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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