

Pungency of Capsicums and Their Oleoresins (HPLC Method - Preferred)

Purpose: To determine pungency levels in crushed red pepper, chili pepper, jalapeno pepper, and red pepper oleoresins.

A. Apparatus:

1. Liquid chromatograph equipped with:
 - a. integrator.
 - b. 20 μ L sample loop injection valve.
 - c. fluorescence detector and/or ultraviolet detector.
2. Chromatographic column stainless steel, 150 x 4.6 mm id packed with 5 μ m LC-18 (available from Supelco, Inc., Bellefonte, PA) or equiv. Use guard column if desired.
3. Balance, readable to 0.01 g.
4. Pipets Volumetric - various sizes, Class A.
5. Glass beads.
6. Water cooled condenser, with 24/40 S/T joint.
7. Syringes, disposable 5 and 10 mL with luer lock tips.
8. Syringe Filters, 0.45 μ m; nylon.
9. Volumetric Flasks, various sizes, Class A.
10. Round bottom flask, 500 mL size with 24/40 S/T joint.

B. Reagents:

1. Ethyl Alcohol (EtOH), 95% or denatured.
2. Acetone, ACS grade.
3. Mobile phase. Use LC grade solvents, or equivalent:
 - a. Deionized H₂O, Add 1% HOAc(v/v).
 - b. Acetonitrile.
 - c. Mix 400 mL of b with 600 mL of a.
 - d. De-gas with helium or by other suitable means.

Pungency of Capsicums and Their Oleoresins (HPLC Method)

4. Standard: N-vanillyl-n-nonanamide, 99 + % (CAS Registry Number 2444-46-4) (available from Penta International Corporation, P.O. Box 1452, West Caldwell, N.J. (201) 740-2300) Code 03-08700 ****CAUTION-----EXTREME IRRITANT-----HANDLE WITH EXTREME CARE-----DO NOT INHALE****.
5. C-18 Sep-pak cartridge, 6 mL capacity (Millipore Inc.), or equivalent.

C. Preparation of Sample:

1. Ground or crushed peppers: Weigh accurately about 25 g pepper into a 500 mL boiling flask. Pipet 200 mL EtOH into the flask and add several glass beads to aid boiling. Reflux gently for 5 hours. Allow to cool. Filter 3-4 mL through a 0.45 μ m syringe filter into glass vial. (Note 1)
2. Oleoresins: Accurately weigh 1 to 2 g oleoresin (increase sample size if total capsaicinoid concentration is below 1%) into 50 mL volumetric flask, being sure not to allow any oleoresin to coat the sides of the flask. Add 5 mL of acetone to flask and swirl acetone until sample is completely dispersed as evidenced by observing no oleoresin coating bottom of flask with neck at 45° angle. Slowly add EtOH, mixing completely by swirling with acetone while adding. Continue adding and mixing until solution becomes cloudy. Dilute to volume and mix well. Place C-18 SEP-PAK on a 10 mL syringe and hold over a 25 mL volume flask. Pipet 5 mL aliquot of sample mixture to syringe. Deliver aliquot to bottom of syringe so that sides of syringe are not coated with sample. Pass aliquot through SEP-PAK and collect in flask. Rinse SEP-PAK through the syringe with three 5 mL portions of EtOH, again collecting washings in flask. Dilute to volume with EtOH and mix. Filter through 0.45 μ m syringe filter into a glass vial.

D. Procedure:

1. Prepare all standard solutions with EtOH. Keep solutions out of direct sunlight.
 - a. Standard solution A-0.15 mg/mL. Accurately weigh and transfer 75 mg of standard into 500 mL volume flask, dissolve, dilute to volume, and mix.
 - b. Standard solution B-0.015 mg/mL. Pipet 10 mL standard solution A into 100 mL volume flask, dilute to volume, and mix. (Use with chili peppers)
 - c. Dilute standard solution C-0.00075 mg/mL. Pipet 5 mL of working standard solution B into 100 mL volumetric flask, dilute to volume, and mix. (Use with samples, which contain capsicum heat levels below 5000.)
2. Chromatographic Conditions:
 - a. Mobile Phase: 40% Acetonitrile and 60% DI - H₂O with 1% Acetic Acid (v/v).
 - b. Flow Rate: 1.5 mL/minute, isocratic.
 - c. Column: LC-18 150 x 4.6 mm i.d., 5 μ m particle size.

Pungency of Capsicums and Their Oleoresins (HPLC Method)

- d. Injection Volume: 20 μ L.
 - e. Detection - Excitation 280 nm, Emission 325 nm for fluorescence or 280 nm for ultraviolet. (Note 2)
Using a sample loop injection valve, inject, in duplicate, 20 μ L of the prepared sample solution onto the column. Inject the appropriate standard solution before first sample injection and after no more than 6 sample injections.
3. Purge the column with 100% acetonitrile for 30 min at 1.5 mL/min after no more than 30 sample injections. Return to previous mobile phase for further determinations.

E. Calculations:

1. Scoville Heat Units (SHU) are the sum of SHU of the three major capsaicinoids. Calculate SHU as follows:
- a) Nordihydrocapsaicin, $SHU_N = (N/A) \times (Cs/C_x) \times (H_N/R_N)$.
 - b) Capsaicin, $SHU_C = (C/A) \times (Cs/C_x) \times (H_C/R_C)$.
 - c) Dihydrocapsaicin, $SHU_D = (D/A) \times (Cs/C_x) \times (H_D/R_D)$.
 - d) Total $SHU_T = SHU_N + SHU_C + SHU_D$.

Where: A = average peak area of standard;
 N, C, and D = average peak areas for respective capsaicinoids (nordihydrocapsaicin, capsaicin and dihydrocapsaicin) from duplicate injections;
 Cs = concentration of std in mg/mL;
 C_x = concentration of sample in extract expressed as, mg of sample/mL;
 H_N, H_C, and H_D = heat factors for respective capsaicinoids;
 R_N, R_C, and R_D = response factors of respective capsaicinoids relative to standard.

2. Accepted heat factors and response factors:

		<u>UV</u>	<u>FLU</u>
Nordihydrocapsaicin (N)	- H _N = 9.3 x 10 ⁶	R _N = 0.98	0.92
Capsaicin (C)	- H _C = 16.0 x 10 ⁶	R _C = 0.89	0.88
Dihydrocapsaicin (D)	- H _D = 16.0 x 10 ⁶	R _D = 0.93	0.93
N-vanillyl-n-nonanamide		R = 1.00	1.00

3. Relative retention times:

Nordihydrocapsaicin	0.90
N-vanillyl-n-nonanamide	1.00
Capsaicin	1.00
Dihydrocapsaicin	1.58

Pungency of Capsicums and Their Oleoresins (HPLC Method)

F. Statistics:

Reproducibility relative standard deviation (RSD_R)

	<u>SHU's</u>	<u>%</u>
Chili Peppers	900-2750	9.6-11.6
Red Peppers	30140-41600	4.5-11.6
Oleoresins	305690-644060	7.6-14.8

G. Note:

- 1.) If directions are followed, there is little to no loss of ETOH.
- 2.) Adjust the detector sensitivity or gain so that eluting peaks are not overloading detector.

H. Reference:

- Official Methods of AOAC International (1996) 995.03 (43.1.43).
 J. AOAC International (1996) 79 (3), 738-745.
 J. Agricultural Food Chemistry (1983) 31, 1326.

I. Appendix: Alternative Rapid Extraction method B, Block Digestion

1. Apparatus:
 - a. Lab-Line Multiblock heater, model 2056, 120V, or equivalent
 - b. Lab-Line multitube module for 6 tubes, 25 mm
 - c. Corning Culture Tube SCP, 25 x 150 mm and screw cap with seal
2. Reagent:
 - a. Ethanol, 95% reagent or equivalent
3. Preparation of Sample:
 - a. This method is only applicable for ground product and samples.

Pungency of Capsicums and Their Oleoresins (HPLC Method)

4. Procedure:
 - a. Preheat the block to $75^{\circ}\text{C} \pm 5^{\circ}\text{C}$.
 - b. Accurately weigh 2.0 ± 0.5 g of sample into the tube.
 - c. Pipet 20.0 mL of ethanol into the culture tube and seal tightly with cap.
 - d. Vortex tube to mix contents.
 - e. Place the tube into the heating block and allow the sample to extract for 1 hour after the solution begins to boil.
 - f. After extraction, remove tube(s) from the block heater, cool tubes to room temperature. **WARNING: DO NOT LOOSEN CAPS AT ELEVATED TEMPERATURE.**
 - g. Filter sample extract through 0.45μ Nylon filter prior to HPLC analysis.

J. Appendix: Alternative Extraction method C, Ethanol saturated with sodium acetate

1. Apparatus:
 - a. Erlenmeyer flask, 125 mL size with standard taper joint
 - b. Stopper to fit flask
 - c. Clamp or fixture to hold stopper
 - d. Clamp to hold flask
2. Reagent:
 - a. 95% ethanol saturated with sodium acetate (ca. 5 g/100 mL at room temperature, a few crystals should remain)
3. Preparation of sample:
 - a. This method is only applicable for ground product and samples
4. Procedure:
 - a. Weight 10.0 ± 0.2 g of sample into the flask.
 - b. Add 100.0 mL of sodium acetate saturated 95% ethanol solution by pipet to the flask and stopper.
 - c. Swirl initially to mix contents, then occasionally during the extraction.
 - d. Place in water bath held at 60°C for three hours.
 - e. Remove from water bath and cool to room temperature.
 - f. Filter sample extract through 0.45μ Nylon filter prior to HPLC analysis.