NOVEL METHOD TO SIMULTANEOUSLY SEPARATE CAPSAICINOIDS AND CAPSINOIDS AND PROFILING OF PHYTONUTRIENTS IN DIFFERENT TYPES OF PEPPER HYBRIDS

A Thesis

by

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ABSTRACT

Fruits and vegetables represent an essential source of phytonutrients, including antioxidants and other health-promoting compounds. Peppers (*Capsicum* spp.) are rich sources of many phytonutrients and accurate, rapid quantitation of the capsaicinoid and capsinoid compounds produced by peppers is essential to assess quality. Here, we developed a rapid ultra-high performance liquid chromatography method for the simultaneous separation of five major capsaicinoids and three major capsinoids from peppers. Optimal chromatographic separation was achieved using a phenyl-hexyl stationary phase with a mobile phase of acidified water and methanol with a flow rate of 0.5 ml/min at a column temperature of 55 °C over 5 minutes. The method was validated by testing linearity, precision, robustness, and limits of detection and quantification. The developed method was successfully employed to profile capsaicinoids and capsinoids from different pepper cultivars. Out of the 10 pepper cultivars analysed, all three major capsinoids were detected in two cultivars. To the best of our knowledge, this is the first report of successful separation of nordihydrocapsiate from capsiate and quantification of nordihydrocapsiate. Even with widely reported health benefits of these phytonutrients, limited studies have compared the composition of different types of peppers and their properties that may improve health. In this study, we measured the phytochemical composition of different pepper types, such as habanero, jalapeño, serrano, and ancho peppers. Significant differences were observed in the concentrations of phytonutrients in the different pepper types. In general, habanero-type hybrids had significantly higher vitamin C content as compared with the jalapeño, and serrano-type hybrids. Seven major

flavonoids were identified using ultra high-performance liquid chromatography-mass spectrometry. Habanero and ancho types had the highest flavonoid concentrations among different pepper hybrids. The ancho-type hybrid ‘TAM EH-227’ showed the highest total phenolics content (1338.13 μg g^{-1}), the habanero-type ‘TAM EH-45’ showed the highest DPPH free radical scavenging activity (850.82 μg g^{-1}), and the habanero type ‘TAM MH’ (1026.32 μg g^{-1}) had the highest ABTS free radical scavenging activity. Taken together, our results show that the phytonutrient contents in pepper hybrids vary substantially; this information lays a foundation for breeding peppers with high concentrations of health-promoting phytonutrients.

DEDICATION

I would like to dedicate this thesis to my parents, who have always supported me and encouraged me to keep pushing forward, for always giving me strength and motivating me to pursue my dreams.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Professor Bhimanagouda S. Patil the committee chair of the Department of Horticultural Sciences, Professor Kevin M. Crosby of the Department of Horticultural Sciences, Professor John Jifon of the Department of Horticultural Sciences, and Professor Giridhar Athrey of the Department of Poultry Science.

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1. INTRODUCTION

Cultivated peppers are all members of the genus *Capsicum* in the family Solanaceae. Peppers comprise five domesticated species: *C. annuum*, *C. baccatum, C. chinense, C. frutescens,* and *C. pubescens* (Pickersgill 1997). Production of peppers has steadily increased globally as they gain popularity as a vegetable and spice. In 2019, the annual global production of chili peppers was around 38 million tons with a cultivated area of 1.9 million hectares (FAO 2019). Peppers have also received attention due to their high levels of phytochemicals with well documented health-promoting properties. Peppers are a rich source of capsaicinoids, carotenoids, flavonoids, ascorbic acid (vitamin C), and tocopherols (vitamin E) (Howard et al. 2000).

Beneficial effects of ascorbic acid as a potent antioxidant and its role in neutralizing cancer-causing free radicals is well reported (Njus et al. 2020). Flavonoids are a class of secondary metabolites also present in peppers with many health-promoting properties including antioxidant, anti-inflammatory, and anti-proliferative activities (Garra et al. 2020). Flavonoids are classified into their subgroups based on the basic structure of aglycone, which are also present as flavonoid glycosides in a conjugated form (Yang et al. 2018).

Capsaicinoids are compounds exclusively produced by the fruits of *Capsicum* that impart characteristic pungency or heat in peppers (Uarrota et al. 2021). Capsaicinoids possess important biologically active functions such as increasing thermogenesis which helps combat obesity (Hernández-Pérez et al. 2020), analgesic effects (Arora, Campbell, and Chung 2021) and anti-tumor properties (Friedman et al. 2019). Capsinoids are analog

compounds that possess similar health benefits without the pungent properties (Gupta et al. 2021). Chemically, capsaicinoids and capsinoids have similar structures, the main difference being the amide bond in capsaicinoids and ester bond in capsinoids (Chen et al. 2019). Capsiate, dihydrocapsiate and nordihydocapsiate are the major capsinoids identified in peppers (Kobata et al. 1998; Kobata et al. 1999). It is critical to have methods that can accurately separate and quantify both classes of compounds due to their structural similarities and important biological functions. However, none of the existing liquid chromatography methods report accurate separation of all known natural capsaicinoids and capsinoids. Also, limited studies have been conducted that compare health-benefitting properties of different pepper types.

OBJECTIVES

1. To develop an ultra high-performance liquid chromatography method to separate and quantify major capsaicinoids and capsinoids in pepper.
2. To assess phytochemical profiles of different types of pepper hybrids.

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2. REVIEW OF LITERATURE

2.1. Health benefits of phytochemicals in peppers

2.1.1. Vitamin C

It is an essential nutrient ingested from a large variety of dietary sources. Vitamin C is and electron donor, which makes it a potent antioxidant (Padayatty et al. 2003). It also has a role in biosynthesis of collagen, tyrosine, and hormones (Levine et al. 1999). Vitamin C is widely present in fruits and vegetables as ascorbic acid and dehydroascorbic acid which are reduced and oxidized forms, respectively. Research has shown that ascorbic acid can prevent cancer by neutralizing the free radicals that cause DNA damage which further leads to tumor growth (Uddin and Ahmad 1995).

2.1.2. Flavonoids

Flavonoids are widely distributed plant secondary metabolites that are a vital part of human diet. Flavonoids are classified into several chemical groups based on their structure such as flavanones, flavones, flavanols, isoflavonoids, etc. (Yao et al. 2004). Chronic diseases such as diabetes, cancer, and cardiovascular diseases are caused by excessive inflammation and oxidative stress (Grivennikov, Greten, and Karin 2010). Flavonoids have shown to have potential in preventing several chronic diseases due to their antioxidant activity and anti-inflammatory properties (Maleki, Crespo, and Cabanillas 2019).

2.1.3. Capsaicinoids and capsinoids

Capsaicinoids are acid amides of vanillylamine that impart the pungency in peppers. Capsaicin and dihydrocapsaicin constitute around 90% of the total capsaicinoids in peppers (Barbero et al. 2014). Research has shown that capsaicinoids possess anti-cancer (Yang et al. 2010; Mori et al. 2006), antioxidant, and pain relief properties (Chung and Campbell 2016). Additionally, they can reduce obesity by enhancing thermogenesis (Li et al. 2020). Capsinoids are non-pungent compounds that have slight differences in their structure and possess similar biological properties (Uarrota et al. 2021). A study conducted in mice showed that capsinoids enhance thermogenesis and increase metabolic rate similar to the effects of capsaicinoids (Kawabata et al. 2009).

2.2. Existing methods to measure capsaicinoids and capsinoids

Dependable analytical techniques are critical to separate and quantify capsaicinoids and capsinoids due to their structural similarities. Capsiate, dihydrocapsiate and nordihydrocapsiate are the major capsinoids identified from peppers (Kobata et al. 1999). Different methods such as gas chromatography (Thomas, Schreiber, and Weisskopf 1998), colorimetric methods (Ryu et al. 2017), liquid chromatography-mass spectrometry (LC-MS) (Thompson et al. 2005), high-performance liquid chromatography (HPLC) (Barbero, Palma, and Barroso 2006) and ultra-high-performance liquid chromatography (UHPLC) (Sganzerla et al. 2014) have been applied to analyze these compounds. Liquid chromatography techniques are by far the most widely used to quantify these groups of compounds. Most reported LC methods use octadecyl (ODS or C-18) columns to separate

capsaicinoids and capsinoids (Barbero et al. 2016) (Singh et al. 2009) (Vázquez-Espinosa et al. 2021).

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3. SEPARATION OF NORDIHYDROCAPSIATE FROM CAPSIATE AND MAJOR CAPSAICINOID ANALOGUES USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

3.1. Introduction

Peppers are members of the *Capsicum* genus in the Solanaceae family. Production and consumption of peppers have increased over time due to their popularity as a vegetable and spice (Crosby, 2008); indeed, peppers have rapidly become an integral part of most cuisines across the globe. In 2019, the annual global production of chili peppers was around 38 million tons with a cultivated area of 1.9 million hectares (FAO, 2019). Popularity of peppers can also be attributed to their potential health benefits, due to the presence of phytochemicals such as carotenoids (provitamin A), ascorbic acid (vitamin C), tocopherols (vitamin E), phenolic compounds, flavonoids, and capsaicinoids (Topuz & Ozdemir, 2007).

Pungency (heat) is a unique characteristic of most hot pepper cultivars and is attributable to capsaicinoids, which are acid amides of vanillylamine and C9 to C11 branched-chain fatty acids exclusively produced by the fruits of capsicum plants (Díaz, Pomar, Bernal, & Merino, 2004). Capsaicin (C) and dihydrocapsaicin (DH-C) are the most abundant capsaicinoids, representing over 90% of the total capsaicinoids (Othman,

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* Reprinted from "Separation of nordihydrocapsiate from capsiate and major capsaicinoid analogues using ultra high performance liquid chromatography" by Kishan Biradar, Jashbir Singh, Syamkumar S Pillai, Kevin M Crosby, Bhimanagouda S Patil, 2022. Food Chemistry, 132585, Copyright (2022) Elsevier.

Ahmed, Habila, & Ghafar, 2011). Capsaicinoids have pharmacological properties such as analgesic (Knotkova, Pappagallo, & Szallasi, 2008), anti-cancer (Mori et al., 2006; Z.-H. Yang, Wang, Wang, Hu, Zheng, & Li, 2010), anti-inflammatory, and anti-oxidative activities (Surh, 2002). Moreover, they can reduce obesity by enhancing thermogenesis and modulating lipid metabolism (Li et al., 2020). Despite the health benefits of peppers, the pungency of capsaicin and other capsaicinoids likely limits the consumption of hot peppers because of its side effects such as irritation and a burning sensation. This has prompted a search for analog compounds called capsinoids that have the potential to induce similar metabolic effects without the pungency or heat properties. The pungency of capsinoids is about 1000 times lower than that of capsaicinoids (Tanaka et al., 2015), but capsinoids have similar biological effects as capsaicinoids (Luo, Peng, & Li, 2011).

Capsinoids and capsaicinoids have similar chemical structures except for their central linkage. Capsaicinoids are vanillylamide moieties with branched-chain fatty acids whereas capsinoids have an ester group instead of the amide group (Tanaka, Hosokawa, Miwa, Watanabe, & Yazawa, 2010). Capsiate (CTE) and dihydrocapsiate (DH-CTE) were the first identified capsinoids from a low-pungency cultivar, 'CH-19 Sweet' (*C. annuum* L.) (Kobata, Todo, Yazawa, Iwai, & Watanabe, 1998). Kobata et al. (Kobata, Sutoh, Todo, Yazawa, Iwai, & Watanabe, 1999) reported nordihydrocapsiate (NDH-CTE) as the third major capsinoid present in fruits of CH-19 Sweet with a ratio of 5:3:1 (CTE: DH-CTE: NDH-CTE).

Capsaicinoids and capsinoids have slight differences in their structures and biological activities, and large variability in the types and amounts present in different

pepper varieties and commercial products. This necessitates dependable analytical techniques for the separation and quantification of both families of compounds. Researchers have used several methods to analyze these compounds, such as gas chromatography (Thomas, Schreiber, & Weisskopf, 1998), colorimetric methods (Ryu, Kim, Kim, & Rhee, 2017), liquid chromatography-mass spectrometry (LC-MS) (Thompson, Phinney, Welch, & White V, 2005), high-performance liquid chromatography (HPLC) (Barbero, Palma, & Barroso, 2006) and ultra-high-performance liquid chromatography (UHPLC) (Sganzerla, Coutinho, de Melo, & Godoy, 2014). Among these methods, reverse phase-HPLC is the most commonly used analytical technique to quantify capsaicinoids and capsinoids. UHPLC significantly reduces solvent use through shorter runtimes and much smaller flow rates while improving resolution and sensitivity compared to HPLC methods.

Most of the reported liquid chromatography (HPLC and UHPLC) methods were used to quantify only one group of compounds, i.e. either capsaicinoids (Barbero, Liazid, Ferreiro-González, Palma, & Barroso, 2016) or capsinoids (S. Singh et al., 2009) from peppers. Recently a rapid UHPLC method was reported for simultaneous quantification of both families of compounds (Vázquez-Espinosa, González-de-Peredo, Espada-Bellido, Ferreiro-González, Barbero, & Palma, 2021). Unfortunately, this method failed to separate and quantify all the three known capsinoids from the pepper samples. Methods used to quantify capsinoids report only CTE and DH-CTE (S. Singh et al., 2009) among the natural capsinoids from peppers, even though standard NDH-CTE is available, and was isolated and reported as the third major natural capsinoid in pepper. To the authors' best

knowledge, none of the reported LC methods have separated NDH-CTE and CTE. Almost all LC methods used a conventional C-18 column to separate these groups of compounds. We noticed that injecting a standard mixture of capsinoids using existing methods gave a merged peak of CTE and NDH-CTE, which could lead to overestimation of CTE concentrations and limit the detection of NDH-CTE in pepper samples. Therefore, the purpose of this study was to develop a reliable, validated method that separates NDH-CTE and CTE. Overall, the validated method will be able to successfully separate and accurately quantify all the five major capsaicinoids (NDH-C, C, DH-C, H-C, and HDH-C) and three major capsinoids (NDH-CTE, CTE, and DH-CTE).

3.2. Materials and methods

3.2.1. Reagents

J.T. Baker water (LC-MS grade) was obtained from Avantor Performance Materials LLC. (Radnor, PA, USA). LC-MS grade methanol and formic acid were purchased from EMD Millipore Corporation (Burlington, MA, USA). Ethyl acetate and reference standards viz., nordihydrocapsaicin, capsaicin, dihydrocapsaicin, nordihydrocapsiate, capsiate and dihydrocapsiate of the highest available purity were purchased from Sigma-Aldrich (St Louis, MO, USA).

3.2.2. Plant materials

Pepper fruits at the green mature stage were collected from the experimental fields of the pepper breeding program at Texas A&M University Horticulture Teaching Research & Extension Center (Somerville, TX). Collected fruits were brought to the Vegetable and

Fruit Improvement Center (TX, USA) and stored at 4 °C until they were processed for further analysis.

3.2.3. Extraction of capsaicinoids and capsinoids

Extraction of capsaicinoids and capsinoids was done according to our previously published protocols with slight modifications (Bae, Jayaprakasha, Jifon, & Patil, 2012). Briefly, fresh pepper fruits were finely chopped, 1 g of fresh pepper sample was weighed, and 4 ml of ethyl acetate was added, homogenized at 15000 rpm for 2 minutes, sonicated for 30 minutes at 4 °C and then centrifuged at 4480 x *g* for 10 minutes. The supernatant was collected, and the residue was re-extracted in 3 ml of ethyl acetate following the steps mentioned above. The two filtrates were pooled, the total volume of the extracts was measured, and 1 ml of the extract was transferred to HPLC vials and stored at -80 °C until analysis.

3.2.4. UHPLC instrumentation and conditions

Capsaicinoids and capsinoids were analyzed with the 1290 Agilent Rapid Resolution LC system coupled to a 1290 Infinity Diode Array Detector (Agilent, Santa Clara, CA). The separation of target compounds was carried out on an Infinity Lab Poroshell 120 Phenyl-Hexyl narrow bore LC column (2.1 x 50 mm, 1.9 µm), Elipse Plus – C18 RRHD column (2.1 x 50 mm, 1.8 µm), and Eclipse Plus-C8 column (3.0 x 50 mm, 1.8 µm; Agilent, Santa Clara, CA) at 55 °C with a flow rate of 0.5 ml/min using a binary mobile phase consisting of (A) 0.2% formic acid in water and (B) methanol. The following gradient program was used to achieve the separation of capsaicinoids and capsinoids: 50–65% B (0–2.3 min),

65% B (2.3–3.3 min), 65–75% B (3.3–3.8 min), 75–80% B (3.8–4.0 min), 80–50% B (4.0–4.8 min) and 4.8–5.0 min B (50%) and the compounds were monitored at 280 nm.

3.2.5. UHPLC-HR-ESI-QTOF-MS based identification of capsaicinoids and capsinoids

Ethyl acetate extracts of pepper samples were analyzed by UHPLC-HR-ESI-QTOF-MS (Bruker Daltonics, Billerica, MA) using electrospray ionisation (ESI) in positive mode according to a previously published method with slight modifications (J. Singh, Jayaprakasha, & Patil, 2018). Briefly, data were acquired using full scan mass spectrum (MS) and broadband collision-induced dissociation (bbCID) mode at *m/z* 50–2000. Calibration of the mass spectrometer was done with sodium formate using high precision calibration mode. Using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) equipped with a Hamilton syringe (Reno, Nevada, USA) the calibration solution was injected at the end of each run directly coupled to the interface. Identification of compounds was done by comparing the mass accuracy, isotopic patterns, adducts, and fragment information obtained through SmartFormula editor.

3.2.6. Validation of the optimized method

Four sets of stock solutions were prepared as follows: A) a standard mixture of available capsaicinoids and capsinoids; B) an ethyl acetate extract of Tabasco pepper cultivar reported to contain five capsaicinoids and CTE spiked with a proportional amount of three capsinoids (NDH-CTE, CTE and DH-CTE); C) an ethyl acetate extract of the Tabasco cultivar without added standards; D) an ethyl acetate extract of pepper 509-45-1 containing all three capsinoids. For each of these samples, the following parameters were

assessed: linearity, limits of detection (LOD) and quantification (LOQ), inter-day and intra-day precision, and robustness.

3.2.6.1. Calibration curve and linearity

Calibration curves were constructed using standard solutions of three capsaicinoids (NDH-C, C, DH-C) and three capsinoids (NDH-CTE, CTE, DH-CTE) at different concentrations (0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125 and 250 µg/ml) prepared by serial dilution. The coefficient of determination (R^2) was calculated from the calibration curves and used to confirm the linear relationship between peak area and concentrations of the compounds. Standards are not available for H-C (quantified in terms of C) and HDH-C (quantified in terms of DH-C); therefore, they were quantified based on compounds with similar molecular structures.

3.2.6.2. Estimation of LOD and LOQ

The lowest concentrations from the standards used for the calibration curve were further diluted until the peak signals of the compounds could not be differentiated from the noise. The limit of detection (LOD) and limit of quantification (LOQ) were determined with signal-to-noise ratios (S/N) of 3 and 10 respectively.

3.2.6.3. Precision

Precision indicates the ability of an analytical method to give consistent results when the method is used to repeatedly measure similar samples. The precision of the optimized method was evaluated by performing repeatability (intra-day precision) and intermediate precision (inter-day precision) tests. Samples were injected ten times (n=10) on the same day for repeatability measurements. For intermediate precision, the samples were injected

ten times on three consecutive days (n=30). All the measurements were taken for standard mixture and samples as mentioned earlier in section 2.6. The relative standard deviation (% RSD) of the peak area and retention time of standards and samples were determined.

3.2.6.4. Robustness of the developed method

Robustness or ruggedness is another measure indicating the reliability of an analytical method, it is the capacity to remain unaffected by small variations in method parameters. To test the robustness of this method, we evaluated peak area and retention time while altering the following parameters: column temperature (45, 50, and 55°C), flow rate (0.45, 0.50, and 0.55 ml/min) and injection volume (0.5, 1, and 1.5 µl).

3.2.6.5. Accuracy

Accuracy or trueness of the method is the closeness between the result obtained by the analytical method and the true value. To test the accuracy of the developed method, an extract of Tabasco pepper was spiked with a known concentration of all six available standards, and their recovery was calculated using the below formula (Peris-Vicente, Esteve-Romero, & Carda-Broch, 2015). Spiking of the real sample was done to take into account the matrix effect of the sample matrix.

$$\%Recovery = \frac{C_S - C_U}{C_{STD}} X100,$$

Where, C_S is concentration in the spiked sample, C_U is concentration in the unspiked sample and C_{STD} is the true concentration of standard added.

3.2.7. Applicability of the developed method

Extracts from green mature fruits of 10 pepper accessions/cultivars were analyzed in triplicate using the developed method. Two commercial pepper products, chilli sauce and

cayenne pepper powder were purchased from local supermarket and analyzed using the developed method. The concentrations of capsinoids and capsaicinoids were quantified by using the optimized method mentioned above.

3.3. Results and discussion

3.3.1. Column selection and optimization of the chromatographic conditions

The objective of this study was to develop a UHPLC method that could separate major capsaicinoids and capsinoids, particularly NDH-CTE and CTE. Different stationary phases were evaluated to determine optimal separation and Table A1 shows the physicochemical properties of the different stationary phases tested. Octadecyl silica (ODS or C18) columns are widely used in reverse-phase liquid chromatography systems due to their ability to resolve a wide range of analytes. However, an appropriate stationary phase with improved selectivity could help separate structurally similar compounds. To overcome coelution of NDH-CTE and CTE, a phenyl-hexyl stationary phase was used, as this system is known to have improved selectivity and retention of aromatic analytes (John & William, 2009). The phenyl-hexyl column is considered a mixed-mode stationary phase in which the phenyl group provides π–π interactions and the hexyl (C6) ligand offers additional hydrophobic interactions (Baek, Lee, & Kim, 2017). In a study that separated hexabromocyclododecane (HBCD) diastereomers, coelution of δ- and ε-HBCD was observed on a C18 column and resolved using a phenyl-hexyl column (Baek et al., 2017). Hu et al. showed that the phenyl-hexyl column improved selectivity and speed in the separation of triacylglycerols compared to a C18 column (Hu, Wei, Lv, Wu, Dong, & Chen, 2014). The rationale behind using this stationary phase was to take advantage of the

presence of aromatic components in the structure of capsaicinoids and capsinoids and enhance their retention due to multiple interactions offered by phenyl-hexyl columns. The optimized method described below for the phenyl-hexyl stationary phase was also applied to C8 and C18 columns and we noticed merging of capsinoid peaks (Figure A1).

Acidified water (0.2% v/v formic acid) was used as solvent A, and methanol and acetonitrile were tested as solvent B. Although acetonitrile eluted the compounds faster and reduced retention times, we observed the coelution of capsinoids, which could be attributed to higher elution strength of acetonitrile as compared to methanol. Additionally, it has been reported that acetonitrile impedes π–π interactions, but methanol does not interfere with these interactions of phenyl groups, potentially making it a better choice (M. Yang, Fazio, Munch, & Drumm, 2005). Methanol was chosen as the organic modifier based on its lower interference and lower elution strength, which are important to avoid co-elution of capsinoids.

Column temperatures of 30 to 55 °C were tested with intervals of 5 °C to study the effect of column temperature on the separation and retention times and consequently choose the optimal temperature. Increasing temperatures led to reduced retention times of capsaicinoids and capsinoids. Additionally, higher temperatures decreased the back pressure, which can be explained by lower viscosity of the mobile phase at higher temperatures. The temperature was not further increased after 55 °C because a column temperature limit of 60 °C was recommended by the manufacturer. Therefore, 55°C was chosen as the optimal temperature, as it facilitated the lowest retention times while maintaining the separation of target compounds.

Reduced column back pressure due to higher temperatures allowed us to increase flow rates, which further helped in reducing retention times. Flow rate was gradually increased from 0.2 ml/min to 0.5 ml/min, which correspondingly reduced retention times. The flow rate was not further increased to avoid operating the system at very high pressure and 0.5 ml/min was chosen as the optimal flow rate.

Several different gradient programs had to be tested once the optimal temperature and flow rate were selected. An increase in the organic modifier (methanol) would elute the compounds earlier, but an excess would lead to overlapping peaks. Meanwhile, very low levels of methanol would lead to longer runtimes. The best resolution of capsaicinoids and capsinoids was achieved using the gradient program mentioned in the Materials and methods section (Figure 1).

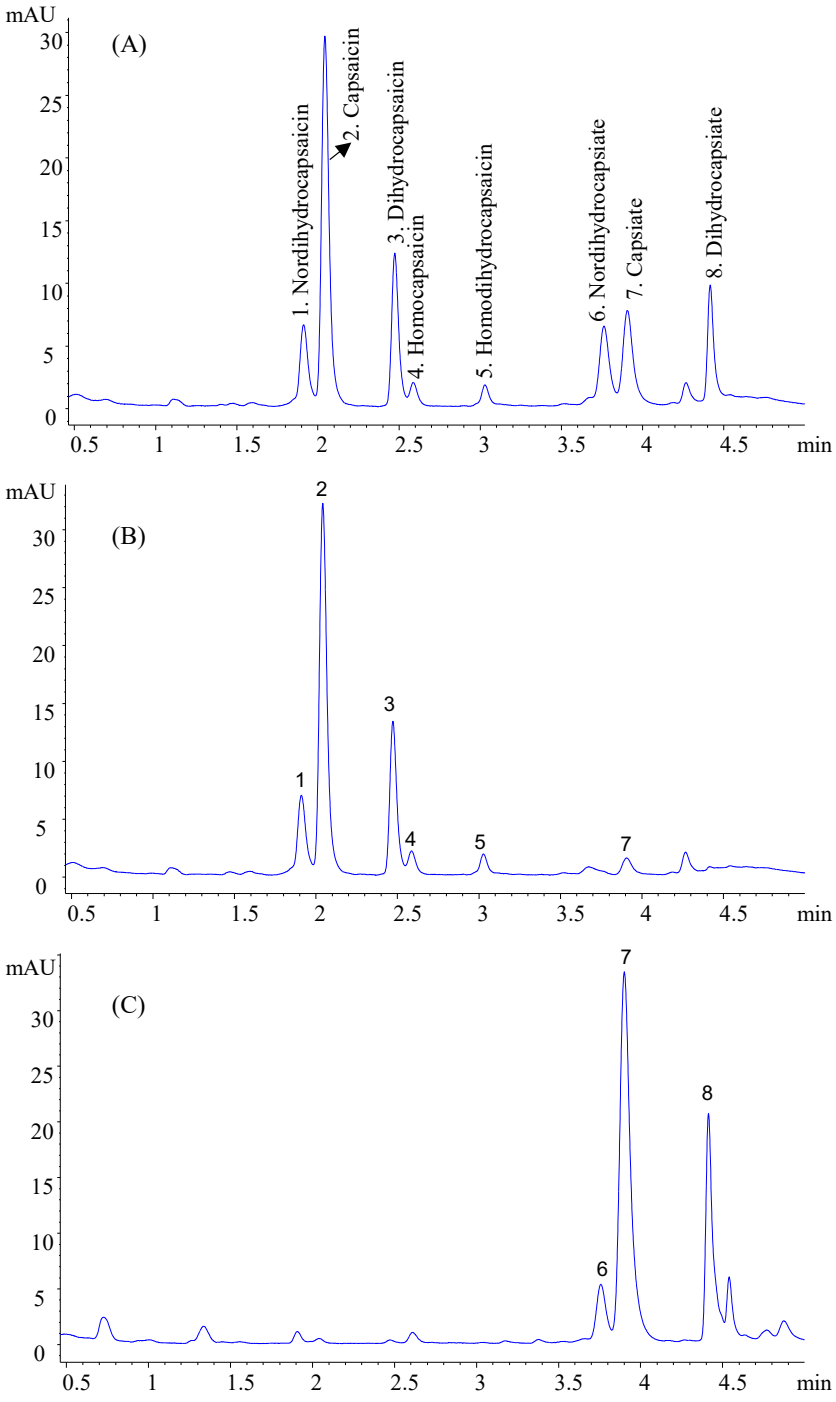

Figure 1. Chromatograms of (A) Tabasco pepper extract spiked with capsinoids; (B) Tabasco pepper extract without added standards; (C) 509-45-1 pepper extract, showing separation of capsaicinoids (Peaks: 1–5) and capsinoids (Peaks: 6–8) at 280 nm.

3.3.2. Identification of capsaicinoids and capsinoids

Ethyl acetate extracts of Tabasco peppers and pepper selection 509-45-1 were used to identify capsaicinoids and capsinoids, respectively. Capsaicinoids identified from extracts of Tabasco pepper in positive ion mode yielded mass spectra with major base peaks of protonated molecular ion $[M+H]^+$ with the following *m/z* ratios: NDH-C: 294.2050; C: 306.2067; DH-C: 308.2218; H-C: 320.2203; HDH-C: 322.2374. Whereas, major capsinoids identified from 509-45-1 pepper mainly showed sodium adducts $[M+Na]^+$ with the following *m/z* ratios: NDH-CTE: 317.1714; CTE: 329.1715; DH-CTE: 331.1865 (Table 1).

Table 1. Capsaicinoids and capsinoids identified in pepper extracts by UHPLC/HR-ESI-QTOF-MS in positive ionization mode.

Compound	Molecular formula	Experimental MS fragment (*m/z*)[a]	Mass error (ppm)[b]
Nordihydrocapsaicin	$C_{17}H_{27}NO_3$	294.2050 $[M+H]^+$	-4.66
Capsaicin	$C_{18}H_{27}NO_3$	306.2067 $[M+H]^+$	1.08
Dihydrocapsaicin	$C_{18}H_{29}NO_3$	308.2218 $[M+H]^+$	-0.71
Homocapsaicin	$C_{19}H_{29}NO_3$	320.2203 $[M+H]^+$	-5.37
Homodihydrocapsaicin	$C_{19}H_{31}NO_3$	322.2374 $[M+H]^+$	-0.84
Nordihydrocapsiate	$C_{17}H_{26}O_4$	317.1714 $[M+Na]^+$	-2.94
Capsiate	$C_{18}H_{26}O_4$	329.1715 $[M+Na]^+$	-2.52
Dihydrocapsiate	$C_{18}H_{28}O_4$	331.1865 $[M+Na]^+$	-4.47

[a]Accurate mass value
[b] Mass error in parts per million (10^6)

3.3.3. Validation of the developed method

Linearity, limits of detection (LOD) and quantification (LOQ): Calibration curves obtained from standards using nine points (0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, and 250 µg/ml) showed excellent linearity with correlation coefficients (R^2) > 0.99 (Table 2). LOD and LOQ were determined as 3 and 10 times the signal-to-noise (S/N) ratio, respectively. Detection and quantification limits were lower than 1 µg/ml (Table 2), which is similar to the results of previously reported methods (Stipcovich, Barbero, Ferreiro-González, Palma, & Barroso, 2018; Vázquez-Espinosa et al., 2021).

Table 2. Linearity, limits of detection (LOD), and limits of quantification (LOQ) of capsaicinoids and capsinoids.

Compound	Regression equation	R^2	LOD (µg/ml)	LOQ (µg/ml)
Nordihydrocapsaicin	y = 1688.8x + 0.7515	0.9999	0.06	0.18
Capsaicin	y = 988.65x + 0.8504	0.9998	0.24	0.72
Dihydrocapsaicin	y = 443.09x + 0.2353	0.9999	0.24	0.72
Nordihydrocapsiate	y = 1139.6x + 0.4169	0.9999	0.24	0.72
Capsiate	y = 1188.9x + 0.7303	0.9998	0.24	0.72
Dihydrocapsiate	y = 1044.7x + 1.2353	0.9992	0.24	0.72

Repeatability and intermediate precision: Repeatability (intra-day precision) and intermediate precision (inter-day precision) tests were conducted by injecting samples ten times for three consecutive days. Table 3 shows %RSD values for peak area and retention times for standard mix and spiked Tabasco sample and Table A2 shows the precision values for non-spiked Tabasco and 509-45-1 samples. The %RSD values for retention

time repeatability and intermediate precision were less than 1% for all peaks. The %RSD values for peak area precision were mostly lower than 3%, except for DH-CTE in spiked Tabasco samples, where it was about 5%. Overall, the method showed acceptable precision in terms of retention time and peak area.

Table 3. Intra- and interday precision of the optimized UHPLC method.

		Intraday*						Interday#	
		Day -1		Day -2		Day -3			
		RT (min)	Area	RT (min)	Area	RT (min)	Area	RT (min)	Area
Standard mixture	NDH-C	0.31	0.80	0.08	0.52	0.09	0.20	0.60	1.38
	C	0.31	0.93	0.06	0.56	0.10	0.27	0.60	1.06
	DH-C	0.28	0.66	0.06	0.51	0.07	0.25	0.56	1.33
	H-C	-	-	-	-	-	-	-	-
	HDH-C	-	-	-	-	-	-	-	-
	NDH-CTE	0.29	0.65	0.05	0.49	0.06	0.31	0.65	1.59
	CTE	0.29	0.62	0.05	0.47	0.06	0.29	0.69	0.92
	DH-CTE	0.12	1.67	0.02	1.86	0.02	0.47	0.31	1.84
Tabasco-spiked	NDH-C	0.05	1.26	0.09	1.48	0.06	1.29	0.54	1.97
	C	0.05	0.29	0.08	0.25	0.05	0.80	0.54	1.03
	DH-C	0.03	0.26	0.08	0.27	0.04	0.53	0.51	1.16
	H-C	0.05	2.06	0.06	1.59	0.05	1.50	0.49	1.89
	HDH-C	0.04	2.42	0.07	1.37	0.04	0.98	0.50	2.04
	NDH-CTE	0.04	0.38	0.07	0.20	0.04	3.95	0.63	3.90
	CTE	0.05	0.34	0.07	0.42	0.04	1.16	0.67	0.93
	DH-CTE	0.03	4.86	0.03	3.77	0.02	5.53	0.30	4.75

*Results show relative standard deviation (%RSD) values of 10 injections within each day, *n*=10

#Results show relative standard deviation (%RSD) values of 10 injections on 3 consecutive days, *n*=30

Abbreviations: NDH-C: nordihydrocapsaicin; C: capsaicin; DH-C: dihydrocapsaicin; H-C: homocapsaicin; HDH-C: homodihydrocapsaicin; NDH-CTE: nordihydrocapsiate; CTE: capsiate; DH-CTE: dihydrocapsiate.

Robustness: Robustness or ruggedness is the ability to remain unaffected by small variations in method parameters. To test the robustness of this method, we evaluated peak area and retention times with six injections each at three levels of the following parameters: column temperature, flow rate, and injection volume. The effects of varying these parameters are summarized in Table 4 for standard mix and spiked Tabasco samples and Table A3 for non-spiked Tabasco and 509-45-1 samples. An increase in temperature and flow rate reduced retention times but varying both parameters did not affect separation. The method remained comparatively unaffected with variation in injection volume in terms of retention time. However, in our preliminary experiments, we noticed distortion of peak shapes when higher injection volumes were used (>2.5µl). This could be attributed to the poor miscibility of ethyl acetate in water. Therefore, it is important to use appropriate injection volumes.

Table 4. Robustness/Ruggedness of the optimized method.

				NDH-C	C	DH-C	H-C	HDH-C	NDH-CTE	CTE	DH-CTE
Flow rate (ml/min)	Standard mixture	RT (min)	0.45	2.087±0.001	2.222±0.001	2.665±0.001	-	-	4.025±0.002	4.156±0.001	4.596±0.001
			0.5	1.917±0.001	2.047±0.001	2.476±0.001	-	-	3.741±0.001	3.879±0.001	4.419±0.001
			0.55	1.778±0.001	1.903±0.001	2.322±0.001	-	-	3.518±0.001	3.643±0.001	4.257±0.001
		Area	0.45	267.6±1.5	220.9±0.4	98.6±2.9	-	-	202.7±0.8	269.9±0.8	221.2±1.1
			0.5	246.9±0.6	205.2±0.5	86.6±0.3	-	-	187.5±0.5	248.2±0.7	203.2±2.4
			0.55	231.7±0.9	192.3±0.7	81.2±0.2	-	-	175.4±0.5	233.9±0.4	190.7±4.5
	Tabasco - spiked	RT (min)	0.45	2.087±0.001	2.222±0.001	2.664±0.001	2.779±0.001	3.239±0.001	4.026±0.001	4.158±0.001	4.597±0.001
			0.5	1.918±0.001	2.047±0.001	2.477±0.001	2.590±0.001	3.032±0.001	3.744±0.001	3.882±0.001	4.421±0.001
			0.55	1.779±0.001	1.904±0.001	2.322±0.001	2.433±0.001	2.865±0.001	3.518±0.001	3.645±0.001	4.258±0.001
		Area	0.45	29.9±0.3	117.0±0.7	45.9±2.6	8.8±0.5	7.5±0.3	31.8±0.9	42.1±1.2	30.9±2.6
			0.5	27.7±0.2	108.0±0.3	41.1±0.4	7.8±0.1	6.9±0.3	30.1±0.2	39.4±0.3	26.9±0.6
			0.55	25.8±0.5	100.5±0.3	38.1±0.4	7.2±0.3	7.1±0.8	28.6±0.5	37.5±0.4	24.7±0.6
Injection Vol (µl)	Standard mixture	RT (min)	0.5	1.916±0.002	2.046±0.002	2.474±0.002	-	-	3.760±0.003	3.902±0.003	4.413±0.001
			1	1.903±0.001	2.034±0.001	2.463±0.001	-	-	3.743±0.002	3.885±0.002	4.405±0.001
			1.5	1.899±0.001	2.029±0.001	2.458±0.001	-	-	3.738±0.001	3.879±0.001	4.403±0.001
		Area	0.5	113.2±0.7	91.8±0.6	39.6±0.2	-	-	85.5±0.3	111.1±0.5	92.8±3.0
			1	241.5±0.7	196.9±0.6	84.6±0.2	-	-	182.7±0.3	237.9±0.4	193.0±1.0
			1.5	377.9±1.7	312.3±1.2	133.5±0.5	-	-	287.5±0.9	374.9±1.0	303.9±1.5
	Tabasco - spiked	RT (min)	0.5	1.914±0.001	2.045±0.001	2.473±0.001	2.586±0.001	3.027±0.001	3.756±0.001	3.899±0.001	4.411±0.001
			1	1.904±0.001	2.035±0.001	2.464±0.001	2.577±0.001	3.018±0.001	3.744±0.001	3.887±0.001	4.406±0.001
			1.5	1.897±0.001	2.029±0.001	2.458±0.001	2.572±0.002	3.013±0.002	3.740±0.001	3.882±0.001	4.405±0.001
		Area	0.5	12.7±0.3	48.8±0.3	18.5±0.2	3.4±0.1	3.1±0.2	14.7±0.1	17.6±0.1	11.8±0.1
			1	26.5±0.2	103.2±0.3	39.6±0.2	7.1±0.1	6.8±0.1	30.9±0.1	37.2±0.2	26.3±0.8
			1.5	39.7±0.1	160.7±0.6	61.3±0.4	10.8±0.2	10.5±0.1	49.1±1.4	58.0±0.5	40.7±0.4

				NDH-C	C	DH-C	H-C	HDH-C	NDH-CTE	CTE	DH-CTE
Temperature (°C)	Standard mixture	RT (min)	45	2.127±0.003	2.268±0.003	2.717±0.003	-	-	4.188±0.004	4.286±0.004	4.628±0.003
			50	2.018±0.004	2.154±0.004	2.593±0.004	-	-	3.988±0.005	4.114±0.004	4.525±0.002
			55	1.901±0.004	2.030±0.004	2.458±0.004	-	-	3.733±0.007	3.873±0.007	4.401±0.003
		Area	45	262.2±1.2	212.5±0.9	91.4±0.4	-	-	198.1±5.0	256.9±0.6	211.4±0.8
			50	272.5±1.3	221.1±1.1	95.0±0.3	-	-	204.2±0.8	266.5±0.8	220.3±1.2
			55	285.5±1.3	232.3±1.3	99.9±0.4	-	-	216.1±0.9	279.9±1.0	227.4±1.1
	Tabasco - spiked	RT (min)	45	2.130±0.001	2.272±0.001	2.722±0.001	2.840±0.001	3.333±0.001	4.193±0.001	4.291±0.001	4.631±0.001
			50	2.014±0.001	2.151±0.001	2.589±0.002	2.706±0.002	3.165±0.001	3.983±0.002	4.111±0.002	4.523±0.001
			55	1.898±0.001	2.029±0.001	2.456±0.001	2.570±0.001	3.009±0.001	3.731±0.001	3.871±0.001	4.399±0.001
		Area	45	27.3±0.1	109.2±0.3	42.0±0.1	7.6±0.1	6.1±0.1	34.3±4.8	38.9±0.1	36.9±0.6
			50	28.3±0.2	111.9±0.5	42.9±0.3	7.3±0.3	5.9±0.3	32.9±0.2	39.6±0.2	43.1±7.2
			55	29.8±0.2	114.9±0.5	44.2±0.3	7.8±0.2	7.5±0.1	36.0±1.1	41.4±0.4	28.2±0.2

Results are represented as mean ± SD of six injections, *n*=6.

Abbreviations: NDH-C: nordihydrocapsaicin; C: capsaicin; DH-C: dihydrocapsaicin; H-C: homocapsaicin; HDH-C: homodihydrocapsaicin; NDH-CTE: nordihydrocapsiate; CTE: capsiate; DH-CTE: dihydrocapsiate.

Accuracy: Accuracy or trueness of the method is another important characteristic of the analytical method that provides information about the closeness of the obtained results to the true reference values. To obtain these results a sample was fortified with a known amount of target analytes and the accuracy is determined as recovery. We observed recoveries ranged between 99-104% of the spiked concentrations (Table A4), indicating that the developed method is efficient in accurately quantifying the capsaicinoids and capsinoids.

3.3.4. Application of the method for analysis of pepper samples

The developed method was used to quantify capsaicinoids and capsinoids from ten accessions of peppers and two commercial products (Table 5). This method can be used to accurately quantify these compounds and, for the first time, separate NDH-CTE from CTE in a total runtime of 5 minutes. All three capsinoids were found only in Himo and 509-45-1 peppers. The 509-45-1 pepper was released as a germplasm with high concentrations of capsinoids to be used in further breeding efforts. However, Jaret et al. only found CTE (757 µg/g) and DH-CTE (256 µg/g) in these fruits (Jarret, Bolton, & Perkins, 2014). Herein, we have successfully shown that all three capsinoids: NDH-CTE (122.76±6.28 µg/g), CTE (838.38±45.27 µg/g), and DH-CTE (372.8±20.59 µg/g) are present (Fig. 1C) and can be accurately quantified. The commercial products chili paste and cayenne pepper powder only had capsaicinoids and none of the capsinoids were detected. The highest concentrations of capsaicinoids were seen in Trinidad scorpion peppers, which also had capsinoids, CTE, and DH-CTE but no NDH-CTE.

Table 5. Levels of capsaicinoids and capsinoids in different pepper cultivars analyzed for method validation (n=3).

Name of hybrid/cultivar	Species	Capsaicinoids (µg/g FW)					Capsinoids (µg/g FW)		
		NDH-C	C	DH-C	H-C*	HDH-C*	NDH-CTE	CTE	DH-CTE
Experimental habanero hybrid - 1	*C. chinense*	6.6±0.9	269.9±15.3	107.8±7.7	nd	nd	nd	11.8±0.9	nd
Experimental habanero hybrid - 2	*C. chinense*	3.6±0.5	65.4±1.3	38.8±2.2	nd	nd	nd	6.2±0.5	nd
Experimental habanero hybrid - 3	*C. chinense*	nd	93.6±4.7	43.9±3.3	nd	nd	nd	28.2±2.2	nd
Megalodon	*C. chinense*	14.9±0.6	367.6±5.6	386.2±5.5	14.1±0.3	22.9±2.9	nd	8.9±0.5	1.9±0.2
Velociraptor	*C. chinense*	7.6±1.0	216.4±1.5	133.1±1.3	8.4±0.3	19.5±1.3	nd	5.1±0.5	nd
Habanero-51	*C. chinense*	nd	nd	nd	nd	nd	nd	61.5±1.9	16.0±0.8
Trinidad Scorpion	*C. chinense*	143.5±7.2	4734.5±231.1	1822.9±89.2	97.8±5.1	33.1±1.7	nd	301.0±15.2	21.1±0.8
Tabasco	*C. frutescens*	101.9±0.7	688.9±10.1	581.4±7.5	49.7±1.7	102.7±4.5	nd	35.3±1.6	8.2±0.9
Himo	*C. annuum*	nd	7.9±2.7	6.8±2.7	nd	nd	6.1±0.8	30.1±1.4	23.6±4.3
509-45-1	*C. annuum*	nd	nd	nd	nd	nd	122.8±6.3	838.4±45.3	372.8±20.5
Commercial products									
Chili sauce	-	3.0±0.8	29.4±2.5	26.7±2.9	nd	nd	nd	nd	nd
Cayenne pepper powder [#]	-	61.4±4.0	897.3±12.5	1303.4±22.1	54.3±3.3	nd	nd	nd	nd

Abbreviations: NDH-C: nordihydrocapsaicin; C: capsaicin; DH-C: dihydrocapsaicin; H-C: homocapsaicin; HDH-C: homodihydrocapsaicin; NDH-CTE: nordihydrocapsiate; CTE: capsiate; DH-CTE: dihydrocapsiate; nd: Not detected.
*H-C: homocapsaicin: quantified in terms of capsaicin; HDH-C: homodihydrocapsaicin; quantified in terms of Dihydrocapsaicin
#Analyzed by dry weight

3.4. References

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4. VARIABILITY OF PHYTONUTRIENT CONTENTS IN DIFFERENT PEPPER HYBRIDS

4.1. Introduction

Peppers (*Capsicum* spp.) are widely consumed worldwide as vegetable or used as a spice in several cuisines. Peppers comprise five domesticated species: *C. annuum*, *C. baccatum, C. chinense, C. frutescens,* and *C. pubescens* (Pickersgill 1997). Peppers are native to the Americas, but are widely cultivated in Asia, Africa, and Mediterranean countries (De Marino et al. 2006). In 2019, the annual global production of chili peppers was around 38 million tons with a production area of 1.9 million hectares (FAO 2019). Fruits of *Capsicum* are a rich source of many health-promoting phytonutrients such as capsaicinoids, carotenoids, flavonoids, ascorbic acid (vitamin C), and tocopherols (vitamin E) (Wahyuni et al. 2013).

Pungency (heat) is an important characteristic of hot peppers attributable to compounds called capsaicinoids, which are exclusively produced by the genus *Capsicum* (Andrews 1995). Capsaicin, dihydrocapsaicin and nordihydrocapsaicin are the primary capsaicinoids found in peppers (Kim et al. 2014). Capsaicinoids are amides of vanillylamine with branched-chain fatty acids (Luo, Peng, and Li 2011), biosynthesized in the placenta, and stored in vesicles on the surface of this tissue (Suzuki et al. 1980). Capsaicinoids have several health benefits such as pain relief, and they increase thermogenesis and body expenditure, thus becoming an effective tool for anti-obesity treatments (Zsiborás et al. 2018). Ascorbic acid is another functional and nutritional

constituent of peppers known for its antioxidant and biologically active functions. Evidence has also indicated that ascorbic acid can neutralize cancer-causing free radicals, reducing the occurrence of different DNA mutations caused by various oxidative stresses (Lutsenko, Cárcamo, and Golde 2002; Rodríguez-Burruezo et al. 2009). Flavonoids are a class of plant secondary metabolites that are divided into flavones, flavanols, flavanones based on the structure of the aglycone. The aglycones are generally coupled with glucosides, aliphatic, and aromatic acids in their natural state (Del Rio et al. 2013). Flavonoid derivatives possess many health-benefitting properties including antioxidant, anti-inflammatory, and anti-proliferative activities (Cho et al. 2020; Panche, Diwan, and Chandra 2016).

Peppers have been studied as rich sources of beneficial phytonutrients and secondary metabolites. For example, one study assessed capsaicinoids and ascorbic acid composition of five *C. annuum* cultivars (Topuz and Ozdemir 2007). In another study, antioxidant profiles and capsaicinoids contents were determined from four *C. annuum* varieties with varying pungency levels (Palma et al. 2020). Another study showed that flavonoids, ascorbic acid, and the antioxidant activity of five bell peppers varied as a function of maturity (Ghasemnezhad, Sherafati, and Payvast 2011). However, only a few studies have reported these compounds and compared antioxidant properties between different types of peppers. In this study, we investigated the content of ascorbic acid, capsaicinoids, and flavonoids, and measured the antioxidant activities of different habanero, jalapeño, serrano, and ancho pepper types.

4.2. Materials and methods

4.2.1. Plant material

Peppers were grown in experimental fields at Texas A&M University Horticulture Teaching Research & Extension Center (Somerville, TX). The details of the different pepper types that were used in the study are provided in Table 6 and the pictures are presented in Figure B1. All the peppers were harvested at their commercial harvest stage: full maturity stage for the habanero types (flesh surface completely yellow, orange, or red); at full size with flesh color still green for jalapeño, serrano, and ancho types. Harvested fruits were brought to the Vegetable and Fruit Improvement Center (TX, USA) and stored at 4 °C until they were processed for further analysis.

Table 6. Details of different pepper hybrids/cultivars used in the study.

S. No.	Pepper hybrid	Species	Pepper type
1	TAM Mild Habanero	*C. chinense*	Habanero
2	TAM Experimental Hybrid-11	*C. chinense*	Habanero
3	TAM Experimental Hybrid-22	*C. chinense*	Habanero
4	TAM Experimental Hybrid-24	*C. chinense*	Habanero
5	TAM Experimental Hybrid-31	*C. chinense*	Habanero
6	TAM Experimental Hybrid-32	*C. chinense*	Habanero
7	TAM Experimental Hybrid-44	*C. chinense*	Habanero
8	TAM Experimental Hybrid-45	*C. chinense*	Habanero
9	TAM Experimental Hybrid-76	*C. chinense*	Habanero
10	TAM Experimental Hybrid-5	*C. annuum*	Jalapeño
11	TAM Experimental Hybrid-189	*C. annuum*	Jalapeño
12	TAM Experimental Hybrid-211	*C. annuum*	Jalapeño
13	TAM Experimental Hybrid-166	*C. annuum*	Serrano
14	TAM Experimental Hybrid-219	*C. annuum*	Ancho
15	TAM Experimental Hybrid-227	*C. annuum*	Ancho

4.2.2. Chemicals and reagents

Methanol (LC-MS grade) and formic acid were purchased from EMD Millipore Corporation (Burlington, MA, USA). Ethyl acetate, meta phosphoric acid, tris (2-carboxy ethyl) phosphine hydrochloride (TCEP), L-ascorbic acid, gallic acid, sodium carbonate, 2,2-diphenyl-1-picryhydrazyl, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), Folin-Ciocalteu reagent, and reference standards (nordihydrocapsaicin, capsaicin,

dihydrocapsaicin, and quercetin) of the highest available purity were purchased from Sigma-Aldrich (St Louis, MO, USA).

4.2.3. Analysis of capsaicinoids

Capsaicinoid analysis was performed as described in our recent publication (Biradar et al. 2022). Briefly, fresh pepper sample (1 g) was weighed, and 4 ml of ethyl acetate was added, then the sample was homogenized at 15000 rpm for 2 minutes (850 Homogenizer, Thermo Fisher Scientific, Waltham, MA, USA), sonicated for 30 minutes at 4 °C and then centrifuged at 4480 x *g* for 15 minutes. The supernatant was collected, and the residue was reextracted in 4 ml of ethyl acetate following the steps mentioned above. The two filtrates were pooled, the total volume of the extracts was recorded, and 1 ml of the extract was transferred to HPLC vials and stored at -80 °C until analysis. Analysis of capsaicinoids was done using a 1290 Agilent Rapid Resolution LC system coupled to a 1290 Infinity Diode Array Detector (Agilent, Santa Clara, CA). The separation of capsaicinoids was carried out on an InfinityLab Poroshell 120 Phenyl-Hexyl narrow bore LC column (2.1 x 50 mm, 1.9 µm). The column temperature was 55 °C with a flow rate of 0.5 ml/min using a binary mobile phase consisting of (A) 0.2% formic acid in water and (B) methanol. The following gradient program was used to separate the capsaicinoids: 50–65% B (0–2.3 min), 65% B (2.3–3.3 min), 65–75% B (3.3–3.8 min), 75–80% B (3.8–4.0 min), 80–50% B (4.0–4.8 min) and 4.8–5.0 min B (50%) and the peaks were monitored at 280 nm.

4.2.4. Determination of ascorbic acid content

Pepper samples (1g) were extracted in 4 ml of 3% meta-phosphoric acid by homogenizing at 15000 rpm (850 Homogenizer, Thermo Fisher Scientific, Waltham, MA, USA) for 2 minutes, sonicated for 30 minutes and centrifuged at 4480 x *g* for 15 minutes. The extract was collected, and the residue was extracted again in 4 ml of 3% meta-phosphoric acid following the steps mentioned above. The two extracts were pooled, and final volume was recorded, 1 ml of the extract was transferred into HPLC vials and used for ascorbic acid quantification. Dehydroascorbic acid (DHA) analysis was done by adding 300 µl of tris(2-carboxyethyl)phosphine to 300 µl of the sample for reduction of dehydroascorbic acid to ascorbic acid as per a previously published protocol (Chebrolu et al. 2012). Analysis was carried out using an Agilent 1220 series HPLC system with an Eclipse plus C18 column (250 x 4.6 mm, 5 µm). A 10 µl sample was injected into the column with a flow rate of 0.5 ml min^{-1} and the peaks were monitored at 243 nm. The elution was carried out using gradient program with 0.3 M phosphoric acid (A) and methanol (B). Initially, elution was carried out at 0% B (0–7.5 min), followed by 0–30% B (7.5–9.0 min), isocratic 30% B (9–10 min) and returned to 0% B at 12 min.

4.2.5. Analysis of flavonoids

The flavonoids were extracted by adding 4 ml of methanol to 1g of fresh pepper sample, the samples were homogenized at 15000 rpm for 2 minutes (850 Homogenizer, Thermo Fisher Scientific, Waltham, MA, USA), sonicated for 30 minutes and centrifuged at 4480 x *g* for 15 minutes. The residue was extracted again by repeating the steps mentioned above to ensure complete extraction from the tissue. Both filtrates were pooled,

and the final volume of the extract was recorded prior to analysis. Analysis of flavonoids was done on 1290 Agilent Rapid Resolution LC system coupled to a 1290 Infinity Diode Array Detector (Agilent, Santa Clara, CA). The separation was achieved using an Agilent poroshell 210 phenyl-hexyl column (3.0 X 100 mm, 2.7 µm) using a gradient mobile phase of (A) acidified water and (B) methanol at a flow rate of 0.4 ml/min. Injection volume was 3 µl and the chromatograms were monitored at 360 nm.

4.2.6. High-resolution mass spectrometry

Mass spectral analyses for identification of flavonoids were performed in the positive ionization mode according to our previously published methodology (Singh, Jayaprakasha, and Patil 2018) with slight modifications. Briefly, the MS and bbCID (broadband collision-induced dissociation) spectra were acquired at the m/z range of 25–2000 amu. For the ion source capillary, the voltage was 4200 V, with the end plate offset at 500 V, and the nebulizer gas pressure was 2.8 bar. Nitrogen was used as a nebulizer and drying gas with the 8.0 l/min flow rate and the temperature was kept at 220 °C. The transfer time of the source was 72 µs and the prepulse storage time was 1 µs. The quadrupole MS and bbCID collision energy were set at 5 eV and 70 eV, respectively. The mass spectrometer calibration was performed with sodium formate solution (1 mM sodium hydroxide and water:isopropanol (1:1) with 0.2% formic acid) at the end of each run using a Cole Palmer syringe pump (Vernon Hills, IL, USA).

4.2.7. Determination of total phenolics

The total phenolic contents of pepper samples were determined using Folin-Ciocalteu (FC) reagent as described in our previous publication with slight modifications

(Singh et al. 2020). Briefly, 5 µl of methanol extract was added to each well of a microplate and adjusted to 200 µl using nanopure water. Standard gallic acid (50 µg ml $^{-1}$) in increasing amounts (10, 20, 30, 40, 50, 75, and 100 µl) was plated alongside the samples to prepare a standard curve. Then, 20 µl of FC reagent was added to all wells and incubated for 10 min; after incubation 50 µl of sodium carbonate was added and incubated for 20 minutes. The absorbance was measured at 760 nm and total phenolic content was expressed as micrograms of gallic acid equivalents per gram of sample.

4.2.8. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH radical scavenging activity was measured according to previously published protocol (Metrani et al. 2020). Briefly, 10 µl of sample was added to a 96-well plate in triplicate and the volume was adjusted to 100 µl using methanol. To each well, 180 µl of DPPH solution (0.1 mM) was added and incubated in the dark for 30 minutes. The absorbance was monitored at 515 nm, standard curve was prepared using ascorbic acid and the results were expressed as µg g^{-1} ascorbic acid equivalent.

4.2.9. 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

ABTS solution was prepared by adding 7 mM and 2.45 mM potassium persulfate, and the solution was kept in the dark for 16 hours before using it for the assay. The ABTS radical scavenging activity was measured according to a previously published method (Ravindranath et al. 2021). An aliquot (10 µl) of pepper extract was added to a 96-well plate, the total volume was adjusted to 100 µl using methanol. ABTS solution (180 µl) was added to each well to initiate the reaction, and the absorbance was measured at 734 nm, and the results were expressed as µg g^{-1} ascorbic acid equivalents.

4.3. Results and discussion

4.3.1. Capsaicinoids

Five major natural capsaicinoids were quantified from the pepper hybrids and there were significant differences in the levels of capsaicinoids (Table 7). In general, capsaicin was the major capsaicinoid in habanero types: however, in the jalapeño type hybrids, dihydrocapsaicin was found at higher levels. The habanero-type hybrid 'TAM EH-76' had the highest total capsaicinoid concentration (191.34 μg g^{-1}) among the hybrids. The jalapeño-type hybrid TAM EH-189 was the only hybrid where all five major capsaicinoids were detected, homodihydrocapsaicin (HDH-C) was not detected in any of the other peppers. None of the capsaicinoids were detected in the ancho type hybrid 'TAM EH-227', indicating that this hybrid could be a good choice for markets preferring mild or non-pungent peppers. In contrast to our results, another study reported capsaicinoids content was higher in serrano peppers than jalapeño peppers (de Jesús Ornelas-Paz et al. 2010). Our results are similar to previous reports which showing that *C. chinense* peppers have higher capsaicinoids contents than *C. annuum* peppers (Cisneros-Pineda et al. 2007).

Table 7. Levels of capsaicinoids quantified by HPLC in pepper samples.

Hybrids	Capsaicinoid concentration (μg g^{-1} fresh weight)					
	nDH-C	C	DH-C	h-C	hDH-C	Total
TAM MH	4.90±0.72^{abcd}	6.44±0.59^{e}	6.36±1.62^{c}	nd	nd	14.52±2.63^{e}
TAM EH-11	6.19±0.61^{a}	73.25±9.14^{ab}	61.71±8.29^{abc}	8.34±1.24^{ab}	nd	149.49±19.02^{ab}
TAM EH-22	2.33±0.23^{cd}	12.16±2.84^{e}	14.33±3.29^{c}	3.16±0.39^{b}	nd	25.66±6.97^{e}
TAM EH-24	4.26±0.63^{abcd}	31.88±9.43^{bc}	27.88±9.14^{bc}	nd	nd	63.49±19.07^{bcde}
TAM EH-31	6.18±1.23^{a}	70.63±15.87^{ab}	63.94±17.83^{abc}	14.79±3.98^{a}	nd	146.6±37.19^{ab}
TAM EH-32	1.92±0.36^{d}	3.90±0.79^{e}	nd	nd	nd	5.58±1.17^{e}
TAM EH-44	3.75±0.41^{abcd}	16.05±3.76^{e}	14.84±3.12^{c}	nd	nd	34.64±6.61^{de}
TAM EH-45	6.22±1.30^{a}	67.01±18.86^{ab}	44.97±11.52^{abc}	9.94±1.91^{a}	nd	124.11±33.68^{abcd}
TAM EH-76	5.25±0.41^{abc}	90.28±17.56^{a}	83.09±13.11^{a}	12.72±1.29^{a}	nd	191.34±32.15^{a}
TAM EH-5	2.26±0.32^{cd}	4.59±0.59^{e}	12.37±0.58^{c}	nd	nd	13.92±2.81^{e}
TAM EH-189	5.50±0.64^{ab}	38.57±7.14^{bc}	75.24±17.69^{ab}	3.21±0.65^{b}	7.44±1.59	129.03±27.25^{abc}
TAM EH-211	5.11±0.78^{abc}	43.50±3.86^{bc}	76.27±7.49^{ab}	nd	nd	124.88±11.99^{abcd}
TAM EH-166	2.67±0.21^{bcd}	10.05±1.80^{e}	16.66±2.62^{c}	nd	nd	29.38±4.44^{e}
TAM EH-219	3.11±0.19^{abcd}	13.28±3.17^{e}	31.37±9.29^{bc}	nd	nd	47.76±12.5^{cde}
TAM EH-227	nd	nd	nd	nd	nd	nd

Abbreviations: NDH-C: nordihydrocapsaicin; C: capsaicin; DH-C: dihydrocapsaicin; H-C: homocapsaicin; HDH-C: Homodihydrocapsaicin;
nd: not detected, different letters indicate significant differences ($p \leq 0.05$).

4.3.2. Ascorbic acid Content

Significant differences in ascorbic acid concentrations were observed among fifteen pepper hybrids tested (Figure 2). The ancho type 'TAM EH-227' had the highest total ascorbic acid content (1736.05 μg g^{-1}), and the jalapeño type 'TAM EH-211' had the lowest ascorbic acid (604.97 μg g^{-1}) content. In general, habanero-type hybrids had significantly higher ascorbic acid contents as compared to the jalapeño and serrano hybrids. This could also be because habanero types are consumed at the mature (red/orange) stage, whereas jalapeño and serrano types are consumed at the immature (green) stage.

An increase in ascorbic acid concentration as a function of maturity has been reported in several previous studies (Bae et al. 2014; Howard et al. 2000; Cisternas-Jamet et al. 2020). US Dietary Reference Intake (DRI) values for ascorbic acid are 75 and 90 mg/day for adult females and males, respectively (Monsen 2000). Considering these DRI values, consuming 100 g fresh fruits of these hybrids would suffice the DRI requirements for females for all hybrids except for 'TAM EH-211' (60.49 mg/100 g). For males, the habanero and ancho type hybrids fulfilled the DRI requirements per serving of 100 g fresh fruits, whereas the serrano and jalapeño types were slightly lower. Overall, the pepper hybrids analyzed in this study can be considered rich sources of vitamin C.

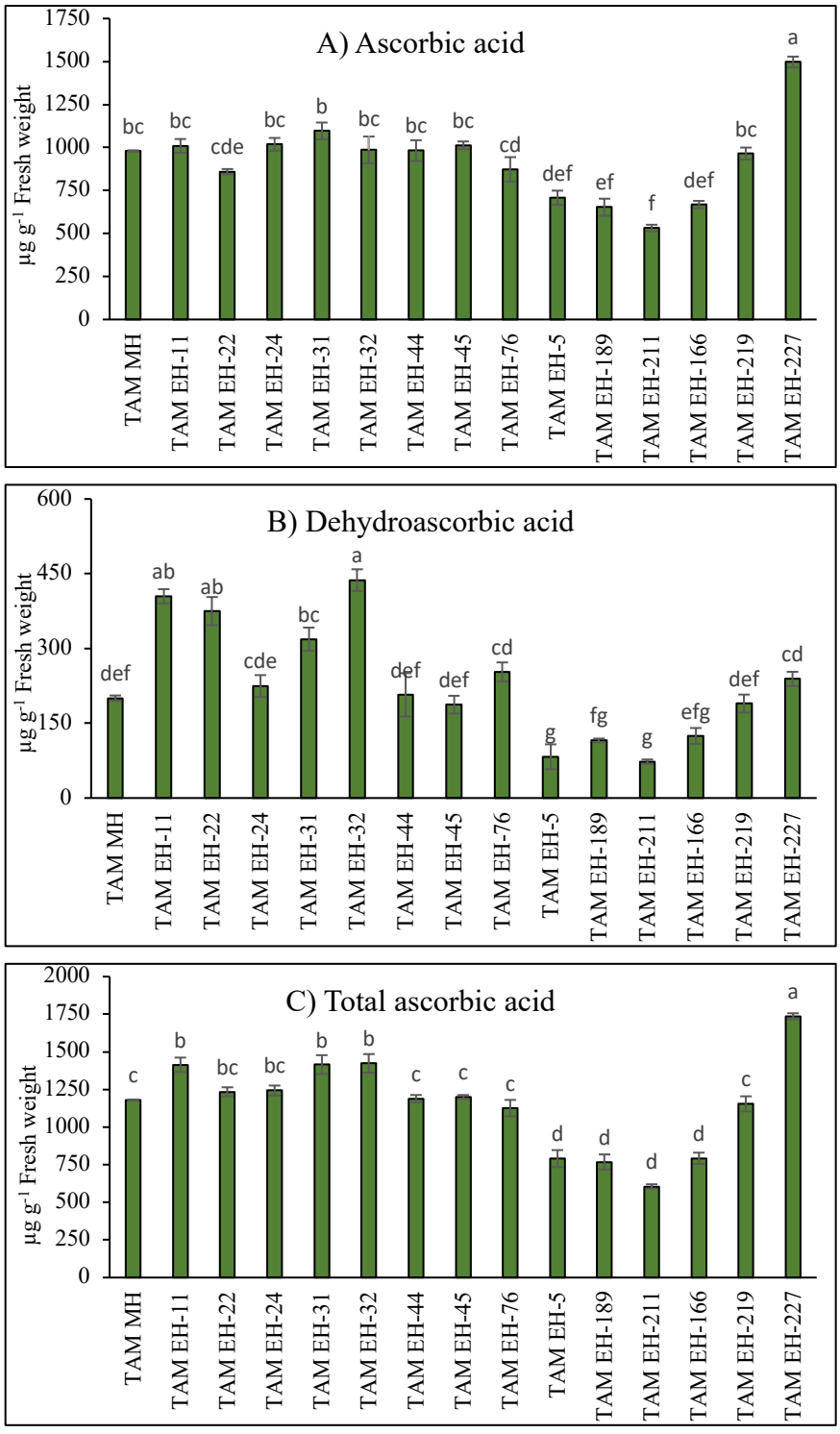

Figure 2. Variation of A) ascorbic acid, B) dehydroascorbic acid, and C) total vitamin C content among pepper hybrids. Values are means of four replications (n=4), different letters indicate significant differences ($p \leq 0.05$).

4.3.3. Identification of flavonoids by ultra high-performance liquid chromatography combined with electrospray ionization quadrupole time of flight mass spectrometry (UHPLC/ESI- QTOF-MS)

Seven major flavonoid compounds were identified in the pepper methanolic extracts. The accurate mass and mass error of the identified flavonoids are presented in Table 8 and the tandem mass spectra of the identified flavonoids are presented in Figure B2.

A peak eluted at retention time (RT) 5.3 min that showed an accurate mass spectrum at *m/z* 743.2021 $[M+H]^+$ (mass error: 1.10 ppm). Neutral loss of 162 mass units from precursor ion gives a product ion at *m/z* 581.1506. It underwent further loss of one molecule of glucose (-162 Da) and one molecule of pentose (-132 Da) and gave a prominent aglycone peak at *m/z* 287.0555 (mass error -1.69 ppm). Therefore, based on mass spectra and literature reports, the proposed compound was identified as luteolin-3-*O*-di-hexose-pentoside (Marín et al. 2004).

Similarly, a peak eluted at RT 5.8 min representing the molecular ion peak at *m/z* 449.1081 $[M+H]^+$ (mass error -0.58 ppm) and *m/z* 471.0897 $[M+Na]^+$. The +bbCID spectra comprised quercetin aglycone base peak at *m/z* 303.0501 (mass error: 1.08 ppm). Therefore, the current peak was identified as quercetin 3-*O*-rhamnopyranoside (Morales-Soto et al. 2013). The peak that eluted at RT 6.2 min displayed a precursor ion at *m/z* 757.2186 $[M+H]^+$ (mass error -0.04 ppm) and *m/z* 479.1996 $[M+Na]^+$. The product ion at *m/z* 595.1664 $[M+H-162]^+$ was yielded by the loss of a glucoside, which further lost one

apiosyl (-132 Da) and a glucoside (-162 Da) to give a prominent agylcone signal at *m/z* 301.0714. Thus, the present peak was identified as chrysoeriol-O-(apiosyl)-dihexoside.

Two peaks that eluted at RT 6.4 and 6.6 min were both identified as luteolin derivatives. The peak eluted at RT 6.4 displays an accurate mass spectrum at *m/z* 829.2043 $[M+H]^+$ (mass error -1.19 ppm). It loses two molecules of glucopyranoside, one apiofuranosyl residue (*m/z*132 Da) and one molecule of malonyl (*m/z* -86 Da) to give a prominent luteolin aglycone at *m/z* 287.0552. Thus, the present peak was identified as luteolin 7-*O*-(2-apiofuranosyl-4-glucopyranosyl-6-malonyl)-glucopyranoside based on mass spectrum and published literature (Morales-Soto et al. 2013). Similarly, a peak that eluted at RT 6.6 min represented the molecular ion peak at *m/z* 667.1495 $[M+H]^+$ (mass error 1.49 ppm) and aglycone *m/z* 287.0541 was identified as luteolin 7-*O*-(2-apiosyl-6-malonyl)-glucoside.

Another peak (RT 7.1 min) displayed a precursor ion at *m/z* 463.1233 $[M+H]^+$ (mass error 0.41 ppm). The product ion at *m/z* 301.0704 (mass error 0.88 ppm) was yielded by the neutral loss of a glucoside (*m/z* -162 Da). Thus, the present peak was identified as diosmetin 7-*O*-β-D-glucoside. Another peak eluted at RT 7.7 min was identified as chrysoeriol derivative, the precursor ion obtained at *m/z* 843.2195 $[M+H]^+$ (mass error -0.64 ppm). Precursor ion underwent sequential losses of 2 molecules of glucose, one apisoyl and a malonlyl residue to give a prominent peak *m/z* 301.0715 (mass error: -2.77 ppm). Thus, based on fragmentation pattern and literature the present peak was identified as chrysoeriol-*O*-(apiosyl-malonyl) dihexoside.

Table 8. Flavonoids identified in pepper extracts by UHPLC/HR-ESI-QTOF-MS in positive ionization mode.

RT (min)	Compound	Experimental MS fragment (*m/z*) [a]	Mass error (ppm)[b]
5.3	Luteolin-O-di-hexose-pentoside	743.2021$[M+H]^+$	1.10
5.8	Quercetin 3-rhamnopyranoside	449.1081$[M+H]^+$	-0.58
6.2	Chrysoeriol-O- (apiosyl)-dihexoside	757.2186$[M+H]^+$	-0.04
6.4	Luteolin 7-O-(2-apiofuranosyl-4-glucopyranosyl-6-malonyl) glucopyranoside	829.2043$[M+H]^+$	-1.19
6.6	Luteolin 7-O-(2-apiosyl-6-malonyl) glucoside	667.1495$[M+H]^+$	1.49
7.1	Diosmetin 7-O-β-D-glucoside	463.1233$[M+H]^+$	0.41
7.7	Chrysoeriol-O- (apiosyl malonyl) dihexoside	843.2195$[M+H]^+$	-0.64

[a]Accurate mass value

[b] Mass error in parts per million (10^6)

4.3.4. Flavonoids

Several studies report flavonoid analyses based on quantification of flavonoid aglycones after acid hydrolysis (Bae et al. 2014; Howard et al. 2000). This is due to flavonoids being present in conjugated forms and use of more sophisticated equipment such as LC-MS is needed to identify compounds in the extracts. In this study, the flavonoids were identified as described previously and quantified by UHPLC. Significant differences were observed in the levels of flavonoids among pepper hybrids (Table 9). The ancho-type hybrids 'TAM EH-219' and 'TAM EH-227' had significantly higher quercetin 3-rhamnopyranoside (51.3 and 42.5 μg g^{-1} respectively) as compared to other pepper types. Our results are similar to a previous report of quercetin 3-rhamnopyranoside in green bell pepper (42.2 μg g^{-1}) (Marín et al. 2004). Interestingly, the flavonoid chrysoeriol-O- (apiosyl)-dihexoside was only detected in habanero type hybrids and the levels were

significantly higher in TAM EH-11 (25.2 μg g^{-1}). The ancho type hybrids also had significantly higher luteolin 7-O-(2-apiosyl-6-malonyl) glucoside as compared to other types and the highest levels were seen in TAM EH-219 (34.1 μg g^{-1}). (Marín et al. 2004) also reported luteolin 7-O-(2-apiosyl-6-malonyl) glucoside in bell peppers (ranging from 3.9 to 41.4 μg g^{-1}), whereas pepper hybrids analyzed in this study ranged between 0.4 and 34.1 μg g^{-1}. The habanero type hybrid 'TAM EH-11' had significantly higher levels of diosmetin 7-O-β-D-glucoside (5.9 μg g^{-1}) and chrysoeriol-O- (apiosyl malonyl) dihexoside (24.8 μg g^{-1}). Based on our results, habanero and ancho type peppers are rich in flavonoids as compared to jalapeño or serrano types.

Table 9. Levels of flavonoids quantified by HPLC in pepper samples.

Hybrids	Flavonoid concentration (μg g^{-1} fresh weight)						
	1	2	3	4	5	6	7
TAM MH	1.6±0.2^{c}	4.2±0.2^{d}	14.1±1.1^{bc}	1.8±0.2^{b}	1.4±0.1^{f}	1.7±0.1^{b}	12.6±0.7^{b}
TAM EH-11	6.0±0.6^{b}	19.5±4.2^{bc}	25.2±2.9^{a}	7.5±0.9^{a}	11.2±2.4^{cd}	5.9±1.2^{a}	24.8±3.2^{a}
TAM EH-22	2.4±0.2^{c}	13.3±2.3^{cd}	10.4±0.8^{cd}	2.3±0.1^{b}	1.7±0.3^{f}	2.1±0.2^{b}	7.0±0.6^{bcde}
TAM EH-24	2.1±0.3^{c}	6.8±1.9^{cd}	11.9±0.9^{bcd}	2.7±0.4^{b}	1.9±0.5^{f}	2.1±0.5^{b}	9.7±1.1^{bc}
TAM EH-31	1.4±0.1^{c}	2.1±0.2^{d}	5.4±0.5^{de}	1.1±0.1^{b}	0.4±0.1^{f}	0.7±0.1^{b}	3.4±0.3^{def}
TAM EH-32	12.2±1.2^{a}	9.6±1.9^{cd}	1.3±0.1^{e}	7.2±0.4^{a}	2.1±0.2^{f}	nd	nd
TAM EH-44	2.4±0.3^{c}	6.9±0.4^{cd}	13.8±1.8^{bc}	1.8±0.1^{b}	1.6±0.2^{f}	1.8±0.1^{b}	7.6±0.6^{bcde}
TAM EH-45	2.2±0.2^{c}	5.1±1.8^{d}	7.5±0.7^{cde}	1.7±0.2^{b}	1.2±0.3^{f}	1.5±0.3^{b}	4.5±0.5^{cdef}
TAM EH-76	2.0±0.3^{c}	1.2±0.2^{d}	17.8±2.0^{b}	1.4±0.2^{b}	1.9±0.2^{f}	1.2±0.2^{b}	8.8±1.0^{bcd}
TAM EH-5	2.4±0.3^{c}	9.5±1.6^{cd}	nd	2.2±0.2^{b}	8.8±1.2^{de}	nd	0.9±0.1^{f}
TAM EH-189	1.5±0.2^{c}	11.3±0.5^{cd}	nd	1.6±0.2^{b}	9.8±1.0^{cde}	nd	1.0±0.2^{ef}
TAM EH-211	1.5±0.1^{c}	8.6±0.6^{cd}	nd	1.8±0.1^{b}	4.0±0.2^{ef}	0.5±0.1^{b}	nd
TAM EH-166	2.7±0.4^{c}	28.2±4.4^{b}	nd	1.8±0.2^{b}	15.3±2.9^{bc}	0.6±0.1^{b}	nd
TAM EH-219	5.8±0.4^{b}	51.3±7.1^{a}	nd	nd	34.1±2.0^{a}	1.0±0.1^{b}	nd
TAM EH-227	2.6±0.2^{c}	42.5±4.5^{a}	nd	nd	17.6±1.6^{b}	0.8±0.1^{b}	nd

Results are expressed relative to quercetin and are means of four replicates. Names of identified flavonoids: 1: Luteolin-O-di-hexose-pentoside; 2: Quercetin 3-rhamnopyranoside; 3: Chrysoeriol-O- (apiosyl)-dihexoside; 4: Luteolin 7-O-(2-apiofuranosyl-4-glucopyranosyl-6-malonyl) glucopyranoside; 5: Luteolin 7-O-(2-apiosyl-6-malonyl) glucoside; 6: Diosmetin 7-O-β-D-glucoside; 7: Chrysoeriol-O- (apiosyl malonyl) dihexoside;
nd: not detected, different letters indicate significant differences ($p \leq 0.05$).

4.3.5. Total phenolic content and antioxidant activities

Pepper hybrids showed significant differences in total phenolic contents and antioxidant activities (Figure 3 A-C). Phenolic compounds possess several beneficial properties such as anti-diabetic, anti-inflammatory, and antioxidant activities (Rodríguez-Pérez, Segura-Carretero, and del Mar Contreras 2019). The Folin-Ciocalteu (FC) method

was used to estimate the total content of phenolics in pepper hybrids and the data is presented in Figure 3A. The ancho-type hybrid 'TAM EH-227' showed significantly higher total phenolics content (1338.13 μg g^{-1}) than all other pepper hybrids. In general, habanero-type hybrids also showed higher total phenolic contents as compared to the jalapeño and serrano types. The jalapeño types had the lowest total phenolic content among which the hybrid 'TAM EH-211' had the lowest content (399.28 μg g^{-1}).

Reactive free radicals are associated with various conditions such as inflammation, damage to cell structures, etc., that can lead to chronic diseases such as cancer, diabetes, and cardiovascular diseases. Free radical scavenging activity or antioxidant activity represents one of the most important health benefitting properties of phytonutrients present in foods that help the body to combat oxidative stress. Therefore, several studies support utilization of antioxidants as a tool for disease management (Lobo et al. 2010).

In the current study, free radical scavenging activity of peppers was determined by performing DPPH and ABTS assays using methanolic extracts. DPPH free radical scavenging activity of the habanero-type hybrid 'TAM EH-45' was significantly higher (850.82 μg g^{-1} ascorbic acid equivalents) than other pepper hybrids. The highest ABTS free radical scavenging activity was seen in habanero-type 'TAM MH' (1026.32 μg g^{-1}ascorbic acid equivalents). In general, the habanero-type peppers had significantly higher antioxidant/free radical scavenging activity compared to other pepper types and the jalapeño types had the lowest antioxidant activity as shown in Figure 3B and 3C. The differences seen in the results obtained from ABTS and DPPH assays are possibly due to the type of reaction mechanisms, single electron transfer and hydrogen atom transfer

(Jayaprakasha, Girennavar, and Patil 2008). DPPH is scavenged by hydrophobic antioxidants and ABTS is scavenged by hydrophilic and hydrophobic antioxidants, which may also lead to the observed differences (Singh et al. 2020).

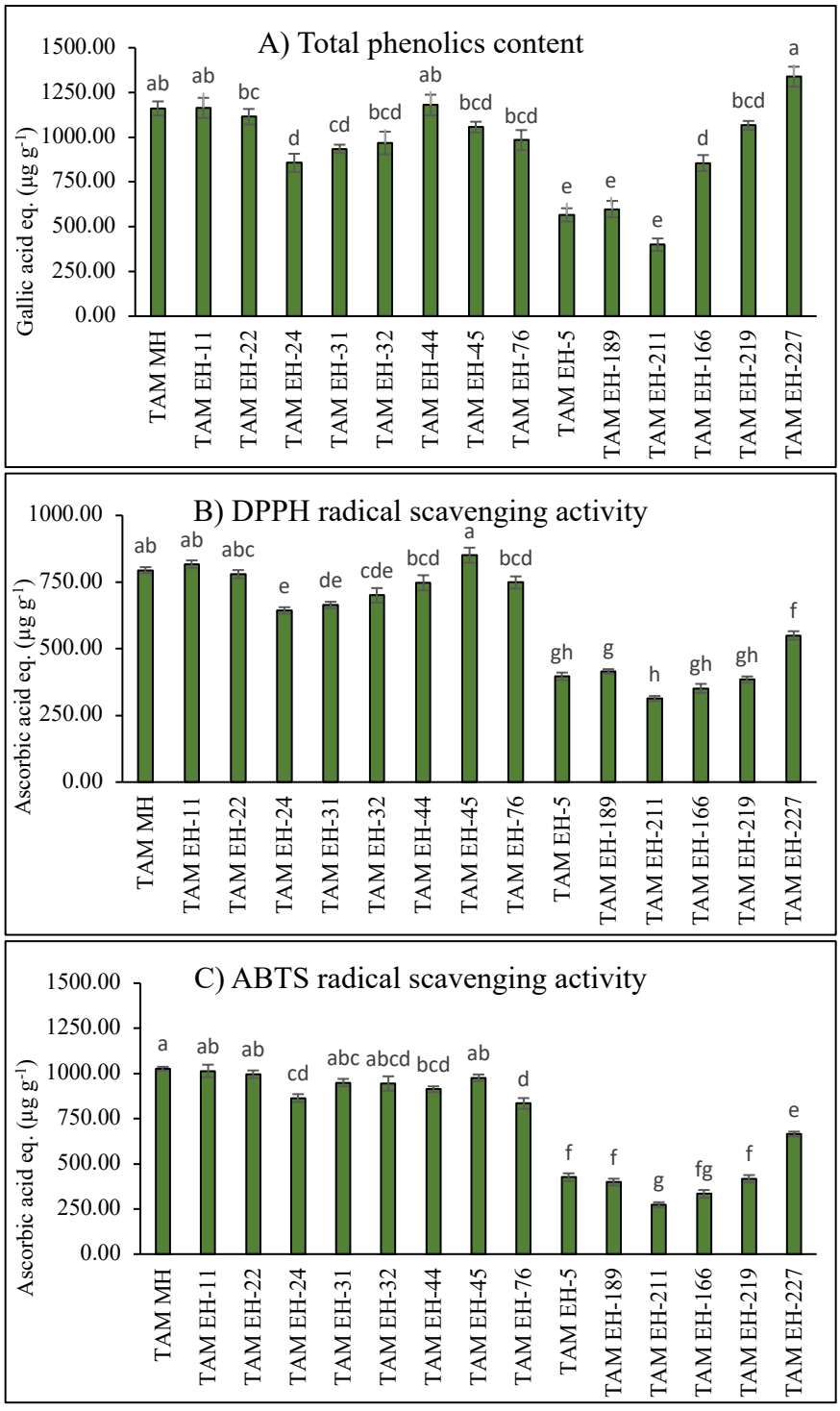

Figure 3. Comparison of A) total phenolic contents, B) DPPH radical scavenging activity, C) ABTS radical scavenging activity of pepper hybrids. The values are means of four replicates (n=4), different letters indicate significant differences ($p \leq 0.05$).

4.4. References

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5. CONCLUSIONS

This work presents the development and validation of a simple and rapid UHPLC method for the simultaneous separation and quantitation of capsaicinoids and capsinoids from peppers. The use of a phenyl-hexyl stationary phase enabled the separation of five major capsaicinoids and all three major capsinoids simultaneously for the first time, within 5 minutes of runtime. The method validation confirmed the linearity, limits of detection and quantification, precision, and robustness of the new method. The successful application of this method on pepper varieties/ selections for concurrent detection of pungent and non-pungent metabolites will be useful for the high-throughput screening of a large number of germplasms for these bioactive compounds, particularly for plant breeders to identify and develop more cultivars with higher levels of these beneficial compounds.

The phytochemical profiling gave us significant insight on the composition of health promoting compounds in different types of pepper hybrids. The concentrations of ascorbic acid were higher in habanero and ancho types as compared to jalapeño and serrano peppers. The pepper hybrids studied in this experiment had varying levels of pungency and we identified hybrids for markets that prefer mild or pungent peppers. Significant variation in the composition of flavonoid compounds and antioxidant capacity was observed among the hybrids. The habanero-type hybrids had significantly higher antioxidant capacity compared to other peppers. The variability among pepper types in phytonutrients found in this study provides a useful tool for breeders to further improve the nutritional quality of peppers.

APPENDIX A

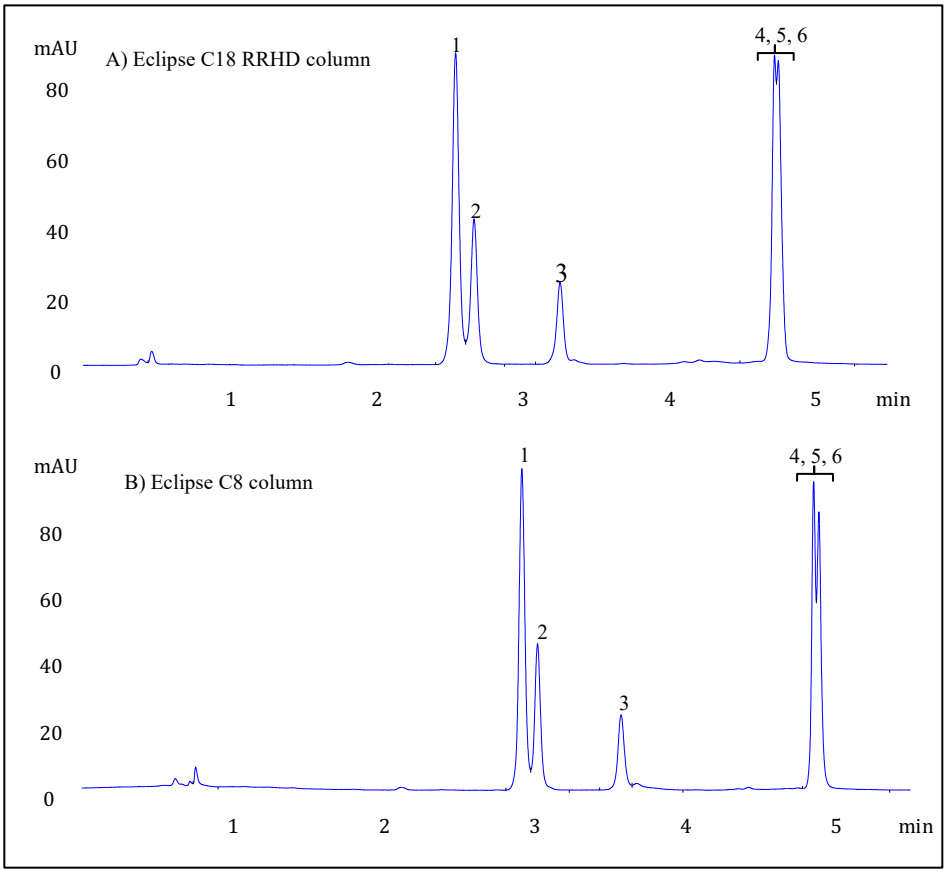

Figure A 1. Chromatograms of standard mixtures of capsaicinoids and capsinoids using A) Eclipse Plus C18 RRHD column; B) Eclipse Plus C8 column. Peak 1: Nordihydrocapsaicin, 2: Capsaicin, 3: Dihydrocapsaicin, 4: Nordihydrocapsiate, 5: Capsiate, 6: Dihydrocapsiate

Table A 1. The physico-chemical properties of stationary phases used for the analysis of capsaicinoids and capsinoids.

Column	Length, i.d (mm)	Particle size (µm)	Pore size (Å)	Carbon load (%)
Eclipse Plus, C-8	50, 3.0	1.8	95	7
Eclipse Plus, C-18 RRHD	50, 2.1	1.8	95	9
Poroshell 120 Phenyl-hexyl	50, 2.1	1.9	120	8

Table A 2. Intra- and interday precision of the developed method.

		Intraday*						Interday#	
		Day -1		Day -2		Day -3			
		RT (min)	Area	RT (min)	Area	RT (min)	Area	RT (min)	Area
Tabasco-Nonspiked	NDH-C	0.07	1.09	0.04	0.78	0.06	0.51	0.55	1.14
	C	0.08	0.65	0.04	0.39	0.05	0.39	0.56	0.52
	DH-C	0.08	0.51	0.04	0.44	0.06	0.58	0.53	0.53
	H-C	0.08	2.26	0.03	2.29	0.07	2.73	0.51	2.71
	HDH-C	0.07	2.59	0.03	2.81	0.05	1.04	0.51	2.25
	NDH-CTE	-	-	-	-	-	-	-	-
	CTE	0.11	1.18	0.02	1.21	0.07	2.82	0.68	2.99
	DH-CTE	-	-	-	-	-	-	-	-
509-45-1	NDH-C	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-
	DH-C	-	-	-	-	-	-	-	-
	H-C	-	-	-	-	-	-	-	-
	HDH-C	-	-	-	-	-	-	-	-
	NDH-CTE	0.05	0.57	0.05	0.71	0.04	2.87	0.66	4.71
	CTE	0.05	0.47	0.05	0.44	0.04	0.45	0.71	0.70
	DH-CTE	0.02	0.76	0.02	0.89	0.03	3.13	0.31	3.55

*Results show relative standard deviation (%RSD) values of 10 injections within each day, n=10

#Results show relative standard deviation (%RSD) values of 10 injections on 3 consecutive days, n=30

Abbreviations: NDH-C: Nordihydrocapsaicin; C: capsaicin; DH-C: Dihydrocapsaicin; H-C: homocapsaicin; HDH-C: homodihydrocapsaicin; NDH-CTE: nordihydrocapsiate; CTE: capsiate; DH-CTE: Dihydrocapsiate.

Table A 3. Robustness of the developed method.

				NDH-C	C	DH-C	H-C	HDH-C	HDH-CTE	CTE	DH-CTE
Flow rate (ml/min)	Tabasco-Non-spiked	RT (min)	0.45	2.086±0.001	2.221±0.001	2.662±0.001	2.778±0.001	3.237±0.001	-	4.157±0.002	-
			0.5	1.918±0.001	2.047±0.001	2.477±0.001	2.590±0.001	3.032±0.001	-	3.883±0.001	-
			0.55	1.778±0.001	1.904±0.001	2.322±0.001	2.433±0.002	2.865±0.002	-	3.645±0.002	-
		Area	0.45	32.2±0.2	126.7±0.9	48.1±0.3	9.1±0.4	7.9±0.4	-	8.3±1.2	-
			0.5	29.2±0.3	115.7±1.2	44.1±0.4	8.6±0.1	7.5±0.2	-	7.3±0.2	-
			0.55	26.9±0.1	106.5±0.6	40.3±0.3	7.5±0.2	6.8±0.4	-	6.6±0.1	-
	509-45-1	RT (min)	0.45	-	-	-	-	-	4.022±0.001	4.153±0.001	4.595±0.001
			0.5	-	-	-	-	-	3.745±0.001	3.882±0.001	4.421±0.001
			0.55	-	-	-	-	-	3.515±0.002	3.641±0.002	4.255±0.001
		Area	0.45	-	-	-	-	-	25.6±0.7	183.5±1.4	72.2±0.3
			0.5	-	-	-	-	-	23.8±1.3	166.0±1.4	66.7±0.5
			0.55	-	-	-	-	-	22.5±0.7	156.8±0.7	62.1±0.3
Injection Vol (µl)	Tabasco-Non-spiked	RT (min)	0.5	1.914±0.002	2.045±0.002	2.472±0.001	2.586±0.002	3.026±0.001	-	3.901±0.003	-
			1	1.904±0.002	2.035±0.002	2.463±0.001	2.577±0.001	3.017±0.001	-	3.888±0.002	-
			1.5	1.897±0.001	2.029±0.001	2.457±0.001	2.571±0.001	3.011±0.001	-	3.881±0.002	-
		Area	0.5	13.5±0.2	53.3±0.3	20.6±0.2	3.7±0.1	3.4±0.2	-	3.4±0.1	-
			1	28.4±0.2	112.1±0.4	43.1±0.2	7.8±0.2	7.7±0.1	-	7.0±0.1	-
			1.5	42.8±0.1	175.1±0.6	66.8±0.4	11.8±0.3	11.3±0.1	-	11.5±0.2	-
	509-45-1	RT (min)	0.5	-	-	-	-	-	3.750±0.003	3.892±0.003	4.408±0.002
			1	-	-	-	-	-	3.743±0.001	3.884±0.001	4.405±0.001
			1.5	-	-	-	-	-	3.737±0.002	3.877±0.002	4.403±0.001
		Area	0.5	-	-	-	-	-	9.9±0.1	76.3±0.4	31.9±0.8
			1	-	-	-	-	-	24.2±0.2	164.2±0.4	66.6±0.5
			1.5	-	-	-	-	-	37.7±0.7	255.4±0.4	102.9±0.6

				NDH-C	C	DH-C	H-C	HDH-C	HDH-CTE	CTE	DH-CTE
Temperature (°C)	Tabasco-Non-spiked	RT (min)	45 °C	2.131±0.001	2.273±0.001	2.721±0.001	2.840±0.001	3.334±0.001	-	4.292±0.001	-
			50 °C	2.013±0.001	2.149±0.001	2.588±0.002	2.704±0.001	3.164±0.002	-	4.109±0.002	-
			55 °C	1.896±0.002	2.026±0.002	2.453±0.002	2.567±0.002	3.005±0.002	-	3.868±0.003	-
		Area	45 °C	29.3±0.1	119.6±0.1	46.1±0.1	8.4±0.2	6.8±0.2	-	6.9±0.1	-
			50 °C	30.4±0.4	123.3±0.4	47.5±0.2	8.4±0.2	6.8±0.5	-	7.3±0.1	-
			55 °C	32.5±0.1	127.3±0.4	49.1±0.1	8.8±0.2	8.3±0.1	-	8.1±0.3	-
	509-45-1	RT (min)	45 °C	-	-	-	-	-	4.194±0.001	4.291±0.001	4.632±0.001
			50 °C	-	-	-	-	-	3.979±0.001	4.106±0.001	4.522±0.001
			55 °C	-	-	-	-	-	3.721±0.001	3.860±0.002	4.394±0.001
		Area	45 °C	-	-	-	-	-	24.2±0.1	173.2±0.4	73.9±1.8
			50 °C	-	-	-	-	-	24.5±1.0	178.8±0.3	76.6±1.7
			55 °C	-	-	-	-	-	26.4±1.6	185.1±0.5	75.9±0.4

Results are represented as Mean ± SD of six injections, *n*=6.

Abbreviations: NDH-C: nordihydrocapsaicin; C: capsaicin; DH-C: dihydrocapsaicin; H-C: homocapsaicin; HDH-C: homodihydrocapsaicin; NDH-CTE: nordihydrocapsiate; CTE: capsiate; DH-CTE: Dihydrocapsiate.

Table A 4. Accuracy of the developed method.

Compound	Recovery (%)
Nordihydrocapsaicin	99.87
Capsaicin	103.14
Dihydrocapsaicin	102.60
Nordihydrocapsiate	102.39
Capsiate	104.02
Dihydrocapsiate	103.34

$$\%Recovery = \frac{C_S - C_U}{C_{STD}} X100$$

Where, C_S is concentration in the spiked sample, C_U is concentration in the unspiked sample and C_{STD} is the true concentration of standard added.

APPENDIX B

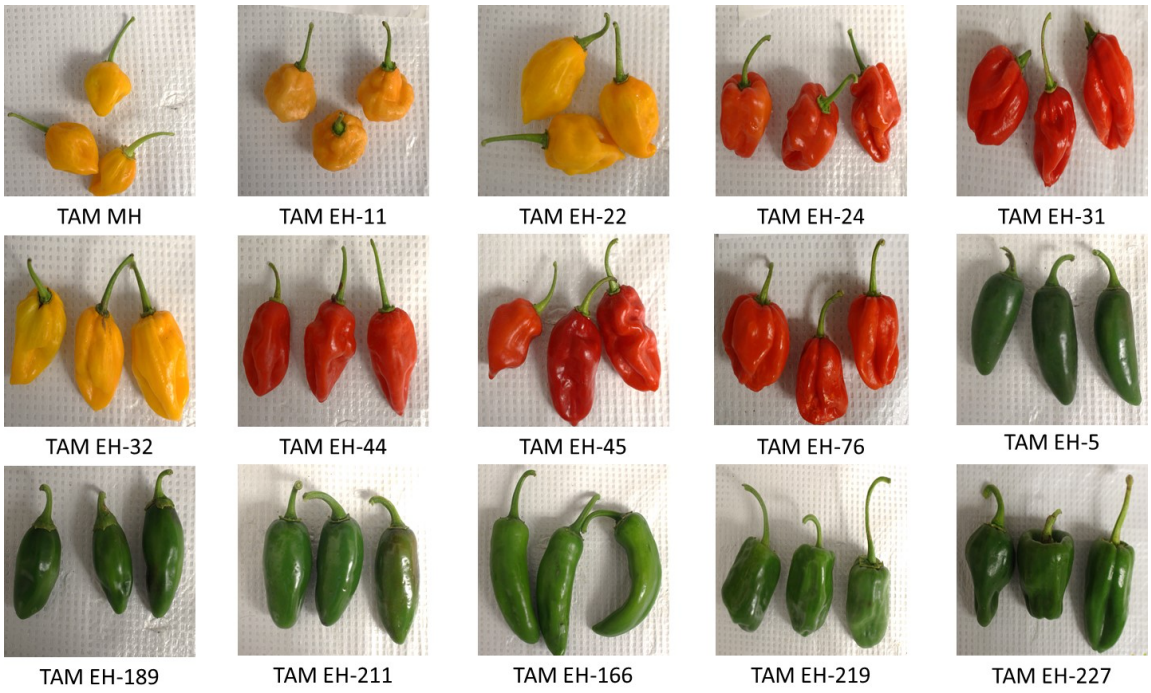

Figure B 1. Pepper hybrids analyzed in the current study.

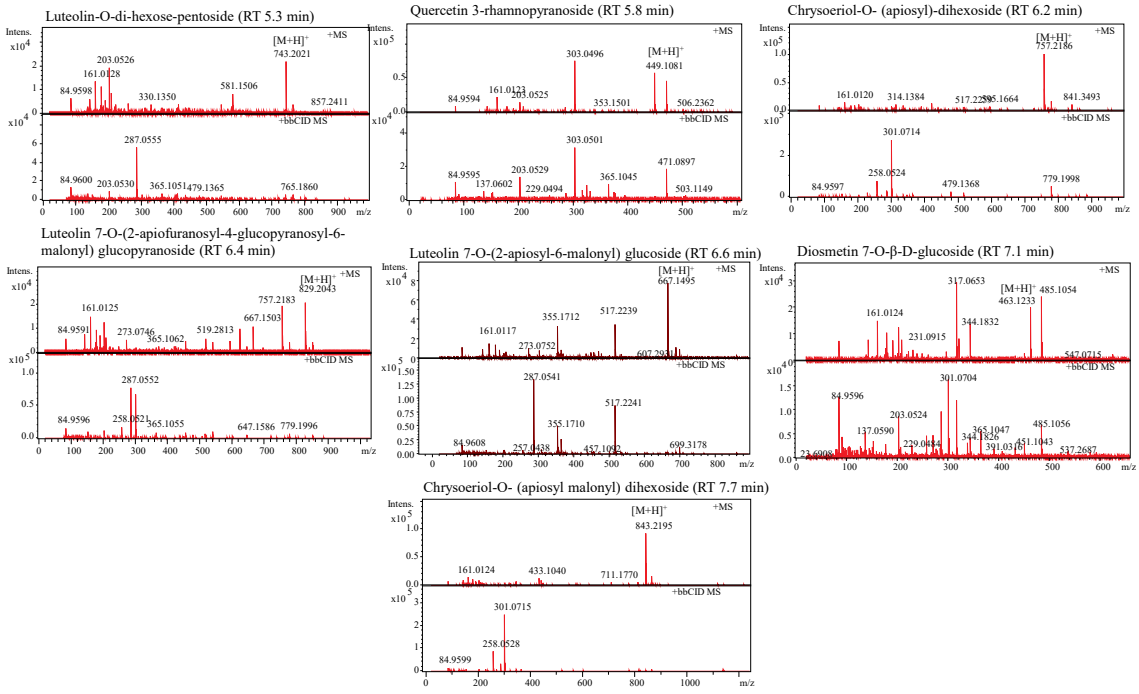

Figure B 2. Tandem mass spectra of identified flavonoids in pepper extracts.