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Determination of Capsaicin and Dihydrocapsaicin in *Capsicum* Fruits by Liquid Chromatography−Electrospray/Time-of-Flight Mass Spectrometry

ANA GARCÉS-CLAVER,† MARÍA S. ARNEDO-ANDRÉS,† JAVIER ABADÍA,§ RAMIRO GIL-ORTEGA,† AND ANA ÁLVAREZ-FERNÁNDEZ*,§

Technology for Plant Production Department, Centro de Investigación y Tecnología Agroalimentaria (CITA), Apartado 727, E-50080 Zaragoza, Spain; and Plant Nutrition Department, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apartado 202, E-50080 Zaragoza, Spain

A simple, highly selective, sensitive, and reproducible liquid chromatography−electrospray ionization/time-of-flight mass spectrometry method has been developed for the direct and simultaneous determination of capsaicin and dihydrocapsaicin in *Capsicum* fruit extracts. Capsaicin and dihydrocapsaicin are the two major members of the so-called capsaicinoid family, which includes other minor analogues, and usually account for at least 90% of the pungency trait in *Capsicum* fruits. Chromatographic separation of capsaicin and dihydrocapsaicin was achieved with a reversed-phase chromatography column, using a gradient of methanol and water. Quantification was done using as an internal standard (4,5-dimethoxybenzyl)-4-methyloctamide, a synthetic capsaicin analogue not found in nature. Analytes were base-peak resolved in less than 16 min, and limits of detection were 20 pmol for capsaicin and 4 pmol for dihydrocapsaicin. The intraday repeatability values were lower than 0.5 and 12% for retention time and peak area, respectively, whereas the interday repeatability values were lower than 0.6 and 14% for retention time and peak area, respectively. Analyte recoveries found were 86 and 93% for capsaicin and dihydrocapsaicin, respectively. The method developed has been applied to the identification and quantification of capsaicin and dihydrocapsaicin in fruit extracts from different *Capsicum* genotypes, and concentrations found ranged from 2 to 6639 mg kg^{-1}.

KEYWORDS: Capsaicin; *Capsicum*; dihydrocapsaicin; liquid chromatography; mass spectrometry; pepper; time-of-flight

INTRODUCTION

Fruits of pepper (*Capsicum*) plants are among the most consumed spices throughout the world. These fruits contain capsaicinoids, a family of compounds that give them the characteristic pungent taste. The two major capsaicinoids, capsaicin and dihydrocapsaicin, are responsible for up to 90% of the total pungency of pepper fruits (*1*, *2*). Besides capsaicin and dihydrocapsaicin, at least nine minor capsaicinoids (**Figure 1**) have been shown to occur in peppers (*3*−*7*). Capsaicinoid compounds have been widely studied and are currently used in the food industry, for medical purposes, as pharmaceuticals, and in defensive sprays (*8*−*15*).

Capsaicinoids are alkaloids produced by a condensation reaction between an aromatic moiety and a C9−C11 branched-chain fatty acid. The aromatic moiety is vanillylamine, a phenylalanine derivative. Branched-chain fatty acids are biosynthesized from valine and leucine, and in the case of capsaicin and dihydrocapsaicin those acids differ in the degree of unsaturation of the 9-carbon fatty acid side chain (*1*, *16*). Suzuki and Iwai (*17*) reviewed aspects related to the chemistry, analysis, biochemistry, and pharmacology of the pungency principle of *Capsicum* spp. Further work on the chemistry and analysis methods for total and individual capsaicinoids has been reviewed by Govindarajan (*18*), Anu et al. (*19*), and Pruthi (*20*).

Capsaicinoids are synthesized exclusively in the epidermal cells of the placenta of *Capsicum* fruits and are accumulated in blisters along the epidermis (*21*−*23*). Their biosynthesis begins approximately 20 days postanthesis (*24*), with a number of enzymes being involved in the biosynthetic pathway. The degree of pungency depends on the *Capsicum* species and cultivars, and the capsaicin and dihydrocapsaicin contents can be affected by different factors such as the developmental stage of the fruit (*25*) and the environmental growth conditions (*26*−*28*). For instance, Kozukue et al. (*29*) reported values for capsaicin and

* Corresponding author (e-mail ana.alvarez@eead.csic.es; telephone +34976716052; fax +34976716145).
† Centro de Investigación y Tecnología Agroalimentaria (CITA).
§ Consejo Superior de Investigaciones Científicas (CSIC).

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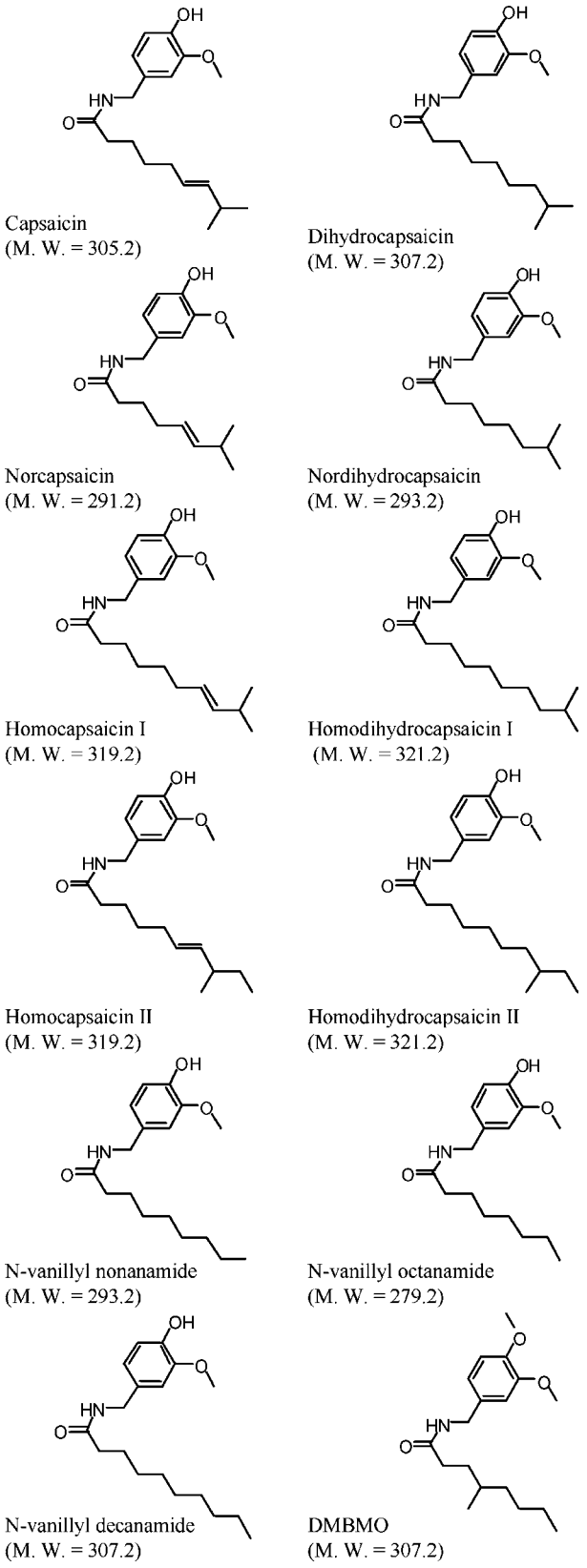

Figure 1. Chemical structures and molecular weight (M.W.) of capsaicinoids and DMBMO.

dihydrocapsaicin, respectively, ranging from a minimum of 2.6 and 8.2 $\mu g\ g^{-1}$ of fresh weight (FW) in canned sliced 'Jalapeño' to maxima of 1187 and 771 $\mu g\ g^{-1}$ of FW in 'Habanero' fresh pepper. The content of capsaicinoids in a given cultivar may differ by as much as 90 mg per fruit depending on environmental factors (*30*). Phenolic intermediates can influence the biosynthesis of capsaicinoids (*31*), and, for instance, it has been demonstrated that 8-methylnonenoic acid could have an important regulatory role in the capsaicinoid biosynthesis pathway (*32*).

The first method developed to assess the pungency of fruits was the Scoville organoleptic test (*33*), which estimates pungency (total capsaicinoid concentration) from the burning mouth sensation, using diluted samples and a tasting panel of five individuals. This test is subjective and does not permit the determination of the levels of individual capsaicinoids. Subsequently, capsaicinoids have been analyzed using spectrophotometry (*34*−*37*) and near-infrared reflectance spectroscopy (NIR) (*38*, *39*). Techniques used to separate capsaicinoids include paper chromatography (*40*), capillary gas chromatography (*41*, *42*), and especially high-performance liquid chromatography (HPLC). The latter technique has been often coupled to UV−vis spectroscopy (*43*−*53*). Although HPLC-UV methods have been used successfully, they have limited selectivity and sensitivity.

The most recent methods for the determination of capsaicinoids have used HPLC coupled to more selective techniques such as mass spectrometry (*29*, *54*−*56*). Mass spectrometry had already been used to determine capsaicinoids in the past using off-line monitoring of HPLC effluents (*57*), HPLC-MS (*58*), and field desorption (*59*). In the past decade, capsaicinoid analytical techniques have been developed by using ionization techniques that permit HPLC-MS coupling, such as atmospheric pressure chemical ionization (APCI) (*56*, *60*) and electrospray (ESI) interfaces (*29*, *61*), using ion-trap or quadrupole mass spectrometers. The high selectivity of HPLC-MS techniques could possibly be applied to determine directly the concentrations of minor capsaicinoids in pepper fruits. Methods have been developed for different matrices, such as self-defense materials (*61*), *Capsicum* oleoresin (*62*), and capsaicinoid-exposed blood (*61*). As far as we are aware, no reports on the determination of capsaicinoids in *Capsicum* fruits have been developed using time-of-flight MS [MS(TOF)], a technique that allows the determination of exact mass and is capable of isotopic resolution in the whole mass range without compromising sensitivity (*63*).

This study was aimed to develop and validate a selective and sensitive HPLC-ESI/MS(TOF) analytical method, allowing the identification and accurate quantification of capsaicin and dihydrocapsaicin, the two major capsaicinoids in *Capsicum* fruits. The method would be particularly suitable to routinely analyze capsaicinoids in breeding programs and may be used to determine the concentrations of other minor capsaicinoids once appropriate standards are available.

MATERIALS AND METHODS

Chemical and Reagents. All eluents, buffers, and standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore, Bedford, MA). Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide) (≥97%), dihydrocapsaicin (8-methyl-*N*-vanillylnonamide) (≥90%), formic acid (50%), methanol (≥99.9%, LC-MS grade), acetonitrile (LC-MS grade), 2-propanol (LC-MS grade), lithium hydroxide monohydrate (99.995%), methionine (99%), and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). The capsaicin analogue (4,5-dimethoxybenzyl)-4-methyloctamide (DMBMO) was synthesized according to the method of Cooper et al. (*62*) and used as an internal standard. The identity of DMBMO was confirmed by nuclear magnetic resonance (NMR) spectrometry, and its purity was >98%. Labeled KNO_3 (10% ^{15}N) and NH_4NO_3 (5% ^{15}N) were purchased from Cambridge Isotopes Laboratory (Andover, MA). $CuSO_2 \cdot 5H_2O$ and Fe(III)-EDTA were purchased from Merck (Darmstadt, Germany), and NH_4NO_3 and Na_2-

$MoO_4 \cdot 2H_2O$ were purchased from Sigma-Aldrich. KNO_3, KH_2PO_4, $Ca(NO_3)_2 \cdot 4H_2O$, $MnCl_2 \cdot 4H_2O$, H_3BO_3, and $ZnSO_4 \cdot 7H_2O$ were purchased from Panreac Química (Barcelona, Spain).

Standard Preparations. Solutions for tuning the ESI-MS(TOF) were (i) 10 mM LiOH, 0.2% (v/v) formic acid, and 50% (v/v) 2-propanol and (ii) 1 μM leucine enkephalin, 20 μM methionine, 5 μM gluthathione, 0.1% (v/v) formic acid, and 50% (v/v) methanol.

Standard 1 mM stock solutions of capsaicin and dihydrocapsaicin were prepared by dissolving the appropriate quantity of each compound in 5 mL of acetonitrile. Stock solutions of 1 mM DMBMO were prepared in 5 mL of methanol. All standards were prepared in triplicate and were stored at −20 °C.

Plant Material. Eleven *Capsicum* spp. genotypes were analyzed, covering the whole range of pepper fruit pungency. These included two nonpungent *C. annuum* genotypes ('Yolo Wonder' and 'Jupiter'), five pungent *C. annuum* genotypes ['Agridulce', 'Sincap', 'Serrano Criollo de Morelos-334' (SCM-334), 'Nigrum' and 'Perennial'], three pungent *C. chinense* cultivars ('Chinense Sweet', 'Orange Habanero', and 'Mild Habanero'), and one pungent *C. frutescens* cultivar ('Tabasco'). Plants were grown in a climatized greenhouse in black plastic pots, 11 cm in diameter (one plant per pot), containing a substrate mixture of peat, sand, clay-loam soil, and Humin Substrat (Klasman-Deilmann, Geeste, Germany) (1:1:1:1, in v). Two grams of a slow-release fertilizer (Osmocote 16N-4P-9K, Scotts, Tarragona, Spain) was top-dressed on each pot. Plants were watered daily to maintain optimum growth, and the average maximum and minimum temperatures in the greenhouse during the growth period were 27.3 and 19.3 °C, respectively.

^{15}N-labeled pepper fruits were obtained in a hydroponics system. Germinated seeds of 'Habanero' were transferred to hydroponic boxes (18 × 18 × 11 cm) filled with a continuously aerated nutrient solution containing 2.25 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1.0 mM $MgSO_4$, 1.0 mM KH_2PO_4, and 0.25 mM NH_4NO_3 and 45 μM Fe(III)-EDTA, 23.1 μM H_3BO_3, 4.6 μM $MnCl_2$, 1.2 μM $ZnSO_4$, 0.19 μM $CuSO_2$, and 0.06 μM Na_2MoO_4. Plants were grown in a growth chamber under a 16/8 h light/dark regime and at 25 °C. To obtain ^{15}N-labeled capsaicinoids some plants were grown in nutrient solution containing 2.5 mM KNO_3 (10% ^{15}N) and 0.25 mM NH_4NO_3 (5% ^{15}N as $^{15}NH_4{}^{15}NO_3$).

Extraction of Capsaicinoids. The fruits were oven-dried at 55 °C for 4−5 days and then ground in a Polytron grinder. One hundred milligrams of dried tissue samples was extracted with 1 mL of pure acetonitrile, containing a small amount of the internal standard DMBMO. Acetonitrile was used for extraction because it gives a high extraction rate while leading to a minimum level of impurities (*52*). The final concentration of DMBMO was 5 μM. The suspension of dried powder in acetonitrile was constantly shaken at room temperature for 1 h (orbital shaker from New Brunswick Scientific, Edison, NJ, operating at 250 rpm) and then heated in a water bath without shaking at 65 °C for 1 h. The mixture was then shaken again at room temperature for another hour in the conditions indicated above. Then, the suspension was centrifuged for 15 min at 16000*g*, and the supernatant was collected and brought to a volume of 1 mL with acetonitrile. Finally, the supernatant was filtered successively through 0.45 and 0.22 μm PVDF membrane filters (Millipore, Bedford, MA) before analysis. Three replicates of each extract were carried out.

HPLC-ESI/MS(TOF) Analysis. Analyses were carried out with a BioTOF II (Bruker Daltonics, Billerica, MA) coaxial multipass time-of-flight mass spectrometer [MS(TOF)] equipped with an Apollo electrospray ionization source (ESI) and coupled to a Waters Alliance 2795 HPLC system (Waters, Milford, MA).

The BioTOF II was operated with endplate and spray tip potentials at 2.8 and 3.3 kV, respectively, in the positive ion mode, and at 3.0 and 3.5 kV, respectively, in the negative ion mode. Drying gas (N_2) and nebulizer pressures were kept at 30 and 60 psi, respectively. The mass axis was calibrated using lithium−formate adducts in negative ion mode and a mixture of 1 μM leucine enkephalin and 20 μM methionine in positive ion mode. Spectra were acquired in the 100−500 mass/charge ratio (*m*/*z*) range.

To optimize the MS signal, direct injection of 10 μM capsaicin and dihydrocapsaicin standards was carried out using a syringe pump (Cole-Parmer Instrument Co., Vernon Hills, IL) operated at 2 μL min^{-1}. Capillary voltage and gas drying temperature were tested in the ranges of 60−120 V and 150−250 °C, respectively, and final values chosen as optimal were 90 V for capillary voltage and 200 °C for gas temperature.

Table 1. Elution Profile for the HPLC-ESI/MS(TOF) Method Developed To Analyze Capsaicinoids

HPLC solvent gradient		HPLC flow gradient	
time (min)	methanol (%)	time (min)	flow (mL min^{-1})
0	70	0	0.9
11	70	14	0.9
12	80	16	1.3
13	100	18	1.6
27	100	22	1.8
30	70	24	1.3
40	70	26	0.9

Samples were chromatographed using a Waters Symmetry C18 column (4.6 × 250 mm; 5 μm) coupled with a Waters Symmetry C18 guard column (3.9 × 20 mm; 5 μm). The autosampler and column were maintained at 4 and 30 °C, respectively, and the injection volume was 20 μL. Capsaicinoids were eluted using a gradient of methanol and Milli-Q water and a flow rate gradient between 0.9 and 1.8 mL min^{-1} (**Table 1**). After each injection, the column was re-equilibrated for 10 min with 30% water/70% methanol, at a flow rate of 0.9 mL min^{-1}. Total analysis run time was 40 min. The gradient was designed to improve the separation of analytes, and a flow rate gradient was included to obtain optimal column cleaning and equilibration. The exit flow from the column was split with a T-connector (Upchurch, Oak Harbor, WA) that led 9.4% of the total efflux (85−170 μL min^{-1}) from the HPLC to the ESI interface of the MS(TOF) apparatus.

The system was controlled with the software packages BioTOF v. 2.2 (Bruker Daltonics, Bremen, Germany) and Hyphenation Star v. 2.3 (Bruker Daltonik). Data were processed with Data Analysis v. 3.2 software (Bruker Daltonik).

Validation was carried out by obtaining calibration curves corrected with DMBMO as an internal standard, limits of detection [LOD, signal-to-noise (S/N) ratio of 3], limits of quantification (LOQ, S/N ratio of 10), and intra- and interday repeatabilities and recoveries using as a matrix an extract of *Capsicum* fruits.

RESULTS

ESI/MS(TOF) Analysis. Negative ion mode ESI/MS(TOF) spectra of standard analyte solutions showed major $[M - H]^{-1}$ ions at *m*/*z* 304.2 for capsaicin (**Figure 2A**), *m*/*z* 306.2 for dihydrocapsaicin (**Figure 2B**), and *m*/*z* 306.2 for DMBMO (**Figure 2C**). The latter compound was used as internal standard in further experiments. Positive ion mode ESI/MS(TOF) spectra showed major $[M + H]^{+1}$ ions at *m*/*z* 306.2 for capsaicin and *m*/*z* 308.2 for dihydrocapsaicin (data not shown). Ionization for both compounds was less intense in the positive than in the negative mode, the intensity of the $[M + H]^{+1}$ ions being 75 and 94% lower than those of the $[M - H]^{-1}$ ions for capsaicin and dihydrocapsaicin, respectively. Therefore, the negative ion mode was chosen for further experiments.

HPLC-ESI/MS(TOF) Analysis. Analytes were separated with a solvent and flow elution gradient in a C18 column, and mass spectra were acquired by using ESI/MS(TOF) in the *m*/*z* 200−400 range during the whole chromatographic run, obtaining three-dimensional (3D) chromatograms (time, *m*/*z*, and intensity). Ion chromatograms were extracted for the exact *m*/*z* values corresponding to the $[M - H]^{-1}$ species of each analyte and the compound used as internal standard (**Figure 3**). Results show that the HPLC-ESI/MS(TOF) method developed is capable of resolving adequately capsaicin, DMBMO, and dihydrocapsaicin when present in a combined standard solution, with retention times of 11.6 (**Figure 3A**), 14.0, and 15.2 min (**Figure**

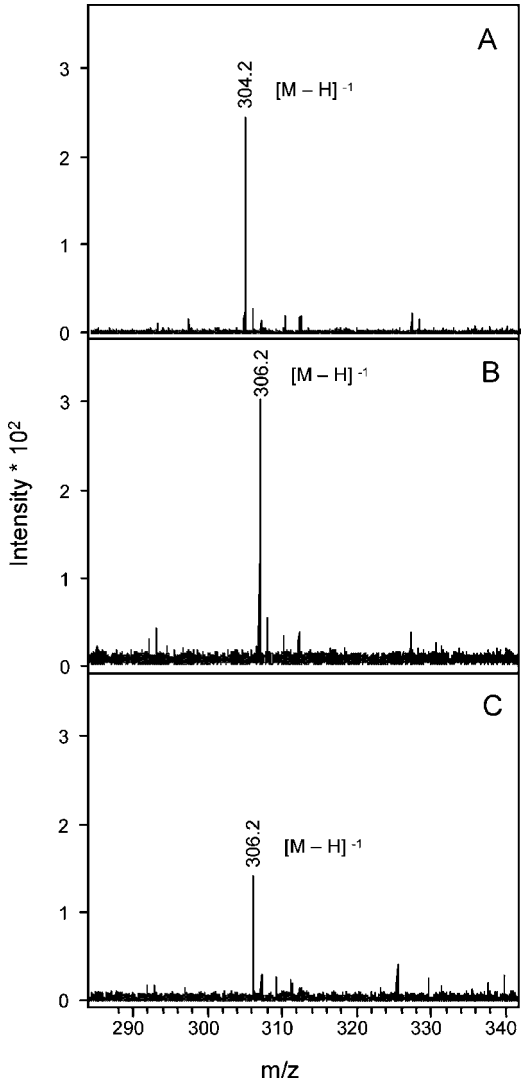

Figure 2. ESI/MS(TOF) mass spectra in negative-ion mode of standard solutions in 70% methanol: 30 μM capsaicin (**A**), 30 μM dihydrocapsaicin (**B**), and 30 μM DMBMO (**C**).

3B), respectively. The resolution (Rs) of dihydrocapsaicin and DMBMO (defined as 2 times the separation between peaks divided by the sum of base peak widths) was 1.2, a value that can be considered as adequate. Separation time for all compounds was approximately 16 min, with column washing and regeneration accounting for 24 min more, leading to a total analysis time of 40 min.

Validation of the HPLC-ESI/MS(TOF) Method. The HPLC-ESI/MS(TOF) method was validated by using analyte solutions in acetonitrile. Calibration curves corrected using DMBMO as internal standard, LODs, LOQs, and intra- and interday repeatability and recoveries were measured. Extracts of fruits from different *Capsicum* genotypes were also analyzed.

Standard solutions were prepared in acetonitrile in the range of 0.5−60 μM. Calibration curves, corrected by internal standardization, were obtained by plotting the peak area ratio (analyte/internal standard) against concentration. Data fit well to a second-degree polynomial regression, both for capsaicin (R^2 of 0.964; **Figure 4A**) and dihydrocapsaicin (R^2 of 0.942; **Figure 4B**). The calibration curves were linear from the LOQ values to 30 μM, for both capsaicin and dihydrocapsaicin.

LOD, defined as the analyte amount giving a S/N ratio of 3, was better for dihydrocapsaicin (4 pmol) than for capsaicin (20 pmol). Using a 20-μL injection volume, these values are

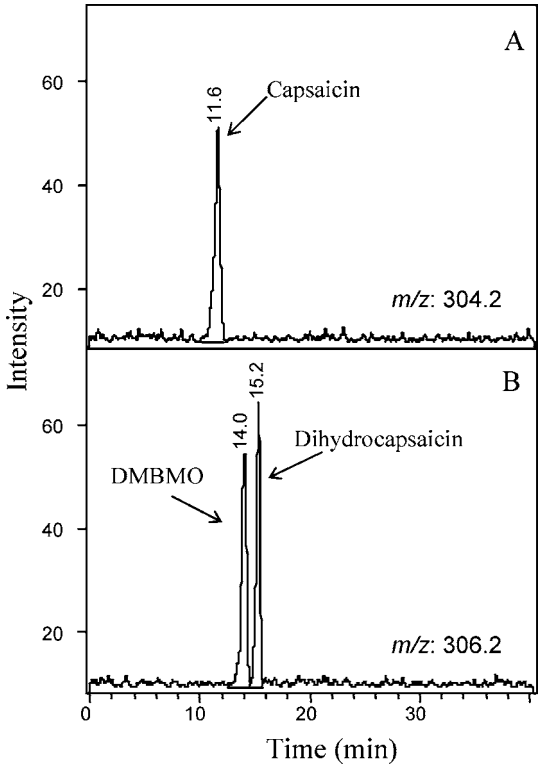

Figure 3. HPLC-ESI/MS(TOF) chromatograms in negative-ion mode of a combined standard solution of 10 μM capsaicin (**A**), 5 μM DMBMO (**B**), and 10 μM dihydrocapsaicin (**B**) in 100% acetonitrile.

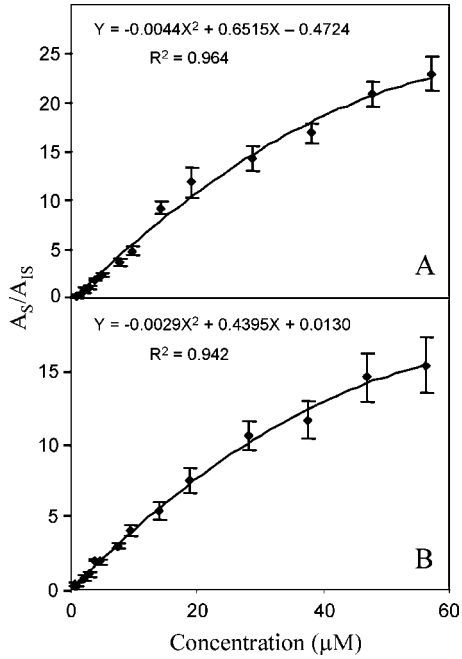

Figure 4. Calibration curves for capsaicin (**A**) and dihydrocapsaicin (**B**) obtained by plotting the peak area ratio (sample area/area of the internal standard; A_S/A_{IS}) versus the analyte concentration injected. The concentration of internal standard was 5 μM. Solutions were made in 100% acetonitrile. Bars are SE for triplicate measurements.

equivalent to analyte concentrations in the injected sample solution of 0.2 μM for dihydrocapsaicin and 1 μM for capsaicin. LOQ values, defined as the amounts giving a S/N ratio of 10, were 60 pmol for capsaicin and 12 pmol for dihydrocapsaicin.

The intraday repeatability for retention time and peak area ratio was evaluated using 10 consecutive analyses with two

Table 2. Intra- ($n = 10$) and Interday ($n = 10$) Repeatability for Retention Times (RT) and Peak Area Ratios (A_S/A_{IS}) of the HPLC-ESI/MS(TOF) Method Developed[a]

		RSD (%)			
		intraday		interday	
analyte	concentration (μM)	RT (min)	A_S/A_{IS}	RT (min)	A_S/A_{IS}
capsaicin	4	0.42	11.9	0.57	9.6
	20	0.36	5.3	0.49	9.2
dihydrocapsaicin	2	0.01	5.4	0.21	13.5
	20	0.01	8.7	0.44	10.2

[a] DMBMO (internal standard) was used at a concentration of 5 μM.

Table 3. Recoveries Obtained for Capsaicin and Dihydrocapsaicin, Using Extracts of Pepper Fruits from the 'SCM-334' Genotype Spiked with Solutions Containing the Amount of Standard Analytes Indicated [Results Are Means ± SE ($n = 5$)]

	amount added (μM)	amount found (μM)	recovery (%)
capsaicin	0	18.0 ± 0.2	
	4.8 ± 0.2	21.8 ± 0.2	78.7 ± 0.8
	21.3 ± 1.9	37.7 ± 0.2	92.4 ± 0.4
dihydrocapsaicin	0	22.0 ± 0.6	
	5.9 ± 0.4	27.2 ± 1.2	90.3 ± 3.1
	37.2 ± 1.5	55.8 ± 0.2	94.9 ± 1.5

different levels of concentrations for each analyte, whereas the interday repeatability was evaluated by analyzing the same standard solutions for 10 consecutive days (**Table 2**). The relative standard deviation (RSD) for peak retention time was always lower than 0.5% in the intraday test and 0.6% in the interday test. The RSD for peak area ratio was in the range of 5.3−11.9% in the intraday test and between 9.2 and 13.5% in the case of the interday test. The RSD for DMBMO peak retention time was always lower than 0.7%.

Recovery assays were carried out for capsaicin and dihydrocapsaicin by spiking fruit extracts of the 'SCM-334' genotype with known amounts of both analyte standards and the internal standard (DMBMO). Recovery was calculated by dividing the amount of analyte found in the spiked sample by the sum of the amount found in the sample plus the amount added. Analyte recoveries found were between 78.7 and 94.9% (**Table 3**).

Analysis of Capsaicin and Dihydrocapsaicin in Fruit Extracts. A chromatogram example of an extract of 'Orange Habanero' fruits grown in normal nutrient solution (not enriched with ^{15}N) is shown in **Figure 5A**. Three peaks, corresponding to capsaicin, DMBMO, and dihydrocapsaicin, were detected at retention times of 11.6, 14.0, and 15.2 min, respectively. The resolution of the MS(TOF) detector used is higher than 10000 fwhm (full width at half mass), and therefore the MS(TOF) spectra provide information on the elemental isotopic distribution of any compound detected in the chromatographic run. The two insets of **Figure 5A** show the zoomed mass spectra of the peaks corresponding to capsaicin and dihydrocapsaicin, with the corresponding major $[M - H]^{-1}$ ions at 304.2 and 306.2. These spectra also show minor peaks at 305.2 and 307.2, due to the natural isotopic composition of the analytes (mainly due to the presence of ^{15}N and ^{13}C isotopes).

When pepper plants were grown in a ^{15}N-enriched nutrient solution, the *m*/*z* signals at 305.2 and 307.2 increased relative to the signals at *m*/*z* 304.2 and 306.2 (**Figure 5B**). The peak area ratios 304.2/305.2 (capsaicin) and 306.2/307.2 (dihydrocapsaicin) were approximately 2-fold lower in fruits from plants grown in ^{15}N-enriched nutrient solution as compared to those from fruits grown in normal nutrient solutions (**Table 4**). The peak area ratios 304.2/305.2 and 306.2/307.2 were not significantly different in standard solutions of capsaicin and dihydrocapsaicin and in extracts of fruits from plants grown in normal nutrient solution.

Although commercial standards of minor capsaicinoids are not available yet, the chromatogram of 'Orange Habanero' fruit extract shows a peak at 16.1 min (*m*/*z* 320.2), which could be putatively attributed to the $[M - H]^{-1}$ ion of any of the homodihydrocapsaicin isomers, and a peak at 17.0 min (*m*/*z* 306.2) that could be putatively attributed to the $[M - H]^{-1}$ ion of *N*-vanillyldecanamide (**Figure 6**). In the latter case, a good separation between this compound, dihydrocapsaicin, and DMBMO, all three having the same molecular mass, was obtained. Of course, other techniques such as nuclear magnetic resonance (NMR) spectroscopy would be necessary to identify unequivocally these two chromatographic peaks.

A number of *Capsicum* genotypes differing in pungency level have been analyzed for capsaicin and dihydrocapsaicin with the method developed, and results are shown in **Table 5**. Concentrations found were in the range of 8−6639 mg kg^{-1} of dried fruit for capsaicin and 2−3727 mg kg^{-1} of dried fruit for dihydrocapsaicin. The genotype 'Orange Habanero' contained the highest concentrations, and, as expected, neither capsaicin nor dihydrocapsaicin was detected in 'Yolo Wonder' and 'Jupiter', because both varieties are considered to be nonpungent. In the case of 'Sincap' only dihydrocapsaicin was detected, and this could be expected because this variety was selected by its low level of pungency (*64*). The capsaicin and dihydrocapsaicin contents are associated with the level of pungency, so these results show that the 11 genotypes studied can be classified into four categories: nonpungent ('Yolo Wonder' and 'Jupiter'), slightly pungent ('Sincap' and 'Agridulce'), pungent ('Sweet Chinense', 'Mild Habanero', 'Perennial', 'Nigrum', 'Tabasco'), and extremely pungent ('Orange Habanero').

DISCUSSION

Two members of the capsaicinoid family, capsaicin and dihydrocapsaicin, are the main compounds responsible for the pungency of *Capsicum* fruits. Pungency is considered to be one of the major quality factors in the pepper industry, and therefore reliable methods aimed to analyze these compounds are needed. We have developed and validated an HPLC-ESI/MS(TOF) method for the determination of capsaicin and dihydrocapsaicin in pepper fruit extracts, which includes separation by reverse phase HPLC, ionization by ESI, and highly selective detection of the analytes, using exact mass measurements with a TOF mass spectrometer. This is the first time, to our knowledge, that capsaicin and dihydrocapsaicin have been determined by an HPLC-ESI/MS method using a TOF mass spectrometer. This method can be applied in routine analysis in breeding programs and also in metabolic tracing studies. The number of analyses per day in routine batches would be approximately 35, with the apparatus running unattended for 5 days (192 samples in total).

The method developed has been validated for capsaicin and dihydrocapsaicin with respect to LODs, LOQs, calibration curves, reproducibility, and analyte recoveries, always using the internal standard DMBMO. Limits of detection obtained were 20 pmol (equivalent to a concentration of 1 μM in the extract) for capsaicin and 4 pmol (equivalent to a concentration of 0.2 μM in the extract) for dihydrocapsaicin. The LODs were better than most of those obtained using HPLC coupled to UV−vis,

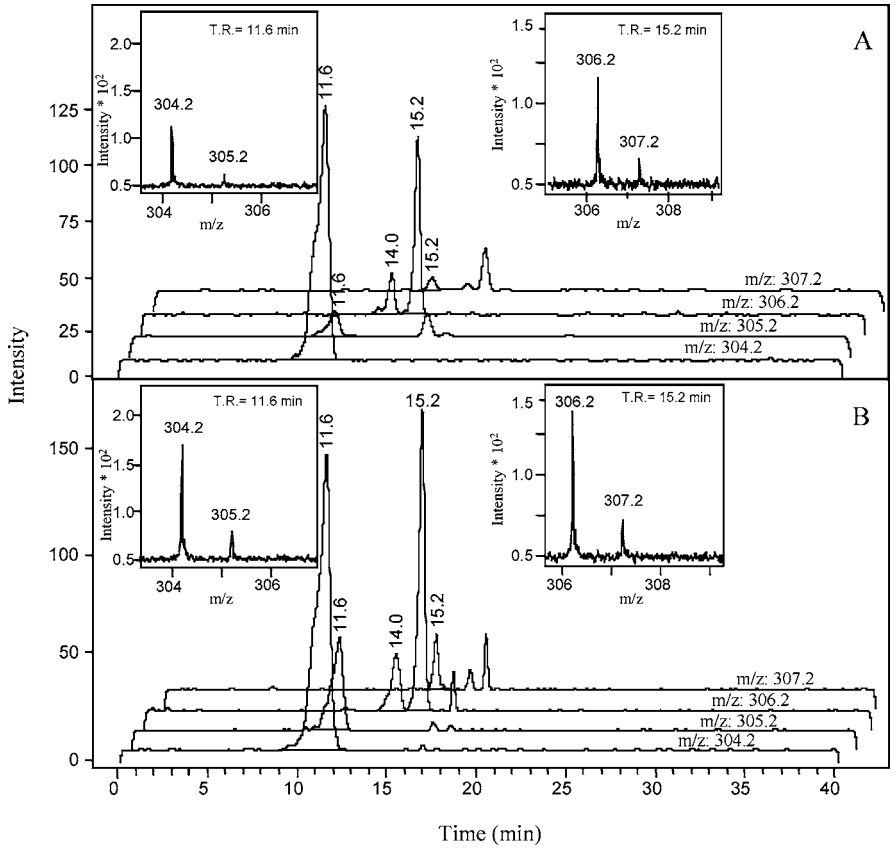

Figure 5. HPLC-ESI/MS(TOF) chromatograms in negative-ion mode of fruit extracts from 'Orange Habanero' plants grown in normal, control nutrient solution (**A**) and in nutrient solution enriched with ^{15}N (**B**).

Table 4. Peak Area Ratios *m/z* 304.2/305.2 (Corresponding to Capsaicin) and *m/z* 306.2/307.2 (Corresponding to Dihydrocapsaicin) for Standard Solutions, 'Orange Habanero' Fruit Extracts from Plant Grown in Normal (Control) Solution and ^{15}N-Enriched Nutrient Solution [Values Are Means ± SE ($n = 3$)][a]

	peak area ratio	
	capsaicin	dihydrocapsaicin
standard	11.2 ± 1.5[a]	9.1 ± 2.0[a]
control	11.1 ± 0.5[a]	10.9 ± 0.8[a]
^{15}N-enriched	5.3 ± 0.3[b]	6.1 ± 0.6[b]

[a] Means followed by different letters in the same column are significantly different ($P < 0.05$).

which are in the range of 6 μM (*52*) to 9 μM (*49*), but not as good as that found with single-quadrupole MS by Thompson et al. (*55*), which are approximately 0.01 pmol (in the range of 3 and 7 pg injected). The lowest LOQ found so far (0.03 μM) was obtained in a HPLC-MS method designed to measure capsaicin and dihydrocapsaicin in forensic sciences by using single-quadrupole MS (*61*). In these particular applications, very low limits of detection are needed to trace the presence of extremely low amounts of biological and nonbiological residues of self-defense spray weapons.

The method repeatability for peak area, with RSD values in the range of 5−14% for intra- and interdays, was similar to those obtained in other HPLC-ESI/MS methods, which were in the ranges of 2−10% (*61*) and 3−10% (*55*) for capsaicin and dihydrocapsaicin, respectively. The recoveries obtained by spiking fruit extracts of the 'SCM-334' cultivar were good, ranging from 79 to 95%, similar to the range (86−114%) found in 'Korean Hansuwi' red pepper using HPLC-ESI/MS with an ion-trap mass analyzer (*29*). Recoveries somewhat higher (99−

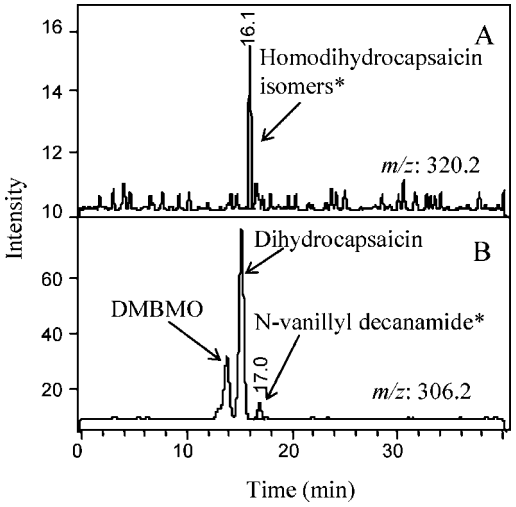

Figure 6. Chromatograms at *m/z* 320.2 (**A**) and *m/z* 306.3 (**B**) of fruit extracts from 'Orange Habanero' plants grown in normal, control nutrient solution. (∗) Putative assignment.

103%) were found by Thompson et al. (*55*) using HPLC-ESI/MS with a single-quadrupole MS analyzer.

The contents of capsaicin and dihydrocapsaicin found here for the different pepper genotypes are in good agreement with those found by other authors. According to our results, 'Orange Habanero', one of the most pungent varieties (*65*), had the highest capsaicinoid contents, with values of 6639 and 3727 mg kg^{-1} of fruit dry weight (DW) for capsaicin and dihydrocapsaicin, respectively. Considering that DW is approximately 10% of FW, these values are very similar to the values reported for 'Habanero' of 660 and 350 $\mu g\ g^{-1}$ of FW for capsaicin and dihydrocapsaicin, respectively, using HPLC-ESI/MS (*55*). Also,

Table 5. Concentrations of Capsaicin and Dihydrocapsaicin in Different *Capsicum* Genotypes Determined by the HPLC-ESI/MS(TOF) Method Developed [Values Are Means ± SE ($n = 3$)]

genotype	capsaicin (mg kg^{-1} of DW[a])	dihydrocapsaicin (mg kg^{-1} of DW)
Yolo Wonder	ND[b]	ND
Jupiter	ND	ND
Sincap	ND	2.3 ± 0.2
Agridulce	8.7 ± 0.1	8.3 ± 0.8
Sweet Chinense	16.5 ± 0.2	12.9 ± 1.8
Mild Habanero	17.7 ± 1.6	13.4 ± 2.4
Perennial	164 ± 7	192 ± 8
Nigrum	905 ± 41	1468 ± 81
Tabasco	3785 ± 235	2461 ± 80
Orange Habanero	6639 ± 348	3727 ± 179

[a] DW, dry weight. [b] ND, not detected, below LOD.

these values are within the range obtained with HPLC-UV methods. For instance, values of 2438 and 1621 mg kg^{-1} of DW for capsaicin and dihydrocapsaicin, respectively, were found by Cázares-Sánchez et al. (*66*), and a capsaicin plus dihydrocapsaicin content of 20069 mg kg^{-1} of DW was measured by Yazawa et al. (*67*) in 'Habanero'. The dihydrocapsaicin content of 'Sincap' (2.3 mg kg^{-1} of DW) is within the range of values previously reported (between 2.7 and 27.4 mg kg^{-1} of DW) by using HPLC-UV (*64*). Although capsaicin is usually the most abundant capsaicinoid, the contents of dihydrocapsaicin were higher than those of capsaicin in the genotypes 'Sincap', 'Perennial', and 'Nigrum'. Similar patterns, with dihydrocapsaicin being the predominant capsaicinoid, have already been previously reported in other cultivars of *C. annuum* and *C. pubescens* (*68*). As far as we know, data for capsaicin and dihydrocapsaicin for the rest of the genotypes analyzed have not been reported before.

This method has significant advantages over HPLC-UV methods commonly used to determine capsaicinoids in plant tissue extracts, which are complex matrices. First, MS(TOF) provides a higher reliability for the identification and quantification of compounds, based on the high selectivity provided by the exact *m*/*z* ratio of each analyte. For instance, this would rule out the possibility of assigning erroneously a peak to a given capsaicinoid, which could occur when identification is based only on retention time and UV spectra. Second, this method has a higher sensitivity (LODs of 0.2 and 1 μM for dihydrocapsaicin and capsaicin, respectively) than those of most HPLC-UV methods (e.g., LODs of 6 and 9 μM) (*49*, *52*). Third, the amount of information obtained for each peak (e.g., high-resolution mass spectra with isotopic signature) is more extensive than that obtained with HPLC-UV only.

The mass analysis technique used in this work (TOF) differs from that used in quadrupole analyzers, which are most widely used for LC-MS instrumentation (*63*). Quadrupole mass analyzers are scanning instruments, where sensitivity (the maximum S/N ratio) decreases with the mass range to be scanned. In this case, however, sensitivity is very high when only a few specific masses (SIM mode) are scanned. In contrast, TOF analyzers are not scanning instruments and characterize simultaneously the masses of all ions in the full mass range with the same sensitivity. The mass resolution of the quadrupole detectors is generally lower than those of the TOF analyzers, although it can be improved to similar values at the expense of overall signal response. To obtain a signal quality similar to that of high mass resolution instruments such as MS(TOF) with low-resolution mass analyzers, it could be often necessary to apply special sample cleanup procedures to reduce the noise introduced by coeluted matrix components (*63*). In the particular case of study of a large number of *Capsicum* genotypes for which a relatively large number of capsaicinoids could be present, TOF-(MS) would offer the possibility of detecting such compounds while still having sufficient sensitivity to adequately determine the major capsaicinoids capsaicin and dihydrocapsaicin in the full range of *Capsicum* fruit pungency. A higher mass resolution, as given by a TOF detector, can improve the quality of the extracted ion chromatograms, allowing more selective signal acquisition and thereby reducing the noise introduced by coeluted matrix components.

An additional advantage of the method presented here is the use as an internal standard of a capsaicin analogue not present in natural *Capsicum* fruits, DMBMO, as first proposed by Cooper et al. (*62*). ESI-MS analysis usually requires the use of internal standards, to control differences in analyte ionization caused by the complexity of the samples, and some studies have used for this purpose minor capsaicinoids present in fruits such as *N*-vanillyloctanamide (*61*). It is unlikely that the chemical differences between DMBMO and capsaicinoids could lead to significant quantification errors, because the ionization behavior of DMBMO was not very different from those of capsaicin and dihydrocapsaicin (**Figures 2** and **3**), and the chromatographic conditions were optimized to obtain retention times similar to those of the analytes (DMBMO must be separated from dihydrocapsaicin, because both compounds have the same molecular mass). In fact, the separation between internal standard and the analytes is smaller in our method (**Figure 3**) than in previous LC-MS methods using minor capsaicinoids as internal standards (*53*, *55*, *61*).

In summary, the method developed permits the direct and simultaneous analysis of capsaicin and dihydrocapsaicin, the two major capsaicinoids in pepper fruits, with high selectivity and sensitivity and sufficient reproducibility. The method developed has wide possibilities of application. So far, it has been tested with several *Capsicum* genotypes, showing its suitability to perform routine analyses in breeding programs aimed to select accessions with high contents of individual capsaicinoids. In addition, the high resolution of the MS(TOF) spectrometer used also gives information on isotopic distribution, allowing its use as a tool in metabolic studies with stable isotopes. For example, plants subjected to different growth conditions (e.g., light, temperature, stress) can be fed with ^{15}N-labeled compounds to follow the capsaicinoid metabolic pathways. Furthermore, the present method could be used to detect simultaneously other minor capsaicinoids by their exact *m*/*z* ratio, once appropriate standards are available.

SAFETY

Capsaicin and dihydrocapsaicin standards are very strong irritants and can be neurotoxic. Always use gloves and a mask when handling these solids and their concentrated solutions.

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