

**Analysis of Caffeine and Chlorogenic Acid in Green and Roast Coffee Beans
by Extraction and High Pressure Liquid Chromatography (HPC)**

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Submission Date: November 12th, 2021

Due Date: November 12th, 2021

CHEM438 - 021L

I submit this laboratory report as an original document. I assert that all ideas and discussion of data contained herein are my own work, unless otherwise referenced.

ABSTRACT

The wavelengths associated with caffeine and chlorogenic acid are 273 and 330 nm respectively. Direct calibration of caffeine standards was generated from the responses of four standard solutions, prepared by dilution in 10-mL volumetric flasks of 0.50, 1.00, 2.00, and 3.00 mL of 0.50 mg/mL caffeine stock solution. The calibration curve has a trendline equation:

$Area = (4,518.8 \pm 49.7) \frac{mAU \cdot s \cdot mL}{mg} [Caffeine] + (1.65 \pm 4.69)mAU \cdot s$. Another set of calibration curves was generated for chlorogenic acid standards, by doing the same procedure to prepare caffeine standard solutions. The resulting calibration curve has the trendline's equation as such: $Area = (2177.4 \pm 323.7) \frac{mAU \cdot s \cdot mL}{mg} [Caffeine] - (29.25 \pm 21.39)mAU \cdot s$.

Green and roasted beans have caffeine responses of 121.58 and 122.92 mAU·s, and the chlorogenic acid responses of 162.63 and 18.47 mAU·s respectively. From the calibration curve, the concentration of caffeine in green and roasted beans are 0.133 ± 0.378 and 0.134 ± 0.382 mg/mL, with a weight percent of 3.60 ± 10.23 and $3.48 \pm 9.91\%$ respectively. On top of that, the chlorogenic acid content in green and roasted beans are 0.441 ± 0.329 and 0.110 ± 0.082 mg/mL, with a weight percent of 3.13 ± 2.34 and $0.75 \pm 0.56\%$ respectively. The separation efficiency was calculated to be 994.56. Roasting decreases the chlorogenic acid content in the original green coffee beans.

INTRODUCTION¹

High-pressure liquid chromatography (HPLC) is a technique used for separation of non-volatile or thermally unstable compounds. A regular gas chromatography is no longer practical. In the lab, HP 1090 chromatography instrument will be used to analyze compounds from a given sample.

Most of the natural products are complex mixtures, which indicates various chemical compounds in the mixtures. Due to that, extraction techniques should be preliminary done to isolate desired compounds from the rest of the mixtures. In order to do so, the understanding of equilibrium partitioning is essential. Extraction aims to selectively remove one or two compounds from a complex mixture by considering the differences in solubility of these compounds in the solvent used. Small differences in equilibrium partitioning constant leads to difficulty in separation, thus using the right solvent, pH, or extraction temperature are crucial so the solubility favors the way we want it.

Chlorogenic acid and caffeine are the two desired compounds that exist in coffee beans. The analysis of the coffee beans cannot be directly performed on the coffee bean itself because of the presence of caffeic acid and quinic acid which will interfere with the responses associated with the desired compounds. Water can act as the solvent used to extract the coffee beans. Both caffeine and chlorogenic acid are polar; thus water-soluble. Addition of heat in the extraction will strongly enhance the extraction process more.

The theory to understand HPLC revolves around the enforcement of equilibrium partitioning constant K. If K of compound A is bigger than that of compound B, B will be more likely to be in the mobile phase of the HPLC, leading to faster separation in the column. In other words, the retention time of compound B, which is the time for compound B to stay in the column, will be much lower than that of A. The selection of solvent used for mobile and stationary phase are important as they determine the retention time for each compound. For this lab, the mobile phase is 5% acetonitrile to 95% water/methanol mixture by volume basis, with water/methanol mixture of 80:20 by volume % ratio, at the same time a slight (<1%) of trifluoroacetic acid.

PROCEDURE ¹

Part I. Direct calibration curve determination

Suitable detection wavelengths for caffeine and chlorogenic acid were determined first prior to collecting measurement for the rest of the standard solutions. To do so, 2 mL of the caffeine (0.50 mg/mL) and chlorogenic acid (0.35 mg/mL) stock solution were diluted in a separate 10.00 mL volumetric flask. The diluent used was acetonitrile:water/methanol solvent. Before dilution, about 1 drop of diluted acetic acid was added into each flask. The spectrum was recorded by filling up the cuvette with respective solutions in the UV-visible spectrometer, a part of the HP 1090 chromatograph. The spectra were used to determine the wavelengths that have maximum spectral resolution of caffeine and chlorogenic acid.

Four 10-mL volumetric flasks were prepared by adding 0.50, 1.00, 2.00, and 3.00 mL of each of the stock solutions into the flask. Each flask should contain the same volume of caffeine and chlorogenic acid, and diluted with the same diluent stated earlier. The samples were eluted in the chromatography column for about 5 minutes, but it would take 15 minutes in total to ensure no cross-contamination between samples. The spectra for each standard solution were collected at both wavelengths determined priorly.

Part II. Coffee beans extraction and analysis

The mass of the green (*San Salvador*) and roasted (*Nicaragua*) beans were measured (about 1.00 g of each). Each bean type was crushed in a mortar before mixing with about 75 mL of distilled water in a 250 mL beaker. A clean stir bar was added into the beaker. The mixture was boiled on the stirrer-hotplate while slowly stirring. The cooled mixture was filtered through a glass wool plug and diluted in a 100-mL volumetric flask with distilled water.

2 mL of the coffee mixture for each bean type was pipetted into a 10-mL volumetric flask and diluted with the diluent mentioned in ***Part I***. 1 mL syringe was used to filter the diluted sample into a vial for spectrum recording. For each coffee sample, the spectra were collected for each of the two predetermined wavelengths.

RESULTS AND DISCUSSION

Table 1. Direct calibration data for caffeine standards.

Sample	^a V _{added} (mL)	Concentrations (mg/mL)	Area (mAU·s)
1	0.5	0.025	118.86
2	1.0	0.050	223.46
3	2.0	0.100	451.17
4	3.0	0.150	681.70

^a Volume of stock solution added; [Caffeine]_{stock} = 0.50 mg/mL

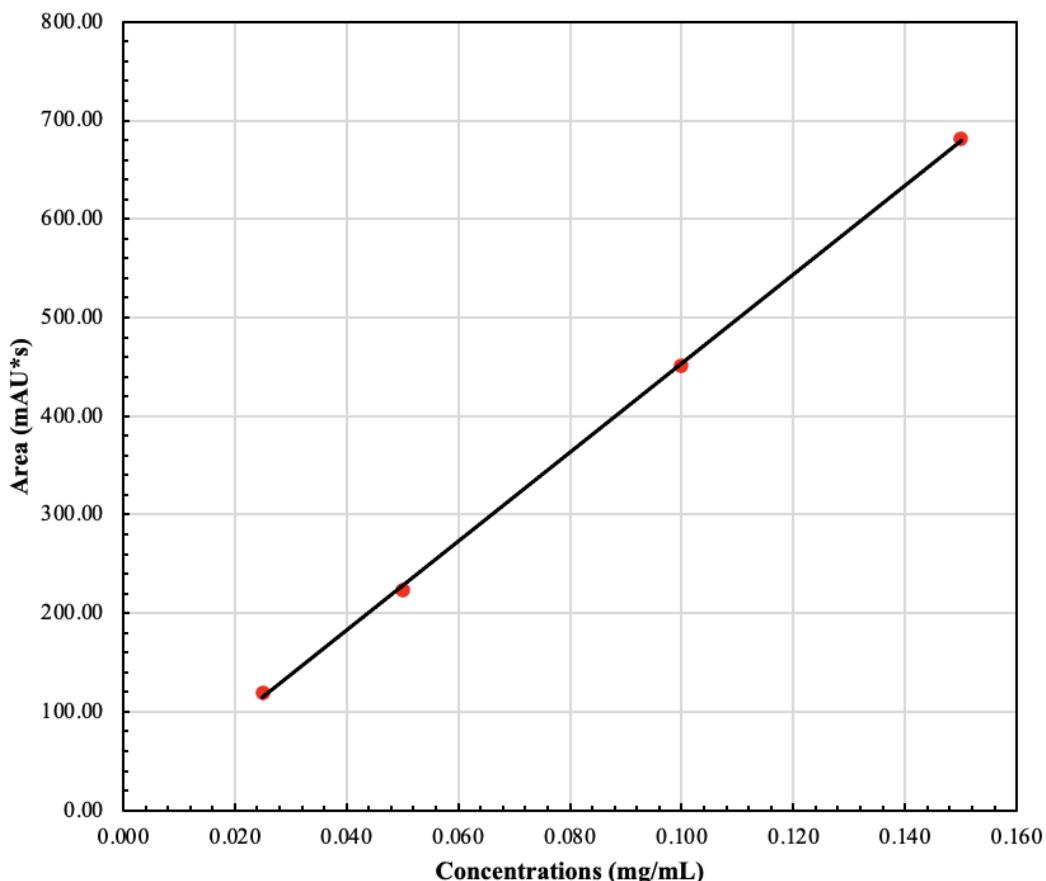


Figure 1. Direct calibration curve of caffeine standards.

The suitable detection wavelengths for caffeine and chlorogenic acid were found to be 273 and 330 nm respectively. The direct calibration curve for coffee standards was as shown in **Figure 1**, with the plotted data tabulated in **Table 1**. The linear trendline's equation was as written in **Eq. 1**.

$$Area = (4,518.8 \pm 49.7) \frac{mAU \cdot s \cdot mL}{mg} [Caffeine] + (1.65 \pm 4.69)mAU \cdot s \quad \text{Eq. 1}$$

The trendline has an R²-value of 0.9998, suggesting a significant linearity in the data. However, the error in the y-intercept of the trendline is significantly bigger than the y-intercept itself. This suggests possible large errors in the concentrations of caffeine in the coffee bean as the errors propagate.

Table 2. Direct calibration data for chlorogenic acid standards.

Sample	^a V _{added} (mL)	Concentrations (mg/mL)	Area (mAU·s)
1	0.5	0.018	24.66
2	1.0	0.035	37.28
3	2.0	0.070	103.01
4	3.0	0.105	213.41

^a Volume of stock solution added; [Chlorogenic acid]_{stock} = 0.35 mg/mL

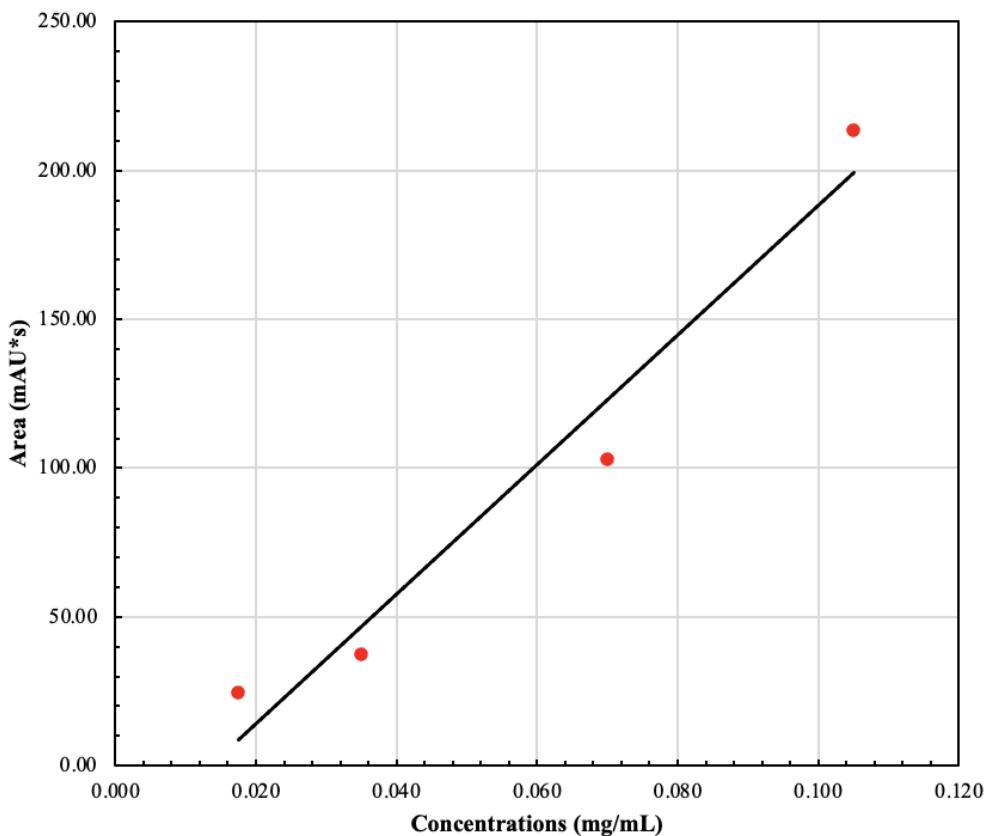


Figure 2. Direct calibration curve of chlorogenic acid standards.

Table 2 tabulated the calibration data for chlorogenic acid standards, resulting in a direct calibration curve as shown in **Figure 2**. The trendline has an equation as shown in **Eq. 2**.

$$Area = (2177.4 \pm 323.7) \frac{mAU \cdot s \cdot mL}{mg} [Caffeine] - (29.25 \pm 21.39)mAU \cdot s \quad \text{Eq. 2}$$

The trendline for chlorogenic acid standard calibration curve however has a lower R²-value, which is 0.958, though it still suggests linearity in data. The error in the y-intercept on the other hand is not significantly bigger than the y-intercept itself, unlike the one observed in the caffeine calibration line.

The response used in the calibration curve is the area under the peak of a chromatograph at a specific retention time of the analyte measured, in unit of mAU·s. The retention time for caffeine and chlorogenic acid in this experiment setting are about 1.78 and 1.71 minutes respectively. The response of caffeine for green and roasted beans were 121.58 and 122.92 mAU·s respectively. Meanwhile, the response of chlorogenic acid in green and roasted beans were 162.63 and 18.47 mAU·s respectively. From **Eq. 1** and **Eq. 2**, the concentration of the two analytes in both bean types can be determined.

Table 3. Concentration of caffeine and chlorogenic acid in green and roast beans.

Type of Beans	[Caffeine] (mg/mL)	^a $\sigma_{[Caffeine]}$ (mg/mL)	[Chlorogenic Acid] (mg/mL)	^a $\sigma_{[Chlorogenic Acid]}$ (mg/mL)
Green	0.133	0.378	0.441	0.329
Roasted	0.134	0.382	0.110	0.082

^a Error in concentration of caffeine/chlorogenic acid (mg/mL)

Tabulated in **Table 3** were the concentration of caffeine and chlorogenic acid in both bean types, measured in mg/mL units. The concentrations were calculated after taking into account the dilution in the 10-mL and 100-mL volumetric flask. As expected, the concentration of caffeine in green and roasted beans have large errors (0.133 ± 0.378 and 0.134 ± 0.382 mg/mL).

Table 4. Weight percent of caffeine and chlorogenic acid in the beans.

Type of Beans	Mass of bean (mg)	(% w/w) _{Caffeine}	^a $\sigma_{(\%w/w), caffeine}$	(% w/w) _{Chlorogenic acid}	^a $\sigma_{(\%w/w), chlorogenic acid}$
Green	1049.9	3.60	10.23	3.13	2.34
Roasted	1096.5	3.48	9.91	0.75	0.56

^a Error in weight percent of caffeine/chlorogenic acid in the beans

The weight percent of caffeine and chlorogenic acid in the beans were calculated and as tabulated in **Table 4**. It could be observed that caffeine concentration in green and roasted beans were similar (3.60 ± 10.23 and 3.48 ± 9.91 %). However, the chlorogenic acid weight percent were significantly different, with 3.13 ± 2.34 and 0.75 ± 0.56 % for the green and roasted beans respectively. From a study conducted by Pilipczuk et al., roasting coffee beans will reduce the chlorogenic acid content by 50 %, and increase the caffeine content by 30 %. ² From the experimental data, the change in chlorogenic acid content was about 76 %, which aligns with the conclusion made in the study (more than 50%, most of the acid removed). Nevertheless, the caffeine content appears to be not changing much, which is the opposite as what was proposed in the study. Assuming that the study was credible and what was proposed is true, the caffeine

content should increase after roasting. The large errors in caffeine weight percent can be one of the reasons the data collected do not align with the study.

The efficiency of the separation in the chromatography can be measured from the number of plates, N. By using the data collected for the caffeine with a retention time, t_R of 1.778 minutes and peak width at half of the height, $W_{0.5H}$ of 0.1327 minute, N was found to be 994.56, calculated from **Eq. 3** as shown below.

$$N = 5.54 \left(\frac{t_R}{W_{0.5H}} \right)^2 \quad \text{Eq. 3}^3$$

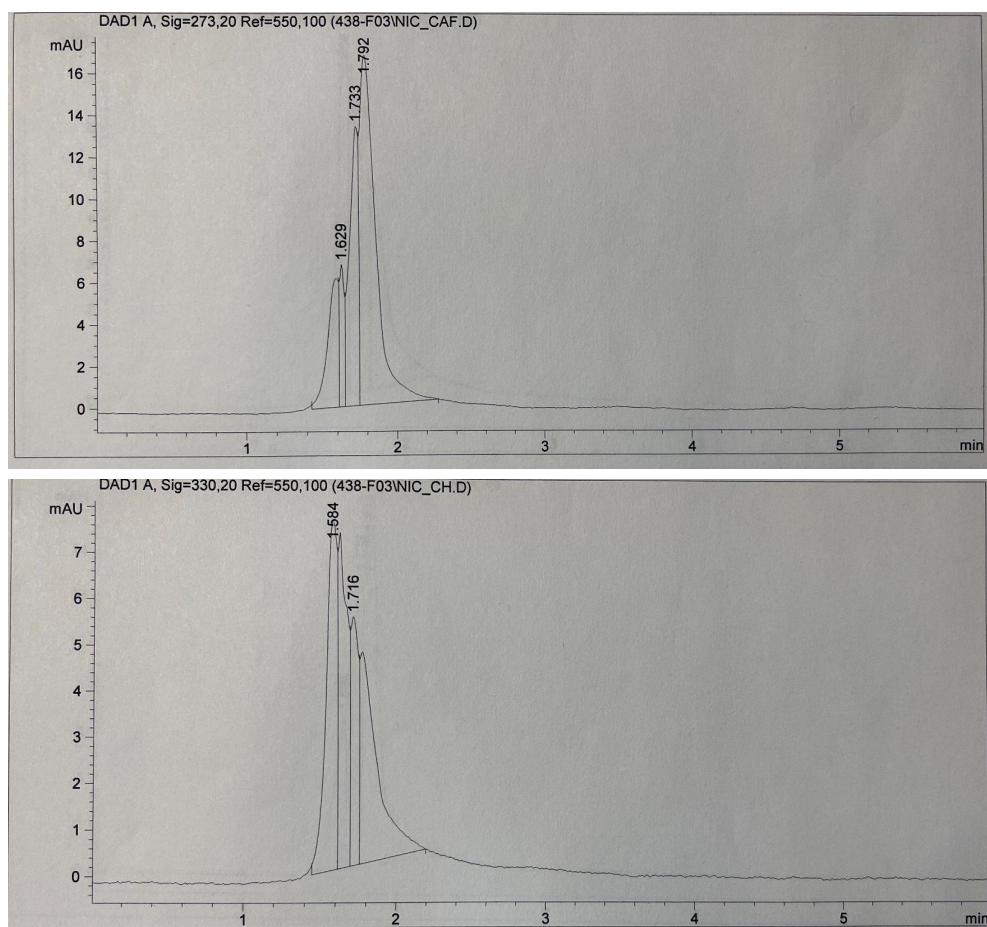


Figure 3. HPLC spectrum of roasted beans measured at 270 nm (top) and 330 nm (bottom).

From **Figure 3**, there are more peaks recorded for the roasted than the green coffee extract. This is associated with the presence of caffeic and quinic acid in the roasted beans. These acids are expected to be present in both green and roasted beans. From the study mentioned earlier, roasting should increase the caffeine content but decrease the content of chlorogenic acid. The change in chlorogenic acid done in this experiment agrees with the conclusion made in the study. Caffeic and quinic acid are believed to increase in concentration, as one of the effects of

roasting coffee beans, the same trend that should be observed for caffeine. Due to that, the peaks associated with these acids are observable, as opposed to less peaks for green beans.

Nicotinic acid is known to be present in a brewed coffee. It is mostly bound up in the coffee bean as nicotinamide adenine dinucleotide, NAD and nicotinamide adenine dinucleotide + hydrogen, NADH^+ .⁴ Upon boiling, both compounds should be released to the air but not entirely.

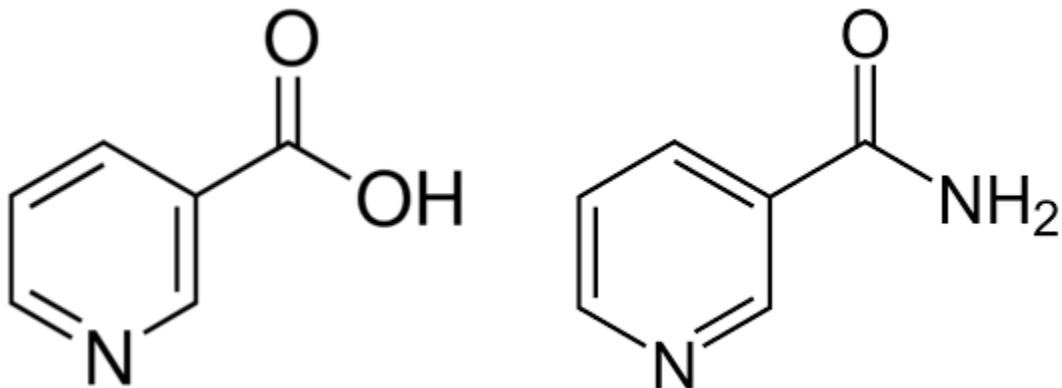


Figure 4. Structure of nicotinic acid (left) and nicotinamide (right).^{5,6}

Figure 4 shows the structure of nicotinic acid and nicotinamide. Both are polar molecules and are expected to be extracted into the aqueous phase under the conditions prepared in this experiment. Nicotinic acid and nicotinamide respectively, are expected to be detected at 330 and 270 nm. This is because they have similar structure as that of chlorogenic acid and caffeine respectively. The release of nicotinic acid/nicotinamide could explain the extra peaks observed for roast coffee. As explained previously, boiling of the coffee extract releases the bounding of these compounds from the coffee bean, making them available in the final extract.

The HPLC spectra for all four standards measured in both wavelengths, alongside the spectra for green and roasted beans, and the determination of detection wavelengths are embedded in the **Appendices** under *Supplemental Figures*.

CONCLUSIONS

Direct calibration approaches can be employed to determine the concentration of caffeine and chlorogenic acid in the coffee beans sample (green and roasted). Two sets of calibration curves will be used, measured at two detection wavelengths. Extraction method is necessary to separate desired compounds from the rest of the coffee beans' matrices, by considering and understanding the equilibrium partitioning constant of the compounds in a particular solvent. Green and roasted beans show similar content of caffeine, though the chlorogenic acid content in roasted beans is significantly lower than in green beans. Roasting should decrease the chlorogenic acid contents, aside from the observable presence of other compounds in the matrix sample for the roasted beans, detected in the HPLC.

REFERENCES

- ¹ Cruz, F. *CHEM438 Instrumental Methods Laboratory - Analysis of Caffeine and Chlorogenic Acid in Green and Roast Coffee Beans by Extraction and High Pressure Liquid Chromatography lab*; University of Delaware: Newark, Delaware, 2020; pp 1-7.
- ² Pilipczuk, T.; Kusznierewicz, B.; Zielińska, D.; Bartoszek, A. The Influence of Roasting and Additional Processing on the Content of Bioactive Components in Special Purpose Coffees. *Journal of Food Science and Technology* **2014**, 52 (9), 5736–5744.
- ³ Skoog, D. A.; Holler, F. J.; Crouch, S. R. *Principles of instrumental analysis*; Cengage Learning: Boston, MA, 2018.
- ⁴ NADH: Overview, uses, side effects, precautions, interactions, dosing and reviews. <https://www.webmd.com/vitamins/ai/ingredientmono-1016/nadh> (accessed Nov 13, 2021).
- ⁵ Nicotinic acid = 98 59-67-6. <https://www.sigmaaldrich.com/US/en/product/sial/n4126> (accessed Nov 13, 2021).
- ⁶ Nicotinamide = 99.5 HPLC 98-92-0. <https://www.sigmaaldrich.com/US/en/product/sigma/72340?context=product> (accessed Nov 13, 2021).

APPENDICES

Lab Notebook

26			
Exp. No.	Experiment/Subject	Date	Course & Section No.
8	CHEM 488 (HPC)	11/1/2021	621 L
Name	Lab Partner	Locker/ Desk No.	
Afzal Fayed	Justin M		

Prelab Questions

- Chlorogenic acid contains ester bond which is very sensitive to basic solution. It will get hydrolyses, producing Caffeic acid and quinic acid. Basic extraction will increase the contamination by Caffeic acid and quinic acid. Slightly acidic solution will ensure the caffeine get removed easily.
- The presence of air bubbles can modify the flow of mobile phase through the column. The pressure in the stream should be maintained all the time.
- Trifluoroacetic acid is a common acidic HPLC mobile phase modifier where it acts as ion-pairing reagent. It equilibrates so quickly in the solution that only a small amount is needed for compounds that act as weak acids/bases.
- Greater flow rate will reduce the retention time, thus causing poor separation. If time were not a factor, it is preferred to have a slower flow for better separation.

Signature	Date	Witness/TA	Date
	11/1/2021		

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Exp. No.	8	Experiment/Subject	CHEM 438 (HPC)	Date	11/1/2021
Name	Abdul Fayed	Lab Partner	Justin M	Locker/ Desk No.	Course & Section No. 021 L

Objective

The purpose of this lab is to analyze the caffeine and chlorogenic acid in green and roast coffee beans by extraction and high pressure liquid chromatography.

Introduction

HPLC is used for mixtures that contain non-volatile or thermally unstable compounds. Aside from caffeine and chlorogenic acid, there are also presence of contaminants: caffeoic acid and quinic acid. Proper choice of eluent can ensure the best separation. The composition of the caffeine / chlorogenic acid might change before and after the roasting.

Procedure

- Measure the detection wavelength for caffeine and chlorogenic acid by using the acetonitrile : water/methanol mobile phase solution (5% acetonitrile : 95% water/methanol, 80:20 v/v of H₂O:Meth)
- Make separate diluted standards for caffeine and chlorogenic acid (stock of caffeine: 0.5mg/ml in solvent, chlorogenic acid: 0.35 mg/ml in solvent).
- Use 2mL of stock solution and dilute with the solvent until to 10mL.
- Add 1 drop of dilute acetic acid to the solution.
- Record the spectrum of UV-vis. If A > 1.0, re-dilute the solution more, then record the spectrum.

mass of roasted coffee beans

$$= 1.0965 \text{ g}$$

mass of green coffee beans

$$= 1.04799 \text{ g}$$

$$\lambda_{\text{cafein}} = 273 \text{ nm}$$

$$\lambda_{\text{chlorogenic acid}} = 330 \text{ nm}$$

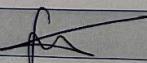
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Exp. No.	8	Experiment/Subject	CHEM 488 (HPC)	Date	11/1/2021
Name	Abdul Fayed.	Lab Partner	Justin M	Locker/ Desk No.	Course & Section No. 021C

6. Determine the wavelengths to be used in HPLC.
7. Record the spectrum for both caffeine and chlorogenic acid.
8. choose the λ where caffeine's absorbance is high but low for chlorogenic acid.
9. Again, choose λ where absorbance of chlorogenic acid is high but low for caffeine.
10. Make 2 runs on each standard and on each coffee sample : λ from caffeine spectrum , and λ from chlorogenic acid .
11. Set detection channel @ selected λ , and select 2nm bandwidth. Set ref. channel to 550 nm, using 10nm bandwidth.
12. Prepare 4 standards in 10mL vols. flask
Add 0.5, 1, 2, 3 mL of each stock solution (0.5 mL of caffeine + 0.5 mL of chlorogenic acid stock solution) ..
13. Flow rate is set to 1 mL/min. Sampler is set to inject 1 μ L sample.
14. Detectable peaks should elute within 5 minutes, But the run will take 15 minutes to elute.
No carry-over of undetected compounds.
15. Weigh about 1 g of the ground green and roasted coffee.
16. Place the sample in 250 mL beaker, then add ~70-75 mL of DI water and clean stir-bar.

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	11/1/2021		

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Exp. No.	8	Experiment/Subject	CHEM 438 (HPC)	Date	11/1/2024
Name	Abdul Fayeed	Lab Partner	Justin M	Locker/ Desk No.	Course & Section No. 524

17. Stir slowly and heat the mixture to boiling on the hot plate. Boil gently for about 10 minutes. then allow to cool.
18. Filter the cooled mixture through a glass wool plug into a clean 100ml volumetric flask. Rinse the coffee on the filter and add the filtrate into the flask. Dilute with DI water to the mark.
19. Take 2ml of coffee extracts into 10ml vol. flask, then dilute with the solvent. #
20. Run the sample unknown sequences after all the standards are done running.
21. Print out & label all 12 chromatographs.
22. Printout & label the UV-vis spectra.

Conclusions

HPLC can be used to analyze the caffeine and chlorogenic acid in green and roasted coffee beans, by using the standard addition method.

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	11/1/2024		

Supplemental Figures

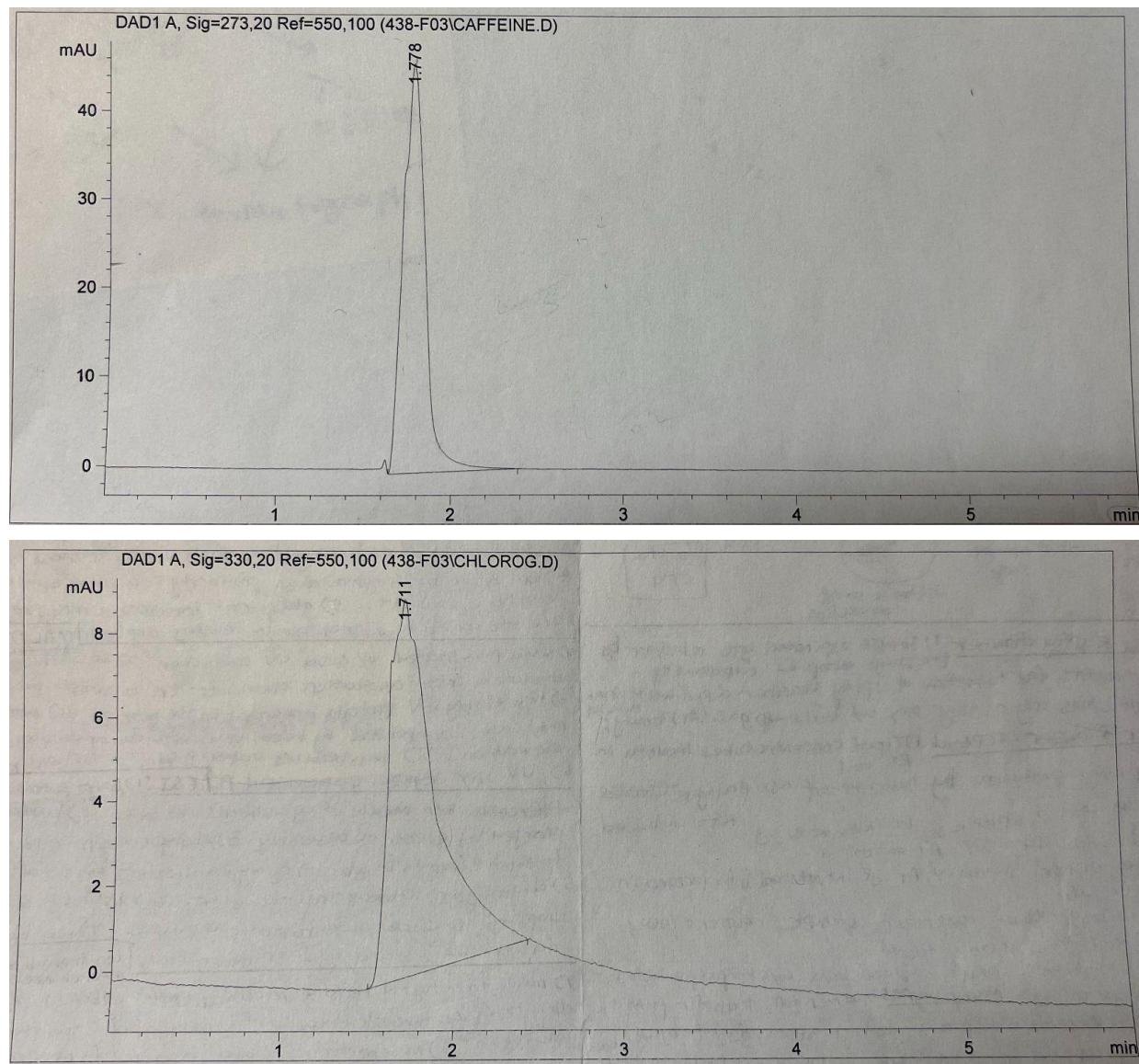


Figure A. HPLC spectrum of caffeine and chlorogenic acid to determine their detection wavelengths.

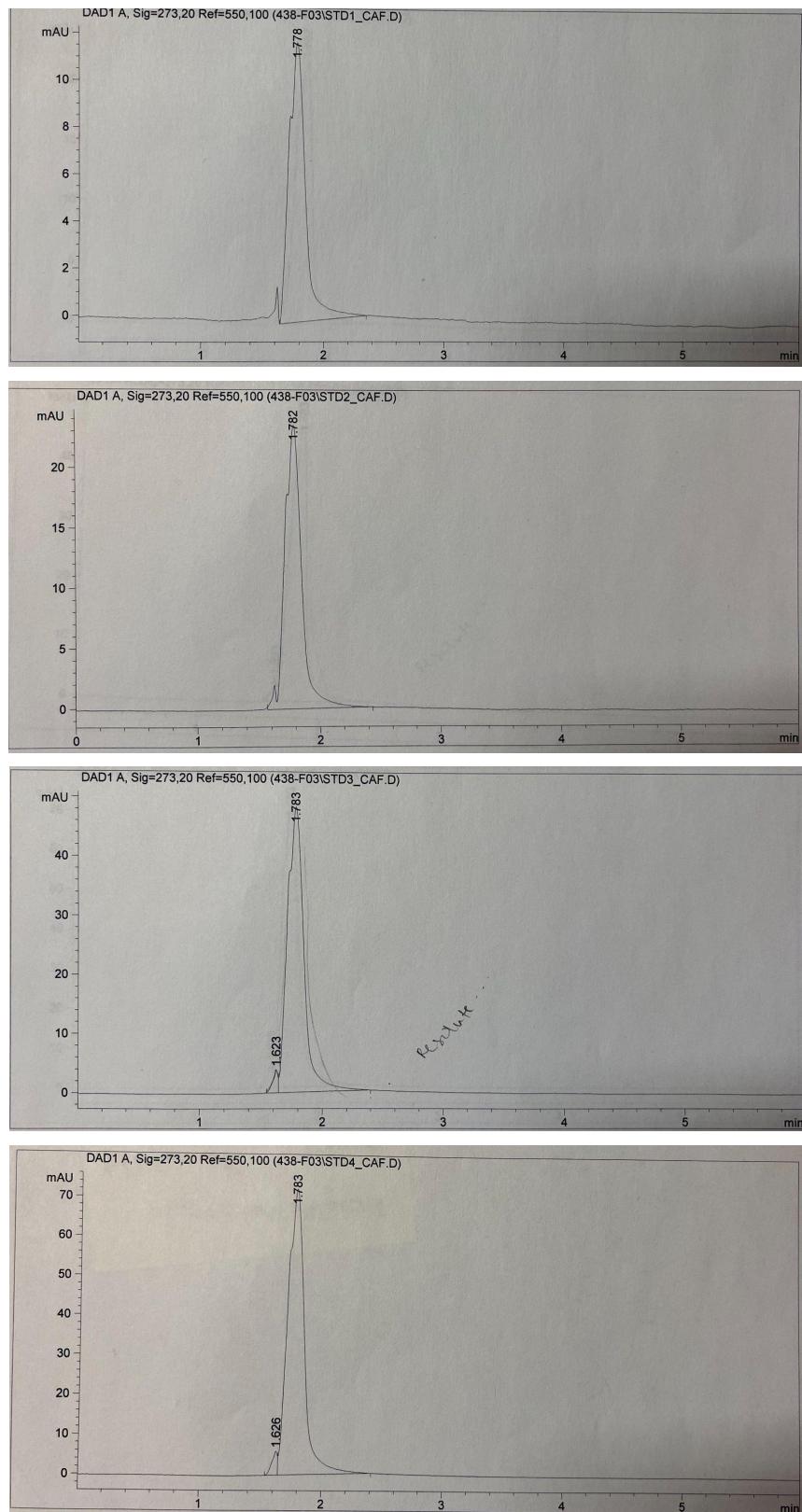


Figure B. HPLC spectrum of 4 standard solutions for caffeine at wavelength of 270 nm.

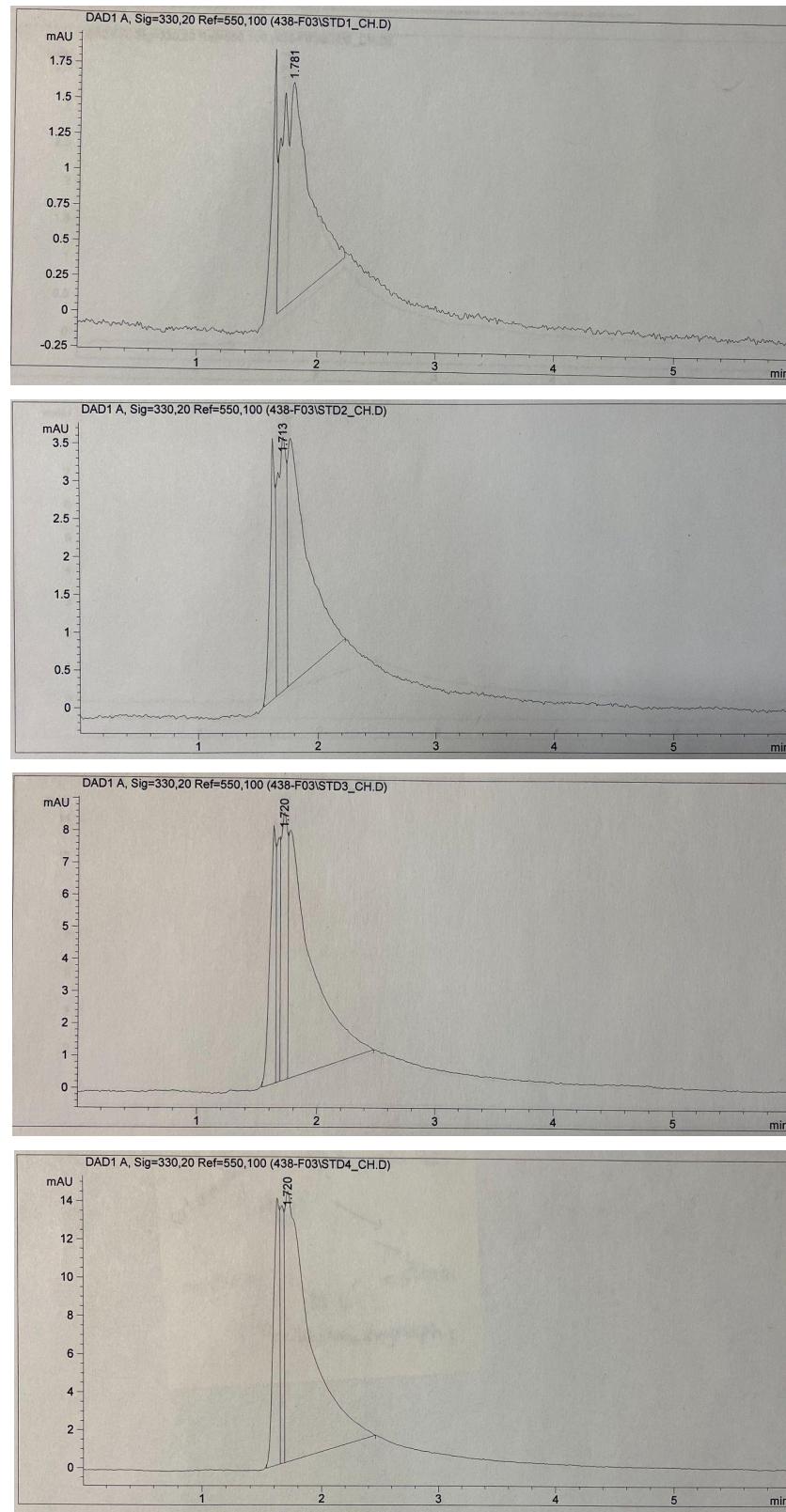


Figure C. HPLC spectrum of 4 standard solutions for chlorogenic acid at wavelength of 330 nm.

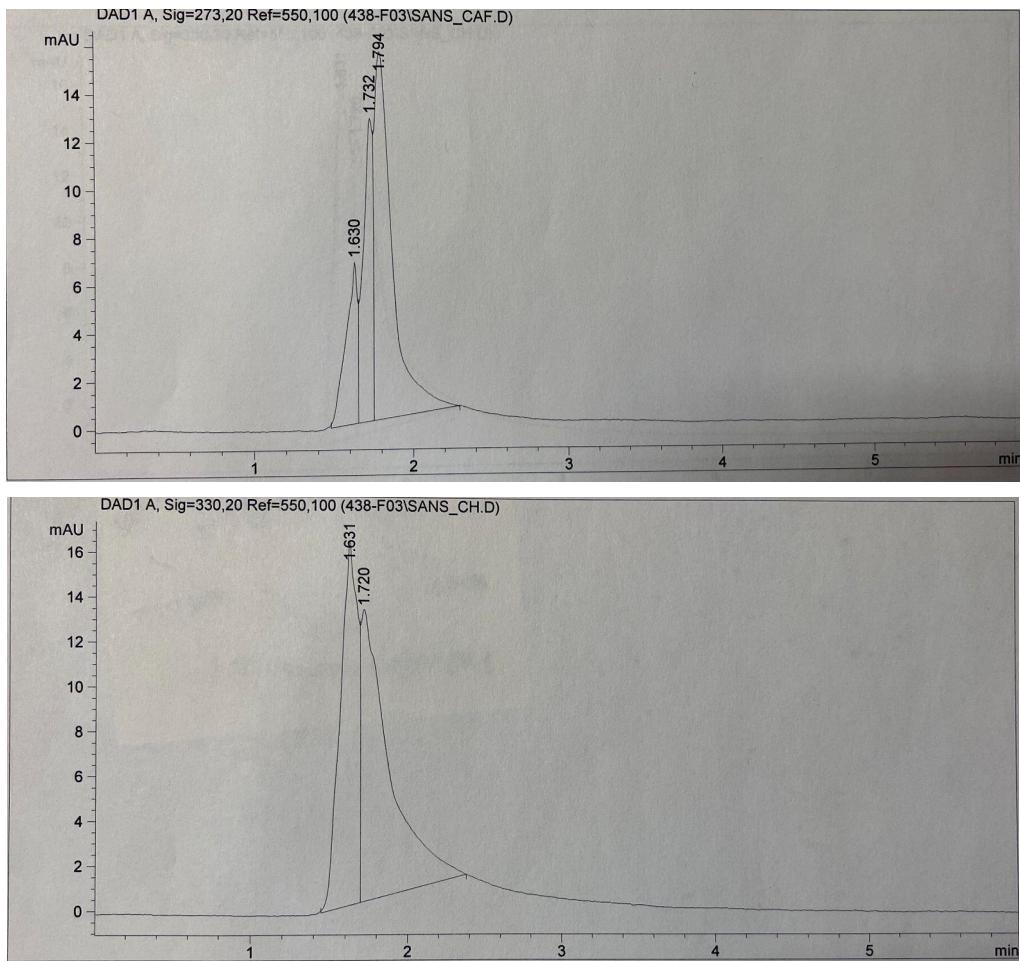


Figure D. HPLC spectrum of green beans measured at 270 nm (top) and 330 nm (bottom).