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ARTICLE

Determination of Capsaicin, Ascorbic Acid, Total Phenolic Compounds and Antioxidant Activity of *Capsicum annuum* L. var. serrano by Mid Infrared Spectroscopy (Mid-FTIR) and Chemometric Analysis

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Abstract Fourier transform mid-infrared (Mid-FTIR) spectroscopy in conjunction with multivariate analysis was used to predict the capsaicin content, ascorbic acid, total phenolic compounds, and antioxidant activity of *Capsicum annuum* L. variety serrano. Two multivariate calibrations, partial least square (PLS), and principal component regression (PCR) were optimized to construct the calibration models. The best models used to quantify the above mentioned compounds were obtained with the PLS algorithm and coefficients of determination (R^2) greater than 0.998 as well as a standard error calibration less than 0.098. The results demonstrated that Mid-FTIR spectroscopy in combination with multivariate analysis can be effectively used for to quantify the capsaicin, ascorbic acid, total phenol content, and antioxidant activity of *Capsicum annuum* var. serrano. Mid-FTIR spectroscopy in combination with multivariate calibration offers rapid, easy sample preparation, is environmentally friendly, and is operationally uncomplicated, demonstrating the significant advantages of the chemometric models compared with conventional methods of analysis.

Keywords *Capsicum annuum* · Mid-Fourier transform infrared · multivariate analysis · serrano chilli

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Introduction

There is evidence that the consumption of fruits and vegetables is associated with decreased risk of cancer, heart disease, and degenerative diseases associated with ageing (Charles, 2013). One such vegetable in which a variety of antioxidants can be found is the chilli. Chillies belong to the genus *Capsicum*, which is an important crop worldwide according to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2011).

Capsicum is widely used due to its strong pungency, aroma, color, and nutritional value. Indian, Chinese, and North American traditional medicines have also used chillies for the treatment of arthritis, rheumatism, stomach aches, skin rashes, dog/snake bites, and flesh wounds (Meghvansi et al., 2010). Presently, the *Capsicum* genus encompasses five well-described domesticated species (*Capsicum baccatum, Capsicum chinense, Capsicum frutescens, Capsicum pubescens*, and *Capsicum annuum*). However, virtually

all commercially cultivated *Capsicum* cultivars in the world belong to *Capsicum annuum,* which includes the following pepper types: ancho/poblano, bell, cayenne, exotics, jalapeño, paprika, pimiento, piquin, serrano among others (Fett, 2003).

In México, it has been estimated that C. *annuum* is the second most consumed after tomatoes, with a consumption rate of approximately 7-9 kg/person per year (Ornelas-Paz et al., 2010). Of this amount, approximately 75% is consumed as a fresh product, being the serrano variety, which is the most consumed (Álvarez-Parrilla et al., 2011).

Fresh *Capsicum annuum* has been recognized to have antioxidant activity due to the presence of metabolites with well-known antioxidant capacities such as ascorbic acid, vitamin E, provitamin A, carotenoids, xanthophylls, and phenolic compounds, which are present in connection with sugars (Materska and Perucka, 2005). In particular, *Capsicum annuum* is considered as the vegetable with the highest content of ascorbic acid (Lee and Kader, 2000) and is useful for prevention of the oxidation of cholesterol and docosahexaenoic acid (Sun et al., 2007). Additionally, it is recognised that *Capsicum* spp. is the only plant that produces capsaicinoids, which are responsible for the characteristic hot taste (pungency) of vegetables. Pungency is caused by the direct effect of capsaicin and its analogues on the pain receptors in the mouth and throat (Diaz, 2004).

Apart from their high antioxidant power (Díaz et al., 2004), capsaicinoids are compounds with notable anti-mutagenic and anti-tumoral properties that are used as topical analgesics to treat many painful diseases, such as post-herpetic neuralgia, arthritis, diabetic neuropathy, among others (Fett, 2003). In recent years, these compounds have been extensively studied and have been proved to be an effective treatment for a number of sensory nerve fiber disorders such as cystitis and the human immunodeficiency virus (Robbins, 2000). Capsaicinoids have also been reported as having antibacterial effects on a particular group of bacteria (Dorantes et al., 2000).

The quantitative determination of bioactive compounds such as ascorbic acid and total phenolic compounds has been reported in several works by using HPLC and spectroscopic techniques (Deepa et al., 2007; Álvarez-Parrilla et al., 2011; Medina-Juárez et al., 2012). The analytical methods employed for the separation and determination of capsaicin include several techniques such as organoleptic methods (Scoville Organoleptic Test) (Topuz and Ozdemir, 2007), spectrophotometry (Deepa et al., 2007), thin layer chromatography (Lee et al., 1976), gas chromatography (Cisneros-Pineda et al., 2007), gas chromatography coupled to mass spectrometry (Pino et al., 2007), pressurised liquid extraction (PLE) (Barbero et al., 2006a), enzyme immunoassay (Brian et al., 2002), liquid chromatography (LC) with fluorescent detection (Brian et al., 2002), liquid chromatography-mass spectrometry (LC/MS) (Kozukue et al., 2005), high performance liquid chromatography (HPLC) (Topuz and Ozdemir, 2007), and HPLC with various detection modes such as MS (Álvarez-Parrilla et al., 2011), UV (Weaver and Awde, 1986), fluorescence (Barbero et al., 2006b), electrospray ionisation/time of flight (ESI/MS) (Garcés-Claver et al., 2007), and electrochemical (Kawada et al., 1985), among others.

However, these techniques are expensive, complicated, laborious, destructive, and time-consuming methods that require skilled personnel and large amounts of solvents and reagents. Fourier Transform Infrared Spectroscopy (Mid-FTIR) is a method that has been proposed and proven to be an excellent alternative to traditional methods due to the low cost, response velocity, simple handling, and the fact that this method allows the use of a small sample without significant previous preparation (Dion and Saskia, 2008).

Mid-infrared spectroscopy in combination with multivariate statistical techniques (chemometrics) makes it possible to obtain specific information on various parameters in a direct, reliable, and rapid way. This method has been applied successfully to the simultaneous evaluation of significant quality parameters in minced beef (Meza-Márquez et al., 2010), avocado oil (Quiñones-Islas et al., 2013), and milk (Iñón et al., 2004), among others.

To the best of our knowledge, there are no reports on the use of Mid-FTIR spectroscopy combined with multivariate analysis for the quantification of the capsaicin, ascorbic acid, total phenolic compounds, and antioxidant activity present in the fruits of *Capsicum annuum* variety serrano. Therefore, the present study examines the application feasibility of Mid-FTIR spectroscopy coupled to chemometrics to quantify the above-mentioned compounds.

Materials and Methods

Chemicals. 2,2'-Azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt radical (ABTS), a Folin-Ciocalteau reagent and standards (capsaicin, ascorbic acid, and gallic acid) were obtained from Sigma-Aldrich (USA). All solvents used were of analytical grade and obtained from J.T. Baker (Baker-Mallinckrodt, Mexico).

Fruits of *Capsicum annuum* variety serrano were obtained from a local market in Mexico City. Serrano chillies without visible damage or physiological defects were selected. The chillies were analysed in various shades of green (dark green, emerald green, dark emerald green and light green), red, green-yellow, green-orange and red-orange to obtain the chemical variability to develop a robust chemometric model.

In all samples, peduncles of the serrano chillies were removed. Because serrano chillies are eaten fresh with seeds, placenta and seeds were considered in the present study. Serrano chillies were washed with distilled water and allowed to dry. Finally, the serrano chillies were stored in air-tight plastic bags at −18°C until analysis.

Capsaicin ([(E)-N-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamide] was extracted and quantified according to the methodology described by the Mexican regulations (NOM-F-389-1982) with a Perkin Elmer UV/VIS lambda 3 spectrophotometer

(Massachusetts, USA). Test tubes were prepared to range from 10 μL to 90 μL mL^{-1} of standard capsaicin in 90% methanol. Absorbance was measured at 281 nm using methanol (90%) as a blank.

Two grams of fresh chilli samples were macerated using a pestle and mortar. The samples were mixed with 25 mL of ethyl acetate and refluxing for 2.5 h, then allowed to cool and filtered. The filtrate was collected in a flask and washed with ethyl acetate until the extract was transparent. Five millilitres of ethyl acetate and 1.5 g of activated alumina were added to a column. The alumina was activated at 100°C for 24 h. The column was drained, and 10 mL of the chilli extract was added to the column. At the end of the washes, capsaicin was eluted with 22.5 mL of methanol (90%). The absorbance at 281 nm was recorded for each sample, and the amount of capsaicin was calculated using the standard curve of pure capsaicin. Determination of ascorbic acid was performed through xylene extraction (Ranganna, 1986) using a Perkin Elmer UV/VIS lambda 3 spectrophotometer (USA). Test tubes were prepared with 0.0, 0.5, 0.75, 1, 1.5, and 2 mL of standard ascorbic acid solution in 3% H_3PO_3 (0.1 mg mL^{-1}) and filled to 2 mL with 3% H_3PO_3 solution. Subsequently, 2 mL of acetate buffer (pH 4), 3 mL of 2,6 dichlorophenol indophenol solution (0.0007 M) and 15 mL of xylene were added in rapid succession. The tubes were capped and stirred for 10–5 s. Phase separation was provided. The xylene phase was extracted, and the absorbance was measured at 520 nm using xylene as a blank.

Sample analysis. Serrano chillies (20 g) were ground in 3% H_3PO_3 and filled to the volume of 200 mL. This solution was filtered, and 2 mL of aliquot was transferred to a test tube, added with 2 mL of the acetate buffer solution (pH 4), 3 mL of 2,6 dichlorophenol indophenol solution (0.0007 M), and 15 mL of xylene in a rapid succession. The tube was capped and stirred for 10–5 s. The xylene phase was extracted, and the absorbance was read at 520 nm. The absorbance of the sample was measured against a blank prepared as described above, but with distilled water instead of the 2,6 dichlorophenol indophenol solution. The ascorbic acid content was expressed as mg acid per gram of dry weight (mg/g dw). Determination was performed according to Sim and Sil (2008) using a Perkin Elmer UV/VIS lambda 3 spectrophotometer (USA).

Standard curve. Solutions containing 0, 0.02, 0.04, 0.06, 0.08, 0.1 mg mL^{-1} gallic acid standard were prepared. From each solution, 100 μL were transferred to test tubes, adding 100 μL deionised water, 1 mL Folin-Ciocalteau reagent, and 0.8 mL sodium carbonate solution (7.5%). Tubes were stirred and allowed to stand in the dark for 30 min. The absorbance at 765 nm was measured against a blank prepared in the same way, substituting the gallic acid solution with distilled water.

Extraction and determination of phenolic compounds. Serrano chillies (20 g) were ground and extracted with 5 mL of 80% methanol, stirred for 1 min, and centrifuged at 500 rpm for 15 min. The resulting supernatant was separated by decantation and placed into an amber glass bottle. The sediment was reconstituted into 5 mL of 80% methanol, and the procedure was repeated. Subsequently, three extractions obtained were combined. The final extract was concentrated in a flash evaporator, and the volume was reduced to 10 mL.

Total phenolic compounds were determined by mixing 2.0 mL extract solution, 1.0 mL of 10% Folin-Ciocalteau reagent, 2.0 mL sodium carbonate (20%), and 2.5 mL distilled water. The reaction mixture was incubated at room temperature for 30 min in the dark. The absorbance of the solution was measured at 765 nm. Total phenol content was subsequently calculated and expressed as μg gallic acid equivalents (GAE) per gram of (μg GAE/g dw).

For the measurement of the antioxidant activity of the chilli extracts, Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) with equivalent antioxidant capacity (TEAC) assay was used. TEAC is based on the reduction of green/blue coloration produced by the reaction of $ABTS^{\bullet+}$ [2,2-azinobis(3-ethylbenzo-thiazoline-6-sulphonic acid)] with the antioxidant present in the sample. $ABTS^{\bullet+}$ is one of the most effective methods for evaluating antioxidant activity in food due to the hydrophilic and lipophilic nature of antioxidant components present in fruits (Arnao, 2000).

$ABTS^{\bullet+}$ radical formation. The $ABTS^{\bullet+}$ radical was generated by reacting an $ABTS^{\bullet+}$ aqueous solution (7 μmol L^{-1}) with $K_2S_2O_8$ (2.45 μmol L^{-1}, final concentration) in the dark for 16 h and adjusting the absorbance to 734±0.02 nm with ethanol.

Sample analysis. A volume of 0.1 mL of chilli extract was mixed with 0.9 mL of the radical solution ($ABTS^{\bullet}$); after 7 min of reaction at room temperature, the absorbance was then measured at 734 nm using ethanol as the control. The difference in absorbance ($Abs_{initial} - Abs_{final}$) was converted to the inhibition percentage, and the antioxidant activity was calculated in μmoles of Trolox equivalent (TE) per gram of dw (μMol TE/g dw) using a calibration curve of Trolox (an analogue of water-soluable vitamin E) from 0.00 to 0.99 μm mL^{-1}.

All determinations were performed in triplicate. The results are expressed as mean values and standard deviation (SD) of the mean. One-way analysis of variance was performed to determine differences between samples. For all analyses, p-values <0.05 were considered statistically significant.

Mid-FTIR spectra acquisition. Spectra were obtained using a PerkinElmer 1600 Series FTIR spectrophotometer (USA) equipped with a deuterated triglycine sulfate detector. The sampling station was equipped with a horizontal attenuated total reflection accessory (PerkinElmer Inc) that comprised transfer optics within the chamber through which infrared radiation was directed to a detachable zinc selenide (ZnSe) crystal mounted into a shallow trough for sample containment. The crystal geometry was a 45° parallelogram with mirrored angled faces, and ten nominal internal reflections.

Approximately 2 g of ground serrano chilli sample was directly applied to the ZnSe crystal and pressed with a press accessory of the spectrophotometer to obtain an intimate contact between the sample and the crystal. The mid-infrared spectrum of each sample

was obtained in triplicate at room temperature over the wavenumber interval of 4000–550 cm^{-1}; each spectrum was an average of 64 scans at a resolution of 4 cm^{-1} and recorded as absorbance units. The triplicates of each sample were averaged to obtain an average spectrum. Single-beam spectra of the samples were obtained against a background of air using the same instrumental conditions. Cleaning of the crystal after each analysis was performed using hexane, Extran® 10% detergent, distilled water, and acetone, and the crystal was finally rinsed with distilled water and allowed to dry at ambient temperature. At the end of the washing stage, the spectrum of the ZnSe crystal was collected and visually inspected to ensure that no residue of the previous sample remained on the crystal. Spectra were collected using the software PE GRAMS (version 3.01B, Galactic Industries Corp. USA) and were directly imported into the software Spectrum (version 3.01.00, PerkinElmer, Inc.) for the successive chemometric analysis.

Spectral pre-processing was used to remove any irrelevant information that could not be handled correctly by the regression technique. The optimal pre-processing treatments included the use of a Savitzky-Golay filter with five smoothing points to remove part of the random noise present in the signal and improve the signal-to-noise ratio. Additionally, normalization was tested using the multiplicative scatter correction, which effectively removes baseline distortions that typically occur due to scattering in samples with variations in particle size; finally, the first derivative with five smoothing points was used to increase spectral differences and remove the baseline offset.

Multivariate analysis. The relationships between each Mid-FTIR chilli spectrum and the parameters (capsaicin, ascorbic acid, total phenolic compounds, and antioxidant activity) were determined by using Spectrum Quant+ software version 4.51.02 (PerkinElmer, Inc.). The software includes principal component regression (PCR) and partial least square (PLS) algorithms, which were used to developed different calibration models with the same training sets.

PCR and PLS are the most widely used multivariate calibration techniques in chemometrics. Both of these techniques make use of the inverse calibration approach, with which it is possible to calibrate for the desired component(s) (in this case, capsaicin, ascorbic acid, total phenolic compounds, and antioxidant activity), while implicitly modelling the other sources of variation. Estimation of the inverse calibration models involves the inversion of a data matrix. PCR and PLS solve this inversion matrix by replacing the original variables with linear combinations of the variables, which are named factors or principal components (PCs). The difference between PCR and PLS is in how the factors (latent variables) or PCs are calculated (Beebe et al., 1998).

To build a regression model, twenty-five averaged Mid-FTIR chilli spectra were used for the calibration set, and five averaged spectra were used for the validation set (samples not included in the calibration set). Samples for the validation set were chosen to cover the entire range of concentrations. In the present study, an external validation was used to assess the calibration accuracy. This type of validation is used when there are sufficient samples available to create separate (independent) training, calibration, and test sets. Therefore, the test set is completely independent of the model building process (variable selection, parameter estimation, and determination of principal components). This procedure has been applied successfully previously (Meza-Márquez et al., 2010; Quiñones-Islas et al., 2013).

The best calibrations models were chosen based on the smallest standard error of calibration (SEC), and the highest coefficient determination (R^2) that indicate the strength and direction of the linear relationship between predicted and the actual values (Beebe et al., 1998). The adequacy of prediction was assessed through the accuracy of predicted values and the standard error of prediction (SEP) of the validation set. The SEP is an estimate of the standard error of prediction (magnitude of the error expected when independent samples are predicted using the model). The model producing the lowest SEP was chosen as the best model.

Additional factors were considered. The optimal factors were established based on the lowest number of factors that gave the closest result to the minimum SEC for each parameter. Factors are linear combinations of the original variables; these quantities represent only significant sources of variation in the characteristics of the spectra and therefore reduce the dimensionality of the spectral data. Factors are used to describe and predict the parameters and to represent the complexity of the chemometric model.

Results and Discussion

The results of the capsaicin content, ascorbic acid content, total phenolic compounds, and antioxidant activity have been expressed in dw. However, to reflect the nutritional values of the fresh *Capsicum* var. serrano samples, the data in fresh weight (fw) has also been included.

Capsaicin content. The capsaicin content, responsible for the pungency of serrano chilli (*Capsicum annuum*), varied between 4.41 and 15.32 mg/g dw (514.83 and 1704.39 μg/g fw, respectively) (Fig. 1A). These results show the different physical and chemical characteristics of the evaluated samples.

All results in Fig. 1A were in the range of those reported by various authors for serrano and other hot peppers (Kozukue et al., 2005; Álvarez-Parrilla et al., 2011; Ornelas-Paz et al., 2010). In an agreement with our results, Álvarez-Parrilla et al. (2011) reported values of 1606.1 μg/g of dw among fresh serrano and 167.5 μg/g dw among pickled serrano; similar results were observed by Kozukue et al. (2005) for jalapeño chilli (ranged from 2.6 to 1187 μg/g fw), showing once again the large variability in phytochemical content due to cultivar, maturity, and pre- and postharvest conditions.

Several studies have been published on the accumulation of capsaicinoids in *Capsicum* fruit in relation to the fruit age, size, and stage of development (Deepa et al., 2007; Pino et al., 2007;

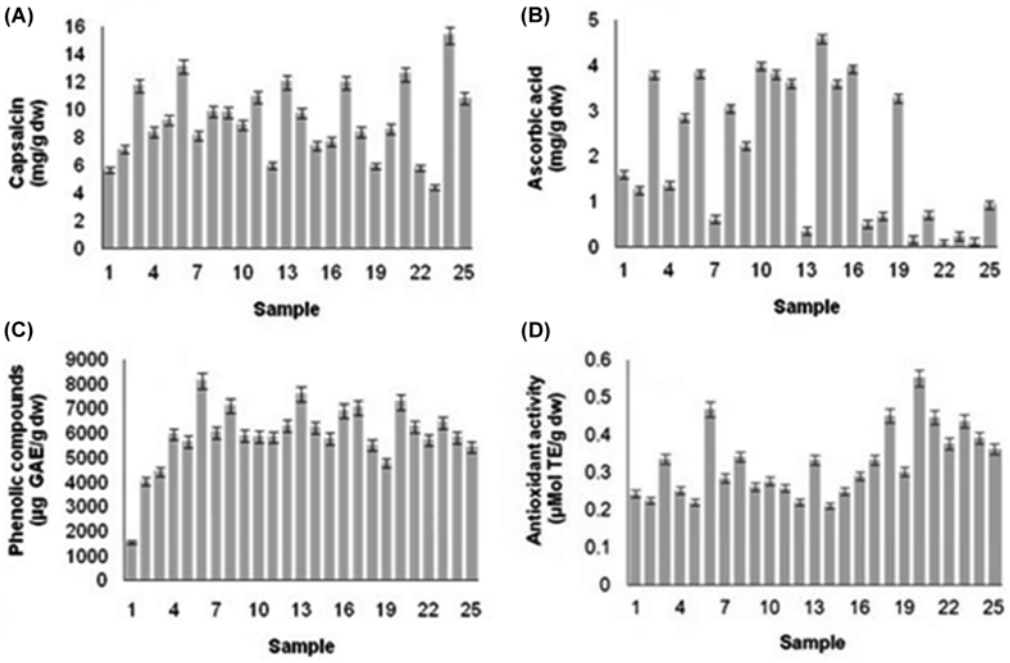

Fig. 1 (A) Capsaicin content, (B) ascorbic acid content, (C) total phenolic compounds and (D) total antioxidant activity of *Capsicum annuum* var. serrano. Values represent means of triplicate, bars show mean ± SD

Cisneros-Pineda et al., 2007). All these papers reported similar results; capsaicin (46–72% of total capsaicinoids) is the primary alkaloid among capsaicinoids found in most of the hot varieties of peppers. Capsaicinoids begin to accumulate in the early stages of the fruit growth and gradually increase until reaching a maximum rate as the fruit approaches the end of the growth phase. However, in some *Capsicum* species, the capsaicinoid concentration decreased slightly in the ripening stage. This decrease in capsaicin content can be due to chemical decomposition by photooxidative reactions or, as has been recently suggested, due to the action of some enzymes such as peroxidases (Díaz et al., 2004).

The variations in the capsaicin quantity found in our study cannot be thoroughly explained; however, this result can be explicated in terms of differences in cultivar, soil, weather conditions, and maturity as well as postharvest manipulation (Fett, 2003).

Ascorbic acid determination. Peppers are, among vegetables with the highest ascorbic acid content. It has been reported that the consumption of 100 g fw of peppers provides 100–200% of the recommended daily administration (RDA) of ascorbic acid (Lee and Kader, 2000). The results of the ascorbic acid content of serrano chilli are shown in Fig. 1B. The ascorbic acid content ranged from 0.07 to 4.58 mg/g dw (0.776 to 64.97 mg/100 g fw, respectively). All results in Fig. 1 were in good agreement with those reported by Muñoz and Ledesma (2002), who suggest a mean value of 65 mg/100 g fw for *Capsicum annuum* var. serrano. The results in this study confirmed that the consumption of 100 g of fresh peppers provides the RDA of ascorbic acid (Lee and Kader, 2000).

Additionally, the ascorbic acid values shown in the present study were in the range of those reported for green hot peppers such as serrano and jalapeño by HPLC and spectroscopic methods (Deepa et al., 2007; Álvarez-Parrilla et al., 2011; Medina-Juárez et al., 2012).

The high variability in the ascorbic acid content among serrano chillies could be due to the differences in growing conditions, environmental factors, maturity, genetic factors, and postharvest manipulations. It has been reported that high storage temperatures can reduce the ascorbic acid content by up to 88% upon storage at 20° for a long time. Additionally, the content of ascorbic acid of pepper fruits increases upon maturation (Howard et al., 2000; Lee and Kader, 2000). In the present study, maturity is the factor that may have a greater impact on the ascorbic acid content, because the serrano chillies were analysed during different maturity stages. *Capsicum* peppers are considered an essential source of ascorbic acid; therefore, these peppers have been attributed health benefits. However, it seems that other compounds with high antioxidant potential such as phenols may also contribute in this context (Charles, 2013).

Total phenolic compounds. Total phenolic content was measured by using Folin-Ciocalteau assay. Although this method overestimates the total phenolic content due to the presence of interfering compounds such as ascorbic acid, this assay is thus far the only single method to determine the total phenols content and has been widely used (Deepa et al., 2007). Generally, the concentration of these chemical constituents increased as the chilli reached maturity regardless of the analytical method employed, as previously reported (Howard et al., 2000).

The results of the total phenols content of serrano chilli are presented in Fig. 1C. Total phenolic concentration ranged from 1549.59 to 8112 μg GAE/g dw (219.84 to 840.95 μg GAE/g fw, respectively). These values are comparable to the values reported by several authors for serrano and other hot chillies (Materska and Perucka, 2005; Ornelas-Paz et al., 2010; Álvarez-Parrilla et al., 2011; Medina-Juárez et al., 2012).

It is well known that the content of phytochemicals, including phenolic compounds present in *Capsicum* spp, is affected by internal factors of the species concerned, agronomic conditions, maturity, postharvest handling, and pre- and postharvest treatments applied to the fruit. For example, an increasing trend in the total phenolic during maturity in the majority of *Capsicum* cultivars has been reported (Howard et al., 2000; Deepa et al., 2007). However, other studies, such as those by Marin et al. (2004) and Estrada et al. (2000) showed that the content of phenolic compounds are significantly higher in the fresh peppers than in the mature ones. Additionally, the plant part used (with or without seeds) may contribute to the higher values. Seeds from various plants have been shown to contribute significantly to high total phenolic content (Velioglu et al., 1998).

The basis for the variation in the total phenol content found in our study cannot be categorically explained, though as mentioned before, it is known that the plant age and maturity are the primary determinants of variation in phenolic content (Deepa et al., 2007).

Antioxidant activity by TEAC assay. The $ABTS^{\bullet+}$ scavenging ability reported as TEAC is presented in Fig. 1D. The results varied from 0.211 to 0.552 μMol TE/g dw. These values were considerably lower than those reported by different authors in Capsicum spp (Hervert-Hernández et al., 2010; Álvarez-Parrilla et al., 2011; Medina-Juárez et al., 2012). Hervert-Hernández et al. (2010) reported activity in the range of 26.6–44.4 μmol TE/g dw for guajillo, morita, chipotle, and árbol chillies. Whereas Álvarez-Parrilla et al. (2011) reported antioxidant activity ranging from 28.64 to 55.41 μmol TE/g dw for chipotle, serrano, and jalapeño chillies from Mexico. On the other hand, Medina-Juárez et al. (2012) reported activity in the range of 19 to 28 μmol TE/g fw, respectively, for serrano and jalapeño chillies from Mexico.

The total antioxidant activity differences reported between our investigation and the above-mentioned results could possibly be due to the diversity and complexity of antioxidants compounds present in chillies. In this sense, many studies showed that differences in the total antioxidant activity in peppers may be explained in terms of growing conditions, maturity, and the cultivar (Álvarez-Parrilla et al., 2011).

The greatest value of antioxidant activity found in the present study (0.552 μMol TE/g dw) represents a 74.7% inhibition on the radical $ABTS^{\bullet+}$. This percentage is greater than that found by Medina-Juárez et al. (2012), who reported a 44.66% inhibition ($DPPH^{\bullet}$ radical) from serrano extracts, and those reported by Sim and Sil (2008), who found a 64% inhibition from red pepper.

According to the literature, several research groups have demonstrated a relationship between the phenolic content and the antioxidant capacity in extracts from plants and vegetables, whereas other researchers found no such correlation. Research reported by Velioglu et al. (1998) demonstrated a strong correlation between the total phenolic content and the antioxidant activity in some fruits, vegetables and grains. In contrast, Kähkönen et al. (1999) did not find a correlation between the antioxidant capacity and the phenolic content in some plant extracts. Additionally, no correlation between the total phenolic content and the radical scavenging activity was found in pepper fruits (C. annuum var. acuminatum) at two maturity stages (small green and green) (Conforti et al., 2006). Our results did not show a correlation between bioactive compounds (total phenolic compounds) and antioxidant activity. This fact may be explained in numerous ways; in fact, the total phenolic content does not incorporate all of the antioxidants (as carotenoids). In addition, the synergism between the antioxidants in the mixture makes the antioxidant action not only dependent on the concentration but also on the structure and the interaction between the antioxidants (Conforti et al., 2006).

The variabilities in the capsaicin content, the ascorbic acid content, the total phenol compounds, and the antioxidant activity of serrano chillies are necessary for the development of chemometric models.

Interpretation of the Mid-FTIR spectra. Fig. 2 depicts the Mid-FTIR spectra of four samples of Capsicum annuum var. serrano in the range of 4000–550 cm^{-1}. As expected, the spectra show absorption bands that correspond to the vibrations of functional groups belonging to carotenoids, phenolic compounds, and ascorbic acid, among others, which are the main components of serrano chilli (Fett, 2003).

The mid-infrared bands were assigned using previous reported studies (Ivanova and Singh, 2003; Chen and Wu, 2009). The first peak (3600–3000 cm^{-1}) was assigned to OH stretching of water, and the two peaks at 2950–2800 cm^{-1} corresponded to the C-H stretching bound of methyl and methylene groups. The stretching vibration absorption peak of carbonyl (C=O) was at 1750–1710 cm^{-1}. In the region called the fingerprint region, the characteristic amide I band at 1650 cm^{-1} (C=O stretching), and the band of OH stretching overtone of water at the same range (1650 cm^{-1}) can be observed. The scissors vibration absorption peak of methylene (CH2) and the anti-symmetric deformation absorption peak of methyl (CH3) were at 1465±20 cm^{-1}. Finally, the bands in the region of 1200–950 cm^{-1} were assigned to the stretching vibration absorption peaks of C-O and C-C (Fig. 2).

Selection of spectral region for calibration. As a first step in the spectral region selection for the quantitative estimation of the capsaicin, ascorbic acid, total phenolic compounds and antioxidant activity in serrano chilli, the wavelength range in which absorbance measurement should be made must be determined. When PCR and PLS methods are employed to develop calibration models, the spectral range dictates the number of spectral points used in the computation of latent variables. The spectral range should include characteristic regions, in which the chemical groups related to the

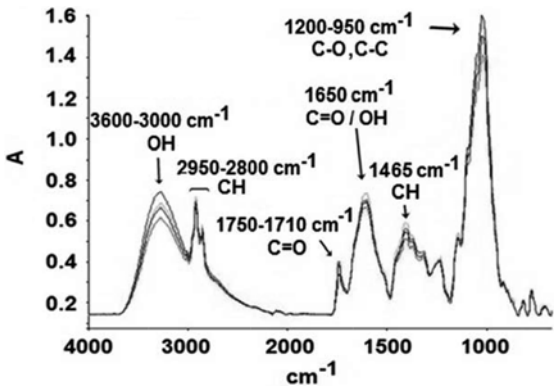

Fig. 2 Mid-FTIR spectra of *Capsicum annuum* var. serrano.

species of interest as well as other matrix constituents absorbed. Most ideally, regions dominated by noise or other artefacts should not be included in the analysis. Although this region rejection may not be entirely possible when absorptions due to artefacts and analytes overlap, even minor considerations will help in developing a robust model. A proper spectral range can be identified by computing the correlation spectrum for the constituents of concern. Regions that show a high positive correlation are ranges that should be chosen, whereas regions that show low or no correlation should be ignored (Tewari and Irudayaraj, 2004). Based on this analysis, the regions at 3600–2800 cm^{-1} and 1800–600 cm^{-1} showing the highest correlation between changes in the composition and spectral response, hence, were selected for calibration.

Calibration and validation models. Table 1 shows the statistics of the developed chemometric models (using PCR and PLS algorithms) to predict the capsaicin content, ascorbic acid, total phenolic compounds, and antioxidant activity of Capsicum annuum var. serrano. The best models were chosen based on the highest R^2 and the lowest SEC, shown in italics and bold type in Table 1.

The values of R^2 exceeded 0.99 for the PLS models and were greater than those obtained by the PCR models (0.331–0.864). The SEC values for all parameters were in the range of 0.003–0.098 for the PLS models and between 0.098 and 20.30 for the PCR models (Table 1). The SEC value is a measure of the average error in the analysis and evaluates the goodness of fit of the regression during calibration. This value has the same units in which the concentration of the property is measured, thus low values are desirable.

The PLS models exhibited lower SEC values and higher R^2 than the PCR models, indicating that the PLS models had better calibration power than the PCR models. Therefore, the best calibration models were obtained with the PLS algorithm (Table 1).Calibration with PLS or PCR captures as much of the variation in the entire spectral range as possible. However, PCR does not consider the concentration values when selecting or constructing spectral components, whereas in the PLS regression model, information about reference values is used in constructing spectral components (Sivakesava and Irudayaraj, 2001). This difference may explain why PLS performed better than the PCR model.

Additionally, Table 1 shows the number of factors. An important parameter of the PCR and PLS algorithms is the number of factors that are included in the calibration model. One difference between the PCR and PLS algorithms is the number of factors that are required to achieve a minimal variation constant; generally, PCR requires more factors than PLS. According to Beebe et al. (1998), the number of factors should be less than half the number of the calibration set samples to avoid noise in the spectrum. The number of factors of each parameter of the optimized chemometric models (PLS models) was approximately 20% of the number of samples used in the calibration (25 samples), which is a suitable number to avoid possible overfitting. Overfitting occurs when the model is closely fit to the data points of the set of calibration. Although the prediction of the calibration point could be a success, the prediction

Table 1 Calibration and validation data of the models developed with PCR and PLS algorithms to predict capsaicin, ascorbic acid, total phenolic compounds, and antioxidant activity of *Capsicum annuum* var. serrano

			Calibration (n = 25)		Validation (n = 5)	
Parameter	Algorithm	Factors [a]	R^2 [b]	SEC [c]	R^2 [b]	SEP [d]
Capsaicin	PCR	15	0.761	20.30	--	---
	PLS	***5***	***0.998***	***0.098***	***0.953***	***0.287***
Ascorbic acid	PCR	16	0.864	1.178	---	---
	PLS	***5***	***0.999***	***0.013***	***0.945***	***0.263***
Phenolic compounds	PCR	10	0.458	9.252	---	---
	PLS	***6***	***0.998***	***0.066***	***0.936***	***0.195***
Antioxidant activity	PCR	10	0.331	0.098	---	---
	PLS	***6***	***0.999***	***0.003***	***0.913***	***0.142***

[a]Optimal factors (PLS) or optimum principal components (PCR).
[b]Coefficient of determination (R^2) should be as close to 1 as possible.
[c]Standard Error of Calibration (SEC) should be as low as possible.
[d]Standard Error of Prediction (SEP) should be as low as possible.
Note: Best model is shown in italics and bold type.

Table 2 External validation data of the PLS chemometric model to determine capsaicin, ascorbic acid, total phenolic compounds, and antioxidant activity of *Capsicum annuum* var. serrano

Parameter	Unit	Actual value	Predicted value	Mahalanobis distance [a]	Residual error [b]
Capsaicin	mg/g dw	10.54	10.72	0.11	0.13
Ascorbic acid	mg/g dw	3.17	3.22	0.06	0.17
Phenolic compounds	µg GAE/g dw	5578	5072	0.04	0.17
Antioxidant activity	µMol TE/g dw	0.21	0.23	0.11	0.17
Capsaicin	mg/g dw	12.47	12.02	0.24	0.12
Ascorbic acid	mg/g dw	4.74	4.36	0.31	0.18
Phenolic compounds	µg GAE/g dw	5148	4997	0.09	0.18
Antioxidant activity	µMol TE/g dw	0.21	0.22	0.14	0.18
Capsaicin	mg/g dw	11.46	11.95	0.51	0.16
Ascorbic acid	mg/g dw	3.02	3.23	0.40	0.19
Phenolic compounds	µg GAE/g dw	5809	5878	0.04	0.20
Antioxidant activity	µMol TE/g dw	0.31	0.36	0.23	0.20
Capsaicin	mg/g dw	7.52	7.55	0.47	0.11
Ascorbic acid	mg/g dw	2.71	2.56	0.11	0.19
Phenolic compounds	µg GAE/g dw	4773	5133	0.16	0.18
Antioxidant activity	µMol TE/g dw	0.21	0.27	0.10	0.19
Capsaicin	mg/g dw	10.75	11.30	0.22	0.14
Ascorbic acid	mg/g dw	2.72	2.87	0.16	0.19
Phenolic compounds	µg GAE/g dw	4797	5555	0.25	0.19
Antioxidant activity	µMol TE/g dw	0.17	0.19	0.21	0.19

Capsaicin = mg per gram of dry weight (mg/g dw).
Ascorbic acid = mg per gram of dry weight (mg/g dw).
Phenolic compounds = µg gallic acid equivalents (GAE) per gram of dry weight (µg GAE/g dw).
Antioxidant activity = µmoles of trolox equivalent (TE) per g of dry weight (µMol TE/g dw).
[a]Mahalanobis distance should be as low as possible, not to exceed 1.
[b]Error Residual should be as low as possible, not to exceed 3.

performance of the new sample is not adequate (Shenk and Westerhaus, 1996). Therefore, optimized chemometric models (PLS models) were built without noise (Table 1).

The robustness of the PLS chemometric models was tested with the prediction of five external samples. Table 1 shows the SEP and R^2 values between the predicted values and the actual values of external samples; these parameters were used to evaluate the accuracy of the prediction of the PLS models. Table 1 shows that the values of R^2 between the predicted and actual values of the external samples were higher than 0.9 (capsaicin, R^2 =0953; ascorbic acid, R^2 =0.945; total phenol, R^2 =0.936; and antioxidant activity, R^2 =0.913). Value of R^2 between 0.82 and 0.90 indicates a good prediction, and a value R^2 above 0.91 is associated with an excellent prediction (Shenk and Westerhaus, 1996). The SEP values were obtained in the range of 0.142–2.87 (Table 1). SEP is indicative of the ability of model to accurately predict unknown samples. A high SEP indicates that the model is not sufficient or effective in predicting samples. According to Rohman and Che-Man (2011), the high values of R^2 and the low values of SEP indicate the success of the PLS regression model.

Table 2 shows the external validation data of the PLS chemometric models showing good correlation between the predicted values and the actual values of all parameters (capsaicin, ascorbic acid, total phenolic compounds, and antioxidant activity). The PLS chemometric models have a good predictive capacity, because the concentrations given by the model were very close to the actual (real) values of the samples, which is reflected in the low Mahalanobis distance and residual error obtained (Table 2). The Mahalanobis distance is a method to determine the similarity of a set of values from an unknown sample with respect to a set of values of known samples. The Mahalonobis distance is measured in terms of the standard deviations from the mean of the training samples (known samples); therefore, the reported matching values give a statistical measure of how well the spectrum of the unknown samples matches the original set of training samples (known samples). The Mahalanobis distance for each validation sample was less than 1.0, indicating that each sample was correctly classified by the model. The residual error was less than 0.20, which is significantly low (Table 2). Based on these results, the PLS models developed in the present study were able to predict all parameters in the serrano chilli samples that were not used for the development of the calibration models (Table 1).

Figure 3 A, B, C, and D exhibits the scatter plots for the relationships between the actual values and the predicted values of the capsaicin content, ascorbic acid content, total phenolic compounds and total antioxidant activity of serrano chilli in the external

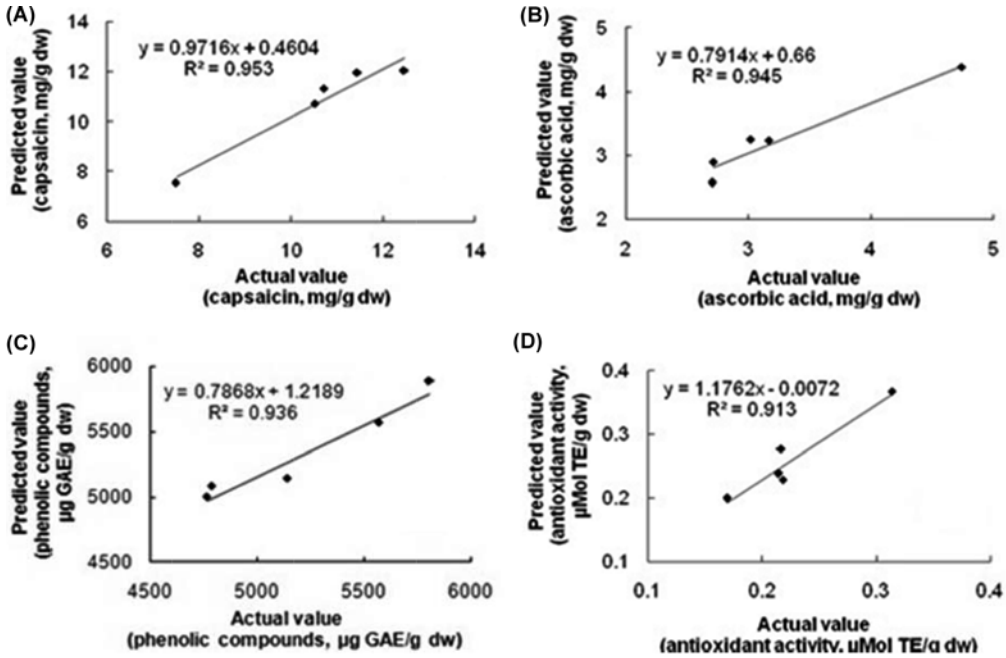

Fig. 3 Plots of predicted values versus actual values of: (A) capsaicin content, (B) ascorbic acid content, (C) total phenolic compounds, and (D) total antioxidant activity of *Capsicum annuum* var. serrano in the external validation determined by the PLS models.

validation. As seen in Fig. 3, independent of the parameter, the predicted values fall very close to the equal concentration line, demonstrating the good prediction capability of the models.

These results indicated that Mid-FTIR spectroscopy combined with chemometrics can be used as an alternative, quick, and low-cost method for the identification of capsaicin, ascorbic acid, total phenolic compounds, and antioxidant activity present in *Capsicum annuum* var. serrano.

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