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Progression of the Total and Individual Capsaicinoids Content in the Fruits of Three Different Cultivars of *Capsicum chinense* Jacq.

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Abstract: The evolution of individual and total capsaicinoids content in three pepper varieties of *Capsicum chinense* Jacq. ('Bode' (B), 'Habanero' (H), and 'Habanero Roxo' (Hr)) during fruit ripening was studied. The five major capsaicinoids (nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin) were extracted using ultrasound-assisted extraction and the extracts were analysed by ultra-performance liquid chromatography with fluorescence detection (UHPLC-Fl). The plants were grown in a glasshouse and sampled every 7 days until over-ripening. As expected, the results indicated that the total capsaicinoids content increases during the ripening of pepper fruits. The maximum contents of capsaicinoids were reached at different fruit development stages depending on the cultivar. The 'Habanero Roxo' pepper presented the greatest total capsaicinoids content (3.86 mg g^{-1} fresh weigh, F.W.), followed by the 'Habanero' pepper (1.33 mg g^{-1} F.W.) and 'Bode' pepper (1.00 mg g^{-1} F.W.). In all the samples, capsaicin represented more than 80% of the total capsaicinoids content. Due to the high variability observed in the evolution of capsaicinoids content over the ripening process, this work intends to contribute to the existing knowledge on this aspect in relation to the quality of peppers.

Keywords: 'Bode' pepper; 'Habanero' pepper; 'Habanero Roxo' pepper; *Capsicum chinense*; pepper ripening; capsaicinoids

1. Introduction

Capsicum ssp. fruits are of great importance in the American continent and in other tropical regions, mainly because of its flavour and taste and for its traditional use in native food. Moreover, peppers have been recognized to contain phytochemicals that are important components in a healthy diet for their capacity to reduce, or to contribute to reducing, the risk of suffering from some degenerative diseases [1,2]. Numerous studies have evaluated some of the properties of pepper phytochemicals as an antioxidant [1,3,4], anti-inflammatory [5,6], anticarcinogenic [7,8], analgesic [9], promoter of energy metabolism, and suppressor of fat accumulation [10] and pro-vitamin A [11]. The most important group of phytochemicals in peppers are capsaicinoids, a nonvolatile alkaloid, which are chemically acidic amides of C9–C11 branched-chain fatty acids and vanillylamine [12]. Capsaicin and dihydrocapsaicin are the main capsaicinoids in peppers and generally represent more than 90% of the total capsaicinoids content [13]. Capsaicinoids content in peppers is one of the principal parameters to determine its commercial quality. The capsaicinoids content in fresh fruit per weight varies substantially depending on its maturity stage and tends to increase as the fruit grows and develops [14]. Capsaicinoids are naturally synthesized in the placenta of peppers by enzymatic condensation of vanillylamine and different fatty acid chains that are elongated by a fatty acid synthase [15–17]. Throughout the maturation, numerous changes occur—biochemical, physiological, and structural—such as the degradation and synthesis of fruit metabolites. For this reason, the characterization of pepper components at different stages of fruit development is essential, since it has an impact on the aroma, taste, and quality of the peppers. The concentrations of capsaicinoids in the hottest spicy varieties, as many *Capsicum chinense* Jacq. varieties, are characterized by a content of capsaicinoids that is more than 0.3% of the total dry weight, with some varieties reaching more than 1% [13,18].

Brazil is considered a centre of diversity for some species of *Capsicum* (domesticated and wild). Of all the species cultivated in Brazil, five of them are also cultivated all over the world: *Capsicum annuum* L., *Capsicum frutescens* L., *Capsicum baccatum* L., *Capsicum pubescens* Ruiz & Pav., and *Capsicum chinense* Jacq. [19]. There are numerous varieties of *Capsicum chinense* Jacq., with all of them appreciated for their flavour, taste, and aroma [20]. The most popular varieties of *Capsicum chinense* pepper from Brazil are 'pimenta-de-bico' (biquinho), 'pimenta-de-cheiro', 'murupi', 'pimenta-de-bode', 'cumari-do-Pará', and 'Habanero' [21]. It is noteworthy that some of the varieties of *Capsicum chinense* are essential ingredients in several traditional recipes from the city of Bahia in Brazil and the consumption of this kind of spice represents a relevant characteristic of the local culture [15]. Of all the pepper varieties of the genus, *Capsicum chinense*, cultivated in Brazil, 'Bode', 'Habanero', and 'Habanero Roxo' are the most often consumed [22,23]. In addition, 'Habanero' pepper also has enormous social and commercial relevance in other American countries, such as Mexico [20,23]. At present, there is a large demand in America and Europe for new pepper varieties that exhibit new attributes, such as colour, aroma, flavour, or shape [23]. Moreover, the current high value of these products in international markets is the result of an offer that has been historically lower than its demand, which has aroused the interest of national private groups to develop processed products in this segment for their peculiar taste [23]. Dried pepper is a conventional way of marketing this product; however, during hot-air drying, vegetables undergo physical, structural, chemical, and nutritional changes that can affect quality attributes, like the texture, colour, flavour, and nutritional value [24–27]. Our previous work [27] showed the effect of the temperature of the drying process on the capsaicinoids content in 'Habanero' pepper. It was observed that capsaicinoids remain constant between 30 and 70 °C during drying. Furthermore, the pungency is an important parameter to establish the quality of fresh hot pepper, and it is equally important for processed products [21,28]. For this reason, it is necessary to establish which factors have an impact on their capsaicinoids concentration level, such as the cultivating conditions [29–32], mineral nutrition [29], watering conditions [31,33], and fruit maturation stage among others [16,34–39]. Several studies were carried out to elucidate the synthesis and accumulation of capsaicinoids in relation to the peppers' ripening stage. These studies found ot that the maximum capsaicinoids content is reached generally between 40 and 60 days post-anthesis (dpa) [37]. On the contrary, there are some works where a constant accumulation of capsaicinoids is observed throughout the maturation [39,40]. It was also observed that capsaicinoids may be degraded in the presence of pepper peroxidases under certain conditions [35,37,41,42]. Some works in the literature deal with the maturation and evolution of biological compounds and capsaicinoids in peppers. As an example, Menichini et al. [28] reported an increase of the antioxidant activity in 'Habanero' peppers when the peppers reached the mature stage. They also concluded that the 'Habanero' pepper has a considerable content of biochemical components, like vitamin C, that reduces the risk of degenerative diseases. Pino et al. [29] evaluated the phytochemical contents in 'Habanero' at two different fruit development stages, immature (green colour) and mature (orange colour). They reported an increase in capsaicinoids content (≈1.2 times) when the ripening stage was reached. However, the volatile compounds decreased with fruit maturation. Nevertheless, since only two maturation stages were considered (immature and mature), additional analyses that comprise the entire fruit maturation stages are needed.

Therefore, the present work intends to evaluate, by means of reverse phase Ultra High Performance Liquid Chromatography with Fluorescence detection (rp-UHPLC-Fl), the accumulation of individual (nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C), and homodihydrocapsaicin h-DHC)) and total capsaicinoids during the different maturation stages of three *Capsicum chinense* Jacq. cultivars ('Bode' pepper (B), 'Habanero' pepper (H), and 'Habanero Roxo' pepper) that are highly valued in Brazil and are currently consumed in many other parts of the world. The results should cast some light on the build-up of capsaicinoids throughout pepper maturation, and particularly in the *Capsicum chinense* cultivars that this work focuses on. This information may be useful to assist breeders in the selection of the pepper fruits on the optimum harvesting time for maximum pungency in breeding programs.

2. Materials and Methods

2.1. Reagents

Both the methanol grade HPLC used for the extraction of capsaicinoids and for the chromatographic separation and the glacial acetic acid grade HPLC were purchased from Merck (Darmstadt, Germany). Water was provided by a Milli-Q water deionization system (Millipore, Burlington, MA, USA). Capsaicin (97%) and dihydrocapsaicin (90%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Pepper Crops

The study was conducted during the spring–summer season (April–September 2016) in an automated glass greenhouse in the "Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA)" in Montañana, Zaragoza, Spain (41°43′29.2″ N, 0°48′30.6″ W) under controlled conditions. The temperature during the study period was 24/12 °C during the spring months and 28/20 °C during the summer months. Seeds of the three varieties were germinated in Petri dishes. When the cotyledons had developed, each plant was transferred to a Jiffy-7 pot (Clause Tezier Iberica, Almeria, Spain). Once the plants had three true leaves (six weeks after sowing), each Jiffy pot was planted into a black plastic pot (one plant per pot; top diameter, 23 cm; bottom diameter, 17 cm; height, 18 cm). The plants were irrigated daily for optimum growth. The plants were irrigated with a hose coupled to a diffuser, according to their needs. The evaluation of the water needs was carried out by checking the humidity of the substrate daily, by manual methods.

2.3. Plant Material

In this study, the five major capsaicinoids were analysed in three Brazilian landraces, 'Bode' (B), 'Habanero' (H), and 'Habanero Roxo' (Hr), from *Capsicum chinense* Jacq. The seeds of the three cultivars were supplied by the Agronomic Institute of Campinas—IAC. Each sample of these three varieties was obtained from 20 pepper plants, all of them cultivated in the same greenhouse and under controlled conditions. The total amounts of peppers collected were in the range of 232–346 g for different ages to avoid any particular effects from individual pepper fruits, as reported previously in the literature [43]. The stem and seeds of the peppers were discarded for the analysis. Only pericarp and placenta were subsequently ground together in a conventional mill to obtain a completely homogeneous sample. Once the peppers had been milled, they were frozen at −32 °C until analysis.

2.4. Fertilization

The three cultivars of peppers were grown in a random distribution inside a controlled atmosphere greenhouse with a substrate mixture made of peat, sand, and clay-loam soil and Humin Substrat (Klasman-Deilmann, Geeste, Germany) (1:1:1:1, v/v). In addition, two grams of Osmocote 16N-4P-9K slow-release fertilizer (The Scotts Miracle-GroCo., Godalming, UK) was top-dressed on each pot at the beginning of growth.

2.5. Monitoring of the Fruit Ripening and Pepper Harvesting

The evolution of individual and total contents of the five major capsaicinoids present in *Capsicum chinense* cultivars, 'Bode', 'Habanero', and 'Habanero Roxo' peppers, was studied at different fruit stages of development [44]. The flowering period starts from mid-July until the end of September. The flowers were labelled, at the time of their anthesis, with a label with the date to determine the fruit stage of development. On Tuesdays and Thursdays, the flowers were labelled, while on Mondays and Wednesdays, all open flowers were removed. This ensured that all flowers were labelled in the same stage of development. Peppers were harvested during the last week in September (plant ≈ 6 months old), and grouped in different stages of fruit ripening at 13, 20, 27, 34, 41, 48, 55, 62, 69, 76, and 83 days post-anthesis (dpa). Sampling was interrupted at that moment since the plants stopped producing new peppers.

2.6. Ultrasound Assisted Extraction of Capsaicinoids

The pepper sample extracts at the different maturation stages were obtained by means of our previously developed technique [45] with an extraction yield >96%. An ultrasonic probe UP200S sonifier (200 W, 24 kHz) (Hielscher Ultrasonics, Teltow, Germany) with the sample immersed in a water bath coupled to a temperature controller (Frigiterm-10, J.P. Selecta, S.A., Barcelona Spain) was used. 0.25 g of *Capsicum chinense* sample was put in contact with 25 mL of methanol for 10 minutes, and was then transferred to the water bath at 50 °C, 200 W output amplitude, and 0.5 s duty cycle. The extracts were filtered through a 0.22 µm nylon syringe filter (Membrane Solution, Dallas, TX, USA) prior to their chromatographic analysis.

2.7. Determination of Total and Individual Capsaicinoids

2.7.1. UHPLC-Fluorescence Analysis

The separation and quantification of capsaicinoids were carried out on UHPLC-fluorescence equipment (ACQUITY UPLC, H-Class, Waters Corp., Milford, MA, USA), equipped with an ACQUITY UPLC Quaternary Pump System (Waters Corp., Milford, MA, USA), an ACQUITY UPLC Auto Sampler (Waters Corp., Milford, MA, USA),with temperature control adjusted at 15 °C, a column oven set at 50 °C for the chromatographic separation, and an ACQUITY UPLC Fluorescence (FLR) Detector (Waters Corp., Milford, MA, USA). Empower 3 software (Waters Corp., Milford, MA, USA) was used to control the equipment and for data acquisition. Capsaicinoids were analysed in a Waters' ACQUITY UPLC BEH C18 column (50 × 2.1 mm I.D., particle size 1.7 µm, Waters Corp., Milford, MA, USA). A previously developed chromatographic method was used [43]. This method involves a gradient with two solvents: Acidified water (0.1% acetic acid, solvent A) and acidified acetonitrile (0.1% acetic acid, solvent B), using a solvent flow of 0.8 mL min^{-1}. The gradient used for the chromatographic separation was (time, solvent B): 0 min, 0%; 0.50 min, 45%; 1.60 min, 45%; 1.95 min, 50%; 2.45 min, 55%; 2.80 min, 63%; 3.00 min, 63%; 4 min, 100%; 6.00 min, 100%. The wavelengths employed for fluorescence detection were 280 nm (excitation) and 305 nm (emission). The injection volume was 3 µL. For the capsaicinoids' quantification, calibration curves for C and DHC were used ($y = 1962318.42x + 64612.54$ for C and $y = 2147922.05x + 48033.91$ for DHC), which are the two capsaicinoids standards that are commercially available. Regression equations and correlation coefficients (r^2) (0.9997 for C and 0.9998 for DHC) were determined. The limits of detection (0.066 mg L^{-1} and 0.050 mg L^{-1} for C and DHC, respectively) were calculated using ALAMIN software [38]. C and DHC were quantified from the calibration curves obtained. Given the structural similarities between the molecules, n-DHC and h-DHC were quantified from the DHC calibration curve and h-C from the C calibration curve. All the analyses were carried out in triplicate.

2.7.2. UHPLC-Q-ToF-MS Analysis

The five principal capsaicinoids present in pepper (n-DHC, C, DHC, h-C, and h-DHC) were identified in these three varieties by UHPLC coupled to quadrupole-time-of-flight mass spectrometry (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA). MassLynx software, version 4.1 (Waters Corp., Milford, MA, USA), was used to control the equipment and for the acquisition and treatment of data. The molecular ions $[M + H]^+$ for the capsaicinoids identified had the following *m/z* ratios: Nordihydrocapsaicin, 294; capsaicin, 306; dihydrocapsaicin, 308; homocapsaicin, 320; homodihydrocapsaicin, 322. A gradient method, using acidified water (0.1% formic acid, solvent A) and acidified methanol (0.1% formic acid, solvent B) working at a flow rate of 0.5 mL min^{-1} was used. The gradient employed (time, solvent B) was as follows: 0 min, 0%; 0.85 min, 55%; 1.60 min, 55%; 1.95 min, 60%; 2.45 min, 63%; 2.80 min, 70%; 3.00 min, 70%; 6.00 min, 100%; 8 min, 100%. The chromatographic separation was performed on an rp-C18 analytical column (Acquity UPLC BEH C18, 2.1 mm × 100 mm and 1.7 μm particle size, Waters Corp., Milford, MA, USA). The capsaicinoids content was determined using an electrospray source operating in positive ionization mode under the following conditions: Desolvation gas flow = 850 L h^{-1}, desolvation temperature = 500 °C, cone gas flow = 10 L h^{-1}, source temperature = 150 °C, capillary voltage = 0.7 eV, cone voltage = 20 V, and trap collision energy = 4 eV. Full-scan mode was used (*m/z* = 100–600).

2.8. Statistical Analysis

Capsaicinoids extraction was carried out in triplicate and the data were expressed as the mean of the replicates. Experimental results were given as mean ± standard deviation and statistical analyses were performed by one-way ANOVA.

3. Results and Discussion

3.1. Evolution of the Total Capsaicinoids Content in Capsicum chinense Cultivars

The three cultivars of pepper plants began to produce peppers in the second week of July. The plants were monitored from 13 days post-anthesis (dpa) and continued until the over-ripening stages were reached (until 83 dpa for ‘Bode’ cultivar, and 76 dpa for ‘Habanero’ and ‘Habanero Roxo’ cultivars). At this final stage, the peppers presented water loss (dehydration) and an intense colour with respect to their previous stages. Table 1 shows the visual appearance of the peppers at different stages of fruit development.

Capsicum chinense varieties are known to have a high content of capsaicinoids per gram of fresh weight (F.W.) fruit, in comparison with other *Capsicum* spp. [13,33]. In this work, ‘Habanero Roxo’ pepper presented the greatest capsaicinoids concentration (3.86 mg g^{-1} F.W.) in comparison to ‘Bode’ (1.00 mg g^{-1} F.W.) and ‘Habanero’ pepper (1.33 mg g^{-1} F.W.).

Table 1. Evolution of fruit colour in the cultivars, 'Bode', 'Habanero', and 'Habanero Roxo', during the pepper fruits' development (dpa).

Dpa *	'Bode'	'Habanero'	'Habanero Roxo'
13	Green	Green	Green
20	Green	Green	Green
27	Green	Green	Green
34	Green	Green	Green
41	Green/yellow	Green/yellow	Green/violet
48	Orange	Orange	Violet
55	Orange	Orange	Violet
62	Orange	Orange	Violet
69	Orange	Orange	Violet
76	Orange	Over-ripening	Over-ripening
83	Over-ripening		

* Days post-anthesis.

Figure 1 and Table S1 show the evolution of the total capsaicinoids content in the three varieties of *Capsicum chinense* peppers studied. Similar behaviour in 'Bode' and 'Habanero' cultivars was observed, where the maximum capsaicinoids content was obtained at 33 dpa. These results are in accordance with those obtained by Fayos et al. [43] for 'Malagueta' pepper, where the maximum capsaicinoids content was registered at 33 dpa, before a slight degradation of capsaicinoids takes place as a result of the action by peroxidases. This behaviour was also reported in relation to other *Capsicum* spp. [46], like 'Cayenne' pepper [47], whose maximum capsaicinoids content level was reached at 40 dpa.

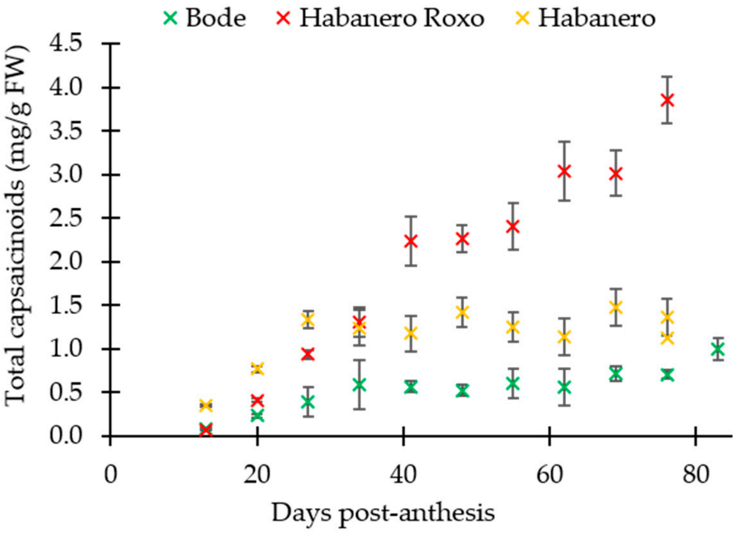

Figure 1. Evolution of total capsaicinoids (mg g^{-1}) in *Capsicum chinense* Jacq. cultivars over the ripening process. Results are presented as mean ± SD ($n = 3$).

After reaching its maximum level, capsaicinoids content remains practically constant during ripening. 'Habanero Roxo' presents its maximum capsaicinoids content at 41 dpa, which coincides with the pepper colour change from green to violet. From that moment, there is a period over which the capsaicinoids concentration remains practically stable until 55 dpa. After that, capsaicinoids concentration increases substantially until the over-ripening stage is reached. Similar behaviour was reported by Barbero et al. [39] for the 'Peter' pepper variety.

In the current study, in contrast to what is generally observed in the literature, a substantial increase in the content of capsaicinoids was observed in the early stages of fruit development (between 13 and 34 dpa for 'Bode' and 'Habanero' pepper, and between 13 and 41 dpa for 'Habanero Roxo' pepper), followed by two more moderate increases at the end of the maturation. This behaviour may be due to the growing conditions in the greenhouse, where the temperature, humidity, irrigation, and fertilization were controlled. A slight reduction in the capsaicinoids content (8.5% and 11.4% for the 'Habanero' and 'Bode' pepper, respectively) was observed from 34 dpa until 48 dpa. This behaviour was associated to the peroxidases action, which may have degraded the capsaicinoids [42].

3.2. Evolution of the Individual Contents of Capsaicinoids in Capsicum chinense Cultivars

The evolution of individual capsaicinoids content in the pepper samples is shown in Table 2 and Figure S1. It was observed that the evolution of the capsaicin content follows a similar pattern to that of the total capsaicinoids content throughout the ripening of the three cultivars of peppers studied. This was a predictable result since capsaicin represents more than 80% of the total capsaicinoids content in these cultivars. Dihydrocapsaicin also exhibits a similar behaviour. The other three capsaicinoids were not considered because of their low proportion.

In addition, the relative percentages of the five principal capsaicinoids (n-DHC, C, DHC, h-C, and h-DHC) were calculated for 'Bode', 'Habanero', and 'Habanero Roxo' peppers at different ripening stages (Table 3 and Figure S2). This study shows that C is the most abundant capsaicinoid present in these *Capsicum chinense* cultivars with rates around 78%–86%. The second one in importance is DHC with values that range around 10%–19%. This differs from other *Capsicum* varieties, such as 'Cayenne' pepper (*Capsicum annuum*) [47], in which C and DHC alternate as the main capsaicinoid during fruit ripening. In addition, there are other pepper species in which DHC is the main capsaicinoid [21]. C and DHC have very similar biological and spicy properties, with small differences in Scoville heat units (SHU). C scores 16,000,000 while DHC scores 15,000,000 (SHU) [30]. The other three capsaicinoids have lower pungency values, i.e., 9,100,000 SHU for n-DHC and 8,600,000 SHU for h-C and h-DHC [12,48,49]. In this study, the maximal SHU value was reached by the 'Habanero Roxo' pepper with 607,400 SHU, followed by the 'Habanero' pepper with 209,438 SHU and the 'Bode' pepper with 157,042 SHU. The capsaicinoids' behaviour was similar in 'Habanero' and 'Habanero Roxo' cultivars. The C relative content is practically constant during the fruit maturation. Unlike the 'Bode' pepper, an increase in the C relative content was observed until 20 dpa, and from then, a practically constant relationship was observed throughout the rest of the maturation. In addition, in the three cultivars studied, the second most abundant capsaicinoid, DHC, decreased when C increased. This result is consistent with the behaviour usually observed for the composition of capsaicinoids in *Capsicum chinense* varieties [13,50]. In general, C is the main capsaicinoid in most varieties of peppers, as observed in the three cultivars studied. There are some varieties that in the early stages of maturation, have a higher concentration of DHC than of C [39].

Table 2. Evolution of individual capsaicinoids (nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C), and homodihydrocapsaicin h-DHC)) (mg g^{-1} F.W.) in *Capsicum chinense* cultivars, during the ripening of pepper fruits (*n* = 3). The development stages of pepper fruits were measured as days post-anthesis (dpa).

	‘Bode’					‘Habanero’					‘Habanero Roxo’				
Dpa	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**
13	0.001 ± 0.000	0.058 ± 0.001	0.024 ± 0.002	0.002 ± 0.001	0.001 ± 0.000	0.001 ± 0.000	0.125 ± 0.002	0.026 ± 0.002	0.002 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.056 ± 0.021	0.014 ± 0.002	0.002 ± 0.001	0.000 ± 0.000
20	0.001 ± 0.000	0.190 ± 0.003	0.039 ± 0.003	0.003 ± 0.001	0.001 ± 0.000	0.002 ± 0.000	0.291 ± 0.024	0.057 ± 0.007	0.005 ± 0.000	0.001 ± 0.000	0.003 ± 0.000	0.324 ± 0.013	0.073 ± 0.007	0.005 ± 0.001	0.002 ± 0.000
27	0.001 ± 0.000	0.325 ± 0.004	0.060 ± 0.003	0.006 ± 0.001	0.001 ± 0.000	0.009 ± 0.000	0.646 ± 0.038	0.120 ± 0.011	0.009 ± 0.001	0.001 ± 0.000	0.003 ± 0.000	0.769 ± 0.043	0.152 ± 0.023	0.008 ± 0.002	0.005 ± 0.000
34	0.001 ± 0.000	0.496 ± 0.002	0.087 ± 0.003	0.007 ± 0.001	0.002 ± 0.000	0.015 ± 0.001	1.063 ± 0.079	0.193 ± 0.004	0.012 ± 0.001	0.001 ± 0.000	0.009 ± 0.001	1.123 ± 0.035	0.168 ± 0.022	0.006 ± 0.001	0.004 ± 0.000
41	0.002 ± 0.000	0.480 ± 0.006	0.077 ± 0.007	0.006 ± 0.001	0.001 ± 0.000	0.017 ± 0.001	0.957 ± 0.049	0.192 ± 0.013	0.011 ± 0.001	0.002 ± 0.000	0.013 ± 0.001	1.926 ± 0.065	0.249 ± 0.016	0.009 ± 0.003	0.005 ± 0.000
48	0.003 ± 0.000	0.434 ± 0.024	0.085 ± 0.006	0.004 ± 0.001	0.001 ± 0.000	0.018 ± 0.002	0.932 ± 0.040	0.182 ± 0.013	0.009 ± 0.000	0.002 ± 0.000	0.014 ± 0.002	1.997 ± 0.046	0.237 ± 0.019	0.015 ± 0.001	0.003 ± 0.000
55	0.003 ± 0.000	0.510 ± 0.010	0.087 ± 0.007	0.005 ± 0.001	0.001 ± 0.000	0.020 ± 0.002	1.110 ± 0.075	0.187 ± 0.009	0.007 ± 0.000	0.002 ± 0.000	0.014 ± 0.002	2.126 ± 0.021	0.249 ± 0.040	0.015 ± 0.003	0.004 ± 0.000
62	0.003 ± 0.000	0.466 ± 0.014	0.094 ± 0.009	0.003 ± 0.001	0.001 ± 0.000	0.019 ± 0.002	1.102 ± 0.025	0.183 ± 0.008	0.009 ± 0.000	0.002 ± 0.000	0.028 ± 0.002	2.630 ± 0.043	0.350 ± 0.032	0.021 ± 0.001	0.006 ± 0.000
69	0.005 ± 0.000	0.596 ± 0.008	0.111 ± 0.008	0.006 ± 0.001	0.001 ± 0.000	0.016 ± 0.002	1.038 ± 0.079	0.165 ± 0.013	0.005 ± 0.001	0.002 ± 0.000	0.027 ± 0.002	2.594 ± 0.054	0.376 ± 0.014	0.020 ± 0.003	0.004 ± 0.000
76	0.005 ± 0.000	0.590 ± 0.014	0.105 ± 0.011	0.004 ± 0.001	0.001 ± 0.000	0.013 ± 0.002	1.051 ± 0.057	0.159 ± 0.008	0.004 ± 0.000	0.001 ± 0.000	0.036 ± 0.002	3.282 ± 0.077	0.511 ± 0.030	0.023 ± 0.004	0.006 ± 0.001
83	0.006 ± 0.000	0.832 ± 0.008	0.150 ± 0.008	0.009 ± 0.001	0.001 ± 0.000										

Table 3. Relative percentages of individual capsaicinoids (nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C), and homodihydrocapsaicin h-DHC)) in *Capsicum chinense* cultivars during pepper fruit ripening. Results are presented as mean ± SD (n = 3). Different letters in the same column indicate statistically significant differences between groups ($p < 0.05$). The development stages of pepper fruits were measured as days post-anthesis (dpa).

			'Bode'					**'Habanero'**					**'Habanero Roxo'**		
Dpa	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**
13	0.60 ± 0.20	67.84 ± 1.24	27.67 ± 0.95	2.70 ± 0.34	1.20 ± 0.06	0.58 ± 0.03	80.64 ± 1.02	17.12 ± 1.04	1.45 ± 0.01	0.21 ± 0.01	0.87 ± 0.03	80.78 ± 1.01	15.93 ± 1.04	2.24 ± 0.06	0.18 ± 0.06
20	0.35 ± 0.07	81.10 ± 1.25	16.64 ± 0.76	1.39 ± 0.38	0.52 ± 0.03	0.58 ± 0.01	81.86 ± 0.51	15.99 ± 0.44	1.42 ± 0.02	0.15 ± 0.01	0.65 ± 0.01	79.70 ± 2.94	17.84 ± 2.81	1.32 ± 0.14	0.49 ± 0.01
27	0.30 ± 0.04	82.65 ± 1.00	15.24 ± 0.44	1.46 ± 0.22	0.36 ± 0.02	1.12 ± 0.01	82.35 ± 0.02	15.27 ± 0.01	1.12 ± 0.03	0.14 ± 0.01	0.37 ± 0.01	82.11 ± 1.40	16.13 ± 1.28	0.82 ± 0.01	0.57 ± 0.03
34	0.24 ± 0.03	83.71 ± 1.64	14.58 ± 0.44	1.22 ± 0.15	0.26 ± 0.01	1.18 ± 0.02	82.79 ± 0.29	15.01 ± 0.23	0.91 ± 0.02	0.11 ± 0.01	0.64 ± 0.01	85.80 ± 1.73	12.76 ± 1.03	0.48 ± 0.01	0.32 ± 0.01
41	0.30 ± 0.03	84.73 ± 1.01	13.63 ± 0.93	1.11 ± 0.16	0.23 ± 0.01	1.45 ± 0.01	81.22 ± 0.44	16.29 ± 0.41	0.90 ± 0.01	0.15 ± 0.01	0.58 ± 0.02	87.53 ± 0.55	11.29 ± 0.28	0.39 ± 0.07	0.21 ± 0.01
48	0.59 ± 0.01	82.48 ± 1.31	16.10 ± 0.74	0.67 ± 0.15	0.16 ± 0.01	1.60 ± 0.01	81.50 ± 0.40	15.92 ± 0.40	0.81 ± 0.00	0.17 ± 0.01	0.62 ± 0.01	88.15 ± 0.66	10.46 ± 0.52	0.66 ± 0.06	0.12 ± 0.01
55	0.45 ± 0.02	84.25 ± 1.00	14.28 ± 0.34	0.88 ± 0.17	0.14 ± 0.01	1.50 ± 0.02	83.74 ± 1.02	14.13 ± 0.05	0.50 ± 0.00	0.13 ± 0.01	0.58 ± 0.02	88.32 ± 1.66	10.30 ± 1.37	0.63 ± 0.05	0.16 ± 0.01
62	0.47 ± 0.02	82.16 ± 1.84	16.62 ± 1.10	0.57 ± 0.12	0.18 ± 0.01	1.47 ± 0.01	83.79 ± 1.00	13.90 ± 0.32	0.68 ± 0.01	0.16 ± 0.01	0.92 ± 0.01	86.67 ± 1.02	11.51 ± 0.73	0.69 ± 0.01	0.21 ± 0.01
69	0.66 ± 0.02	83.04 ± 1.71	15.37 ± 0.64	0.78 ± 0.12	0.15 ± 0.01	1.33 ± 0.01	84.62 ± 1.20	13.44 ± 0.27	0.43 ± 0.01	0.19 ± 0.01	0.89 ± 0.01	85.86 ± 0.43	12.45 ± 0.14	0.66 ± 0.02	0.14 ± 0.01
76	0.67 ± 0.02	83.64 ± 1.89	14.94 ± 0.80	0.63 ± 0.08	0.12 ± 0.01	1.04 ± 0.02	85.56 ± 0.32	12.95 ± 0.21	0.35 ± 0.01	0.11 ± 0.01	0.93 ± 0.02	85.09 ± 0.62	13.23 ± 0.37	0.60 ± 0.03	0.15 ± 0.01
83	0.59 ± 0.09	83.37 ± 3.17	15.04 ± 1.34	0.85 ± 0.04	0.14 ± 0.01										

3.3. Evolution of the Relative Percentage of Capsaicinoids in Capsicum chinense Cultivars

The relative percentage of capsaicinoids in the *Capsicum chinense* Jacq. cultivars was determined using the following Equation (1):

$$RP = \frac{(Cap_x)}{(CapT_x)} \times 100 \tag{1}$$

where: RPi is the relative percentage of each individual capsaicinoid, (Cap_{ix}) is the concentration of an individual capsaicinoid at time "x", and ($CapM_i$) is the maximum concentration of this individual capsaicinoid during ripening. Table 4 and Figure S3 show the evolution of the different capsaicinoids' standardized contents in 'Bode', 'Habanero', and 'Habanero Roxo' peppers. The behaviour of the capsaicinoids varied depending on the pepper cultivar. Nevertheless, the standardized capsaicinoids content in 'Habanero' and 'Habanero Roxo' pepper tended to increase until 80 dpa. All the capsaicinoids in 'Habanero' peppers showed a fairly similar behaviour and reached their maximum content level of C, h-C, and DHC at 34 dpa, while n-DHC and h-DHC reached their maximum content level some days later. With respect to the 'Habanero Roxo' pepper, the progression of the relative percentage of h-DHC was different to that of C, h-C, n-DHC, and DHC all of which presented a more or less ascending evolution throughout the whole fruit maturation. The 'Bode' pepper, on the other hand, showed a different behaviour, where the h-C and h-DHC reached their peak at the early stages of maturation (34 dpa) while the rest of the capsaicinoids exhibited an irregular ascending progression throughout the whole fruit maturation, similar to the 'Habanero Roxo' pepper.

Several works in the literature present the evolution of individual groups of phytochemicals present in the fruits of *Capsicum* (volatile compounds, phenolics, carotenoids, and capsaicinoids) during maturation [28,29,38]. However, these works only analyse a stage of the specific maturation and do not address several growing cycles. In fact, due to the difficulty, duration, and cost of carrying out this type of work, a single period of maturation usually occurs, in which the effect that environmental variations have on the evolution of these compounds is not evaluated. In this sense, in these works, it is necessary to describe perfectly the cultivation conditions (fertilization, irrigation, temperatures, etc.) to which the crop has been subjected. The evolution of this type of compound is influenced by many environmental factors, such as the temperature, humidity, fertilization of plants, temperature differences throughout the day, light received, etc. [31,51,52]. For this reason, these parameters must be perfectly controlled and described in this type of work so that they can be comparable and reproducible with other published references.

Table 4. Relative percentages (standardized values) of individual capsaicinoids (nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C), and homodihydrocapsaicin h-DHC)) in *Capsicum chinense* cultivars during pepper fruit ripening. Results are presented as mean ± SD ($n = 3$). Different letters in the same column indicate statistically significant differences between groups ($p < 0.05$). The development stages of pepper fruits were measured as days post-anthesis (dpa).

	'Bode'					**'Habanero'**					**'Habanero Roxo'**				
Dpa	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**	**n-DHC**	**C**	**DHC**	**h-C**	**h-DHC**
13	8.7 ± 1.1	7.0 ± 0.1	15.8 ± 0.2	26.8 ± 0.6	66.3 ± 1.2	4.5 ± 0.1	11.1 ± 0.4	13.6 ± 0.3	19.3 ± 0.3	13.4 ± 0.5	1.6 ± 0.1	1.7 ± 0.6	2.6 ± 2.2	7.2 ± 3.7	2.2 ± 1.5
20	13.8 ± 2.9	22.8 ± 0.1	25.9 ± 0.1	37.8 ± 3.3	79.4 ± 1.3	10.4 ± 0.2	25.9 ± 1.0	29.2 ± 0.4	43.2 ± 0.1	21.9 ± 0.8	7.6 ± 1.4	9.9 ± 0.2	14.3 ± 2.5	23.3 ± 2.3	30.7 ± 0.6
27	19.6 ± 2.7	39.0 ± 0.1	39.9 ± 0.9	67.0 ± 3.9	91.9 ± 0.5	44.1 ± 1.	57.5 ± 0.9	61.6 ± 1.4	75.1 ± 2.6	44.9 ± 0.9	9.8 ± 2.0	23.4 ± 0.8	29.6 ± 2.8	33.3 ± 5.8	82.0 ± 1.0
34	23.6 ± 2.6	59.7 ± 0.3	57.6 ± 0.4	84.5 ± 1.8	100.0 ± 0.0	76.3 ± 2.0	94.5 ± 2.7	100.0 ± 0.0	100.0 ± 0.0	57.4 ± 1.7	21.9 ± 2.1	34.2 ± 0.3	32.7 ± 2.4	27.1 ± 5.0	64.9 ± 1.1
41	28.3 ± 2.4	57.7 ± 0.2	51.5 ± 2.1	73.6 ± 3.1	83.7 ± 1.1	86.0 ± 3.7	85.1 ± 1.0	98.6 ± 2.1	90.6 ± 1.1	73.1 ± 2.7	35.2 ± 2.4	58.7 ± 0.6	48.7 ± 0.3	37.0 ± 9.8	70.1 ± 0.6
48	52.4 ± 1.6	52.1 ± 2.4	56.4 ± 2.5	41.3 ± 4.5	53.6 ± 3.0	92.4 ± 2.2	82.9 ± 1.0	93.6 ± 3.2	79.0 ± 0.4	79.9 ± 2.8	39.4 ± 1.6	60.8 ± 0.0	46.4 ± 1.0	64.6 ± 1.3	40.9 ± 1.8
55	46.2 ± 1.8	61.3 ± 0.6	57.6 ± 1.1	62.1 ± 2.4	55.5 ± 2.9	100.0 ± 0.0	100.0 ± 1.0	96.2 ± 3.1	56.8 ± 2.1	75.1 ± 2.6	38.5 ± 4.4	64.8 ± 0.9	48.5 ± 5.0	65.9 ± 3.7	60.1 ± 0.3
62	44.6 ± 1.9	56.0 ± 1.1	62.9 ± 1.1	37.5 ± 4.0	67.2 ± 2.1	97.6 ± 0.3	98.1 ± 2.3	94.0 ± 1.3	76.1 ± 0.1	88.6 ± 0.3	78.1 ± 3.0	80.1 ± 0.6	68.4 ± 2.3	90.5 ± 5.9	100.0 ± 0.0
69	79.5 ± 0.7	71.7 ± 0.3	73.5 ± 3.2	65.7 ± 5.7	70.1 ± 1.9	81.8 ± 3.7	92.2 ± 2.8	84.6 ± 1.3	44.9 ± 1.2	100.0 ± 0.0	75.0 ± 2.2	79.1 ± 0.2	73.8 ± 1.6	85.5 ± 4.6	65.8 ± 0.5
76	79.5 ± 0.7	70.9 ± 1.0	70.2 ± 1.1	52.1 ± 2.3	53.8 ± 3.0	64.1 ± 1.3	93.5 ± 1.2	81.7 ± 2.3	36.5 ± 1.7	57.6 ± 3.9	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	89.5 ± 5.0
83	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	93.0 ± 0.5										

4. Conclusions

The five principal capsaicinoids (n-DHC, C, DHC, h-C, and h-DHC) were found in the three *Capsicum chinense* Jacq. cultivars ('Bode', 'Habanero', and 'Habanero Roxo' peppers) and studied at different ripening stages. Capsaicin represents the main capsaicinoid in the three species studied (78%–86%), followed by dihydrocapsaicin (10%–19%), and this relationship remains practically constant during all stages of maturation. A similar behaviour in 'Bode' and 'Habanero' cultivars was observed, where the maximum capsaicinoids content was reached at 33 dpa. The concentration of total capsaicinoids in 'Habanero Roxo' tended to increase until the over-ripening stages were reached. The capsaicinoids relative content remained practically constant during pepper fruit ripening in the three cultivars. The most pungent pepper cultivar was the 'Habanero Roxo' pepper (607,400 SHU) followed by the 'Habanero' pepper (209,438 SHU) and finally the 'Bode' pepper (157,042 SHU). This work deepens the knowledge on the evolution of the content of capsaicinoids in peppers, specifically of the varieties of *Capsicum chinense*. Given the important biological activity of capsaicinoids, the information described in this work allows the harvesting of *Capsicum chinense* pepper at the moment when the peppers' capsaicinoids content is the greatest. In any case, harvesting should be completed before the over-ripening stages of the fruit are visibly reached.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/3/141/s1, Figure S1: Evolution of individual capsaicinoids (mg g^{-1} F.W.) in Capsicum chinense cultivars: a) 'Bode'; b) 'Habanero'; c) 'Habanero Roxo', during pepper fruits ripening (*n* = 3). The development stages of pepper fruits were measured as days post-anthesis (dpa), Figure S2: Relative percentages of individual capsaicinoids in *Capsicum chinense* cultivars: a) 'Bode'; b) 'Habanero'; c) 'Habanero Roxo', during pepper fruit ripening (*n* = 3). The development stages of pepper fruits were measured as days post-anthesis (dpa), Figure S3: Relative percentages of individual capsaicinoids (standardized values) in *Capsicum chinense* cultivars: a) 'Bode'; b) 'Habanero'; c) 'Habanero Roxo' during pepper fruit ripening (*n* = 3). The development stages of pepper fruits were measured as days post-anthesis (dpa). Table S1: Evolution of total capsaicinoids (mg g^{-1}) in Capsicum chinense Jacq. cultivars over the ripening process. Results are presented as mean ± SD (*n* = 3). Different letters in the same column indicate statistically significant differences between groups ($p < 0.05$).

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