August 10, 2017

Mr. Ed Currie
[redacted]

Dear Mr. Currie,

First, let me thank you for providing our laboratory with samples of your unique peppers. The peppers provide a great opportunity to train Winthrop University chemistry students in the principles and operation of High Performance Liquid Chromatography (HPLC) and apply those principles to a real-world analysis such as the determination of Scoville Heat Units (SHU) for your peppers. Students are often excited by projects that directly relate to "real world" applications.

The purpose of this letter is to report the methodology our students used to determine the Scoville Heat Units and subsequent results. I am pleased to send this report outlining our methods and results that span the last 3 years by two undergraduate chemistry majors under my direction. These two students have since graduated and are currently working for local chemical industries, one using HPLC as his primary analysis tool. Their exposure to this project has certainly had a significant impact on each student's career and career objectives.

For this report, I will focus on the hybrid pepper you coded HP22B as requested. During the past 3 year period (2014 – present), you have provided the lab with approximately 58 grams total HP22B pepper weight, along with several other varieties, hybrids and products. Our analysis method is based on the Association of Official Analytical Chemists International (AOAC) Official Method, 995.03, "Capsaicinoids in Capsicums and Their Extractives, Liquid Chromatographic Method". Of the twenty or so known capsaicinoids, this method focuses on capsaicin, dihydrocapsaicin and nordihydrocapsaicin. Your HP22B hybrid produces, almost exclusively, capsaicin and dihydrocapsaicin, with little or undetectable amounts of nordihydrocapsaicin.

Also, the AOAC method specifies 25 grams of dried pepper be extracted with 200 mL of ethanol. However, given the high concentration of capsaicinoids in your peppers and since we are looking at individual pepper SHUs, the proportions were changed to approximately 0.5 grams of dried pepper extracted with 100 mL of ethanol. The AOAC proportions would produce concentration responses that would require further dilution prior to quantitative calculation of concentration. In addition, a single dried pepper will produce a little less than one gram of dry mass. Otherwise, the students followed the AOAC method as specified.

Samples: The peppers were initially weighed as received and then dried to constant mass using a Labconco FreeZone 2.5L freeze dryer system available in our department.
To freeze dry, peppers were stored at -80^{o}C for at least 24 hours using one of our -80^{o}C freezers, followed by 96 hours freeze dry time in a vacuum desiccator attached to the freeze dry system, operating at -49^{o}C and 0.050 mBar temperature and pressure, respectively. No more than 10 peppers were processed at a time. Peppers were weighed each 24 hour period

until constant mass was achieved, using liquid nitrogen to flash re-freeze the peppers before reattaching to the freeze dry system.

After 96 hours, peppers were stored in the vacuum desiccator until extraction/analysis.

Extraction: Dried peppers were weighed, ground using a mortar & pestle and quantitatively transferred to a boiling flask with approximately 40-50 mL of 100% ethanol (Fisher Scientific, Inc. Absolute Ethanol 200 proof – Molecular Biology Grade). The solution was allowed to gently boil for approximately 5 hours. After cooling, the sample was gravity filtered into a 100 mL volumetric flask, washing and diluting with additional 100% ethanol to a final volume of 100.00 mL. No further sample preparation was needed.

Analysis: Capsaicinoid compounds were separated from other mixture components using High Performance Liquid Chromatography (HPLC) with Ultraviolet (UV) detection at 280 nanometer wavelength. Concentrations of the capsaicinoid compounds in the pepper extract solutions were determined using signal peak area integration and the calibration curve method of quantitative analysis.

Apparatus: The HPLC system we use in our lab is a Dionex Corp. ICS-3000, purchased in 2008. The system consists of a 4-solvent gradient pump, single temperature zone column compartment with fixed sample loop injection valve, photodiode array (PDA) and fluorescence detectors, in tandem and AS40 auto-sampler, running Chromeleon v.6.8 control software on a local Dell Optiplex 960 PC computer. The HPLC column utilized for this project is a Dionex Acclaim 120 C-18 4.6 mm i.d. x 150 mm length column with 5 µm/120 Å particle/pore size, respectively. The injection valve is configured with a 25 µL injection loop and the AS40 auto-sampler filter caps remove particles larger than 20 µm on 0.5 mL total sample volume. The gradient pump was operated at a flow rate of 1.00 mL/min and the column compartment was held at 35^{o}C. The PDA detector was used for this project. This detector can monitor 280 nm wavelength UV absorbance, as is commonly recommended in relevant capsaicin analysis literature for quantitative analysis, as well as collect complete spectra from 200 to 400 nm wavelength at a rate of 10 points per second….useful for qualitative analysis. Microsoft's Excel spreadsheet software was used for processing data and calculation of SHUs.

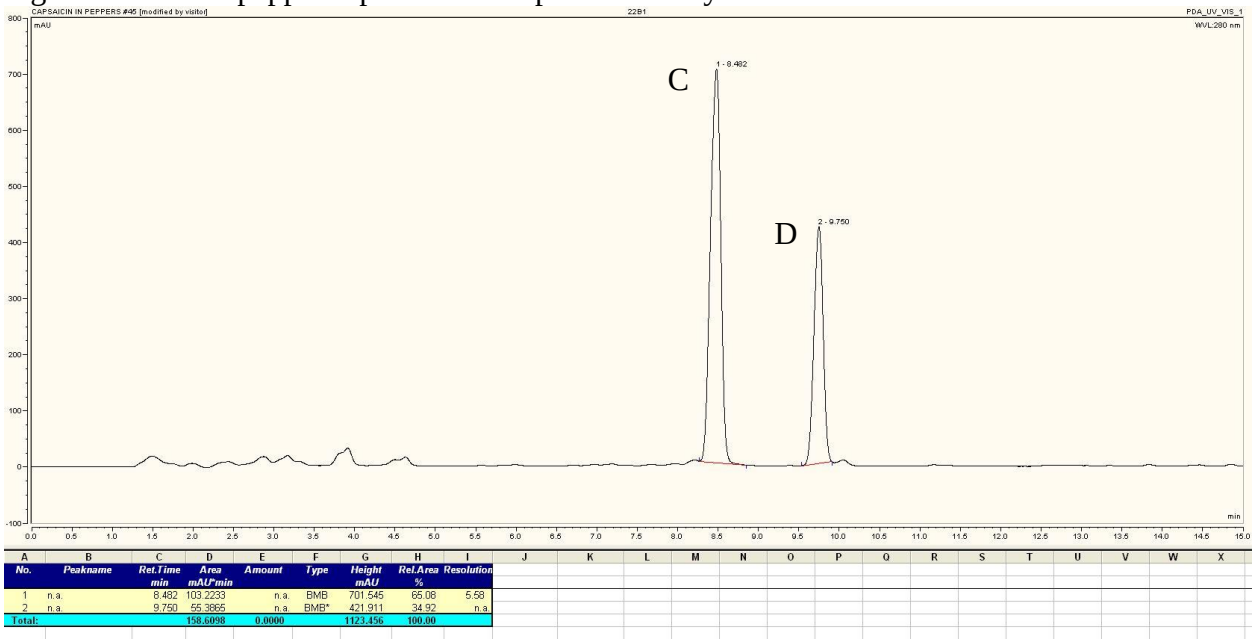
Reagents: HPLC-grade acetonitrile, water, ammonium acetate and acetic acid (Thermo Fisher) were used for the HPLC mobile phase. The HPLC used a gradient elution from 40%/60% acetonitrile/aqueous to 90%/10% acetonitrile/aqueous over a 15 minute period. The aqueous phase contains 50 millimolar ammonium acetate and 0.1% acetic acid.

Calibration: To calibrate the HPLC and relate peak area to capsaicinoid concentration, AOAC suggests using a single synthetic capsaicin compound, N-Vanillyl-n-nonanamide, standard solution and relative UV response factors of 1.000 for capsaicin (CP), 1.101 for nordihydrocapsaicin(NC) and 1.045 for dihydrocapsaicin(DC). We used Capsaicin – Analytical Standard for Food Analysis (Fluka - 12084) and these response factors to quantify a natural capsaicin standard (Aldrich - 360376), separate and produce calibration curves for each capsaicin compound, using six known standard solutions for each of the three primary capsaicinoids of interest. This procedure is also found in the literature and is better practice for

the students. We have used N-Vanillyl-n-nonanamide standard solutions and find good agreement with the Capsaicin – Analytical Standard for Food Analysis standard solutions.

Results: A typical separation for an HP22B pepper is shown in Figure 1, with capsaicin (CP) eluting about 8.5 minutes after injection and dihydrocapsaicin (DC) eluting about 9.75 minutes after injection. As noted above, nordihydrocapsaicin (NC) is negligible, but elutes at about 8.0 minutes after injection using the natural capsaicin standard reference solution. Other components of the pepper mixture can be seen eluting earlier, but are well separated from the capsaicinoids of interest.

Figure 1. HP22B pepper separation of capsaicinoids by HPLC



No.	Peakname	Ret.Time min	Area mAU*min	Amount	Type	Height mAU	Rel.Area %	Resolution
1	n.a.	8.482	103.2233	n.a.	BMB	701.545	65.08	5.58
2	n.a.	9.750	55.3865	n.a.	BMB*	421.911	34.92	n.a.
Total:			158.6098	0.0000		1123.456	100.00	

Figures 2-4 are typical calibration curves produced by the system for concentration range of interest for the three primary capsaicin compounds of interest. Integrated areas of the peak are used to determine the capsaicin concentration in the 100 mL pepper extract solution.

Figure 2. NC Calibration

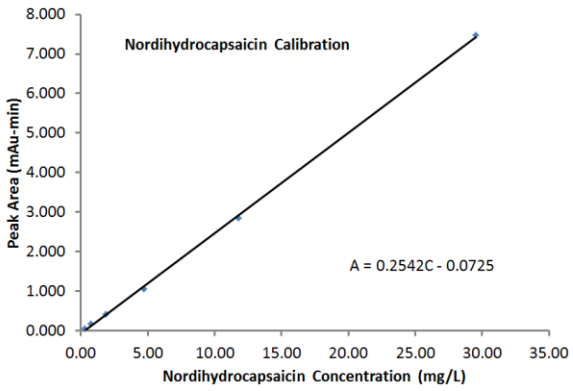


Figure 3. CP Calibration

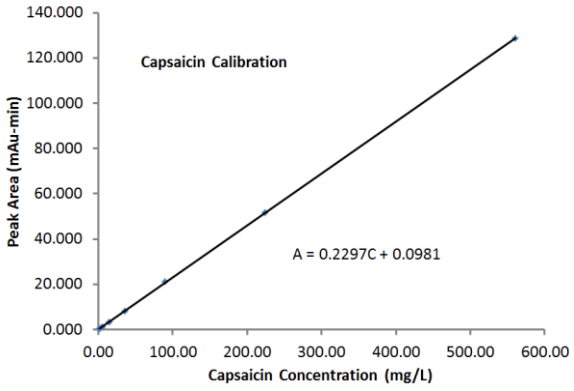
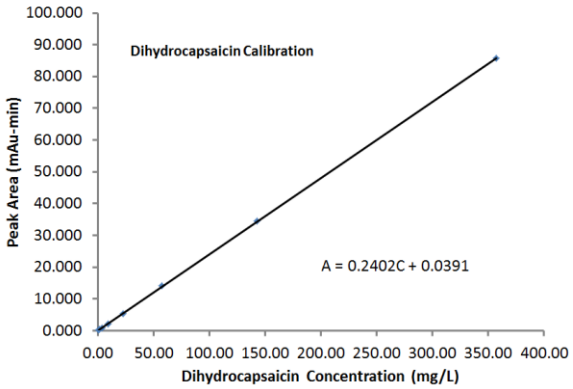


Figure 4. DC Calibration



Scoville Heat Unit calculation: For any pepper extract injected through the HPLC, the concentration of a capsaicin compound (using the retention time to identify the appropriate compound and the peak area/calibration curve to determine concentration) can be determined. With a known solution volume of 100 mL (0.100L), we can calculate the mass (in milligrams) of the capsaicin in the mass of dry pepper used to make the solution. The mass ratio of grams of capsaicin compound per gram of dry pepper can be multiplied by each compound's pungency value, as specified in AOAC and originally determined by P.H. Todd, then summed to determine the SHU value:

Compound	Pungency Value
Nordihydrocapsaicin	9,300,000
Capsaicin	16,100,000
Dihydrocapsaicin	16,100,000

Here is an example calculation using Figure 1 areas from the 0.697g dry pepper:

Capsaicin: A = 0.2297C + 0.0981
Area (A): 103.2233 mAu•min
C(mg/L) = (103.2233-0.0981)/0.2297
= 448.96 mg/L

(448.96 mg/L)(0.100L)= 44.896 mg
0.044896 g Capsaicin

(0.044896g CP/0.697g*16,100,000) = 1,037,000

Dihydrocapsaicin: A = 0.2402C + 0.0391
Area (A): 55.38659 mAu•min
C(mg/L) = (55.38659 – 0.0391)/0.2402
= 230.42 mg/L

(230.94 mg/L)(0.100L) = 23.092 mg
0.023092 g Dihydrocapsaicin

(0.023094g DC/0.697g*16,100,000) = 532,000

SHU = 1,037,000 + 532,000 = 1,569,000 (1.569 million)

HP22B Results: A sampling of the HP22B results…areas, concentrations, mass ratios and SHUs for the last three years is shown in Table 1, below. Again, this variety of pepper did not routinely produce a measurable amount of nordihydrocapsaicin, so we simply noted the amount was below our detection limit (BDL).

Table 1. Concentrations, Mass Ratios and SHUs for HP22B variety peppers

Pepper Mass	NC area	CP area	DC area	NC conc	CP conc	DC conc	Mass ratio	Mass Ratio	Mass Ratio	SHU
g	mAu-min	mAu-min	mAu-min	mg/L	mg/L	mg/L	gNC/g	gCP/g	gDC/g	
0.475	BDL	82.9927	21.9101		360.88	91.07		0.0760	0.0192	1531869
0.510	0.999	96.349	31.192	4.22	419.03	129.72	0.00089	0.0882	0.0273	1868207
0.599	BDL	74.985	21.174		326.02	88.00		0.0686	0.0185	1403318
0.601	BDL	69.735	25.479		303.16	105.93		0.0638	0.0223	1386608
0.583	BDL	58.123	22.481		252.61	93.45		0.0532	0.0197	1172950
0.621	BDL	102.445	29.225		445.56	121.53		0.0938	0.0256	1922145
0.699	0.566	99.314	28.875	2.51	431.93	120.07	0.00053	0.0909	0.0253	1875923
0.588	BDL	91.515	27.996		397.98	116.41		0.0838	0.0245	1743517
0.602	BDL	88.112	24.112		383.17	100.24		0.0807	0.0211	1638485
0.611	BDL	97.344	30.779		423.36	128.00		0.0891	0.0269	1868808

BDL – below detection limit NC – Nordihydrocapsaicin CP – Capsaicin DC – Dihydrocapsaicin

High 1,922,145
Low 1,172,950
Range 749,195

Conclusion: Based on our sampling/analysis during the past three growing seasons, your HB22B hybrid pepper has an SHU value of 1.64 million with a standard deviation of about 0.26 million Scoville Heat Units. Published literature has suggested variations as much as 15-20% variation, depending on the growing season and within a single crop, which is also what we've observed. I look forward to continuing to train and involve students in the characterization of your hybrid peppers.

Best Regards,

Clifton P. Calloway

Cliff Calloway, Ph.D.
Professor of Chemistry