Utilizing LC/UV and LC/MS for the Characterization, Isolation, and Quantitation of Capsaicinoids in Chili Peppers and Hot Sauces

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Abstract

Hot and spicy food has dramatically increased in popularity over the past 10-20 years. Capsaicin is the most abundant compound found in chili peppers giving them their fiery flavor. Capsaicin is formed when vanillylamine is coupled to a 10 carbon fatty acid through an amide linkage. However, there are other related compounds often called capsaicinoids. These compounds have the same vanillylamine group but differ by the associated fatty acid chain and are responsible for the perception of different heat profiles for different chili peppers. Some peppers are described as having a high initial flash of heat while other peppers are described by a long and late burning profile.

The work presented here, initially uses HPLC with UV detection to profile capsaicinoids extracted from several different chili peppers and commercially available hot sauces. Prep HPLC is then used to isolate individual capsaicinoids from the pepper extracts. Finally, a triple quadruple MS system is employed to identify and quantitate the observed capsaicinoids.

Capsaicin was found to be the most prevalent capsaicinoid species in all of the studied matrices. Significant amounts of Nordihydrocapsaicin were found in a cayenne hot sauce and in Thai chili pepper extract. Dihydrocapsaicin and Homodihydrocapsaicin were also identified in many of the investigated chili extracts and hot sauces but at lower levels. The typical concentration of these compounds were found to be in the µg/g range but varied widely among the different chili peppers and hot sauces.

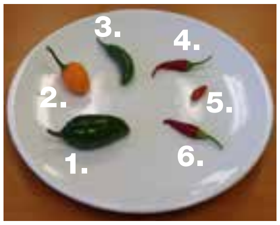
Introduction

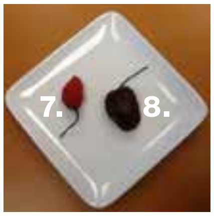
Capsaicin is an amide derived from vanillylamine and a short chain fatty acid. A variety of capsaicin related compounds are formed when different fatty acid chains form this amide. This group of compounds are responsible for the pungency of chili peppers. Wilber Scoville established a scale to demonstrate the pungency of chili peppers based on taste-tests[1] . This scale was later named in his honor. The pungency of chili peppers and hot sauces (or any other food) are currently determining the combined concentration of the three major capsaicinoids; capsaicin, dihydrocapsaicin and nordihydrocapsaicin. This determination is typically accomplished by a reversed phase chromatographic method (RP-HPLC). After this combined concentration value is determined by the chromatographic method (in ppm), it is then multiplied by a conversion factor of 15 to arrive at a corresponding Scoville Heat Unit (SHU) value.

Although in high concentrations, all capsaicinoids produce burning sensations throughout the mouth and throat (and mucous membrane), in low concentrations they affect different areas of the mouth and throat. It is unknown how the low level addition of these pungent compounds can enhance the food flavor profile[1-3].

The work presented here has 3 distinct parts. Initially a 3 minute analytical scale HPLC-UV method was developed utilizing a Kinetex® 5 µm C18 50 x 4.6 mm column. This methodology was employed to profile capsaicinoids from several different chili peppers and commercially available hot sauces. A fixed amount of ethanol and one whole pepper was used for the pepper extractions except for the Ghost Chilis, where only ½ of the pepper was used. This allows for a direct comparison of the amount of capsaicin related components on a per pepper basis. This analytical methodology was then scaled to Prep HPLC on a Kinetex 5 µm C18 100 x 21.2 mm AXIA™ packed column and utilized to isolate individual capsaicinoids from pepper extracts. Finally, a triple quadruple LC/MS system is employed to identify and quantitate the observed capsaicinoids.

Figure 1. Pepper Identification





Materials and Methods

Pepper samples and salsa samples were home grown and homemade. Hot sauce samples were purchased at stores within the Los Angeles area. Reagents were purchased from Sigma Aldrich, including Acetonitrile (ACN), Ethanol (EtOH), Formic Acid, Methanol (MeOH), Perchloric Acid, Trifluoroacetic Acetic Acid (TFA)

Capsaicin, Dihydrocapsaicin and Novanimide were purchased (ChromaDex®, Irvine, CA)

Sample Preparation

Bulk Chili Pepper Extraction (used for capsaicinoid profiling and LC/MS analyses)

1. 250 g of fresh chili peppers were blended for an extended period of time with DI water and EtOH to contain approximately 65 % alcohol
2. An aliquot of the puree was removed and filtered prior to analysis.

Single Chili Pepper Extraction (used only for capsaicinoid profiling)

1. An entire pepper (except for the stem) was finely chopped (for Ghost Peppers, ½ a pepper was used)
2. The chopped pepper was placed in a 20 mL scintillation vial with 10 mL EtOH
3. The vial was capped and the contents were sonicated for 20 min
4. The remaining solids were allowed to settle and a portion of the liquid was filtered and used directly

Chili Pepper Sauce Extraction

1. Various national and local brand chili pepper sauces (picante form) were obtained and 0.5 g were placed in separate 20 mL scintillation vials
2. To each vial, 3 mL DI water and 7 mL EtOH were added
3. The vials were capped and sonicated for 20 min at room temp
4. The mixture is then centrifuged and an aliquot of supernatant is collected
5. For HPLC-UV, the supernatant is used directly
6. For LC/MS/MS the supernatant is diluted with 30 % MeOH containing 0.1 % formic acid

Capsaicinoids Profiling/Characterization

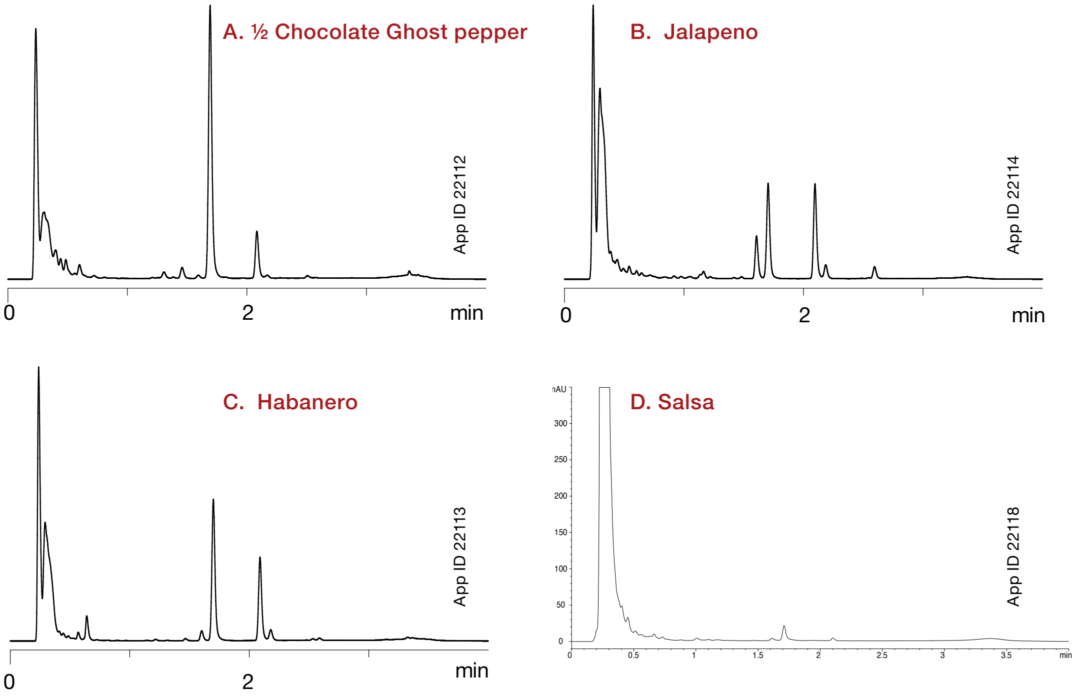
LC/UV Conditions

Column: Kinetex 2.6 µm C18
Dimensions: 50 x 4.6 mm
Part No.: 00B-4462-E0
Mobile Phase: A: 0.1 % Perchloric Acid in Water
B: Acetonitrile
Gradient:

Time (min)	B (%)
0.0	40
2.25	60
2.50	70
2.65	90
2.85	40
3.00	40

Flow Rate: 2.0 mL/min
Temperature: 30° C
Detection: UV @ 228 nm
System: Agilent® 1100 Quaternary
Detector: Agilent 1100 PDA

Figure 2. LC/UV example chromatograms of representative chilies and sauces


A. ½ Chocolate Ghost pepper

B. Jalapeno

C. Habanero

D. Salsa

Capsaicinoid Individual Isolation

LC/UV Preparative Chromatography Conditions

Column: Kinetex 5 µm C18 Axia Packed

Dimensions: 100 x 21.2 mm

Part No.: 00F-4601-P0-AX

Mobile Phase: A: 0.1 % TFA in Water
B: 0.1 % TFA in Acetonitrile

Gradient:

Time (min)	B (%)
0.0	40
4.5	60
5	70
6	40

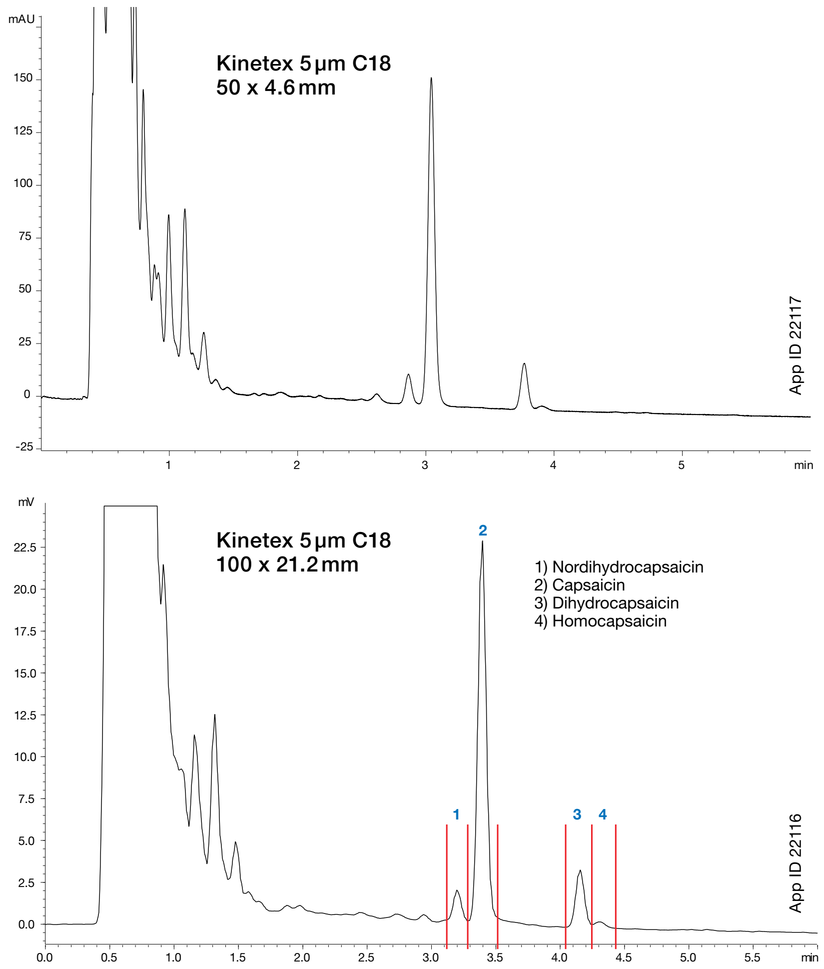
Flow Rate: 2.0 mL/min

Temperature: Ambient

System: Shimadzu® LC20

Detection: UV @ 228 nm

Figure 3. Analytical and Corresponding Preparative Chromatogram



Identification and Quantification of Major Capsaicinoids

LC/MS Conditions

Column:	Kinetex 5 µm C18
Dimensions:	100 x 2.1 mm
Part No.:	00D-4601-AN
Mobile Phase:	A: 0.1 % Formic in Water B: 0.1 % Formic Acid in Methanol
Flow Rate:	0.6 mL/min
Temperature:	Ambient
System:	Agilent® 1200SL (binary pump)
Detection System:	AB SCIEX API 4000™ LC/MS/MS
Ionization Source:	ESI in Positive Polarity

Gradient:

Time (min)	B (%)
0.0	30
7.0	90
9.0	90
9.1	30
12.0	30

MS/MS Conditions

Precursor Method

Precursor mass:	136.9 amu
Q1 Mass Range:	150 to 350 amu
Scan time:	2 sec
Scan mode:	Profile @ 0.25 amu step size

IDA Parameters:

1 to 2 most intense peaks exceeding 49000 cps
MS/MS scan at 20 and 30 V from 100-350 Da

Ionization Source Parameters

Gas 1 & Gas 2	50
CAD	5
Cur	35
IS	3500
Temp	600

Table 2. MRM Transitions and Ionization Source Parameters

Compound	Q1, Da	Q3, Da	DP, V	CE, V
Capsaicin	306.1	136.9	75	40
Dihydrocapsaicin	308.1	136.9	42	25
Nordihydrocapsaicin	294.1	136.9	45	18
Homodihydrocapsaicin	322.2	136.9	70	20
Homocapsaicin	320.2	136.9	47	20

Figure 4. Fragmentation Comparison of Capsaicin and Dihydrocapsaicin at Collision Energy (CE) of 15 V

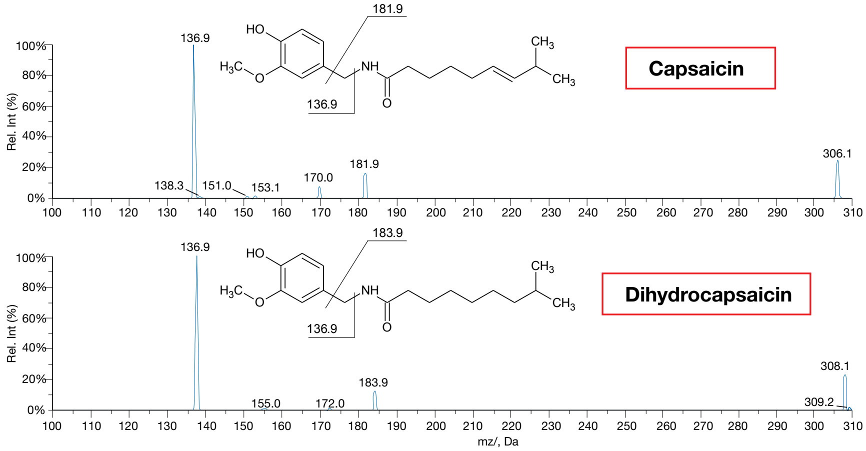


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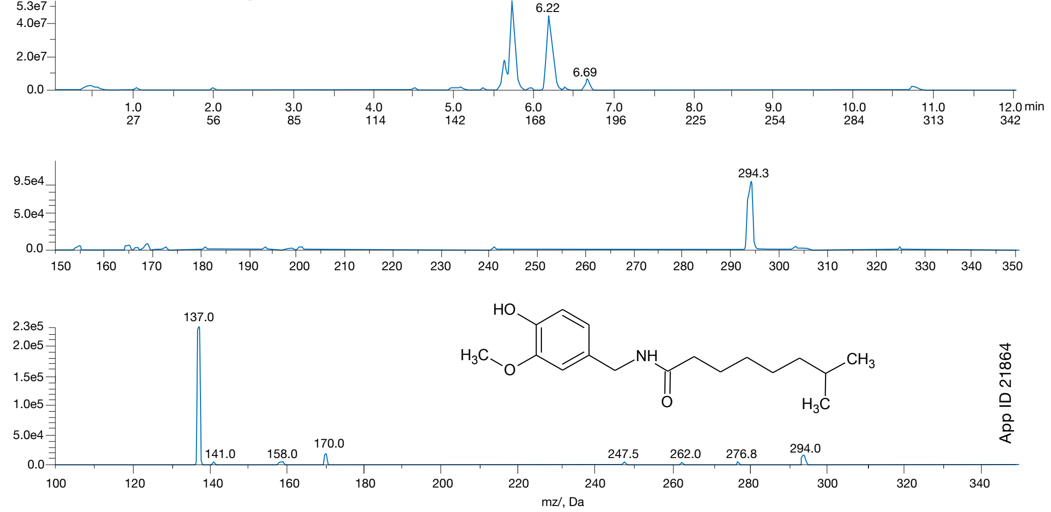
Figure 5. Precursor Scan of Nordihydrocapsaicin in Serrano Chili Pepper and MS/MS Spectrum of Nordihydrocapsaicin at CE = 20 V



Results and Discussion

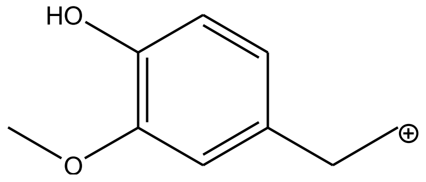
Analysis - Bulk Chili Pepper Extract Comparison

Extracts from Habanero, Serrano, Jalapeno and Thai chili peppers were analyzed by a precursor scan method. MS/MS spectra of suitable candidate peaks were collected as a further measure of identification of the capsaicinoids (**Table 3**). The possible capsaicinoids are listed in **Table 1**. Surprisingly, based on ion intensities, Thai chili peppers had a large overall concentration of capsaicinoids. In addition, the ratio these other capsaicinoids to capsaicin was higher than the rest of the chili peppers we investigated, see **Table 3**.

The presence of multiple peaks for some of these compounds, most noteworthy nordihydrocapsaicin, suggests the presence of isomers, possibly on the fatty acid tail side. The presence of branched (e.g. isopropyl tail) and straight chain acid could be one of the possible isomeric forms (**Figure 5**). Due to poor fragmentation pattern of these molecules, a confirmation will require additional techniques such as H-NMR. Such an endeavor might be pursued in the future work.

Capsaicins Fragmentation Profile

Capsaicin, dihydrocapsaicin, and novanimide were purchased and analyzed to determine their ionization properties and fragmentation patterns. The fragmentation pattern indicated the amide bond breaks to form a predominant fragment for all of these related compounds (**Figure 4**). The most abundant fragment has a mass of 136.9 and is displayed below. This mass was used in the MRM and Precursor scan analysis, see below.



Quantitation of Capsaicinoids in Hot Sauces

Following the extraction and purification of the various capsaicinoids from Thai chili peppers, 0.1 to 7 mg of various species were obtained (**Figure 3**). After a quick infusion study and MS analysis, the material purity deemed sufficient to setup a semi-quantitative method with an external calibration.

An MRM method was quickly established with the various precursor masses established earlier and the common fragment as the Q3 mass (**Table 2**). Furthermore, due to high concentration of these compounds, some of the ion source and mass-dependent parameters were detuned to reduce overall signal intensities. The identity of the specific octanoic acid vanillinamide were not known at this time and these peaks were only monitored (**Table 1**). The extracts from the chili sauces were analyzed with a faster LC gradient under the MRM method (**Table 4**).

Evaluation of Capsaicinoids from Single Peppers

Bulk extracts are a fair way to compare different types of peppers but it is not very practical from a consumer perspective. When purchasing peppers to prepare a dish, it would be useful to know the typical amount of capsaicinoids each pepper would contribute to that dish. **Table 3** summarizes the capsaicinoid results for individual peppers. From the Peak Area data, the total amount of capsaicinoids can be evaluated. From the Peak Area % data, the relative amount of each capsaicinoid in that pepper is described.

Table 3. HPLC-UV Individual Pepper Results

Peak Area	1	2	3	4
	Nordihydrocapsaicin	Capsaicin	Dihydrocapsaicin	Homocapsaicin
1/2 Chocolate Ghost	7.71	638.91	106.14	7.56
1/2 Ghost	9.29	280.06	162.14	8.02
Pequin	12.75	262.14	53.34	2.87
Habanero	14.68	217.2	126.97	14.05
Jalapeno	73.42	170.89	170.22	19.84
Serrano	11.18	53.85	38.36	2.13
Cayenne	19.56	39.91	47.7	26.81
Thai chili	5.63	9.5	11.77	3.42

Peak Area %	1	2	3	4
	Nordihydrocapsaicin	Capsaicin	Dihydrocapsaicin	Homocapsaicin
1/2 Chocolate Ghost	1.0	84.0	14.0	1.0
1/2 Ghost	2.0	60.9	35.3	1.7
Pequin	3.9	79.2	16.1	0.9
Habanero	3.9	58.2	34.0	3.8
Jalapeno	16.9	39.3	39.2	4.6
Serrano	10.6	51.0	36.4	2.0
Cayenne	14.6	29.8	35.6	20.0
Thai chili	18.6	31.3	38.8	11.3

Table 4. Concentration of Various Capsaicinoids in Hot Sauces, Reported in PPM Levels and Subsequent Scoville Heat Unit Calculations

Compound	Sauce M	Sauce T	Sauce H	Sauce S	Sauce C
Nordihydrocapsaicin (1)	1600	40	60	60	20
Capsaicin (2)	14000	140	600	140	80
Dihydrocapsaicin (3)	12000	200	400	200	80
Homocapsaicin	6000	60	0	60	40
Homodihydrocapsaicin	< 1	< 1	< 1	< 1	< 1
Total 1+2+3	27,600	380	1,060	400	180
Scoville Heat Unit, SHU	414,000	5,700	15,900	6,000	2,700
Published SHU	600,000	N/A	N/A	N/A	N/A

Conclusion

The 3 minute analytical scale HPLC-UV method developed with a Kinetex 5 µm C18 50 x 4.6 mm column was very effective for the evaluation of a variety of capsaicinoids in pepper extracts, hot sauces, and salsa. The analytical methodology was then scaled to a Kinetex 5 µm C18 100 x 21.2 mm Axia prep column for the isolation of these capsaicinoids. A precursor scan was an effective way to identify the various capsaicinoids species in plant matrix. A fast LC/MS/MS method then efficiently separated all capsaicinoids, including all isomeric species in both quantitative and qualitative mode. The Ghost Chili peppers had significantly more capsaicin (and other capsaicinoids) than any other single pepper. The Pequin pepper contained a surprisingly large amount of capsaicin for such a small sized pepper and Thai chili peppers had the highest ratio of nordihydrocapsaicin to capsaicin than any other chili peppers we tested. Finally Jalapeno and Cayenne peppers had the most evenly dispersed profiles for these capsaicinoids.

References

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