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Evolution of total and individual capsaicinoids in peppers during ripening of the Cayenne pepper plant (*Capsicum annuum* L.)

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ABSTRACT

The evolution of total capsaicinoids and the individual contents of the five major capsaicinoids: nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin present in the Cayenne pepper (*Capsicum annuum* L.), during fruit ripening, has been established. Capsaicinoids begin to accumulate gradually in the peppers from the beginning of its development up to a maximum concentration (1789 μmol/Kg FW). From this time there is initially a sharp decrease in the total capsaicinoid content (32%), followed by a gradual decrease until day 80 of ripening. The two major capsaicinoids present in the Cayenne pepper are capsaicin and dihydrocapsaicin, which represent between 79% and 90%, respectively, of total capsaicinoids depending on fruit ripening. The relative content of capsaicin differs from the evolution of the other four capsaicinoids studied.

1. Introduction

Hot or spicy peppers are savoury food additives that are widely utilized in many parts of the world and they are highly valued for their attributes of colour, pungency and flavour. Capsaicinoids are the compounds responsible for the spicy flavour of peppers. Among these compounds there are two major capsaicinoids, capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide), which represent about 77–98% of capsaicinoids present in peppers (Govindarajan, Rajalakshmi, & Chand, 1987). Besides these two major capsaicinoids, other minor capsaicinoids have been found in chilli peppers and these include nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, nonivamide among more than twenty reported compounds (Constant, Cordell, & West, 1996; Giuffrida et al., 2013; Huang, Xue, Jiang, & Zhu, 2013).

Capsaicinoids are responsible for the spiciness of peppers and are widely used in food in most parts of the world due to their pungent properties. These compounds also have many other properties and biological effects (Kaale, Van Schepdael, Roets, & Hoogmartens, 2002). This fact has led to the extensive study of these compounds because of the large number of benefits associated with them. These include their properties as chemopreventive and anticarcinogenic compounds (Chanda et al., 2004; Surh & Lee, 1995), their antioxidant properties (Alvarez-Parrilla, de la Rosa, Amarowicz, & Shahidi, 2011), the regulation of the energetic metabolism of fats (Bloomer, Canale, & Fisher-Wellman, 2009), anti-inflammatory properties (Spiller et al., 2008), protection of the gastric mucosa (Abdel Salam, Szolcsanyi, & Mozsik, 1997) and antimicrobial properties (Careaga et al., 2003), among others.

Capsaicinoids are synthesized and accumulated in the placenta of peppers, as confirmed by tracer experiments (Iwai, Suzuki, & Fujiwake, 1979). It was observed that the radioactivity of capsaicinoids was much higher in the placenta than in the pericarp of the pepper, at all stages of maturation. Later, the cellular structure of the placenta was examined using a light microscope (Fujiwake, Suzuki, & Iwai, 1980) and it was noted that morphological changes occurred in the epidermal tissue of the placenta during the maturation. It was concluded that the epidermal cell of the placenta seems to be the accumulation site of capsaicinoids in peppers. In addition to the placenta, capsaicinoids have been found in other parts of the fruit, such as the pericarp, albeit always in smaller quantities, and even in vegetative organs of the plant such as the leaves and stem (Estrada, Bernal, Diaz, Pomar, & Merino, 2002). In an effort to ascertain whether the capsaicinoids present in the vegetative organs came from the fruit, the floral buds were removed and fruit formation was prevented. In this case, capsaicinoids were not found in either the stem or leaves, suggesting that they originated in the fruit.

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Several studies have been carried out on the accumulation of capsaicinoids in Capsicum fruits in relation to fruit age, size and stage of development (Estrada, Bernal, Diaz, Pomar, & Merino, 2000; Mueller-Seitz, Hiepler, & Petz, 2008). All results were similar and showed that the capsaicinoids begin to accumulate in the early stages of fruit development, continuing their accumulation during ripening until reaching a maximum. At this moment there is a rapid turnaround in the trend, with a capsaicinoid degradation greater than 60%. This fact was demonstrated by *in vitro* assays in which the pepper peroxidase was able to oxidize both capsaicin and dihydrocapsaicin (Bernal et al., 1993a, 1993b). The oxidation of capsaicinoids by *Capsicum* peroxidase is strictly dependent on the presence of H_2O_2. Subsequent studies related the variation of capsaicinoid content depending on the activity of peroxidases, showing that an increased activity of peroxidase means a decrease in the capsaicinoid content of peppers (Estrada et al., 2002).

It has been reported that the production of capsaicinoids is influenced both by genetics and by environmental aspects (Garces-Claver, Gil-Ortega, Alvarez-Fernandez, & Arnedo-Andres, 2007; Zewdie & Bosland, 2000a). It has also been shown that pepper crops with a water deficit produce an increase in the capsaicinoid content of the fruit (Ruiz-Lau et al., 2011; Sung, Chang, & Ting, 2005). On the other hand, the addition of mineral supplements to the pepper cultivation causes an increase in the capsaicinoid content (Estrada, Pomar, Diaz, Merino, & Bernal, 1998) and that nitrogen supply is essential for their synthesis (Monforte-Gonzalez, Guzman-Antonio, Uuh-Chim, & Vazquez-Flota, 2010). Infections of pepper plants also cause an increase in the capsaicinoids content (Tahboub, Sanogo, Bosland, & Murray, 2008). Furthermore, it is known that the capsaicinoid content of peppers can vary between different fruits within the same plant, even when harvested at the same time after flowering (Kirschbaum-Titze, Mueller-Seitz, & Petz, 2002) and in peppers that have different node positions (Zewdie & Bosland, 2000b).

Methods used for the determination of capsaicinoids in pepper samples, as well as in other types of matrices, have been very varied and have included techniques such as thin layer chromatography (Suzuki, Kawada, & Iwai, 1980), gas chromatography (Mullerst et al., 1971) and high performance liquid chromatography (Garces-Claver, Arnedo-Andres, Abadia, Gil-Ortega, & Alvarez-Fernandez, 2006; Schweiggert, Carle, & Schieber, 2006). By far the most common technique used for the determination and quantification of this type of compound has been reversed phase HPLC, and a large number of separation methods have been used with different equipment, columns, separation solvents and gradients (Kirschbaum-Titze et al., 2002).

The aim of the work described here was to determine the evolution of both the total capsaicinoid content and the individual contents of the five major capsaicinoids (capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin) present in Cayenne pepper (*Capsicum annuum* L.) during fruit ripening.

2. Materials and methods

2.1. Chemicals

The reference standards of capsaicinoids, i.e. capsaicin (97%) and dihydrocapsaicin (90%), were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). The water was obtained from a Milli-Q water deionization system (Millipore, Bedford, MA, USA). The methanol used both for the extraction of capsaicinoids and for the chromatographic separation and the glacial acetic acid were HPLC grade and were purchased from Merck (Darmstadt, Germany). The internal standard 2,5-dihydroxybenzaldehyde was obtained from Sigma–Aldrich.

2.2. Pepper crops

The cultivation of this variety of pepper was carried out in a greenhouse. The seeds of this variety were planted in the seedbeds in September and the pepper plants were transplanted to the greenhouse in November. The plants were watered by a drip system, with water enriched in nutrients.

The greenhouse temperature was controlled. During the winter months, at temperatures below 10 °C, a heating system was activated to ensure that the temperature did not fall below this level. During hot weather (spring–summer), the greenhouse walls were raised to allow air exchange and to prevent excessive heating of the greenhouse (to maintain temperatures below 35 °C).

2.3. Fertilization of the plants

Before planting the peppers in the greenhouse a deep fertilization of the ground was carried out. The part of the greenhouse where the Cayenne peppers were cultivated had an area of 17×70 m. The initial fertilization of the greenhouse was carried out with the following quantities of fertilizers: 20 kg of magnesium sulfate, 15 kg of superphosphate of lime, 20 kg of potassium sulfate, 20 kg of ammonium sulfate.

Once the pepper plants had been planted, a fertigation regime was applied (Table 1). This fertilization depended on the stage of plant development at each time.

2.4. Monitoring of the ripening and harvesting of peppers

The evolution of total and individual contents of the five major capsaicinoids present in Cayenne peppers was studied. Peppers were marked at the end of flowering and hence the age of each pepper at the time of collection was known.

Plants began to flower in mid-February. From this date, the new peppers that grew were marked with a temporal spacing of 10 days. The collection of peppers was carried out in the first week of June (plant $\approx$ 8 months old). From this date, the plant stopped producing peppers and sampling was therefore discontinued.

2.5. Plant material

The Cayenne pepper variety (*Capsicum annuum* L.) was used in this study. Samples were taken from 296 pepper plants cultivated in a greenhouse. Samples for different ages were obtained from all of them. Total amount of peppers ranged from a minimum of 232–346 g for different ages, to avoid particular effects from individual pepper fruits previously reported in the literature (Kirschbaum-Titze et al., 2002). For the analysis, the stem and seeds of the peppers were discarded. Pericarp and placenta were subsequently ground together in a conventional mill to obtain a completely homogeneous sample. Aliquots of this sample were used for subsequent analyses. Once the peppers had been milled, they were frozen at −32 °C until analysis.

2.6. Extraction procedure

The extracts from the pepper samples were obtained using an ultrasound-assisted extraction technique, according to our previously developed method (Barbero, Liazid, Palma, & Barroso, 2008a, 2008b).

The extraction by ultrasound was performed in an ultrasonic bath (360 W, J.P. Selecta, Barcelona, Spain) coupled to a temperature controller, which allowed the water in the bath to be renewed.

For the extraction of the capsaicinoids present in peppers, the following extraction parameters were used: extraction solvent: methanol; temperature: 50 °C; power: 360 W; solvent volume: