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ARTICLE

Determination of Capsinoids by HPLC-DAD in *Capsicum* Species

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Capsicum fruits contain a newly discovered phytochemical called capsinoids. Because little is known about the quantities of these compounds in both sweet and pungent pepper fruits, a high-performance liquid chromatography (HPLC) method was developed to identify and quantify the capsinoids (naturally present *E*-capsiate and dihydrocapsiate) utilizing fruit obtained from a variety of *Capsicum* spp. in the U.S. Department of Agriculture's *Capsicum* germplasm collection. Capsinoids were extracted with acetonitrile, filtered, and analyzed using an HPLC system equipped with a C_{18} monolithic column, gradient pump, and diode array detector. The elution solvents were acetonitrile and water (60:40) with an isocratic flow rate of 1.0 mL/min. Forty-nine samples representing distinct morphotypes of four cultivated species (*C. annuum* var. *annuum*, *C. annuum* var. *glabriusculum*, *C. baccatum*, *C. chinense*, and *C. frutescens*) contained detectable levels (11−369 μg/g) of *E*-capsiate quantified at a wavelength of 280 nm. Nine of the *E*-capsiate-containing samples also had dihydrocapsiate (18−86 μg/g). Gas chromatography with a mass spectrometry detector (GC-MS) confirmed the presence of these compounds in the *Capsicum* spp.

KEYWORDS: *Capsicum*; capsinoids; *E*-capsiate; dihydrocapsiate

INTRODUCTION

Pepper (*Capsicum* spp.) is an herbaceous plant belonging to the Solanaceae family (*1*) and is native to Central and South America. Its fruits are cultivated for use as spices, which add aroma and flavor to foods. Peppers are also a source of nutrients such as carbohydrates, sugars, calcium, magnesium, phenolic acids, β-carotene, ascorbic acid, and tocopherols (*2*). In recent years, pharmaceutical, antimicrobial, and insecticidal properties associated with capsaicinoids found in the pungent *Capsicum* spp. have garnered increasing attention (*3*−*5*), in part because the capsaicinoids are natural compounds. They provide pungency or "heat" to hot peppers. The popularity of hot peppers as a food or spice has increased significantly over the years with the discovery of these benefits. Commercial pepper-based food products, such as salsa, now account for an estimated $500 million in annual sales (*6*).

The capsaicinoids, vanillylamide moieties with C_9−C_{11} branched-chain fatty acids, exhibit antioxidant activity (*7*). However, their use as ingredients in certain foods and pharmaceuticals has been limited by pungency, the very characteristic that makes them popular as a spice. The recently discovered nonpungent capsinoids, which were first isolated from the sweet pepper cultivar CH-19 Sweet (*8*), may offer similar benefits with a milder flavor. They are similar in structure to the capsaicinoids with an ester group instead of the amide moiety. Known capsinoids include capsiate, dihydrocapsiate, and nordihydrocapsiate (**Figure 1**). Research with cv. CH-19 Sweet has shown that capsinoids are biosynthesized from phenylalanine and valine (*9*). In addition to antioxidant activity, capsinoids enhance adrenal catecholamine secretion, promote energy metabolism, and suppress body fat accumulation (*10*−*15*). Although research indicates that the capsinoids provide health benefits (*10*−*15*), no published data characterizing the relative concentrations of these compounds in different *Capsicum* spp. are available. Identification of peppers high in capsinoids may permit the capsaicinoid-intolerant portion of the population to access the healthful effects associated with these types of compounds.

The extensively studied capsaicinoids have been quantified using a variety of analytical methodologies such as gas chromatography with electron capture or mass spectrometry detectors, as well as high-performance liquid chromatography equipped with a photodiode array detector or a mass spectrometer (*11*−*16*). Hitherto, thin layer chromatography has been used to successfully separate and identify capsinoids (*8*). Nuclear magnetic resonance spectroscopy and infrared spectroscopy were used to elucidate the structures of capsiate, dihydrocapsiate, and nordihydrocapsiate (*8*, *17*). The contents of capsaicinoid-like substances have been determined in CH-19 Sweet (*18*).

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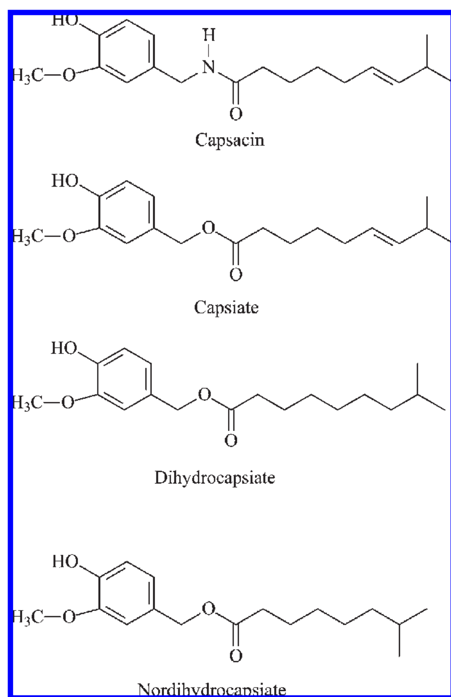

Figure 1. Structures of three capsinoids and one capsaicinoid (capsaicin).

With limited data available, the objectives of the research were to identify and quantify capsiate and dihydrocapsiate by high-performance liquid chromatography-diode array detector (HPLC-DAD) in a variety of *Capsicum* spp. and to develop a gas chromatography−mass spectrometry (GC-MS) method to identify and confirm the presence of these compounds in the peppers. With the increased interest in pharmacologically active compounds in foods, accurate measurement of capsinoids in a wide variety of *Capsicum* spp. will benefit the food and pharmaceutical industries, as well as consumers.

MATERIALS AND METHODS

Plant Material. All fruits utilized in this study were harvested from plants for which seeds were obtained from the USDA/ARS *Capsicum* spp. germplasm collection in Griffin, GA. Twenty-five seeds of each genotype were sown in the greenhouse before being transplanted to the field at either the Georgia Experiment Station (Griffin, GA) or the USDA Fruit and Nut Research Station (Byron, GA). Mature fruits were harvested from a single plant of each accession and sent via overnight carrier to the University of Maine (Orono, ME) for analysis.

Reagents. The solvents and reagents used were of the highest available purity. Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA). Thionyl chloride, 8-methylnonanoic acid, (*E*)-8-methyl-6-nonenoic acid, sodium borohydride, vanillin, vanillyl chloride, pyridine, hydrochloric acid, ethyl acetate, and calcium chloride were obtained from Acros Organics (Fair Lawn, NJ). Silica gel (70−230 mesh; 60 Å) and sodium sulfate were purchased from Sigma-Aldrich (St. Louis, MO).

Extraction of Capsinoids. The extraction procedure was adapted from that of Perkins et al. (*6*). Fresh peppers were homogenized using a Waring blender. A subsample of the macerated pepper (5.0 g) was placed into a 50.0 mL disposable polyethylene centrifuge tube, followed by addition of acetonitrile (25.0 mL). The unused pepper was stored at −83 °C for later use. The mixture was refrigerated at 4 °C for 24 h, after which it was homogenized using a Polytron (Kinematica Inc., Bohemia, NY) for 2 min. The finely ground sample was centrifuged for 10 min at 5000*g*. Approximately 1.5 mL of supernatant was transferred to an autosampler vial and analyzed by HPLC-DAD. In a preliminary study, fruit tissue samples were extracted twice for capsinoids. Because the initial trial extraction contained 92 and 98% of capsiate and dihydrocapsiate, respectively, a single extraction was utilized in subsequent analyses. Samples were analyzed either in duplicate or in triplicate depending on the quantity of sample available. For the GC-MS analysis, approximately 1.5 mL of the supernatant was dried under a stream of nitrogen, and the residue was resuspended in 1.5 mL of methylene chloride. After the insoluble materials were removed by filtration using a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter (Fisher Scientific, Hampton, NH), the samples were analyzed using GC-MS to confirm the presence of capsinoid.

HPLC-DAD Analysis of Capsinoids. Capsinoids were identified and quantified using a Hewlett-Packard (HP) HPLC series 1050 that was equipped with an automatic sampler and DAD. A 100 × 4.6 mm i.d. C_{18} monolithic column (Phenomenex, Torrance, CA) was used for the separation of the compounds. The elution solvents were acetonitrile and water (60:40) with an isocratic flow rate of 1.0 mL/min. The column was held at ambient temperature. Absorbance of the eluate was monitored at 280 nm, and ultraviolet (UV) spectra were recorded in the range of 220−350 nm at an acquisition rate of 1.25 scan/s. Injection volume was 20 μL. Synthesized capsiate and dihydrocapsiate standards were used for compound identification and for preparation of calibration curves.

GC-MS Analysis of Capsinoids. Identification of capsiate and dihydrocapsiate was conducted using a method modified from that of Manirakiza et al. (*19*). An Agilent 6890 series GC system (Agilent, Wilmington, DE) interfaced to an Agilent 5973 mass selective detector was used with a 30 m × 0.25 mm i.d., 0.25 μm, DB-5 MS 5% phenyl methyl siloxane capillary column (Agilent, St. Louis, MO). Helium (grade 5.0) was the carrier gas with a flow rate of 2.0 mL/min. An Agilent 7683 autoinjector was also used. The inlet heater was set at 275 °C. A 2.0 μL sample was injected using the splitless mode. Initial oven temperature was 90 °C, followed by a 25 °C/min increase to 290 °C. This temperature was held for 5 min. The total run time was 14.00 min. The MS acquisition parameters used were the same as in the previous method (*19*).

Synthesis of Capsiate Standard. Capsiate was synthesized from the esterification of vanillyl alcohol onto (*E*)-8-methyl-6-nonenoic acid. Vanillin (1 mol equiv) was dissolved in ethanol and chilled at 0 °C. Sodium borohydride (1 mol equiv) was added, and the reaction was allowed to come to room temperature. The solvent was removed under reduced pressure, and the solid was suspended in ether, followed by treatment with aqueous ammonium chloride. The aqueous layer was separated and extracted twice more. Organic layers were then combined and washed with water and brine before being dried over magnesium sulfate. The solvent was removed under reduced pressure, followed by recrystallization from ethanol to yield pure vanillyl alcohol. The method used to synthesize (*E*)-8-methyl-6-nonenoic acid was adapted from that of Kaga et al. (*20*). The synthesized (*E*)-8-methyl-6-nonenoic acid was then dissolved in dry tetrahydrofuran (10 mL/g) and chilled at 0 °C. Vanillyl alcohol (1 equiv) was then added to this solution, followed by 1 equiv of triphenylphosphine (PPh_3). Diethylazodicarboxylate was added dropwise, and the solution was removed from the ice bath. The reaction was allowed to proceed for several hours after the solution became cloudy. The solvent was removed under reduced pressure, and the residue was dissolved in ether. Ether was removed, followed by further additions of ether until no cloudy precipitate was seen. Aqueous sodium bicarbonate was added to remove the acid residue. A short plug of silica was used to remove phosphine residue and other impurities. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy (Varian Inc., Palo Alto, CA) was used for the structural elucidation of the capsiate. The solvent for the NMR analysis was $CDCl_3$.

Synthesis of Dihydrocapsiate Standard. The procedure for the synthesis of dihydrocapsiate was taken from that of Kobata et al. (*8*). Thionyl chloride, 8-methylnonanoic acid, and vanillyl alcohol were the starting materials used in the synthesis. The residue was purified using silica gel (70−230 mesh; 60 Å).

RESULTS AND DISCUSSION

The intent of the study was to identify and quantify capsinoids present in *Capsicum* spp. fruit using an efficient analytical process. Thus, the method developed by Perkins et al. (*6*) was modified using a diode array detector, which gave a larger dynamic range when compared to the fluorescence detector used in that study. Because the capsinoids elute after capsaicinoids and other coextractants, we were able to gain separation of the compounds of interest.

Isomers of capsiate (*E*-capsiate and *Z*-capsiate), with purities of 90%, were provided by the University of Georgia for use as analytical standards. The NMR data for the capsiate matched the results obtained by Kobata et al. (*8*).

Dihydrocapsiate was synthesized according to the method developed by Kobata et al. (*8*). There was no significant difficulty during the synthesis of dihydrocapsiate, which had a yield of 36%. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and infrared (IR) spectroscopy were used for the structural elucidation of the dihydrocapsiate, which was used as an analytical standard. The results from the ^{1}H NMR and IR for dihydrocapsiate were compared to data obtained by Kobata et al. (*8*) for confirmation.

After experimentation with several reversed-phase columns, a monolithic silica C-18 column was selected for capsinoid separation. The advantage of this column is its increased permeability, which allows flow rates of up to 5.0 mL/min and, consequently, higher throughput. Flow rates higher than 1.0 mL/min resulted in band broadening of peaks. The selectivity of this column is similar to that of packed columns.

Acetonitrile was selected as the solvent for capsinoid extraction because it will not solubilize large quantities of lipids in the pepper tissue. This reduced the interference levels in the matrix and facilitated detection of the analyte. The capsinoids were extracted twice, and it was found that the first extraction contained 92% capsiate and 98% dihydrocapsiate, respectively. Hence, only a single extraction of the capsinoids was conducted. A mobile phase of acetonitrile/water (60:40, v/v) was utilized. Higher volumes of acetonitrile resulted in distorted peak shapes.

The capsinoids in the samples identified were *E*-capsiate and dihydrocapsiate. There was no *Z*-capsiate present, which eluted approximately 1 min before the *E*-capsiate, in any of the samples analyzed. Nordihydrocapsiate was not analyzed in this study. The *E*-capsiate eluted at approximately 6.5 min, whereas the dihydrocapsiate's retention time was 8.5 min (**Figure 2**). The chromatogram of the capsinoids in a typical pepper sample is shown in **Figure 3**. Initial confirmation of the capsinoids was achieved by conducting a UV scan from 220 to 350 nm on each capsinoid in the sample, with the sample spectra compared to those of the respective standards. The distinctive UV spectrum for *E*-capsiate and dihydrocapsiate is shown in **Figure 4**. Maximum absorbance occurs at 228−230 and 280 nm for both compounds. This is similar to the capsaicinoids because both classes of compounds are remarkably similar (*21*).

Identification of the capsinoids was followed by quantification of these compounds in the peppers. Signal response for *E*-capsiate was linear from 1 to 300 μg/mL with a correlation coefficient (r^2) of 0.9999. *E*-Capsiate was present in 49 samples (**Table 1**). Sample PB 26 consistently showed the highest levels

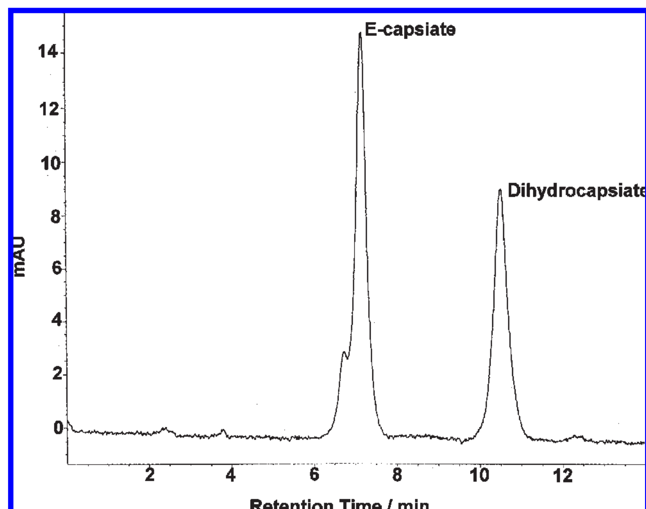

Figure 2. HPLC chromatogram of *E*-capsiate and dihydrocapsiate standards.

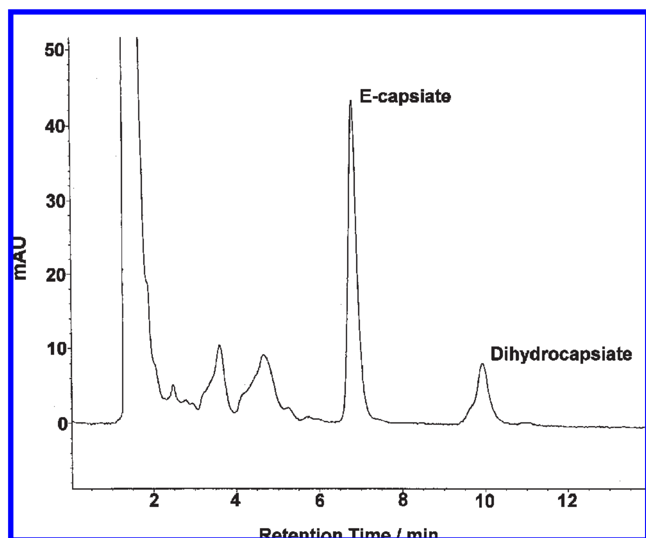

Figure 3. HPLC chromatogram of two capsinoids in a pepper sample.

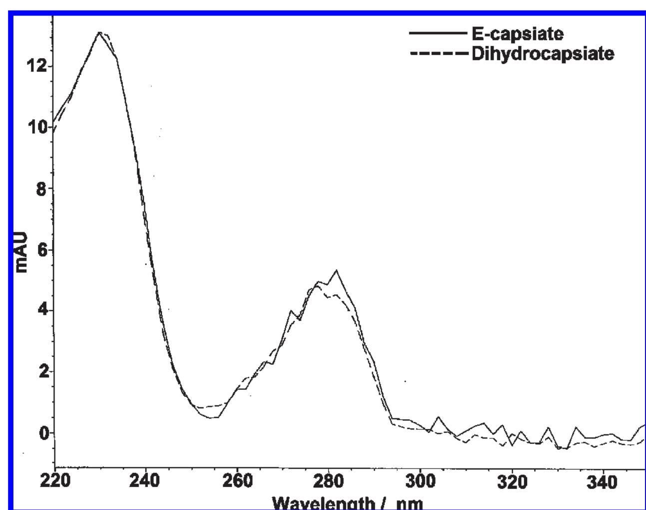

Figure 4. Graph showing UV spectrum of *E*-capsiate and dihydrocapsiate at 280 nm (wavelength vs absorbance).

Table 1. Concentrations of *E*-Capsiate and Dihydrocapsiate in Pepper Samples[a]

sample ID	genus/species	origin	*E*-capsiate (μg/g of FW)	SD	dihydrocapsiate (μg/g of FW)	SD
PB 26	*C. annuum* var. *annuum*	Nigeria	369.1	35.5	86.9[b]	
PE 56	*C. annuum* var. *annuum*	Venezuela	246.9	5.9	73.3	0.9
PE 84	*C. annuum* var. *glabriusculum*	Mexico	189.7[c]	33.3	nd[d]	
NP 30	*C. chinense*	USA	166.7	1.9	49.8	0.1
NP 42	*C. chinense*	Brazil	166.3	4.4	18.0	1.2
PE 86	*C. annuum* var. *glabriusculum*	Mexico	166.2	19.3	33.7	1.2
PE 31	*C. annuum* var. *glabriusculum*	Mexico	158.7	20.3	21.4[c]	
NP 37	*C. chinense*	Venezuela	137.5	5.2	34.8	1.2
GRIF 9302	*C. chinense*	Colombia	132.3	11.5[c]	nd	
PE 66	*C. frutescens*	Ecuador	122.6	19.5	35.5	0.9
PE 67	*C. chinense*	Ecuador	116.4	17.9	nd	
NP 28	*C. chinense*	Costa Rica	110.3	6.1	nd	
PB 35	*C. annuum* var. *annuum*	Malaysia	109.6	14.3	45.5	0.8
PB 31	*C. annuum* var. *annuum*	unknown	105.7	20.1	nd	
PE 12	*C. annuum* var. *annuum*	Guatemala	83.8	15.4	nd	
PE 50	*C. annuum* var. *annuum*	India	81.4	27.6	nd	
PE 75	*C. annuum* var. *glabriusculum*	Mexico	80.0	2.5	nd	
PI 438644	*C. chinense*	Mexico	79.9	3.1	nd	
PB 22	*C. annuum* var. *annuum*	Thailand	75.7	16.9	nd	
PE 16	*C. annuum* var. *annuum*	Colombia	71.5	3.6	nd	
PE 23	*C. annuum* var. *annuum*	USA	64.7	2.5	nd	
PE 59	*C. annuum* var. *annuum*	Honduras	60.0	4.3	nd	
PE 80	*C. annuum* var. *glabriusculum*	Mexico	57.8	9.1	nd	
NP 13	*C. annuum* var. *annuum*	Korea	55.4	12.5	nd	
PE 82	*C. annuum* var. *glabriusculum*	Mexico	53.1	3.2[c]	nd	
PE 33	*C. annuum* var. *annuum*	India	46.0	9.4	nd	
PB 38	*C. annuum* var. *annuum*	USA	45.8	5.5	nd	
P 21	*C. annuum* var. *annuum*	Mexico	43.3[b]		nd	
NP 47	*C. frutescens*	Bolivia	40.6	20.5	nd	
PI 224424	*C. chinense*	Costa Rica	40.4	20.1	nd	
NP 1	*C. annuum* var. *annuum*	Mexico	38.3	2.8	nd	
PE 37	*C. annuum* var. *annuum*	Yugoslavia	35.5	3.4	nd	
PI 438535	*C. chinense*	Belize	32.4	1.7	nd	
NP 32	*C. chinense*	USA	31.5	1.8	nd	
PB 93	*C. frutescens*	Thailand	30.7[b]		nd	
PB 18	*C. annuum* var. *annuum*	France	29.9	9.3	nd	
NP 4	*C. annuum* var. *annuum*	Cuba	22.2	10.9[c]	nd	
PE 06	*C. annuum* var. *annuum*	Afghanistan	20.8	2.9	nd	
PE 05	*C. annuum* var. *annuum*	Afghanistan	20.6	1.9[c]	nd	
NP 23	*C. baccatum*	Peru	19.5	2.9	nd	
PE 79	*C. annuum* var. *glabriusculum*	Nicaragua	19.0	7.4[c]	nd	
NP 5	*C. annuum* var. *annuum*	USA	17.8	3.1	nd	
PE 85	*C. annuum* var. *glabriusculum*	Mexico	17.7	3.9[c]	nd	
PE 54	*C. annuum* var. *annuum*	China	16.7	2.9	nd	
PI 439476	*C. chinense*	Puerto Rico	14.7	0.9	nd	
PB 25	*C. annuum var. annuum*	Bolivia	13.5	0.4	nd	
NP 35	*C. chinense*	Peru	12.9	2.0	nd	
PE 61	*C. annuum* var. *annuum*	USA	11.7	4.6	nd	
PE 7	*C. annuum* var. *annuum*	Afghanistan	11.5	5.4[c]	nd	

[a] Peppers were in the mature stage of growth when analyzed. [b] Only a single analysis was conducted. [c] Only duplicate analysis was done. [d] Not determined.

of *E*-capsiate (369.1 ± 35.5 μg/g). This was followed by PE 56 (246.9 ± 5.9 μg/g) and PE 84 (189.7 ± 33.4 μg/g). Some of the samples showed large variability among the replicates, which may be attributed to fruit-to-fruit variability. Significant levels of fruit-to-fruit variability in capsaicinoid concentrations have been reported (*22*).

Nine of the cultivars containing the highest levels of *E*-capsiate, of which sufficient sample was available, were analyzed for dihydrocapsiate. The correlation coefficient of the calibration curve for dihydrocapsiate was 0.9998, with a linear response from 1 to 200 μg/mL. Sample PB 26, which showed the highest levels of *E*-capsiate, also contained the highest concentration of dihydrocapsiate (86.9 μg/g) (**Table 1**). Due to limited material available for this sample, only a single analysis was performed.

The modified GC-MS method was used to confirm the presence of the two capsinoids. Capsinoids were easily separated on the DB-5 column, with minimal cleanup required for the analysis. The *E*-capsiate standard had a retention time of 9.08 min, whereas the dihydrocapsiate standard had a retention time of 9.14 min. The identifying peaks of *E*-capsiate and dihydrocapsiate from the pepper extract are shown in **Figure 5** with retention times of 9.09 and 9.16 min, respectively. There was no quantification of these compounds. Using analytical standards, three capsaicinoids (capsaicin, dihydrocapsaicin, and nordihydrocapsaicin) were also identified (**Figure 5**). The retention times for the capsaicin, dihydrocapsaicin, and nordihydrocapsaicin were 10.27, 10.34, and 9.83 min, respectively. On the DB-5 column, the capsinoids eluted before the capsaicinoids.

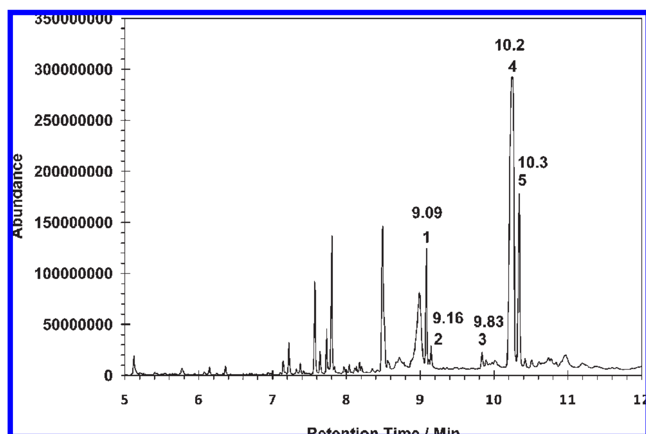

Figure 5. Gas chromatogram of capsinoids and capsaicinoids found in pepper sample: 1, *E*-capsiate; 2, dihydrocapsiate; 3, nordihydrocapsaicin; 4, capsaicin; 5, dihydrocapsaicin.

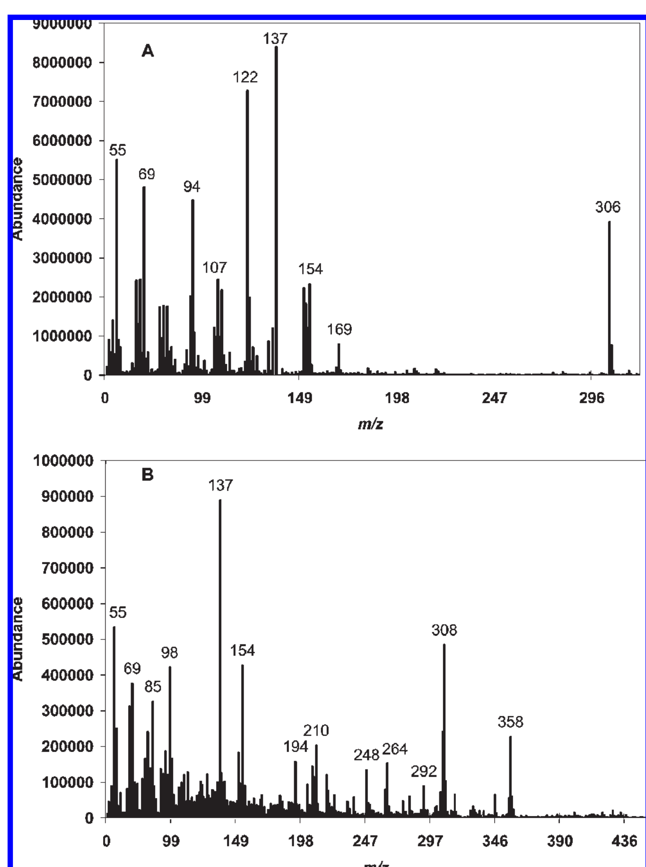

Figure 6. Mass spectra of (**A**) *E*-capsiate and (**B**) dihydrocapsiate.

The mass scan spectrum for *E*-capsiate is shown in **Figure 6 A**. Electron impact ionization resulted in several fragmentation ions. The molecular ion for *E*-capsiate had *m*/*z* 306. The *m*/*z* ion with the highest abundance was at *m*/*z* 137 (base peak). On the basis of the fragmentation pattern seen for capsaicinoids, we are postulating that the base peak is the vanillyl moiety (*21*, *23*). In **Figure 6 B** is shown the mass spectrum for dihydrocapsiate. The molecular ion for this compound was at *m*/*z* 308, with a similar base peak of *m*/*z* 137. Again, we hypothesize that this peak represents the vanillyl amide moiety.

The rugged and rapid HPLC-DAD method described in this study, which requires little sample preparation or cleanup, will be widely used in a variety of applications including ascertaining optimum fruit maturity, developing capsinoid-rich cultivars, and advancing food and ingredient processing methods that retain functional capsinoid content. Documenting the presence of capsinoids in all *Capsicum* species tested will facilitate the characterization of the biochemical pathways that produce capsiate in peppers and yield propagation strategies that ultimately produce peppers with elevated capsiate content.

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