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Fast method for capsaicinoids analysis from *Capsicum chinense* fruits

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ABSTRACT

Chili peppers are widely utilized in the world as savory food additives due the pungency induced by the capsaicinoids. Also, these compounds have functional properties as antimutagenic, antitumoral, antioxidant and analgesic. These characteristics increase the interest in this compound class, hence the capsaicinoid analysis must be reproducible and accurate. This study aimed to develop and validate a fast, efficient and reproducible method to analyze capsaicinoids in Brazilian *Capsicum chinense* fruits. The extracts were obtained after an optimization step that indicated the condition 100% of methanol and 10 min on ultrasound assisted extraction. The analyses were carried out in an ultra high performance liquid chromatographic system with detection by a photo diode array and mass spectrometer. The analytical method developed permits the separation of 8 capsaicinoids in 4 min of time analysis expending only 2 mL of solvent as mobile phase. The validation parameters evaluated for the method show the effectiveness and satisfactory performance to answer the analytical needs of this research area, presenting low values to relative standard deviation in repeatability and reproducibility and recoveries ranged from 88 to 112% for capsaicin and 89 to 109% for dihydrocapsaicin. In the extracts from different accessions of *C. chinense* fruits analyzed, the contents of capsaicin and dihydrocapsaicin were in the range of 156–1442 μg g^{-1} and 26–478 μg g^{-1} of fresh fruit, respectively, showing the large application of this method for quantification of the two major capsaicinoids in fast routine analysis and may be used to determine the concentrations of other minor capsaicinoids once appropriate standards are available.

1. Introduction

Fruits of chili pepper plants that belong to the family *Solanaceae*, genus *Capsicum* are among the most consumed spices throughout the world (Garcés-Claver, Arnedo-André, Avier Abadía, Gil-Ortega, & Álvarez-Fernández, 2006) and are very important commercially. Brazil, a center of genetic diversity, is one of the world's largest producers of *Capsicum* peppers. In the year 2005, chili peppers of this genus were the second-most exported vegetable from Brazil, with an exportation volume of 9222 t (Ribeiro, Lopes, Carvalho, Henz, & Reifschneider, 2008). Some of the most popular domesticated varieties of peppers cultivated in the Brazilian territory belong to the specie *Capsicum chinense*, that includes innumerous morphotypes, which fruits have different characteristics of color and aroma and can be of low as well as high pungency.

The consumption of chili peppers is due mainly to their very pungent flavor. The pungency is caused by capsaicinoids and is proportional to the combined concentrations of the various vanillyl amides that are collectively referred to as capsaicinoids (Reilly, Crouch, & Yost, 2001). Among the most abundant of these components are capsaicin (trans-8 methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8 methyl-N-vanillylnonanamide), which are responsible for about 90% of the spiciness (Barbero, Liazid, Palma, & Barroso, 2008a; Laskaridou-Monnerville, 1999). Besides these two major capsaicinoids, other minor ones have been shown to occur in peppers (Barbero, Liazid, Palma, & Barroso, 2008b; Garcés-Claver et al., 2006; Jin, Pan, Xie, Zhou, & Xia, 2009; Zewdie & Bosland, 2001), including nordihydrocapsaicin, norcapsaicin, homocapsaicin I and II, homodihydrocapsaicin I and II, nornorcapsaicin, nornornorcapsaicin, and nonivamide, among others. The relative concentrations of these analogues vary with taxa and genotype (Jarret et al., 2003; Zewdie & Bosland, 2001).

The interest in these compounds extends far beyond their roles as flavor ingredients in food; they have also medical, toxicological, and forensic implications. Capsaicinoids are known for their pharmacological properties for instance as chemoprotectors against mutagenesis or tumorigenesis (Surh et al., 1995), as antimicrobials (Careaga et al., 2003; Cichewicz, 1996; Graham, Anderson, & Lang, 1999; Molina-Torres, Garcia-Chavez, & Ramirez-Chavez, 1999), as antioxidants (Hendersen & Slickman, 1999), for their analgesic effects (Kaale, Van Schepdael, Roets, & Hoogmartens, 2002), their effect on the neuronal responsiveness for pain transmission and neurogenic inflammation (Szolcsányi, 2004), and their anticancer effect that is closely related to their ability to prevent cell proliferation and migration and to induce cell apoptosis (Luo, Peng, & Li, 2011). In addition, these compounds are discussed as

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a way to manage obesity (Mueller-Seitz, Hiepler, & Petz, 2008; Reilly et al., 2001) and capsaicin is currently used for the treatment of diabetic neuropathy, osteoarthritis, post-herpetic neuralgia, and psoriasis (Davis, Markey, Busch, & Busch, 2007).

Due their properties and current application in the food industry, in the medical area as pharmaceuticals, and in defensive sprays (Daood et al., 2002), capsaicinoid compounds have been widely studied and for this purpose diverse procedures have been reported for the isolation and analysis of these secondary metabolites (Kozukue et al., 2005).

In the last decade, there has been an increasing demand for new analytical methods that are more reliable and accurate, with short operational time and reduced cost, as well as with minimized use and generations of hazardous substances. Accordingly, many studies have been published that report advances in the extraction techniques and instrumental analysis applied to the measurement of pungency (Barbero et al., 2008a; Ha et al., 2010; Thompson, Phinney, Welch, & White, 2005).

The extraction of capsaicinoids from chili peppers has been conducted using different techniques, including maceration (Kirschbaum-Titze, Hiepler, Mueller-Seitz, & Petz, 2002), magnetic stirring (Contreras-Padilla & Yahia, 1998), enzymatic extraction (Salgado-Roman et al., 2008), solid-phase microextraction (SPME) (Tapia, Garcia, Escamilla, Calva, & Rocha, 1993), accelerated solvent extraction (ASE) (Chantai, Juangsamoot, Ruangviriyachai, & Techawongstien, 2012), ultrasonic-assisted extraction (UAE) (Barbero et al., 2008b), Soxhlet (Korel, Bagdatlioglu, Balaban, & Hisil, 2002), supercritical fluid extraction (SFE) (Duarte et al., 2004; Sato et al., 1999), pressurized liquids (Barbero, Palma, & Barroso, 2006a), and microwave-assisted extraction (MAE) (Barbero, Palma, & Barroso, 2006b). Among these extraction techniques, the UAE method is particularly commended for its simplicity and low equipment cost (Boonkird, Phisalaphong, & Phisalaphong, 2008; Deng, Gao, Huang, & Liu, 2012).

Techniques used to separate capsaicinoids include thin layer chromatography (Lee, Suzuki, Kobashi, Hasegawa, & Iwai, 1976), capillary gas chromatography (Ha et al., 2010), micellar electrokinetic capillary chromatography (Laskaridou-Monnerville, 1999), supercritical fluid chromatography (SFE/SFC) (Sato et al., 1999), and especially liquid chromatography (LC), the method most frequently used for analysis of capsaicinoids because of its rapidity, reliability, accuracy and precision (Barbero et al., 2008a,b; Chantai et al., 2012; Davis et al., 2007; Garcés-Claver et al., 2006).

Methods using liquid chromatography with ultraviolet (UV) detection have been used successfully, although they have limited selectivity and a correct identification of individual compounds solely based on chromatographic behavior and UV spectrophotometric data, due to the complexity of the matrix and structural similarity between the capsaicinoids, is impracticable. The most recent methods for the determination of capsaicinoids have used LC coupled to more selective techniques such as mass spectrometry (Alothman et al., 2012; Garcés-Claver et al., 2006; Jin et al., 2009; Kozukue et al., 2005; Schweiggert, Carle, & Schieber, 2006; Thompson et al., 2005).

Nowadays, high speed and low cost of analysis are increasingly being demanded in many areas where liquid chromatography is applied in order to increase throughput and reduce costs (Barbero et al., 2008a). In this connection, the ultra high performance liquid chromatography (UHPLC) technique has been known to be economical and environmentally friendly due to extremely rapid analysis and the low consumption of solvent for mobile phase, reduced up to 5 to 10 fold, comparing with the conventional HPLC (Ha et al., 2010). Recently, the UHPLC method coupled with mass spectrometry has been adopted in many areas of food and pharmaceutical analysis.

This study reports a new method using the UHPLC technique, rapid and reproducible, completely validated and optimized since extraction step for capsaicinoid determination applied to Brazilian *C. chinense* fruits that have not yet been sufficiently investigated.

2. Material and methods

2.1. Plant material

For this study were used fruits from 9 accessions of *C. chinense* (Table 5) from a chili pepper germplasm bank of the Agronomic Institute of Campinas (IAC). The plants grown in field conditions during the 2011 summer season in IAC (Campinas, SP, Brazil, 22°54′S, 47°05′W, 674 m of elevation). The fruits were harvested during spring season at the ripening stage and preserved in the freezer at −20 °C until analysis. About 2 kg of ripe fruits was harvested from 40 plants of each accession. Of these *C. chinense* accessions, four accessions were the color orange, two the color red, and three the color yellow.

'Cumari do Pará' chili pepper (*C. chinense*) was used for the development of the UAE and UHPLC methods and fruits of 'Malagueta' chili pepper (*Capsicum frutescens*) purchased on the local market were used to show the separation of minor capsaicinoids, because in the *C. chinense* specie only capsaicin and dihydrocapsaicin were found.

2.2. Chemical and reagents

The solvents methanol, acetone and acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) utilized were of HPLC-grade. The water was obtained from a Milli-Q water bidistillation system (Millipore, Bedford, MA, USA). The reference standards of capsaicinoids, capsaicin and dihydrocapsaicin (more than 95% of purity) were obtained from Cayman Chemical Company (Arbor, MI, USA).

All solvents used as mobile phase were filtered and degassed using Millipore filters (0.22 μm pore size, filter type GV (Durapore) PVDF for water and FG (Fluoropore) PTFE for organic solvents).

2.3. Analysis of capsaicinoids

Analysis of the capsaicinoids was performed using an UHPLC–DAD–MS/MS Thermo LCQ Fleet system (Thermo Fisher, San Jose, CA, USA). The separation of capsaicinoids was achieved with a Hypersil Gold C18 column with pore size 175 Å (1.9 μm, 3 mm × 100 mm) (Part number: 0606943X9, Thermo Scientific, Waltham, MA, USA) and mobile phase consisting of water (A) and acetonitrile (B) (A:B (40:60, v/v)) in isocratic mode at 0.5 mL min^{-1} of flow rate. Capsaicinoids in the sample were indicated by the relative retention time to standards and by comparing the mass spectra between standards, library and samples. The MS was equipped with an APCI (atmospheric pressure chemical ionization) source in positive mode of ionization, working with vaporizer temperature set at 300 °C, sheath gas pressure at 50 units, auxiliary gas pressure at 5 units (arbitrary units of the equipment), a corona needle voltage of 6 kV and an ion trap detection system operating in selected monitoring mode for ions *m/z* 80–310 and the fragments for each capsaicinoid. Data handling was performed with the Xcalibur software package.

2.4. Validation of analytical procedures

To determine that the proposed method provides suitable aspects for quantitative analysis of the capsaicinoids, the following validation data are commonly investigated. The linearity of the UHPLC method was determined through external calibration curves obtained with a series of standard solution which were prepared covering a concentration range of 0.0055–66.0 μg mL^{-1} for capsaicin and 0.0044–60.0 μg mL^{-1} for dihydrocapsaicin by serial dilution of the stock standard solutions. The limit of detection (LOD) was calculated as the analyte concentration giving a signal to noise ratio (S/N) of 3 and limit of quantitation (LOQ) was determined giving a signal to noise ratio (S/N) of 6. The precision of the method was presented as the repeatability and reproducibility of retention time and peak area. The repeatability (intra-day precision) was deduced from ten replicates within a day ($n = 10$) and

reproducibility (inter-day precision) was calculated from the experiments carried out in three consecutive days ($n = 3 \times 10$). Recovery experiments were carried out with the standard addition in the sample matrix method using 'Cumari do Pará' pepper in three concentration levels for capsaicin (118.4, 236.8, and 355.2 $\mu g \cdot mL^{-1}$) and dihydrocapsaicin (42.7, 85.5, and 128.2 $\mu g \cdot mL^{-1}$).

2.5. Extraction procedure

2.5.1. Ultrasound assisted extraction (UAE) optimization

The influence of operating parameters (solvent type, solvent ratio methanol:acetone and time of ultrasonically assisted extraction) on extraction of capsaicinoids from 'Cumari do Pará' was studied, employing different extraction conditions according an experimental design (2^2, with central and axial points, totalizing 11 assays): where the variable 1 was methanol proportion in relation to acetone (0–100%); and variable 2 was the sample extraction time in ultrasound bath (0–20 min). Sample quantity (1 g) and solvent volume (25 mL) in the extraction are previously established according to the study of Barbero et al. (2008b). The UAE process was performed in a Unique UC1400 ultrasonic bath (Indaiatuba, SP, Brazil) working at a frequency of 40 kHz and at room temperature (24 °C ± 1 °C), which allowed the water in the bath to be renewed.

2.5.2. Sample extraction

The experiment was carried out in triplicate. For the sample preparation chili pepper fruits were blended in a grinder Turratec TE102 (Tecnal, Piracicaba, SP, Brazil) for 3 min at 20,000 rpm until a homogeneous sample was obtained and, immediately, submitted to the extraction step using the conditions established in the UAE optimization step.

2.6. Quantification of capsaicinoids

Quantification was based on the UV response at 280 nm and recoveries from spiked samples in the UHPLC system working in the same conditions previously described for separation of compounds. Quantification was performed on the capsaicinoids (capsaicin and dihydrocapsaicin) present in nine accessions of peppers (*C. chinense*) through calibration curves obtained from the standard solutions. Since there are no commercial standards of another capsaicinoid as nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, homocapsaicin and considering that these compounds are not representative in the samples valuated, the structural similarities between these molecules and standards were not considered for tentative quantification.

Calibration graphs were constructed by the external standard method by plotting the ratio between peak areas of analyte versus analyte concentration. The curves were prepared by injecting 10 μL in real triplicates of 0.0055, 13.2, 26.4, 39.6, 52.8 and 66 $\mu g\ mL^{-1}$ for capsaicin and 0.0044, 12, 24, 36, 48, and 60 $\mu g\ mL^{-1}$ for dihydrocapsaicin, and these solutions are prepared by dilution of capsaicin and dihydrocapsaicin stock solutions with methanol:water (70:30, v/v).

2.7. Statistical analysis

Statistical analysis and design generation were performed using the software Statistica 7.0. The models were validated by means of the Analysis of Variance (ANOVA) at the 95% confidence interval ($p < 0.05$). A lack of fit test and regression tests for each calibration curve were performed.

3. Results and discussion

3.1. Analytical conditions

The optimization for UHPLC analysis of capsaicinoids was investigated by varying the composition of mobile phase whereas the other conditions used throughout were as follows: flow rate of 0.5 mL min^{-1}, ambient temperature, and PDA detector set at 280 nm.

In this study, the organic solvent selected for the preliminary experiments was methanol due to the solubility of capsaicinoids. The mobile phases containing various percent of methanol:water were investigated and with the addition of acetonitrile a better resolution of peaks was achieved. After, the separation of capsaicinoids in shorter time was obtained by excluding the methanol of mobile phase and the application of a mobile phase of less complexity that constituted only of water and acetonitrile (40:60, v/v) in mode of isocratic elution at 0.5 mL min^{-1} was verified.

Initially, methanol was employed as sample solvent injection for the analysis of capsaicinoids using the extract obtained immediately after the extraction procedure, however it was observed that the injection solvent had a greater chromatographic strength than mobile phase (water and acetonitrile, 40:60, v/v) causing an effect of enlargement of peak base. Therefore, water was added in the injection solvent, to decrease the chromatographic strength without reducing the capsaicinoid solubility. The increasing water percentage provided a decrease in the base peak and gain on detected signal and chromatographic resolution (Fig. 1). Thus, a step for dilution of the capsaicinoid extract with water prior to the analysis was established, changing the composition of sample solvent injection for 70% of methanol and 30% of water. A partial insolubility of the capsaicinoids was observed in percentages of water greater than 30%.

The method developed is rapid and efficient with the separation of 8 capsaicinoids in a very short time of analysis (4 min). The speed of this method is evidenced for comparison with other methods in the

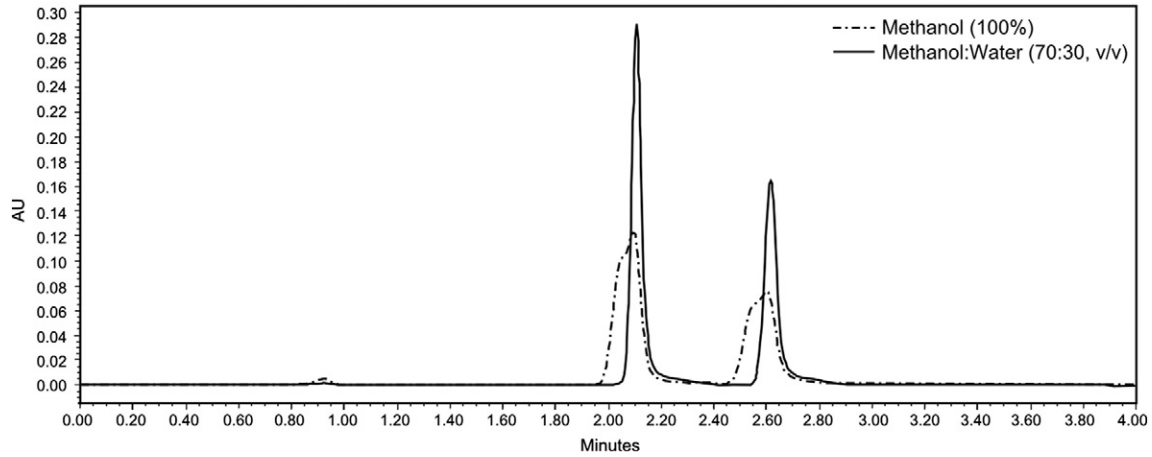

Fig. 1. Effect of sample solvent injection composition.

Table 1
Comparison among different methods of capsaicinoid analysis.

Method	Flow rate (mL min^{-1})	Run time (min)	Volume of MP for analysis (mL)	Mobile phase (MP) composition	Instrumentation devices	Compounds analyzed	References
1	0.5	4	42	(Acetonitrile:water) (60:40) isocratic	UHPLC–DAD–MS/MS Thermo LCQ Fleet system. Column Hypersil Gold C18 (100 × 3 mm, 1.9 μm) (Thermo Scientific)	Capsaicin, dihydrocapsaicin, nornorcapsaicin, nornordihydrocapsaicin, nordihydrocapsaicin, norcapsaicin, I-dihydrocapsaicin, homodihydrocapsaicin	This work
2	1	14	14	A:B (50:50) isocratic A (acetonitrile) B (water–acetonitrile 90:10)	Waters LC616 System Spherisorb ODS2 C18 column (150 mm × 4.6 mm, 5 μm)	Capsaicin, and dihydrocapsaicin	Estrada, Bernal, Pomar, and Merino (2002)
3	1	70	70	A:B (31:69) A (acetonitrile) B (water with 0.5% formic acid)	LC–MS Finnigan LCQ Advantage MAX. Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 3.5 μm) (Agilent Technologies)	Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin (I and II), homodihydrocapsaicin (I and II), nonivamide	Kozukue et al. (2005)
4	2	11	22	Gradient: 50% of A, changing to 80% of A in 7 min and return to 50% of A in 8 min, with 3 min of equilibrium. A (acetonitrile) B (water with 1% of acetic acid)	Hewlett-Packard liquid chromatograph (model 1090) Pinnacle II C-18 column (250 mm × 4.6 mm, 5 μm)	Capsaicin Dihydrocapsaicin	Davis et al. (2007)
5	0.2	6	1.2	Water:acetonitrile (45:55) with 0.1% of acetic acid, isocratic.	LC–MS/MS (Thermo, USA). Zorbax SB-C18 column (100 × 2.1 mm, 3.5 μm Agilent, USA)	Capsaicin, dihydrocapsaicin	Zhang et al. (2010)
6	0.6	7	4.2	Water with 1% of formic acid:acetonitrile (60:40)	U–HPLC (LaChromUltra Hitachi-High Technologies). C18 (2 mm × 50 mm, 2 μm) Hitachi-High Technologies column	Capsaicin and dihydrocapsaicin	Ha et al. (2010)
7	6	15	90	Gradient: 10% of B, change to 100% of B in 15 min. A (water/acetic acid 0.01%) B (methanol/acetic acid 0.01%)	The HPLC–fluorescence (Sunnyvale, CA, USA), (PDA-100), a fluorescence detector (RF 2000), C18 (100 mm × 4.6 mm Merck)	Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homonordihydrocapsaicin	Barbero et al. (2008b)
8	0.4	44	17.6	Gradient: 100% of A, changing to 100% B in 42 min. A (water, 0.01% acetic acid) B (methanol, 0.01% acetic acid)	HPLC Waters, with column C18 (Luna, 150 × 3 mm, 5 μm) Phenomenex	Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, I-dihydrocapsaicin, and homodihydrocapsaicin	Barbero et al. (2006b)
9	0.4	30	12	Gradient: 20% of B changing to 100% of B in 24 min. A (water/acetonitrile 90:10 with 0.5% acetic acid) B (Acetonitrile/Water 90:10, with 0.5% acetic acid)	Agilent HPLC (Agilent, Germany) column C18 (150 mm × 3.0 mm, 4 μm) Phenomenex LC–MS (Bruker-Bremen, Germany)	Nornorcapsaicin, nornordihydrocapsaicin, N-vanillyl-octamide, norcapsaicin, nordihydrocapsaicin, capsaicin, N-vanillyl-nonamide, dihydrocapsaicin, N-vanillyl-decamide	Schweiggert et al. (2006)
10	1	30	30	Gradient: 100% of A changing to 100% of B in 30 min A (Water/acetonitrile 90:10) B (Acetonitrile/water 90:10)	Knauer Chromatograph, detector UV. Column Eurospher 80 (C18) (Dimensions not specified)	Capsaicin, and dihydrocapsaicin	Perucka & Oleszek (2000)
11	1	70	70	Acetonitrile/water (40:60) with acetic acid (pH 3.0)	HPLC Shimadzu series VP, PDA (UV–vis). Column C18 250 × 4.6 mm	Capsaicin, dihydrocapsaicin, and nordihydrocapsaicin	Poyrazoğlu, Yemis, Kadakal, and Artik (2005)
12	0.8	30	24	Water/acetonitrile/tetrahydrofuran/acetic acid (55:40:5:1)	HPLC, pump M-510, auto sampler WISP-712, fluorescence detector (Waters) Column YMC-Pack ODS-A (150 × 4.6 mm, 3 μm)	Capsaicin, and zucapsaicin	Lu and Cwik (1997)
13	1.5	8	12	Acetonitrile/water (50:50) isocratic	Thermo HPLC system, photodiode array (PDA) detector, column Betasil C18 (150 × 4.6 mm × 3 μm)	Capsaicin, dihydrocapsaicin, and nordihydrocapsaicin	Al Othman et al. (2011)
14	0.5	8	4	Acetonitrile (A)/water 0.1% formic acid (B). Gradient started with 40% A changing to 50% of A in 8 min	UPLC–MS Acquity Waters Column BEH C18 (100 × 2.1 mm; 1.7 μm)	Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin	Alothman et al. (2012)
15	0.2	4	0.8	Methanol/water/acetic acid (90:9:1) isocratic	HPLC (Waters GmbH, Eschborn, Germany) 3C8 column (125 × 2 mm, 3 μm)	Capsaicin and dihydrocapsaicin	Wolf et al. (1999)

Table 2
Calibration curves of capsaicin (CAP) and dihydrocapsaicin (DHC).

		CAP	DHC
Equation		y = 13,257x − 2337.3	y = 16,255x − 3741
R^2		0.999	0.998
ANOVA (p ≤ 0,05)	Regression	$F_{(1,4)}$ = 15,800.22 Significant	$F_{(1,4)}$ = 11,474.08 Significant
	Lack of fit	$F_{(4,12)}$ = 0.66 Not significant	$F_{(4,12)}$ = 0.62 Not significant
Linearity (μg mL^{-1})		0.0055–66	0.0044–60

literature in relation to time analysis and number of compounds separated (Table 1). The method proposed in this work also follows the trend of a decrease in the solvent consumption, expending 2 mL for each analysis and resulting in little generation of residue.

According to Table 1, other works showed a similar short time of analysis (Ha et al., 2010; Wolf, Huschka, Raith, Wohlrab, & Neubert, 1999; Zhang, Hu, Sheng, & Li, 2010), but shown separation only for capsaicin and dihydrocapsaicin, minor capsaicinoids that cannot be separated in chromatographic stage, were detected with a mass spectrometer in the case of the study of Zhang et al. (2010). On the other hand, the methods able to separate also the minor compounds were performed in a larger time of analysis using the HPLC technique (Al Othman, Ahmed, Habila, & Ghafar, 2011; Barbero et al., 2006b; Schweiggert et al., 2006) and using the UHPLC technique, Alothman et al. (2012) achieved the separation of five compounds in 8 min of run time.

3.2. Validation of the analytical method

The method developed has been validated for capsaicin and dihydrocapsaicin with respect to limit of detection (LOD), limit of quantification (LOQ), linearity ranges, repeatability, reproducibility, and accuracy through analyte recoveries.

LOD and LOQ were estimated by signal to noise ratios of 3 and 6, respectively. In this condition LOD was 0.0027 μg mL^{-1} for capsaicin and 0.0022 μg mL^{-1} for dihydrocapsaicin. LOQ was 0.0055 μg mL^{-1} and 0.0044 μg mL^{-1} for capsaicin and dihydrocapsaicin, respectively. The linearity was determined for LOQ of 66 and 60 μg mL^{-1} for capsaicin and dihydrocapsaicin, respectively. The analytical curves were constructed with six different concentrations of standard solution for capsaicin and dihydrocapsaicin, each solution was prepared and injected in triplicate and the results were submitted to ANOVA (Table 2).

The precision was evaluated by RSD (Relative Standard Deviation) of peak area and retention time, using solution standard in three levels of concentration. Repeatability (Intra-day precision) was determined by 10 injections on the same day, and reproducibility (Inter-day precision) by the 10 injections in each day for three consecutive days. The low RSD of peak area (less than 6.11%) and retention time (less than 0.32%) showed a good repeatability and reproducibility. The accuracy was carried out by recovery of capsaicin and dihydrocapsaicin with the standard addition method on the sample 'Cumari do Pará' in three concentration levels. Recoveries ranged from 88 to 112% for capsaicin and 89 to 109% for dihydrocapsaicin, indicating satisfactory accuracy of the method (Table 3).

3.3. Extraction optimization

The experimental results obtained by this initial model demonstrated how the variable concentration of methanol:acetone and time extraction in ultrasound bath influenced the extraction efficiency. Other parameters considered in the extraction were kept constant, namely the amount of sample (1.0 g) and the solvent volume (25 mL) according Barbero et al. (2008b).

The extraction yields were found in the ranges of 1.55 to 2.88 mg g^{-1} for capsaicin in fresh weight of 'Cumari do Pará', with RSD less than 0.5% in the central points. Capsaicin and dihydrocapsaicin showed a similar behavior in the extraction assays in accordance to Chantai et al. (2012), thereby different conditions do not give priority to either compound. The experimental design parameters and the elemental response are shown in Table 4.

The quadratic model was shown to be more appropriate to represent the responses of optimization of extraction (Eq. (1)).

$$\hat{y} = b_0 + b_m x_m + b_t x_t + b_{mm} x^2{}_m + b_{tt} x^2{}_t + b_{mt} x_m x_t \quad (1)$$

where, $\hat{y}$ is the predicted response, b is the coefficient of model and x is the variable values. The indexes m and t correspond to values of methanol and time, respectively. The last term of Eq. (1) was not considered in the predictions of this model since the effect of the interaction between the two variables was not significant. Analysis of variance (ANOVA) was performed on the experimental design to assess the significance of the model. The generated model showed a lack of fit with a value of $F_{(3,2,95\%)}$ calculated of 3359.78, greater than $F_{(3,2,95\%)}$ tabulated, which was 19.16. As a model cannot be used to predict the optimum point of extraction, the optimum point of extraction was defined by the analysis of real results and practical considerations as in the results reported by Meinhart et al. (2010).

According to the assays performed in the extraction optimization step, an increase in the amount of capsaicin extracted in assays 2 and 6 (Table 4) was observed, the condition of assay 6 using 100% methanol and 10 min of ultrasound extraction was selected to carried out the experiments based on the fact that methanol used as solvent extractor for capsaicinoids from chili pepper fruits showed an important advantage

Table 3
Precision and accuracy of method developed for capsaicinoid determination.

Parameter of validation		Capsaicin levels (μg mL^{-1})	Dihydrocapsaicin levels (μg mL^{-1})	Capsaicin (%)[a]	Dihydrocapsaicin (%)[a]
Repeatability (Intra-day precision)	Area	0.0055	0.0044	3.58	5.86
(RSD %)		33	30	0.40	0.27
n = 10		66	60	0.40	0.21
	Retention time	0.0055	0.0044	0.16	0.12
		33	30	0.025	0.02
		66	60	0.024	0.01
Reproducibility	Area	0.0055	0.0044	5.28	6.11
(Inter-day precision)		33	30	0.9	0.97
(RSD %)		66	60	0.8	0.73
n = 30	Retention time	0.0055	0.0044	0.30	0.32
		33	30	0.12	0.14
		66	60	0.09	0.13
Accuracy	Quantity on sample (μg)	940.59	155.43		
n = 3 (%, mean value ± sd)	Quantity added (μg)	118.4	42.7	103.27 ± 7.72	100.3 ± 8.42
		236.8	85.5	100.86 ± 6.48	96.59 ± 9.84
		355.2	128.2	92.66 ± 3.57	95.79 ± 7.95

[a] Values of % RSD to repeatability and reproducibility parameters and % recovery to accuracy, expressed through mean values ± standard deviation (sd) to triplicate analysis.

Table 4
Experimental design and capsaicin amount extracted.

Assay	Extraction conditions		Capsaicin concentration[a]
	% of methanol in acetone (v/v)	Ultrasound time (minutes)	
1	14.5 (−1)	2.9 (−1)	1.87
2	14.5 (−1)	17.1 (+1)	2.88
3	85.5 (+1)	2.9 (−1)	1.78
4	85.5 (+1)	17.1 (+1)	2.72
5	0 (−1.41)	10 (0)	1.55
6	100 (+1.41)	10 (0)	2.88
7	50 (0)	0 (−1.41)	2.04
8	50 (0)	20 (+1.41)	2.07
9	50 (0)	10 (0)	2.21
10	50 (0)	10 (0)	2.22
11	50 (0)	10 (0)	2.23

[a] mg g^{-1} of fresh 'Cumari do Pará' pepper.

because it reduces the amounts of pigments and oils extracted simultaneously with the capsaicinoids according to Attuquayefio and Buckle (1987) and is compatible with the UAE process and subsequently chromatographic analysis. In relation to extraction time, these experiments indicated that an intermediary value (10 min) of the range studied is the most suitable. Besides making working with a shorter time of sample preparation possible, this fact can be supported by authors that suggest the possibility of degradation occurrence in function of a long time of conventional liquid–solid extraction (Ya-Qin, Jian-Chu, Dong-Hong, & Xing-Qian, 2009).

Thus, the suitable condition for extraction of capsaicinoids by indirect sonication in an ultrasonic bath was at a ratio of 1 g of sample material:25 mL of methanol, with 10 min of extraction time. These conditions were very similar to those established by Barbero et al. (2008b), which in his study of extraction optimization, also found methanol as the most efficient extractor solvent with an optimal range time of ultrasound between 10 and 20 min.

With regard to extraction solvent for capsaicinoids, the mostly used organic solvents that are reported as the most efficient in the capsaicinoid extraction are methanol and acetone (Attuquayefio & Buckle, 1987; Barbero et al., 2008b; Boonkird et al., 2008; Deng et al., 2012), but these results were found in studies where these two organic solvents are not confronted or had their performance evaluated in the form of mixture. This is the first time that these solvents were assessed in the same study. The effectiveness of UAE depends on the capacity of the extraction solvent for absorbing and transmitting the energy of ultrasounds (Barbero et al., 2008b). Thus, selecting an appropriate solvent for analyte extraction from matrix of samples is an important step in the development of the UAE method (Deng et al., 2012). Finally, 100% of methanol and 10 min in ultrasound bath were chosen as the extraction conditions for the following experiments.

The enhancement of extraction efficiency of organic compounds by ultrasound is attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave. Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid and liquid phases (Barbero et al., 2008b; Deng et al., 2012).

3.4. Identification of capsaicinoids

The identification of capsaicinoids was carried out in 'Malagueta' pepper (*C. frutescens*) sample. Capsaicin and dihydrocapsaicin were identified by the comparison of retention time, UV–vis and mass spectrum obtained with commercial standards. The minor capsaicinoids were identified according to their chromatographic behavior and by comparison of mass spectrum with library data. Fig. 2 shows the chromatogram for separation of capsaicinoids present in the extract from "Malagueta" pepper.

The protonated molecule $[M + H]^+$ for the capsaicinoids found presented the following *m/z* ratios: nornorcapsaicin, 278; nornordihydrocapsaicin, 280; nordihydrocapsaicin, 294; capsaicin, 306; norcapsaicin, 292; dihydrocapsaicin, 308; isomer of dihydrocapsaicin, 308; and homodihydrocapsaicin, 322. In the mass spectra of these eight capsaicinoids, the *m/z* peak (137) characteristic of the fragmentations of capsaicinoids appears clearly. The $[M + H]^+$ and fragments identified were compatible to standards of capsaicin and dihydrocapsaicin and literature data (Kozukue et al., 2005; Barbero et al., 2006a; Schweiggert et al., 2006).

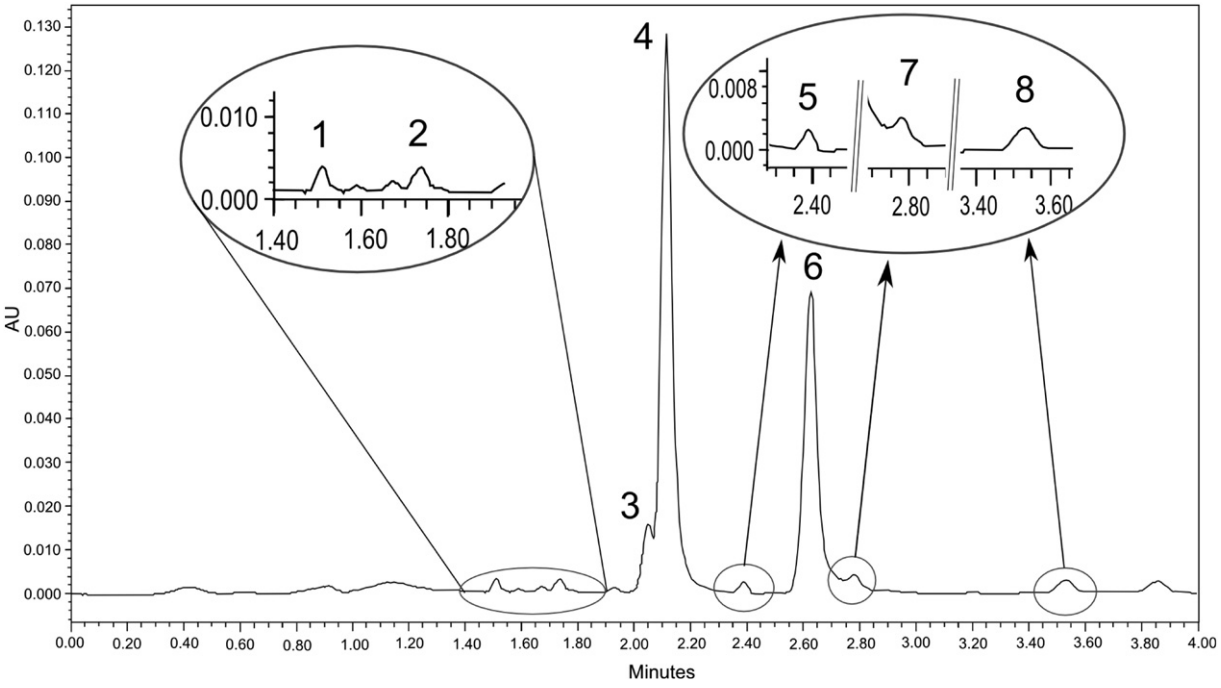

Fig. 2. Separation profile of capsaicinoids from 'Malagueta' chili pepper (*C. frutescens*). 1 — nornorcapsaicin, 2 — nornordihydrocapsaicin, 3 — nordihydrocapsaicin, 4 — capsaicin, 5 — norcapsaicin, 6 — dihydrocapsaicin, 7 — i-dihydrocapsaicin, 8 — homocapsaicin.

Table 5
Amounts of capsaicin and dihydrocapsaicin in the *C. chinense* chili peppers.

Sample	Capsaicin[a] (μg g−1 fresh sample)	Dihydrocapsaicin[a] (μg g−1 fresh sample)
IAC 1573 'Cumari-do-Pará'	798 ± 56	143 ± 9
IAC 1552 'Murupi amarela'	1270 ± 80	206 ± 9
IAC 1638 'De cheiro'	656 ± 13	153 ± 12
IAC1642 'Habanero'	1442 ± 3	478 ± 5
IAC 1644 'Fidalga'	156 ± 24	26 ± 9
IAC 1647 'De cheiro'	182 ± 41	36 ± 2
IAC 1648 'De cheiro'	267 ± 59	53 ± 6
IAC 1641 'Murupi vermelha'	303 ± 40	82 ± 10
IAC 1643 'Biquinho'	nd	nd

[a] Mean values ± standard deviation to triplicate analysis. nd = not detected.

3.5. Quantification of capsaicinoids in C. chinense fruits

The capsaicin and dihydrocapsaicin levels were determined in 9 different accessions of *C. chinense*. The levels of capsaicin and dihydrocapsaicin were between 156 and 1442 μg g^{-1} and 26 and 478 μg g^{-1} of fresh pepper, respectively (Table 5).

The chili pepper capsaicinoid contents showed varied pungency levels in the peppers used for this study that evidences the large application of the method developed. The contents of capsaicin and dihydrocapsaicin found in this work for the different chili pepper accessions are in good agreement with those found by other authors. According to our results, 'Habanero' chili pepper, one of the most pungent varieties (Garcés-Claver et al., 2006), had the highest capsaicinoid content.

4. Conclusions

The method described for capsaicinoid extraction, chromatographic separation is fast, efficient and reliable. In addition, the method shows large application for the quantification of capsaicin and dihydrocapsaicin in different concentration levels. Total sample preparation takes about 15 min, with reduced requirements for sample, solvents and instrumentation, also resulting in reduced chromatographic interferences. UAE, by means of the method developed, allows the quantitative and reproducible extraction of the capsaicinoids present in chili peppers, employing methanol as solvent extractor.

Due to its simplicity and its analytical capabilities, the method developed can be applied for the fast routine analysis of capsaicinoids in chili peppers and 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.

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