Name \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

 **Some Like It Hot: HPLC Determination of Capsaicinoids in Hot Peppers**

**Introduction**

The “hotness” of some peppers comes from a family of organic molecules contained in peppers known as capsaicinoids. In this lab, you will use liquid chromatography to quantify the amounts of capsaicinoids in three different foods: hot sauce, puréed fresh chili pepper, and ground dried chili pepper. The three capsaicinoid compounds you will quantify (molecular structures shown below) are: nordihydrocapsaicin, capsaicin, dihydrocapsaicin. Prior to lab, you will qualitatively estimate the “hotness” of each food using the Scoville Scale. During the lab, you will compare your qualitative estimate to the quantitative Scoville value determined by liquid chromatography.



*Capsaicinoids and the Scoville Scale*

The Scoville Scale was developed by pharmacist Wilbur Scoville in the early 20th century. The “hotness” of peppers is rated on this scale using Scoville Heat Units (SHUs). A pepper with no “hotness,” such as a bell pepper, has an SHU of 0. The hottest known pepper in the world is the Carolina Reaper with an SHU of 2,200,000. Traditionally, Scoville ratings are determined by a panel of tasters. As such, they are largely subjective.

Quantitative high pressure liquid chromatography (HPLC) analysis of capsaicinoids assigns Scoville ratings in a more objective manner. Capsaicinoids are first extracted from a known mass of a food using a known volume of ethanol solution. The concentration of each capsaicinoid in the liquid extract is then determined by HPLC analysis. Sample volume (mL) and concentration (μg/mL = ppm) are used to determine the mass of capsaicinoid compound per gram of food (μg/g = ppm). Once this value is known for all three capsaicinoid compounds, the SHU for a food is calculated as follows:

SHU = N + C + D *(Equation 1)*

where N = (μg of nordihydrocapsaicin/g of food) x 9.3

 C = (μg of capsaicin/g of food) x 16.1

 D = (μg of dihydrocapsaicin/g of food) x 16.1

The uncertainty of the SHU value for a food is determined using rules of error propagation for sums and differences: The uncertainty of a sum or difference is the sum of the individual uncertainties of the values being added or subtracted.

*HPLC Analysis of Capsaicinoids*

In this lab, the concentration of three capsaicinoid compounds will be determine using reverse-phase HPLC analysis with gradient elution and UV detection. An autosampler will be used. The three capsaicinoids elute in the following order: nordihydrocapsaicin, capsaicin, dihydrocapsaicin. The HPLC is run under the conditions shown below for all standards and samples. The HPLC routine for these conditions is stored as Method X.

|  |  |
| --- | --- |
| Column: | 150 mm x 4.6 mm C-18 |
| Mobile Phase A: | 0.5 % (v/v) phosphoric acid |
| Mobile Phase B: | acetonitrile (HPLC grade) |
| Gradient Elution: | 50 – 80 % acetonitrile in 12 minutes |
| Detector: | UV at 205 nm |

*Calculating Uncertainty of HPLC-Determined Capsaicinoid Concentrations*

The uncertainty in the concentration of a sample (*sx*) determined using a calibration curve depends on the number of data points used for the calibration curve and their precision, the slope of the least squares regression line, and the number of replicate measurements made on the unknown. It is calculated using the following equation:

$$s\_{x}=\frac{s\_{y}}{\left|m\right|}\sqrt{\frac{1}{k}+\frac{1}{n}+\frac{(y-\overbar{y})^{2}}{m^{2}\sum\_{}^{}(x\_{i}-\overbar{x})^{2}}} (Equation 2)$$

where $m$ = slope of least squares regression line

 $k$ = number of replicates of sample analyzed

 $n$ = number of data points on calibration curve

 $y$ = average of corrected peak areas for the sample

 $\overbar{y}$ = average of corrected peak areas for standards used\*

 $x\_{i} $= concentration of each standard used\*

 $\overbar{x} $= average concentration of standards used\*

sy is the standard deviation of the vertical deviations in each standard, yi, from the ordinate point y (where x = xi) on the least squares regression line. It is calculated using the following formula:

$$s\_{y}=\sqrt{\frac{\sum\_{}^{}(y\_{i}-mx\_{i}-b)^{2}}{n-2}} (Equation 3)$$

where $y\_{i}$ = corrected peak area for each standard used\*

 $m$ = slope of the least squares regression line

$x\_{i }$= concentration of each standard used\*

 $b$ = y-intercept of the least squares regression line

 $n$ = number of data points for calibration curve

\*It is important to include only values of standards actually used to construct the calibration curve. Any standards data excluded from the calibration curve (e.g. outliers) should not be included in these calculations.

These calculations are best done using an Excel spreadsheet because sx is calculated for every sample concentration determined. Samples are run in triplicate (see **Experimental Procedure** below). This means that three sx calculations are necessary for each sample.

 **Safety**

Have you ever touched your skin, eyes, nose, or other sensitive areas after handling hot peppers? If so, you know that it stings and burns, sometimes severely! This lab is no different. The capsaicinoids in the hot sauce, puréed fresh chili pepper, and ground dried chili pepper that you will be handling during the extraction are irritants to the skin and eyes. This is also true of the 1000 ppm standard solution containing the three capsaicinoids nordihydrocapsaicin, capsaicin, and dihydrocapsaicin. **To avoid getting concentrated capsaicinoid compounds on your skin or in your eyes, you must wear goggles and gloves at all times.** You may want to consider a lab coat for skin protection.

 **Experimental Procedure**

 Part I. Capsaicinoid Extraction

1. Weigh 10 g of hot sauce, 5 g of puréed fresh chili pepper (fresh chili), and 2 g of ground chili pepper (dry chili). Record the exact weights in Data Table VII.
2. Place each sample in a separate 50 mL Erlenmeyer flask with 15 mL of 95 % HPLC grade ethanol and cover each flask with a watch glass.
3. Extract samples by heating on a hot plate inside a fume hood. Heat samples to a gentle boil while continuously stirring. Once a gentle boil is reached, cover with a watch glass to minimize ethanol evaporation and heat for an additional 30 minutes.
4. Cool the samples to room temperature.
5. Transfer cooled samples into a 25 mL volumetric flask by gravity filtration through filter paper. Rinse three times with ethanol to ensure full sample transfer and complete the volume to 25 mL with ethanol. (If storing samples for analysis on another day, place them in a refrigerator.)

 Part II. HPLC Quantification of Capsaicinoids

1. Prepare and clearly label three blanks in autosampler vials by filtering HPLC grade 95 % ethanol directly into vials through 0.45 μm syringe filters.
2. Use volumetric flasks to prepare five standard concentrations (5 ppm, 10 ppm, 25 ppm, 50 ppm, 100 ppm) from the 1000 ppm (or μg/mL) standard solution containing the capsaicinoid compounds. **The exact concentration of the stock solution might be slightly different than 1000 ppm. Note this and use it to calculate the exact concentration of each standard.** Enter the exact standard concentrations into the blanks provided at the heads of Data Tables II through VI. **Use the HPLC grade 95 % ethanol, NOT distilled water, as your solvent.** Once prepared, you should have a total of five volumetric flasks.
3. Standards are run in triplicate, so prepare and clearly label three autosampler vials for each standard concentration. Filter each standard directly into autosampler vials through 0.45 μm syringe filters. **Be sure to push some of each standard through the filter and into a waste container first to initially rinse the filters.** Also, **use a new 0.45 μm syringe filter for each standard**. Once prepared, you should have fifteen autosampler vials for your standards.
4. Obtain the three samples extracted in Part I (hot sauce, fresh chili, and dried chili). Filter them through 0.45 μm syringe filters directly into autosampler vials, but **be sure to push some extract through the filter and into a waste container first to initially rinse the filters**. Also, **use a new 0.45 μm syringe filter for each sample type**. Samples are run in triplicate, so you should have nine autosampler vials once all samples are prepared.
5. When all standards and samples are prepared, take them to the laboratory technician for analysis on the HPLC.

 **Data Tables**

Data Table I. Peak Areas for Reagent Blanks

|  |  |  |
| --- | --- | --- |
| *Blank Concentration**(ppm or ug/L)* | *Replicate* | *Peak Area* |
| 0 ppm | 1 |  |
| 0 ppm | 2 |  |
| 0 ppm | 3 |  |

Average Reagent Blank Peak Area \_\_\_\_\_\_\_\_\_\_

For Data Tables II through VI below, subtract the Average Reagent Blank Peak Area from the Peak Area of each standard to obtain the Corrected Peak Area.

Data Table II. Calibration Curve, 5 ppm standard, Exact Concentration \_\_\_\_\_\_\_\_\_\_\_

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Standard Concentration**(ppm or ug/L)* |  *Replicate* | *Capsaicinoid Compound* | *Peak Area* | *Corrected Peak Area*  |
| 5 ppm | 1 | nordihydrocapsaicin |  |  |
| 5 ppm | 1 | capsaicin |  |  |
| 5 ppm | 1 | dihydrocapsaicin |  |  |
| 5 ppm | 2 | nordihydrocapsaicin |  |  |
| 5 ppm | 2 | capsaicin |  |  |
| 5 ppm | 2 | dihydrocapsaicin |  |  |
| 5 ppm | 3 | nordihydrocapsaicin |  |  |
| 5 ppm | 3 | capsaicin |  |  |
| 5 ppm | 3 | dihydrocapsaicin |  |  |

Data Table III. Calibration Curve, 10 ppm standard, Exact Concentration \_\_\_\_\_\_\_\_\_\_\_

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Standard Concentration**(ppm or ug/L)* |  *Replicate* | *Capsaicinoid Compound* | *Peak Area* | *Corrected Peak Area*  |
| 10 ppm | 1 | nordihydrocapsaicin |  |  |
| 10 ppm | 1 | capsaicin |  |  |
| 10 ppm | 1 | dihydrocapsaicin |  |  |
| 10 ppm | 2 | nordihydrocapsaicin |  |  |
| 10 ppm | 2 | capsaicin |  |  |
| 10 ppm | 2 | dihydrocapsaicin |  |  |
| 10 ppm | 3 | nordihydrocapsaicin |  |  |
| 10 ppm | 3 | capsaicin |  |  |
| 10 ppm | 3 | dihydrocapsaicin |  |  |

Data Table IV. Calibration Curve, 25 ppm standard, Exact Concentration \_\_\_\_\_\_\_\_\_\_

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Standard Concentration**(ppm or ug/L)* |  *Replicate* | *Capsaicinoid Compound* | *Peak Area* | *Corrected Peak Area*  |
| 25 ppm | 1 | nordihydrocapsaicin |  |  |
| 25 ppm | 1 | capsaicin |  |  |
| 25 ppm | 1 | dihydrocapsaicin |  |  |
| 25 ppm | 2 | nordihydrocapsaicin |  |  |
| 25 ppm | 2 | capsaicin |  |  |
| 25 ppm | 2 | dihydrocapsaicin |  |  |
| 25 ppm | 3 | nordihydrocapsaicin |  |  |
| 25 ppm | 3 | capsaicin |  |  |
| 25 ppm | 3 | dihydrocapsaicin |  |  |

Data Table V. Calibration Curve, 50 ppm standard, Exact Concentration \_\_\_\_\_\_\_\_\_\_\_

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Standard Concentration**(ppm or ug/L)* |  *Replicate* | *Capsaicinoid Compound* | *Peak Area* | *Corrected Peak Area*  |
| 50 ppm | 1 | nordihydrocapsaicin |  |  |
| 50 ppm | 1 | capsaicin |  |  |
| 50 ppm | 1 | dihydrocapsaicin |  |  |
| 50 ppm | 2 | nordihydrocapsaicin |  |  |
| 50 ppm | 2 | capsaicin |  |  |
| 50 ppm | 2 | dihydrocapsaicin |  |  |
| 50 ppm | 3 | nordihydrocapsaicin |  |  |
| 50 ppm | 3 | capsaicin |  |  |
| 50 ppm | 3 | dihydrocapsaicin |  |  |

Data Table VI. Calibration Curve, 100 ppm standard, Exact Concentration \_\_\_\_\_\_\_\_\_

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Standard Concentration**(ppm or ug/L)* |  *Replicate* | *Capsaicinoid Compound* | *Peak Area* | *Corrected Peak Area*  |
| 100 ppm | 1 | nordihydrocapsaicin |  |  |
| 100 ppm | 1 | capsaicin |  |  |
| 100 ppm | 1 | dihydrocapsaicin |  |  |
| 100 ppm | 2 | nordihydrocapsaicin |  |  |
| 100 ppm | 2 | capsaicin |  |  |
| 100 ppm | 2 | dihydrocapsaicin |  |  |
| 100 ppm | 3 | nordihydrocapsaicin |  |  |
| 100 ppm | 3 | capsaicin |  |  |
| 100 ppm | 3 | dihydrocapsaicin |  |  |

Data Table VII. Sample Data

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| *Sample Type* | *Sample**Weight (g)* | *Replicate* | *Capsaicinoid Compound* | *Peak Area* | *Corrected Peak Area* |
| Hot sauce |  | 1 | nordihydrocapsaicin |  |  |
| Hot sauce |  | 1 | capsaicin |  |  |
| Hot sauce |  | 1 | dihydrocapsaicin |  |  |
| Hot sauce |  | 2 | nordihydrocapsaicin |  |  |
| Hot sauce |  | 2 | capsaicin |  |  |
| Hot sauce |  | 2 | dihydrocapsaicin |  |  |
| Hot sauce |  | 3 | nordihydrocapsaicin |  |  |
| Hot sauce |  | 3 | capsaicin |  |  |
| Hot sauce |  | 3 | dihydrocapsaicin |  |  |
| Fresh chili |  | 1 | nordihydrocapsaicin |  |  |
| Fresh chili |  | 1 | capsaicin |  |  |
| Fresh chili |  | 1 | dihydrocapsaicin |  |  |
| Fresh chili |  | 2 | nordihydrocapsaicin |  |  |
| Fresh chili |  | 2 | capsaicin |  |  |
| Fresh chili |  | 2 | dihydrocapsaicin |  |  |
| Fresh chili |  | 3 | nordihydrocapsaicin |  |  |
| Fresh chili |  | 3 | capsaicin |  |  |
| Fresh chili |  | 3 | dihydrocapsaicin |  |  |
| Dried chili |  | 1 | nordihydrocapsaicin |  |  |
| Dried chili |  | 1 | capsaicin |  |  |
| Dried chili |  | 1 | dihydrocapsaicin |  |  |
| Dried chili |  | 2 | nordihydrocapsaicin |  |  |
| Dried chili |  | 2 | capsaicin |  |  |
| Dried chili |  | 2 | dihydrocapsaicin |  |  |
| Dried chili |  | 3 | nordihydrocapsaicin |  |  |
| Dried chili |  | 3 | capsaicin |  |  |
| Dried chili |  | 3 | dihydrocapsaicin |  |  |

**Calculations**

1. Use Excel and the Corrected Peak Area data from Data Tables II through VI to plot a separate calibration curve for each of the three capsaicinoid compounds (nordihydrocapsaicin, capsaicin, and dihydrocapsaicin).
2. Are there data points you will exclude from the calibration curves? **Explain why or why not**.
3. After excluding any data points, display the least squares regression equation on each graph. **Print all three graphs and hand them in with your lab report.**
4. Use the Corrected Peak Area data for your samples from Data Table VII to calculate the Average Peak Area for each compound in each of the three samples. Enter these values into Results Table I.
5. Use the least squares regression equation from the calibration curve for each capsaicin compound to calculate the Concentration of each compound in each of the three samples. Enter these values into Results Table I.
6. Use Equations 2 and 3 to calculate the uncertainty in the concentration of each compound in each of the three samples. Enter these values into Results Table I.
7. Write the qualitative SHV number that you estimated in Pre-lab Question 1 into Results Table II. Use Equation 1 to calculate the quantitative Scoville Heat Value for the three foods analyzed. Enter these values into Results Table II.
8. Use the rules of error propagation for sums and differences to estimate the uncertainty in the quantitative SHV value for each of the three food analyzed. Enter these values into Results Table II.

**Results and Discussion**

Results Table I.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *Sample Type* | *Capsaicinoid Compound* | *Average Peak Area* | *Concentration**(ppm or ug/L)* | *Uncertainty in Concentration (sx)* |
| Hot Sauce | nordihydrocapsaicin |  |  |  |
| Hot Sauce | capsaicin |  |  |  |
| Hot Sauce | dihydrocapsaicin |  |  |  |
| Fresh Chili | nordihydrocapsaicin |  |  |  |
| Fresh Chili | capsaicin |  |  |  |
| Fresh Chili | dihydrocapsaicin |  |  |  |
| Dried Chili | nordihydrocapsaicin |  |  |  |
| Dried Chili | capsaicin |  |  |  |
| Dried Chili | dihydrocapsaicin |  |  |  |

Results Table II. Comparison of Qualitative and Quantitative Scoville Heat Units

|  |  |  |  |
| --- | --- | --- | --- |
| *Sample* | *Qualitative SHU* | *Quantitative SHU* | *Uncertainty in Quantitative SHU* |
| Hot sauce |  |  |  |
| Fresh chili |  |  |  |
| Dried chili |  |  |  |

*Discussion Questions*

1. The uncertainty in the concentration of some capsaicinoid compounds in your samples is likely to be higher or lower than others.
2. Which capsaicinoids have higher or lower uncertainty compared to others?
3. Explain why the uncertainty is higher or lower than the others.
4. (a) Describe how your qualitative SHU estimates compared to the quantitatively determined SHU values.

(b) Which method for determining SHU is more accurate? Explain.

1. Compare your quantitative SHU values with those in the literature. How do they compare? Cite your sources.

**Post-Lab Questions**

1. The C-18 column used for this lab is for reversed-phase chromatography. If the C-18 column were replaced by a column for molecular exclusion chromatography, would the capsaicinoid analysis still work? Why or why not?

1. The acetonitrile mobile phase used for this lab works for reverse-phase chromatography. If the acetonitrile mobile phase were replaced by a hexane mobile phase, would the capsaicinoid analysis still work? Why or why not?
2. If the peak area precision for the standards was less, how would this affect the uncertainty of the capsaicinoid concentrations in your samples? **Explain.**
3. (a) If you used thin layer chromatography (TLC) for the capsaicinoid analysis instead of HPLC, how would your results be the same or different?

(b) If you used gas chromatography (GC) for the capsaicinoid analysis instead of HPLC, how would your methods be the same or different?

Name \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Pre-Lab Questions**

1. Obtain a sample of hot sauce, fresh chili pepper, and dried ground chili from your instructor the week before the lab. Taste each sample and estimate a Scoville Heat Unit (SHU) for each. **Sample tasting must occur outside of the laboratory*.***

|  |  |
| --- | --- |
| *Sample* | *Qualitative Scoville Heat Unit* |
| Hot sauce |  |
| Fresh chili |  |
| Dried chili |  |

1. You use 25 mL volumetric flasks to prepare five standard concentrations (5 ppm, 10 ppm, 25 ppm, 50 ppm, 100 ppm) from a 500 ppm (or μg/mL) standard stock solution. What volume of 500 ppm stock solution will you add to each volumetric flask? **Show one example calculation below and enter all results in the Table.**

|  |  |
| --- | --- |
| *Standard Concentration* | *Volume of 500 ppm Solution Added to Flask (mL)* |
| 5 ppm |  |
| 10 ppm |  |
| 25 ppm |  |
| 50 ppm |  |
| 100 ppm |  |

1. Uncertainty in sample concentration is calculated using Equations 2 and 3. What happens to the uncertainty in the sample concentration if the number of replicate measurements of the sample is increased? **Explain.**

Adapted From: Batchelor, J.D. and B.T. Jones. 2000. Determination of the Scoville Heat Value for hot sauces and chilies: an HPLC experiment, *J Chem Ed*, 77(2): 266 – 267