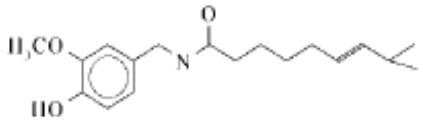
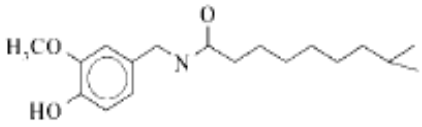
High Performance Liquid Chromatography

I. Introduction

In the last twenty years, the development of low-cost, high performance pumps, efficient columns and versatile detector interfaces has made liquid chromatography an essential tool in any analytical laboratory. Applications cover wide-ranging areas, including biochemistry, environmental analysis, and food science. For this experiment, you will analyze peppers for their capsaicinoid content using high performance liquid chromatography HPLC. Capsaicinoids are the compounds responsible for the pungency (i.e., spiciness) of pepper fruits and their products. Capsaicin and dihydrocapsaicin comprise over 90% of the "heat" in peppers; their structures are shown below. The degree of hotness of spicy foods is typically reported in Scoville Heat Units (SHU). The various capsaicin compounds have been assigned reference SHU values, corresponding to the pure component. The capsaicinoids in your samples will be separated using reversed-phase conditions and will be detected spectrophotometrically. By comparing the results for capsaicin standards to those for your samples, a quantitative measurement will be made, and using the reference SHU for each compound, the total Scoville Heat Value (SHV) will be calculated.



capsaicin



dihydrocapsaicin

II. Equipment

A. Hewlett Packard Series 1050 HPLC System with Diode-Array Detector and HP ChemStation Computer Interface
B. 25-µL **blunt-nose** syringe (obtain from the instructor)
C. 0.45-µm filter disks (obtain from the instructor)
D. 10-mL plastic syringes (obtain from the instructor)
E. 30-mL glass vials (obtain from the instructor)
F. Mortar and pestle
G. 50-mL centrifuge tubes (obtain from the instructor)

III. Reagents

A. HPLC grade water and methanol
B. Stock capsaicin solution (65% capsaicin and 35% dihydrocapsaicin)
C. Pepper sample (habanero or jalapeño)
D. Liquid N_2

IV. Procedure

A. Unknown Preparation

1. Weigh about 10 g of pepper and place it in a mortar. Add liquid N_2 to the mortar and allow the pepper to freeze. Crush the pepper using the pestle and grind until it becomes a powder.

2. Weigh a 50-mL flat-bottom centrifuge tube (orange cap) and mini stir bar.

3. Place the powdered pepper into the centrifuge tube and weigh.

4. Add 35 mL of methanol to the tube and stir for about 1hr.

5. Refrigerate your labeled centrifuge tube until the next lab period. Reweigh the tube plus its contents.

6. Filter about 5 mL of the supernatant through a 0.45-µm filter.

B. Standard Preparation

1. A stock solution of capsaicin (in methanol) will be provided. Calculate the concentration of your stock solution in ppm ($d_{methanol}$ = 0.791 g/mL). Using successive dilutions, prepare four standards (in HPLC-grade methanol) between approximately 10 and 100 ppm. If necessary, store these solutions in a labeled beaker in the refrigerator until the next lab period.

2. Your instructor will demonstrate the use of the chromatograph and ChemStation software. Ensure that the “chem422" method is loaded. Take careful note of the mobile phase composition, flow rate, and the detection wavelength (write these down in your lab notebook).

3. Fill the sample loop with the 100-ppm capsaicin standard and inject the sample. When the run is completed, run a replicate sample. Note and compare the retention times and areas of the observed peaks. Examine the UV spectra acquired during the elution of the capsaicin and dihydrocapsaicin peak and print out a representative sample of each for either run. Compare the detection wavelength with the maxima in these spectra. Note which capsaicinoid elutes first.

4. Run each the remaining standards once. (Note: It is not necessary to print UV spectra for each standard concentration.)

C. Quantitative Capsaicin Analysis

1. Run the filtered extract (IV.A.6.) using the same HPLC conditions as for the standards.

2. If the extract peak area(s) of capsaicinoid(s) are greater than that of the highest concentration standard, then dilute the pepper extract appropriately and run the sample again.

3. Run a replicate sample of the appropriately diluted extract.

V. Treatment of Data

A. Tabulate the standard concentrations, standard peak areas and retention times.

B. Prepare a calibration curve based on the capsaicin standards and another one based on the dihydrocapsaicin standards.

C. Report the capsaicin and dihydrocapsaicin peak areas, retention times and calculated concentrations in your pepper sample (g capsaicinoid/g sample) based on the standard calibration curves.

D. Calculate the SHU for each capsaicinoid in your pepper on the basis of dry weight (assume 85% water content). The SHU for pure capsaicin and dihydrocapsaicin are 1.6×10^7 each. Multiply this factor by the capsaicinoid concentration (g/g dry weight) to yield the SHU.

E. Calculate the Scovile Heat Value for your pepper sample by adding the SHUs for capsaicin and dihydrocapsaicin.

F. Include with your report the standard calibration curves, a representative chromatogram for the standards and the unknown, representative UV spectra for the standard and unknown peaks and a complete description of the analysis conditions.

VI. Questions

1. Provide a rationale for the relative elution order of capsaicin and dihydrocapsaicin. Does this order make sense with regard to the chromatography conditions used?

2. How does the ratio of capsaicin to dihydrocapsacin in your pepper sample compare to that in the standard.

Lab Cautions for High Performance Liquid Chromatography

1. If water is added to its mobile phase reservoir, purge this reservoir with argon for at least 5 minutes. Use only HPLC-grade solvents in the reservoirs.

2. The capsaicin standard in higher concentrations can cause irritation. Wear appropriate eye protection and be very careful with it.

3. Liquid N_2 can cause freeze burns and must be handled with care. Always wear appropriate eye protection and avoid direct contact with anything contacting the liquid nitrogen.

4. AFTER COMPLETION OF THE EXPERIMENT

a. Check the level of the mobile phase component reservoirs. If the level is low for any of the components, notify your lab instructor.

b. TURN OFF THE PUMP AND DETECTOR.

c. Recover all sample vials.

d. Return all items borrowed from the instructor.