Determination of Caffeine by Solid Phase Extraction and High Performance Liquid Chromatography (SPE – HPLC)

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Introduction:

This experiment is designed to introduce the use of the technique of Solid Phase Extraction (SPE) to clean up samples and the technique of HPLC for performing a separation and quantification of caffeine in beverages.

Beverages consist of many components that cause chromatographic interferences with caffeine. For this reason the sample treatment is done by SPE with Bakerbond C-18 sorbent (500 mg) cartridges that enable separation of caffeine and remove most of the interfering components.

Solid-phase extraction (SPE) is a separation technique used to extract compounds from a mixture of impurities. SPE is used to concentrate and purify samples for analytes of interest from several matrices. The separation ability of solid phase extraction is based on the preferential affinity of desired analyte, usually, to a solid phase through which the test sample is passed. The solid phase is selected so that the impurities in the sample are un-retained on the solid phase (adsorbent/stationary phase) while the analyte of interest is retained on it. Analytes that are retained on the stationary phase can then be eluted from the solid phase extraction cartridge with the appropriate solvent.

HPLC High-performance liquid chromatography (HPLC) is a separation technique used to separate components of a mixture from each other by taking advantage of a variety of physio-chemical interactions of analytes in the mixture between two phases. One phase is held stationary in a column while the mixture to be analyzed is introduced into another phase that is moved over the stationary phase.

All standards and replicate samples will be analyzed using reverse phase HPLC with a filtered and degassed mobile phase and absorbance detection. Quantitation will be done by first generating a calibration curve prepared using known caffeine standards run under the same conditions as the samples. Before preparing the calibration curve, separations conditions must be optimized to ensure that the caffeine peak is adequately resolved from other components in the sample in minimal time.

Optimization of the method involves changing parameters like mobile phase composition and flow rate until the required separation quality is reached.

Prior to start of the separation, a detection wavelength must be chosen to give optimum sensitivity for your caffeine analyte. Using a UV-Vis spectrophotometer, collect a qualitative spectrum of caffeine and determine the optimum wavelength for the LC detector. Once a detection wavelength has been set, separations can begin. Choose an initial flow rate in the range of 0.5-1.5 mL/min and an initial mobile phase composition in the range given below. Sample sizes should be consistent and should be within the range of 5-50 µL. Be sure to record all

conditions. Allow at least 5 minutes for the column to equilibrate to the solvent (conditioning) and inject the highest concentration caffeine standard,

Chemicals and Solutions:

1. A caffeinated beverage or dry soluble coffee (for analysis)
2. HPLC grade methanol, acetonitrile and methanol (for mobile phase)
3. 18 MΩ resistive water
4. Caffeine aqueous stock standard solution at 1000 μg/mL (ppm).

HPLC mobile phase and solvent: 50 mM potassium dihydrogen phosphate (pH=2), acetonitrile and methanol (40:8:2) which strictly is also the solvent for standards and 'test' material (filter a sufficient volume of all solutions with 0.45μm syringe filter before injections).

Method:

Preparation of Standards:

Dilute the stock solution appropriately with the *mobile phase* to make 10.00 mL each of 10, 20, 30, 40 and 50 ppm caffeine standard solutions.

Preparation of the Beverage Laboratory Sample.

Obtain sample of the 'test' material (commercial instant coffee) containing caffeine. A measured mass of the dry coffee sample will be used to make a laboratory sample of it after cleaning the sample by Solid Phase Extraction (SPE), as follows.

Measure out *accurately* ~0.1g of the dry caffeine containing sample in a 50mL beaker. Pipette 10.00mL of de-ionized water into the 50mL beaker and dissolve the measured sample (may use the sonicator). Filter *all* of the resulting solution with a 0.45μm filter; the filtrate would be the laboratory sample.

Conditioning the SPE sorbent: Conditioning step - activate an SPE cartridge stationary phase, the sorbent (Bakerbond – C18) to by passing 6mL 50% methanol through the sorbent slowly; use a vacuum manifold. Make sure to keep the sorbent wet. *Turn off the vacuum as methanol level approaches the top of packing.*

Equilibration - pass 3mL of water through the packing; keep the sorbent wet and turn off the vacuum as water level approaches the top of packing.

Loading - Introduce 1.00mL (micro-pipette) of the laboratory sample made above to the *preconditioned* SPE cartridge/tube/column and apply a vacuum, *make sure to keep the sorbent wet*. Do not discard the collected filtrate.

Move the SPE tube to a different location with a test tube set up to collect the filtrate. Reintroduce the above filtrate into the same SPE tube. Apply a vacuum, this time draw all liquid through. Draw air through the SPE tube for 3-5 minutes until the sorbent is dry.

Elution – Move the SPE tube with the adsorbed analyte (caffeine) from the SPE column to a new location in the manifold setup with clean dry test tube. Introduce 10 mL (5mL×2 aliquots) of ethyl acetate (eluate) into the SPE tube, apply a vacuum slowly, and collect the eluate in the fresh clean dry test tube.

Transfer all of the solution from the preceding step into a 50mL clean, dry round bottom flask quantitatively. This may require collecting of a few ethyl acetate washings into the 50mL flask.

Dry the solution thoroughly using a rotor-evaporator; re-dissolve the residue in 10.00 mL (pipette) of the mobile phase of the HPLC to be used for the separation. Filter the solution (≤0.45μ). This is the laboratory sample to be chromatographed.

Chromatographic Analysis:

HPLC Parameters
Mobile phase: 50 mM potassium dihydrogen phosphate (pH=2): acetonitrile: methanol (40:8:2)
Column: Phenomenex MAXSIL 5 RP-2; 250×4.60mm
Flow rate; 0.8 mL/min
Pressure: 1400psi.
UV detector wavelength: 254nm
Injection volume: 50μL

Obtain ~3mL of the standard into a 5mL syringe. Introduce about 2mL of the standard into the sample injector of the HPLC via a filter disc (≤0.45μ). Run the chromatogram (takes ~9 min to elute). Record the retention time (t_r), peak height (h) and peak area (A) for the caffeine peak. Repeat the procedure with the rest of the standards. Transfer ***all*** HPLC output data on to a flash drive.

Next, inject the prepared laboratory sample of the beverage and note the quality of the separation of the caffeine peak from any other components in the sample.

If the caffeine peak is completely resolved, record the peak t_r, h, and A. If the caffeine peak height from the sample is larger than the 50 ppm standard, the beverage sample is too concentrated. Dilute quantitatively to bring the concentration into the calibration range and re-inject the diluted sample. (triplicate).

Treatment of Results:

Generate calibration plot(s) using peak areas and/or peak heights (as dependent variable) vs. concentration of the standards. Use the calibration plot with the least square fit to determine a) the concentration of caffeine in the analyzed laboratory sample and b) mass% of caffeine the original solid coffee sample.

Report the concentration and the confidence limits at 95% confidence level.