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Improved Method for Quantifying Capsaicinoids in *Capsicum* Using High-performance Liquid Chromatography

Margaret D. Collins[1], Loide Mayer Wasmund[2], and Paul W. Bosland[3]
Department of Agronomy and Horticulture, New Mexico State University, Las Cruces, NM 88003

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Abstract. **An improved high-performance liquid chromatography (HPLC) method for analysis of capsaicinoids in dried *Capsicum* fruit powder, involving changes in extraction, mobile phase, flow rate, and excitation and emission spectra and resulting in reduced analysis time, increased sensitivity, and safety, is reported. Extraction of *Capsicum* fruit powder using acetonitrile proved to be the best capsaicinoid extractor in the shortest time interval. Solvents used for HPLC separation and quantification of capsaicinoids include methanol and water at 1 ml·min^{-1} flow rate. Instrument sensitivity is enhanced by altering the fluorescence detector excitation and emission wavelengths. Two analytical methods have been developed. One method determines total amount of heat units in 7 minutes, while the other provides total amount of heat units as well as separation of all present major and minor capsaicinoids in 20 minutes. These improved techniques provide inexpensive and rapid methods for quantitative and qualitative analysis of capsaicinoids in *Capsicum* fruit samples along with good sensitivity and no interference or confounding peaks.**

Capsaicinoids, alkaloid compounds that produce the hot flavor or pungency associated with eating chiles, are commonly found in the genus *Capsicum*. The first reported reliable measurement of chile pungency is the Scoville Organoleptic Test (Scoville, 1912). This test uses a taste panel of five individuals who evaluate a chile sample and then record the hot flavor level. A sample is then diluted until pungency can no longer be detected. This dilution is referred to as the Scoville Heat Unit. This test is subjective, and members of the taste panel cannot determine the amount of each of the capsaicinoids present in the sample. There are seven different capsaicinoids, and the hot flavor is generally made up of at least two and perhaps all of these compounds.

Accurate measurement of pungency has become important because of the increased demand by consumers for southwestern foods; moreover, accurate determination of levels of various capsaicinoids is also needed due to their increased use in pharmaceuticals (Carmichael, 1991). Food industry researchers need reliable, safe, and standard analytical procedures that are useful for comparing pungency levels among different samples. Therefore, the Scoville Organoleptic Test has since been replaced with instrumental methods. Currently, analysis of capsaicinoids is conducted by using spectrophotometric (Bajaj, 1980; Ramos, 1979; Rymal et al., 1984), gas chromatographic (Krajewska and Powers, 1987), and high-performance liquid chromatographic (HPLC) procedures [American Spice Trade Association (ASTA), 1985; Attuquayefio and Buckle, 1987; Cooper et al., 1991; Hoffman et al., 1983; Law, 1983; Saria et al., 1981; Woodbury, 1980]. Techniques using HPLC provide accurate and efficient analysis of content and type of capsaicinoids present in a chile sample.

The most commonly used HPLC procedure for determining capsaicinoid content has been outlined in a manual (ASTA, 1985). The ASTA method involves drying and grinding a chile sample, followed by extracting capsaicinoids with 95% ethanol saturated with sodium acetate, and injecting the sample extract into the HPLC instrument. Three peaks are observed corresponding to the pigment, capsaicin, and dihydrocapsaicin. However, it is still necessary to refine this method to identify each of the remaining closely related capsaicinoids in the extract. Therefore, studies have been conducted to optimize techniques for separating and quantifying all known natural capsaicinoids. Several preliminary experiments have been conducted to establish appropriate methods and techniques; however, here we describe only the recommended.

Materials and Methods

Plant material. Fully mature chile pods with stems removed were dried and ground with seeds. Chile pods were grown under normal greenhouse and farming practices at either the Fabian Garcia Science Center located 2 km west of Las Cruces, N.M., or at the Leyendecker Plant Science Center located 12 km south of Las Cruces.

Apparatus. A Waters (Waters Corp., Milford, Mass.) HPLC instrument equipped with two 501 solvent delivery pumps, a U6K universal liquid injector, a 484 UV absorbance detector set at 280 nm, a 420 fluorescence detector with excitation at 280 nm and emission at 338 nm, and a system interface module was used. The reverse-phase chromatographic column was a radial compression Nova-Pak (Waters Corp.) C-18, 100×5-mm size, packed with silica/C18. A precolumn guard cartridge, Nova-Pak C-18, was also used.

Reagents. HPLC-grade methanol and acetonitrile were used. Water was purified by running it through reverse osmosis and then Corning Mega-Pure water distillation units (Baxter Healthcare Corp., Tempe, Ariz.). All solvents were filtered and degassed using a 47-mm, all-glass filter holder (Millipore Corp., Bedford, Mass.). Millipore methanol filters (0.5-µm pore size, filter type FH) and Millipore water filters (0.45-µm pore size, filter type HA) were used.

Procedure. Whole or sliced chile fruit were oven-dried at 58 to 60C for 2 to 5 days (depending on sample size), ground using a Wiley laboratory mill (Baxter Healthcare Corp.) equipped with a 1-mm screen, and stored in sealed plastic bags at 20C until processed. All samples were processed within 7 days of grinding.

Preliminary trials showed that as little as 1 g of powder produced consistently reliable capsaicinoid measurements. Samples <1 g showed statistically significant differences in capsaicinoid levels (data not shown) and therefore could not be accurately used to measure capsaicinoid levels.

For capsaicinoid extraction, a 1:10 (gram : milliliter) ratio of dried chile powder to acetonitrile was placed in 120-ml glass bottles with Teflon-lined lids. The quantity of chile powder ranged between 1 and 5 g, depending on the amount of sample available for processing. Bottles were capped and placed in an 80C water bath for 4 h; they were swirled manually every hour. Samples were removed from the water bath and cooled to room temperature. Two to 3 ml of supernatant was extracted and filtered (0.45-µm Waters Millex-HV filter unit on a 5-ml disposable syringe) into a 2-ml glass sample vial, capped, and stored at 5C until analyzed. A 10-µl aliquot was used for each HPLC injection.

HPLC operating conditions to determine total heat units (designated "short" run) included ambient temperature, a flow rate of 1 ml·min^{-1}, and a run duration of 7 min. The

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[1]Research Specialist.
[2]Assistant Professor. Current address: Dept. of Physical Sciences, Southeastern Oklahoma State Univ., Durant, OK 74701.
[3]Professor.

Table 1. Capsaicinoid parts per million heat in *Capsicum* species and cultivars.

Species	Pod type	Unidentified capsaicinoid[z]	Unidentified capsaicinoid[z]	Nordihydro-capsaicin	Capsaicin	Dihydro-capsaicin	Isomer of dihydrocapsaicin[y]	Homodihydro-capsaicin
C. annuum	Pasilla	---	---	18	195	144	6	7
	Cascabel	---	---	7	88	42	---	---
	Cubanella	---	---	55	834	291	19	18
	Jalapeño	---	---	107	1307	595	---	28
	New Mexican	---	---	---	39	42	---	---
	Yellow mushroom	---	---	92	1196	627	44	34
C. baccatum		---	---	79	558	352	---	12
C. cardenasii		---	46	706	984	934	113	67
C. chinense	Habanero	---	---	279	10951	3002	131	60
C. pubescens		30	119	300	401	487	58	14
		37	197	412	502	441	104	22

[z]Determination of compounds under way.
[y]Possibly *n*-vanillyl-*n*-decanamide.

mobile phase was isocratic, with 70% solvent B (100% methanol) and 30% solvent A [10% methanol (by volume) in water]. For individual capsaicinoid peak detection (designated "long" run), operating conditions included ambient temperature, a flow rate of 1 ml·min^{-1}, and a run duration of 20 min. The mobile phase was a gradient consisting of 57% solvent B and 43% solvent A for 10 min followed by 68% solvent B and 32% solvent A for an additional 10 min.

Capsaicinoid standards. Standards of 8-methyl-*n*-vanillyl-6-nonenamide (capsaicin) and 8-methyl-*n*-vanillyl-nonanamide (dihydrocapsaicin) were obtained from Sigma Chemical Co. (St. Louis) and were used for retention-time verification and instrument calibration. Standard solutions of 1000, 500, 100, 50, 25, 10, 5, and 1 ppm were prepared in 100% methanol by dilution of a 2000-ppm stock solution. Quantification of unknowns was achieved by the external standard method.

Results and Discussion

Using the outlined methods, reproducible results were obtained for total heat units and individual capsaicinoid peaks for more than 1400 samples of various *Capsicum* species tested, including *C. annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L., *C. pubescens* R. & P., *C. cardenasii* Heiser & Smith, *C. chacoense* A.T. Heinz, and *C. tovarii* Eshbaugh, Smith & Nickient (Table 1). Repeated injections of a given sample were typically within 1% to 2%, and the separation was observed with no interference or confounding peaks (Figs. 1 and 2). The baseline separation shown in Fig. 1 was obtained for total capsaicinoid heat units using a short run. The retention times for capsaicin and dihydrocapsaicin were 3.2 and 4.3 min, respectively.

The baseline separation for identifying minor capsaicinoid peaks using a long run is shown in Fig. 2. Retention time for the constituents is 8.4 min for nordihydrocapsaicin, 9.3 min for capsaicin, 14.3 min for dihydrocapsaicin, and 17.7 min for homodihydrocapsaicin. These peaks have been verified using an HPLC–mass spectrometry (LC–MS) analysis conducted at McCormick and Co. (Hunt Valley, Md.). The peak immediately following dihydrocapsaicin is presumed to be either *n*-vanillyl-*n*-decanamide, as reported in other studies (Attuquayefio and Buckle, 1987),

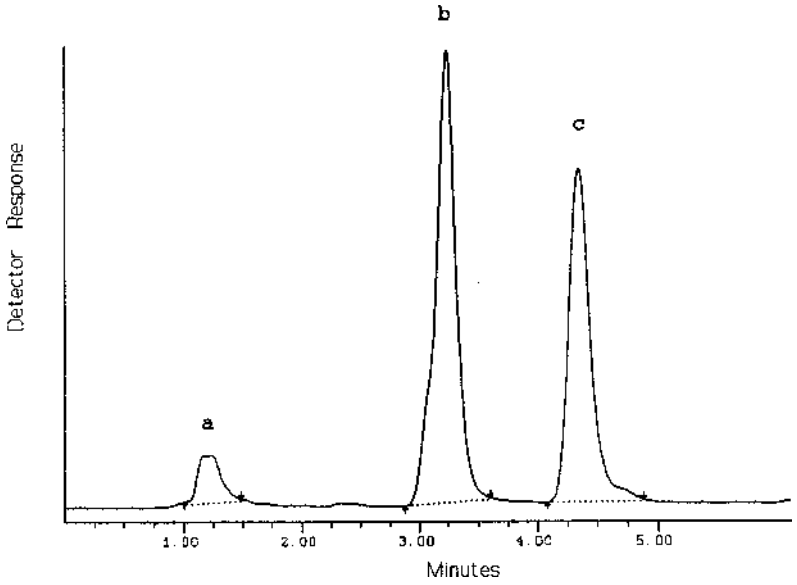

Fig. 1. High-performance liquid chromatography (HPLC) chromatogram of *Capsicum annuum* 'Punjab Lal' showing baseline separation for a "short" HPLC run. Illustrated is the separation of (**a**) pigment, (**b**) capsaicin, and (**c**) dihydrocapsaicin. Peaks were identified by comparing retention times to those of standard compounds (capsaicin and dihydrocapsaicin) and by HPLC–mass spectrometry.

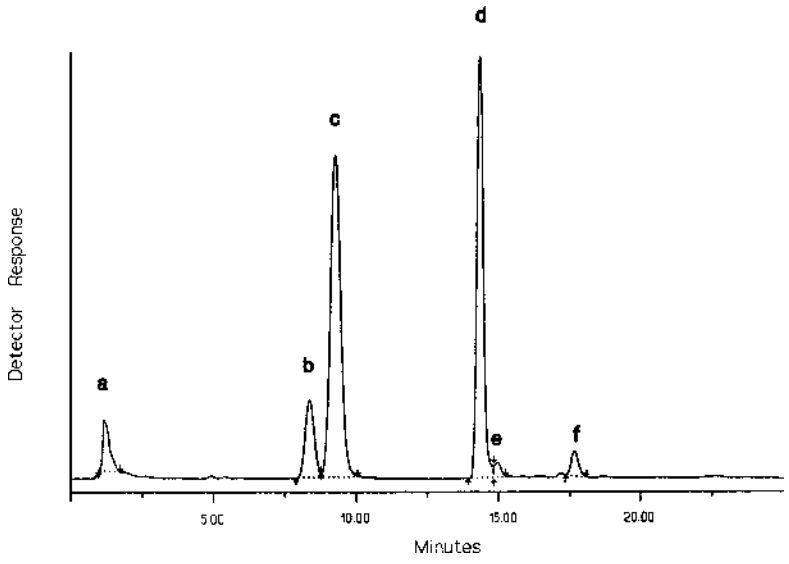

Fig. 2. High-performance liquid chromatography (HPLC) chromatogram of *Capsicum annuum* 'Punjab Lal' showing baseline separation of (**a**) pigment, (**b**) nordihydrocapsaicin, (**c**) capsaicin, (**d**) dihydrocapsaicin, and (**f**) homodihydrocapsaicin using a "long" HPLC run. The minor peak (**e**) directly following dihydrocapsaicin is either *n*-vanillyl-*n*-decanamide or an isomer of dihydrocapsaicin. Peaks were identified by comparing retention times to those of standard compounds (capsaicin and dihydrocapsaicin) and by HPLC–mass spectrometry.

Table 2. Comparison of methods for capsaicinoid detection.

Protocol (year)	Extraction method	Cleanup	Mobile phase	Flow rate (ml·min^{-1})	Fluorescence detector (nm) Excitation	Emission
ASTA (1985)	Sodium-acetate-saturated 95% ethanol, 3-h hot plate or water bath	Allow solids to settle	Acetonitrile, dioxane, perchloric acid, water methanol	0.6–1.8	288	320
ASTA (1993)	95% ethanol, 5-h refluxing	0.45-µm syringe filter[z]	Acetonitrile, water, acetic acid, column purge used	1.5	280[z]	325
Attuquayefio and Buckle (1987)	Acetonitrile[z]	Sep-pak filtration	Methanol, water[z]	3.5	N/A[y]	N/A
Cooper et al. (1991)	Method 1: methanol and centrifugation	0.45-µm filter	Methanol, water, citric acid	1.5	229	320
	Method 2: silica gel, hexane, methanol, water bath	N/A	Methanol, water, citric acid	1.5	229	320
Hoffman et al. (1983)	95% ethanol, 5-h heating	Allow solids to settle	Acetonitrile, water	1.5	N/A	N/A
Woodbury (1980)	Sodium-acetate-saturated 95% ethanol, 5-h hot plate	Allow solids to settle	Acetonitrile, water, dioxane, methanol, perchloric acid	1.0[z]	228	320
Collins et al. (1994)	Acetonitrile, 4-h water bath	0.45-µm syringe filter	Methanol, water	1.0	280	338

[z]Similar to Collins et al. (1994) method.
[y]N/A = not applicable.

or an isomer of dihydrocap-saicin. The molecular weight of this compound ($C_{18}H_{29}NO_3$, 307 g/mol) determined by LC–MS is correct for either of these two chemical structures (Linda Rouse, personal communication).

Major aspects of the proposed procedure and previously reported techniques are listed in Table 2. In our protocol, capsaicinoids were extracted using acetonitrile instead of 95% ethanol saturated with sodium acetate, as required in the ASTA (1985) procedure. Several extraction solvents were evaluated, including 95% ethanol, 95% ethanol saturated with sodium acetate, chloroform, ethyl acetate, and acetonitrile, by extracting capsaicinoids from the same chile sample for varied lengths of time. Acetonitrile yielded the highest amount most quickly (data not presented). The extraction procedure of ASTA (1993) requires refluxing the sample for 5 h, while our protocol requires maintaining the sample in a bath for only 4 h, with no refluxing. The mobile phase in the ASTA (1985) technique requires methanol, water, dioxane, acetonitrile, and perchloric acid, while that described in the ASTA (1993) protocol requires acetic acid (HOAc), water, and acetonitrile. Our mobile phase uses only methanol and water. Methanol is less expensive than the other chemicals. The flow rate in our protocol was 1 ml·min^{-1}, while it is 1.5 ml·min^{-1} in Cooper et al. (1991), 3.5 ml·min^{-1} in Attuquayefio and Buckle (1987), 0.6 to 1.8 ml·min^{-1} in ASTA (1985), and 1.5 ml·min^{-1} in ASTA (1993). Cooper et al. (1991) and ASTA (1993) use a column purge following a certain number of samples, whereas this is not necessary in our protocol. While internal standardization is needed in other procedures (ASTA, 1993; Cooper et al., 1991), only external standardization is necessary for our protocol. Furthermore, sample run lengths, fruit sample preparation routines, and extraction time all differ from other published HPLC techniques (ASTA, 1985; Attuquayefio and Buckle, 1987; Cooper et al., 1991; Hoffman, 1983; Woodbury, 1980).

The fluorescence detector filter settings of Cooper et al. (1991) were 229 nm excitation and 320 nm emission. The 1985 ASTA suggested settings were 288 nm excitation and 320 nm emission and the 1993 ASTA suggested settings were 280 nm excitation and 325 nm emission. We found using fluorescence detection wavelengths of 280 nm excitation and 338 nm emission provided the highest level of sensitivity. Using ASTA (1985) protocols, the detection limit was 10 ppm, whereas the detection limit was reduced to 3 ppm in our protocol.

Scoville Heat Units are calculated in parts per million of heat (ppmH) based on sample dry weight according to the following equation from ASTA (1985): ppmH = [Peak area of capsaicin + (0.82)(peak area of dihydrocapsaicin)](ppm standard)(ml acetonitrile) ÷ (Total capsaicin peak area of standard)(g sample).

Conversion to Scoville Heat Units can be made by multiplying ppmH by a factor of 15. In the course of this study, the ASTA formula was used to calculate heat for all samples tested.

The protocols described for quantitative and qualitative analysis of capsaicinoids are simple, highly sensitive, and time- and cost-effective. Extraction is easy and results in optimum capsaicinoid extraction. Cleanup is rapid and complete, and because filtering equipment is disposable, chances of contamination between samples is eliminated. Fluorescence detector settings result in greatly improved sensitivity. These procedures enable the calculation of total capsaicinoid amount and the detection of minor capsaicinoids producing rapid, reliable, and consistent results.

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