

SOP: Quantification of Caffeine in Coffee using HPLC

Approvals

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Date: 23OCT13

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Date: 30OCT13

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Date: 31OCT13

1. Purpose

- 1.1. Quantify concentration of caffeine in a coffee sample using isocratic reverse phase high performance liquid chromatography (RP-HPLC) configured with a C18 column and a UV-Vis detector set for 275 nm.

2. Scope and Applicability

- 2.1. High performance liquid chromatography (HPLC) is an analytical chemistry technique for separating the components of a liquid sample and for identifying and quantifying the components of the sample. This SOP uses an HPLC to quantify the caffeine concentration in generic brewed coffee by assaying a series of caffeine standards in order to construct a calibration curve, and then assaying coffee samples and calculating caffeine concentration against the calibration curve. This SOP provides the details of HPLC column selection, mobile phase solution preparation, sample preparation, flow rates, and run times. Refer to the SOPs listed below for step-by-step HPLC operation instructions.

3. Summary of Method

- 3.1. Prepare mobile phase solution
- 3.2. Prepare caffeine standards and coffee samples
- 3.3. Power up the HPLC system and equilibrate with mobile phase solution
- 3.4. Run an assay for each of the caffeine standards and coffee samples
- 3.5. Wash the system with mobile phase solution
- 3.6. Graph the calibration curve
- 3.7. Compute the caffeine concentration of the coffee samples
- 3.8. Power down the system

4. References

- 4.1. SOP: Buck Scientific BLC-20P HPLC Operation, document QCB 7, revision 0, effective
- 4.2. SOP: Degassing a Solution by Helium Sparge, document number QCB 6, revision 0, effective 25SEP13.

5. Definitions

<i>CV</i>	Column Volume; the volume (mL) of the column containing the stationary phase; CV=2.91 mL for a standard size (4.6 X 250 mm) column
<i>Equilibration</i>	Running the mobile phase solution through the column prior to injecting the sample in order to bring the system into equilibrium
<i>Flow rate</i>	The rate (mL/min) that solution is pumped through the column. The operating flow rate is determined by the assay protocol.
<i>Helium sparge</i>	Using a stream of helium bubbles to sweep dissolved air out of liquids (helium is virtually insoluble in most HPLC solvent solutions, so very little helium replaces the air)
<i>HPLC</i>	High Performance Liquid Chromatography

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<i>Isocratic</i>	The composition of the mobile phase solution is constant; the system has only one pump.
<i>Mobile phase</i>	The solvent solution used to carry the sample through the column
<i>PeakSimple</i>	Software used to collect and display data
<i>PSI</i>	Pounds per Square Inch
<i>Reverse phase chromatography</i>	Separation based on hydrophobicity under conditions where the stationary phase is more hydrophobic than the mobile phase.
<i>Stationary phase</i>	The chromatography matrix through which the sample travels.

6. Precautions

- 6.1. HPLC systems operate at high pressures. Personnel injury and equipment damage can result if maximum pressure is exceeded or the pump runs dry. Monitor pressure readings and solution level whenever the pump is running. If pressure exceeds 2500 psi or if the solution runs out, stop the pump immediately by pressing the RUN/STOP button. Do not set the flow rate higher than 1.5 ml/min with a 250 mm column.
- 6.2. Flow rate consistency is affected by the quality of the solutions. Use HPLC-grade solvents and filter solutions using a sub-micron filter (preferably 0.22 μm). Degas solutions prior to use.
- 6.3. To avoid microbial growth, do not leave the system in a high aqueous solution for a prolonged period. The system should be washed with a storage solution of 50% Methanol/H₂O or 50% Acetonitrile/H₂O if it is to be idle more than a few hours.
- 6.4. Methanol is flammable. Can cause blindness if swallowed. Vapor is harmful. Irritating to skin and eyes.

7. Responsibilities

- 7.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
- 7.2. It is the responsibility of the students/technician to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

8. Equipment and Materials

- 8.1. Buck Scientific BLC-20P HPLC system pre-configured with:
 - 8.1.1. UV-Vis detector
 - 8.1.2. PeakSimple Chromatography Data System
 - 8.1.3. Computer system with PeakSimple software installed
 - 8.1.4. Haisil 100 C18 5 μm 250 X 4.6mm HPLC column
- 8.2. HPLC-grade methanol
- 8.3. HPLC-grade water
- 8.4. Laboratory-grade caffeine
- 8.5. 2 different samples of generic-brewed regular coffee
- 8.6. Sample overflow waste beaker
- 8.7. Analytic balance
- 8.8. 250 mL or 500 mL graduated cylinder
- 8.9. 500 mL volumetric flask
- 8.10. 100 mL volumetric flask

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- 8.11. 1 mL volumetric pipette
- 8.12. Stirring plate
- 8.13. 2 – 500 mL laboratory bottles (for mobile phase solution and waste)
- 8.14. Small laboratory bottle (for mobile phase to rinse the sample syringe)
- 8.15. 100 mL laboratory bottle (for stock caffeine solution)
- 8.16. Nalgene Rapid-flow filtration unit
- 8.17. 2 – 0.45 μm or 0.22 μm syringe filters
- 8.18. 2 – small beakers (for filtered coffee)
- 8.19. 6 – microfuge tubes (for caffeine standards and diluted coffee samples)
- 8.20. 5 mL Luer-Lok syringe
- 8.21. 100 μL HPLC sample syringe
- 8.22. Parafilm
- 8.23. Timer

9. Procedure

- 9.1. Prepare 500 mL 50% methanol/H₂O mobile phase solution (also to be used as storage solution):
 - 9.1.1. Measure 250 mL HPLC-grade methanol using a graduated cylinder into a 500 mL volumetric flask.
 - 9.1.2. Bring to volume 500 mL with HPLC-grade H₂O. Cover with parafilm and invert to mix. Check the volume and repeat. (When methanol and water combine, the total volume may be slightly less than the sum of the original volumes.)
 - 9.1.3. Filter the mobile phase solution using a Nalgene Rapid-flow filtration unit. Transfer approximately 10 mL of mobile phase solution to a small labeled bottle to be used for rinsing the sample syringe. Transfer remaining solution to a labeled 500 mL laboratory bottle.
 - 9.1.4. Label an empty bottle as mobile phase solution waste.

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- 9.2. Prepare 100 mL 1000 ppm caffeine stock solution:
 - 9.2.1. Weigh 100 mg of caffeine on weighing paper or weighing boat using an analytic balance.
 - 9.2.2. Transfer caffeine to a 100 mL volumetric flask.
 - 9.2.3. Bring to volume 100 mL with mobile phase solution.
 - 9.2.4. Rinse a stir bar, insert it into the flask, cover the flask with parafilm, and mix on a stir plate for 10 minutes. Remove the stir bar.
 - 9.2.5. Filter the caffeine stock solution using the Nalgene Rapid-flow filtration unit. Transfer caffeine stock solution to a labeled 100 mL bottle.
- 9.3. Prepare 500 μ L each of 200, 100, 50, and 25 ppm caffeine standards in labeled microfuge tubes as a serial dilution from 1000 ppm caffeine stock solution and mobile phase solution using a micropipette. Pipette up and down, then cap and vortex to mix each standard.
- 9.4. Filter approximately 5 mL of each coffee sample into a small labeled beaker using a 0.45 μ m or 0.22 μ m syringe filter, one beaker and filter per sample.
- 9.5. Prepare 1000 μ L 20% coffee samples in labeled microfuge tubes by diluting the filtered coffee with mobile phase solution using a micropipette. Pipette up and down, then cap and vortex to mix each sample.
- 9.6. Degas the mobile phase solution per the Degassing a Solution SOP.
- 9.7. Power up the HPLC system and equilibrate with mobile phase solution for 30 minutes at the flow rate 0.5 mL/min:
 - 9.7.1. Power up the HPLC system components and start the PeakSimple data collection software per the HPLC Operation SOP.
 - 9.7.2. Switch the system to mobile phase solution per the HPLC Operation SOP.
 - 9.7.3. Purge the intake line and prime the pump per the HPLC Operation SOP.
 - 9.7.4. Start the pump and gradually increase the flow rate to 0.5 mL/min over 5 minutes per the HPLC Operation SOP.
 - 9.7.5. Set the UV-Vis detector wavelength to 275 nm and autozero the detector per the HPLC Operation SOP.
 - 9.7.6. Equilibrate the system with mobile phase solution at the flow rate 0.5 mL/min for 30 minutes per the HPLC Operation SOP. Monitor the UV-Vis detector readings.
 - 9.7.7. Ensure that the UV-Vis detector warms up for 60 minutes prior to collecting data per the HPLC Operation SOP.
- 9.8. For each of the 25, 50, 100 and 200 ppm caffeine standards, run an assay for 10 minutes at 0.5 mL/min per the HPLC Operation SOP:
 - 9.8.1. Use PeakSimple to start a new 10 minute run.
 - 9.8.2. Autozero the UV-Vis detector.
 - 9.8.3. Load and inject a caffeine standard.
 - 9.8.4. Operate the pump for 10 minutes at 0.5 mL/min.
 - 9.8.5. Note the time at the center of the caffeine peak on the chromatograph.
 - 9.8.6. Save the data to a separate chromatogram file.
 - 9.8.7. View the results and copy the data to a separate sheet in an Excel workbook.

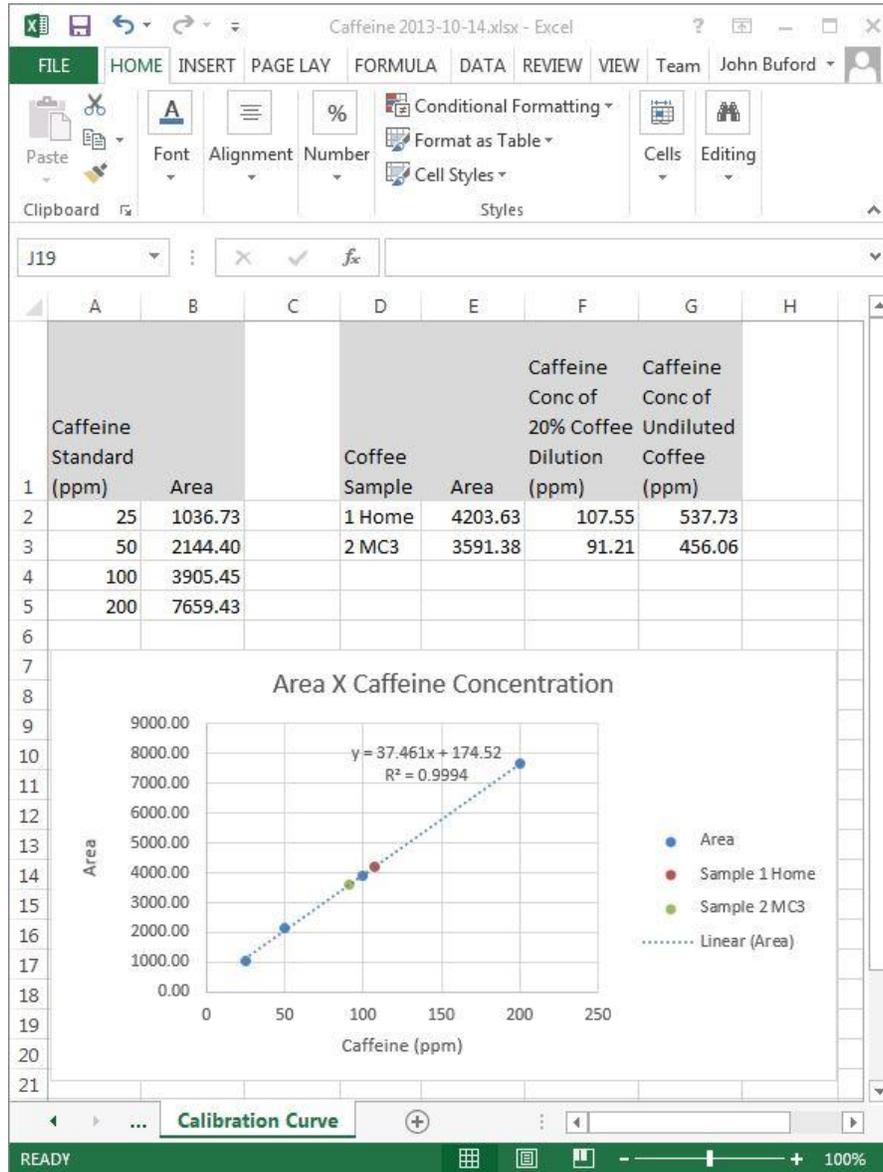
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- 9.9. For each of the coffee samples, run an assay for 10 minutes each at 0.5 mL/min per the HPLC Operation SOP:
 - 9.9.1. Use PeakSimple to start a new 10 minute run.
 - 9.9.2. Autozero the UV-Vis detector.
 - 9.9.3. Load and inject a coffee sample.
 - 9.9.4. Operate the pump for 10 minutes at 0.5 mL/min.
 - 9.9.5. Identify the caffeine peak on the chromatograph at the time noted above.
 - 9.9.6. Save the data to a separate chromatogram file.
 - 9.9.7. View the results and copy the data to a separate sheet in an Excel workbook.
- 9.10. Wash the system with mobile phase solution for 30 minutes at the flow rate 0.5 mL/min.
- 9.11. Graph the calibration curve using Excel:
 - 9.11.1. Add a new sheet to the Excel workbook with the columns "Caffeine Standard" and "Area".
 - 9.11.2. Fill in the Caffeine Standard column with the values 25, 50, 100, and 200.
 - 9.11.3. Fill in the Area column with the peak area values from the corresponding caffeine standard data collected above.
 - 9.11.4. Use a scatter graph to display Caffeine Standard values on the x-axis and Area values on the y-axis.
 - 9.11.5. Add a linear trendline to the graph, displaying the equation and R-squared value on the graph.
- 9.12. Compute the caffeine concentration of the coffee samples using the Excel TREND function.
- 9.13. Stop the pump and allow the pressure to decrease to 0 per the HPLC Operation SOP.
- 9.14. Power down the system per the HPLC Operation SOP.

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10. Attachments

Figure 1. Example Calibration Curve



11. History

Revision Number	Effective Date	Preparer	Description of Change
0	31OCT13	John Buford	Initial release