Hot Peppers: IV. HPLC Determination of the Relative Pungency and Fruit Quality Attributes of Eight (8) Caribbean Hot Pepper Landraces

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Abstract: **- The hot pepper trade for the food processing industry is faced with the issue of variability and consistency in fruit quality in terms of pungency. The Caribbean is recognized as the producers of some of the world's hottest and most pungent peppers but these have not been adequately quantified. This study evaluated eight of the landraces cultivated in the Caribbean and compared these with three of the international varieties. The analysis was conducted on the quality attributes and the determination of both the capsaicin and dihydrocapsaicin were analysed using the U3000HPLC high performance liquid chromatography. The results indicated that the Trinidad Scorpion had the highest capsaicinoids content (2.079 mg.ml) compared to Seven Pot (1.1029 mg.ml) and Carvalho hot (1.070 mg.ml). Further when the capsaicinoids content was converted to the pungency based on Scoville scores, the Trinidad Scorpion (30,000,00SHUs) was twice as hot as both of them. The Carvalho Hot pepper plant has the highest total fixed oil (9.0ml) and capsaicinoids (75.27 mg) and was two to three folds higher than the nearest rival landrace**.

I. INTRODUCTION

There is an International passion about the world's hottest pepper, and pepper enthusiasts and plant breeders are working to develop and select varieties with increased pungency and organoleptic quality attributes including sensation of heat. Hot peppers or Capsicum spp fruits, whether fresh or processed give food that pungent flavor and color. It has pharmaceutical applications and anti-oxidant properties (ILida, et al., 2003; Backonja, et. al., 2010, Reyes-Escogido, et. al. 2011) , as well as non-lethal and self-defence values (Sanatombi and Sharma, 2008).

Pungency in peppers is associated with an alkaloid (capsaicinoids) found only in the genus Capsicum, and is influenced by genotype, fruit maturity and development, (Contreras-Padilla et. al., 1998, Estrada, et al., 2000), and nitrogen and potassium fertilizers (Monforte-González, et. al . 2010) Mohamed and Bridgemohan (2014) successfully developed Caribbean hot peppers with higher capsaicinoid content by reciprocal crossing and selection at The University of Trinidad and Tobago (UTT).

Capsaicinoids are a group of 12 or more related alkaloids in fruits of the genus Capsicum. Capsaicin [(E)-N-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamide] and dihydrocapsaicin (N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonanamide) (Cisneros-Pineda, et al , 2007, Peña-Alvarez et al 2009). Among the capsaicinoids, capsaicin and dihydrocapsaicin together account for about 90% of pungency (Kawada et al., 1985., Govidajaran, 1986;). Capsaicinoids are unique to the genus Capsicum (Govindarajan and Sathyanarayana, 1991). Capsaicin (Hoffman et al., 1983), is restricted to the fruits of hot cultivars and is bio-synthesized and stored in the placenta (Suzuki, Fujiwake, & Iwai (1980).

Different methods have been used to determine and quantify the relative pungency of capsaicinoids and oleoresins of peppers, including organoleptic methods (Govindarajan et al., 1977; Scoville, 1912). The Scoville heat units (SHU) hedonically rated as non-pungent (0–700 SHU) to very highly pungent (>80,000 SHU) (Scoville, 1912, Weiss, 2002) and can be tedious and subjective. Analytical techniques are now used to determine very low amounts of capsaicinoids as they have very similar chemical structures. Spectrophotometry (Anan et al., 1996) and thin-layer chromatography (Sankarikutty et al., 1978) were later conducted. The application of gas–liquid chromatography (Todd et al., 1977), and high-performance liquid chromatography (HPLC) (Weaver and Awde, 1986) have significantly improved the quantification and identification of the various capsaicinoids. The HPLC-MS (mass spectrometry) (Reilly et al., 2002, Thompson, et al., 2005) as the main analytical approach is beneficial to the food processing industry with preparation of hot sauces and pepper mash as it ensures the correct level of pungency. The HPLC is considered the most reliable and rapid method (Mohammed, et al, 2008, Yao et al., 1994)

available for the identification and quantification of capsaicin analogues.

In the Caribbean, there are several superhot chilies eg. the Trinidad / Moruga Scorpion (2,000,00 SHUs) (Torrisi, 2012) and Trinidad chocolate pepper (577,00 SHUs), with the latter culturallylater culturally described as providing enough heat to flavor 7 pots of stew (Bridgemohan, 2010). It has been one of the more common cultivated pepper, and it was suggested that it may be the source of many other landraces [Mohamed, pers.comm, 2016). It has Scoville heat units similar to Bhut Jolokia (1,041,427 SHUs) and the skin resembles that of Bhut Jolokia and Naga Morich (1,359,000 SHUs), but has more ribs and is more plump (Anon, 2012; Reilly et al., 2001; Mohamed and Bridgemohan, 2014).

The indigenous Trinidad Scorpion cultivar (*Capsicum chinense* cvs) is among the most piquant and is referred as such as its tail end is similar to a scorpion's stinger (Anon, 2013). In Trinidad, a new selection of hot pepper was discovered after years of selection and purification by an amateur agronomist and avid home gardener and called Carvahlo Hot (Bridgemohan, 2010). It is described as different from the infamous Trinidad Seven Pot, as the plant is more vigorous and hardy, and the pungency as much as three folds hotter and more piquant compared to other hot pepper landraces grown in the Caribbean and Latin America.The relative pungency was determined by a method developed for rapid assessment of the total fixed oil yield or capsaicinoid using paper chromatography (Mohamed and Bridgemohan, 2014).

This is the first scientific and therefore pioneering investigation to verify and quantify the relative pungency of Caribbean hot pepper landraces using HPLC and to compute Scoville units. The objective of this study is to determine the levels of capsaicin and dihydrocapsaicin and the degree of pungency as well as to and quantify the fixed oil yield for use in the food industry.

II. MATERIALS AND METHODS

The University of Trinidad and Tobago has an extensive program (2010 to 2016) of hot peppers research of more than 24 lines and land races [agronomy, plant breeding and postharvest physiology and biochemistry] at the Waterloo Research Campus. In this particular study, eight (8) of the more popular Caribbean hot peppers landraces were cultivated together with 3 international established varieties from Mexico and India under full sunlight in plastic containers [0.70m3]. The plants were 'fertigated' daily [2.0 kg.200L l of water/day] with a N.P.K. nutrient mix [9:18:36] using a drip irrigation. Pests and diseases were controlled using a judicious spray program of Obberon 24 SC® and Consento 45 SC® at weekly intervals. At maturity, all the ripe peppers of ten plants of each landrace were harvested weekly up to 5 weeks, washed in tap cold water, air–dried in a single layer at 28-30C and then stored in a refrigerator for subsequent analysis. The fruit dimensions included length (cm), width [cm], number of lobules, seed number per fruit placenta wt. [g], skin thickness [mm],color, and skin description. The fixed Oil yield [ml/100g] was quantified after the preceding parameters were recorded. Fruits were chopped and air–dried for 72hrs at room temperature (28-300C). The extraction method used was the solvent extraction technique using both ethanol and acetone as described by Krishna (2004). The Capsaicin and Dihydrocapsaicin were analyzed using the U3000_HPLC high performance liquid chromatography (HPLC) equipped with a the ODS-2 Beckmann Column [250mm x 4.6mm; 5µm] (Table 1). The Solvent was filtered using Reverse Osmosis water and Methanol (HPLC grade – BDH) at 60% Methanol/H2O (0-2 minutes), 60%-99% Methanol/H2O (2-6 minutes), 99% Methanol/H2O (6-8 minutes), and 99%-60% Methanol/H2O (8-10 minutes) (Mohammed et. al 2008) . The Standard solutions were prepared from a stock solution of capsaicin and dihydrocapsaicin using six serial dilutions (0.50 to 0.5 µg/g) (Table 1), exhibited a linear response for both compounds (Table 4, Plate 2). Each solution was injected three times and standard solutions were run on the HPLC and the standard curves were generated by plotting peak area against concentration (Table 1). The external calibration curves were found at r2 = 0.9982 for capsaicin and r2 = 0.9996 for dihydrocapsaicin, and values of r^2 were highly significant confirming the good linearity of the method. The Capsaicin and Dihydrocapsaicin values are presented in Plate 2.

The quality descriptors used to differentiate each landrace were developed by Valls (2007). The pungency in the capsicum samples was computed based on the conversion of the total concentration of capsaicinoids by a factor of approximately 15,000 Scoville Heat Units (SHU)/µg of total capsaicinoids according to Canto-Flick et al. (2008). All field experiments were laid out as completely randomized design with three replicates and at all times the experimental harvested plots were maintained as 10 plants. For analytical purposes from each pooled landrace per harvest samples were taken using three replicates with each replicate consisting of 10 fruits. All data were analysed using the MINTAB statistical package.

Table 1. Summary of the HPLC conditions used for the determination of capsaicinoids (µg/g) in hot peppers.

HPLC Conditions	
Instrument:	Dionex Ultimate 3000
Diode array detector	Diode array detector
Instrument Method:	**Capsaicin method_20160524A**
Processing Method:	**pepper samples_20160530A**
Analysis Time:	4.0 min.
Flow Rate:	1.0 mL/min. (3000 psi)
Oven Temp.:	25 ºC
Run Time (min):	**10.00**
Calibration Level:	**04**
Channel:	**UV_VIS_3**
Wavelength:	280.0
Bandwidth:	1
Sample Weight:	1.0000
Detection:	Altus A-10 PDA ; Excitation: 222 nm
Injection Volume:	10 µL
Injection name	**CAL Mix 4**
Vial Number:	**GA4**
Injection Type:	**Calibration Standard**
Sampling (Data) Rate:	10 pts./sec
Injection volume:	20 µl
Samples:	Stored at 4°C until analyzed
Samples injected	1:1 dilution with MeOH
Standards:	Capsaicin (Sigma-Aldrich) stock solution (1.85 mg/ml)
Dehydrocapsaicin (Sigma-Aldrich)	stock solution (1.91 mg/ml)
Calibration range:	Capsaicin (0.0833mg/ml – 1.850/
Dehydrocapsaicin	(0.0847 mg/ml – 1.910 mg/ml)

III. RESULTS AND DISCUSSION

The quality attributes such as colour, skin texture, fruit shape varied among landraces. The characteristic colour of the nine (9) landraces showed that 7 were red, and one was green and one yellow (Plate 1). The skin texture varied between smooth and very rough. It was observed that the rough skin was related to flavour and pungency, as the rougher the skin appeared the more pungent and flavourful were the fruits (Table 2). Most of the fruits were globular and rounded, with cv. Scotch Bonnet having a distinct bonnet at the base of the fruit. Three of the pepper landraces had tapered ends (Carvahlo, Bhut Jalokia, and Scorpion) and these were classified as very hot to extremely hot. The tapered ends are described as the 'scorpion tail' and is observed to be the hottest peppers. The fruit length varied between 11.3 cm (Chilli) to 2.8 cm (Seven Pot). The elongated fruits had less widths compared to the rounded – globular fruits, and were usually single lobed. All the other fruits had 3 or more lobules. Carvalho had the highest placenta weight (9g) compared to Scorpion and Chilli. While the mean number of seed per fruit was 58, Seven Pot, Carvalho and Habanero produced in excess of 69 seeds / fruit (Table 3). The fixed oil yield in the Carvalho (9.0 ml) and Bhut Jalokai (6.0 ml) were higher than the other seven pepper landraces.

Table 2. Fruits quality characteristics of hot pepper landraces.

Characteristics	Hot pepper Landraces								
	Scotch Bonnet	**Seven Pot**	**'Carvahlo Hot'**	**Bhut Jolokia**	**Habanero**	**Scorpion**	**Bird**	**Chilli**	**Jalapeno**
Colour	yellow	red	red	red	red	red	Red	green	red
Skin texture	Smooth, firm	Rough, firm	Very rough, firm	Rough, thin membrane	Smooth, firm	Rough, thin membrane	smooth	Smooth, firm	Smooth, firm
Appearance	Vivid, glossy	Vivid	Vivid	Vivid	Waxy, glossy	Vivid	Slight sheen	Vivid, waxy, glossy,	Waxy, glossy,
Shape	Globular	Globular	Globular tapered end	Triangulate elongated, tapered end	Globular	Triangulate elongated, tapered end	Conical Tapered end	Elongated, cylindrical, tapered end	Elliptical, elongated, tapered end
Flavour	Hot	Very hot	Extremely hot	Very hot	Hot	Very hot	hot	Hot, fruity	Medium heat, fruity
Aroma	Mild	Pungent	Very pungent	Pungent	Mild	Pungent	Medium	Medium	Medium, fruity

Table 3. Selected fruit characteristics of landraces of hot peppers

Characteristics	Hot pepper Landraces									
	Scotch Bonnet	Seven Pot	'Carvahlo Hot'	Bhut Jolokia	Habanero	Scorpion	Chilli	Bird	Jalapeno	$\bar{x}$ [S.E ±]
Length [cm]	3.1	2.8	2.9	7	3.2	5.3	11.3	3.68	5.7	5.07 [1.062]
Width [cm]	2.7	2.6	2.8	2	2.6	1.62	1.8	0.8	1.8	2.16 [0.367]
Nos. Lobules	3	4	3	3	3	4	1	1	1	2.75 [0.817]
Nos. Seed.fruit^{-1}	118	72	69	27	79	26	44	29	36	58.87 [33.5]
Placenta wt. [g]	0.34	0.41	0.9	0.32	0.39	0.61	0.79	0.07	0.21	0.49 [0.055]
Skin thickness [mm]	1	1	2	1.82	1.4	1.1	1.9	0.11	1.7	1.49 [0.286]
Oil yield.100g^{-1}	3.5	5.0	9.0	6.0	4.0	5.0	1.0		1.0	4.31 [1.021]

Table 4. Calibration data of the HPLC method for the determination of capsaicinoids (µg/g).

Capsaicinoids	Linear Range	R^2	Ret. Time	Average Peak Area
Capsaicin	0.05–0.50	0.9982	8.327	219.1647
Dihydrocapsaicin	0.05–0.50	0.9996	8.320	0.9212

Table 5. Selected fruit characteristics of three landraces of hot peppers

Landraces	code	Area mAU*min	Height mAU	Rel. Area %	Capsaicin concentration (mg/ml	Dihydrocapsaicin concentration (mg/ml
Scorpion	SC	146.70	1946.70	78.02	1.61	0.46
Chilli	Ch	10.52	154.24	78.22	0.12	0.04
Cherry	CPl	7.28	107.33	86.88	0.09	0.02
Scotch Bonnet	SB	15.52	229.03	77.99	0.18	0.05
Carvahlo Hot'	Cl	71.13	1034.16	74.11	0.79	0.28
Jalapeno	J	8.00	113.00	57.74	0.09	0.07
Kiri-kiri	KK	40.93	620.29	82.83	0.45	0.10
Bird	BPl	8.69	127.28	78.31	0.10	0.03
Bhut Jolokia	BJl	33.04	477.37	73.32	0.37	0.14
Seven Pot	SPl	70.35	1010.02	71.99	0.78	0.30
Habanero	SPl	23.93	359.67	78.03	0.27	0.08
Capsaicin	CAP neat	219.16	2388.90	100.00	1.20	
Dihydrocapsaicin	DHCAP neat	0.921	13.567	0.41	0.01	
$\overline{x}$		50.47	660.12	72.14	0.47	0.14
[S.E ±]		37.439	434.493	13.601	0.286	0.084

Table6. The relative Pungency of hot pepper landrace using the Scoville Heat Units (SHU)/mg of total capsaicinoids .

Landrace	[capsaicinoid] mg	Scoville Heat Units (SHU)
Scorpion	2.0815	31,222,500
Chilli	0.167	2,505,000
Cherry	0.1109	1,663,500
Scotch Bonnet	0.2379	3,568,500
Carvahlo Hot'	1.0712	16,068,000
Jalapeno	0.1682	2,523,000
Kiri-Kiri	0.561	8,415,000
Bird	0.1413	2,119,500
Bhut Jolokia	0.5138	7,707,000
Seven Pot	1.0912	16,368,000
Habenero	0.3558	5,337,000

The Capsaicin and Dihydrocapsaicin values for all 9 landraces are presented in Plate 3. concentration varied between 1.119 to 0.106 (mg/ml) and 0.04625 to 0.0353 (mg/ml) for Scorpion and Bird peppers, respectively (Table 5). Scorpion had the highest concentration of both capsaicinoids (more than twice) the second candidate Carvalho for both Capsaicin and Dihydrocapsaicin concentration (0.790 and 0.TT Table 1)2812mg/ml), respectively. The peppers classed as very hot and pungent all had capsaicinoids in excess of 1.0 (mg/ml) viz. Scorpion (2.01 mg/ml), Carvalho (1.07 mg/ml), and Seven Pot (1.091mg/ml). There was no correlation between seed numbers and placenta size (-0.04) or placenta and total oil yield ((0.04).

The capsicum pungency [capsaicinoids] was converted to Scoville Heat Units (SHU)/g (Canto-Flick et al., 2008). It showed that Scorpion had in excess of 30, 000,000 SHUs, compared to Seven Pot and Carvalho at second place with 16,000,000SHUs (Table 6). However, at the plant level (Table 7), Carvalho had the ability to produce twice the yield of Capsaicinoid /tree [75.2mg] compared to Scorpion (32.27mg). This suggested that Scorpion is the hottest Caribbean pepper landrace (Bridgemohan, 2010). For food processing and the pharmaceutical industry, ergonomically, it is more feasible to cultivate Carvalho to achieve the highest yield of Capsaicinoid.

This study confirms that the Caribbean possesses Capsicum chinense landraces which includes some of the most pungent chili peppers in the world. Bosland and Baral (2007) reported the two hottest peppers were Habanero pepper (100,000 to 350,000 SHU) and Bhut Jolokia (879,953 to 927,199 SHU). However, when these same cultivars were grown under the experimental conditions in Trinidad, the pungency were much higher for both Habanero pepper (5,337,000 SHU) and Bhut Jolokia (7,707,000 SHU). The capsaicinoids is responsible for the pungency and the characteristic sharp taste or sensation of heat caused by the fruit when consumed (Zewdie and Bosland, 2000; Mohammed et. al. 2008). The capsaicinoids are biosynthesized and stored in the vegetative organs, and small amounts in the seeds (Balbaa et al., 1986; Estrada et al., 2002; Ohta and Chuong, 1975). While there were no significant differences in seed production between Seven Pot and Carvalho, they were three time higher than Scorpion. Further, scorpion had a placenta weight 2 to 3 times greater than all other peppers. This suggest that within the pepper fruit, as in the case of Seven Pot, capsaicinoids can accumulate along the epidermal cells of the interlocular septum, derived from the tissue connecting the placenta to the pericarp (Judd et al., 1999).

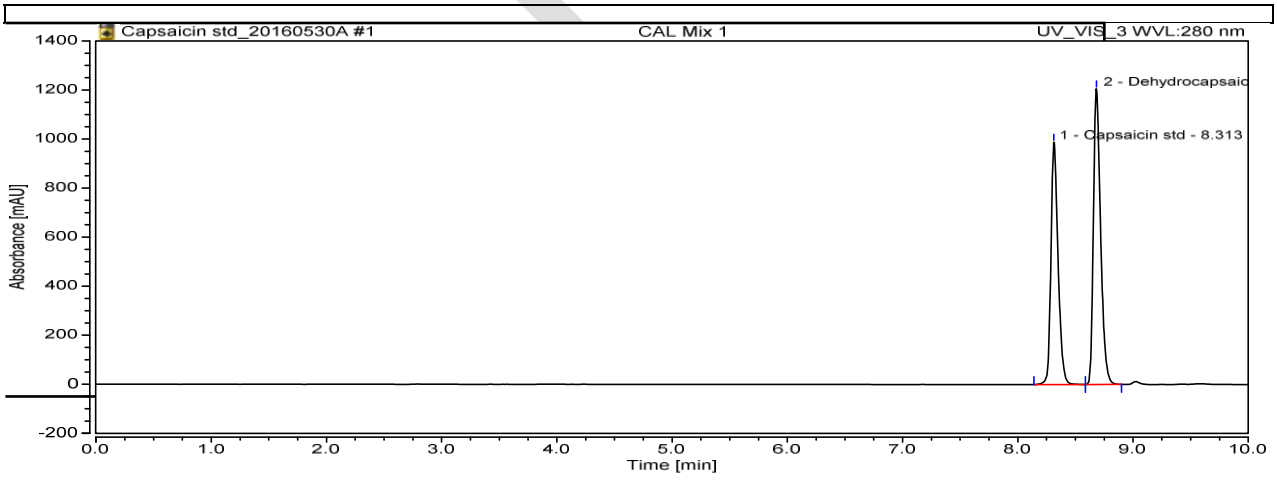
In the more pungent landraces, there were marked epidermal protrusions or rough skin which arises from the lifting of the cuticle layer from the cell wall during the filling of subcuticular cavities with capsaicinoids (Rao and Paran, 2003).

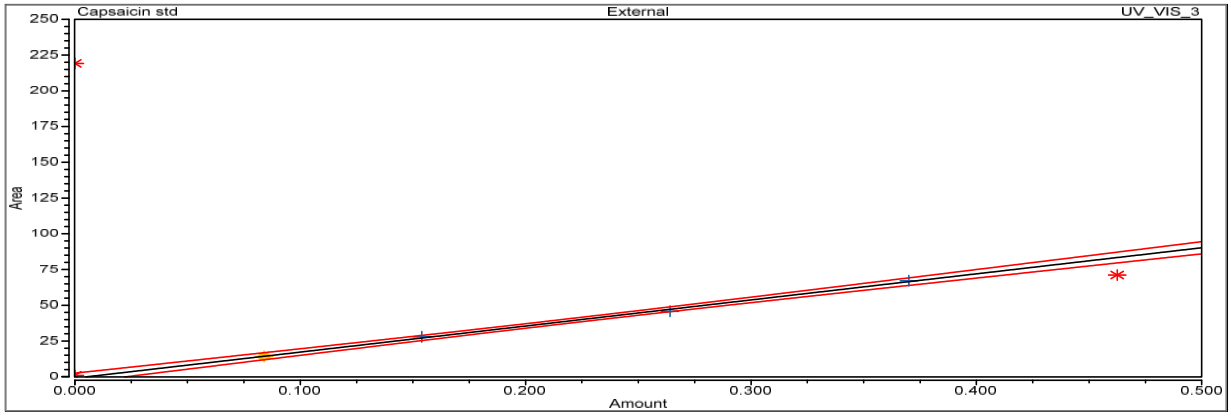
The Fixed Oil content which is a complex of all the capsaicinoids capsaicin, homo-, dihydro-, nordihydro- and homodihydro-capsaicins, include all other aromatic compounds which add to the flavour and aroma of the fruit. 'Carvahlo hot' has the potential to produce approximately the highest pepper-oil yield in the Caribbean (4.15 to 5.05g at 450bar) using SFE and Gibbs and O'Garro [2004] (37.6 to 497mg/100g) using HPLC).

Capsaicinoids have significant pharmaceutical and non-lethal force potential. It is used in topical ointments to relieve pain of peripheral neuropathy at low concentrations [0.025 to 0.075%], as a treatment in apoptosis of prostate cancer cell, and is being tested for the prevention of pain post-surgery (Swaminathan, 2007). Whilst most of the varieties / landraces cultivated are considered 'hot' based on the Scoville Unit [SU] test e.g. Scotch Bonnet (300,000SU), Seven Pot (750,000SU), and Scorpion, (>1,000,000SU) little effort was pursued in producing varieties for high oil yield for the food industry.

Brown Congo **Habaner** **Jalapeño**

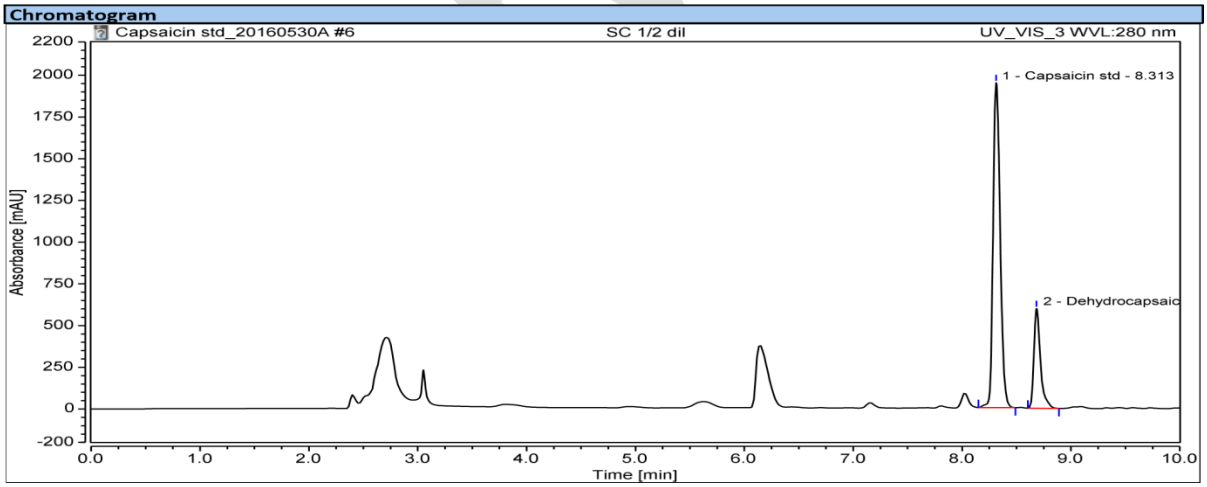
Plate1. Popular Caribbean landraces and international varieties of hot peppers.





Calibration Results	**Capsaicin std**						
No.	Injection Name	Calibration	X Value UV_VIS_3 Capsaicin	Y Value UV_VIS_3 Capsaicin	Y Value UV_VIS_3 Capsaicin	Area UV_VIS_3 Capsaicin	Height UV_VIS_3 Capsaicin
1	CAL Mix 1	01	0.4625	71.16123012	71.16123012	71.1612	988.2997
2	CAL Mix 2	02	0.37	67.16146395	67.16146395	67.1615	958.5976
3	CAL Mix 3	03	0.264	45.86304326	45.86304326	45.8630	668.5309
4	CAL Mix 4	04	0.154	27.95982128	27.95982128	27.9598	408.7052
5	CAL Mix 5	05	0.084	14.22432475	14.22432475	14.2243	208.5893
17	CAP neat	06	0	219.1646636	219.1646636	219.1647	2388.8995
18	DHCAP neat	06	0	0.9212290072	0.9212290072	0.9212	13.5675

Plate 2. Calibration data of the HPLC method for the determination of capsaicin and dihydrocapsaicin (µg/g).



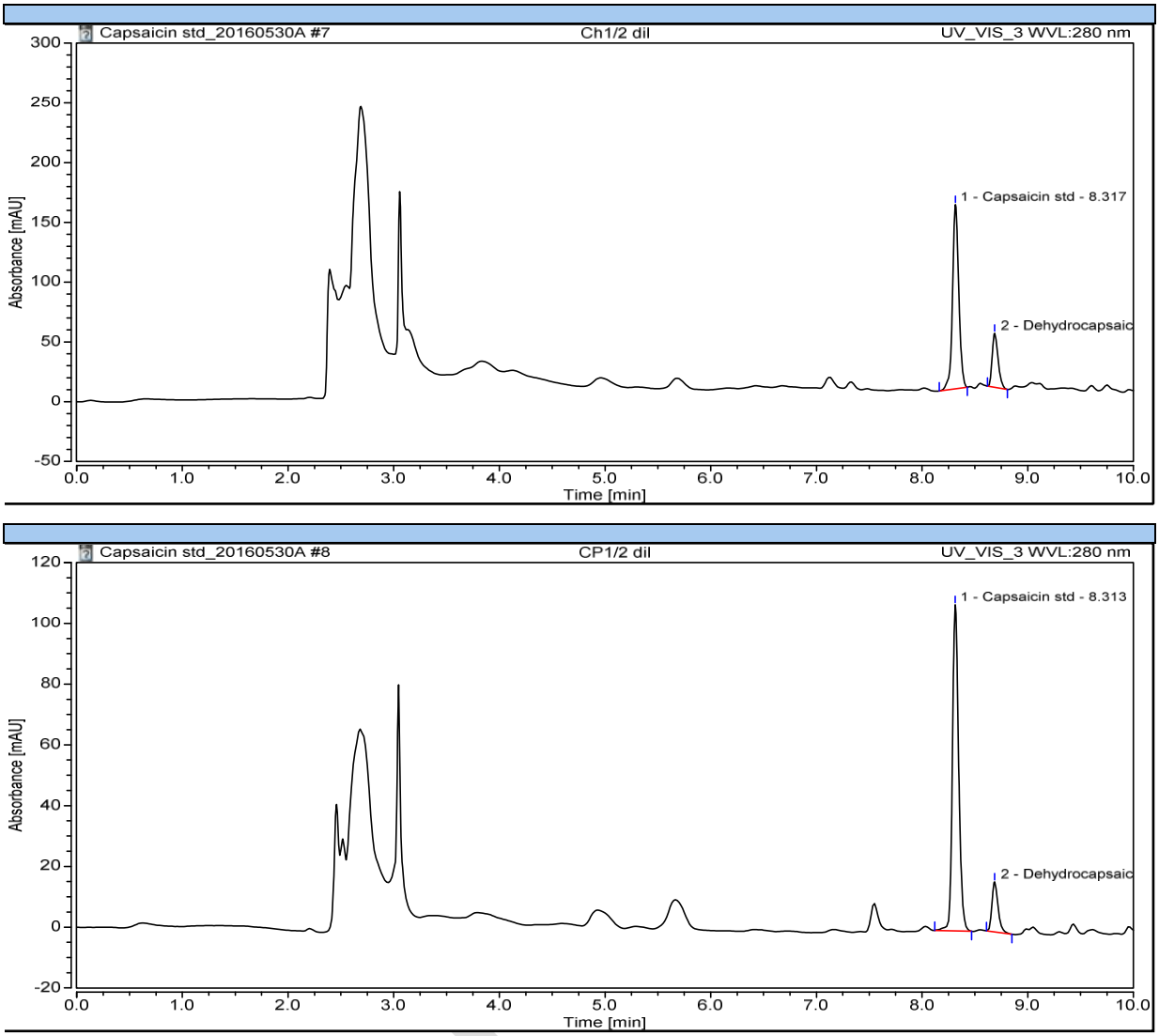
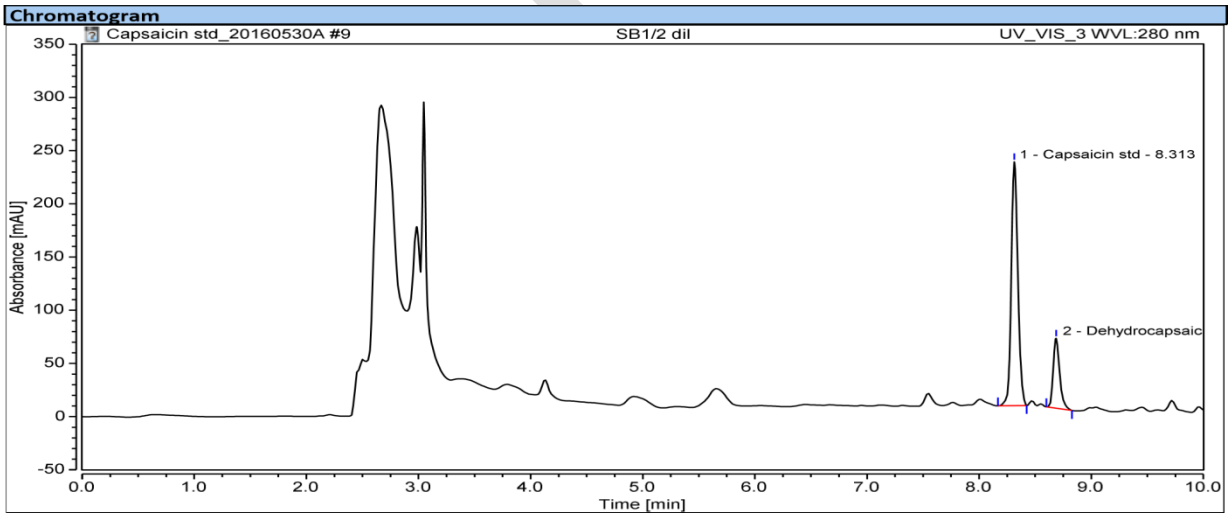


Plate 3a. Capsaicin and dihydrocapsaicin (µg/g) contents of the Trinidad Scorpion, Chilli and Cherry pepper landraces using the HPLC method .



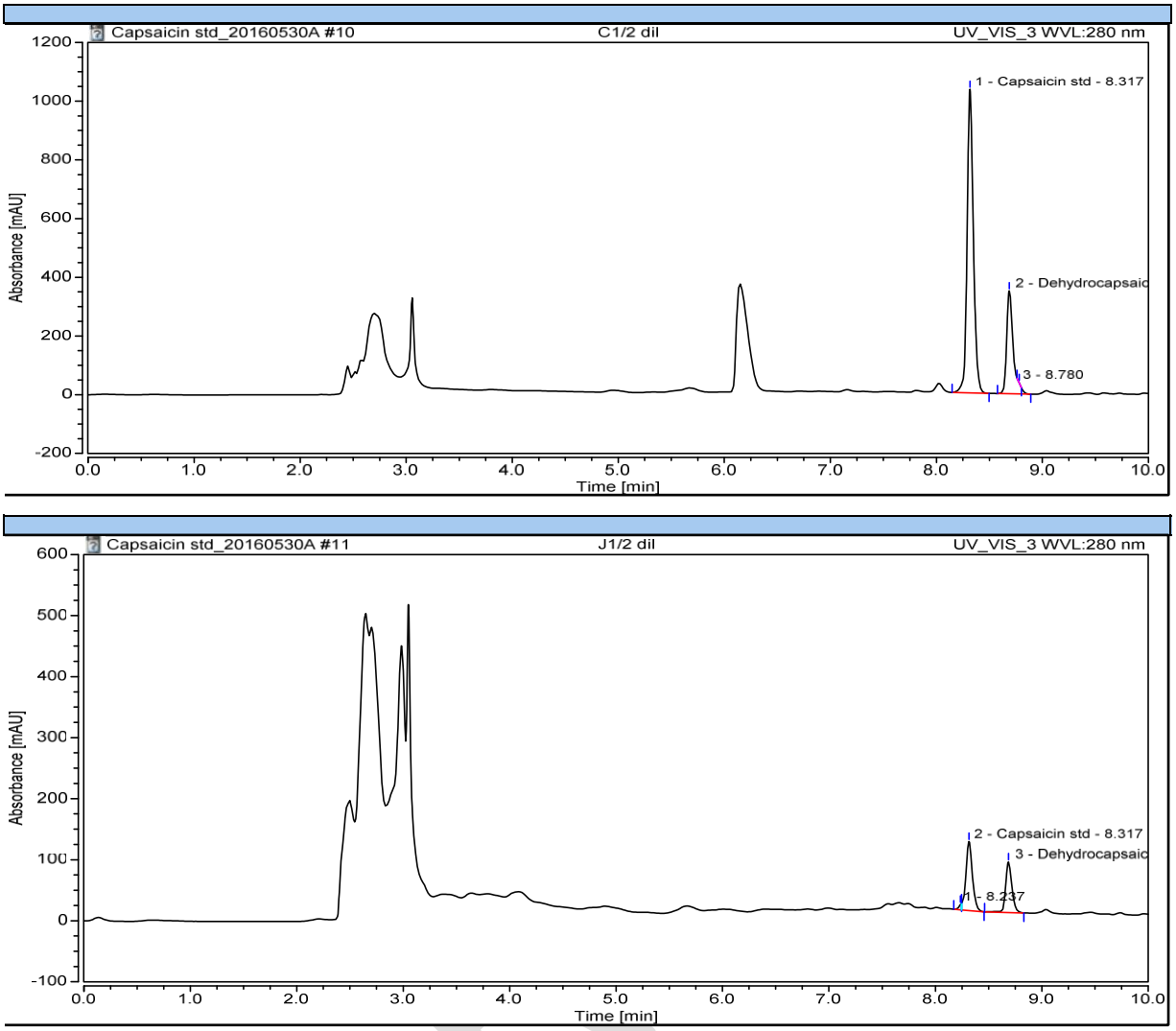
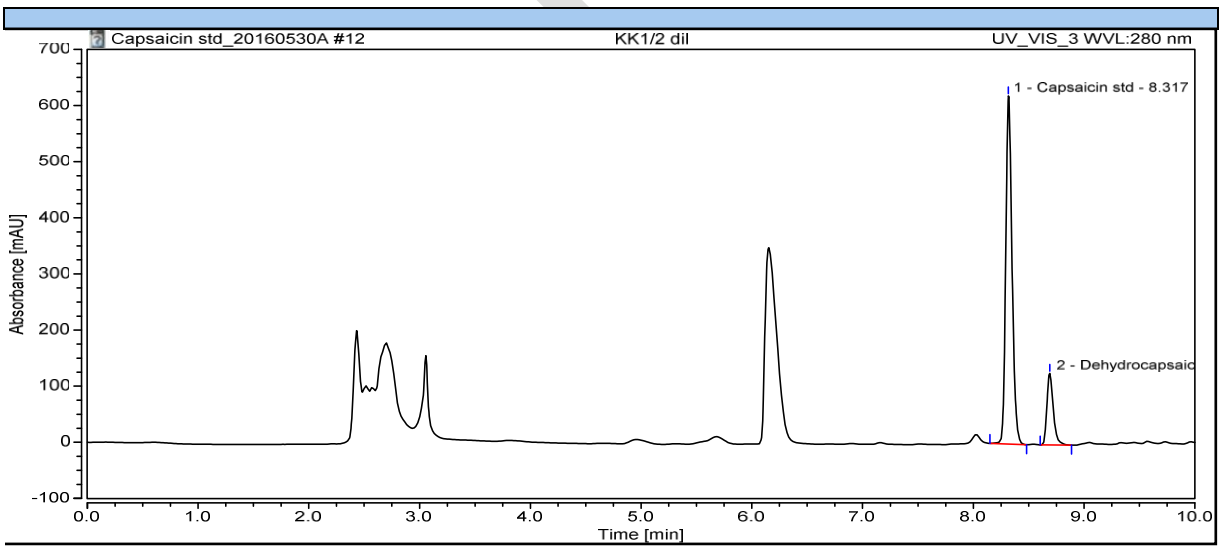


Plate 3b. Capsaicin and dihydrocapsaicin (µg/g) contents of the Scotch Bonnet, Carvalho Hot and Jalapeno pepper landraces using the HPLC method .



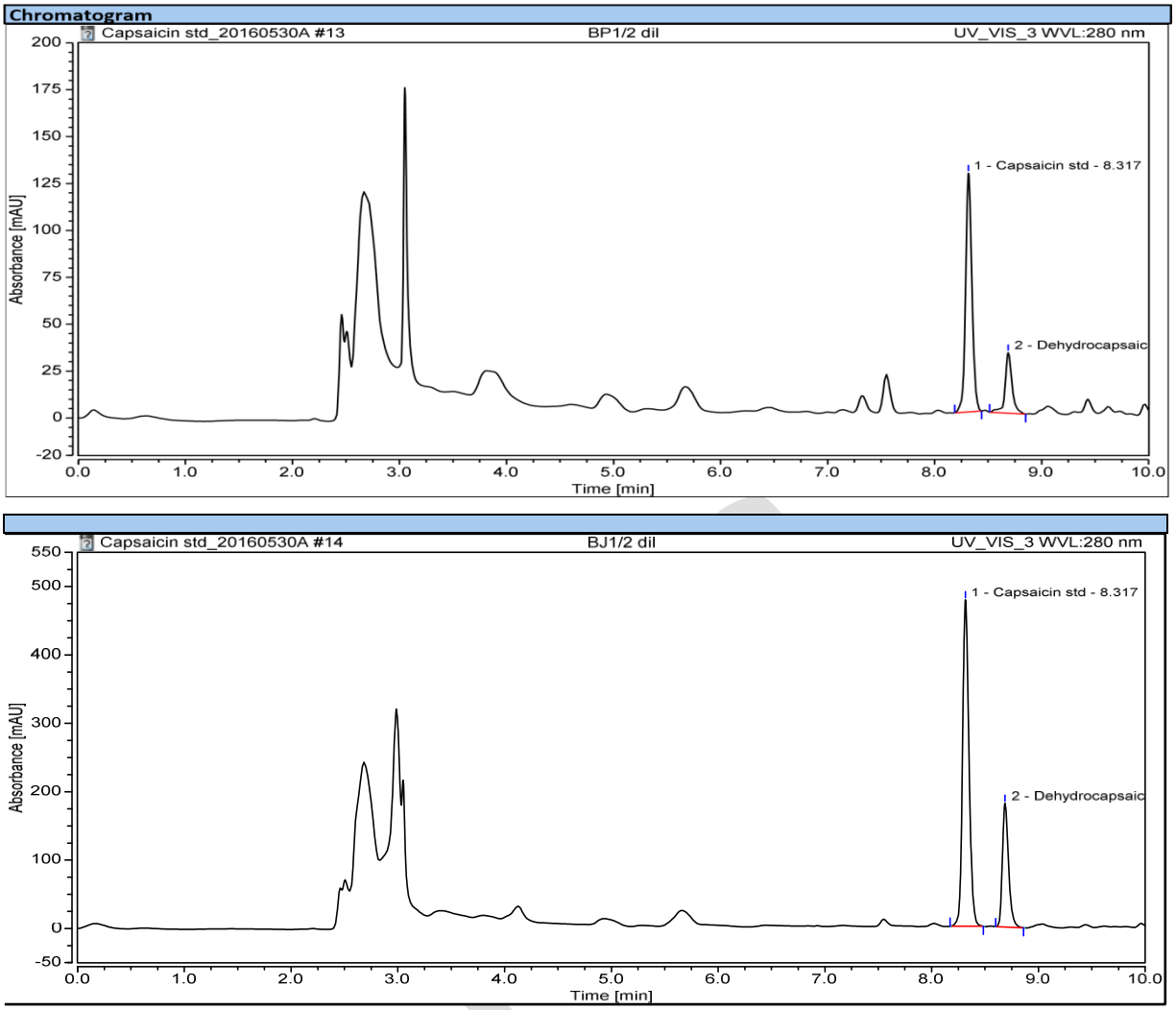
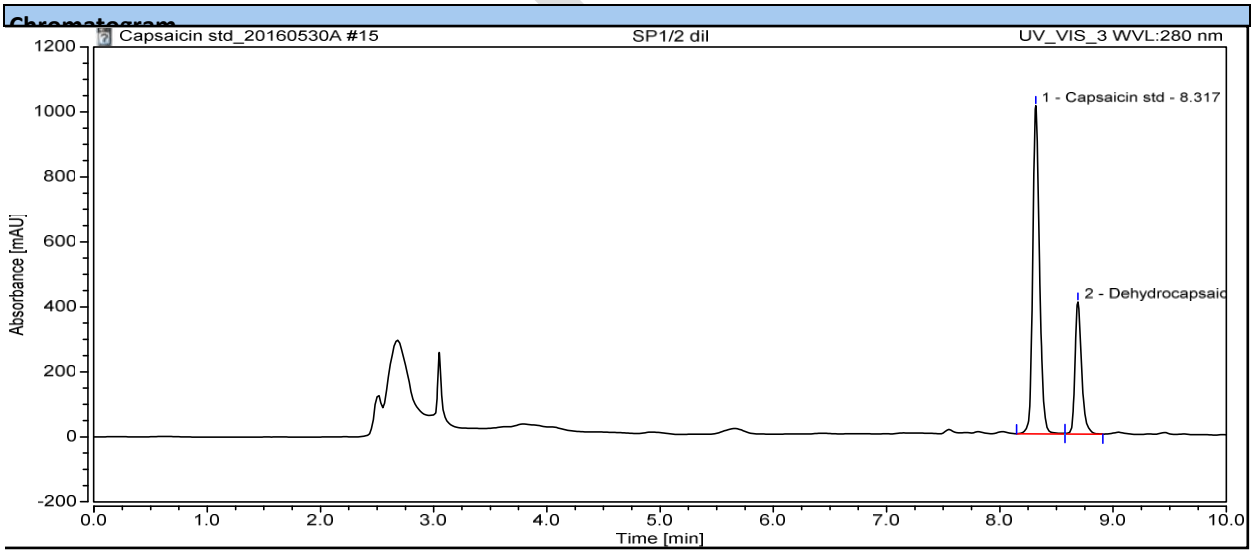


Plate 3c. Capsaicin and dihydrocapsaicin (µg/g) contents of the Kiri Kiri, Bird and Bhut Jalokia peppers using the HPLC method .



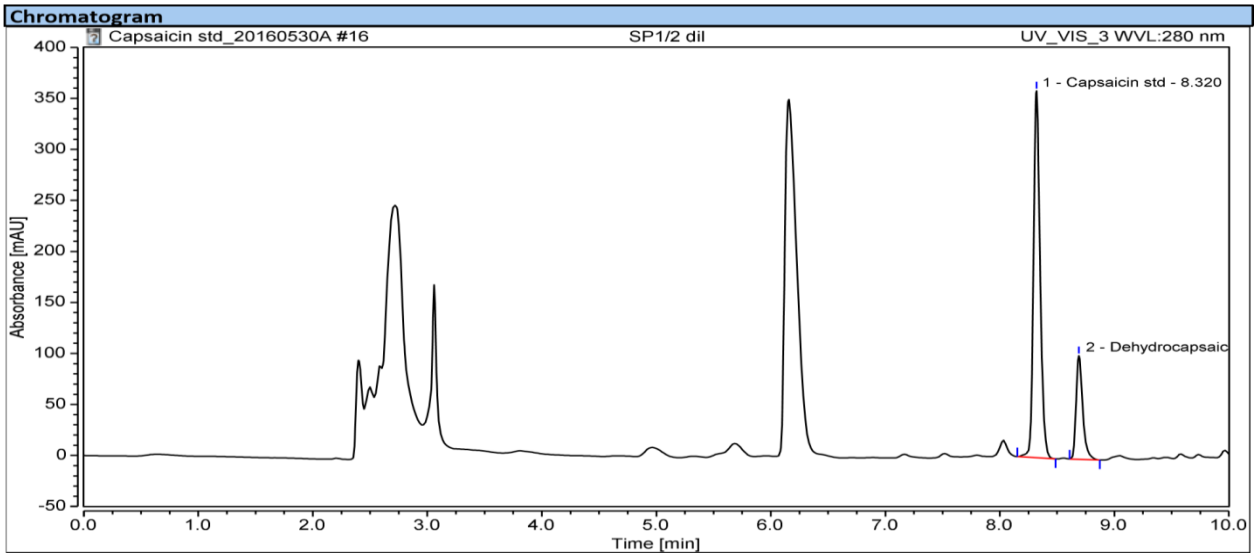


Plate 3d. Capsaicin and dihydrocapsaicin (µg/g) contents of the Seven Pot, Bird and Habanero peppers using the HPLC method.

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