAGE

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Abstract: The three known civet coffee varieties are Robusta, Excelsa and Liberica.Proteins from the coffee beans were extracted via maceration and were subjected to two different analyses namely Bradford Assay and SDS-PAGE. Protein concentrations were obtained from Bradford Assay and Rf values from SDS-PAGE. A significant decrease in protein concentration was observed after irradiation. Concentrations of the resolving gels were varied to 12%,15% and 17.5% to fully optimize gel runs. The concentration that resulted to more resolved bands was 17.5%. All proteins had low Rf values thus having high molecular weights.

Key Words: SDS-PAGE; Gamma-irradiation; Civet Coffee; Bradford assay

1. INTRODUCTION

One popular type of coffee that is known in the Philippines despite its high cost is Civet Coffee. This type of coffee comes from the Philippine civet cat which are fed with selected coffee beans and processed in their stomach, which alters its chemical composition prior to excretion. Since this is one of the types of coffee that is naturally processed, analysis of the proteins it contains became a subject or topic of interest. Gamma irradiation of natural products such as rice and fruits has been studied and is commercially applied. Researchers have found out that subjecting a natural product or processed foods to gamma irradiation has improved the storage life of these products thus became an effective preservation method for some businesses. The strong energy of gamma rays eliminates microorganisms that cause food spoilage. Several studies of coffee being irradiated with gamma rays have been conducted focusing on how it will affect the taste and aroma of the coffee. However, the effects of gamma irradiation on its protein content have not been further studied and there are only limited publications about it.

The research aimed to study and differentiate the effect of gamma irradiation to the proteins of the coffee beans particularly Kape Alamid with different varieties of coffee beans using Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE). This paper is part of an ongoing study on the Philippine civet coffee. Previous studies have researched on the different protein profiles of Civet and non-Civet coffee and found that there was only a slight difference of its protein composition. Through this study, the difference of the protein profiles of irradiated civet coffee bean varieties were identified by comparing the Rf values of its protein bands from the SDS-PAGE analysis. Also, the effect of irradiation was analyzed by comparing irradiated coffee beans with non-irradiated beans.

The samples were from a single batch of harvest obtained from a coffee plantation at Alfonso, Cavite. Irradiation of coffee beans was done at PNRI for 33.6 hours of irradiation with 1 kGy gamma rays of dose rate of 29.77 Grays per hour. Although Robusta and Excelsa types are the most abundant coffee variety in the area, Liberica samples were also obtained. Rf values were analyzed with multiple trials. Non-Irradiated civet beans were done with only one trial due to contamination of sample.

2. METHODOLOGY

Irradiated and non-irradiated of the raw civet coffee samples were tested for their protein composition using two methods: Bradford Protein

Assay and SDS-PAGE (Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis).

2.1 Sample Preparation

One kilogram of the raw civet coffee beans obtained from the supplier in Alfonso, Cavite was harvested in March 2013. It was stored in polyethylene in room temperature. The beans were thoroughly cleaned using tap water. After cleaning, it was sundried for 3 hours and oven dried at 40 degrees Celsius for 3 hours. Since the sample was a mixture of Excelsa, Robusta and Liberica, these were sorted. Sorted samples were stored in a tightly sealed container which was then wrapped with Parafilm®. Beans that were difficult to identify were discarded. After cleaning, half of the samples were sent to PNRI (Philippine Nuclear Research Institute) Quezon City for gamma irradiation. For each subsequent analysis, approximately 1 gram of the sample was used.

A measured amount of 5g of irradiated coffee beans from each coffee variety and Kape Barako beans were macerated separately using a mortar and pestle. The solvent that was to be mixed with the macerated beans was prepared by mixing 10 mL of 50 mM EDTA, 10 mL10x phosphate buffer, 5 mg PVPP, and 80 mL deionized water. From the prepared solvent, 10 mL was transferred to each mortar and pestle during maceration. One mL of each extract was then obtained and filtered using a filter paper. These were then added to separate microcentrifuge tubes. The samples were centrifuged at 13, 200 rpm for 3 mins. After this, samples were stored for 1 day at 4°C.

2.2 Preparation of Standard Curve

In the preparation of the standard curve, varying amounts of Chicken egg white albumin standards were mixed with certain amounts of Bradford's reagent and 10X Phosphate buffer saline in test tubes.

The mixtures were vortex mixed for 5 seconds and were left to stand for 10 minutes. These were then transferred to separate polystyrene cuvettes and was analyzed using Hitachi U-2900 Spectrophotometer at 595 nm. A standard calibration curve was constructed from the absorbance and concentration data of the standards using Microsoft Excel 2011.

The cooled extracts that were obtained from maceration were thawed and then vortexed for 5 seconds. Volumes of each extract were transferred to individual test tubes and then mixed with Bradford's reagent and PBS. Each mixture was vortexed and was left to stand for 10 minutes and were analyzed using Hitachi U-2900 Spectrophotometer at 595 nm. The concentrations of the individual samples were calculated using the equation of the best-fit line of the plotted standard calibration curve.

2.3 SDS-PAGE Analysis

Protein samples were steamed for 10 minutes in boiling water in order to ensure complete denaturation. In order to prevent errors from time exposure, preparation of the castings of the gel was first performed. A gel cassette was assembled by placing the glass plates against each other with the short plate on the inner side and was locked tightly on a casting frame. This was tightened into position on top of a silicon pad which was placed on the base of the casting stand.

The samples each composed of 6.0 µL of protein sample and 1.0 µL of bromophenol blue dye (4.00 mL glycerol, 2.00 mL 2-mercaptoethanol, 1.2 g SDS, 5.00 mL of 4x stacking tris, 0.03 g bromophenol blue) were then loaded to each well. This gel cassette sandwich was then unlocked from the casting stand and transferred to the tank with the lid placed on the same side as the corresponding plug jacks. The tank buffer (4x tris-glycine tank buffer: 36.00 g Trizma® base and 172.8 g glycine dissolved with distilled water to a total volume of 3.000 L) (1x trisglycine tank buffer: 750.00 mL 4x tris-glycine tank buffer and 30.00 mL of 10% SDS diluted with distilled water to 3.000 L) was poured in the gel cassette sandwich so that the entire glass plates were covered with the tank buffer. The black electrode was attached to negative plug jack and red electrode to positive plug jack. The power supply (external DC voltage power) was turned on and was initially set to 200 V until the samples reached the boundary of the two gels. The power was then shifted to 180 V to ensure that the samples cease to travel further down the lanes.

The power was turned off, the electrodes were carefully detached from the plug jacks and the gel cassette assembly was removed. The glass plates were removed by unsnapping the handles. The glass plates were then laid down and were set apart by the use of two spatulas. The gel was then transferred to a microwavable container and completely submerged in a fixing agent (500.00 mL methanol, 100.00 mL glacial acetic acid, 400.00 mL distilled water). After an hour of submersion, the fixing agent was poured off and was replaced by a staining agent (100.00 mL glacial acetic acid in 450.00 mL distilled water mixed with 3.00 g Coomassie® brilliant blue R250 dye dissolved in 450.00 mL methanol) for another hour. After this, the staining agent was poured off and then

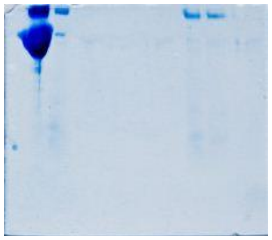
replaced with a destaining agent (150.00 mL ethanol, 100.00 glacial acetic acid and 300.00 mL distilled water) for another hour, until the bands became clearly visible and distinct. Gels were stored in microwavable plastics while submerged in water to avoid shrinking of the gel.

3. RESULTS AND DISCUSSION

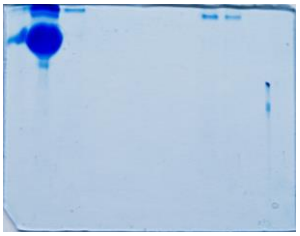
3.1 Optimization of the SDS – PAGE Gels

Low concentration of the gels in early runs of electrophoresis showed no distinct bands after being destained. The concentration of the gel used for the initials trial was 12%. For the first trial, no bands were seen in the gel except for the standard Bovine Serum Albumin. This may be because the samples were not steamed for 10 minutes before running the experiment. The second the third trial produced bands contrary to the first trial. The gels were destained and no bands were seen thus the assumption that the 12% concentration of gel is not effective and is possible that the proteins are eluting fast. As suggested by Ong and Cruz, in order for the bands to be seen, the concentration of the gel should be increased. Therefore, in order to address the problem, the gels were increased to 15% and 17.5% percent. Roasted Barako beans were also subjected to SDS-PAGE run in order to verify if the gel is effective. Resolved bands were seen when it was done using gels with higher concentration thus verifying that the gel preparation is effective. The bands of the gel are somewhat similar and did not migrate lower. Increasing the concentration of the gel is more effective as it yielded more resolved bands. The 17.5% concentration of the gel is the best concentration used in running the samples.

12% gel. Bands are now visible not only for the standard but also for the coffee samples. From left to right: BSA, Non – irradiated Robusta (3 trials), Non – irradiated Excelsa (3 trials) and Non – irradiated Liberica (3 trials).



15% gel. From left to right: BSA, Irradiated Robusta, Irradiated Excelsa and Irradiated Liberica.



17.5% gel. From left to right: BSA, Irradiated Robusta, Excelsa and Liberica

3.2 Quantitative Speculative Analysis

3.2.1 Computation of Molecular Weights of the Protein

A standard calibration curve was constructed to aid in calculating the different molecular weights of protein samples. Molecular weights of BSA marker were generated by comparing the best BSA run from a source with established molecular weights of BSA. This was then plotted versus the Rf value to be able to obtain an equation of the line (graph 13) $y = -2.764x + 2.400$. From the equation of the line, different protein concentrations were calculated by manipulating the equation.

Graphs of each trial were constructed to see different trends and. It is evident from the graphs that the molecular weight is inversely proportional to the Rf value. This is because compounds with higher molecular weights are harder to migrate than that of lower compounds. In addition, Molecular weights of coffee samples ranges only from 820 to 868 g/mol showing that all samples have high molecular weight. This can also be observed from the gel trials wherein the bands are mostly located on top of the gel. Overall, there were no definitive differences among the three varieties of civet coffee. It is thus needed to take into consideration that all accounts made for this analysis were speculative due to the estimation of the molecular weight of the BSA marker.

3.2.2 Bradford Protein Assay

One of the easiest ways in determining protein concentration is by performing Bradford Protein Assay. This is mainly due to its specificity for proteins because Bradford reagent is unreactive with non-protein substances. This makes Bradford

assay it an accurate method in determining the amount of protein in the coffee.

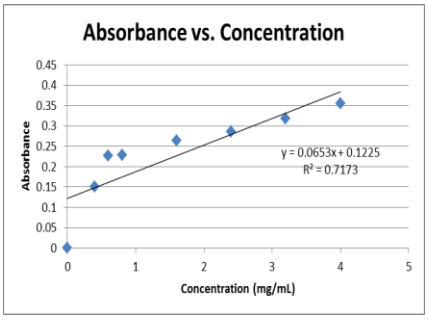


Figure 1. Standard Calibration Curve

A Standard calibration curve (figure 1) was constructed from varying concentrations of albumin. From the equation of the line, protein concentrations of different coffee samples were calculated. This was done by measuring the absorbance of the coffee extracts and calculating the concentration from the equation of the line.

The following (table 1) shows the absorbance and corresponding protein concentration present in each civet coffee sample:

Sample	Absorbance	Concentration (mg/mL)	Average
Non-irradiated Robusta trial 1	0.912	12.1	7.21
Non-irradiated Robusta trial 2	0.638	7.89	
Non-irradiated Robusta trial 3	0.230	1.65	
Non-irradiated Excelsa trial 1	0.223	1.54	2.06
Non-irradiated Excelsa trial 2	0.380	3.94	
Non-irradiated Excelsa trial 3	0.170	0.720	
Non-irradiated Liberica trial 1	0.200	1.19	1.95
Non-irradiated Liberica trial 2	0.327	3.13	
Non-irradiated Liberica trial 3	0.223	1.54	

Table 1. Bradford Protein Assay Results for Non-Irradiated Samples

From the table above, it is seen that the Robusta coffee variety contained the most amount of protein. Next is the Excelsa, then the Liberica. However, this was not seen in the results of the SDS-PAGE analysis because the intensity of the protein bands were too faint which was due to mold formation that may have hydrolyzed the protein present in the Non-irradated coffee beans.

Concentration of proteins in the irradiated coffee samples were also analyzed and shown in the table below:

Sample	Absorbance	Concentration (mg/mL)	Average
Irradiated Robusta trial 1	0.214	1.4	1.39
Irradiated Robusta trial 2	0.257	2.06	
Irradiated Robusta trial 3	0.168	0.697	
Irradiated Excelsa trial 1	0.151	0.436	1.36
Irradiated Excelsa trial 2	0.302	2.75	
Irradiated Excelsa trial 3	0.18	0.881	
Irradiated Liberica trial 1	0.212	1.37	1.37

Table 2. Bradford Protein Assay Results for Irradiated Samples

From the table above (table 2), the irradiated Robusta samples resulted the most amount of proteins. Overall, the protein concentration decreased after irradiation.

3.3 Qualitative Analysis based on Speculative Quantitative Results

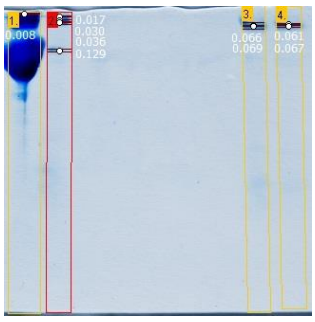


Figure 2.Trial 1 15% gel

In this trial (figure 2), the Bovine Serum Albumin (BSA) standard did not elute well. Lane

2, where the irradiated Robusta sample was loaded had the highest Rf values while Lanes 3 and 4 (irradiated Excelsa and irradiated Liberica) had closer Rf values. However, all samples showed that it had all high molecular

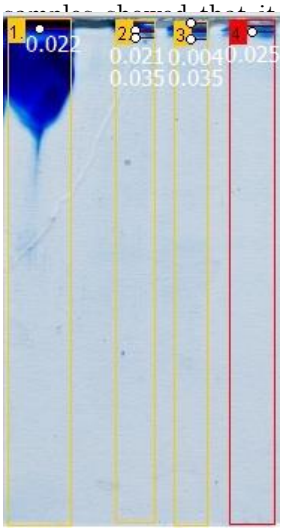


Figure 3.Trial 2 15% gel

The same with the first trial, the bands (figure 2) in all the lanes exhibited higher bands and did not travel further thus the conclusion that it has high molecular weights. However, it is noticeable that Lane 2, where irradiated Robusta was loaded, had the same Rf value with lane 3 where irradiated Excelsa was loaded.

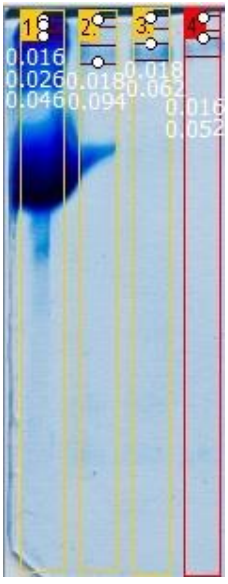


Figure 4. Trial 3 15% gel

The third trial (figure 4) for the BSA was not also successful and did not travel downward as expected based on the first and second trial for this concentration. Lanes 2, 3 and 4 where irradiated Robusta, Excelsa and Liberica was loaded respectively, had almost similar bands. Also, the Rf value for lane 2 and 3 had the same numbers.

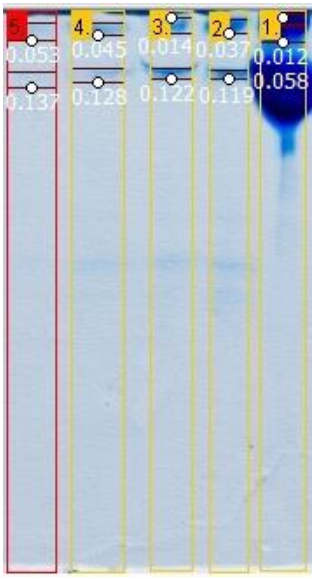


Figure 5.Trial 1 17.5% gel

All of the lanes in this gel (figure 5) only exhibited two bands and the BSA did not elute properly just like the previous trials. Non-roasted Robusta in Lane 2 and Roasted Robusta in Lane 5 showed that there is a difference in the Rf value and that roasting affected the molecular weight of the samples but not very significant.

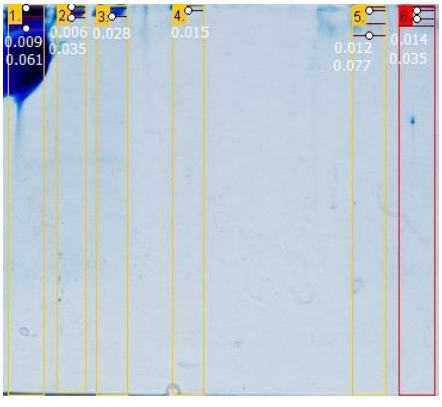


Figure 6. Trial 2 17.5% gel

As showed in Figure 6 of the gel sample, lanes 4, 5, and 6 only exhibited high bands thus expected to be high molecular weights.

Figure 7.Trial 3 17.5% gel

As compared to the first and second trial of the 17.5% gel, there are a few bands that appeared in this gel (figure 7). This may be because the samples were boiled multiple times. When samples are boiled or exposed to heat that is more than 100 degrees celcius, this causes the peptide bonds to break[31]. No fresh samples can be prepared since the samples ran out because of its limited availability. Lane 3 and 4 where irradiated Excelsa and Liberica was loaded are the same in Rf values.

Figure 8.Trial 1 12% gel

For the first trial in the 12% gel (figure 8), no bands were detected for the samples but instead only the BSA sample appeared. This may be because the samples were not boiled for the proteins to be denatured. However, the separation of the bands for the standard is not distinct and easy to determine.

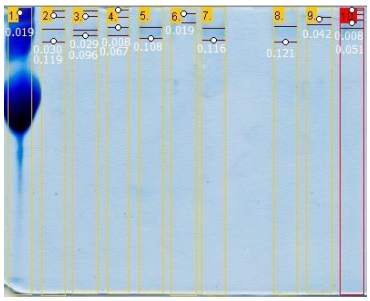


Figure 9.Trial 2 12% gel

For this second trial (figure 9), three trials per variation of the non-irradiated coffee beans were done. All the samples loaded in this gel were raw non-irradiated coffee bean samples. Lanes 2, 3 and 4 where non-irradiated Robusta was loaded did not show a pattern to the trials because lane 4 is different from lane 2 and 3. For the Excelsa samples loaded in lanes 5, 6, and 7, lane 2 is the outlier Rf value. Same with the Liberica samples in lanes 8, 9 and 10, lane 3 is the most different among the three. Overall, Robusta samples had consistent Rf values with two bands, one band for Excelsa and one lane of two bands for Liberica.

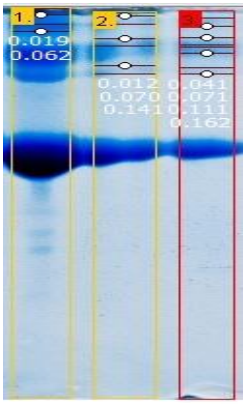


Figure 10.Trial 3 12% gel

For this last trial of the 12% gel (figure 10), three BSA samples were loaded in order to check if lessening the amount of samples loaded will make it elute better. However, no distinct changes were seen. Lane 3 of the BSA sample was used for the standard curve to determine the molecular weights of the samples using the molecular weight markers.

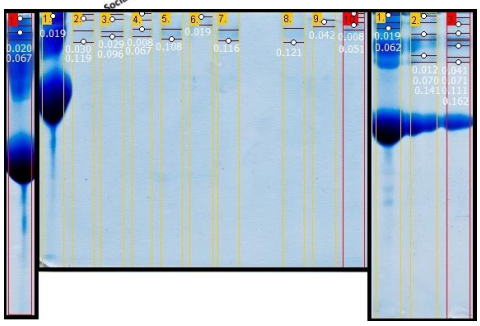


Figure 11. 12% gels combined

Comparing the three trials (figure 11), Trial 3 is the best trial among the three even though the BSA sample did not elute properly and did not show a good band separation. Robusta trials had the most number of Rf values thus had the most number of heavy molecular weights.

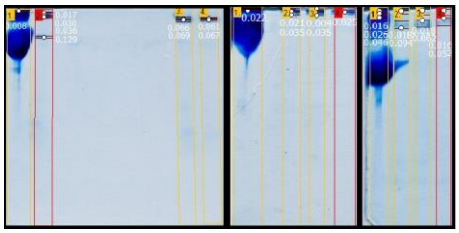


Figure 12. 15% gel combined

Comparing all the trials for the 15% gel (figure 12), trial 3 is the best run because the BSA sample is more migrated compared to trial 1 and 2 and the bands for the samples were more distinct. Trial 2 did not show any good data to be interpreted because the bands were only on top. No pattern can be derived from the results of the trials even though the samples were run on the same condition and parameters. For trial 1, Robusta is the heaviest for trial 2, Excelsa and trial 3, Liberica.

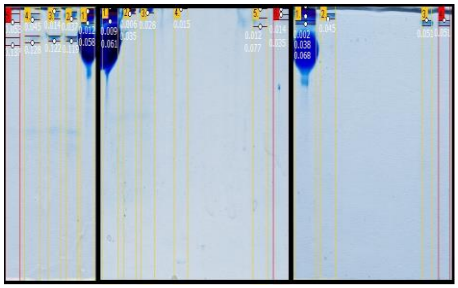


Figure 13. 17.5% gel combined

The BSA sample (figure 13) did not elute properly in any of the trials. Although among the three, trial 1 can be considered the best trial among all and the heaviest molecular weight is Excelsa. Robusta has the heaviest molecular weight for trials 2 and 3.

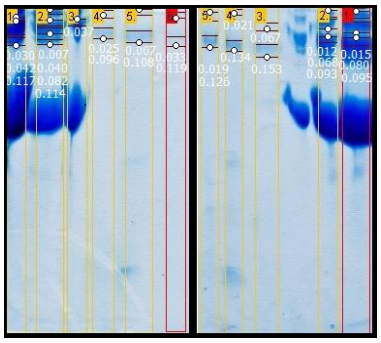


Figure 14.BSA Variation Combined

In order to improve the elution of the BSA samples, several amounts of BSA samples were loaded (figure 14). The amount of the samples was decreased in order for the elution of the bands to be more distinct but from the results even though two trials were done, the width of the BSA samples only changed and there was no improvement in the elution. Comparing lane 4 Robusta from the first trial with lane 3 Robusta on the second trial, lane 4 is heavier in weight. Another comparison with lane 5 Excelsa first trial with lane 4 Excelsa second trial, lane 5 showed to be the heaviest molecular weight. Lastly, comparing lane 6 Liberica first trial with lane 5 Liberica second trial, the second band of lane 6 and the first band of lane 5 were considered heavy in weight.

As a general conclusion, the proteins present in the coffee are high in molecular weight even in non-irradiated samples. Therefore, it can be said that the irradiation did cause a great effect on the molecular weight of the proteins because the bands of the irradiated samples are the somewhat the same with the bands of the irradiated samples. The gel with concentration 17.5% gave the most resolved bands and the Robusta type of bean projected the most number of bands in all the runs and the gel concentration.

4. CONCLUSIONS

Different varieties of Philippine Civet Coffee beans (Robusta, Excelsa and Liberica) were differentiated and quantitated using SDS-PAGE and Bradford Protein Assay. Half of the coffee bean

samples were sent to PNRI for irradiation of gamma rays ast 1 kGy with a dose rate of 29.77 Grays per hour and was irradiated for 33.6 hours long. As proven by the previous study, the method of the protein extraction that worked best is thru maceration. This technique enabled the thorough extraction of proteins for easier detection when run for SDS-PAGE. Optimization was done in order to find out what is the best concentration of gel that will give the most resolved and distinct band for elution. From all the trials, 17.5% gel concentration gave the best bands because it was more resolved, easy to distinguish and did not aggregate on one part of the gel.

Bradford Protein Assay gave the concentration of the proteins present in the coffee beans. It was consistent in the data that irradiated Robusta and non-irradiated Robusta gave the highest concentration of proteins. However, the concentration gradually decreased when it was irradiated. Even though the molecular weights calculated in the SDS-PAGE were almost the same, it is not consistent with the concentrations in the Bradford Protein Assay. This may be because the coffee samples are not mainly composed of proteins and when the proteins are extracted during the process, proteins and nucleic acids are also released30. Therefore, irradiation has caused a significant decrease in the concentration of the protein concentration.

For the SDS-PAGE results, no significant difference in the molecular weights can be distinguished between the irradiated and non-irradiated samples and the different types of coffee bean. Therefore, it will be hard to identify which type of bean is present just by looking at the Rf values.

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