Adv. Hort. Sci., 2021 35(3): 305-318 DOI: 10.36253/ahsc-10630

AHS
Advances in Horticultural Science

Comparison of wild and domesticated hot peppers fruit: volatile emissions, pungency and protein profiles

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Citation:
COMPARINI D., TAITI C., LANZA M., VITA F., PANDOLFI C., LUTI S., SPINELLI F., PAZZAGLI L., MANCUSO S., 2021 - *Comparison of wild and domesticated hot peppers fruit: volatile emissions, pungency and protein profiles.* - Adv. Hort. Sci., 35(3): 305-318.

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 9 March 2021
Accepted for publication 3 August 2021

Key words: Artificial Neural Network, *Capsicum spp*. classification, capsaicin, SDS-PAGE, VOCs.

Abstract: *Capsicum* plant species are globally cultivated in warm and temperate regions, being important for agro-economic, biological and cultural aspects. While their worldwide spread and their ability of cross-pollination to easily hybridize play an important role in the formation of numerous species and varieties but also create confusion for their classification. For this reason, the categorization of species and varieties is complex and several methods have been used to evaluate pepper plant origin and evolution. Therefore, the objectives of this study were to compare a wild pepper (*Capsicum chacoense*) with other two domesticated cultivars belonging to different species such as *Capsicum annuum* and *C. baccatum* and draw conclusions about their origins using different approaches. For this purpose three methodologies have been used and compared: the comparison of their fruits volatile organic compounds (VOCs) emissions , their capsaicin and dihydrocapsaicin content and the leaves proteomic profiles. The VOCs analysis has been conducted by a time-of-flight mass spectrometry (ToF-MS) with an innovative approach to better identify all the compounds detected, in particular using two different ionization agents (H_3O^+ and NO^+) to better identify all the compounds detected. The VOCs and pungency analyses were then used to build back propagation neural networks (BPNN) and a Random Tree classifier to conduct a multivariate analysis and evaluate the most species-specific volatiles. The outcomes appeared to be a most accurate approach with respect to the traditional varieties descriptors used for peppers discrimination. The BPNN led to the identification of several putative volatiles as good candidates for the recognition of these species or significant nodes in a decision learning tool. Finally, protein profiles have been obtained by SDS-PAGE analysis on the leaves to perform a fast proteomic comparison among the species. The protein profiles showed the *C. baccatum* and *C. chacoense* were more similar to the domesticated pepper *C. annuum*.

1. Introduction

Origin and classification of Capsicum

The origin of chili peppers has been located in several locations of Latin America as testified by archeological records and pepper is usually classified as one of the first new world domesticated plants (Pickersgill, 1969; Long-Solis, 1986; Perry *et al*., 2007). Peppers represent one of the most ancient plants cultivated in America together with the *Phaseolus* L., maize and other plants of the Cucurbitaceae family, fundamental ingredients of natives' diet. About 36 different species belong to the genus Capsicum and even today there are many wild species to be defined from the taxonomic point of view, consequently, we cannot exclude the existence of new individuals currently unknown (Davenport, 2004). The identification and maintenance of the genetic diversity in *Capsicum* are important to avoid genetic erosion.

Several species exist and *Capsicum* species have recently been described from Bolivia (Nee *et al*., 2006). Among these, only five species respectively C. *annuum* (variety *annuum)*, C. *chinense*, C. *frutescens*, C. *baccatum* (variety *pendolum* and *umbelicatum)* and C. *pubescens* have been domesticated in the past by the American natives and later, in the post-Colombian period, they have been widely imported and cultivated in temperate and tropical regions for the characteristics of their fruits (McLeod *et al*., 1979; McLeod *et al*., 1983). The broad geographical distribution of this genus, usually used as feeds not only by humans but also by birds that don't have receptors for capsaicin, associated with the antiquity of the origin and the high frequency of hybridization, created a broad genetic variability during the evolution and resulted in many morpho-qualitative differences among cultivars of homologous species (Pozzobon *et al*., 2006). In fact peppers plants have been used for several reasons, starting from their high nutritional value, good content in vitamins but also as medicine or mystic rituals. This determined a rapid diffusion of these plants in the old continent, stimulated also by their spicy flavour similar to the black pepper, a very valued spice, whilst peppers presented higher production and more flexibility as food. Furthermore the mechanism of cross-pollination and the ability of peppers to easy hybridize played another role in the formation of numerous varieties with specific features. During the last centuries, botanists have been active to cross-pollinate creating confusion for the classification of the varieties and the identification of indigenes species. For these reasons the zone where each species originated is still subjected to debate. Brazil is considered as the center of origin of the genus capsicum and currently represents the most important source of genetic diversity (Buso *et al*., 2001). For example, a recent survey of chili cultivars from the state of Roraima in northwestern Brazil noted 60 distinct landraces of peppers from four different species: *Capsicum annuum; C. frutescens, C. baccatum and C. chinense* (Barbosa *et al*., 2006). Therefore Brazil and Bolivia possess the highest number of wild species (Pickersgill, 1984), however, a broad and complete study about diversity has not been done yet for the native species. It is anticipated that continuing, plant exploration in southern Peru, Bolivia and Brazil, will yield additional new narrow endemic pepper species (Russo, 2012).

As consequence, a taxonomic classification of peppers and the determination of all relations among species are challenging and created many debates about the origin and evolution of the *Capsicum genus*. Most current peppers are derived from domesticated species. The difficulties in identifying them lie in dialectal names linked with the local tradition, making challenging their classification. For example, a study among the Mexican peppers population underlined that among about 200 common names used to refer to different peppers, only approximately 15 types were different commercial pods (Russo, 2012). This situation contributes in the last filthy years several authors to focus on the taxonomy and origin of the species of this genus without coming out with a complete and flawless analysis (Heiser and Smith, 1953; Pickersgill, 1988; Hunziker 2001; Barboza and Bianchetti, 2005).

It has been proposed that the *C. chacoense* in Bolivia is one of the most ancestral nuclear centers for the origin of *Capsicum* (2n=24) and it is considered as the basal for the evolution of the species (McLeod *et al*., 1982). Furthermore, a study based on karyotype and other considerations of different *Capsicum* by Moscone *et al*. (2007) concluded the existence of a triple origin of domesticated *Capsicum* species. The whole genus has been hypothesized to be originated in the tropical regions of the Americas and the ancestral species should be born in the Chaco zone which extends into several parts in the country of Argentina, Bolivia, Brasil and Paraguay and propose that the genus "chacoense" as one of the most primitive origi-

nated pepper taxon. All the lines that we know today derived from this first line of evolution and have developed and differentiated according to the characteristics of the new habitat: in the north of the Amazon to the ancient forms of *C. annuum,* on the north coast of Brazil and Venezuela in the forms of *C. chinense* and *C. frutescens*; and in a subsequent period from the same ancestral form were differentiated to *C. rhomboideum* (Colombia, Ecuador) and *C. galapagoense* (Galapagos Islands) (Moscone *et al*., 2007).

Starting from a second evolutionary line instead, they are originated different species: in subtropical areas of Brazil and later the *C. baccatum* and *C. eximium*; in the arid regions of Peru the *C. cardenasii, C. tovarii* and *C. pubescens*, while in Paraguay *C. flexuosum* and *C. praetermissum*. Finally, it was hypothesized that the migration of ancestral forms of *C. flexuosum* and *C. praetermissum* gave rise, in some areas of Brazil, the largest center of diversification of the genus represented by the large group of wild species in 26 chromosomes (Moscone *et al*., 2007). Currently, the species that best reflects the ancestral morphologically and physiologically, is the *C. chacoense* (Hunziker, 2001). *C. chacoense* has also been used in breeding *C. annuum* programs, focusing on tobacco mosaic virus resistance (Boukema, 1982). The wild species with 24 and 26 chromosomes present different morphological traits and geographical distribution (Pozzobon *et al*., 2006). The differences that arise are probably related to the different agents of seed dispersal, birds for the first whilst bats and other small mammals for species of 26 chromosomes that present hanging fruit, inconspicuous, and a little spicy. In a second time, the domestication and subsequent human selection caused a selective pressure in favor of large hanging fruits, less attractive for birds with small exception (e.g. *C. frutescens* "Tabasco" varieties), (AISPES, 2010). The classification of chilies, like that of any multifarious group of cultivars, is confusing. For example, a very large amount of pod types exist in *C. annuum*, *C. chinense* and *C. baccatum* (Bosland and Votava, 2012).

Usually, the classification of the genus *Capsicum* and the varieties, belonging to each species, is carried out based on morphological descriptors that define the shape of flowers and fruits (Pickersgill, 1971; Moscone *et al*., 2007; Ince *et al*., 2009; Sudré *et al*., 2010) and the current system for classifications involved genus, species, variety, pod type and cultivars (Bosland and Votava, 2012). However, other descriptors are considered essential for an accurate germplasm characterization, such as those indicated by IPGRI (International Plant Genetic Resources Institute). Furthermore, the characterization and evaluation of the species belonging to the genus *Capsicum* are particularly interesting for breeders, gene banks, because of the large genetic variability available (Guzmán *et al*., 2005; Sudré *et al*., 2006; Ince *et al*., 2009). They can furthermore be identified from the different flavors of the fruits and two main factors that contribute to the aroma perception are pungency and aroma, and these are associated with the fruit volatile compounds (Taiti *et al*., 2015).

With the advancement of computer technology, multivariate methods have become an important tool for taxonomic classification (Ortiz *et al*., 2008). However, the procedures of statistical classification require a data set based on a large number of variables. Thus, the paper has aimed to analyze and characterize the wild *C. chacoense* and to compare its profile of volatile emissions, pungency, and proteins with two domesticated species i.e. *C. annuum* and *C. baccatum* to elucidate the evolution of the *Capsicum* genus. For this reason, the volatiles compounds emissions profile of each species has been used to build an Artificial Neural network and differentiate and identify the species. Finally, an additional proteomic analysis of the leaves of the peppers has been used to evaluate the relationships among the species and compare the results obtained from the volatiles emission, pungency and protein expression profiles of each species.

2. Materials and Methods

Fresh pepper material

Ripe pepper fruits belonging to 3 different species were used in this study: *Capsicum annuum* var. Ciliegino, *C. baccatum* var. Brasileiro and *C. chacoense* (wild accession). The fruits were collected from ten plants, for each species, grown into greenhouse (Florence, Italy). Fully matured uniform-sized fruits were collected within 24h after the 100% color surface was reached. All plants were obtained from seeds, germination and growing phases were made following the same system used in the previous work of Taiti *et al*., 2015. All plants belonging to each species were grown in the greenhouse in three different rooms to avoid the effects of cross-pollination.

Capsaicin and dihydrocapsaicin quantification

Capsaicinoids are a group of alkaloids produced as

secondary metabolites by chili peppers responsible for the pungency. Among several structural analogs capsaicinoids, capsaicin and dihydrocapsaicin are the two most potent and abundant compounds accounting for more than 90% of total capsaicinoids in chili pepper (Ziino *et al*., 2009). Capsaicin and dihydrocapsaicin have been extracted from whole frozen fruits and calculated as the average of five extraction for each species (n=5, SD). About 2 g of frozen fruits have been weighted and then pulverized in 10 mL of cold acetone at 4°C and kept overnight. Then 50 µl of the sample have been collected by using a 0.22 µm filter syringe and used for the quantification. All data have been calculated as µg of capsaicin or dihydrocapsaicin content per gram of fresh weight after normalization based on the exact weight of the initial fresh sample.

RP-HPLC quantification of capsaicin and dihydrocapsaicin was performed by using a C18 column, 3 µm, 15x4.6 cm (Supelco, Bellefonte, Pennsylvania, USA). The stock solution containing capsaicin and dihydrocapsaicin (cod, 360376 Sigma-Aldrich, St Louis, MO, USA) was prepared in 20% Acetonitrile at concentrations of 7.5 mg/mL. For calibration curves construction 30 µl, 60 µl, 90 µl, 120 µl, 150 µl, 300 µl and 500µl of stock solution were analyzed obtaining a linear curve for both capsaicin and dihydrocapsaicin (R^2=0.9912 and R^2=0.9923, respectively). Elution gradient was performed at a flow rate of 0.8 ml min^{-1} with the following solvent system: 10mM trifluoroacetic acid (TFA) in acetonitrile (solvent A); 10mM TFA in water (solvent B). The gradient used was 20% A for 2 min, from 20% to 100% A in 15 min, holding at 100% A for 10 min, from 100% A to 20% A in 2 min, and detection was based on UV absorbance at 280 nm. Under these conditions, the capsaicin peak appeared at a retention time (Rt) of 10.6 min and dihydrocapsaicin at Rt of 11.3 min. Quantification was calculated using the Chromeleon software.

Volatiles organic compounds (VOCs) analysis

SRI-MS ToF protocols. For headspace analysis, pepper fruits have been selected among ten plants and the uniform-sized fruits were collected at the optimal ripening stage (100% of coloration). For each species ten samples were analyzed, each constituted of 10 grams of fresh pepper fruits. Using a commercial PTR-TOF 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) instrument with SRI-MS (Switchable Reagent Ions - Mass Spectrum) upgrade, the analysis of the samples was carried out following a similar procedure described in Taiti *et al*. (2015). In short, 10 g of freshly cut chili pepper (including the seeds) without any pre-treatment were placed in a glass jar (750 mL at 25°C, with a dynamic headspace flushing flow rate of 200 mL min^{-1}) equipped with two Teflon inlet and outlet tubes on the opposite side, which were respectively connected to a zero-air generator (Peak Scientific) and the PTR-TOF MS instrument. Moreover, for the first time, the Switchable Reagent Ion System (H_3O^+ and NO^+) has been used to produce different ionization agents for capsicum study. Using the additional precursor (reagent) ions as NO^+, besides the usual H_3O^+, improved the analytical possibilities of this technique (Wang *et al*., 2004; Mochalski *et al*., 2014). In particular, the SRI system allows: (1) the analysis of VOCs which are not detectable with the reference ion H_3O^+ (e.g. alkanes); (2) the separation of isobaric compounds as in the case of aldehydes and ketones using NO^+ (Jordan *et al*., 2009; Del Pulgar *et al*., 2013). For a detailed explanation of the system see Blake *et al*. (2006) and Blake *et al.* (2009). All samples were analyzed using the same procedure and the VOCs were assessed with H_3O^+ and NO^+ as reagent ions; the drift conditions for each primary ion used are reported in Table 1. The sampling time for each channel of TOF acquisition was 0.2 ns, for a mass spectrum comprised between *m/z* 20-210. The duration of a single sample measurement was 120 seconds, which corresponds to 60 mass spectra. All the samples were analyzed in an air-conditioned room, with a constant temperature of 25±1°C (Mancuso *et al*., 2015). The SRI-MS upgrade consists of an additional mass flow controllers for the respective reagent gases (water vapor for H_3O^+, charcoal filtered air for NO^+); for a detailed explanation see Jordan *et al*. (2009). In short, the use of NO^+ and the H_3O^+ as a reagent ion

Table 1 - Instrumental condition throughout the experiment

Primary Ion	Drift voltage (V)	Pressure (mB)	Temperature (°C)	Us (V)	Uso (V)	Ihc (mA)	Udx (V)	E/N (Td)	Mass calibration	Mass calibration	Mass calibration
H_3O^+	594	2.25	110	110	85	4.0	35	140	21.022	29.997	59.041
NO^+	600	2.30	110	25	80	5.0	36	137	21.022	32.002	47.997

improves the analytical performance of the tool, particularly for the separation of isobaric compounds and for the detection of compounds with proton affinities lower than that of water. Furthermore, as reported by Edtbauer *et al.* (2014) when the PTR-MS instrument works in NO^+ mode can improve the selectivity of compounds detection.

Mass data statistical analysis. Since the external calibration provided by the tool gave a poor mass accuracy, it has been performed off-line thus ensuring, a high mass accuracy generally than 0.001Th, which in most cases allowed the formula identification (Taiti *et al*., 2017). The raw data of each peak spectrum (calculated as number of counts per second, cps) were acquired with the software TOFDaq (TOFwerk AG, Switzerland) by setting a dead time of 20 ns for the Poisson correction, instead, for peak quantification, the resulting data were corrected according to the duty cycle and the signals were normalized (ncps "normalized count per second") as described by Herbig *et al*. (2009). Moreover, the Poisson correction has been applied to correct all spectra for any count losses. Finally, VOCs putative identification was based on a high instrumental mass resolution and the fragmentation patterns of pure standards available in the bibliography (Buhr *et al*., 2002; Lee *et al*., 2006; Maleknia *et al*., 2007; Kim *et al*., 2009; Tani, 2013; Aprea *et al.*, 2015; Taiti *et al.*, 2019) and integrated with previously detected VOCs emitted from *Capsicum* fruit available in literature (Table 2 and 3).

Data processing and classification methods

Back propagation neural network. In this study, the capsaicinoid contents and the 52 volatile signals detected by the PTR-ToF-MS by using H_3O^+ as reagent ion were used as input layers, and the 3 pepper species represented the output (30 single pepper fruits analysis, ten for each species). The BPNN was built using a data mining software (Weka 3.6.14) and the Multilayer Perceptron classifier was used for the classification.

Two BPNN were made, one with only the VOCs emission profiles, and another with VOCs and capsaicinoids. The number of hidden neurons and the number of iterations was adjusted to optimize the neural network activity. Many factors, such as learning schemes, numbers of nodes, and connections between them, play an important role in determing of the best configuration of the hidden layers (Zurada

Table 2 - List of the average m/z-signals that can be specifically assigned using H_3O^+ as reagent ion: Volatile Organic Compounds headspace intensity expressed in ncps (n=10; ±SD); chemical formulae and tentative identifications for each signals detected; the compounds identification was linked to the PTR-ToF-MS pattern fragmentation references [(a)] or previously reported in *Capsicum* species [(b)]

	Measured mass (*m/z*)	*Capsicum Annuum* Ciliegino	*Capsicum Baccatum* Brasileiro	*Capsicum Chacoense* Wild pepper	Sum formula	Tentative identification	PTR Pattern fragmentation [a]	Capsicum Literature [b]
1	27.022	799.86±234.50	200.93±109.28	496.95±140.33	$C_2H_3^+$	Acetylene		
2	31.018*	615.25±205.40	184.12±62.55	81.22±21.30	CH_3O^+	Formaldehyde	[2]	
3	33.033	1712.59±220.12	415.39±167.27	1067.70±200.55	CH_5O^+	Methanol	[1]	
4	41.038	2612.818±638.34	983.12±320.11	378.78±100.33	$C_3H_5^+$	Alkyl fragment (Alcohols and esters)	[1]	
5	43.054	578.30±190.22	133.23±33.90	101.94±25.90	$C_3H_7^+$	Alkyl fragment (Alcohols)	[1]	
6	45.033	3049.50±982.28	529.04±67.40	5638.53±1230.45	$C_2H_5O^+$	Acetaldheyde	[2]	
7	47.010	353.83±111.89	224.89±51.58	636.43±160.55	$CH_3O_2^+$	Formic Acid/Formates	[2]	
8	47.049	4.80±1.20	3.50±0.80	2.95±1.00	$C_2H_7O^+$	Ethanol	[1]	Rodriguez-Burruezzo *et al*., 2010
9	53.030	102.26±35.40	50.90±22.33	17.53±5.00	$C_4H_5^+$	Cyclobutadiene		
10	55.050	6.82±1.90	1.99±0.50	3.32±1.30	$C_4H_7^+$	C4 aldehydes fragment	[1]	
11	57.033*	2910.50±1109.12	366.28±78.54	179.55±49.50	$C_3H_5O^+$	C3 aldehydes and ketones fragments	[6]	
12	57.069	274.43±85.40	45.91±17.22	33.02±9.25	$C_4H_9^+$	Alcohol fragments (1-Butanol, 1-Pentanol, 1-Hexanol, 2-Methyl-1-propanol, Pentanol, 1-Heptanol, Octanol, Nonanol)	[1]	
13	59.049*	553.06±133.09	196.04±65.90	459.80±180.44	$C_3H_7O^+$	Propanal, Acetone	[2]	Ziino *et al.*, 2009
14	61.028	303.46±44.65	120.22±39.10	355.05±44.44	$C_2H_5O_2^+$	Acetates	[6]	Ziino *et al*., 2009
15	63.027	4.45±1.30	4.64±1.20	5.05±0.80	$C_2H_7S^+$	Dimethylsulfide		Taiti *et al*., 2015

* The signals that mostly contributed to the BPNN classification have been marked.

to be continued...

Table 2 - List of the average m/z-signals that can be specifically assigned using H_3O^+ as reagent ion: Volatile Organic Compounds headspace intensity expressed in ncps (n=10; ±SD); chemical formulae and tentative identifications for each signals detected; the compounds identification was linked to the PTR-ToF-MS pattern fragmentation references [(a)] or previously reported in *Capsicum* species [(b)]

	Measured mass (*m/z*)	*Capsicum Annuum* Ciliegino	*Capsicum Baccatum* Brasileiro	*Capsicum Chacoense* Wild pepper	Sum Formula	Tentative identification	PTR Pattern fragmentation [a]	Capsicum Literature [b]
16	65.038	4.87±3.25	2.49±0.80	3.45±1.11	$C_5H_5^+$	Alkyl fragment/ Terpenes fragment	[1/2]	
17	67.050	61.35±16.66	13.86±6.40	6.77±1.40	$C_5H_7^+$	Terpenes fragment	[2]	
18	69.033	80.90±21.98	17.82±4.50	11.92±4.10	$C_4H_5O^+$	Furan		
19	69.069	395.06±133.90	139.11±80.33	92.50±18.44	$C_5H_9^+$	Isoprene/Alkyl fragment (e.g. 2-methylbutanal, 1-octen-3-ol)	[2/6]	
20	71.049*	21.29±7.10	6.52±2.12	8.70±2.50	$C_4H_7O^+$	2-Butenal		Taiti *et al.*, 2015
21	71.086	15.92±5.90	4.71±190	7.65±1.90	$C_5H_{11}^+$	Alchol (3-methyl-1-butanol, Pentanol, Iso-pentanol, 2-ethyl-1-hexanol,		Eggink *et al.*, 2012 a
22	73.060*	43.02±12.35	6.62±2.10	15.45±4.90	$C_4H_9O^+$	Isobutanal/Butanone/Butanal	[1]	Ziino *et al.*, 2009
23	75.044	57.76±22.25	14.33±4.50	12.16±6.00	$C_3H_7O_2^+$	Butanol/Methyl acetate/Propanoates	[1]	
24	77.038	7.09±2.79	7.77±2.20	14.53±3.50	$C_6H_5^+$	Alkyl fragment		
25	79.054	134.86±48.60	22.84±7.98	15.7±4.10	$C_6H_7^+$	Benzene/Alkyl and terpenes fragment	[2]	
26	81.068	949.61±301.39	178.02±68.78	152.06±50.30	$C_6H_9^+$	Terpenes fragment/Aldehydes fragment (trans- 2-hexenal)	[4]	Taiti *et al.*, 2015
27	83.049	76.90±19.75	32.36±9.94	13.69±2.90	$C_5H_7O^+$	2-Methylfuran		Rodriguez-Burruezzo *et al.*, 2010
28	83.086	175.59±50.10	92.78±29.50	16.29±4.90	$C_6H_{11}^+$	C6 compounds/ Hexenol fragment	[6]	
29	85.064	46.91±15.40	9.90±3.10	18.20±4.30	$C_5H_9O^+$	Methyl-butenal/1-penten-3-one		Ziino *et al.*, 2009
30	85.101*	32.25±12.30	2.08±0.94	7.41±2.00	$C_6H_{13}^+$	Alcohol (1-Hexanol/Nonanol)		Ziino *et al.*, 2009
31	87.045*	25.21±5.40	6.92±3.20	12.65±4.10	$C_4H_7O_2^+$	Diacetyl /2,3-butanedione	[1]	Ziino *et al.*, 2009
32	87.080	10.23±1.21	6.44±0.95	2.01±0.55	$C_5H_{11}O^+$	2,3-Methylbutanal /(Z)-2-penten-1-ol/3-Pentanone	[1]	Rodriguez-Burruezzo *et al.*, 2010
33	91.075	22.38±5.40	17.68±4.40	6.35±1.10	$C_4H_{11}O_2^+$	2,3-Butanediol/Monoterpene ketone	[5]	Ziino *et al.*, 2009
34	93.069	7.07±1.95	12.12±5.40	7.59±1.50	$C_7H_9^+$	Terpene fragments (e.g. cymene, limonene)	[3]	Rodriguez-Burruezzo *et al.*, 2010
35	95.086	27.45±6.99	4.18±2.20	14.97±1.10	$C_7H_{11}^+$	1-Methyl-1,4-cyclohexadiene		Eggink *et al.*, 2012 b
36	97.064	50.45±10.50	14.90±4.80	10.18±2.50	$C_6H_9O^+$	2-Ethylfuran		Rodriguez-Burruezzo *et al.*, 2010
37	99.080*	60.65±20.30	8.62±2.75	15.98±6.30	$C_6H_{11}O^+$	cis-3-Hexenal/ (E)-2-Hexenal		Rodriguez-Burruezzo *et al.*, 2010
38	101.096	33.66±5.70	9.62±3.55	17.02±5.50	$C_6H_{12}O^+$	Hexanal/ (E)-2-Hexenol	[1]	Ziino *et al.*, 2009
39	103.075	54.22±20.44	18.20±6.50	9.28±2.80	$C_5H_{11}O_2^+$	3-Methylbutanoic acid	[6]	Zimmermann and Schieberle, 2000, Azcarate *et al.*, 2010
40	105.069	4.98±0.65	5.23±0.78	5.96±1.12	$C_8H_9^+$	Styrene/Styrol/Phenylethanol	[6]	Rodriguez-Burruezzo *et al.*, 2010
41	107.085	9.50±5.44	3.65±2.30	12.85±7.20	$C_8H_{11}^+$	p-Xilene	[6]	Eggink *et al.*, 2012 a
42	109.101	13.85±4.50	3.15±0.90	6.06±2.20	$C_8H_{13}^+$	Terpenes fragments	[3]	
43	115.111*	17.27±4.30	6.04±2.50	1.36±0.50	$C_7H_{14}O^+$	Heptanal	[6]	Ziino *et al.*, 2009
44	117.091	6.35±1.60	14.05±3.00	3.96±1.30	$C_6H_{13}O_2^+$	Hexanoic acid/Hexanoates	[6]	Eggink *et al.*, 2012 b
45	119.085	7.75±3.70	3.26±1.35	4.89±2.40	$C_9H_{11}^+$	Terpenes fragment	[3]	Ziino *et al.*, 2009
46	121.101	6.40±2.50	15.08±3.50	6.10±2.00	$C_9H_{13}^+$	Terpenes fragment	[3]	
47	123.120	8.40±2.80	1.65±0.60	1.72±0.85	$C_9H_{15}^+$	Sesquiterpene fragments	[4]	
48	135.117*	7.15±1.83	2.69±1.40	6.62±1.30	$C_{10}H_{15}^+$	p-Cymene/Monoterpene ketone fragment	[6/5]	Ziino *et al.*, 2009
49	137.132*	17.12±4.05	9.75±1.10	23.88±4.35	$C_{10}H_{17}^+$	Monoterpenes (e.g. (Z)-b-ocimene)	[5]	Eggink *et al.*, 2012 a
50	149.132*	7.13±4.40	3.91±1.05	9.65±3.20	$C_{11}H_{17}^+$	Sesquiterpenes fragments (e.g. Ectocarpene)	[4]	Taiti *et al.*, 2015
51	205.195	25.44±7.10	11.90±3.33	9.59±2.65	C15H25+	Sesquiterpenes	[4]	Eggink *et al.*, 2012 b
TOTAL VOCs EMISSION (average ncps)		22116	4850	13214				

* The signals that mostly contributed to the BPNN classification have been marked.

Table 3 - SIFT-MS signals obtained using NO+ as reagent ions, in the range between to m/z 20-200. The analysis showed only the signals with intensity expressed in ncps higher than 1 (n=10; ±SD)

Number of compounds	Measured mass (m/z)	*Capsicum Annuum* Ciliegino	*Capsicum Baccatum* Brasileiro	*Capsicum Chacoense* Wild pepper	Sum Formula	Tentative identification
1	41	10.91±3.10	45.53±16.60	19.47±8.85	$C_3H_5^+$	Alkyl fragment (Alcohols and esters)
2	45	18.72±2.10	25.67±8.40	21.61±6.80	$C_2H_5O^+$	C6 fragment (e.g. (E)-2-Hexenol)
3	57	7.41±2.98	48.61±18.07	9.68±1.55	$C_3H_5O^+$	C3 Aldehyde
4	58	50.01±8.86	39.10±3.25	25.63±9.52	$C_3H_6O^+$	C3 ketones
5	69	13.07±2.33	45.73±18.80	20.95±8.55	$C_4H_4O^+$	Furan
6	83	15.11±2.23	79.05±20.44	13.85±0.60	$C_6H_{11}^+$	C6 aldehydes (e.g (E)-2-Hexenol)
7	84	4.17±0.88	3.64±0.55	2.94±0.90	$C_5H_8O^+$	C5
8	85	7.62±3.20	27.85±7.22	10.69±3.33	$C_5H_{11}O^+$	Valeraldehyde
9	88	59.06±24.44	44.29±27.70	61.12±20.32	$C_3H_6O\ NO^+$	Acetone
10	99	11.12±2.33	15.24±3.33	10.76±2.22	$C_6H_{13}O^+$	Hexanal/ C6 aldehydes (e.g (E)-2-Hexenol)
11	106	3.67±0.78	2.87±0.30	2.780±6.52	$C_8H_{11}^+$	Xylene
12	113	1.05±0.33	6.65±.55	1.34±0.55	$C_7H_{13}O^+$	Heptenal
13	114	2.60±0.44	10.15±1.1	2.16±0.30	$C_5H_8NO_2^+$	Cluster C5 unsaturated ketones
14	116	9.47±2.63	26.33±8.33	6.39±1.20	$C_5H_{10}NO_2$	Cluster C5 ketones/Pentanone
15	128	3.34±0.81	2.04±1.01	1.88±0.72	$C_8H_{16}O$ +	6-Methyl-5-hepten-2-ol
16	136	17.85±1.66	7.78±0.95	26.57±6.77	$C_{10}H_{16}^+$	Monoterpene compounds
17	144	0.80±0.62	3.21±0.81	0.70±0.55	$C_6H_{10}NO_3^+$	Cluster hexanedione/heptanone
18	166	0.73±±55	2.38±0.35	0.80±0.68	$C_{10}H_{16}NO^+$	Monoterpenes fragment
Total VOCs emission average (ncps)		236.71	436.09	239.29		

1992; Zurada and Malinowski, 1994). In our case, the minimum error was reached with a network composed of 29 hidden neurons for both BPNN, positioned on one level, with the hidden layer activated by a logistic sigmoid activation function:

$$f(x) = 1/(1+e^{-x}) \quad (1)$$

These sigmoid functions fix the output signal limit between 0 and 1. The resulting function works as an output logic-gate that can be opened (1) or closed (0). Also, as part of a continuous function it can happen that a gate is partially opened (i.e., its value results between 0 and 1). Ideally, only a group of outputs, which represents an accession, would express a value of 1 (meaning correct identification) while the remaining groups would show a value of 0 (incorrect identification). In reality, this take place rarely, for this reason it is usually considered as "incorrect" a value closer to zero (wrong identification), while "correct" when the resulted value is close to 1 (correct identification) (Pandolfi *et al*., 2009).

A 10-folds cross-validation was applied to test the performance of the model. The original dataset was essentially randomly segregated into 10 equal-sized groups. Each set is divided into two groups: 90% of data are used for training the network and 10% of data are used for the validation test. The cross-validation process is then repeated 10 times (the folds), in which every time a subsamples is validated. The results deriving from all folds are finally averaged to result in a single evaluation of the network's performance.

The values of the identification for each species were highlighted using a misidentification matrix. All identification processes executed by the network were averaged and the results represented in Table 4. The rows refer to the species in the test set, the columns report the species to which the test plants are referred by the neural network. An "Attribute selection filter" provided by Weka was also applied to the two sets of data, to determine the more discriminant parameters.

Random tree. The 51 volatile signals detected by the PTR-ToF-MS using H_3O^+ (30 single pepper fruits analysis, ten for each species) were also analyzed using the "Random tree" algorithm, a decision tree

Table 4 - Confusion Matrix derived from the Random tree analysis from the aromatic profiles of the pepper species obtained using a decision tree learning tool

	C. baccatum	*C. chacoense*	*C. annuum*
C. baccatum	9	1	0
C. chacoense	0	10	0
C. annuum	0	0	10

learning tool provided by the software (Weka 3.6.14). In classification trees, each internal node is labeled with an input feature, which can be informative to detect similarities or differences among the pepper species.

Similarly to the BPNN, a 10-folds cross validation was applied to test the performance of the model, and the results from the folds are then averaged to produce a single estimation of the performance of the algorithm.

SDS-page protein analysis

Protein extraction and quantification. Soluble proteins were extracted from leaves of three Chili pepper plants of each species (*Capsicum Chacoense, C. annuum* and *C. Baccatum)* according to Vita *et al*. (2013), with some modifications. In short, for each analysis 100 mg of fresh leaves were grounded in liquid nitrogen and homogenized with 1 mL of extraction buffer (5 M urea, 2 M thiourea, 40 mM Tris-HCl, 2% CHAPS, 50 mM DTT). The homogenates were centrifuged for 15 min at 15,000 rpm. Supernatants were precipitated using TCA (15%, v/v) containing 0.007% β-mercaptoethanol in acetone at -20°C for 2 h and successively at 4°C for a minimum of 2 h. Samples were centrifuged at 4°C for 15 min at 14,000 rpm, supernatants were discarded and pellets were washed twice with ice-cold acetone containing 0.007% β-mercaptoethanol. Pellets were dissolved in a rehydration buffer (5 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). Protein quantification was performed using a Bradford-based assay kit assay (Bio-Rad Hercules, CA), using bovine serum albumin as a standard.

SDS-PAGE. The protein separation was carried out by the established technique of SDS-PAGE. In detail, the gels used (size 20 * 20 cm) had a thickness of 1 mm and were constituted by a stacking gel (4.8% T, 1.3% C, pH 6.8) and a running gel (15%, 1.3% C, pH 8.5). SDS-PAGE analyses were performed 4 times (n=4). Electrophoresis runs were carried out using the Protean XI cells (Bio-Rad Laboratories, Inc, Hercules CA) with specific parameters (for each gel 25 mA, 8h running time, temperature 15°C). Precision Plus Protein™ Unstained was the molecular marker used for the essay (Bio-Rad Laboratories Inc., Hercules, CA). The protein samples were analyzed by SDS-PAGE on gels stained with the Brilliant Blue G-Colloidal Concentrate Coomassie (Sigma-Aldrich) according to the manufacturer's instructions.

The images of each gel were acquired using a Bio-Rad densitometer GS-800 ™ in greyscale colors, with a definition of 300 dpi. The images were analyzed using the software Quantity One 1-D Analysis ™ software (Bio-Rad Laboratories, Inc, Hercules CA). Dendrogram based on signal quantities was created using correlation-based distances and Ward's method of agglomeration was used in the present analysis (Ward, 1963).

3. Results and Discussion

Capsaicinoid

In all the species analyzed both the capsaicin and the dihydrocapsaicin (DHC) contributed to the pungency of the fruits. As expected (Stoica *et al*., 2016), the capsaicin content was higher than the DHC in all species and C. *chacoense* resulted in the higher content of about 300 µg/g of fresh weight (FW) compared to C. *baccatum* whilst C. *annum* have been the most variable samples with an high standard deviation that made it not statistical different from the other two species (Fig. 1). Interestingly the C. *chacoense* resulted in the higher content of DHC of 243±36 µg/g FW, followed by the C. *annum* and C. *baccatum* that did not resulted statistical different

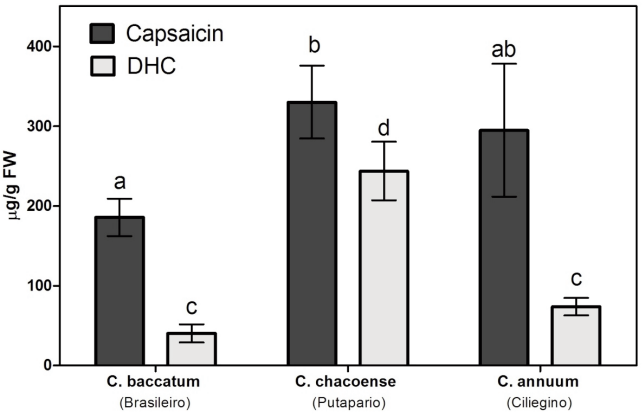


Fig. 1- Capsaicin and dihydrocapsaicin (DHC) content obtained from ripe fresh pepper fruits for each species analyzed, using HPLC detection (n=5; SD). Different letters represent statistical significance (ANOVA, p<0.05).

from each other with respectively 73±11 µg/g FW and 40±11 µg/g FW. The capsaicin content influences more the spiciness followed by the DHC, therefore *C. baccatum* have showed the lowest level of spiciness.

PTR-ToF-MS volatiles compounds analysis

The volatiles compounds analysis by SRI-ToF-MS revealed the volatile profile of each pepper species. The extraction of every single peak permitted the detection and the quantification of specific signals resultant of the protonation of numerous VOCs (*m/z* range = 20-210). From the volatile fraction composition of different pepper species, were detected many volatile compounds specifically alcohols, aldehydes, esters, ketones, hydrocarbons and terpenes compounds. For each peak identified using H_3O^+ and NO^+ as ion reagents, all of the *m/z* detected have been assigned to the mass formulas reported respectively in Table 2 and 3 and expressed in ncps higher than 1 (n=10; ±SD). In particular, by trusting the high accuracy and resolution of this tool, the chemical compounds have been tentatively proposed and matched with the existing documentation of VOCs in literature and by the acknowledged VOCs emitted by peppers. Thus, the identification of the compounds has been further improved by the use of two different reagents ions. All the peaks obtained have been filtered and 51 mass spectral peaks have been detected when H_3O^+ was the reagent ion and 18 using NO^+ as reagent ion.

Remarkably even if the pepper analyzed derived from different species (in particular *Capsicum Chacoense*), all the peaks identified using H_3O^+ or NO^+ as reagent ions were always present in the three species of chili pepper included in this study. Moreover, since the aroma is linked to the species and varieties (Taiti *et al*., 2019) the differences of VOCs emission among these three hot peppers were expected (Table 2 and 3). Above all, by using H_3O^+, *C. annuum* showed the highest total VOCs emission (22,116 ncps) followed by C. *chacoense* (13,214 ncps) and C. *baccatum* (4,850 ncps) as reported in Table 2. In particular, C. *annuum* ("Ciliegino" var.) seems to have a richest volatile profile compared to the other ones and showed the highest VOCs intensity for the compounds linked to the herbaceous notes. This trend could be confirmed by the high intensity of compounds detected at m/z 81.069, 83.086, 85.101, 99.080 and 101.096 all identified as C6 compounds (Table 2). Notably, peaks that confirmed this trend were detected at $C_4H_9^+$ (measured at *m/z*=57.069) probably derived from Alkyl fragment (Hexanol/valeric acid), $C_5H_7^+$ (measured *m/z*=67.055) and $C_6H_9^+$ (measured *m/z*=81.069) likely correspond to Alkyl fragment (isoprene and terpenes or aldehydes fragments respectively), $C_6H_{13}^+$ (measured *m/z*=85.101) probably refers to fragments of 1-Hexanol and/or Nonanol, $C_5H_{11}O_2^+$ (measured *m/z*=103.075) refers as 3-Methylbutanoic acid and $C_{15}H_{25}^+$ (measured *m/z*=205.195) attributed to Sesquiterpene compounds. On the contrary, the VOCs emission observed in the wild accession (*C. chacoense*) is characterized by the high emission of signals detected at m/z 33.033, 45.033 and 137.132, with the first two compounds which are linked to the ripening process whilst the last compound belongs to the terpene class. Moreover, it is interesting to note that the signal intensity of monoterpenes (*m/z* 137.132) is higher in *C. chacoense* than that observed for sesquiterpenes (*m/z* 205.101), in contrast to the emissions of *C. annum* and *C. baccatum* (Fig. 2, Table 2). Regarding the wide chemical classes of terpenes, common compounds which strongly contributes to the aroma of fruits and vegetables (Rodrîguez-Burruezo *et al.*, 2010), in this study were identified several peaks belonging to monoterpenes (C-10) and sesquiterpenes (C-15) while no one was detected as oxygenated terpenes. This result is also confirmed by what was already reported (Rodrîguez-Burruezo *et al*., 2010), where the oxygenated terpenes were found at only traces and mainly in *C. chinense*.

Finally, the aroma of "Brasileiro" variety (C. *baccatum*) was characterized by a lower signals intensity, except for the compounds detected at m/z 117.091 (TI: Hexanoic acid/Hexanoates) and m/z 121.101 (TI: Terpenes fragment). Additional representative chemical classes of the volatile fraction were aliphatic

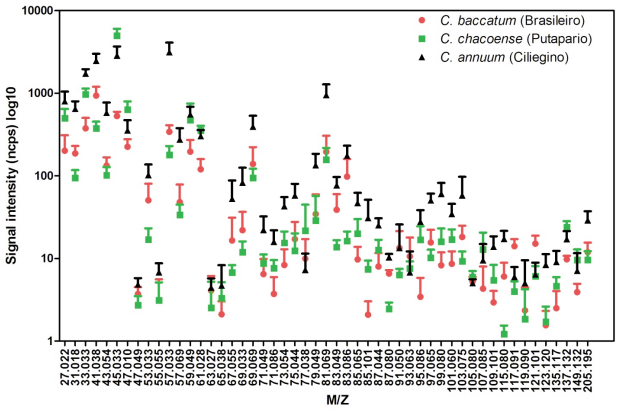


Fig. 2 - Example of schematic chart of mass peaks detected with H_3O^+ ion as reagent for each capsicum species used in this paper. Signal intensities are given in normalize count per second (ncps) log^{-1} and higher than 1.

aldehydes, alcohols, and branched hydrocarbons. In particular, Hexanal, Hexanol, cis-2-Hexanal and cis-2-Hexenol were the main compounds that contribute to the flavour note described as the odour of freshly cut grass or ground leaves, which are typically produced in fresh *Capsicum* fruits later on the tissue destruction (Ziino *et al.*, 2009). On the contrary by using NO^+ as donor ion, the differences of VOCs emission among all varieties studied were smaller as well as the number of peaks detected (Table 3). Sometimes these spikes were different, while sometimes were identical to those obtained using H_3O^+ as a reagent ion and this behavior is usual and has been already reported elsewhere (Jordan *et al*., 2009). Indeed, even if we use NO^+ as reagent ion in a complex matrix, H_3O^+ could occur in the ionization of the compounds present in the sample headspace (Jordan *et al*., 2009, Del Pulgar *et al*., 2013). Interesting, in contrast to what was observed with H_3O^+ , when using NO^+ the Capsicum species *C. baccatum* showed for the majority of signals the highest intensity detected (Table 3). Moreover, Table 3 shows significant differences in the concentration of many peaks between the three pepper species, especially as far as they are concerned with protonated aldehydes and ketones.

Artificial neural networks

Two neural networks have been built: the first one uses only VOCs emission, the second one combines the pungency with capsaicin and DHC analysis as inputs layers. Both the ANN (Artificial Neural Network) were able to discriminate among the accession with 100% accuracy. Thus, the confusion matrix assigns all the aromatic profiles correctly to the related pepper species (Table 4). According to the attribute selection filter applied, 12 VOCs profile and DHC content were the most discriminant parameters. In particular, the *m/z* signal of the VOCs and the tentative identification are reported here below and are marked in Table 2 with the asterisk symbol: *m/z* 31.018 (Formaldehyde), *m/z* 57.033 (C3 aldehydes and ketones), *m/z* 59.049 (Propanal, Acetone), *m/z* 71.049 (2-Butenal), *m/z* 73.060 (Isobutanal/Butanone), *m/z* 85.101 (1-Hexanol/Nonanol), *m/z* 99.080 (cis-3-Hexenal/(E)-2-Hexenal), *m/z* 115.111 (Heptanal), *m/z* 135.117 (p-Cymene), *m/z* 137.132 (Monoterpenes) and *m/z* 149.132 (Sesquiterpenes fragments).

Random tree

The data coming from the aromatic profiles of the pepper species were also analyzed using a decision tree learning tool. The identification was successful in 96.6% of the cases, and, as shown in the confusion matrix, Table 4, only one instance belonging to *C. baccatum* was incorrectly attributed to *C. chacoense.* The decision tree showing the two significant nodes is reported in figure 3. According to the tree, only two VOCs are fundamental to discriminate among the three species: *m/z* 81.068 and *m/z* 85.064.

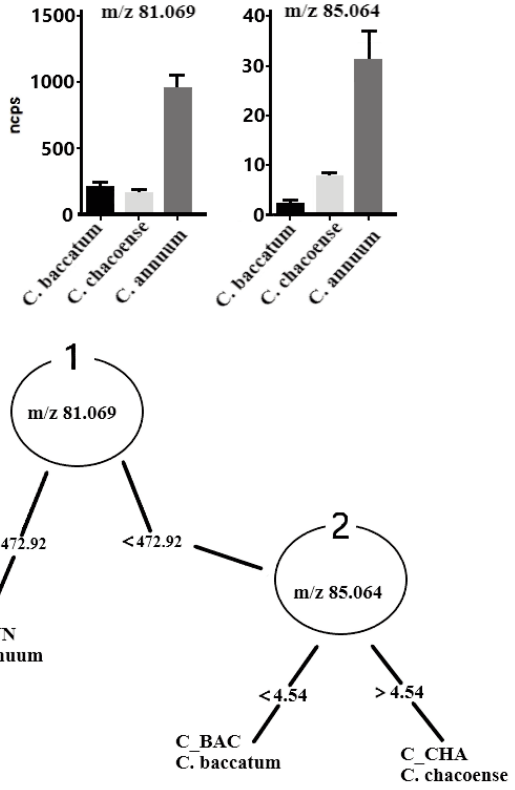


Fig. 3 - On the top the VOCs signal of the two most significant masses that help the identification of the species using a decision tree learning tool (n=10; SD). On the bottom, the two significant nodes have been reported.

Protein profiles

SDS-PAGE analyses performed on *Capsicum* species (Fig. 4) identified specific profiles linked to each sample. Gel images were then analyzed to generate data like a phylogenetic tree based on similarity comparison (Fig. 4) to graphically display relationships among samples. Dendrograms results showed as two samples, *C. chacoense* and *C. baccatum,* clusterized independently from the third sample *C. annuum.* Differences detected in the protein profiles could be associated with quantitative differences in the band densities as soon as some quantitative differ-

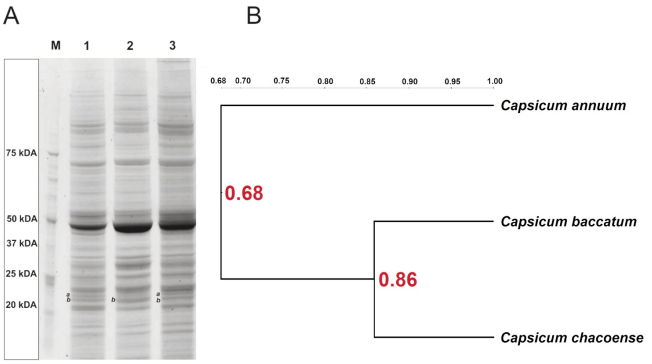


Fig. 4 - (A) SDS-PAGE analysis of proteins getting from *Capsicum* samples, where 1= *C. chacoense*; 2= *C. annuum*; 3= *C. baccatum*; M= molecular marker. (B) Phylogenetic tree resulting from the analysis of protein by SDS-PAGE analysis. Image data were processed with Quantity One software (Bio-Rad Laboratories, Inc, Hercules CA) using Ward's method for clustering (Ward, 1963).

ences. *C. chacoense* and *C. baccatum* showed a band (a) with a molecular weight slightly higher 20 kDa that were not detected in the *C. annuum* sample (Fig. 4).

4. Conclusions

Our study underlined the high potential of the using the PTR- SRI-MS to obtain a species-specific fingerprinting of the volatile compounds emitted by the pepper fruits. This technology can help to highlight particular VOCs signals that are specific of species or specific growing conditions of chili pepper fruits with a rapid analysis without any pre-treatment of the samples. Indeed, the switching reagent ion system in PTR-MS instrumentation was applied for the first time to analyze pepper fruits by using not only H_3O^+ but also NO^+ as precursor ions. This tool has permitted to find the VOCS able to discriminate among the species and by using two ionization agents a more accurate identification of the volatile compounds has been possible. In particular, the PTR-ToF-MS analysis with NO^+ as reagent ion has allowed (i) the detection of aldehydes and ketones in separated peaks, (ii) to detect some molecules not found using H_3O^+, (iii) to confirm the results obtained using H3O+ as reagent ion for VOC analysis. VOCs results were thus confirmed by protein analysis according to qualitative and quantitative differences, which turn out to be able to differentiate samples within the capsicum genus. Moreover, the multivariate statistical approach revealed that some of these compounds can be successfully used for the species recognition in our artificial neural networks. The BPNN classifier utilized in the work had always 100% of success, both for H_3O^+ and NO^+ (data not shown) and this was probably due to the marked differences in the volatiles emissions of the three species. Further studies will aim to use the same method for a higher number of species, to challenge the analysis. Our investigation permitted the identification of 12 promising VOCs as more discriminant for each species and, among them, the masses *m/z* 81.068 and *m/z* 85.064 have been recognized as the most promising volatile markers of these species (Fig. 3). In addition to 12 VOCs, regarding the capsaicinoids, the DHC content was a more effective parameter to distinguish among domesticated and wild species of the genus *Capsicum* with respect to the capsaicin. The results from the random tree have been according with the protein analysis, which showed that the *C. chacoense* and *C. baccatum* have more similarities with respect to the C. annuum, although this was a preliminary analysis and additional analysis need to be done to support our hypothesis. According to our findings, both domesticated species, i.e. *C. baccatum* and *C. annuum* differ from the wild species *C. chacoense*, in particular, the *C. annuum* resulted in being the most dissimilar from the wild species. This was probably due to a more strict genetic selection that the *C. annuum* faced during years of domestication and supports the hypothesis that C. chacoense is one of the most ancient species of the genus and that the *C. baccatum* and *C. annuum* evolved separately from this common predecessor.

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