Presented at the Research Congress 2013
De La Salle University Manila
March 7-9, 2013

A BRADFORD ASSAY AND SDS-PAGE ANALYSIS OF PROTEIN EXTRACTS OF EXCELSA AND ROBUSTA CIVET COFFEE BEANS

Emmanuel V. Garcia, Christian Alfredo K. Cruz; and Deorela F. Ong
Chemistry Department, De La Salle University
2401 Taft Avenue, Manila, 1004 Philippines

ABSTRACT

Abstract: The protein profiles of Excelsa and Robusta Civet and non-Civet coffee bean extracts were determined using Bradford protein assay and Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE). The profiles of the different coffee bean varieties that came from the feces of *Paradoxurus Hermaphroditus* or the Civet cat were compared with the naturally grown and normally processed coffee beans. Extraction methods were also modified to be specific for the extraction of the proteins from the Civet coffee beans. Physical extraction via maceration and solvent extraction using PVPP, PBS buffer, and EDTA were efficient in extracting the proteins since both yielded comparable protein concentrations. The Bradford protein assay showed that the respective soluble protein concentration in the crude samples of Robusta and Excelsa Civet coffee beans, using the modified extraction methods, are 2.390×10^{-3} %(w/w) and 2.383×10^{-3} % (w/w). Meanwhile, the protein concentration of the corresponding normally processed coffee beans are 2.338×10^{-3} %(w/w) and 2.392×10^{-3} %(w/w), respectively. Qualitative differences of the protein content of the coffee bean samples were determined using SDS-PAGE using a 12% polyacrylamide gel. Differences were found by comparison against a mixture of the samples. The analysis of the bands of the Robusta varieties did not show any significant differences in protein content. In contrast, the Excelsa varieties showed unique bands for both the Civet and non-Civet coffee bean samples. The band unique to the normal Excelsa variety had an R_f value of 0.309 and the unique band of the Excelsa Civet coffee beans had an R_f value of 0.285. Both analyses show that there is, indeed, a difference between the Civet and non-Civet processed coffee beans. To further improve on these results, statistical analysis and further modification of the extraction method are recommended.

Keywords: Bradford protein assay, Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE), *Paradoxurus Hermaphroditus*

1. INTRODUCTION

Coffee produced from the feces of the Palm Civet, *Paradoxurus Hermaphroditus*, is one of the most expensive Coffees in the world. In the Philippines, this coffee is called *Kape Alamid. Coffea canephora* (Excelsa) and *Coffee robusta* (Robusta) are the two species of coffee that are commonly cultivated in the

Philippines; thus, these can be considered as the species wherein the Civet cat consumes in order to produce the feces used in making the *Kape Alamid*. One of the unique characteristics of the Civet coffee is its aroma; thus, chemical components responsible for this can be used to differentiate the Civet coffee from the normally processed coffee. Among all the chemical constituents of coffee, protein has been pinpointed as the component responsible for coffee quality (Bau *et. al.*, 2001), which includes the aroma.

In order to examine the protein profiles of the different varieties of coffee and compare the Civet and non-Civet types, certain analytical techniques can be used. Out of all the possible techniques that can be used, SDS-PAGE can produce viable results that would show significant differences of the protein profiles (Marcone, 2004). In line with SDS-PAGE, Bradford protein assay has been used as a protocol in order to determine the protein concentration of samples that will be used since the amount of buffer for SDS-PAGE that would be mixed with the samples are dependent on the amount of protein in the sample (Grabski and Burgess, 2001).

The study aimed to differentiate the protein profile of the Civet and non-Civet coffee beans of the Excelsa and Robusta variety through the results of SDS-PAGE and Bradford protein assay analyses. To efficiently perform the analysis, a proper protein extraction method specific to the coffee bean samples was developed by the determination of a physical method, maceration or grinding, that would yield an extract with higher protein concentration and by the modification of a solvent formulated by Bau, *et. al.* (2001) used to extract soluble proteins from the coffee beans. After extraction has been done, the protein content will be quantified through the Bradford protein assay and qualitative analysis of the protein components will be done through SDS-PAGE. The results of the analyses of the Civet and non-Civet coffee samples would be compared. The difference can be determined by pinpointing several bands, obtained in SDS-PAGE, that are responsible for the difference and by calculating the percentage difference of the protein concentration of each variety.

2. METHODOLOGY

Sample collection

Approximately 1g of crushed and peeled Coffee beans of Excelsa and Robusta varieties of Civet Coffee and Coffee beans that were harvested, on January 7, 2012, and sun-dried were used for the subsequent analyses. The Civet Coffee beans were obtained from a reliable supplier in Alfonso, Cavite. These were already sun-dried but with the whole bean structure still intact, meanwhile, the Excelsa and Robusta Coffee beans that came from the Coffee cherries were further purified and cleaned in the laboratory.

Protein extraction

The solvent used for extraction was modified from the formula used by Bau *et. al.* (2001). A 100-mL stock solution of the solvent was made by mixing 50 mM EDTA (10 mL), 10X PBS (10 mL), PVPP (5mg), and distilled deionized water (80 mL).

Equal amounts of each coffee bean sample were subjected to two different physical extraction methods: grinding and maceration. A KRUPS Type 203 Coffee grinder was used to grind the coffee beans. After, the ground samples were mixed with the solvent in individual vials. These were centrifuged at 1163 g for 3 minutes and stored for 1 day at 4^{o}C. Maceration was done using a mortar and pestle. The solvent was slowly added to the sample while it was crushed. The samples were, then, placed into microcentrifuge tubes, centrifuged at 13, 200 rpm for 3 min., and stored for 1 day at 4^{o}C.

Bradford protein assay

The samples for each extraction method were subjected to Bradford protein assay using different standards and dilution (**Tables 1 and 2** show the volumes of samples and Bradford reagents used for each of the samples). Chicken egg albumin was used for the extracts obtained by maceration, while bovine serum albumin was used for the extracts that were ground (**Tables 1 and 2** show the concentration of standards produced). The absorbance of the macerated and ground samples and the corresponding standards with the proper amount of Bradford reagent was obtained using Spectronic GeneSys 10 UV-Vis Spectrophotometer and Spectronic 20 UV-Vis spectrophotometer, respectively, at 595 nm. The protein concentrations for each sample were determined from the standard calibration curve using Beer's Law.

Table 1 Volume of samples and Bradford reagent and Bovine Serum Albumin (BSA) stock solution needed to plot a standard calibration curve

Standard Concentration (mg/mL)	Bradford's Reagent (µL)	BSA Stock solution (µL)
Blank	1.0	0.0
1.0	1.0	1.0
2.0	1.0	2.0
4.0	1.0	4.0
8.0	1.0	8.0

Table 2 Volume of samples and Bradford reagent needed for sample analysis for extracts obtained via maceration

Sample	Bradford Reagent (µL)	PBS (µL)	Sample (µL)
Civet Excelsa	250	150	100
Excelsa	250	150	100
Civet Robusta	250	150	100
Robusta	250	150	100

SDS-PAGE

The samples obtained from the grinding method were analyzed using SDS-PAGE analysis using the standard method as indicated by Bio-Rad. The respective voltages used for stacking and running are 200 V and 180 V. In order to maintain the leakage of the dye, the gel was continually exposed to 200 V after the dye ceased to travel. The buffer used in the system was 4x Tris solution for the resolving and stacking gel. The tank buffer was 4x Tris-glycine solution.

3. RESULTS AND DISCUSSION

Protein extraction

The extraction solvent modified was efficient in extracting the proteins since the extracts were able to produce results from the Bradford protein assay, which is specific for proteins only. As seen from the Bradford protein assay results **(Table 3)**, the maceration method was able to extract more soluble proteins than the grinding method since this had a higher percentage difference.

Table 3 Comparison of maceration and grinding methods via Civet and non-Civet Coffee protein concentrations through percentage difference

Sample	Concentration of extracts via maceration (mg/mL)	Concentration of extracts via grinding (mg/mL)	Percent protein in crude extracts via maceration	Percent protein in crude extracts via grinding	Average	Percent Difference
Civet Excelsa	0.1195	0.1185	2.383×10^{-3}	0.190×10^{-3}	0.001290	170.60
Excelsa	0.1196	0.1243	2.392×10^{-3}	0.195×10^{-3}	0.001296	169.30
Civet Robusta	0.1196	0.1298	2.39×10^{-3}	0.199×10^{-3}	0.001300	168.04
Robusta	0.1169	0.1219	2.338×10^{-3}	0.191×10^{-3}	0.001267	169.21
Comparison of CE and E						0.4676
Comparison of CR and R						2.5990

Bradford protein assay

The soluble protein concentrations of each sample are shown in **Table 3**. These were obtained using Beer's Law by plotting a standard calibration curve. The percentage differences calculated relative to the percent of soluble protein in the non-Civet coffee extracts show that there is a difference in the soluble protein concentrations of the Civet coffee extracts and the non-Civet coffee extracts. In comparing the species of coffee, there are more differences in the Robusta variety than the Excelsa with respect to the non-Civet Coffee bean protein concentration since the percentage difference is greater: 2.5990% and 0.4676%, respectively. Although proteins have been extracted, it was present only in low amounts since the percent proteins by weight of each sample are expressed as notations at 10^{-3}. This is far from the soluble protein composition reported by Esquivel *et. al.* (2011), wherein the different anatomical parts of the Coffee bean would have around 10% soluble protein. This discrepancy may be due to the lack of the extraction of other biomolecules such as lipids that can interrupt protein extraction by interacting with the soluble proteins, which should have been extracted.

SDS-PAGE

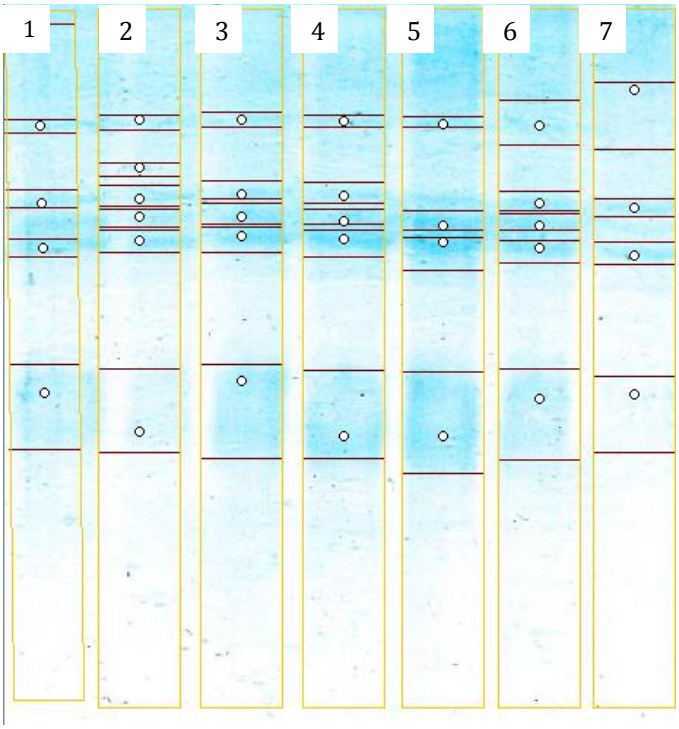

Figure 1 SDS-PAGE of Coffee samples and mixtures - Civet excelsa + civet robusta (lane 1); Robusta (lane 2); Robusta + civet robusta (lane 3); Civet robusta (lane 4); Excelsa (lane 5); Civet excelsa + excelsa (lane 6); Civet excelsa (lane 7)

Each extract obtained from the grinding method was placed in corresponding lanes in a polyacrylamide gel (**Figure 1)**. R_f values and relative intensities (raw volume) of the bands obtained formed in each lanes were compared. All comparisons were done relative to the lanes containing the mixture of the extracts being compared. The bands that correlate with each other are shown in **Tables 4 and 5**. Additionally, some bands also had the same relative intensities as their corresponding bands in the mixture lanes, which can be seen as colored cells in **Tables 4 and 5**. The similar R_f values of the bands of the individual varieties with the bands in the mixture can be interpreted as proteins normally present in the coffee specie that are not affected by the chemical processes in the digestive tract of the Civet cat. Unique bands can be explained by the possibility that the proteins may have been lost or modified during the digestion of the coffee via the Civet cat.

Table 4 Comparison of the SDS-PAGE bands of Robusta extracts (lanes 2,3, and 4)

Band number	Robusta	Robusta + Civet Robusta	Civet Robusta
1	0.158	0.158	0.16
2	0.227/0.27	0.264	0.267
3	0.298	0.296	0.304
4	0.331	0.325	0.328
5	0.603	0.531	0.61

Table 5 Comparison of the SDS-PAGE bands of Excelsa extracts (lanes 5,6, and 7)

Band number	Excelsa	Excelsa + Civet Excelsa	Civet Excelsa
1	0.165	0.166	0.016
2		0.277	0.285
3	0.309	0.31	
4	0.333	0.342	0.352
5	0.611	0.557	0.55

Individual SDS-PAGE analysis of each coffee specie provided insight about the variability between the natural coffee beans and the coffee beans digested by the Civet cat. The SDS-PAGE analysis of Robusta Coffee was unable to identify unique bands to the Robusta varieties. Furthermore, it only provided data that displayed fusion of what should have been distinct bands as seen in the second band (R_f = 0.264) of the mixture lane (Lane 3). Meanwhile, the Excelsa variety showed two unique bands in the Civet (Lane 7) and non-Civet (Lane 5) lanes with R_f values of 0.285 and 0.309, respectively.

4. CONCLUSIONS

Civet and non-Civet Coffee of the varieties Excelsa and Robusta was differentiated using SDS-PAGE and Bradford protein assay. In order to obtain valid results, proper protein extraction was done. Since none have determined the proper protein extraction method, specifically for Civet Coffee beans, maceration and grinding methods were compared. Based on the high percentage difference of soluble protein concentration, the maceration method would enable the extraction of more proteins. Chemical extraction was modified and a formula for the solvent was deduced based on the most common reagents used as said in literature. Such reagents are PVPP, PBS buffer, and EDTA. Results of the Bradford protein assay of the extracts showed the presence of protein; thus, indicating that the methods used were efficient.

Bradford protein assay and SDS-PAGE analysis of the Coffee varieties of Civet and non-Civet Coffee samples showed that each contained very similar proteins that differ mainly in the concentration. Despite the presence of a difference, the validity and significance cannot be accurately stated since the SDS-PAGE results showed that there may be non-protein entities in the samples. This is seen in various bands that do not reflect expected relative intensities. These components have molecular weights that coincide with that of the proteins in the bands; thus. possess the same R_f values but cause the relative concentrations to change. It is worthy to note that nucleic acids and proteins are released simultaneously in most extractions and obtaining a pure protein sample may be difficult since the former adds to the viscosity of the sample. These discrepancies can also be the result of the interaction of the non-protein components with the Coffee bean protein prior to analysis, which affected fragmentation. Thus, the method used should be polished in order to obtain accurate results in order to fully understand the difference in protein content of Civet and non-Civet Coffee.

5. REFERENCES

A Guide to Polyacrylamide Gel Electrophoresis and Detection. Retrieved 21 August 2012 from the World Wide Web: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040A.pdf

Bau, S. M. T.; Mazzafera, P.; Santoro, L. G. Seed Storage Proteins in Coffee. *Rev. Bras. Fisiol. Veg* **2001**, *13*, 33-40.

Bonner, P. L. R. *Protein Purification*; Taylor & Francis Group: New York, 2007; pp 2-9.

Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle. *Anal. Biochem.* **1974**, *72*, 248-254.

Coffeechemistry.com. *Protein content in Coffee*; http://www.Coffeechemistry.com/index.php/Chemistry/Proteins/protein-content-in-Coffee.html (accessed August 21, 2012).

Coffee Production (Philippines). Retrieved 20 August 2012 from the World Wide Web: http://pinkhawk.hubpages.com/hub/Coffee-production-philippines

Doonan, S. *Protein Purification Protocols*; Humana Press Inc: New Jersey, 1996; pp 23-25.

Esquivel, P.; Jimenez, V.M. Functional properties of Coffee and Coffee by-products. *Food Res. Int* **2011**, *46*, 488--495.

Espresso & Coffee Guide. *Vietnam Civet Coffee;* http://www.espressoCoffeeguide.com/gourmet-Coffee/asian-indonesian-and-pacific-Coffees/civet-Coffee/vietnam-civet-Coffee/ (accessed August 20, 2012).

Grabski, A. C.;Burgess, R. R. Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: procedures and tips. Retrieved 21 August 2012 from the World Wide Web: http://wolfson.huji.ac.il/purification/PDF/PAGE_SDS/NOVAGEN_Prepare_Sample_PAGE_SDS.pdf

Gel Electrophoresis of Proteins; Hames, B. D.; Rickwood, D.; Oxford University Press: New York,1994.

Innovations-report. *New Research Explains Structure, Tase of Kopi Luwak Coffee.* Retrieved 20 August 2012 from the World Wide Web: http://www.innovations-report.de/html/berichte/biowissenschaften_chemie/bericht-31682.html

Massimo, F. M. Composition and properties of Indonesian palm civet Coffee and Ethiopian civet

Coffee. *Food Res. Int.* **2004**, *37*, 901-912.

Murkovic, M.; Derler, K. Analysis of amino acids and carbohydrates in green Coffee. *J. Biochem. Biophys.* **2006**, *69*, 25-32.