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Received May 09, 2012
Revised July 02, 2012
Accepted July 11, 2012

Research Article

Determination of capsaicinoids in Capsicum species using ultra performance liquid chromatography-mass spectrometry

In the present work, a rapid and sensitive ultra performance liquid chromatography-mass spectrometry method has been proposed for the analysis of capsaicinoids (nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin) present in different Capsicum samples. Extraction of capsaicinoids was carried out by liquid–liquid extraction using ethanol as an extracting solvent, while the chromatographic separation was achieved by reversed phase C_{18} column with gradient mobile phase (solvent A: acetonitrile and solvent B: water with 0.1% formic acid). Under the optimum experimental conditions, the linear ranges were 0.5–50 μg/g with correlation coefficient (r^2) >0.999 for each capsaicinoids and detection limits were 0.15, 0.05, 0.06, 0.2, and 0.1 μg/g for nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin, respectively. Run-to-run and day-to-day precisions of the method with relative standard deviations <1.5% were achieved for all analyzed capsaicinoids. The robustness of the method was determined by utilizing different injection volumes of the extracts. Furthermore, to validate the system robustness, a run of high number of capsaicinoids present in different varieties of Capsicum samples was performed in this study. All the capsaicinoids were separated in a time of less than 9 min by employing the proposed method.

Keywords: Capsaicinoids / Capsicum / Chili / Peppers / Ultra performance liquid chromatography-mass spectrometry
DOI 10.1002/jssc.201200459

1 Introduction

Hot peppers are among the most popular food additives around the world because of their sensory attributes of pungency, aroma, and color. They are also commercially very important since large quantities are consumed around the world. The consumption is due mainly to their very pungent flavor. The pungent metabolites in the fruits of Capsicum species are called capsaicinoids, which are a group of 12 or more alkaloids with a structure of vanillylamide of branched fatty acids with 9–11 carbons [1, 2]. The most abundant capsaicinoids are capsaicin (C; *trans*-8 methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (DHC; 8 methyl-N-vanillylnonanamide), which are responsible for about 90% of the spiciness [3] and the less-abundant capsaicinoids are nordihydrocapsaicin (n-DHC), norcapsaicin, homocapsaicin (h-C), homodihydrocapsaicin (h-DHC), nornorcapsaicin, nornordihydrocapsaicin, nonivamide, etc. [4]. An accurate determination of the levels of various capsaicinoids has become important because of the increasing demand by consumers for spicy foods, and the increasing use in pharmaceuticals [5].

A variety of methods have been used for the analysis of capsaicinoids. These include spectrophotometry [1, 6, 7]. In recent years, reversed-phase high performance liquid chromatography (HPLC) has become the method most frequently used for analysis of capsaicinoids because of its rapidity and reliability. HPLC methods with UV [8], fluorescence [9], electrochemical [10], and MS [11–15] detection and gas chromatography with flame ionization detection [16, 17] have also been developed for the determination of capsaicinoids. These developed methods have drawback in longer analysis time and high solvent consumption. Nowadays, high speed and low sample consumption of analysis is increasingly being demanded in many areas where HPLC is applied, including pharmaceutical and food analysis, in order to increase throughput and reduce loss of time and extra sample volume. The rapid separation of samples is an analytical stage that requires high efficiency as well as speed, due to the complexity of sample matrix, and hence is particularly challenging to achieve. Therefore, in this study,

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Abbreviations: C, capsaicin; **DHC**, dihydrocapsaicin; **h-C**, homocapsaicin; **h-DHC**, homodihydrocapsaicin; **n-DHC**, nordihydrocapsaicin; **SHU**, Scoville heat units; **SIR**, selected ion reaction; **UPLC-MS**, ultra performance liquid chromatography-mass spectrometry

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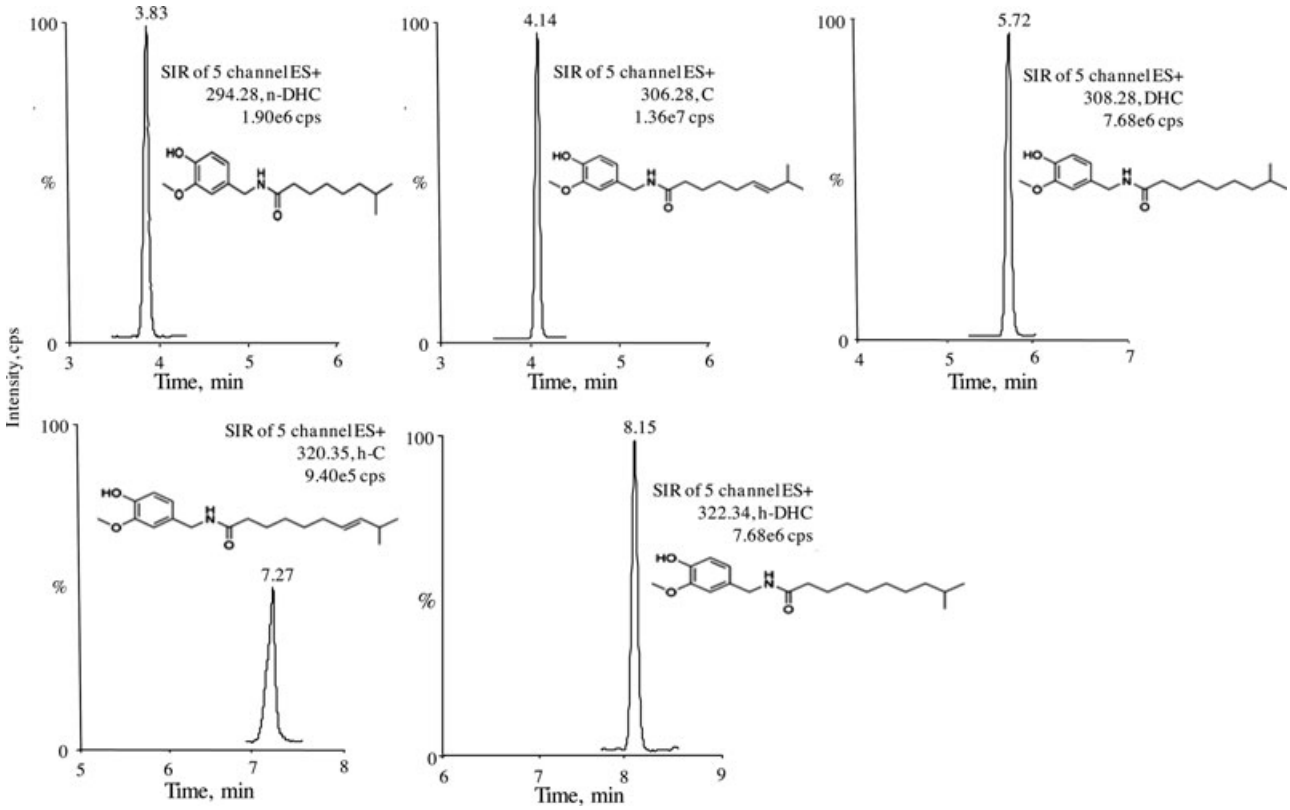

Figure 1. Chromatogram of the separation in standard mixture of capsaicinoids–SIR record is displayed from calibration level 50 μg/mL, each chromatogram displays SIR transition for individual compound.

the development of a rapid, sensitive, and reproducible ultra performance liquid chromatography-mass spectrometry (UPLC-MS) method has been presented. The proposed method showed some complementary advantages to the conventional HPLC-MS method, such as shorter analysis times and improved sensitivity. The developed method was successfully applied for the determination of n-DHC, C, DHC, h-C, and h-DHC, present in the hot varieties of peppers. To the author's knowledge, this is the first description relating to the UPLC-MS method development and its application to the analysis of capsaicinoids in Capsicum samples.

2 Materials and methods

2.1 Chemicals and reagents

All the capsaicinoid standards, n-DHC, C, DHC, h-C, and h-DHC (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and ethyl acetate were obtained from BDH Chemicals (Poole, England). Formic acid for mobile-phase preparation was obtained from Panreac (Barcelona, Spain). Water was purified through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Stock standard solutions of capsaicinoid (100 μg/mL) were prepared in mobile phase (acetonitrile/water, 50:50, v/v). Standard mixture of capsiacinoids at different concentration levels (0.1–100 μg/mL) was prepared for calibration curve. All the standards and samples were filtered through a 0.22-μm PVDF syringe filter (Membrane Solutions, TX, USA) before being injected into the UPLC-MS system. All solvents used for capsaicinoid analysis were of HPLC grade.

2.2 Instrumentation

2.2.1 Ultra performance liquid chromatography

The chromatographic separation of capsaicinoids (Fig. 1) was performed under ambient temperature condition with Waters Acquity UPLC system equipped with a quaternary pump (Waters, Mildford, MA, USA) and Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7-μm particle size; Waters). Optimum separation of capsaicinoids was achieved with a binary mobile phase at flow rate of 0.5 mL/min – solvent A: acetonitrile; solvent B: water with 0.1% formic acid, the gradient elution program was 0–8 min, 60–50% B; equilibration time 2 min. The sample injection volume was 5 μL.

2.2.2 Mass spectrometry

The UPLC system was coupled to a Quattro Premier triple quadrupole mass spectrometer (Micromass, Milford, MA,

USA) using electrospray ionization source (Z-spray) operating in positive ionization mode. The analysis was performed in selected ion reaction (SIR) mode. The parameters affecting the ion transmission were optimized by infusing a standard solution of capsaicinoids at 5 μg/mL. The optimized source working conditions were as follows: cone voltage, 20 V; capillary voltage, 3.0 kV; source temperature, 120°C; desolvation temperature, 300°C; cone gas flow rate, 60 L/h; desolvation gas flow rate, 600 L/h. Nitrogen (99.99% purity), produced using a Peak Scientific nitrogen generator model NM30LA (Inchinann, UK), was used as cone gas. An Oerlikon rotary pump, model SOGEVAC SV40 BI (Cologne, Germany) provided the primary vacuum to the mass spectrometer. Data acquisition was carried out with MassLynx V4.1 software.

2.3 Sample analysis

2.3.1 Extraction of capsaicinoids

Capsicum samples used for this study were purchased from local markets in Riyadh city consisting of green chili, hot chili, red chili, green pepper, red pepper, and yellow pepper. The capsaicinoids were extracted from the Capsicum samples using previously published paper [18, 19]. The extraction method was as follows. First, the peppers were peeled, and the peduncle and seeds were separated. Only the pericarp and the placenta of the pepper were studied. Both the pericarp and the placenta were triturated with a conventional beater to obtained homogeneous sample for the analysis. After that, the dried pepper was placed in 120 mL glass bottles with Teflon-lined lids in a ratio of sample/ethanol (1:1; g/mL). Bottles were capped and placed in an 80°C water bath for 4 h; swirled manually after every hour. Samples were removed from water bath and cooled at room temperature. The supernatant content of samples (5 mL) was filtered through 0.45-μm filter paper using a 5 mL disposable syringe (Millipore Corporation) into a UPLC sample vial, capped and stored at 4°C in refrigerator until analysis. The capsaicinoid concentrations in samples were expressed as microgram per gram pepper.

3 Results and discussion

3.1 Optimization of UPLC-MS method

First, an optimization process (Quan-optimization) for each analyte was performed to optimize the data acquisition parameters under highly sensitive SIR mode. Cone voltage was optimized for all the individual analytes. This process was performed by infusing individual capsaicinoid standard solutions (50 μg/mL) to the ion source of the MS detector. The tuning of the method was performed to obtain the maximum intensity of the precursor ions. Optimal MS conditions for each capsaicinoid were obtained using intellistart software program and the values are summarized in Table 1.

Table 1. SIR parameters used with MS instrument

Capsaicinoids	Molecular formula	MS parameters[a)]	
		Precursor ion $[M+H]^+$	Cone voltage (V)
n-DHC	$C_{17}H_{27}NO_3$	294.2	38
C	$C_{18}H_{27}NO_3$	306.2	36
DHC	$C_{18}H_{29}NO_3$	308.2	38
h-C	$C_{19}H_{31}NO_3$	320.3	40
h-DHC	$C_{19}H_{29}NO_3$	322.3	36

a) Dwell time = 0.025 sec in all cases.

Acquity BEH C_{18} analytical column was chosen for the separation of capsaicinoids. In this proposed method, the temperature was held constant at 25°C. By employing the gradient elution profile, separation of the capsaicinoids was obtained. The method has achieved good resolution of the chromatographic peaks and notably the time taken for the analysis was very short in comparison with the analysis time needed in conventional reversed-phase HPLC techniques [20–22]. The obtained chromatogram and the resulting retention times for capsaicinoids present in the extract of hot chili are shown in Fig. 2. The developed separation method addressed so many important issues, such as, good resolution, and very sharp and symmetric peak shape without any tailing. Although the efficient separation is not definitely necessary in MS detection however it brings further enhancement of sensitivity and selectivity of the analysis [23].

The addition of formic acid to the mobile phase was played an important role in the enhancement of ionization efficiency of the analytes. The capsaicinoids were eluted according to their molecular size (Figs. 1 and 2), since the relatively small molecules with polar groups are not strongly retained on C_{18} stationary phase. The identification of the compounds was performed based on the retention times as

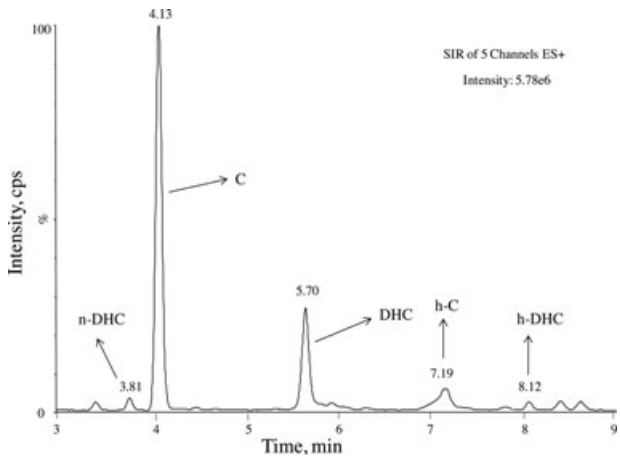

Figure 2. Chromatogram of the red chili extract obtained by using optimized UPLC-MS method.

Table 2. Quality parameters of UPLC-MS method

Compound	r^2	LOD (μg/g)	LOQ (μg/g)	Run-to-run precision (RSD%)[a)]	Day-to-day precision (RSD%)[a)]
n-DHC	0.999	0.15	0.47	1.07	1.20
C	0.999	0.05	0.16	1.16	1.40
DHC	0.999	0.06	0.19	0.89	1.02
h-C	0.999	0.2	0.62	1.22	1.50
h-DHC	0.999	0.1	0.31	1.11	1.27

a) $N = 5$.

well as molecular mass of each component obtained under identical UPLC-MS conditions.

3.2 Performance of the proposed method

The quantification of all capsaicinoid was performed by comparison of analyte peak area with standard calibration curve. Calibration standards at 0.1, 0.3, 0.6, 1, 5, 10, 15, 50, and 100 μg/mL were prepared in 50% of acetonitrile in ultrapure water solution. Under the optimal experimental conditions, a linear relationship between peak and capsaicinoid concentrations was established. The linear regression equations were obtained from the calibration curves, which are the five standards of commercially available capsaicinoids. The regression equation for all analyzed compounds has shown correlation coefficient values higher than 0.999. Linear response was found in the range of 1–50 μg/mL. The limit of detection (signal-to-noise ratio, 3:1) and limit of quantification (signal-to-noise ratio, 10:1) values for all the analyzed compounds were calculated. Run-to-run and day-to-day precisions of the method were carried out on standard solution (1 μg/mL) of all capsaicinoid by injecting five replicates on the same day and 15 replicates over three consecutive days (five replicates each day), respectively. High run-to-run and day-to-day precisions were achieved with RSD values lower than 1.5% for all capsaicinoids. All obtained values are listed in Table 2. The lower than 1.5% RSD values confirm that the proposed analytical method is successful in providing acceptable values of run-to-run and day-to-day precisions required for an accurate analysis of capsaicinoids. From these obtained results, it was concluded that the UPLC-MS method can be successfully used in the routine analysis of capsaicinoids present in peppers sample.

3.3 Application: Analysis of capsaicinoids in peppers

The optimized UPLC-MS method was applied to quantify the major capsaicinoids present in six varieties of peppers (hot chili, red chili, green chili, red pepper, green pepper, and yellow pepper). The results for the concentration of capsaicinoids in the analyzed pepper samples have been shown in Table 3. From the obtained results, it can be easily concluded that C and DHC are the major capsaicinoids in all of the six varieties of analyzed peppers, which are in agreements with the reported results [18]. Figure 2 shows, as an example, the chromatograms obtained by the proposed UPLC-MS method for capsaicinoids present in the extract of hot chili. The chromatograms did not show any interference, as no detectable matrix peak was eluted in the retention time of the target capsaicinoids. The recovery test was carried out by standard addition method. Five replicate samples at low-, medium-, and high-quality control were prepared for recovery determination. The recoveries were obtained between 88 and 94%.

Additionally, the corresponding pepper contents were calculated in microgram per gram and converted to Scoville heat units (SHU) according to the reported paper [24] in order to classify them with respect to their various pungency levels. The conversion to SHU was done by multiplying the C content in pepper dry weight (g/g) by the coefficient corresponding to the heat value for pure C, which is 1.6×10^7. There are five levels of pungency classified using SHU, which are nonpungent, mildly pungent, moderately pungent, highly pungent, and very highly pungent [25]. The values of SHU and corresponding pungency level of each sample are listed in Table 4. Thus, on the basis of above classification, it is obvious that hot chili is highly pungent compared to the other samples studied.

Table 3. Level of capsaicinoids (μg/g) and recovery rates (*R*, %) in the analyzed pepper samples ($n = 3$)

Sample	Hot chili μg/g ± SD[a)]	*R*	Green chili μg/g ± SD[a)]	*R*	Red chili μg/g ± SD[a)]	*R*	Red pepper μg/g ± SD[a)]	*R*	Green pepper μg/g ± SD[a)]	*R*	Yellow pepper μg/g ± SD[a)]	*R*
n-DHC	140.3 ± 3.5	88	12.2 ± 1.6	90	152.9 ± 3.7	90	4.0 ± 0.2	94	6.3 ± 0.3	92	1.1 ± 0.1	93
C	4795.5 ± 30.5	90	687.2 ± 9.5	90	1941.2 ± 20.6	91	148.9 ± 3.6	94	199.8 ± 4.3	93	26.2 ± 2.2	94
DHC	1399.3 ± 15.5	89	399.7 ± 7.1	89	1585.3 ± 16.6	90	41.6 ± 2.8	94	58.4 ± 3.1	93	7.8 ± 0.5	93
h-C	207.8 ± 4.1	89	527.4 ± 7.3	90	404.5 ± 7.0	90	2.2 ± 0.1	93	3.9 ± 0.2	92	nd	nd
h-DHC	139.4 ± 3.5	91	75.3 ± 2.5	89	118.4 ± 3.3	89	1.3 ± 0.1	93	6.1 ± 0.3	92	nd	nd

SD, standard deviation; nd, not detected.
a) Mean of three measurements.

Table 4. The Scoville heat units and the pungency level of the sample analyzed

Sample	Capsaicine (μg/g $\pm$ SD)	Scoville heat units (SHU)	Pungency
Hot chili	4795.5 $\pm$ 30.5	76727.81	Very highly pungent
Green chili	687.2 $\pm$ 9.5	10995.23	Moderately pungent
Red chili	1941.2 $\pm$ 20.6	31058.38	Highly pungent
Red pepper	148.9 $\pm$ 3.6	2382.72	Mildly pungent
Green pepper	199.8 $\pm$ 4.3	3196.99	Moderately pungent
Yellow pepper	26.2 $\pm$ 2.2	419.472	Nonpungent

SD, standard deviation.

4 Concluding remarks

A rapid and sensitive method based on UPLC-MS has been proposed for determination of capsaicinoids in peppers. The quantification of the capsaicinoids was calculated in terms of SHU. The analysis results have shown that hot chili is the most pungent (76727.81 SHU) among the peppers studied. The pungency level of analyzed samples was decreased as follows: hot chili (very highly pungent), red chili (highly pungent), green chili (moderately pungent), green pepper (moderately pungent), red pepper (mildly pungent), and yellow pepper (nonpungent). The developed method has shown excellent sensitivity, good linearity of response, and good precisions. All the good performance of the proposed method comes from different relevant aspects. The UPLC offers the reducing chromatographic run time and the improved sensitivity due to the narrower chromatographic peaks compared to conventional HPLC. The MS allowed the acquisition of SIR monitoring of the compound with good sensitivity, providing a reliable confirmation of capsaicinoids detected in samples. The results of the capsaicinoids suggest that the method could be applicable for further evaluation and application in agricultural and biomedical sciences.

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project no. RGP-VPP-043.

The authors have declared no conflict of interest.

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