August 7, 2013

Mr. Ed Currie

Dear Mr. Currie,

Thank you for providing our laboratory with samples of your unique peppers. This provides 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.

The purpose of this letter is to report the methodology our students used to determine the Scoville Heat Units with subsequent results. For the purpose of this report, I will focus on the hybrid pepper you coded HP22B. I am pleased to send this report outlining our methods and results that span the last 3 years by three undergraduate chemistry majors under my direction. Two students have graduated and are working for chemical industries. The third is a rising junior. The exposure to this project has certainly had a significant impact on each student's career and career objectives.

Our 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".

The AOAC method specifies 25 grams of dried pepper 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 1 gram 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 the SHU. In addition, a single dried pepper will produce only about one gram of mass. Otherwise, the students followed the AOAC method as specified.

Samples: The peppers were initially weighed as received and then dried to constant mass.

Our first year student used an air drying scheme, suspending the pepper in an open beaker with a wooden rod pierced through the pepper cap. This scheme required about 3 weeks to reach constant mass, so subsequent students used a Labconco FreeZone 2.5L freeze dryer system available in our department.

To freeze dry, peppers were frozen at -80^{o}C for at least 24 hours using one of our -80 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, the sample was gravity filtered into a 100 mL volumetric flask, 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. Concentrations of the capsaicinoid compounds in the pepper extract solutions were determined using signal peak area integration and 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, four-channel variable wavelength and photodiode array detectors (PDA) in tandem and AS40 auto-sampler, running Chromeleon v.6.8 control software on a local Dell GX755 PC. 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°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. Excel 2007 spreadsheet software was used for processing the data and calculation of SHUs.

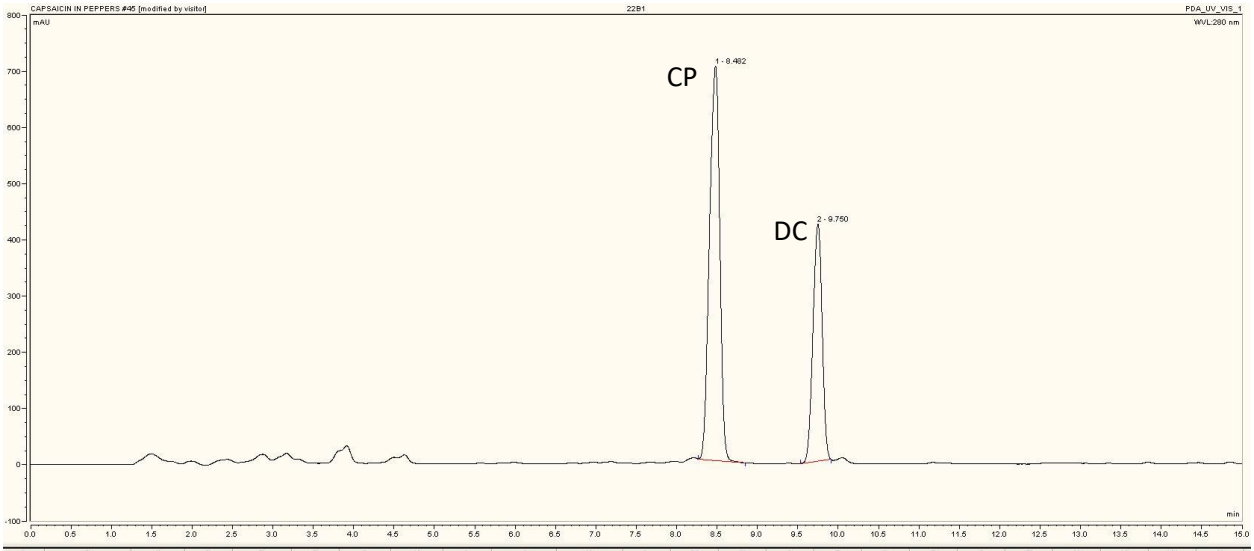
Reagents: HPLC-grade acetonitrile, water, ammonium acetate and acetic acid (Thermo Fisher) were used for the mobile phase. The HPLC used a gradient elution from 40%/60% acetonitrile/aqueous to 90%/10% acetonitrile/aqueous over a 15 minute period. The aqueous phase contains 50 millimolar ammonium acetate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

Calibration: To calibrate the HPLC and relate peak area to capsaicinoid concentration, AOAC suggests using a single synthetic capsaicin, 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 using six known standard solutions for each of the three primary capsaicinoids of interest, as also found in literature and 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 8.48 minutes after injection and dihydrocapsaicin (DC) eluting 9.75 minutes after injection. Nordihydrocapsaicin (NC) in this pepper sample is negligible, but elutes 8.17 minutes after injection

using the natural capsaicin standard solution. Other components of the mixture are seen eluting earlier and 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 of the calibration curves produced by the system for concentration range of interest for the three primary capsaicin compounds of interest. We use the integrated area of the capsaicin peak to determine the capsaicin concentration in the 100 mL pepper extract solution.

Figure 2. NC Calibration

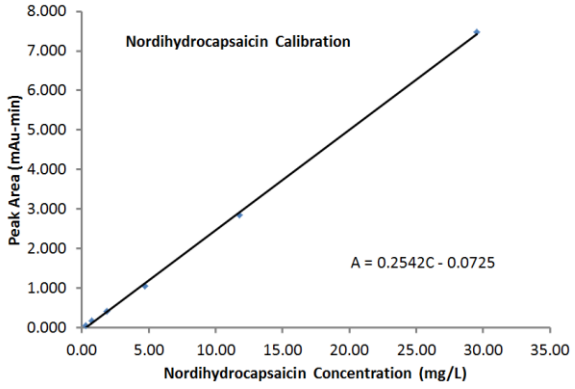


Figure 3. CP Calibration

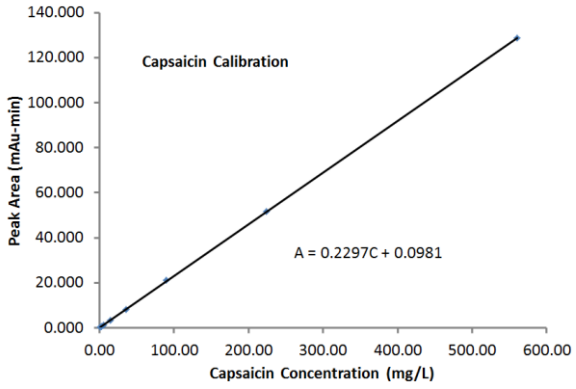
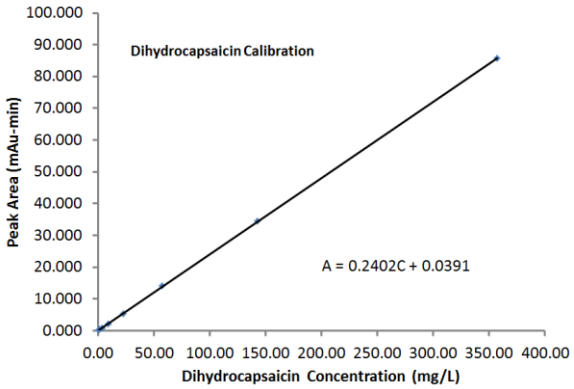


Figure 4. DC Calibration



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 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

(0.044896 g CP/0.697*16,100,00) = 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

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

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

HP22B Results: HPLC resulting areas, concentrations, mass ratios and SHUs for peppers over the last three years are shown in Table 1. This variety of pepper did not 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

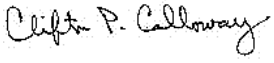
Date	PepperID	Pepper Mass	NC area	CP area	DC area	NC conc	CP conc	DC conc	Mass ratio	Mass Ratio	Mass Ratio	SHU
		g	mAu-min	mAu-min	mAu-min	mg/L	mg/L	mg/L	gNC/g	gCP/g	gDC/g	
Nov-10	HP22B1	0.697	BDL	103.2233	55.3865		448.9521	230.4639		0.0644	0.0331	1,569,383
	HP22B2	0.657	BDL	91.8846	49.0032		399.5895	203.8842		0.0608	0.0310	1,478,832
	HP22B3	0.718	BDL	92.2893	50.8706		401.3513	211.6599		0.0559	0.0295	1,374,580
Nov-12	HP221	0.625	BDL	82.9927	21.9101		360.8789	91.06991		0.0577	0.0146	1,164,220
	HP222	0.655	BDL	96.3495	31.1920		419.0272	129.7193		0.0640	0.0198	1,348,827
	HP223	0.622	BDL	74.9847	21.1740		326.0163	88.00483		0.0524	0.0141	1,071,662
	HP224	0.609	BDL	69.7353	25.4786		303.1632	105.929		0.0498	0.0174	1,081,508
Jan-13	HP22B1	0.738	BDL	148.493	43.468		646.0322	180.8359		0.0751	0.0210	1,547,974

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

Average	1,329,000
Std Dev	201,000
High	1,569,000
Low	1,071,000
Range	498,000

Conclusion: You will note three peppers above the 1.47 million SHU with a high of 1.569 million, low of 1.071 million and a range of 498,000 SHUs. I look forward to continuing to train and involve students in the characterization of your hybrid peppers.

Best Regards,



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