Bioactive Compound Variability in a Brazilian *Capsicum* Pepper Collection

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ABSTRACT

This report aims to characterize the bioactive content of pepper (*Capsicum* L., Solanaceae) accessions from a Brazilian germplasm collection. Peppers are currently a major vegetable crop worldwide, with 31 Tg produced in 2013, according to FAO. Seventy-two accessions of *Capsicum annuum* L., *C. baccatum* L., *C. chinense* Jacq., and *C. frutescens* L. were evaluated and high variability was observed in soluble solids, pH, acidity, fruit color, total phenolic content, total carotenoid content, antioxidant potential, and capsaicinoid content. Accessions P119, P302, and P189 had the highest phenolic compound contents, which ranged from 225.16 to 243.47 mg gallic acid equivalent 100 g^{-1}. The highest antioxidant potential observed ranged from 2.71 to 3.03 mmol Trolox equivalent 100 mg^{-1} in accessions P287, P50, and P25. Maximum carotenoid content was seen in accessions P269, P280, and P179, which contained between 53.42 and 54.11 mg β-carotene equivalent 100 g^{-1}. Capsaicinoid content was highest in accessions P120, P246, and P236 and ranged from 6132.00 to 19,543.70 mg 100 g^{-1}. Capsaicin and dihydrocapsaicin represented, on average, 79% of the total capasaicinoid content. No one accession contained high levels of multiple bioactive compounds.

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Abbreviations: DDPH, 2,2-diphenyl-1-picrylhydrazyl; FLD, fluorescence detector; GAE, gallic acid equivalents; HPLC, high performance liquid chromatography; QTOF, quadrupole time-of-flight; SSC, soluble solids content; UHPLC, ultra-high performance liquid chromatography.

BRAZIL is a secondary center of diversity for *Capsicum* L. peppers, and many landraces are cultivated in small farms from north to south of the country. The genus *Capsicum* belongs to the Solanaceae family and contains more than 20 species, five of which (*Capsicum annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L., and *C. pubescens* Ruiz & Pav.) are domesticated and grown commercially, primarily for culinary use in the food industry for their unique flavor, as a coloring and flavoring agent, and in the making of a variety of pepper-containing food products such as jellies, jams, and sauces. Additionally, oleoresin derived from *Capsicum* is used to produce pepper spray (Pino et al., 2007; Bae et al., 2012). In the last few years, the market for *Capsicum* has grown such that, in 2013, the world's *Capsicum* (chilies and fresh pepper) production reached 1.9 million ha and >31 Tg of harvested crop (FAOSTAT, 2016). *Capsicum* peppers may be sweet as with bell peppers or hot, like jalapeño and habanero. In hot *Capsicum* peppers, capsaicin ($C_{18}H_{27}NO_3$), derived from homovanillic acid, is the predominant pungent alkaloid (Appendino, 2008). In addition to capsaicinoids, the fruit of *Capsicum* is an important

source of carotenoids (some with pro-vitamin A activity), flavonoids, ascorbic acid (vitamin C), and tocopherols (vitamin E) (Howard and Wildman, 2006). A number of health benefits have been associated with the compounds present in *Capsicum* peppers and include antioxidant, antimutagenic, and antitumor activities (Topuz and Ozdemir, 2007; Meghvansi et al., 2010; Zimmer et al., 2012). The development of cultivars rich in bioactive compounds has been cited as one of the main objectives of breeding programs (Lannes et al., 2007; Wahyuni et al., 2011). Therefore, the characterization of available germplasm is essential to increase conservation of genetic diversity and support breeding efforts (Hayano-Kanashiro et al., 2016). Brazilian germplasm collections conserve many accessions of *Capsicum* peppers, including landraces selected by farmers for several generations. However, in order for these accessions to become commercially available and be further explored, they need to be properly characterized (Rêgo et al., 2011; Padilha et al., 2015). The success of a breeding program is increased with germplasm characterization, particularly in terms of genetic variability. This study reports the chemical characterization of targeted specialized metabolites and groups of compounds present in *Capsicum* pepper accessions from a Brazilian genebank.

MATERIALS AND METHODS
Germplasm
Fruit of 72 accessions of *C. annuum*, *C. baccatum*, *C. chinense*, and *C. frutescens* of the Embrapa Temperate Agriculture (Pelotas, RS, Brazil) germplasm collection were evaluated. Of these 72 genotypes, 51 were analyzed for total phenolic content, total carotenoid content, and antioxidant potential, and 40 were evaluated for capsaicinoid content (Table 1). Seeds from each genotype were obtained from plants grown under greenhouse conditions at Embrapa. Seedlings were transplanted to the field at the five- to seven-leaf stage in October in a randomized complete block design with three replicates and 72 treatments (genotypes). The experimental unit consisted of one row containing five plants of one genotype. Rows covered in black plastic were spaced 1.3 m apart, with plants spaced 0.5 m apart. Plants were irrigated by drip irrigation. Fruit from five plants per genotype from each replicate were harvested from February through May at the mature stage between 9:00 and 10:00 AM and pooled for a total of 216 samples. Seeds were removed and fresh fruit were evaluated for pH, acidity, soluble solids, and color. The remaining fruit were stored at −76°C for the other analyses. Each sample consisted of 10 g of fruit ground in liquid nitrogen in a ball mill (Marconi, MA350, Brazil).

Analyses
All analyses were performed in triplicate for each genotype replicate, and the mean of the three repeats was used for statistical analysis.

Table 1. Accessions of *Capsicum* (Solanaceae) analyzed for total phenolic content, total carotenoid content, antioxidant potential, and capsaicinoid content.

Accession	Species	Color of mature fruit
P9	Capsicum baccatum	red
P14	Capsicum baccatum	red
P25	Capsicum baccatum	red
P27	Capsicum baccatum	yellow
P28	Capsicum baccatum	red
P34	Capsicum baccatum	red
P46	Capsicum annuum	red
P49	Capsicum baccatum	red
P50	Capsicum baccatum	yellow
P59	Capsicum baccatum	red
P61	Capsicum baccatum	yellow
P72	Capsicum chinense	red
P76	Capsicum annuum	yellow
P79	Capsicum baccatum	red
P87	Capsicum baccatum	red
P89	Capsicum baccatum	red
P92	Capsicum baccatum	red
P100	Capsicum annuum	red
P103	Capsicum baccatum	red
P105	Capsicum baccatum	red
P108	Capsicum baccatum	red
P110	Capsicum baccatum	yellow
P116	Capsicum chinense	pale yellow
P117	Capsicum baccatum	red
P119	Capsicum annuum	red
P120	Capsicum baccatum	red
P169	Capsicum baccatum	red
P179	Capsicum baccatum	red
P189	Capsicum annuum	red
P197	Capsicum baccatum	red
P203	Capsicum annuum	orange
P213	Capsicum baccatum	red
P219	Capsicum baccatum	red
P235	Capsicum baccatum	red
P236	Capsicum chinense	orange
P239	Capsicum baccatum	red
P241	Capsicum baccatum	red
P242	Capsicum chinense	pale orange
P246	Capsicum chinense	pale orange
P258	Capsicum annuum	red
P259	Capsicum annuum	yellow
P269	Capsicum chinense	red
P270	Capsicum baccatum	red
P274	Capsicum baccatum	orange
P278	Capsicum baccatum	red
P280	Capsicum baccatum	red
P283	Capsicum baccatum	red
P284	Capsicum baccatum	red
P287	Capsicum baccatum	red
P295	Capsicum chinense	red
P302	Capsicum annuum	red

Soluble Solids, pH, Acidity, and Color

Soluble solids content (SSC) was determined by refractometry (digital refractometer, Atago, PR–32α), and the results were expressed as a percentage (w/w). Acidity and pH were measured directly from the extracted fruit juice. Acidity was determined by titration and results were expressed as milligrams of citric acid per 100 g of fresh fruit (mg 100 g^{-1}). Skin color was measured with a colorimeter (Minolta, CR–300). Colors are specified in the CIE $L\star a\star b\star$ system. The hue angle (°Hue) was calculated using the formula °Hue = $\tan^{-1} b\star / a\star$. The $L\star$ axis represents lightness, the $a\star$ axis represents redness ranging from negative values for green to positive values for red, and the $b\star$ axis represents yellowness ranging from negative values for blue to positive values for yellow.

Total Phenolic Content, Total Carotenoid Content, and Antioxidant Potential

Total phenolic content was determined using the Folin–Ciocalteu reagent. Two grams of ground fruit flesh without seeds was homogenized with 20 mL methanol for 1 h and centrifuged for 20 min at 5000 rpm in a centrifuge refrigerated at 4°C. A 250-μL aliquot of the supernatant was diluted in 4 mL of water, and a control was also prepared containing 250 μL of methanol. Both the samples and the control were combined with 250 mL of 0.25 N Folin–Ciocalteu reagent (Swain and Hillis, 1959). After 3 min of reaction, 500 mL 1N Na_2CO_3 were added, the mixture was incubated for 2 h at room temperature, and the absorbance was measured at 725 nm with results expressed as mg of gallic acid equivalents 100 g^{-1} of fruit fresh wt. (mg GAE 100 g^{-1} fresh wt.). Total carotenoid content was evaluated using the Association of Official Analytical Chemists method (970.64; AOAC, 2005) modified for the purpose of reducing the amount of sample used. One gram of ground fruit flesh was homogenized with 15 mL solvent extractor (hexane:ethanol:acetone:toluene at 10:6:7:7). Then aliquots of 1 mL of 10% methanolic potassium hydroxide were added and mixed for an additional 1 min. The flasks were capped and placed in a 56°C water bath for 20 min and allowed to cool to room temperature for 1 h. Aliquots of 15 mL hexane were pipetted into each flask. The flasks were brought to 50 mL with a 10% sodium sulfate solution. The flasks were shaken thoroughly and placed in a dark area for approximately 1 h to allow for phase separation. Absorbance was measured at 454 nm (spectrophotometer, JENWAY 6705 UV-Vis) and results were expressed as micrograms of β-carotene equivalents per gram of fruit fresh wt. (μg β-carotene g^{-1} fresh wt.). Antioxidant potential was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Brand-Williams et al., 1995). One hundred microliters of the methanolic fruit extract was added to 3.9 mL of DPPH solution in methanol (100 mM). The solution was then stirred and kept in a closed flask in the dark. Absorbance was measured at 517 nm after 24 h of reaction. The antioxidant potential was expressed as millimoles of Trolox equivalent 100 mg^{-1} fresh wt. (mmol Trolox 100 mg^{-1} fresh wt.).

Capsaicinoid Content by HPLC-FLD

For the analysis of capsaicinoids, 300 mg of lyophilized pepper fruit without seeds were extracted with 1 mL of acetonitrile and sonicated (sonicator, Unique, Ultracleaner 1400 A) for 10 min twice. The two extracts were combined, centrifuged (centrifuge, Eppendorf, 5430) for 5 min at 13,000 rpm, and filtered through a 0.2-μm polytetrafluoroethylene membrane filter into an amber vial. Ten microliters of sample were injected in a high performance liquid chromatography (HPLC) (Thermo Scientific) equipped with a Luna C18 precolumn (2.0 × 4 mm) and analytical column (2.0 × 150 mm, 100 Å, particle size 3 μm) from Phenomenex (Torrance) for chromatographic separation. Degassed solutions of acetic acid:ultrapure water (1:10^3, v/v, eluent A) and acetic acid:acetonitrile (1:10^3, v/v, eluent B) were used as mobile phase. The flow rate of the mobile phase was 0.2 mL min^{-1} using isocratic elution at 52% B. The column temperature was kept at 40°C. Capsaicinoids were detected by fluorescence (at λ Ex = 280 nm and λ Em = 354 nm) and quantified by comparison with capsaicin and dihydrocapsaicin standards from Sigma-Aldrich.

Capsaicinoid Identification by UHPLC-QTOF MS

Ten microliters of the acetonitrile extract of genotypes P28 and P61 were injected in the ultra-high performance liquid chromatography (Shimadzu, Nexera) coupled to a high-resolution quadrupole time–of–flight (QTOF) mass spectrometer (maXis Impact). Capsaicinoids were separated using a Kinetex C18 column (50 × 2.1 mm, 1.7 μm; Phenomenex), using a gradient of formic acid solution in water (0.1% v/v, A), formic acid in acetonitrile (0.1% v/v, B), with 2% B for 7 min, followed by 98% B for 2 min and 2% B for 1 min, for a total of 10 min at a flow rate of 0.4 mL min^{-1}. The mass spectrometer was operated in the electrospray ionization positive mode with a capillary voltage of 3500 V, a nebulizer gas (N_2) pressure of two bars, drying gas at 10 L min^{-1}, and a source temperature of 200°C. The equipment was calibrated using sodium formate (10 mM) covering an acquisition mass/charge (m/z) range from 50 to 1000 amu. The following protonated molecules were monitored: capsaicin (m/z 306.2064), dihydrocapsaicin (m/z 308.2220), homocapsaicin (m/z 320.2220), homodihydrocapsaicin (m/z 322.2377), and nordihydrocapsaicin (m/z 294.2064).

Statistical Analysis

Data were analyzed for normality using Shapiro–Wilk's test, for homoscedasticity by Hartley's test, and the independence of residuals was graphically verified. Data were then analyzed using ANOVA ($p \leq 0.05$). When significant, the effects were compared by LSD ($p \leq 0.05$). Statistical analyses were performed using SAS software 9.2 (SAS Institute, 2008).

RESULTS AND DISCUSSION

High variability in soluble solids, pH, acidity, color of fruit, total phenolic content, total carotenoid content, antioxidant potential, and capsaicinoid content was observed in the pepper accessions evaluated.

Compositional Variation

Pepper fruit take ~90 d from fruit set to maturity. They have an indeterminate pattern of production and seasonal variation in the rate at which maturation can occur. Peppers are generally harvested with at least 85% of the fruit surface having achieved the characteristic varietal color of red, yellow, or orange. There is also an associated increase in soluble solids and decrease in acidity at this stage (Lin and Saltveit, 2012). Total SSC of the studied genotypes varied from 5.8 (P228, *C. chinense*) to 15.0°Bx (P9, *C. baccatum*) and averaged 9.1 ± 1.8 (SD) (Table 2). In a study by Lannes et al. (2007) with 49 pepper accessions of *C. chinense*, SSC varied between 6 and 10°Bx. An elevated SSC is of interest to the food industry since it is responsible for a higher fruit yield in food processing. Fruit acidity is another relevant technological parameter that determines the choice of thermal treatment applied during processing. In the Embrapa pepper collection evaluated, acidity varied from 0.1 (P161) to 0.6 g (P203) citric acid equivalents 100 g^{-1} of sample. Both extreme values occurred in genotypes of *C. annuum*, demonstrating within-species variability. *Capsicum frutescens* were on average more acidic than *C. chinense* and *C. baccatum*. pH varied from 5.2 (P100, *C. annuum*) to 6.5 (P27, *C. baccatum*) and averaged 5.8 ± 0.3. pH has an expected inversely proportional relation to acidity, where the higher the acid concentration, the lower the pH. However, this is true only if the acids are found dissociated. Within the cell, acids are generally associated with potassium salts constituting buffer systems. The buffer capacity of some fruit allows for great variation in acidity without significant changes in pH (Powers and Shinholser, 1979). In addition to SSC, acidity, and pH, color is another relevant parameter that influences fruit quality. Hue angle is determined by coordinates *a*★ and *b*★ and defines fruit color tone. Yellow, orange, and red were the characteristic colors observed in mature fruit in this germplasm collection. Among accessions evaluated, hue angle varied from 6.73 to 102.19°, which indicated that fruit color ranged from purplish red to light yellow.

In *Capsicum* peppers, carotenoids are the compounds responsible for fruit color (Duarte et al., 2004). Carotenoid content (Fig. 1 and 2) varied from 1.54 (P116, *C. chinense*) to 54.11 mg β-carotene 100 g^{-1} fresh wt. (P179, *C. baccatum*) and averaged 32.16 ± 15.77 mg β-carotene 100 g^{-1} fresh wt. *Capsicum baccatum* showed, on average, the highest carotenoid content when compared with the other species in this germplasm collection (Table 3). Rodriguez-Uribe et al. (2012) studied six cultivars of *C. annuum* with orange-colored fruit and found carotenoid concentrations that ranged from 1 to 16 mg 100 g^{-1}. Although genetically similar, fruit composition may vary due to extrinsic factors such as biotic and abiotic stresses. While studying the chemical composition of 32 *Capsicum* accessions (*C. annuum*, *C. frutescens*, *C. chinense*, and *C. baccatum*), Wahyuni et al. (2011) observed an average total carotenoid content of ~40 mg 100 g^{-1} fresh wt., similar to results found in this study. Padilha et al. (2015) found large variation for carotenoid content in *Capsicum annuum* genotypes ranging from 1.5 to 147.7 mg 100 g^{-1}.

Regarding fruit phenolic compound content in the Embrapa accessions (Fig. 1 and 2), the concentration ranged from 54.4 (P179) to 243.47 (P189) mg of GAE 100 g^{-1}. The genotypes with the highest and lowest phenolic compound contents were found in *C. baccatum*, and the grand mean of all accessions tested in this study was 122.08 mg GAE 100 g^{-1}. These results are in agreement with Bae et al. (2012), who reported fruit phenolic compound contents of 91 to 138 mg 100 g^{-1} for *Capsicum* spp. Among species, the unidentified accessions of *Capsicum* spp. showed the highest mean phenolic compound content (200.53), followed by *C. annuum* (175.52), *C. chinense* (144.39), and *C. baccatum* (100.67) (Table 3). Perucka and Materska (2007) observed *Capsicum* pepper fruit phenolic contents that ranged from 37.54 to 67.35 mg 100 g^{-1}, which are closer to the lower end of the results found here.

Antioxidant potential of pepper fruit evaluated was found to vary from 0.5 to 3.0 mmol Trolox 100 g^{-1} and averaged 1.3 ± 0.7 mmol Trolox 100 g^{-1}. On average, *C. annuum* accessions had the highest antioxidant potential, followed by *C. chinense* (Table 3). Studies have shown a positive direct correlation between the antioxidant potential and the phenolic content of fruit (Bae et al., 2012). In this study, the observed correlation between antioxidant potential and *Capsicum* fruit phenolic content was very low (R^2 = 0.1121). After removal of five outlying accessions (P25, P287, P50, P100, and P246) from the dataset, the correlation coefficient increased to 0.448, which still does not indicate a direct relationship between the two variables in

Table 2. Physiochemical characteristics of 72 pepper genotypes grouped by species.

Species	pH	Soluble solids	Acidity†	Hue angle
		°Bx	g 100 g^{-1}	°
Capsicum annuum (14 accessions)	5.7 ± 0.3b‡	9.7 ± 1.6a	0.29 ± 0.1b	42.6 ± 24.6ab
Capsicum baccatum (39 accessions)	5.9 ± 0.3a	9.0 ± 1.9ab	0.23 ± 0.1c	35.6 ± 20.9b
Capsicum chinense (16 accessions)	5.8 ± 0.2ab	8.7 ± 1.9ab	0.19 ± 0.1c	58.8 ± 27.2a
Capsicum frutescens (1 accession)	5.7 ± 0.0b	8.3 ± 0.0b	0.42 ± 0.0a	27.6 ± 0.0b
Capsicum spp. (2 accessions)	5.8 ± 0.0ab	9.7 ± 1.4a	0.22 ± 0.1c	38.9 ± 30.4b

† Expressed as grams of citric acid 100 g^{-1} of fruit on a fresh-weight basis.

‡ Values are mean ± standard deviation. Means followed by the same letter within a column are not significantly different at $p \leq 0.05$.
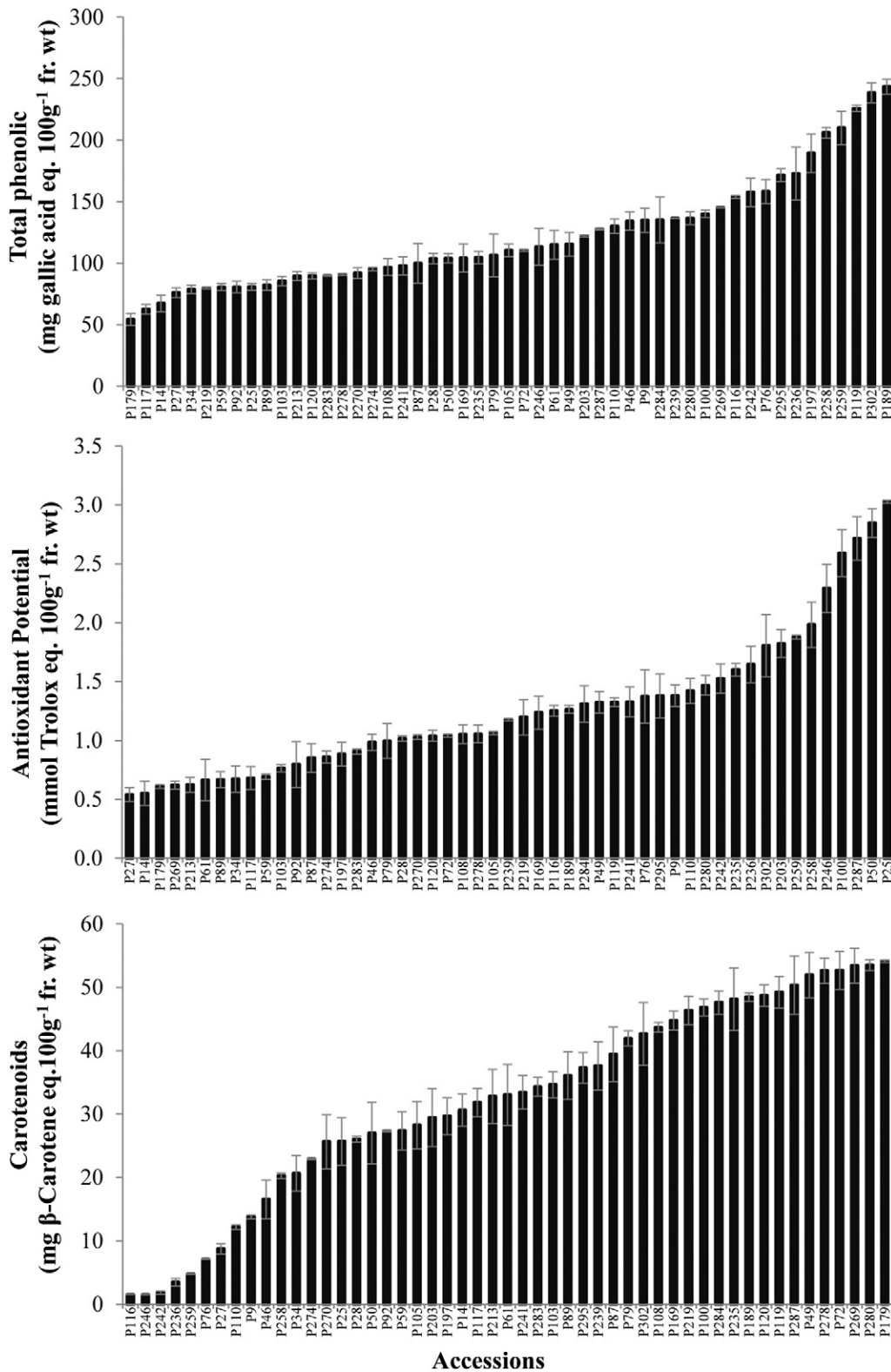
Fig. 1. Total phenolic content, antioxidant potential, and total carotenoid content of 51 pepper accessions (*Capsicum* spp.). Bars indicate standard error of the mean (*n* = 3).

this study. Other compounds including ascorbic acid may have contributed more strongly to the observed antioxidant potential and therefore may explain the low correlation seen between antioxidant potential and phenolic content.

Consumers are looking for foods more nutritionally and functionally rich. On the other hand, growers are looking for more productive cultivars with improved tolerance to biotic and abiotic stresses. The breeders must take both

requirements into consideration when developing new cultivars. In this study, the observed antioxidant potential and total phenolic content found for *Capsicum* genotypes is higher than those found in other commonly consumed vegetables and grains, including rice (*Oryza sativa* L.), beans (*Phaseolus coccineus* L.), beets (*Beta vulgaris* L.), carrots (*Daucus carota* L.), potatoes (*Solanum tuberosum* L.), and pumpkins (*Cucurbita maxima* Duch.) (Sreeramulu and Raghunath,

Fig. 2. Pepper accessions that showed the highest phenolic compounds content (P119, P302, and P189), the highest antioxidant potential (P287, P50, and P25), and the highest carotenoid content (P269, P280, and P179). Images: Rosa Lía Barbieri, Henrique Padilha, and Juliana Castelo Branco Villela.

Table 3. Mean total phenolic content (TP), antioxidant potential (AOP), and total carotenoid content (TC) of 51 pepper genotypes grouped by species.

Species	TP†	AOP‡	TC§
	mg 100 g^{-1}	mmol 100 g^{-1}	mg 100 g^{-1}
Capsicum annuum	175.52 ± 44.2ab¶	1.63 ± 0.59a	28.47 ± 19.34a
Capsicum baccatum	110.67 ± 26.34b	1.14 ± 0.61b	34.97 ± 34.94a
Capsicum chinense	144.39 ± 30.61b	1.53 ± 0.48a	23.21 ± 23.55a
Capsicum spp.	200.53 ± 60.73a	1.39 ± 0.19ab	25.17 ± 32.98a

† Total phenolic content expressed as milligrams gallic acid equivalents 100 g^{-1} of fruit on a fresh-weight basis.

‡ Antioxidant potential expressed as millimoles Trolox equivalent 100 g^{-1} fresh wt.

§ Total carotenoid content expressed as milligrams β-carotene 100 g^{-1} fresh wt.

¶ Mean ± standard deviation. Means followed by the same letter within a column are not significantly different at $p \leq 0.05$.

potential. Some carotenoids, for example, are also vitamin A precursors, an essential nutrient not produced by the human body but essential for growth and development, maintenance of epithelial tissues, reproduction, and proper functioning of the visual system in the regeneration of photoreceptors (Dutta et al., 2004) In another context, specialized metabolites serve as protection against stresses in plants (Schulze and Spiteller, 2009). For example, Park et al. (2012) reported the induction of polyphenol accumulation in *C. annuum* infected with the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., the causal agent of anthracnose disease and the major pathogen found in pepper. Another group of specialized metabolites with limited occurrence within *Capsicum* is the capsaicinoids. These are alkaloids that include capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin, among others, which are responsible for the characteristic pungency of *Capsicum* spp. (Kozukue et al., 2005). Whether these compounds play any role in plant defenses against pathogens or provide a fitness advantage under specific environmental conditions has not yet been established. Capsaicinoids have, however, been associated with anti–inflammatory and analgesic properties (Meghvansi et al., 2010; Zimmer et al., 2012).

Method Optimization and Validation for Determination of Capsaicinoids
Calibration Curve and Merit Figures
Table 4 shows the parameters of the analytical curves for capsaicin and dihydrocapsaicin analyzed by HPLC with a fluorescence detector (FLD), with the respective concentration intervals and interday variation. Calibration curves had high linear coefficients ($R \geq 0.998$) and similar interday sensitivity for both compounds. The limit of detection was 2.5 ng mL^{-1} for capsaicin and 0.6 ng mL^{-1} for dihydrocapsaicin, indicating that the optimized method has a good detection limit when compared with methods employed in other studies (Karnka et al., 2002; Juangsamoot et al., 2012). Limits of quantitation were 8 ng mL^{-1} for capsaicin and 3 ng mL^{-1} for dihydrocapsaicin. The interday variation

2010). Phenolic compounds are important not only for their antioxidant potential but also for their antiallergenic (Chung and Champagne, 2008), antimicrobial (Viswanath et al., 2009), antithrombotic, and anti–inflammatory activities (Singh et al., 2009). There are numerous compounds with antioxidant potential capable of countering biological oxidative processes within the human body (Rahman et al., 2006). For example, carotenoids are among the compounds present in significant amounts in pepper with elevated antioxidant potential. Carotenoid content found in the studied germplasm collection was higher than that observed by Reif et al. (2013) for vegetables such as tomato (*Lycopersicon lycopersicum* L.) and carrots, widely known and recommended for consumption as being a rich source of carotenoids.

Although a specific daily–recommended intake for phytonutrients such as carotenoids and phenolic compounds does not currently exist, it is known that their consumption provides relevant health benefits beyond their antioxidative

Table 4. Method validation parameters from the high performance liquid chromatography–fluorescence detector analysis of capsaicin and dihydrocapsaicin.

| Analyte | CR | Parameters† | | | | | Day to day |
| | | *a* | LD | LQ | *R*² | RSD | RSD |
	——— µg mL⁻¹ ———		——— ng mL⁻¹ ———			——— % ———	
Capsaicin	1–10	37031	2.38	7.95	0.998	0.19	0.28
Dihydrocapsaicin	1–10	94329	0.54	3.12	0.999	0.19	0.59

† CR, concentration range; *a*, slope; LD, limit of detection; LQ, limit of quantification; *R*², linear regression coefficient; RSD, relative standard deviation.

was low, with a relative standard deviation of 0.28% for capsaicin and 0.59% for dihydrocapsaicin.

Optimization of Extraction Solvent and Sonication Time

Although different solvents are mentioned in the literature for the extraction of capsaicinoids, methanol (Antonious et al., 2009; Chinn et al., 2011; Wahyuni et al., 2011) and acetonitrile (Singh et al., 2009) are most frequently used. When comparing different solvents (methanol, acetone, and acetonitrile) for the extraction of capsaicinoids, acetonitrile was found to be the most efficient and allowed for extracts with higher purity compared with other solvents (Karnka et al., 2002). In this study, methanol and acetonitrile were tested for capsaicinoid extraction efficiency. Acetonitrile was found to be better than methanol for capsaicin and dihydrocapsaicin extraction (Fig. 3).

To improve solvent extraction, some techniques such as ultrasound (Wahyuni et al., 2011), heating (Chinn et al., 2011), or soxhlet (Peña-Alvarez et al., 2012) are employed. However, methods such as soxhlet have disadvantages when compared with ultrasound, since they require the use of a larger quantity of sample, solvent, and time of extraction (Peña-Alvarez et al., 2012). Moreover, higher temperatures can promote degradation of the target compounds.

Upon choosing acetonitrile as the extraction solvent, the effect of sonication for different time periods was tested and 10 min was found to be the optimal treatment duration (Fig. 4). Once the extraction conditions were

established, the samples were spiked with the standards to determine the recuperation yield that accounted for 75.7% for capsaicin and 79.8% for dihydrocapsaicin, with a relative standard deviation of 2.8 and 3.4%, respectively.

Capsaicinoid Analysis
HPLC-FLD

Capsaicin content of *Capsicum* accessions determined by HPLC-FLD ranged from 0.10 mg 100 g⁻¹ (P105, *C. baccatum*) to 1935.77 mg 100 g⁻¹ (P236, *C. chinense*) and averaged 191.4 mg 100 g⁻¹ of fruit on a dry-weight basis. The average capsaicin concentration found in *Capsicum* fruit studied here is on the same order of magnitude of that found for 16 *Capsicum* fruit from Nepal (Thapa et al., 2009), which had up to 1973 mg 100 g⁻¹ on a dry-weight basis. The large variability observed here was also seen in 63 accessions of *C. chinense* (Antonious et al., 2009), whereby the maximum value was 100 times greater than the minimum. When comparing among species (Table 5), on average, fruit of *C. chinense* showed the highest capsaicin concentration (640.81 mg 100 g⁻¹), followed by *C. annuum* (229.09 mg 100 g⁻¹) and *C. baccatum* (114.05 mg 100 g⁻¹). Antonious and Jarret (2006) also reported a higher capsaicin concentration in *C. chinense* when compared with *C. frutescens* and *C. baccatum*. *Capsicum annuum*, on the other hand, had significantly lower capsaicin content than the other species tested (0.93 mg 100 g⁻¹). Dihydrocapsaicin content (Fig. 5) varied from 0.03 (P116) to 186.00 mg 100 g⁻¹ (P236), and the extreme values were found in *C. chinense*, while the average
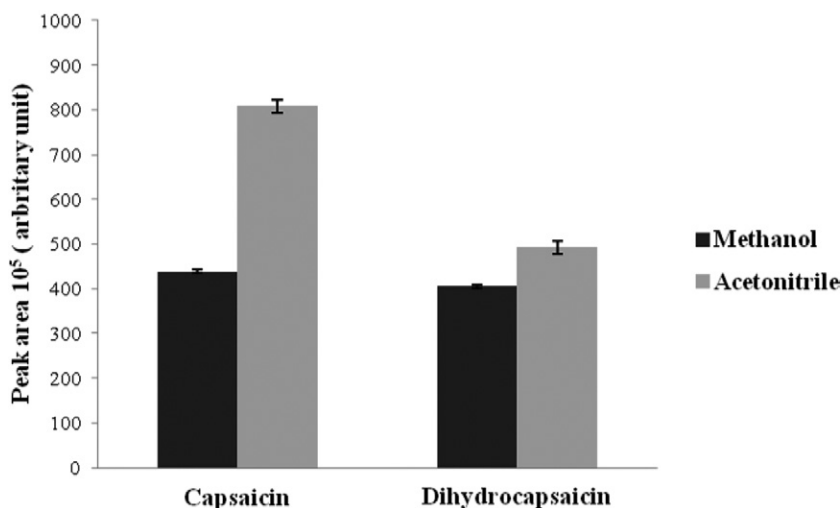
Fig. 3. Comparison of the extraction potential of capsaicinoids using methanol and acetonitrile. Bars indicate standard error of the mean (*n* = 3).
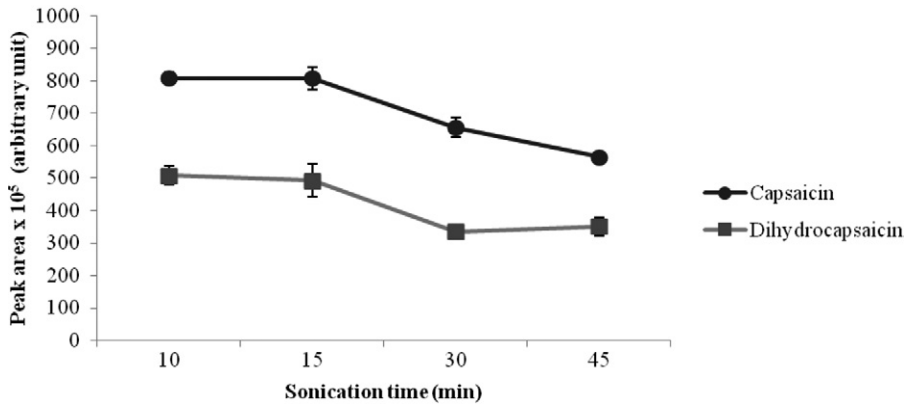
Fig. 4. Capsaicin and dihydrocapsaicin extracted with acetonitrile sonicated for different time periods. Bars indicate standard error of the mean ($n = 3$).

Table 5. Mean ($\pm$ SD) capsaicin and dihydrocapsaicin content of pepper accessions (*Capsicum* spp.).

Species	Capsaicin	Dihydrocapsaicin
	mg 100 g⁻¹ dry wt.	
Capsicum annuum	229.09 ± 284.40b	50.55 ± 59.23a
Capsicum baccatum	114.05 ± 162.86b	19.46 ± 26.74a
Capsicum chinense	640.81 ± 888.71a	64.33 ± 84.89a

content of dihydrocapsaicin was 28.18 mg 100 g^{-1}. Similar to capsaicin content, *C. chinense* fruit had higher average dihydrocapsaicin content (64.33 mg 100 g^{-1}) than *C. annuum* (50.55 mg 100 g^{-1}) and *C. baccatum* (19.46 mg 100 g^{-1}). In a study of 11 *Capsicum* genotypes, Garcés–Claver et al. (2006) reported capsaicin concentrations ranging from 0.8 to 663.9 mg 100 g^{-1} and dihydrocapsaicin concentrations from 0.2
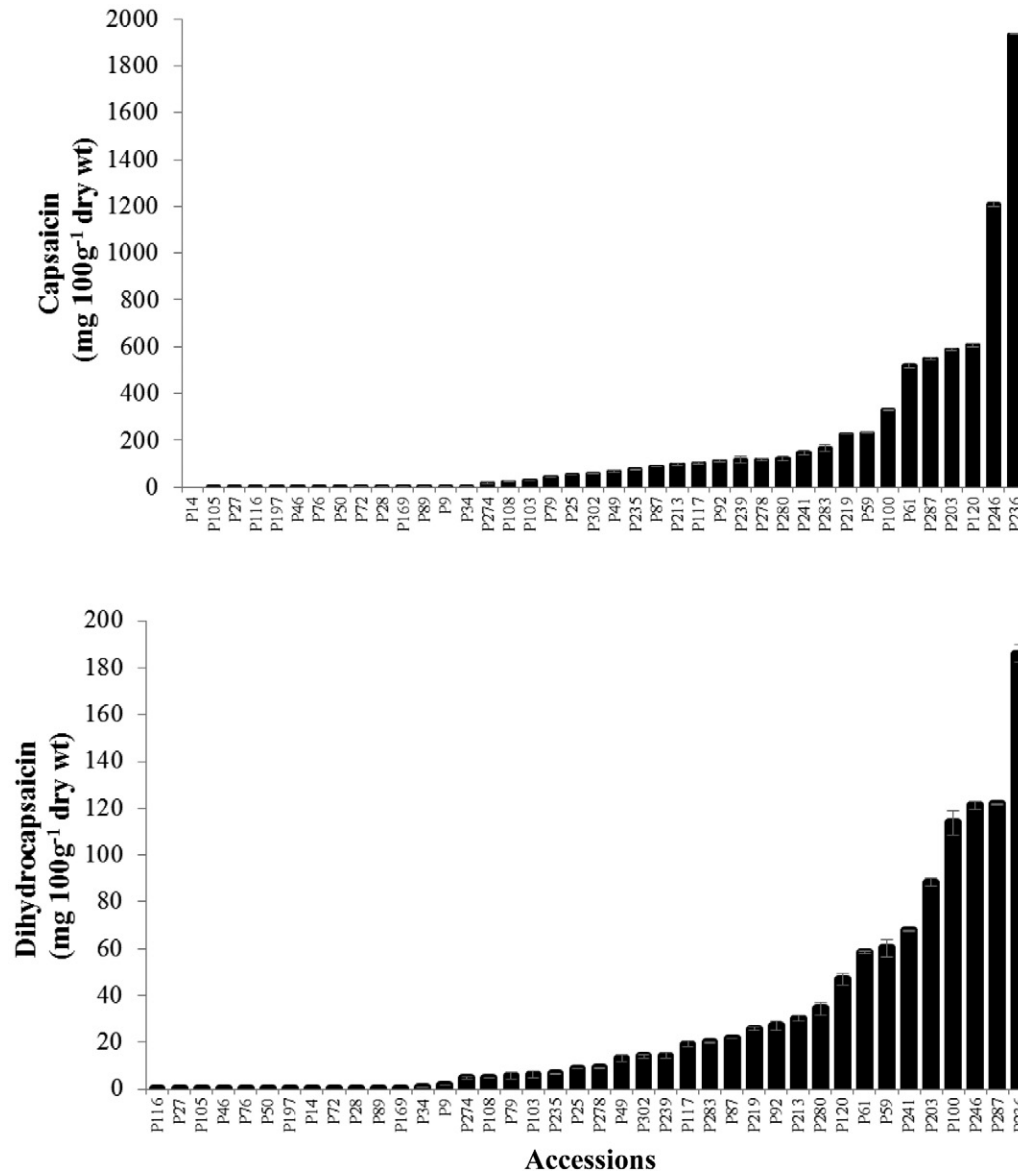

Fig. 5. Capsaicin and dihydrocapsaicin content of 40 pepper accessions (*Capsicum* spp.). Bars indicate standard error of the mean ($n = 3$).

Fig. 6. Accessions with the highest capsaicinoid content. Images: Carla Sigales de Vasconcelos and Juliana Castelo Branco Villela.

to 372.7 mg 100 g^{-1}. Despite *C. annuum* being known as sweet and nonpungent, studies have demonstrated the presence of capsaicinoids in *C. annuum* fruit (Estrada et al., 2002; Garcés-Claver et al., 2006). Therefore, this species contains genotypes with and without pungency. This phenomenon may be related to possible changes in the genome during the evolution of the genus *Capsicum* caused, for example, by the action of retrotransposons, pieces of DNA able to move throughout the genome and alter genome structure and gene expression (Tam et al., 2009). Capsaicinoid variability, as well as variability of other metabolites in a

germplasm collection, is desirable, demonstrating potential for the development of new cultivars that are more productive, adapted, tolerant to diseases, and have characteristics to satisfy the consumer, such as high bioactive compound content. In this study, genotypes P14, P105, and P27 (all genotypes of *C. baccatum*) are candidates to become low-pungency pepper cultivars since they showed the lowest concentration of capsaicinoids, which was a few thousand times less than the average of all genotypes evaluated. On the other hand, genotypes P236 (*C. chinense*), P246 (*C. baccatum*), and P120 (*C. baccatum*) showed the greatest potential to be developed into pungent cultivars (Fig. 6), with capsaicinoid concentration three times higher than the average.

UHPLC-QTOF Mass Spectrometry

To confirm the identity of the measured capsaicinoids, 2 of the 40 samples were also analyzed by UHPLC-QTOF mass spectrometry. On the basis of the total ion chromatogram of each sample, some capsaicinoids were identified within a 5-mDa error based on the exact mass of each compound. Five different capsaicinoids were identified: capsaicin, nordihydrocapsaicin, dihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin (Fig. 7).
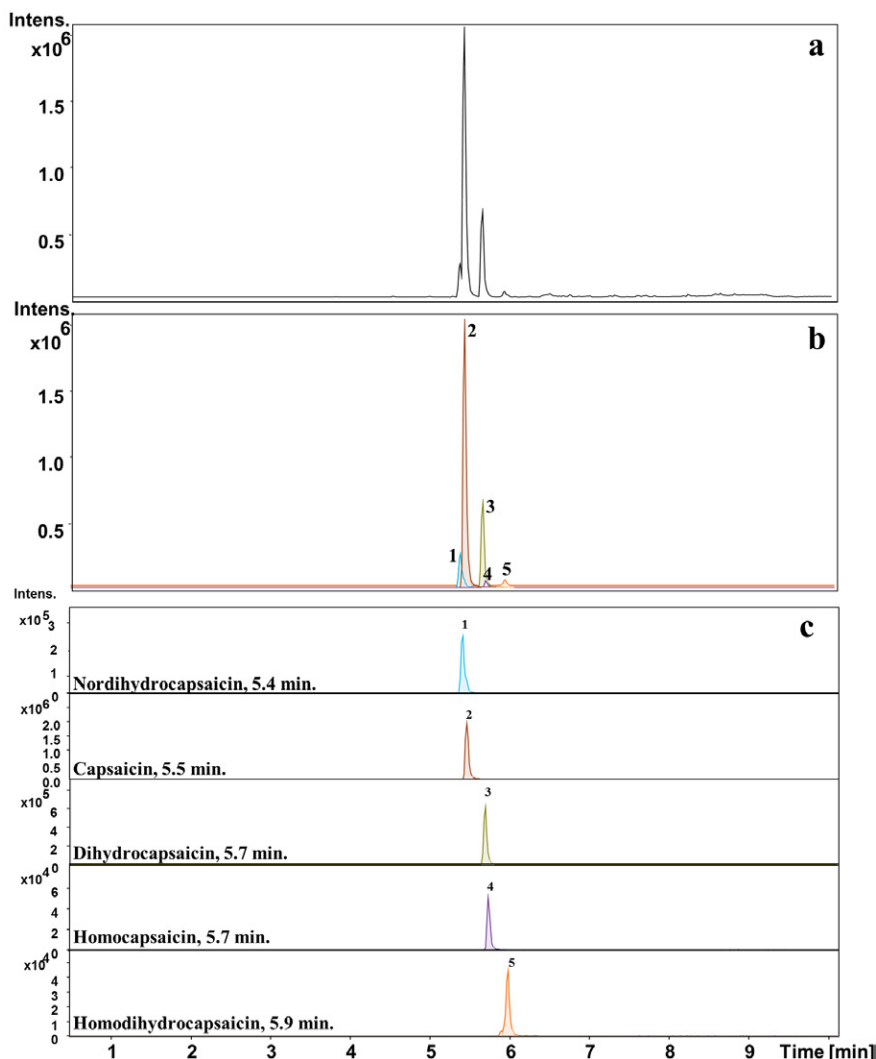
Fig. 7. (a) Total ion chromatogram and (b,c) extracted ion chromatograms of capsaicinoids extracted from *Capsicum* genotype P61.

The mass spectra of the peak with retention time of 5.4 min showed a signal with m/z 294.2069 ([M + H]$^+$: $C_{17}H_{28}NO_3$) corresponding to the molecular ion and base peak at m/z 293.1991, indicating the presence of nordihydrocapsaicin, with an error of 1.9 ppm, compared with the theoretical spectrum (Fig. 8). The mass spectrum of the peak at retention time 5.5 min showed a molecular ion and base peak at m/z 306.2068 ([M + H]$^+$: $C_{18}H_{28}NO_3$), indicating the presence of capsaicin, with an error of -1.4 ppm compared with the theoretical spectrum of this compound (Fig. 8). The compounds with a retention time of 5.7 min showed mass spectra with a molecular ion and a base peak at m/z 308.2218 ([M + H]$^+$: $C_{18}H_{30}NO_3$) and 320.2231 ([M + H]$^+$: $C_{19}H_{30}NO_3$), respectively, indicating the presence of dihydrocapsaicin and homocapsaicin, with errors of 0.6 and -3.5 ppm compared with the respective theoretical spectra (Fig. 8 and 9). The mass spectrum of the peak at retention time 6.0 min presented a base peak at m/z 322.2380 ([M + H]$^+$: $C_{19}H_{32}NO_3$) that confirmed homodihydrocapsaicin, with an error of 0.9 ppm compared with its theoretical m/z 322.2377 (Fig. 9).

The chromatogram in Fig. 5 shows the retention time of the identified capsaicinoid nordihydrocapsaicin as being 5.4 min, followed by capsaicin with a retention time of 5.5 min, dihydrocapsaicin and homocapsaicin with a retention time of 5.7 min, and homodihydrocapsacin with a retention time of 6.0 min. Meanwhile, by HPLC-FLD, only two capsaicinoid peaks were observed that matched the retention time of the standards, capsaicin with a retention time of 8.3 min and dihydrocapsaicin with a retention time of 11.3 min.
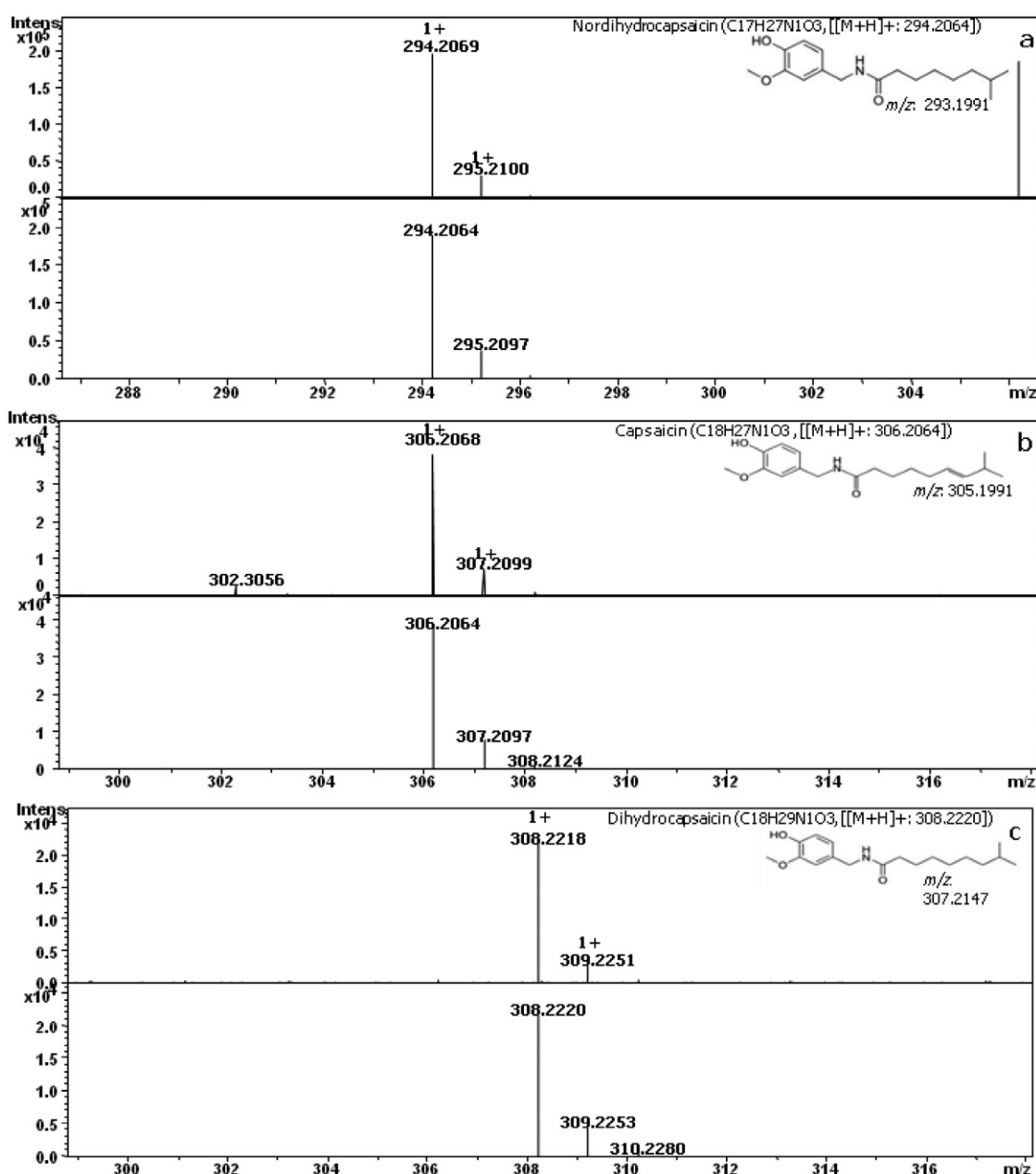

Fig. 8. Experimental and theoretical mass spectrum of (a) nordihydrocapsaicin, (b) capsaicin, and (c) dihydrocapsaicin.
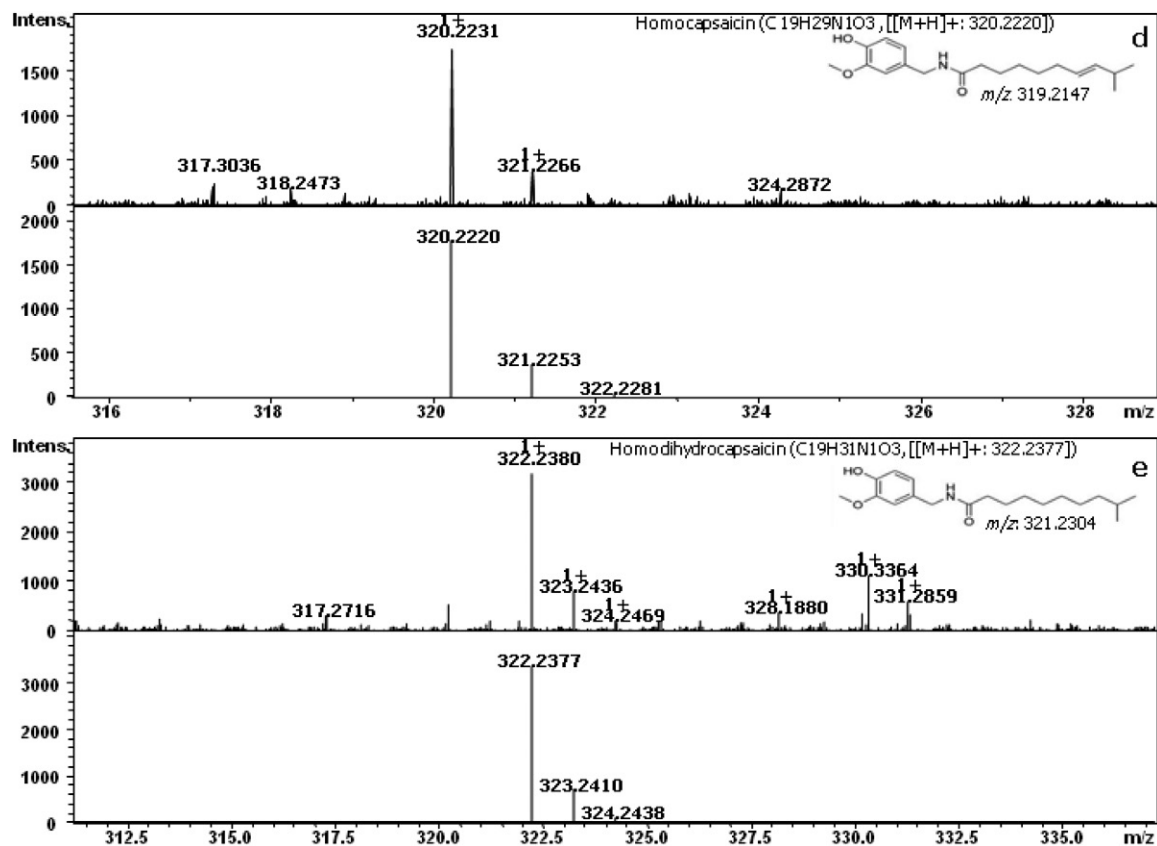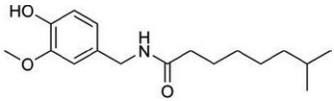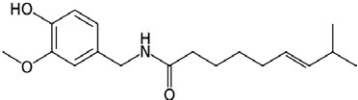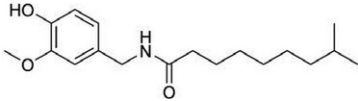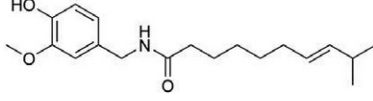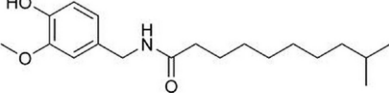
Fig. 9. Experimental and theoretical mass spectra of (d) homocapsaicin and (e) homodihydrocapsaicin.

Capsaicin and dihydrocapsaicin are considered the most pungent capsaicinoids and constitute up to 90% of the total capsaicinoid content found in peppers (Topuz and Ozdemir, 2007). In this study, capsaicin and dihydrocapsaicin, in a relative comparison with the other capsaicinoids found in the samples, accounted for ~80% of total capsaicinoid content (Table 6).

CONCLUSIONS

Data presented here highlighted the variability of physi-cochemical parameters measured in *Capsicum* pepper and demonstrated the potential of individual accessions in the Embrapa Temperate Agriculture *Capsicum* germplasm collection for breeding efforts guided not only by agronomic

Table 6. Relative quantitation (%) of capsaicinoids in pepper genotypes P28 and P61 analyzed by ultra-high performance liquid chromatography–quadrupole time-of-flight mass spectrometry.

tR†	Compound		P28	P61
min			——— % ———	
5.4	Nordihydrocapsaicin		16	18
5.5	Capsaicin		45	60
5.7	Dihydrocapsaicin		33	20
5.7	Homocapsaicin		3	2
6.0	Homodihydrocapsaicin		3	2

† tR, retention time.

characteristics but also by the bioactive compounds composition of the fruit for development of new cultivars.

The optimized extraction protocol for capsaicinoid analysis devised here was quick and simple, and the quantification method by HPLC-FLD showed low detection and quantitation limits for capsaicin (2.5 and 8 ng mL^{-1}) and dihydrocapsaicin (0.6 and 3 ng mL^{-1}) and had high reproducibility with limited variation. Analysis with UHPLC-QTOF mass spectrometry allowed for unequivocal confirmation of the quantified capsaicinoids, as well as the identification of other minor capsaicinoids present in *Capsicum* pepper fruit.

Acknowledgments

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