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^{1}H NMR-based metabolomic fingerprinting to determine metabolite levels in serrano peppers (*Capsicum annum* L.) grown in two different regions

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ABSTRACT

Chili pepper (*Capsicum annuum*) is the most important and emblematic condiment in Mexican food. Serrano pepper is a variety of *C. annuum* that is traditionally cultivated in Mexico and commercialized in local markets. The aim of this study was to describe the ^{1}H NMR metabolomic profiling of the aqueous phase of serrano peppers harvested from two distinct regions, in the states of Veracruz and Oaxaca, Mexico. According to the current results, aspartate citrate, lactate, leucine and sucrose were found at higher amount in the serrano peppers from Veracruz. On the other hand, acetate, formate, fumarate, malonate, phosphocholine, pyruvate and succinate showed the highest abundance in this product from Oaxaca. These are the main metabolites that distinguish one group from the other. The spectrometric method reported presently is characterized by great simplicity, robustness and reproducibility. Thus, this technique can be used for establishing reliable metabolomic fingerprints of serrano peppers grown under different environmental conditions.

1. Introduction

Green chili pepper (*Capsicum annuum* L.) has been a popular food condiment in Mexico since pre-Hispanic times and is considered almost imperative for Mexican dishes today (Aguilar-Rincón, 2012). Its use in preparing exotic dishes around the world has increased in recent years. In addition to the culinary application of chili peppers, they are employed in traditional ceremonies and as a natural analgesic.

Although the pungent activity of chili pepper is the most attractive property for consumers, the nutritional and anti-nutritional content should also be considered. This is particularly important in Mexico, as it is the sixth largest chili exporter in the world, making the marketing of this product a valuable economic resource for the country (Gaytan & Benita, 2014). In order to maintain quality for export, reliable and fast analytical methods must be available to estimate the nutritional properties of chili peppers.

The chemical composition of *C. annuum* has been extensively studied. Glycosides, fatty acids, carotenoids, anthocyanins, volatile organic compounds, aldehydes, organic acids, ketones, alcohols, ethers, and sulfur compounds have been identified from the epicarp, mesocarp and endocarp of green chili peppers (Pino et al., 2007). Capsaicinoids, responsible for the characteristic pungency of the fruit, are biosynthesized in the placenta (González-Zamora et al., 2013; Kim, Ha, & Park, 2008; Maji & Banerji, 2016; Pino et al., 2007). Bromatological analyses suggest that chili peppers contain an acceptable amount of fat, protein, assimilable carbohydrates, fiber, thiamine, riboflavin, niacin and ascorbic acid, as well as high levels of calcium and sodium (Bijttebier et al., 2014; Conforti, Statti, & Menichini, 2007; Hervert-Hernandez, Sayago-Ayerdi, & Goñi, 2010; Howard, Smith, Wagner, Villalon, & Burns, 1994; Jeun & Hwang, 1991; Okunlola, Akinwale, & Adelusi, 2016). The presence (and relative abundance) of these metabolites in *C. annum* is strongly linked to genetic variability and to the effect of biotic and abiotic factors, such as the ripeness of the fruit and the conditions of the open fields in which it is cultivated (Kim et al., 2008).

Nuclear magnetic resonance (NMR), gas chromatography–mass

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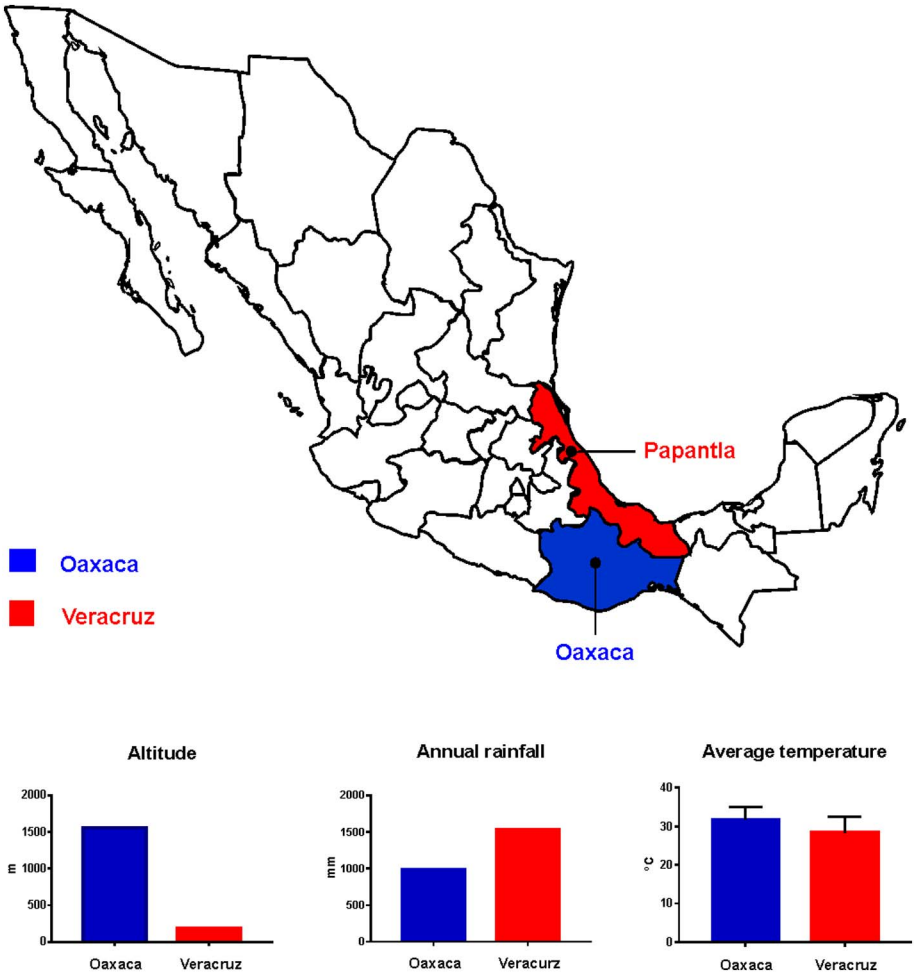

Fig. 1. Geographical distribution of the two regions in Mexico where the serrano pepper samples of the current study were collected, with differences in altitude (m), annual rainfall (mm), and average temperature in January and August (°C).

Table 1
Size and weight of the serrano peppers (*Capsicum annum* L.) harvested in the states of Veracruz and Oaxaca, Mexico.

Species	Variety	Region of origin	Weight (g)	Length (cm)	Width (cm)
Capsicum annum L.	Serrano	Oaxaca, Oaxaca	14.85 ± 1	6.93 ± 1	1.1 ± 0.2
Capsicum annum L.	Serrano	Papantla, Veracruz	15.11 ± 1	7.24 ± 1	1.2 ± 0.2

spectrometry (GC–MS) and Liquid chromatography-mass spectrometry (LCMS) are the routine analytic techniques used to identify the small molecules that comprise chili peppers (Shulaev, 2006). The high reproducibility of NMR-based techniques gives this method many advantages over other analytical techniques in large-scale and long-term metabolomic studies. Although MS is more sensitive than NMR, the later has the advantages of being relatively robust across many samples (no part of the sample becomes contaminated during the process), it is relatively fast (with spectra acquired within a few minutes) and is a non-destructive analytical tool (Emwas, Salek, Griffin, & Merzaban, 2013; Gebregiworgis & Powers, 2012; Larive, Barding, & Dinges, 2015). The inherent limitation of NMR spectroscopy is its low sensitivity. Despite the significant enhancement of NMR sensitivity resulting from continuous developments in hardware, software and applications, such as the introduction of *para*-hydrogen-induced hyperpolarization (PHIP) and spin hyperpolarization via dynamic nuclear polarization (DNP), MS remains superior in sensitivity (Bjerrum, 2015). When combined with multivariate statistical analysis, NMR has proven to be a powerful tool for evaluating the chemical profiling of several types of vegetables growing in different geographical regions, such as cabbage, grapes, green coffee and green tea (Hong-Seok et al., 2009; Jahan et al., 2013; Jang-Eun et al., 2010; Wei et al., 2012).

The information obtained from an NMR study can help determine the nutritional properties of a cultivated vegetable species as well as the specific chemical fingerprint of these species from distinct geographical origins. To our knowledge, there are no reports on the NMR metabolomic fingerprint of the *Capsicum* species cultivated in Mexico. Hence, the main goal of this work was to develop an ^{1}H NMR-based protocol to differentiate the metabolomic profiling of serrano peppers cultivated in Veracruz and Oaxaca, Mexico.

2. Materials and methods

2.1. Plant source

On June 6th, 2016, 20 serrano peppers (*Capsicum annum* L.) were harvested in Oaxaca, Oaxaca (Mexico, 17°04′01″N 96°43′13″O), and on June 8th another 20 were picked in Papantla, Veracruz (Mexico, 20°26′56″N 97°19′28″O). The data on average temperature (°C) and annual rainfall (mm) were obtained from the Mexican Meteorological Administration (Fig. 1). Healthy fruits were chosen considering a similar color, size, texture and weight. Similarity in color and texture among samples was estimated by the individual perception of the harvester. The length, width and weight of the peppers were determined in accordance to the Mexican Official Norms NMX-FF-025-SCFI-2014 (Normas Oficiales Mexicanas, 2015). Measurement of the length was made from the base (excluding the peduncle) to the apex,

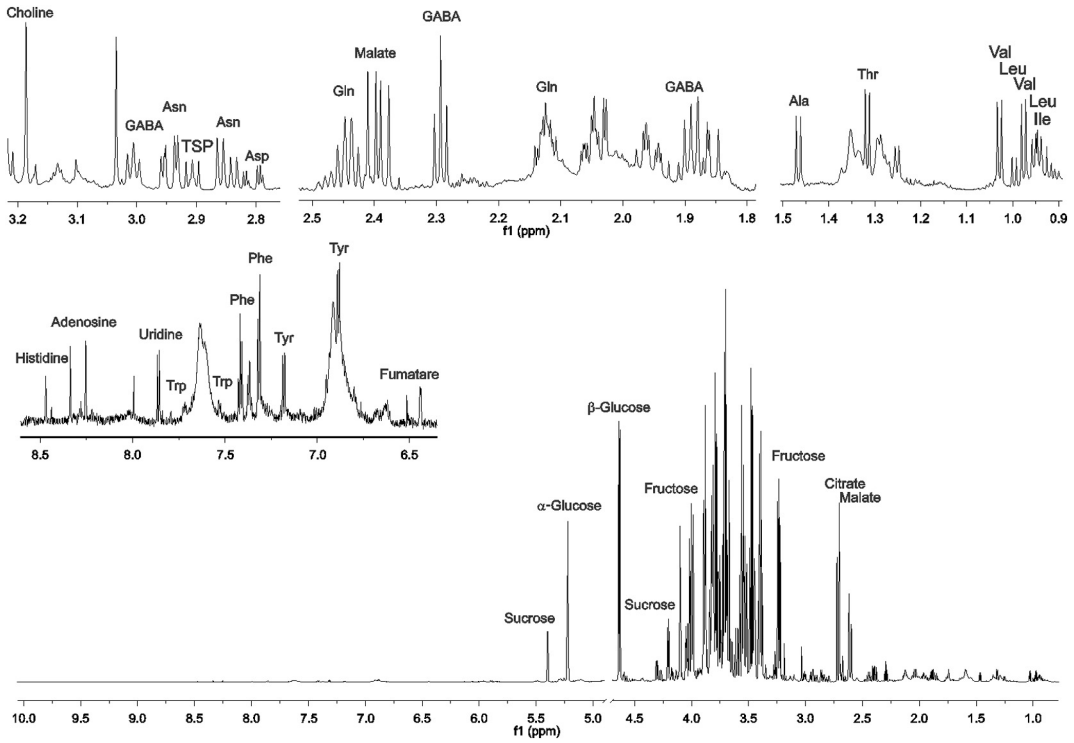

Fig. 2. ^{1}H NMR spectrum (750 MHz) of the aqueous extract of serrano peppers from Veracruz, Mexico. Signal assignments are based on reference spectra and the literature (Aizat et al., 2014; Ritota et al., 2010).

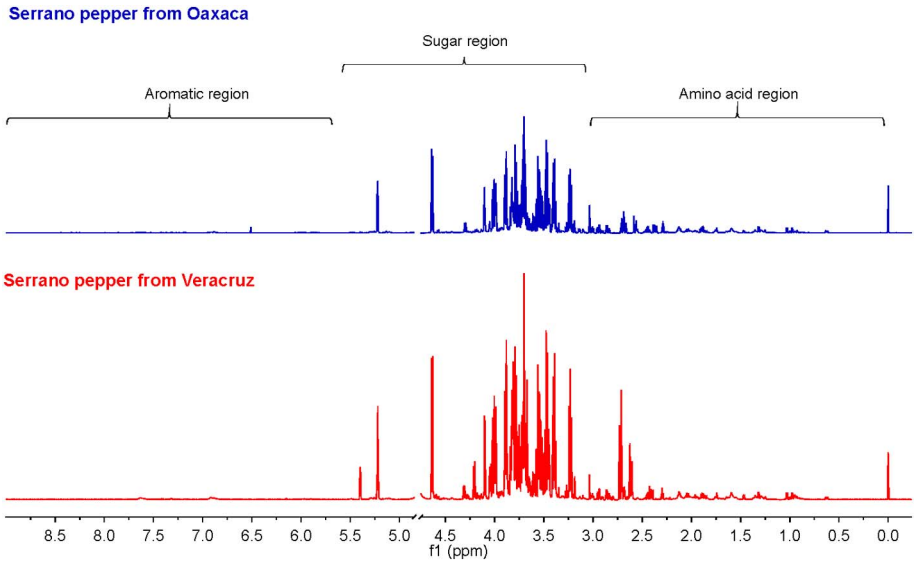

Fig. 3. Stacking of the 750 MHz ^{1}H NMR spectra of serrano peppers harvested in the states of Veracruz and Oaxaca, Mexico.

and of the width at the widest point. The weight was evaluated on an analytical balance (Table 1) (Troconis-Torres et al., 2012).

2.2. Chemicals

Deuterium oxide (D_2O, D 99.9 at.%) was purchased (Cambridge Isotope Laboratories, Inc.) and used as solvent. For ^{1}H NMR analysis, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (Sigma-Aldrich Co.; TSP, 97%) was the internal standard, while EDTA (ethylenediaminetetraacetic acid) and sodium azide (NaN_3) (Merck™) were also added to samples. NaOH and HCl (Sigma-Aldrich Co.) were employed to adjust pH.

2.3. Sample preparation

Each pepper was squeezed in a mortar and the juice was centrifuged for 20 min at 15900*g*. For subsequent ^{1}H NMR analysis, 900 μL of the aqueous upper phase was mixed with 100 μL of a solution containing 7 mM TSP, 10 mM EDTA, and 2 mM NaN_3 in D_2O, at pH 5.42 ± 0.05. Finally, 600 μL of this solution were placed into 5 mm NMR tubes (Hohmann, Christoph, Wachter, & Holzgrabe, 2014).

2.4. Nuclear magnetic resonance (NMR) experiments

NMR experiments were carried out by using a Bruker 750 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm TXI cryoprobe. All the aqueous extracts from the serrano peppers

Table 2
Metabolites identified by ^{1}H NMR in serrano peppers from Oaxaca (O) and Veracruz (V).

Compound	O	V
Amino acids		
Alanine	x	x
Asparagine	x	x
Aspartate	x	x
4-Aminobutyric acid	x	x
Glutamine	x	x
Histidine	x	x
Isoleucine	x	x
Leucine	x	x
Phenylalanine	x	x
Threonine	x	x
Tryptophan	x	x
Tyrosine	x	x
Valine	x	x
Carboxylic acids		
Acetate	x	x
Citrate	x	x
Formate	x	x
Fumarate	x	x
Lactate	x	
Malate	x	x
Malonate	x	x
Pyruvate	x	x
Succinate		x
Alcohols		
Ethanol	x	x
Ethanolamine	x	x
Methanol	x	x
Glycerol	x	x
Sugars and sugars derivates		
Fructose	x	x
Galactose	x	x
Glucose	x	x
Maltose	x	x
Myo-inositol	x	x
Sucrose	x	x
Nucleosides		
Adenosine	x	x
Cytidine	x	x
Guanosine	x	x
Uridine	x	x
Organic compounds		
2-Hydroxybutyrate	x	x
2-Hydroxyisobutyrate	x	x
Choline	x	x
Phosphocholine	x	x

were measured at 298.1 ± 0.1 K, without rotation and with 4 dummy scans prior to 64 scans. Acquisition parameters were set as follows: FID size = 64 K, spectral width = 19.9967 ppm, receiver gain = 1, acquisition time = 2.18 s, relaxation delay = 10 s, and FID resolution = 0.45 Hz. Data acquisition was achieved by NOESY presaturation pulse sequence (Bruker 1D noesypr1d) with water suppression via irradiation of the water frequency during the recycle and mixing time delays (Hohmann et al., 2014).

Several NMR experiments were performed to corroborate signals assignments. Such experiments were based on J-resolved spectroscopy (2D ^{1}H JRES), homonuclear correlation spectroscopy (2D ^{1}H–^{1}H COSY), total correlation spectroscopy (2D ^{1}H–^{1}H TOCSY), heteronuclear single quantum correlations (2D ^{1}H–^{13}C HSQC) (Table S1). The 2D NMR experiments were performed using a Varian NMR system 500 spectrometer (now Agilent, Santa Clara, CA, US) operating at 499.8 MHz (^{1}H frequency) and at 298 K. The parameters for one dimensional ^{1}H NMR spectrum were: number of scans = 8, acquisition time = 2.04 s, relaxation delay = 2 s, spectral width = 8012.8 Hz and FID size = 32 k data. For ^{13}C NMR spectrum the number of scans = 3000, acquisition time = 1.04, relaxation delay = 2 s, spectral width = 31,250.0 Hz, and FID size = 64 k data. COSY measurements were performed with spectral width = 8012.8 Hz in either dimension; 2 k × 128 were acquired with number of scans = 8 per increment and relaxation delay = 2 s. TOCSY experiment was acquired with a spectral width of 8012.8 Hz in both dimensions, and a mixing time of 80 ms; 4 scans per increment were collected into 256 increments with a relaxation delay of 1 s and acquisition time of 0.15 s. The 2D ^{1}H JRES spectrum was acquired using a HOMO2DJ pulse sequence using 16 scans per increment and 128 increments with an acquisition time of 0.625 s, and a relaxation delay of 1 s. The spectral widths in F1 and F2 were 64 Hz and 7 kHz, respectively. For gHSQC, the number of scans = 8 and 256 increments, acquisition time = 0.15 s, relaxation delay = 1 s, spectral width = 8012.8 and 25,133.5 Hz for the ^{1}H and ^{13}C dimensions, respectively, $^{1}J_{CH}$ = 145 Hz.

2.5. Metabolite profiling

Metabolites identification was essentially achieved by consulting the literature, (Clausen, Bach, Edelenbos, & Bertram, 2012; Deborde et al., 2009; Hamida et al., 2017; Le Gall, Colquhoun, Davis, Collins, & Verhoeen, 2003; Ritota, Marini, Sequi, & Valentini, 2010; Tarachiwin, Masako, & Fukusaki, 2008) and were corroborated by performing the above mentioned 1D and 2D NMR experiments (Table S1). Firstly, the ^{1}H NMR spectra were automatically phased and the baseline was corrected and calibrated to the TSP signal at 0.0 ppm by using the MestReNova program (version 6.0.2; MestReC, Santiago de Compostela, Spain) (Hohmann et al., 2014). The resulting ^{1}H NMR spectra processed in MestReC were also imported into the Processor module of Chenomx NMR Suite version 8.2 (Chenomx, Edmonton, Canada), where they were subject to baseline correction, line broadening, phase correction and shim correction. In this module, the spectra were calibrated to the signal of internal standard (TSP) and the pH was entered within a certain range (pH 4–9). The Profiler module was used to determine the relative metabolites abundance. A list of compounds and their relative abundance is produced from each spectrum and was subjected to statistical analysis for a given sample (Duynhoven, Van As, Belton, & Webb, 2013; Eun-Jeong, Shaykhutdinov, Weljie, Vogel, & Facchini, 2009).

2.6. Multivariate statistical analysis

Chemometric analysis was performed with SIMCA version 13.0.3 (Umetrics, Kinnelon, NJ, USA) in the form of an unsupervised principal component analysis (PCA). This is a widely used multivariate analysis method in metabolomics studies and chemometrics in general. Its purpose is to reduce the number of predictive variables and solve the multi-collinearity problem (Maitra & Yan, 2008). With PCA, class differences were established from a multivariate dataset (Fig. S1-A to S1-C). To maximize the separation of class, orthogonal projections to latent structures discriminant analysis (OPLS-DA) was applied (Worley & Powers, 2013) (Fig. S2-A to S2-D). The quality of the model was described by the Rx^2 and Q^2 values. Rx^2 was defined as the proportion of the variance in the data observed in the model, and this parameter indicated goodness of fit. Q^2 was defined as the proportion of variance in the data that was predictable by the model, and it denoted predictability (Jung et al., 2010; Wei, Furihata, Zhang, Miyakawa, & Tanokura, 2016).

3. Results and discussion

The characteristic one-dimensional ^{1}H NMR profiling of the aqueous phase of the serrano peppers is shown in Fig. 2. There were 40 metabolites identified in the ^{1}H NMR spectra of aqueous extracts, and most of these possess nutritional relevance.

In the chemical profiling, glucose, fructose and sucrose were the predominant carbohydrates observed (3.0 to 5.5 ppm). These

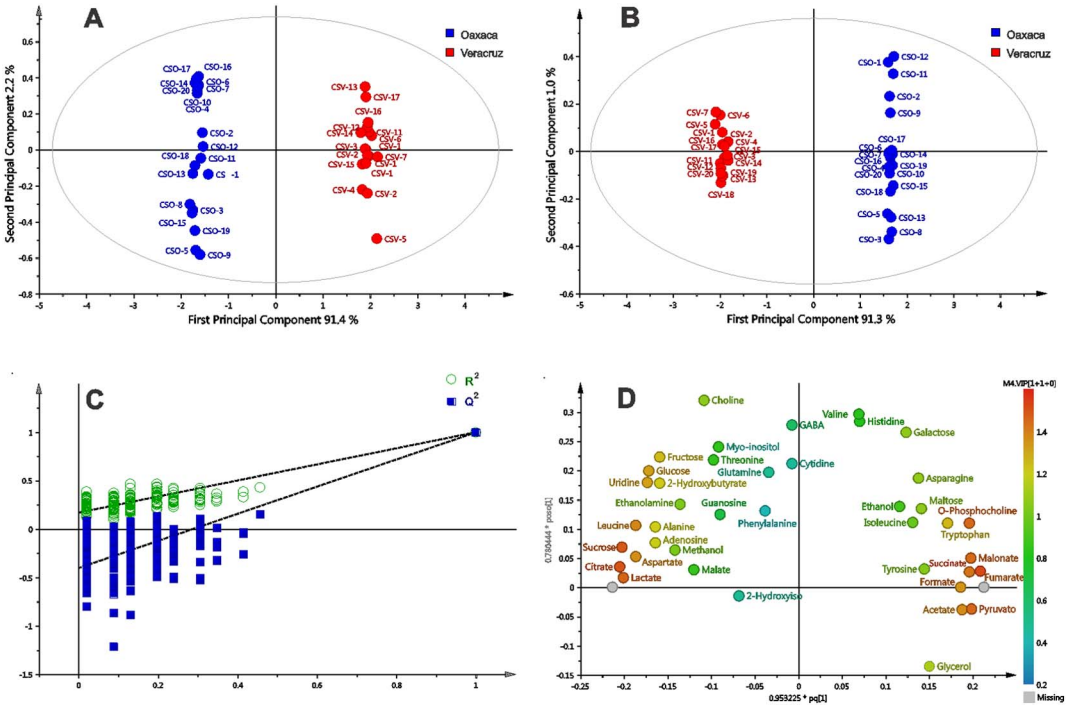

Fig. 4. A) PCA and B) OPLS-DA score plots derived from the ^{1}H NMR spectra of *C. annuum*, serrano type, cultivated in the State of Veracruz (red) and Oaxaca (blue). C) Statistical validation of the OPLS-DA model using permutation analysis for discrimination. D) Corresponding loading scatter plots derived from targeted profiling of mostly primary metabolites. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this chapter.)

compounds are easily recognizable in the anomeric region at 4.01, 4.63, 5.22 and 5.40 ppm, for fructose, β-glucose, α-glucose, and sucrose, respectively. Amino acids and carboxylic acids were detected in the high field region (0.5–3.0 ppm), while only a few low intensity signals (corresponding to the presence of aromatic compounds) were found in the low field region (6.0–10.0 ppm). The comparison between the chemical profiling of serrano peppers from Oaxaca and Veracruz is shown in Fig. 3.

Visual inspection of the ^{1}H NMR spectra revealed clear differences between the two groups in the levels of citrate, fructose, glucose and sucrose. The qualitative results of the metabolic profiling from the two representative locations are listed in Table 2. According to the chemical profiling, the serrano peppers contained 13 amino acids (including seven essential amino acids for human nutrition), 9 carboxylic acids, 4 alcohols, 6 sugars and 4 nucleosides. Interestingly, the main differences were that the peppers from Oaxaca had lactate but no succinate, while those from Veracruz presented succinate without the presence of lactate.

PCA is a well-known unsupervised pattern-recognition method that does not require prior knowledge. It is employed in chemometric analysis to visualize grouping trends and atypical values. With the binning integrals of the metabolites measured in the NMR spectra, the PCA and OPLS-DA score plots were constructed. In Fig. 4A and B can be seen the separation of the groups corresponding to the evaluated serrano peppers from two different regions of Mexico. Based on these plots, the metabolic fingerprints of the two groups of samples were compared and the metabolites were determined.

The PCA score plots demonstrate statistically significant differences in metabolite composition between the two groups. The PCA model (Fig. 4A) for distinguishing serrano peppers from different origins, established with three components, revealed Rx^2 and Q^2 values of 0.936 and 0.875, respectively. To better observe differences between groups, OPLS-DA was applied. The OPLS-DA model (Fig. 4B), consisting of one predictive and two orthogonal components, revealed Rx^2, Ry^2 and Q^2 values of 0.923, 0.999 and 0.998, respectively.

A random permutation test (200 times) was performed on the corresponding OPLS-DA model to validate the differences between the serrano peppers harvested in each of the two regions. In the plots of the permutation test, the regression line for R^2 is in green and that for Q^2 is in blue. The y-axis intercepts of R^2 and Q^2 were 0.17 and -0.402, respectively (Fig. 4C), indicating the validation of the model (since the intercept of R^2 was less than 0.4 and the intercept of Q^2 was less than 0.05) (Eriksson, Johansson, Trygg, & Vikström, 2013; Wang et al., 2012).

To further understand the variables contributing to the differentiation, loadings plot from OPLS-DA was constructed. It turns out that aspartate, citrate, lactate, leucine and sucrose showed higher presence in the serrano peppers grown in Veracruz. On the other hand, acetate, formate, fumarate, malonate, phosphocholine, pyruvate and succinate exhibited the highest abundance in the product from Oaxaca (Fig. 4D). In the fruit from Veracruz, sucrose was more abundant, and levels of aspartate and leucine were elevated. Organic acids displayed a variable composition in the samples, with citrate, and lactate being higher in the peppers from Veracruz, while acetate, formate, fumarate, malonate and succinate were predominant in those from Oaxaca (Fig. 5), where relative abundance is expressed as normally used (Eun-Jeong et al., 2009; Jung et al., 2010). Relative abundance of other significant metabolites is shown in Fig. S3.

Overall, the main differences between the metabolic profiling of the two groups of serrano peppers were related to organic acids. As is well known, organic acids and sugars are the main hydrophilic components of ripe fruits. These compounds are associated with organoleptic properties such as sweetness and sourness. *Capsicum chinense* contains moderate levels of sugars and relatively elevated levels of organic acids, a specific chemical composition that generates its characteristic non-sweetness (Famiani, Battistelli, Moscatello, Cruz-Castillo, & Walker, 2015; Hockema & Etxeberria, 2001; Jarret, Berke, Baldwin, & Antonious, 2009).

The presence of organic acids is correlated with maturity levels in hot pepper “CM334”. Significant changes in the endogenous levels of oxalic, malic and succinic acids are associated with the expression of certain genes in six developmental stages of the hot pepper fruit (Jang,

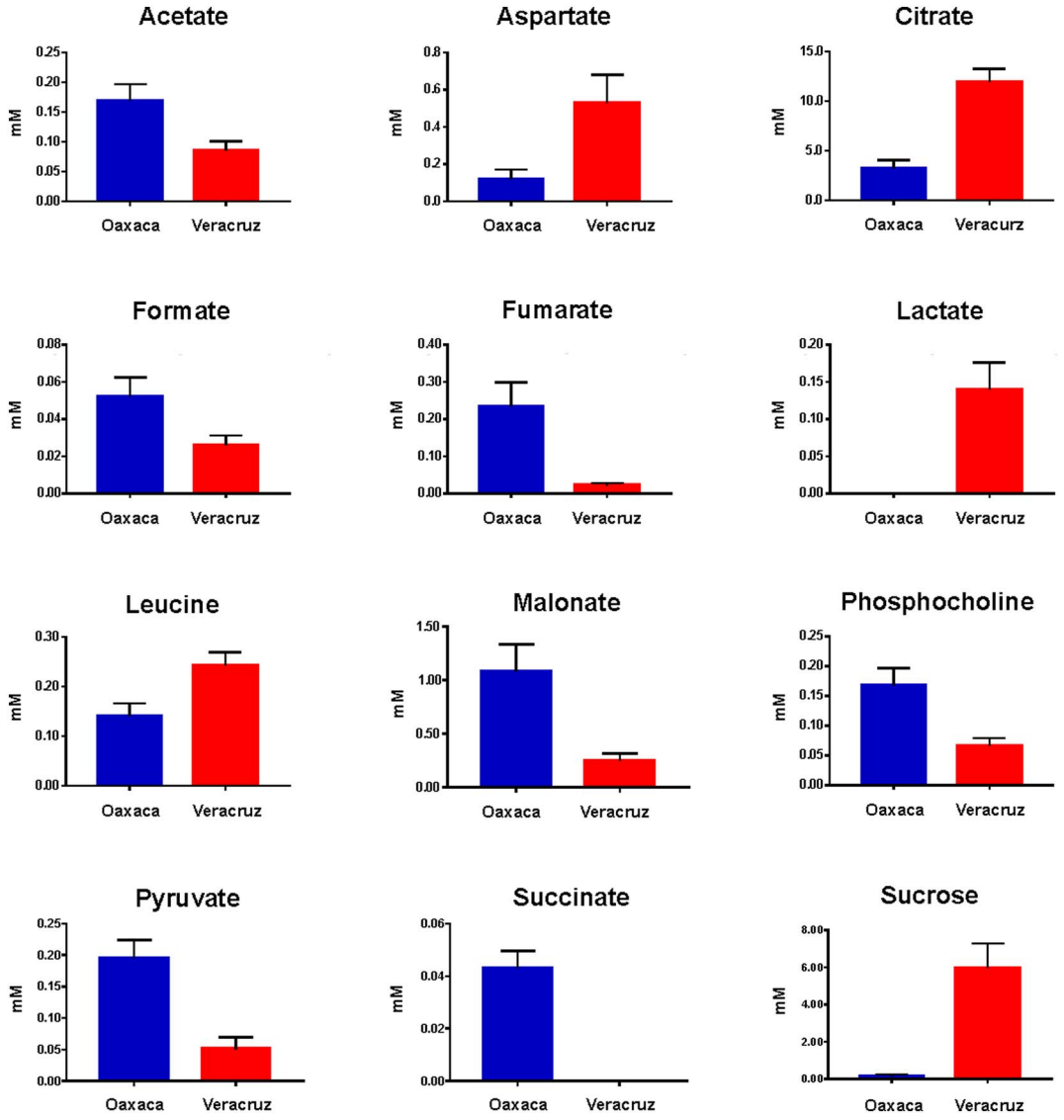

Fig. 5. Relative abundance of metabolites based on the mean peak area of the associated signals from 750 MHz ^{1}H NMR spectra of serrano peppers from Oaxaca and Veracruz, Mexico. Data are expressed as the mean ± standard error calculated using twenty replicates.

Jung, Hun-Ah, Choi, & Lee, 2015). Chili pepper is one of the richest fruits in vitamin C, and the accumulation of this macronutrient is directly linked to a high degree of maturity (Kumar & Tata, 2009). The conversion of oxalic acid into ascorbic acid has been classically reported in green tissues that accumulate keto acids (Yang & Loewus, 1975). Conversely, the putative degradation of ascorbic acid into keto acids was observed in the latter stages of maturity of tomatoes (Truffault, Fry, Stevens, & Gautier, 2017). Therefore, the determination of elevated levels of keto acids may be considered as a possible chemical fingerprint of a high degree of maturity.

According to the current results, most of the differential organic acids found in the two groups of serrano peppers (from Oaxaca and Veracruz) are part of the Krebs cycle. It has been demonstrated that this amphibolic pathway can be altered in fruits by several factors, including temperature (Famiani et al., 2015), water availability, and the practices used in cultivation, harvesting and storage. The data from the present study suggest that geographical origin could be determinant in the production of organic acids in serrano peppers (Aizat et al., 2014; Araújo, Nunes-Nesi, Nikoloski, Sweetlove, & Fernie, 2012; Kallsen, Sanden, & Arpaia, 2011; Vega-Gálvez et al., 2009).

In the central valley of Oaxaca, there is mostly vertisol soil and a semi-dry, semi-warm climate with rain in the summer. Contrarily, Papantla, Veracruz, has regosol soil and warm weather with abundant rain in the summer and early autumn. The average annual temperature in January and August is 31.8 °C and 28.3 °C, respectively (SMN, Comisión Nacional del Agua, reporte del clima en México, 2016).

The temperature at which fruits are grown affects both their titratable acidity and content of stored organic acids (Jarret et al., 2009). In fruits, as in all plant tissues, a rise in temperature increases their metabolic rate, and hence the consumption of compounds such as sugars and stored Krebs cycle acids that serve as metabolic substrates. In this sense, higher levels of fumaric, pyruvic, malonic and succinic acids were observed in the *C. annuum* from the Oaxaca area, which had the higher average temperature.

On the other hand, excess water supply, whether from abundant rainfall or irrigation, can affect the organic acid composition of fruit (Famiani et al., 2015). In this case, the effect is not very clear because of the interaction with other factors, such as climatic conditions, cultivation techniques, and the time during fruit development when the water stress occurs. However, many studies have shown an indirect relationship between the water supply and the titratable acidity and organic acid content of fruits (Kallsen et al., 2011), findings in agreement with the current results.

4. Conclusion

The ^{1}H NMR chemical profiling of the aqueous extracts from serrano peppers is described for the first time. The two groups of chili peppers, from Oaxaca and Veracruz, exhibited a significant chemical variance in keto acid content. Due to its simplicity and robustness, the method herein developed can be used to perform further studies on serrano peppers grown in diverse geographical locations and/or under different environmental conditions and cultivation practices. The present results demonstrate that the *C. annuum* metabolomic profiling depends on the growing area and hence is influenced by climatic conditions such as temperature and pluvial precipitation. Furthermore, the current data may be useful for gaining insight into chili pepper metabolism, and this information could possibly improve its nutritional value.

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Appendix A. Supplementary data

Supplementary data associated with this article is available free of charge. Supplementary data associated with this article can be found in the online version, at doi: https://doi.org/10.1016/j.foodres.2017.10.005.

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