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Quantitative determination of capsaicinoids by liquid chromatography–electrospray mass spectrometry

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Abstract Eight naturally occurring capsaicinoids have been determined in *Capsicum* by use of high-purity standards, with norcapsaicin as an internal standard. The solid standards were rigorously checked for purity. The sensitivity of electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), and coordination ion-spray (CIS; with silver) toward the capsaicinoids were measured and compared. The highest sensitivity was found for positive-ion ESI. Method validation of the liquid chromatography–ESI-mass spectrometry (LC–ESI-MS) determination is reported, including tests for repeatability (4%), detection limit (5 pg injected), linear range (20–6 ng injected), quantitation (excellent linearity; $<2\%$ relative standard deviation), and recovery (99–103%). The major and minor capsaicinoids in a commercial plant extract and in chili pepper fruits were quantified.

Keywords Capsaicinoids · Chili pepper · LC–MS · Mass spectrometry · Electrospray ionization · Atmospheric-pressure chemical ionization

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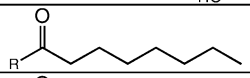
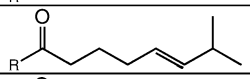
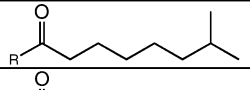
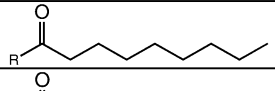
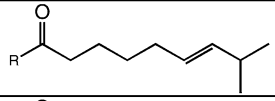
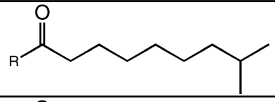
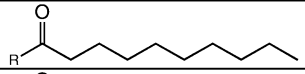
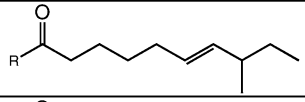
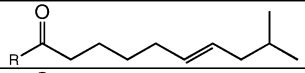
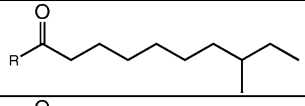
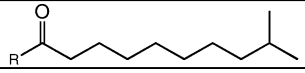
Introduction

Capsaicinoids from chili peppers (genus *Capsicum*) are noteworthy for their pungent taste and for pain production. The common naturally occurring capsaicinoids, *N*-vanillyl amides of fatty acids, are listed in Table 1. The most abundant in chilis, the major capsaicinoids, are 6-ene-8-methylcapsaicin, 8-methyldihydrocapsaicin, and 7-methylnordihydrocapsaicin. A previous paper [1] focused on the chromatographic separation of the other, minor capsaicinoids.

Although there is a sizable literature on liquid-chromatographic analysis, with ultraviolet absorbance detection (LC–UV), of capsaicinoid mixtures and extracts of chili peppers (reviewed in Ref. [1]), reports of the determination of capsaicinoids by liquid chromatography–mass spectrometry (LC–MS) are few. A comprehensive literature search yielded about 30 citations, only 11 of which employed LC–MS for chemical analysis. The references reveal the historical development of the technique. In 1979 Heresch and Jurenitsch [2] first used mass spectrometry (off-line) in combination with liquid chromatography to determine capsaicinoids; a moving-belt interface was applied in the mid-1980s [3, 4]; thermospray ionization was reported in 1993 [5]; and finally atmospheric-pressure chemical ionization (APCI) [6] and electrospray ionization (ESI) were introduced to chili pepper analysis beginning in 1999.

Most of the recent references are from a single laboratory (Reilly et al. at the University of Utah) and focus on the nociceptive agents of defense sprays. LC–ESI-MS measurements were made of capsaicinoids in the liquid contents of pepper spray cans [7, 8], on fabrics as spray residues [9], in the blood and tissues of rats after exposure [10], and in cell extracts to identify capsaicin metabolites [11]. Wolf et al. [12] sampled human skin for the pungent compounds in dermatological creams. Some of this recent work involved tandem mass spectrometry and selected-reaction monitoring [8, 10–12]. The previ-

Table 1 Naturally-occurring capsaicinoids

Number	Compound Name	Structure
1	N-vanillyl octanamide	
2	5-ene-7-methyl norcapsaicin	
3	7-methyl nordihydrocapsaicin	
4	N-vanillyl nonanamide	
5	6-ene-8-methyl capsaicin	
6	8-methyl dihydrocapsaicin	
7	N-vanillyl decanamide	
8	6-ene-8-methyl homocapsaicin	
9	6-ene-9-methyl homocapsaicin	
10	8-methyl homodihydrocapsaicin (tentative identification)	
11	9-methyl homodihydrocapsaicin	

ous paper [1] is the only report on the coordination ion spray (CIS) mass spectrometry of capsaicinoids.

The literature on fully validated quantitative analyses of capsaicinoids by LC–MS is very sparse and essentially has been limited to the three compounds for which there are readily available standards—6-ene-8-methylcapsaicin, 8-methyldihydrocapsaicin, and *N*-vanillylnonanamide. Other capsaicinoids—7-methylnordihydrocapsaicin, homocapsaicin (undefined isomer), and a homodihydrocapsaicin (undefined isomer)—have been determined [7, 9], but the purity of the standards (compounds isolated from a plant extract by liquid chromatography) upon which the quantitative analysis was based is uncertain. In addition, *N*-vanillyloctanamide was used as an internal standard even though it is a significant natural component of chili peppers [1, 13–15].

In this report the quantification of eight capsaicinoids in a commercial capsaicinoid mixture and in the fruits of two different chili peppers using an LC–ESI-MS method [1] is described. Method validation studies, including tests for repeatability, linear range, detection limit, quantitation, and recovery, were performed. The analysis employed pure solid standards of each of the analytes and norcapsaicin as internal standard. The identities of the standards were confirmed by NMR, and their purity was assessed by a suite of techniques, including UV spectrophotometry, LC–UV, LC–MS, differential scanning calorimetry (DSC), and direct insertion probe electron ionization mass spectrometry. During method development comparison of ESI, both positive-ion and negative-ion modes, APCI, both positive-ion and negative-ion modes, and CIS with silver ion for their sensitivity toward the capsaicinoids was made.

Experimental

Caution

The pure capsaicinoids and concentrated extracts of chili peppers are very strong irritants and must be used with care. They can cause burning, pain, coughing, and lachrymation upon skin exposure and inhalation. Use gloves and a mask when handling these solids or their concentrated solutions.

Materials and methods

Disclaimer

Certain commercial equipment, instruments, or materials are identified in this report in order to adequately

specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Reagents

A commercial capsaicinoid mixture (Sigma–Aldrich, Taufkirchen, Germany; #M3403), isolated from plant tissue and containing the major and minor capsaicinoids, served as the primary sample in this investigation. Solid standards of *E*-6-ene-8-methylcapsaicin (USP), 8-methyldihydrocapsaicin (USP), and *N*-vanillylnonanamide (Sigma–Aldrich) were obtained commercially. Solid standards of *E*-5-ene-7-methylnorcapsaicin, 7-methylnordihydrocapsaicin, *E*-6-ene-8-methylhomocapsaicin, *E*-7-ene-9-methylhomocapsaicin, and 9-methylhomodihydrocapsaicin, and their NMR spectra, were obtained from Dr Kazuhiko Orito at Hokkaido University in Japan. *N*-vanillyloctanamide and *N*-vanillyldecanamide were synthesized by a procedure adapted from the literature [16]; the procedure is described in detail in a previous paper [1]. Because no isotope-labeled capsaicinoid was available, norcapsaicin was used as the internal standard for quantitative analysis. Earlier work demonstrated that only a trace of norcapsaicin was present in the commercial capsaicinoid mixture [1].

Stock solutions of each of the solid standards were prepared gravimetrically by dissolving several milligrams of capsaicinoid (weighed to ± 10 μg) in approximately 1 g methanol (weighed to ± 1 mg). Working solutions were made by diluting a known mass of stock solution to a known volume with 50:50 water–methanol. After this stage any serial dilutions were prepared volumetrically. All solutions were stored in sealed, amber glass vials at 4°C. Under these conditions no change in solution concentration was noticed over a period of 6 months.

HPLC-grade solvents were used to prepare the mobile phases. Water was distilled deionized water, measured at 18 MΩ, produced by a bench-top water purifier. Acetic acid, silver nitrate, and other reagents were of ACS Reagent Grade or better.

The capsaicinoids were separated isocratically at 30°C on an Ace Phenyl column of dimensions 4.6 mm×250 mm and packed with 5-μm particles. For positive-ion MS detection, the aqueous portion of the mobile phase contained either acetic acid (for ESI, APCI) or silver nitrate (for CIS), and the organic modifier was acetonitrile (45% volume fraction) or methanol (65% volume fraction). The aqueous portion of the mobile phase for negative-ion ESI or APCI contained ammonium acetate or ammonium hydroxide. Other conditions were identical with those used in positive-ion mode. Mobile phase flow rate for LC–MS was 0.80 mL min^{-1}. The mobile phase composition is abbreviated *w*:*o* throughout this report, where *w* is the volume fraction of aqueous solution (in %) and *o* is the volume fraction of organic solvent (in %), selected by use of the proportioning valve on the LC pump.

LC–MS instrumentation

The LC–MS (Agilent 1100 series) consisted of a vacuum degasser, an autosampler with variable injection volume, a high-pressure pump set at 0.80 mL min^{-1}, a thermostatted column compartment set at 30°C, an absorbance detector set at 280 nm, an interchangeable ESI/APCI interface, and a single-quadrupole MS detector. The two most important settings for the electrospray ionization interface—spray capillary voltage and fragmentor voltage—were adjusted to maximize the mass spectrometer sensitivity for the various ionization modes. In addition for APCI, the corona current was optimized. Other variables were drying gas flow rate (12 L min^{-1} for ESI/CIS; 4 L min^{-1} for APCI), nebulizer pressure (0.0087 Pa (60 psig) for ESI/CIS; 0.0073 Pa (50 psig) for APCI), drying gas temperature (350°C for ESI/CIS; 300°C for APCI), and vaporizer temperature (300°C for APCI).

Sample preparation: chili peppers

A jalapeno pepper (home-grown; frozen for a year) and a habanero pepper (from a grocery store) were each processed in the following manner. Pieces of the fruit weighing about 7 g and 15 mL water were blended for 2 min at low speed in a commercial blender. The suspension was transferred to a plastic centrifuge tube, and 5 mL water, used to rinse the blender, plus 20 mL methanol were added to the tube. The tube and its contents were shaken for 30 min. The tube was centrifuged for 10 min at high speed and the supernatant was filtered through a 0.2-μm nylon filter. The clear yellow liquid was diluted volumetrically 1:200 with 50:50 water–methanol and internal standard was added before injection into the LC–MS instrument.

Results and discussion

Purity of the standard compounds

The purity of each of the solid standard materials was assessed by use of four different techniques: differential scanning calorimetry (DSC), LC with UV absorbance detection at 210 nm, LC with electrospray ionization and mass spectrometric detection, and ultraviolet absorbance at 280 nm. Details are given in the supplementary material, and the results are described in Table 2. All of the standards were of very high purity. The three *N*-vanillyl-*n*-acylamides were also tested by direct insertion probe electron ionization mass spectrometry. Molecular ions and the expected fragments

were found, and no significant impurity peaks (solvent, precursors, etc.) were evident.

Comparison of ionization modes: mass spectra and analytical sensitivity

ESI (Positive Ion)

The commercial capsaicinoid mixture served as the sample in most of the studies of LC–MS ionization modes. Protonated molecules of the capsaicinoids were readily produced in the electrospray interface. The response toward the capsaicinoids, measured by the intensity of the $(M+H)^+$ ion for capsaicin, was maximized separately for three variables—spray capillary voltage (+2500 to +5000 V), fragmentor voltage (+40 to +150 V), and percentage of acetic acid (0.1–2% volume fraction)—through a series of flow-injection experiments. The response was relatively insensitive toward capillary voltage and acetic acid content. Increasing the fragmentor voltage from +40 to +90 V gave a slightly increasing response, but greater voltages significantly depressed the $(M+H)^+$ signal. At +150 V the response was only 20% of the maximum value. From these data, settings for further experiments were chosen: +4000 V for the spray capillary (actually 0 V on the spray capillary and −4000 V on the spray shield), +90 V for the fragmentor (cone/skimmer) voltage, acetic acid (0.2% volume fraction, pH 2.9) in the aqueous portion of the mobile phase, and other settings—12.0 L min^{-1}gas flow, 0.0073 Pa (50 psig) nebulizer pressure, 350°C spray chamber. The quantitative response to the capsaicinoids varied little with organic modifier; total MS counts were similar with either methanol or acetonitrile in the mobile phase.

Representative background-subtracted, full-range mass spectra (m/z 100–700) for 6-ene-8-methylcapsaicin and 8-methyldihydrocapsaicin are shown in Fig. 1. (Tabulated mass spectral data may be found in the supplementary material.) Peaks at $(M+H)^+$ for each compound were most pronounced, but sodium ion and potassium ion adducts also were apparent. Using an acetonitrile-containing mobile phase, the $(M+K)^+$ signal varied between 10 and 40% relative intensity whereas $(M+Na)^+$ was typically 5% or less. In contrast, methanol furnished sodiated ions with relative intensities from 70 to 105%, potassium adducts at 15–85%, and some dimeric adducts $(2M+Na)^+$. The use of acetonitrile gave much cleaner spectra.

The mass spectra also contained a small peak at m/z 137. This ion is derived from the vanillyl ring common to all of the capsaicinoid compounds and is produced by collision-induced dissociation (CID) in front of the skimmer in the ESI interface [3, 9]. As such, the ion intensity is greatly influenced by the fragmentor voltage. Flow-injection studies revealed the presence of the fragment ion beginning at +70 V and increasing in intensity in a fairly linear fashion up to +150 V. A concomitant decrease in the $(M+H)^+$ signal, due to the fragmentation, was measured as described earlier. Based on these data, a fragmentor voltage of +150 V was used to monitor the vanillyl ring fragment ion. The intensity of the m/z 137 fragment was also affected by the length of the acyl chain. The extent of fragmentation was greater in capsaicinoids with shorter chains (e.g. nor-

Table 2 Purity of capsaicinoids standards

Compound (see Table 1)	% Purity DSC (temp)[a]	% Purity UV @ 280 nm	% Purity LC–UV @ 210 nm	% Purity LC–MS	Other capsaicinoids present[b]
1	91 ± 2 (45°C)	99 ± 2	98 ± 2	> 99	< 0.3% *N*-Vanillylnonanamide < 0.2% *N*-Vanillyldecanamide
2	90 ± 6 (58°C)	102 ± 2	98 ± 2	> 99	< 0.2% Dihydrocapsaicin
3	– (59°C)	100 ± 2	100 ± 2	~100	None evident
4	100 ± 1 (63°C)	100	99 ± 2	> 99	< 0.2% *N*-Vanillyloctanamide < 0.4% *N*-Vanillyldecanamide
5	100 ± 1 (64°C)	99 ± 2	98 ± 2	> 98	< 0.1% Norcapsaicin < 0.2% Nordihydrocapsaicin < 0.1% *N*-Vanillylnonanamide < 0.8% Dihydrocapsaicin < 0.8% Homocapsaicin
6	94 ± 5 (55°C)	103 ± 2	98 ± 2	> 99	< 0.6% Capsaicin
7	94 ± 2 (54°C)	99 ± 2	99 ± 2	> 99	< 0.2% *N*-Vanillylnonanamide < 0.2% *N*-Vanillyloctanamide
7-ene-9-methylhomocapsaicin	101 ± 2 (61°C)	102 ± 2	100 ± 2	> 99	< 0.3% Capsaicin < 0.2% Norcapsaicin
8	99 ± 4 (63°C)	99 ± 2	98 ± 2	> 99	< 0.2% Capsaicin < 0.2% Norcapsaicin
11	100 ± 1 (69°C)	98 ± 2	99 ± 2	> 99	< 0.2% Nordihydrocapsaicin < 0.1% *N*-Vanillyldecanamide

Uncertainties listed are the population standard deviations. The uncertainties for DSC were obtained from replicate experiments; the others were estimates

[a]Temperature of peak heat-flow during the differential scanning calorimetry experiment

[b]Compounds identified from the masses of protonated molecular ions and retention times. Quantities estimated from the ratios of peak areas

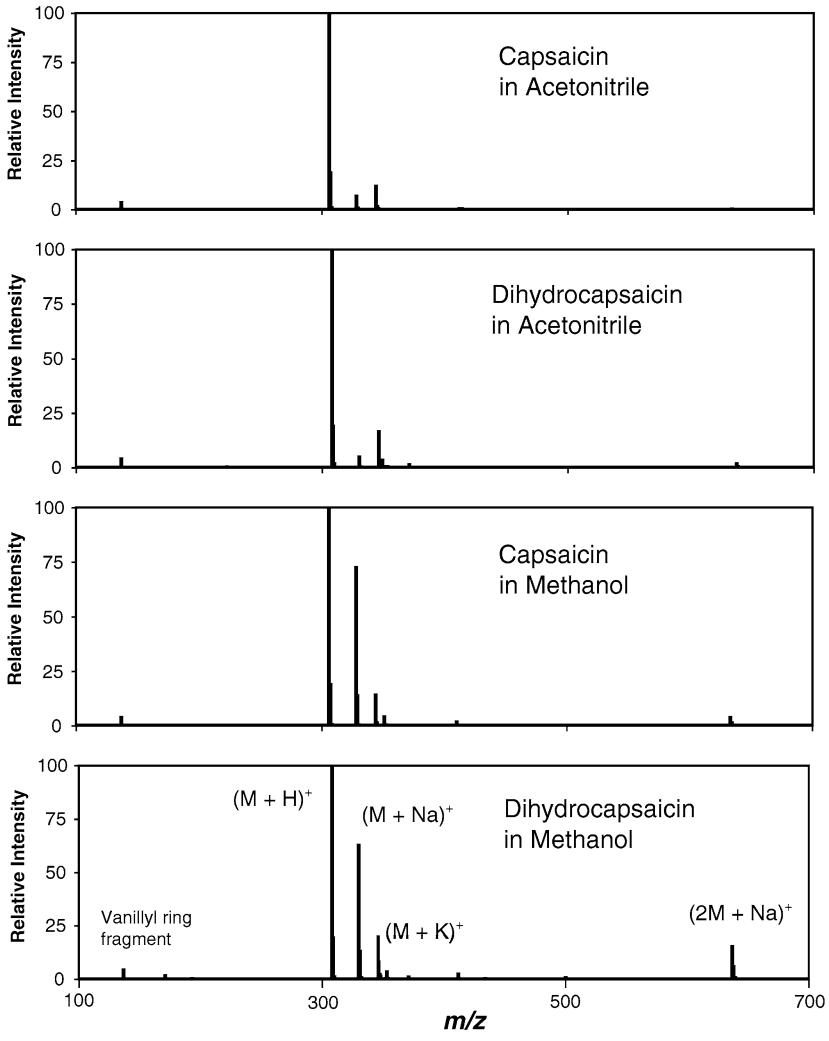


Fig. 1 Full-range (*m/z* 100–700), background-subtracted, positive-ion ESI mass spectra for capsaicin and dihydrocapsaicin. The spectra were collected with voltage settings of +4000 V (electrospray capillary) and +90 V (fragmentor) and with either 35:65 aqueous 0.2 vol.% acetic acid–methanol or 55:45 aqueous 0.2 vol.% acetic acid–acetonitrile as mobile phase. Tabulated *m/z* values and ion intensities for the spectra in methanol are given in the electronic supplementary material

capsaicin) compared with longer molecules in the same series (e.g. homocapsaicin).

ESI (negative ion)

Flow injection of the commercial capsaicinoid mixture into aqueous ammonia (0.2% volume fraction, pH 10.0) mixed with acetonitrile or methanol was performed. Similar results were obtained with the two organic modifiers. Optimization studies suggested settings of −4000 V for the spray capillary and −90 V for the fragmentor, and other settings were the same as for positive-ion mode. The sensitivity of the instrument in negative-ion mode was much less than in positive-ion mode, probably because of the weakly basic character of the vanillylamide portion of the capsaicinoid structure. Consequently, no further experimentation was performed in this mode.

APCI (positive ion)

The response toward the mixture of capsaicinoids was optimized separately for five variables—spray chamber and vaporizer temperatures (200–350°C), nebulizer pressure (30–80 psig), spray capillary voltage (+2000 to +5000 V), fragmentor voltage (+80 to +160 V), and corona current (2–6 μA)—by means of a series of flow-injection experiments. The temperatures, gas pressure, and capillary and fragmentor voltage settings had only weak effects on the ion intensities whereas the corona current setting had a more significant effect, doubling the signal when changed from 2 to 4 μA. Higher currents produced smaller ion intensities. From these data, settings for further experiments were chosen: +4000 V for the spray capillary, +135 V for the fragmentor voltage, 4 μA corona current, 4 L min^{-1} gas flow, 0.0073 Pa (50 psig) nebulizer pressure, 300°C spray chamber, and 300°C drying gas.

Full-range mass spectra (*m/z* 100–700) were collected under the optimal conditions, and the resulting representative, background-subtracted spectra for 6-ene-8-methylcapsaicin and 8-methyldihydrocapsaicin are shown in Fig. 2. The spectra are dominated by the protonated molecules of each compound and show few other ions. One other ion common to all of the mass spectra was the vanillyl ring fragment at *m/z* 137. The

APCI spectra of the capsaicinoids differed from the ESI spectra of the capsaicinoids in three major ways: (1) sodium ion and potassium ion adducts were missing; (2) for the capsaicins only, a significant peak at $(M–11)^+$ was apparent. This was certainly not an artifact from co-eluting compounds, but its genesis is unknown. A strong possibility is a hydronium ion adduct (+19) that lost its methoxy group from the vanillyl ring (elimination of $CH_2=O$; −30); (3) APCI spectra generated from methanol-containing and acetonitrile-containing mobile phases were similar, except that the spectra from methanol were more intense.

APCI (negative ion)

The response toward the mixture of capsaicinoids was optimized in a manner similar to that described above. The settings giving rise to the highest sensitivity were: −4500 V for the spray capillary, −90 V for the fragmentor voltage, 35 μA for the corona current, drying gas and vaporizer temperatures of 250°C, and a nebulizer pressure of 0.0087 Pa (60 psig). Even under these optimum conditions the response toward the capsaicinoids was very poor in negative APCI, and no further experiments were performed.

CIS $(M+Ag)^+$

CIS is an atmospheric-pressure ionization technique in which charged complexes between added aqueous metal ions and the separated sample components are formed in solution and analyzed by the mass spectrometer. A number of recent literature articles report success with coordination ion spray of molecules such as triglycerides [18], endocannabinoids [19], isomers of vitamin E [17], ginsenosides [20], polyaromatic hydrocarbons [21, 22], and petroleum products [23], all using silver ion (most often the nitrate salt) as the complexing agent.

Chromatographic and flow-injection studies in our laboratory using an aqueous silver nitrate–methanol mobile phase demonstrated that a spray capillary voltage of +3000 V and a fragmentor voltage of +180 V yielded the highest sensitivity toward the capsaicinoids.

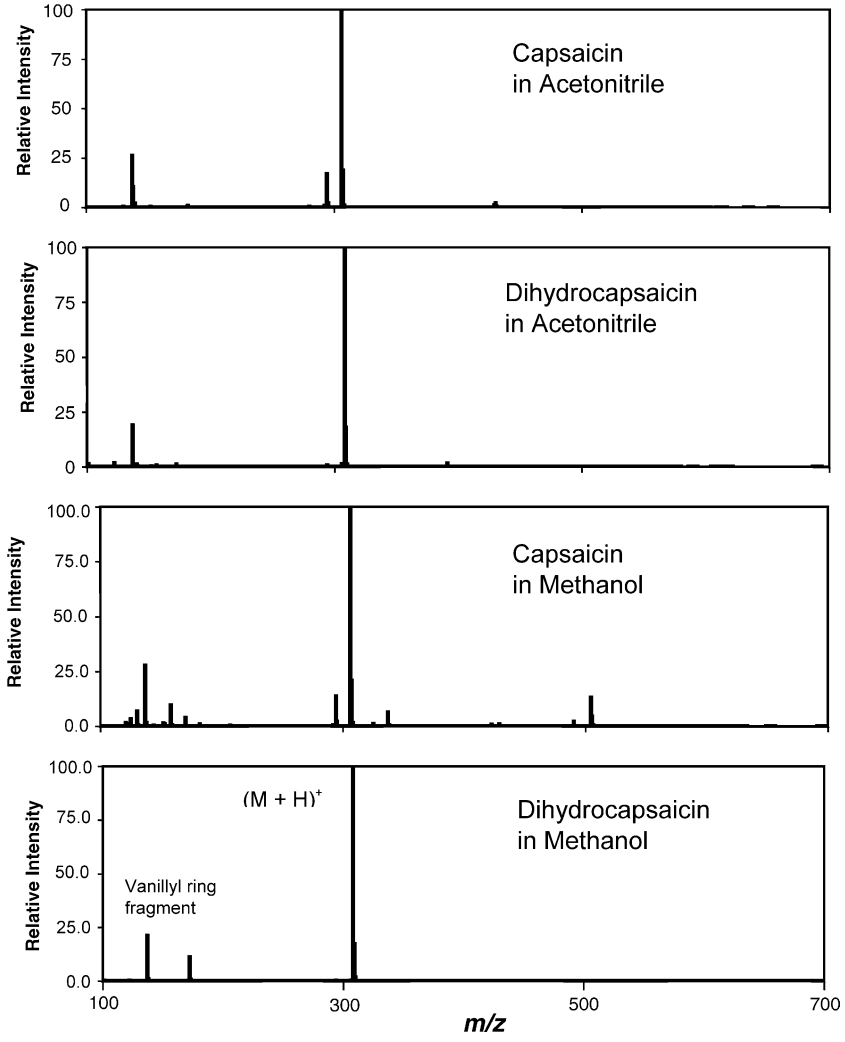


Fig. 2 Full-range (*m/z* 100–700), background-subtracted, positive-ion APCI mass spectra for capsaicin and dihydrocapsaicin. The spectra were collected with voltage settings of +4000 V (electrospray capillary) and +135 V (fragmentor), a corona current of 4 μA, and with either 35:65 aqueous 0.2 vol.% acetic acid–methanol or 55:45 aqueous 0.2 vol.% acetic acid–acetonitrile mobile phase. Tabulated *m/z* values and ion intensities for the spectra in methanol are given in the supplementary material

Detector response had only a weak, positive correlation with capillary and fragmentor voltages. Other settings were 12 L min^{-1} gas flow, 0.0073 Pa (50 psig) nebulizer pressure, and 300°C spray chamber. Silver nitrate concentration in the aqueous portion of the mobile phase, varied from 10 to 250 μmol L^{-1}, had a small positive effect on the MS detector response, and a concentration of 100 μmol L^{-1} was chosen for most experiments.

The optimum conditions were used to obtain mass spectra from *m/z*200 to 800. In aqueous acetonitrile mobile phase the silver-adduct ions in the mass spectra of the capsaicinoids were mostly $(M+Ag)^+$, <20% relative intensity $(M+AgNO_3+Ag)^+$, and <5% $(2M+Ag)^+$ (Fig. 3). The silver adducts came in two isotopic forms (+107 and +109) with a nearly 1:1 intensity ratio as predicted by the natural abundance of the silver isotopes. A small proportion of protonated molecule persisted, particularly for the compounds with saturated acyl chains. As with electrospray ionization, switching to methanol led to significantly greater formation of metal-ion adducts and fragments.

Analytical sensitivity comparison

To measure and compare method sensitivities, about 400 pg 6-ene-8-methylcapsaicin and 8-methyldihydrocapsaicin, dissolved in methanol, were injected separately into 50:50 mixtures of aqueous organic mobile phase (0.30 mL min^{-1} for ESI/CIS, 0.50 mL min^{-1} for APCI), and the selected ion monitoring (SIM) peak areas were measured. For 6-ene-8-methylcapsaicin, monitoring occurred at *m/z* 306 for positive-ion ESI/APCI or *m/z* 412 plus 414 for CIS. For 8-methyldihydrocapsaicin, monitoring occurred at *m/z* 308 for positive ESI/APCI or *m/z* 414 plus 416 for CIS. The mass window for SIM was one unit wide. The sensitivities of the various methods were compared by normalizing against the peak areas produced in the positive electrospray ionization, aqueous acetonitrile mobile phase experiment. Generally, electrospray ionization produced the most intense signal, followed by silver coordination ion spray and atmospheric-pressure chemical ionization (Table 3).

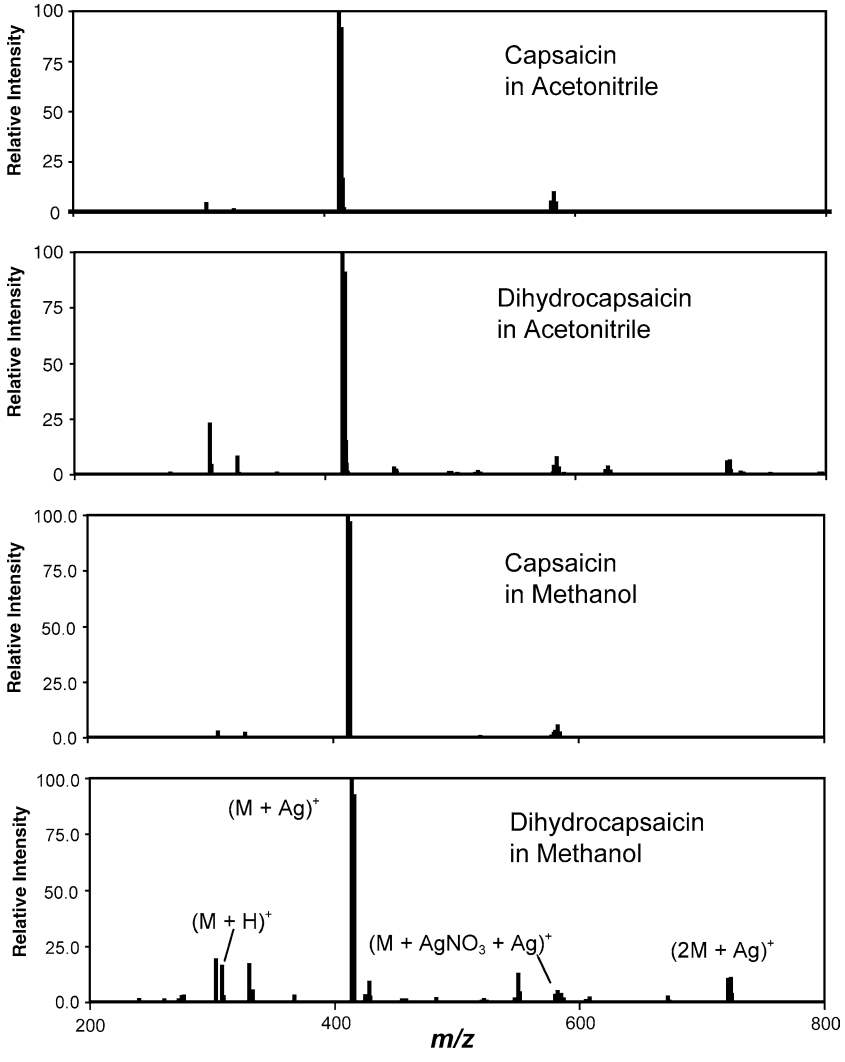


Fig. 3 Full-range (*m/z* 200–800), background-subtracted, CIS (silver ion) mass spectra for capsaicin and dihydrocapsaicin. The spectra were collected with voltage settings of +3000 V (electrospray capillary) and +150 V (fragmentor) and with either 35:65 aqueous 0.10 mmol L^{-1} silver nitrate–methanol or 55:45 aqueous 0.10 mmol L^{-1} silver nitrate–acetonitrile mobile phase. Tabulated *m/z* values and ion intensities for the spectra in methanol are given in the supplementary material

The nature of the organic component of the mobile phase had little effect on ESI sensitivity, but had a significant impact on APCI and CIS. The presence of acetonitrile in the mobile phase attenuated the peak area of 8-methyldihydrocapsaicin by factors of 1.5 for APCI and 3 for CIS relative to the experiments with methanol. It may be that the same strong interaction of silver ion and acetonitrile demonstrated in liquid chromatographic phases in our earlier studies [1] also occurred in the ionization source of the mass spectrometer. Acetonitrile-containing mobile phases cannot be recommended for silver CIS, thereby limiting the utility of the technique. This is particularly unfortunate because chromatographic separation is better with acetonitrile than with methanol [1].

Silver ion and alkenes (e.g. capsaicin) are known to form strong complexes. Thus, it was not surprising to find higher sensitivity for 6-ene-8-methylcapsaicin than for 8-methyldihydrocapsaicin in silver CIS. The MS signal intensity produced by capsaicin was at least double that produced by dihydrocapsaicin, and response to the other capsaicins was similar. The sensitivity of silver CIS for capsaicin matched that of positive-ion ESI. Nevertheless, positive-ion ESI was the simplest and most effective ionization method of those studied for the capsaicinoids as a group, and it was used for all subsequent experiments.

LC–ESI-MS method

Chromatographic separation of capsaicinoids on a phenyl stationary phase with an aqueous acetonitrile mobile phase and the mass spectrometric detection by electrospray ionization and selected-ion monitoring was fully described in the previous article [1].

Method validation studies

Detection limit

For each standard two very dilute solutions were prepared such that the signal-to-noise ratios (S/N) generated were between 5 and 10 for the less concentrated and between 50 and 100 for the more concentrated. From these results a line was constructed through zero, and the concentration that would have produced a S/N equal to 3 was calculated. The detection limits for the nine standard compounds were all quite similar, ranging from 3 to 7 pg injected.

Linear range and sensitivity

A set of seven solutions, ranging in concentration from 0.010 to 20 ng μL^{-1} (0.020–40 ng injected; about 5–1000 times the detection limit) for each standard, was prepared and analyzed. The solutions did not contain any internal standard. The LC–MS peak area for each analyte was plotted versus concentration. Figure 4 is the plot for 8-methyldihydrocapsaicin and was typical of the others. The data became non-linear above 5 ng μL^{-1} (15 μmol L^{-1}). All of the plots were linear between about 10 pg μL^{-1} (quantitation limit; $S/N = 10$) and 3 ng μL^{-1}, with correlation coefficients above 0.998 and relative standard errors of the line about 6%. The slopes of the calibration lines (analytical sensitivity) varied between 2.4×10^6 and 2.8×10^6 $\mu L\ ng^{-1}$. The small range indicated that the combined electrospray ionization efficiency, interface and quadrupole throughput, and ion detector response were very much the same among the set of capsaicinoids tested.

Repeatability

The commercial capsaicinoid mixture was diluted with water–methanol solvent to give solutions of four different total concentrations—0.042, 0.85, 8.2, 40.6 ng μL^{-1}. No internal standard was added. Three 2-μL injections of each of the test solutions were made on three consecutive days and the data were collected and analyzed. Analysis of variance of the peak areas revealed no significant differences between the uncertainties within one set of data and between sets (days). The aggregate relative standard deviation (RSD) values for the repeatability study varied by compound and by concentration level (Table 4). The RSD values generally improved as the amount injected was increased. An RSD of 3–4% was typical for the higher concentrations. The sample components were identified by their retention times and mass spectra as described in the previous article [1].

Quantitative analysis

A standard mixture that approximated the composition of the commercial plant extract was prepared by

Table 3 Comparison of the ionization modes and their sensitivities

Ionization mode	Aqueous phase	Organic phase	Relative SIM peak area	
			Capsaicin	Dihydrocapsaicin
ESI $(M+H)^+$	0.2 vol.% HOAc[a]	CH_3CN	1.00	1.00
	0.2 vol.% HOAc	CH_3OH	1.0	0.9
CIS $(M+Ag)^+$	0.10 mmol $L^{-1}AgNO_3$	CH_3CN	0.49	0.18
	0.10 mmol $L^{-1}AgNO_3$	CH_3OH	1.0	0.52
APCI $(M+H)^+$	0.2 vol.% HOAc	CH_3CN	0.18	0.23
	0.2 vol.% HOAc	CH_3OH	0.27	0.37

[a]HOAc is acetic acid

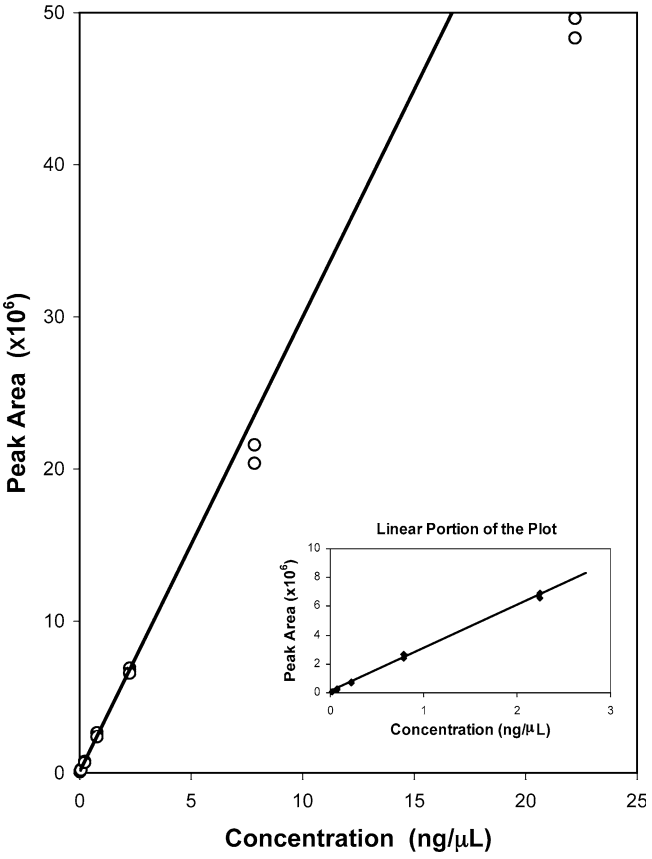


Fig. 4 LC–ESI-MS calibration plot for dihydrocapsaicin showing the linear range and beyond. The *circles* are the experimental data. For the linear regression line through the lowest four analyte concentrations (*inset*) $r^2 = 0.9989$ and *rel* $s_E = 6.2\%$. No internal standard was used

appropriate dilution with water–methanol of stock solutions of the eight standard compounds (1, 3–8, 11; Table 1). Further dilutions with aqueous methanol were made and internal standard (norcapsaicin, 2) was added to prepare three solutions in a molar ratio of 1:7:20 within the linear range. These solutions were injected in duplicate and peak areas measured on two consecutive days. Linear regression of peak-area ratio (analyte area/internal standard area) versus concentration (ng μL^{-1}) for each of the compounds showed excellent linearity (Table 5) with regression coefficients $r^2 > 0.9996$ and *rel* $s_E < 2\%$, except for *N*-vanillyldecanamide, 7.

A solution of the commercial capsaicinoid mixture was spiked with internal standard, diluted appropriately, and tested with the working standard solutions described above. Comparison of peak-area ratios with the calibration lines gave the sample component concentrations, and further calculations gave the fractional composition of the mixture (Table 5). To estimate the amount of 10, the calibration line for its isomer 11 was used. The sum of the masses of the individual components determined experimentally, 11.95 ± 0.09 mg, was in reasonable agreement with the actual mass of sample, 11.66 ± 0.02 mg, and the fractional composition was confirmed by LC–UV analysis at 280 nm. The LC–UV

Table 4 Results of repeatability study

Sample concentration	Compound			
	8	3	6	5
Low level				
Mean peak area	0.0294	0.0557	0.358	0.868
% rsd	15.3	9.9	10.3	7.3
Mid level				
Mean peak area	0.339	0.597	3.79	9.42
% rsd	10.3	6.4	7.4	6.6
High level				
Mean peak area	3.19	5.66	34.6	80.3
% rsd	4.7	3.9	3.5	4.4
Highest level				
Mean peak area	15.5	27.0	145	306
% rsd	3.8	3.7	3.5	3.3

results were: 1 (< 1%), 2 (trace), 3 (4%), 4 (1.5%), 5 (63%), 6 (25%), 7 (1.5%), 8 (2%), 10 and 11 (2%). In the LC–UV method the fractional composition was calculated by dividing component peak areas by the summed peak areas of the capsaicinoids. The accuracy of this calculation depends on identical molar absorptivities at 280 nm, a fact demonstrated by experiment ($\epsilon = 2890 \pm 10$ mol^{-1} L cm^{-1}). Details are given in the supplementary material.

Recovery study

The sample stock solution was spiked with internal standard and the mixture of standards, diluted appropriately, and tested with the working standard solutions described above. Comparison of peak-area ratios with the calibration lines gave the total amount of each component. From these values were subtracted the known sample amounts to give the amounts of compounds added and recovery values (Table 5). Recovery was defined as the ratio of the experimentally determined result to the known added amount of the compound. Percent recovery was very good, ranging from 99 to 103%, except for 7 at 108%. Remember that the calibration line for 7 had the largest uncertainty. The highest uncertainty for a recovery value was ±8% for 11, probably because of the poor chromatographic resolution of the homodihydrocapsaicin isomers.

Analysis of pepper fruits

Jalapeno and habanero peppers were processed as described in the experimental section, extracts were injected, and the LC–ESI-MS results were obtained (Fig. 5). Peak identifications were made by matching retention times and masses. A large amount of more hydrophilic compounds eluted much earlier than the capsaicinoids and did not interfere with the analysis. Quantitative analysis of the chilis was performed in duplicate, and, as expected, the habanero fruit contained more total capsaicinoids (0.113% mass fraction) than

Table 5 Results of the quantitative studies

	Compound									
	1	3	4	5	6	7	8	10	11
Calibration slope (ng $\mu L^{-1})^{-1}$	2.202 ± 0.009	2.114 ± 0.007	2.103 ± 0.007	1.84 ± 0.01	1.869 ± 0.004	1.92 ± 0.04	2.297 ± 0.009		1.75 ± 0.02
R^2	0.99993	0.99995	0.99996	0.99978	0.99998	0.99820	0.99994		0.99966
% *rel* s_E[a]	0.9	0.7	0.7	1.5	0.5	4.4	0.8		1.9
Sample concentration (ng μL^{-1})	0.0087 ± 0.0005	0.1258 ± 0.0007	0.0414 ± 0.0006	2.15 ± 0.02	0.882 ± 0.007	0.050 ± 0.003	0.0649[b] ± 0.0003	0.02[c]	0.0439 ± 0.0007
% Total capsaicinoids	0.26 ± 0.01	3.71 ± 0.03	1.22 ± 0.02	63.5 ± 0.9	26.0 ± 0.3	1.49 ± 0.08	1.91[b] ± 0.02	0.6[c]	1.29 ± 0.02
% Recovery	101 ± 2	103 ± 2	102 ± 2	99 ± 2	101 ± 2	108 ± 4	102 ± 4		100 ± 9

Uncertainties listed are the population standard deviations and were calculated by propagation of uncertainty
[a]*rel* s_E is the standard error of the linear regression line expressed in the *Y* dimension divided by the average *Y* value
[b]This value includes contributions from other homocapsaicin isomers, such as 9, that co-elute with compound 8
[c]This value is an estimate. No pure standard was available, so the calibration plot for compound 11 was used to calculate concentrations

the jalapeno (0.068%) (see Table 6). These values match well with reported values in the literature—up to 0.01% in hot peppers, up to 0.3% in chilis, and as high as 1% in very strong chilis [24]. The amount of 10 was estimated using the calibration line for 11, and the values reported for 8 include contributions from co-eluting homocapsaicin isomers, such as 9.

The distribution of capsaicinoids varied with chili type (Table 6). Both chilis contained significant (≥0.5% composition) amounts of 7-methylnordihydrocapsaicin 3, *N*-vanillylnonanamide 4, 6-ene-8-methylcapsaicin 5, 8-methyldihydrocapsaicin 6, 6-ene-8-methylhomocapsaicin 8, and 9-methylhomodihydrocapsaicin 11, with 5 and 6 dominating. The relative amounts of 3 and 11 were much higher in the jalapeno pepper. Three capsaicinoids appeared in significant quantities only in the habanero pepper: *N*-vanillyloctanamide 1, *N*-vanillyldecanamide 7, and 10, probably 8-methylhomodihydrocapsaicin [1]. The $(M+H)^+$ ions of mass 334 and 336, corresponding to bishomocapsaicins and bishomodihydrocapsaicins, were not detected in the pepper extracts. However, traces of what was thought to be

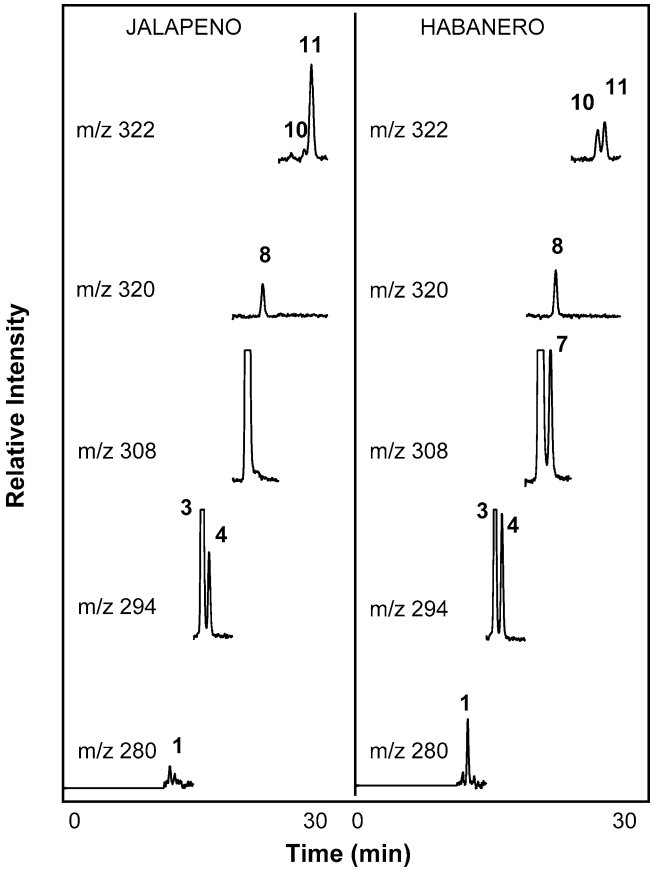


Fig. 5 Selected ion chromatograms for the *minor* capsaicinoids identified in pepper fruits. (Data for norcapsaicin (added internal standard) and capsaicin are not shown, and the large peak for dihydrocapsaicin (*m*/*z* 308) is not labeled.) The number labels were defined in Table 1. Chromatographic conditions: 250 mm×4.6 mm×5 μm particle Ace Phenyl column (30°C); 55:45 0.2 vol.% acetic acid–acetonitrile (0.8 mL min^{-1}). ESI and SIM mass settings were as described in the text

Table 6 Capsaicinoid content of pepper fruits

Compound	Jalapeno		Habanero	
	μg g^{-1}fruit	% composition	μg g^{-1}fruit	% composition
Bisnordihydrocapsaicin?	2.6[a]	0.4[a]	0.9[a]	0.08[a]
1	0.8	0.1	5.5	0.5
3	80	12	38	3.4
4	9.6	1.4	14	1.2
5	310	46	660	59
6	250	37	350	31
7	0	0	18	1.6
8	4.4[b]	0.7[b]	23[b]	2.1[b]
10	1.5[c]	0.2[c]	5.8[c]	0.5[c]
11	20	2.9	7.4	0.7
Total	680	100	1130	100

The relative uncertainty in all values is ±5%
[a]This value is an estimate. No pure standard was available, so the calibration plot for compound 2 was used to calculate concentrations
[b]This value includes contributions from other homocapsaicin isomers, such as 9, that co-elute with compound 8
[c]This value is an estimate. No pure standard was available, so the calibration plot for compound 11 was used to calculate concentrations

bisnordihydrocapsaicin (elution just before peak 1; retention time = 11.0 min; $(M+H)^+$ at m/z 280) were found in both fruits.

Conclusions

Of ESI, APCI, and silver CIS, positive-ion electrospray ionization provided the highest response with the capsaicinoids. Subsequently, a quantitative LC–ESI-MS method for the major and minor capsaicinoids found in plant extracts was developed. Baseline resolution of nearly all of the sample components in selected-ion traces was achieved [1], MS response was linear with capsaicinoid concentration, and as little as 5 pg injected could be detected. Recovery of capsaicinoids added to the plant extract was excellent, and the fractional composition of the mixture was accurately determined. Similar LC–UV analysis confirmed the quantitative values. The method also proved useful for the quantitative determination of the major and minor capsaicinoids found in the fruits of chili peppers.

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