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Review

Effect of solvent polarity on the Ultrasound Assisted extraction and antioxidant activity of phenolic compounds from habanero pepper leaves (*Capsicum chinense*) and its identification by UPLC-PDA-ESI-MS/MS

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ABSTRACT

Phenolic compounds are secondary metabolites involved in plant adaptation processes. The development of extraction procedures, quantification, and identification of this compounds in habanero pepper (*Capsicum chinense*) leaves can provide information about their accumulation and possible biological function. The main objective of this work was to study the effect of the UAE method and the polarity of different extraction solvents on the recovery of phenolic compounds from *C. chinense* leaves. Quantification of the total phenolic content (TPC), antioxidant activity (AA) by $ABTS^{+}$ and DPPH radical inhibition methods, and the relation between the dielectric constant (ε) as polarity parameter of the solvents and TPC using Weibull and Gaussian distribution models was analyzed. The major phenolic compounds in *C. chinense* leaves extracts were identified and quantified by UPLC-PDA-ESI-MS/MS. The highest recovery of TPC (24.39 ± 2.41 mg GAE g^{-1} dry wt) was obtained using MeOH (50%) by UAE method. Correlations between TPC and AA of 0.89 and 0.91 were found for both radical inhibition methods ($ABTS^{+}$ and DPPH). The Weibull and Gaussian models showed high regression values (0.93 to 0.95) suggesting that the highest phenolic compounds recovery is obtained using solvents with "ε" values between 35 and 52 by UAE. The major compounds were identified as N-caffeoyl putrescine, apigenin, luteolin and diosmetin derivatives. The models presented are proposed as a useful tool to predict the appropriate solvent composition for the extraction of phenolic compounds from *C. chinense* leaves by UAE based on the "ε" of the solvents for future metabolomic studies.

1. Introduction

The habanero pepper (*Capsicum chinense* Jacq.) is a representative crop of the Mexican Yucatan Peninsula with a great cultural and commercial importance [15]. As this region is recognized since 2010 by the protection designation of origin (PDO) as the center of habanero pepper production and domestication, the measurements of regulated parameters are necessary to guarantee its quality [22,41]. Plant growth

Abbreviations: UPLC, Ultra-high performance liquid chromatography; PDA, Photodiode detector array; MS, mass spectrometry; ε, dielectric constant; CE, Conventional extraction; UAE, Ultrasound-Assisted extraction; TPC, Total phenolic content; GAE, Gallic acid equivalent; Eq CA, Caffeic acid equivalents; Eq L, Luteolin equivalents; Eq A, Apigenin equivalent; Eq Q, Quercetin equivalents; Dry wt, Dry weight; HT, Proton transfer; SET, Simple electron transfer; Hx, Hexane; AcOEt, Ethyl acetate; Ace, Acetone; 80% Ace, 80% Acetone; MeOH, Methanol; 80% MeOH, 80% Methanol; 50% MeOH, 50% Methanol; 20% MeOH, 20% Methanol; 1% AcOH, Acetic acid; W, Water.

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can be affected by different factors such as soil moisture, nutrient deficiency, exposure to UV-B radiation, temperature, and pathogen attack that can significantly reduce the quality and productivity of the habanero pepper crops [8,10,17,32,39]. The production of phenolic compounds during plant growth is one of the main triggered responses to different biotic and abiotic stress factors and strongly influences the plant adaptation processes and interactions with the environment [4,14,47]. It has been found the presence of chlorogenic acid and flavonoids (apigenin and luteolin) in *C. annuum* leaves as a response to oxidative stress cause by UV-B radiation [27]. Phenol polyamides have been observed in *C. annuum* fruits produced in response to *Colletotrichum gloeosporioides* infection suggesting its effect as a physical barrier [39].

On the other hand, different traditional methods and solvents have been evaluated for phenolic compound extraction from *Capsicum* fruits, such as Soxhlet and maceration methods [7]. Nevertheless, low recovery of phenolic compounds has been obtained. Nowadays green technologies such as Ultrasound assisted extraction (UAE) for phytochemical compounds recovery has been evaluated for *Capsicum* fruits. Dias et al. [18] reported higher recovery of total phenolic content (TPC) by UAE than Soxhlet extraction on *C. baccatum* fruits, and a significant effect of the extraction solvent is also mentioned. It has been reported that UAE promotes cell wall decomposition and allows mass transfer of the solutes into the solvent preventing the degradation of phenolic compounds producing an increment on TPC recovery with respect to other methods [31]. At the same time, the recovery of phenolic compounds is strongly correlated with the biological activity of the extracts such as antioxidant activity and it is also affected by the polarity of the solvent used during extraction [29]. Based on the properties of the solvents, the dielectric constant (ε) has been proposed as a good polarity parameter considering solvent temperature and composition [24] that can help to predict the performance and profile of extracted phenolic compounds [6,37]. Additionally, thermodynamic models of solubility of phenolic compounds based on experimental results and empirical thermodynamic models such as the conductor-like screening model for realistic solvation (COSMO-RS) have been reported [12,19].

Extraction protocols with green technologies in combination with analytical tools such as liquid chromatography (LC) coupled to mass spectrometry (MS) have allowed the identification of phenolic compounds related to the complex metabolic responses caused by changes or alterations in various plant species [25] including the *Capsicum* genus [27,39]. Nevertheless, the majority of the studies have focused on the characterization of bioactive compounds in *Capsicum* fruits [11]. Phenolic characterization from other organs such as the leaves of *C. chinense* can provide useful information for the understanding of possible metabolic responses of the crop to different environmental conditions, and it could facilitate the development of engineering strategies for crop production and protection. Furthermore, solvent selection is a key factor for the application of phenolic compound extraction protocols that will be targeted to metabolomics studies. Therefore, the main objective of this work was to study the effect of UAE method and the polarity of the solvents according to its "ε" on the total phenolic content (TPC) and its antioxidant activity in extracts of habanero pepper leaves (*C. chinense*). Additionally, the relation between the "ε" of the solvent and the TPC with the Weibull and Gaussian distribution models was analyzed. Ultra-high-performance liquid chromatography (UPLC) coupled with a photodiode array detector (PDA) and a mass spectrometer was used to obtain the profile of phenolic compounds and their content in the extracts using different solvents.

2. Materials and methods

2.1. Chemicals

The reagents, Folin-Ciocalteu (2 N), gallic acid monohydrate (≥98.0%), formic acid (≥95.0%), acetonitrile (≥99.9%), acetic acid (AcOH; ≥99.7%) and ethyl acetate (AcOEt; ≥99.9%); and analytical standards, caffeic acid (≥98.0%), luteolin (≥99.7%), apigenin (≥95.0%) and quercetin (≥95.0%) were purchased from SIGMA-Aldrich (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3), hydrochloric acid (≥36.5%), methanol (MeOH; ≥99.90%) and acetone (Ace; ≥ 99.60%) were purchased from Avantor J. T. Baker (Radnor, PA, USA), n-hexane (Hx; ≥95%) were purchased from Fermont (Monterrey, NL, MX) and ultrapure water (W) was obtained through a Milli-Q water filtration system (Millipore, Bedford, MA, USA.

2.2. Vegetal material and sample pretreatment

C. chinense seedlings of the Chichén Itzá variety with 45 days of post-germination were obtained from a local producer in the community of Suma, Yucatán (Mexico) in March 2018.

The leaves of the seedlings were carefully separated with scissors and frozen in liquid nitrogen. They were then cold pulverized in a mill (KRUPS, Model GX41000, Mexico) to a powder with a particle size of < 500 μm. Finally, the samples were freeze dried using a freeze dryer (FreeZone 6 Liter Benchtop, Labconco, USA) to obtain a moisture percentage of 10% determined by a thermobalance (OHAUS, MB45, USA).

2.3. Empirical determination of dielectric constant (ε) of the solvents

The "ε" of pure solvents at different temperatures reported by Akerlof (1932) were used to calculate the empirical "ε" of binary solvent mixtures according to equation developed by Jouyban et al. [24]. This is an empirical model to determine the contribution of solvent composition and temperature on the "ε" and has been compared with experimental data. (**Eq. 1**):

$$ln\varepsilon_{m,T} = \phi_1 ln\varepsilon_{1,T} + \phi_2 ln\varepsilon_{2,T} + \phi_1\phi_2 \sum_{j=0}^{2}\left[\frac{A_j(\phi_1 - \phi_2)}{T}\right] \quad (1)$$

Where:

$\varepsilon_{m,\ T}$: Dielectric constant of the mixture at the temperature evaluated.

$\varepsilon_{i,\ T}$: Dielectric constant of the pure solvent at the temperature evaluated.

ϕ_i: Proportion of each solvent in the mixture

A_j: Model constant to calculate dielectric constant of the solvent mixture at different temperatures [24].

2.4. Phenolic compounds recovery by Ultrasound-Assisted extraction (UAE) and Conventional extraction (CE)

A 2 × 10 factorial design was performed to evaluate the effect of solvent and extraction method on the recovery of phenolic compounds. Factor A (extraction methods) were defined as: Conventional Extraction (CE) and Ultrasound-Assisted Extraction (UAE), and Factor B (different solvents or concentrations of the solvents) were defined as: hexane (Hx), ethyl acetate (AcOEt), acetone (Ace), methanol (MeOH), 80% acetone (80% Ace), 80% methanol (80% MeOH), 50% methanol (50% MeOH), 20% methanol (20% MeOH), 1% acetic acid (1% AcOH) and water (W). For all treatments, four replicates were performed. See Additional information (Table S1) for experimental design table.

For the extraction, the lyophilized powder (200 mg) was placed in 15 mL conical tubes (Falcon™) with 10 mL of solvent. CE was carried out in a hot water bath using a heating plate (Thermo Scientific, Model No.: SP131325Q, China) at 50 °C (323.15 K), with magnetic stirring for 4 h. The volume of solvent remained without change during extraction due to the conical tube was previously sealed. (scheme of the CE method is shown in Figure S1, Supplementary material). UAE methodology was performed using the same weight-volume ratio (1:50 g/mL) and ultrasonic extraction conditions reported by Covarrubias-Cardenas et al. (2018). The extraction was carried out using a 3 mm (1/8″) high intensity probe (operating volume range: 0.25 to 10 mL) coupled to an

ultrasound processor (GEX130PB, Sonics and Materials Inc., Newtown, USA) at 80% amplitude for 15 min. In addition to the reported method, an ice bath was used to minimize solvent losses during extraction by the effect of the increment of the temperature during sonication. The temperature was found in a range of 20 to 50 °C (293.15 to 323.15 K) (scheme of the UAE method is shown in Figure S2, supplementary material). For all treatments, the extracts were centrifuged (Centrific 225, Fisher Scientific, USA) at 6,500 rpm for 15 min and supernatant was graduated in a 10 mL volumetric flask. The samples were stored in amber vials at – 40° C until further analysis.

2.5. Determination of total phenolic content and antioxidant activity

TPC determination was performed by the Folin-Ciocalteu method according to the modifications used by Covarrubias-Cárdenas et al. [16]. The TPC of samples was expressed in mg equivalents of gallic acid (GAE) g^{-1} of dry weight (dry wt).

The antioxidant activity was determined by the ABTS and DPPH radical assays. ABTS was performed according to described by Alonso-Carrillo et al. [5] and DPPH radical assay according to described by Covarrubias-Cárdenas et al. [16]. The antioxidant activity of samples was expressed in μEq of Trolox g^{-1} dry wt.

2.6. Weibull and Gaussian distribution model adjustment of TPC and dielectric constant

TPC was correlated with dielectric constant of the solvents using a Weibull (**Eq. 2**) and Gaussian distribution (**Eq. 3**) model equation:

$$f = a\left(\frac{c-1}{c}\right)^{\frac{1-c}{c}}\left[\frac{x-x_0}{b}+\left(\frac{c-1}{c}\right)^{\frac{1}{c}}\right]^{c-1} e^{\left[\frac{x-x_0}{b}+\left(\frac{c-1}{c}\right)^{\frac{1}{c}}\right]}+\frac{c-1}{c} \quad (2)$$

$$f = ae^{\left[-0.5\left(\frac{x-x_0}{b}\right)^2\right]} \quad (3)$$

Where:

a: Represents the maximum peak or the highest TPC of the curve centered with respect to the parameter "X_0".

b: Represents the distribution coefficient or standard deviation related to the width of the Weibull and Gaussian bell.

X: Value of the dielectric constant of the pure solvent or solvent mixture.

X_0: Value of the dielectric constant in which the "a" or higher TPC value is obtained in the curve.

2.7. Chromatographic analysis by UPLC-PDA-ESI-MS/MS

Phenolic compounds were identified from the 50% MeOH extract obtained by the UAE and its hydrolyzed extract. Chromatographic profiles were obtained using a Waters Acquity H Class UPLC (Milford, MA, USA) with a quaternary pump (UPQSM), an automatic injector (UPPDALTC) and a PDA λ photodiode array detector (UPPDALTC). The chromatographic separation was carried out with a Waters Acquity UPLC BEH C18 column, 1.7 μm, 100 × 2.1 mm ID (Milford, MA, USA) using a mobile phase of 0.1% formic acid in ultrapure water (A) and 0.1% formic acid in acetonitrile (B), with the conditions reported by Covarrubias-Cárdenas et al. [16]. The PDA reading λ was performed in a range of 190 to 400 nm. The analytical response absorbance was taken at 290 nm. The quantification of phenolic compounds identified in the samples was expressed as μmol Eq of luteolin, apigenin, quercetin, and caffeic acid g^{-1} dry wt according to their similarity with λ of the analytical standards used.

For the mass spectrometry (MS/MS) analysis, a Waters Xevo TQ-S micro instrument was used. Conditions were used as reported by Covarrubias-Cárdenas et al. [16]. The collision energy used was 10 eV for scanning in negative ion mode and 3 eV in positive ion mode. The mass spectra were recorded in full scan mode in a range of 50 *m/z* to 700 *m/z*. The MassLynx V4.1 software (Waters, Milford, MA, USA) was used for data acquisition and processing. The tentative identification was assigned by comparing fingerprint and MS data of the compounds detected with the reported in the literature and in public European Mass Bank database (https://massbank.eu/MassBank/index.html) and ReSpect for phytochemicals (http://spectra.psc.riken.jp/menta.cgi/respect/index).

2.8. Analysis of phenolic compounds in aglycone form by acid hydrolysis

Phenolic compounds in plants are commonly found in glycosylated forms [26]. The analysis of the phenolic compounds in their aglycone form provides complementary information such as the structure of the flavonoid-based skeleton, which supports the identification of their possible substitutes. To release the substituents, present in the glycosylated flavonoids from the UAE extract obtained by the 50% MeOH concentration was subjected to acid hydrolysis. The extract (2 mL) was treated with 2 N HCl (1 mL) at 90 °C for 1 h according to the modified methodology of Bae *et al.*, (2012b). Subsequently, the extracts were neutralized using 2 N NaOH/MeOH solution and centrifuged at 6,500 rpm for 15 min using a refrigerated centrifuge (Centrific 225, Fisher Scientific, USA) (schematic of hydrolysis shown in Figure S3, Supplementary material). The supernatant was concentrated in a rotary evaporator (BUCHI, Model: R-215, Switzerland) at 50 °C and 250 mbar vacuum. Finally, samples were filtered using acrodiscs with 0.2 μm membranes (Millex - FG, PTFE 0.2) and stored at – 40 °C until further analysis. The chromatographic analysis of the hydrolyzed extract was performed according to the conditions already detailed above.

2.9. Statistical analysis

The results of all treatments were expressed as the mean ± standard deviation. The factorial design was analyzed as a multifactorial ANOVA ($p > 0.05$) and to determine the effect of each factor on the analysis of variance components (AVC). The correlation between TPC and Antioxidant Activity ($ABTS^+$ and DPPH assay) was analyzed by a linear model to obtain the Pearson correlation coefficient. To perform both analyses was used the software Statgraphics Centurion Version XVI (Manugistic Inc., Rockville MD, USA). The Weibull and Gaussian distribution model were generated using the Sigma Plot software (Systat Software Inc., USA).

3. Results and discussions

3.1. Empirical determination of the dielectric constant "ε" of solvents

The "ε" is a macroscopic physical parameter and a relative measure of polarity related to the molecular interaction between the solvent and solutes. For binary mixtures, the "ε" value is calculated by additive function according to the concentration of each component. However, this property is also affected by changes in temperature and solvent composition. In this sense, an increase of temperature decreases the "ε" value of the solvents due to the weakening of the intermolecular interactions, an opposite effect is reported when the water is presented showing a non-ideal behavior due to higher intermolecular interactions among solvent components [24]. Herein the effect of temperature and solvent composition were considered to calculate the "ε" values by the Jouyban equation described in the previous section. The temperature used for the calculation of the "ε" was 323.15 °K for CE as process temperature remains constant during extraction (Table 1). In the case of the UAE, averages of temperatures from 293.15 to 323.15 K were considered for the "ε" calculation according to the different solvents used, as cavitation produced by ultrasonic waves generates a wide range of temperature (Table 1). Based on the numerical values obtained for the

Table 1
Total phenolic content (TPC) and antioxidant activity of *C. chinense* leaves extracts obtained by UAE and CE using different pure solvents and aqueous binary mixtures.

Solvent	Polarity				Phenolic content		Antioxidant Activity			
	UAE		CE		TPC (mg GAE g^{-1} dry wt)		DPPH (μEq Trolox g^{-1} dry wt)		$ABTS^+$ (μEq Trolox g^{-1} dry wt)	
	T (K)[A]	ε	T (K)	ε	UAE	CE	UAE	CE	UAE	CE
W	323.15	76.73	323.15	69.98	5.75 ± 1.85^{cd}	11.93 ± 0.94^{c}	19.74 ± 2.36^{c}	18.16 ± 1.04^{e}	41.97 ± 0.37^{c}	41.61 ± 0.46^{d}
1% AcOH	313.15	73.12[C]	323.15	69.85[C]	8.91 ± 3.06^{c}	14.19 ± 1.11^{c}	22.13 ± 1.58^{bc}	26.60 ± 1.09^{d}	44.90 ± 1.06^{bc}	50.70 ± 0.74^{c}
20% MeOH	313.15	66.51	323.15	63.31	15.32 ± 1.56^{b}	18.31 ± 1.16^{b}	23.08 ± 2.04^{bc}	22.75 ± 1.60^{d}	46.86 ± 1.92^{bc}	42.82 ± 1.97^{d}
50% MeOH	313.15	52.09	323.15	46.99	24.39 ± 2.41^{a}	22.77 ± 1.34^{a}	28.46 ± 1.87^{ab}	32.21 ± 0.01^{bc}	64.06 ± 2.88^{a}	67.39 ± 0.52^{ab}
80% MeOH	303.15	35.91	323.15	32.39	21.21 ± 1.70^{a}	18.14 ± 2.79^{b}	32.66 ± 1.35^{a}	36.27 ± 1.84^{ab}	61.42 ± 0.10^{a}	71.61 ± 1.17^{a}
80% Ace	303.15	22.45	323.15	20.62	16.44 ± 0.55^{b}	14.85 ± 1.69^{bc}	33.03 ± 2.43^{a}	37.48 ± 1.87^{a}	63.29 ± 4.31^{a}	61.88 ± 0.51^{b}
MeOH	303.15	30.68	323.15	27.44	15.82 ± 0.70^{b}	15.31 ± 2.11^{bc}	23.90 ± 1.21^{bc}	31.14 ± 0.46^{c}	51.43 ± 1.05^{b}	54.19 ± 3.04^{c}
Ace	303.15	18.67	323.15	16.98	3.68 ± 0.73^{de}	6.30 ± 0.61^{d}	7.03 ± 1.09^{d}	7.90 ± 0.85^{f}	19.11 ± 0.12^{d}	24.08 ± 1.04^{e}
AcOEt[B]	293.15	6.02	323.15	6.02	3.26 ± 0.55^{de}	4.40 ± 0.46^{d}	2.49 ± 1.06^{de}	0.00 ± 0.00^{g}	13.33 ± 3.55^{de}	21.94 ± 4.22^{e}
Hx[B]	293.15	1.89	323.15	1.89	1.72 ± 0.19^{e}	2.92 ± 0.20^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{g}	4.89 ± 0.79^{e}	13.57 ± 0.59^{f}

Different letters by column indicate significant differences ($p < 0.05$).
[A] The average temperature during UAE was used to calculate the dielectric constant.
[B] Low polarity solvents dielectric constants were not calculated for different temperatures as they are poorly affected.
[C] Temperature effect was the only consideration for dielectric constant for the low acid solution as the Jouyban equation did not was applied for this solution.

pure and the binary mixtures of solvents, the order from the lowest to the highest polarity values expressed as "ε" was: Hx < AcOEt < Ace < 80% Ace < MeOH < 80% MeOH < 50% MeOH < 20% MeOH < 1% AcOH < W. "ε" values from the different solvents were considered to correlated solvent polarity with phenolic compounds recovery.

3.2. Total phenolic content in C. Chinense leaves extracts obtained by Ultrasound Assisted extraction (UAE) and Conventional extraction (CE) with different solvents.

The TPC values obtained with the different solvents and both extraction methods (UAE and CE) were in a range of 1.70 to 24.40 mg GAE g^{-1} dry wt (Table 1). A higher TPC was observed using 50% MeOH, 24.39 ± 2.41 and 22.77 ± 1.34 mg GAE g^{-1} dry wt for UAE and CE method, respectively. A better TPC extraction by UAE can be explained by the rupture of the cell wall and cell vesicles that contain phenolic compounds produced by cavitation and allowing solvent penetration favoring solvation process [31]. In general, solvents of low polarity (Hx, AcOET and Ace) showed low response for TPC recovey (1.72 to 6.30 mg GAE g^{-1} dry wt), while solvents of higher polarity (MeOH, MeOH 80%, MeOH 50%, MeOH 20% and Ace 80%) are more effective for the recovery of phenolic compounds (14.85 to 24.39 mg GAE g^{-1} dry wt). However, some exceptions were observed using solvents such as water and 1% AcOH, where low response for TPC was observed (5.75 to 14.19 mg GAE g^{-1} dry wt) independently of the extraction method used. These observations are summarized through the multivariate Analysis of Variance (ANOVA) that indicated a significant effect of both factors (solvent and extraction method) on the TPC. The percentage of the contribution of these factors was estimated through a variance components analysis (VCA) that showed a higher contribution (88.44%) of the extraction solvent than the extraction method (7.32%). The differences in the response for TPC among treatments using different extraction solvents are more evident than those observed between extraction methods. However, some advantages of the UAE method over CE should be considered, i.e., a high response for TPC is obtained in a short extraction time (15 min) and cavitation produced during the UAE improves the mass transfer of solutes in the solvent due to cell wall disruption and microstreaming effect.

In the studies conducted by Dias et al. [18] and Bae *et al.* (2012a), a clear contribution of the extraction solvent in the recovery of phenolic compounds is showed. Dias et al. [18] reported that a higher TPC response is obtained from *C. baccatum* fruit extracts using MeOH as solvent and Soxhlet extraction method (4.93 mg GAE g^{-1} of raw material), while lower polarity solvents such as Ace, AcOEt and Hx showed a low response for TPC. Phenolic compounds are generally polar; therefore, they can be recovered with solvents of high polarity. However, in the study conducted by Bae *et al.* (2012a) a different behavior of the effect of the solvent on the TPC response was observed. They reported a TPC between 24.80 and 68.90 mg of catechin equivalents (CAE) g^{-1} dry wt in extracts of *C. annuum* fruit varieties obtained by Soxhlet extraction using different solvents; and the highest TPC was obtained using AcOEt, while higher polarity solvents such as Ace, MeOH and 80% MeOH showed a lower response for TPC. The results reported by Bae *et al.* (2012a) may be due to the presence of capsaicinoids, which are predominantly hydrophobic and mainly found in the fruits of *Capsicum* species that are not synthesized in other tissues such as leaves and stems [21]. Additionally, other factors could be related to the different responses for TPC, i.e., the profile and content of bioactive compounds in the different species, varieties and tissues of the genus *Capsicum*, stage of growth and development, agricultural practices, exposure to biotic and abiotic stresses, processes of harvesting, pretreatment of plant material, method and conditions for the extraction of phenolic compounds and overestimation of phenolic compounds by the Folin-Ciocalteu method caused by capsaicinoids, sterols and carotenoids contained in the extracts.

Focusing on the method and conditions for the extraction of phenolic compounds different parameters can be improved, i. e., Dias et al. [18] evaluated the effect of solvent, temperature and ultrasonic intensity on TPC and capsaicinoids content in extracts of *C. baccatum* fruits. Additionally, they presented a mathematical model that correlates the different parameters evaluated with the recovery of bioactive compounds. Nevertheless, the effect of the UAE method and solvent selection for the recovery of phenolic compounds from habanero pepper leaves is presented herein for the first time.

3.3. Antioxidant activity of C. Chinense leaves extracts.

The antioxidant activity evaluated by the $ABTS^+$ assay was in the range of 4.89 to 71.61 μEq Trolox g^{-1} dry wt, while for the DPPH assay it was in the range of 0.00 to 37.48 μEq Trolox g^{-1} dry wt (Table 1). The multivariate ANOVA determined that the method and solvent had a significant effect on the antioxidant activity of the extracts, however, as expected the solvent has a greater contribution on antioxidant activity by both assays ($ABTS^+$ and DPPH and assay). The highest values of antioxidant activity were obtained using 50% MeOH, 80% MeOH and 80% Ace as extraction solvents. Commonly, extracts obtained with high polarity solvents show higher antioxidant activity, the polar phase of the extract contributes to the inhibition of $ABTS^+$ and DPPH radicals through simple electron transfer (SET) and proton transfer (HT) [43]. Both methods are reliable for the measurement of antioxidant activity, however, the $ABTS^+$ assay has advantages over DPPH assay, i.e., the $ABTS^+$ radical is soluble in aqueous and organic solvents; therefore, the

antioxidant activity of both hydrophilic and lipophilic compounds can be evaluated. Moreover, results obtained through the DPPH are difficult to be interpreted when the compounds in the extracts have UV spectra that overlap the DPPH at 515 nm (i.e., carotenes and xanthophylls) [36,43].

Both antioxidant activity assays showed a high correlation with the TPC (Fig. 1). The TPC and antioxidant activity by $ABTS^{+}$ assay showed a Pearson correlation coefficient of 0.8773 and 0.8871 for extracts obtained by CE and UAE, respectively. A similar correlation was observed between the TPC and antioxidant activity by DPPH assay (0.9064 and 0.9080, for CE and UAE, respectively). Finally, the correlation coefficients between $ABTS^{+}$ and DPPH assay were 0.9908 and 0.9705 by CE and UAE, respectively. The high correlation between the antioxidant activity measured by $ABTS^{+}$ and DPPH assay could be attributed to the fact that both are methods of antioxidant capacity mainly classified as SET mechanism. Although, these radicals can also be inhibited by HT [43]. Antioxidant activity is commonly correlated with TPC, this has been shown by some studies on the extraction of bioactive compounds from fruits of different *Capsicum* species [7,18,29], however, in pepper fruits, the antioxidant activity could be attributed to other secondary metabolites such as capsaicinoids, carotenoids, and organic acids, therefore, the multiple linear correlations are more appropriate to explain antioxidant activity in pepper fruits, similar to presented by Sora et al. [45] to chemometric studies applications. A high linear correlation between TPC and antioxidant activity suggests that the principal antioxidant compounds present in *C. chinense* leaves correspond to phenolic compounds.

3.4. Weibull and Gaussian distribution model for the correlation of solvent dielectric constant and total phenolic content

For the recovery of phenolic compounds from *C. chinense* leaves by UAE and CE, the TPC increased as the solvent polarity increased, however, a low TPC response was observed when the higher polarity solvents (20% MeOH, 1% AcOH and W) were used. Oreopoulou et al. [37] reported changes in TPC as a function of the composition of the extraction solvent, this phenomenon is described in more detail by Catena et al. [12], they reported similar behavior in the extraction of phenolic compounds and anthocyanins from rice (*Oryza sativa* L. 'Violet Nori'), an increase in the recovery of phenolic compounds and anthocyanins as a function of solvent composition was observed; a higher concentration of ethanol in the hydroalcoholic solutions improved the recovery of phenolic compounds, however, at very high ethanol concentration (70 to 100% EtOH v/v) a lower response for TPC was observed. This phenomenon is attributed to a change of polarity of the solvent, therefore, by modifying the composition of the solvent it is possible to increase and/or decrease the solvent polarity to improve the recovery of phenolic compounds. This behavior can be correlated with an asymmetric or normal distribution model such as the Weibull (Eq. 2) and Gaussian (Eq. 3) distribution models, respectively. These models were proposed to predict the best condition for obtaining the highest phenolic content and to describe the recovery of phenolic compounds as a function of solvent polarity during UAE extractions.

The graphical models that show the correlation of the TPC with the "ε" are presented in Fig. 2. The equation constants ("a", "b", "c" and "X_0"), the R and R^2 of the models are presented in Table 2. Both distribution models showed a high correlation (R: 0.93 to 0.95). The coefficient "a" represents the maximum value of TPC that can be obtained according to the models, similar values of 25.20 and 23.07 mg of GAE g^{-1} dry wt, were obtained by UAE and CE, respectively, for the Weibull and the Gaussian distribution models. The values of coefficient "b", related to the width of the bell behavior of the models, were 18.59 and 22.11 for UAE and CE methods respectively, for the Gaussian distribution model, while for the Weibull distribution model were 72.82 (UAE) and 76.97 (CE). This coefficient was lower by UAE than CE in both models and low values indicate reduced a narrow spectrum of polarity values. The X_0 ("ε") values to the Weibull distribution model were 47.93 and 47.57 for UAE and CE, respectively, while for the Gaussian distribution model values were 46.57 (UAE) and 46.85 (CE). The coefficient values (X_0) were similar between the Weibull and Gaussian distribution model. This coefficient represents the "ε" of the solvent that provides the highest TPC response according to the models, 50% MeOH had a similar "ε" (46.99 to 52.09) and was the solvent that showed the highest capacity for the recovery of phenolic compounds by UAE. Finally, all

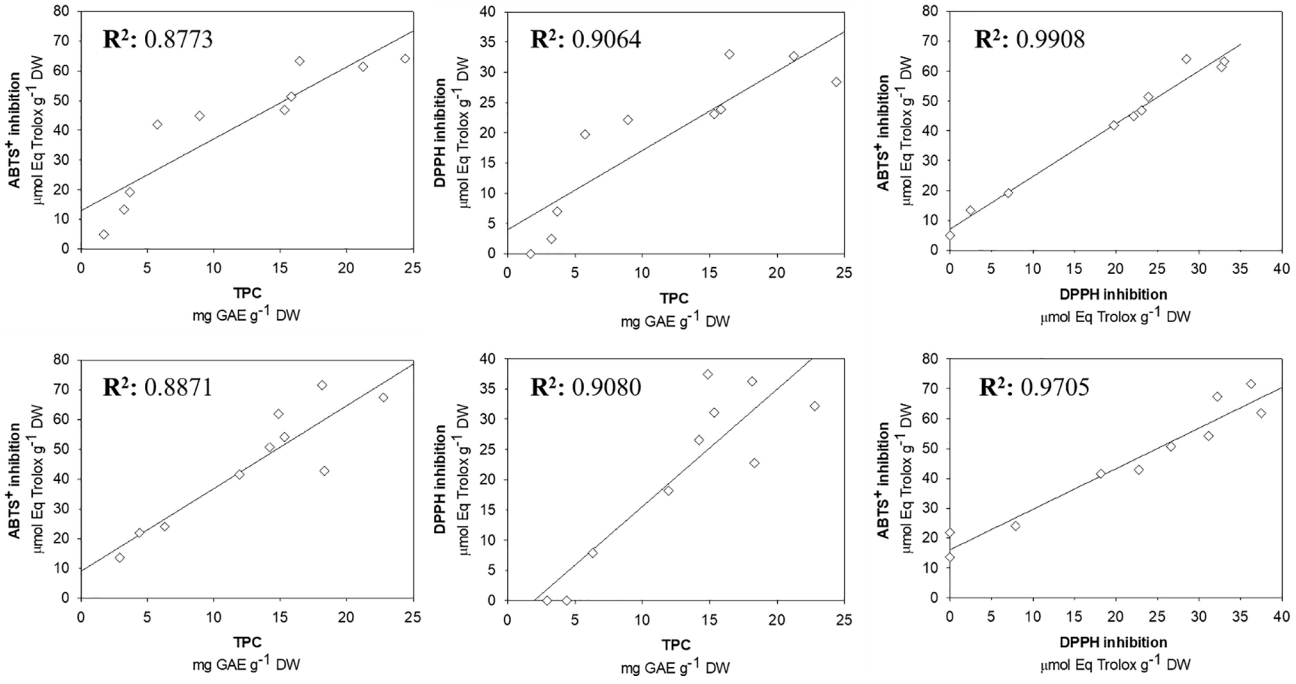

Fig. 1. Pearson correlation matrix between total phenolic content (TPC) and antioxidant activity ($ABTS^{+}$ and DPPH assays) in *C. chinense* leaves extracts obtained by UAE and CE.

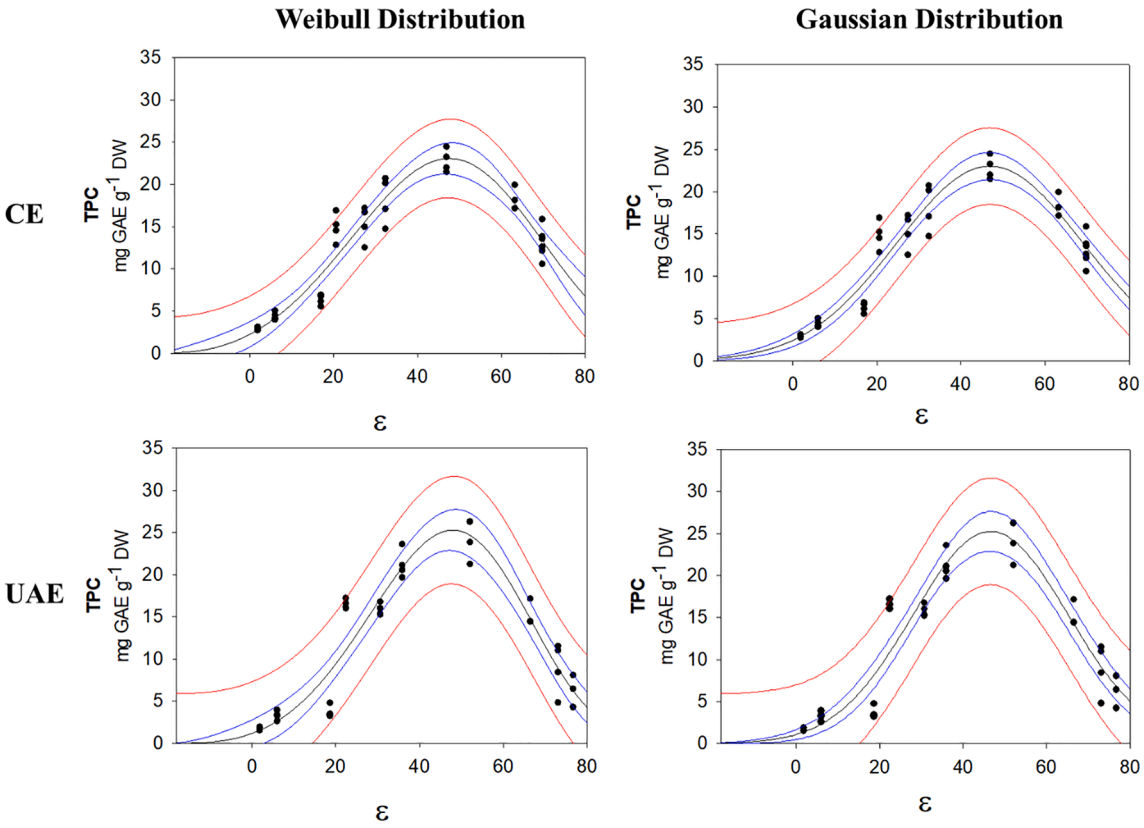

Fig. 2. The Weibull and Gaussian distribution models to the correlation between TPC and the "ε" of the solvent and solvent mixtures evaluated by CE and UAE.

Table 2
Equation constants, Pearson correlation, determination coefficients, and Normality Test (Shapiro-Wilk) of Gaussian and Weibull distribution model: Total phenolic content (TPC) vs dielectric constants (ε) as polarity parameter.

Model	Extraction method	Equation constants [B]				R	R^2	SD	Normality Test (Shapiro-Wilk) [A]	Constant Variance Test
		a	b	c	X_0					
Gaussian Distribution	*UAE*	25.26 (1.18)	18.59 (0.83)	–	46.57 (0.77)	0.93	0.87	2.90	0.3777	0.2761
	CE	23.01 (0.73)	22.11 (0.87)	–	46.85 (0.66)	0.95	0.90	2.08	0.6036	0.1000
Weibull Distribution	*UAE*	25.29 (1.20)	72.82 (16.43)	4.15 (0.99)	47.93 (1.28)	0.94	0.85	2.91	0.2970	0.2225
	CE	23.07 (0.92)	76.97 (14.01)	3.73 (0.82)	47.57 (1.09)	0.95	0.90	2.11	0.5337	0.1417

[A] Normality test (Shapiro-Wilk) significance level to p-value > 0.05.
[B] Equation constant (SD: standard deviation).

correlations presented a Normal distribution due to the models passed normality test of Shapiro-Wilk.

The determination of the relation between solvent polarity and the recovery of phenolic compounds using mathematical models is useful for understanding and improving extraction procedures. Oreopoulou et al. [37] applied a kinetic model for the recovery of phenolic compounds from oregano (*Origanum vulgare*) controlling as main parameters, particle size, time, and extraction solvents. Dong et al. [19] evaluated the empirical model COSMO-RS and an experimental model to determine the solubility of daidzein in different solvents and discussed the importance of solvent polarity and hydrogen bonding in the dissolution of these molecules and their application to extraction and purification methods. Álvarez et al. [6] developed a semi-empirical model for the recovery of grape pomace compounds based on the dielectric properties of the solvents. In summary, through the understanding of the processes of solubilization and solvation of phenolic compounds using mathematical models it is possible to obtain information to improve extraction methodologies and propose purification strategies.

The results suggest that the "ε" can be considered as a suitable polarity parameter to evaluate the effect of a pure solvent or binary mixture on the extraction of phenolic compounds from *C. chinense* leaves and it can be used as criteria for selection of the suitable solvent in phenolic extraction protocols. However, to have a complete understanding of the solvent effect on the extraction procedure it is necessary to evaluate the effect that it produces on the profile and content of each phenolic compound extracted.

3.5. Phenolic compounds identification by UPLC-PDA-ESI-MS/MS

The chromatogram in Fig. 3 shows the compounds identified by UPLC-PDA-ESI-MS/MS in an extract of *C. chinense* leaves obtained by UAE using 50% methanol and in the hydrolyzed extract. A total of 15 phenolic compounds and 6 flavonoid compounds were identified,

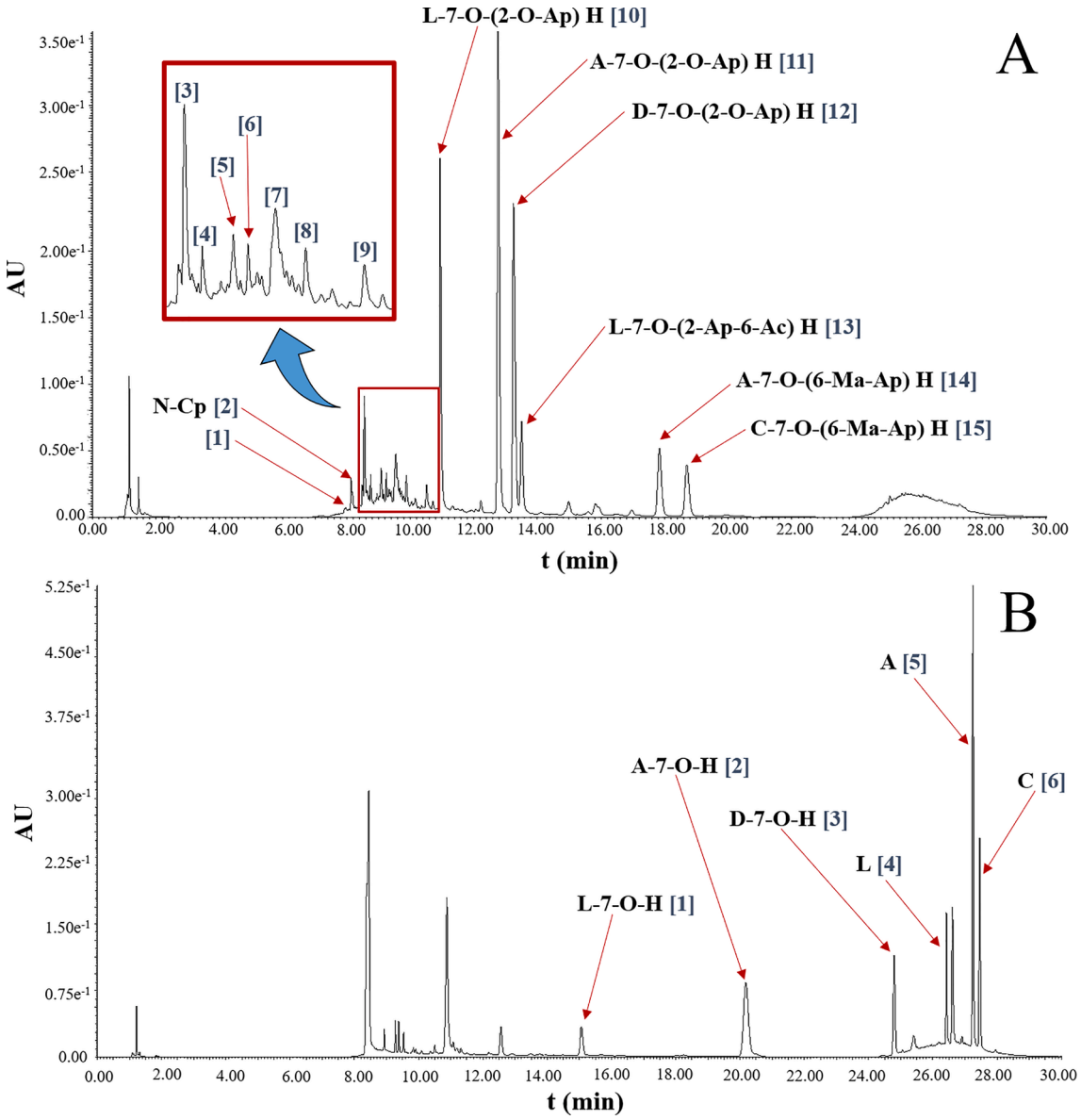

Fig. 3. Chromatogram of phenolic compounds identified in *C. chinense* leaves extract obtained by UPLC-PDA analysis (A: 50% MeOH by UAE; B: hydrolyzed extract obtained from A; Compounds was showed according to Table 3).

respectively (Table 3).

Compounds 1 (Isomer I; R_T: 7.941 min; λ_{max}: 194, 213, 293, 317 nm) and 2 (Isomer II; R_T: 8.130 min; λ_{max}: 194, 213, 293, 317 nm) were identified as N-caffeoyl putrescine (N-Cp). A molecular ion at *m/z* 251 ($[M + H]^+$) was found in positive ion mode. The fragments at *m/z* 234 ($[M - H - 17\ (NH_3\ loss)]^+$), *m/z* 163 ($[M - H - 86\ (putrescine\ loss)]^+$), and *m/z* 89 (putrescine) ($[M + 3H - 164\ (loss\ of\ caffeoyl)]^+$) were identified. Additionally, it is suggested that the identified fragment at *m/z* 72 ($[M - 178]^+$) represent the loss of an NH_3 group from putrescine (Fig. 4). This compound has been reported in *Nicotiana tabacum* L. [9,10]. Furthermore, Park et al. [39] have also identified this compound in *C. annuum* fruits in response to the anthracnose infection produced by *Colletotrichum gloeosporioides*.

Compound 3 (R_T: 9.550 min; λ_{max}: 197, 218, 278 nm) was tentatively identified as protocatechuic acid hexoside (PAH). The molecular ion at *m/z* 315 ($[M - H]^-$) and a fragment at *m/z* 203 in negative ion mode were found. In positive ion mode, the fragment at *m/z* 227 ($[M + H - 90\ (3 \cdot HCHO)]^+$) was detected, which suggests that correspond to the loss of three aldehyde groups, which is usual in glycosylated compounds. This compound has been reported by Vallverdú-Queralt et al. [46] in tomato sub-products by HPLC-ESI-QTOF and by Moco et al. [33] as aglycone in tomato fruits (*Solanum Lycopersicum*).

Compound 4 (R_T: 8.740 min; λ_{max}: 212, 299, 325 nm) was identified as 5-caffeoylquinic acid (5-CQA) with a molecular ion at *m/z* 353 ($[M - H]^-$) and a fragment at *m/z* 191 that corresponds to the quinic acid ($[M - H - 162]^-$) which is a characteristic fragment of this compound. It has been identified in tomatoes [33], cherry tomatoes [44], tomato-based by-products [46], and in *C. annuum* var. Lemeška and Lakošnička [35]. Mikulic-Petkovsek et al. [32] have reported the presence of this compound in response to the infection produced by *Colletotrichum coccodes*.

Compound 5 (R_T: 9.080 min; λ_{max}: 209, 284 nm), 6 (R_T: 9.235 min; λ_{max}: 205, 270, 297, 338 nm) and 8 (R_T: 9.860; λ_{max}: 272, 320 nm) were no identified. However, according to its UV spectrum is suggested that compounds 5 and 8 correspond to phenolic acids, while compound 6 correspond to a flavonoid.

Compound 7 (R_T: 9.540; λ_{max}: 195, 213, 312 nm) presented a molecular ion at *m/z* 329 ($[M - H]^-$). This compound was identified as vanillic acid-4-β-D-glucoside (VAG) and is reported by Vallverdú-Queralt et al., [46] in tomato-based by-products, and in *C. annuum* fruits

Table 3
Phenolic compounds identified in *C. chinense* leaves by UPLC-PDA-ESI-MS/MS in the extract obtained using 50% MeOH by UAE and hydrolyzed extract.

Number	TR	PDA UV bands (nm)	Experimental Accurate Mass		Molecular Formula	Fragments (*m/z*)		Tentative identification
			$[M–H]^-$	$[M+H]^+$		Ion negative	Ion positive	
1	7.941	194, 213, 293, 317	249.0850	251.1308	$C_{13}H_{18}N_2O_3$	249 (1 0 0)	251 (95) 234 (21) 163 (1 0 0) 72 (80)	N-caffeoyl putrescine (Isomer I)
2	8.130	194, 213, 293, 317	249.0850	251.1308	$C_{13}H_{18}N_2O_3$	249 (1 0 0)	251 (92) 234 (21) 163 (1 0 0) 72 (61)	N-caffeoyl putrescine (Isomer II)
3	8.550	197, 218, 278	315.0823	227.1421	$C_{13}H_{16}O_9$	315 (1 0 0) 203 (54)	227 (18) 188 (1 0 0) 146 (91) 118 (28) 100 (17)	Protocatechuic acidhexoside
4	8.740	212, 299, 325	353.0936		$C_{16}H_{18}O_9$	353 (1 0 0)215 (13) 293 (19) 191 (82)		5-Caffeolquinic acid
5	9.080	209, 284	215.0827	217.1303	$C_{12}H_{12}N_2O_2$	215 (1 0 0)	217 (47) 200 (95) 88 (1 0 0)	Unknown
6	9.235	205, 270, 297, 338	593.1991	595.2100	$C_{27}H_{30}O_{15}$	593 (72) 431 (9) 302 (9) 177 (1 0 0)	595 (7) 471 (1 0 0) 144 (57) 100 (31)	Unknown
7	9.540	195, 213, 312	329.0902		$C_{14}H_{18}O_9$	329 (55) 265 (62) 177 (1 0 0)		Vanillic Acid-4-β-D-glucoside
8	9.860	272, 320	597.2781		$C_{27}H_{34}O_{15}$	387 (1 0 0) 597 (9)		Unknown
9	10.485	210, 306	337.1141		$C_{16}H_{18}O_8$	337 (1 0 0) 191 (89)		Coumaroylquinic acid
10	10.904	205, 254, 348	579.1603	581.1707	$C_{26}H_{28}O_{15}$	579 (1 0 0)	581 (1 0 0) 287 (18)	Luteolin-7-O-(2-O-apiosyl) hexoside
11	12.730	199, 266, 336	563.1839	565.1846	$C_{26}H_{28}O_{14}$	563 (1 0 0)	565 (1 0 0) 433 (9) 271 (29)	Apigenin-7-O-(2-O-apiosyl) hexoside
12	13.212	206, 251, 266, 347	593.1651	595.2073	$C_{27}H_{30}O_{15}$	593 (1 0 0)	595 (1 0 0) 387 (25) 301 (14)	Diosmetin-7-O-(2-O-apiosyl) hexoside
13	13.466	206, 253, 348	665.1592	667.1755	$C_{26}H_{34}O_{20}$	621 (1 0 0) 665 (57)	667 (1 0 0)	Luteolin-7-O-(2-apiosyl-6-malonyl) hexoside
14	17.761	208, 266, 336	649.1833	651.1900	$C_{29}H_{30}O_{17}$	649 (9) 635 (32) 605 (1 0 0)	651 (1 0 0)	Apigenin-7-O-(6-malonyl-apiosyl) hexoside
15	18.603	210, 251, 266, 347	679.1975	681.1900	$C_{23}H_{40}O_{20}$	679 (12) 635 (1 0 0)	681 (1 0 0)	Chysoeriol-7-O-(6 malonyl-apiosyl) hexoside
1*	15.148	207, 253, 347	447.1658	449.1168	$C_{21}H_{20}O_{11}$	447 (1 0 0) 285 (90) 284 (80)	449 (1 0 0) 287 (61)	Luteolin-7-O-hexoside
2*	20.271	206, 266, 336	431.1199	433.1222	$C_{21}H_{20}O_{10}$	431 (88) 268 (1 0 0)	433 (1 0 0) 271 (79)	Apigenin-7-O-hexoside
3*	24.880	205, 251, 265, 347	461.1377	463.1222	$C_{22}H_{22}O_{11}$	461 (1 0 0) 297 (6) 283 (32) 255 (22)	463 (1 0 0) 453 (30) 301 (44)	Diosmetin-7-O-hexoside
4*	26.505	210, 252, 267, 347	285.061	287.0468	$C_{15}H_{10}O_6$	285 (68) 133 (1 0 0)	287 (1 0 0)	Luteolin
5*	27.359	214, 267, 336	269.0797	271.0555	$C_{15}H_{10}O_5$	269 (27) 117 (1 0 0)	271 (1 0 0) 246 (34)	Apigenin
6*	27.529	210, 247, 267, 346	299.0699	301.0551	$C_{16}H_{12}O_6$	299 (1 0 0) 284 (49) 227 (46) 151 (30) 107 (35)	301 (1 0 0)	Chrysoeriol

* Identified in the hydrolyzed extract.

by Morales-Soto et al. [34]. In addition, has also been detected in several vegetables sources such as artichoke [3], cucumber [2] and araceae leaves [1].

Compound 9 (Isomer II, R_T: 10.485 min, λ_{max} = 210, 306 nm) showed molecular ion at *m/z* 337 in negative ion mode. It was identified as coumaroylquinic acid (CQA). The fragment at *m/z* 191 was also detected, this corresponds to quinic acid ($[M – H – 146]^-$ or [192 (quinic acid) – $H]^-$) and is a fragment commonly reported for CQA [3,35,40,42,44,46].

Compound 10 (R_T: 10.904 min; λ_{max}: 205, 254, 348 nm) showed molecular ions at *m/z* 579 ($[M – H]^-$) and *m/z* 581 ($[M + H]^+$) in negative and positive ion mode, respectively. It was identified as luteolin-7-O-(2-O-apiosyl) hexoside (L-7-(2-Ap) H) [30,32,39]). The fragment at *m/z* 287 in positive ion mode corresponding to the protonated luteolin molecule ($[M + 2H – 295]^+$) was also detected. In the hydrolyzed extract, the molecular ions at *m/z* 447 ($[M – H]^-$) and *m/z* 449 ($[M + H]^+$) were found in negative and positive ion mode, respectively. It was identified as luteolin-7-O-hexoside (L-7-H) [1,34,35,39] and is suggested that its presence is derived from to the loss of pentose belonging to the L-7-(2-Ap) H by acid hydrolysis. The fragments at *m/z* 285 ([M – 163 (hexoside)]$^-$), *m/z* 284 ([M – H – 163 (hexoside)]$^-$), and *m/z* 287 were identified ($[M + H – 163 (hexoside)]^+$) to correspond to the luteolin after the loss of a hexose during ionization (Fig. 4).

Compounds 11 (R_T: 12.730 min; λ_{max}: 199, 266, 336 nm) was identified as apigenin-7-O-(2-O-apiosyl) hexoside (A-7-(2-Ap) H) with a molecular ions at *m/z* 563 and *m/z* 565 in negative ion mode and positive ion mode, respectively [35]. The fragment at *m/z* 433 was also found in positive ion mode that may correspond to the loss of a pentose ($[M + 2H – 133]^+$) and the fragment at *m/z* 271 to the protonated apigenin molecule ($M + H – 294]^+$) after the loss of both sugars. Additionally, the molecular ions at *m/z* 431 ($[M – H]^-$) and *m/z* 433 ($[M + H]^+$) were found in the hydrolyzed extract (Table 3). This compound was identified as apigenin-7-O-hexoside (A-7-H) [1,34,39] after the loss of a pentose as a result of acid hydrolysis. The fragments at *m/z* 268 ($[M – H – 163]^-$) and *m/z* 271 ($[M + 2H – 163]^+$) are suggested to correspond to the apigenin molecule after the loss of both sugars after ionization (Fig. 4).

Compound 12 (R_T: 13.212 min; λ_{max}: 206, 251, 266, 347 nm) showed molecular ions at *m/z* 593 ($[M – H]^-$) and *m/z* 595 ($[M + H]^+$) in negative and positive ion mode, respectively. It was identified as diosmetin-7-O-(2-O-apiosyl) hexoside (D-7-(2-Ap) H) [2]. In the hydrolyzed extract, the molecular ions at *m/z* 461 ($[M – H]^-$) and *m/z* 463 ($[M + H]^+$) in negative and positive ion mode were observed, respectively (Table 4). It is suggested its identification as diosmetin-7-O-hexoside (D-7-H) [44]). It is also suggested that is the result of the release of the pentose from 2-O-glucosidic bond of D-7-(2-Ap) H after acid hydrolysis. For this same compound were identified the fragment at *m/z* 446 corresponding to the loss of a methyl group ([M – H – 15 $(CH_3)]^-$), fragment *m/z* 297 possible attributed to the loss of the hexose of

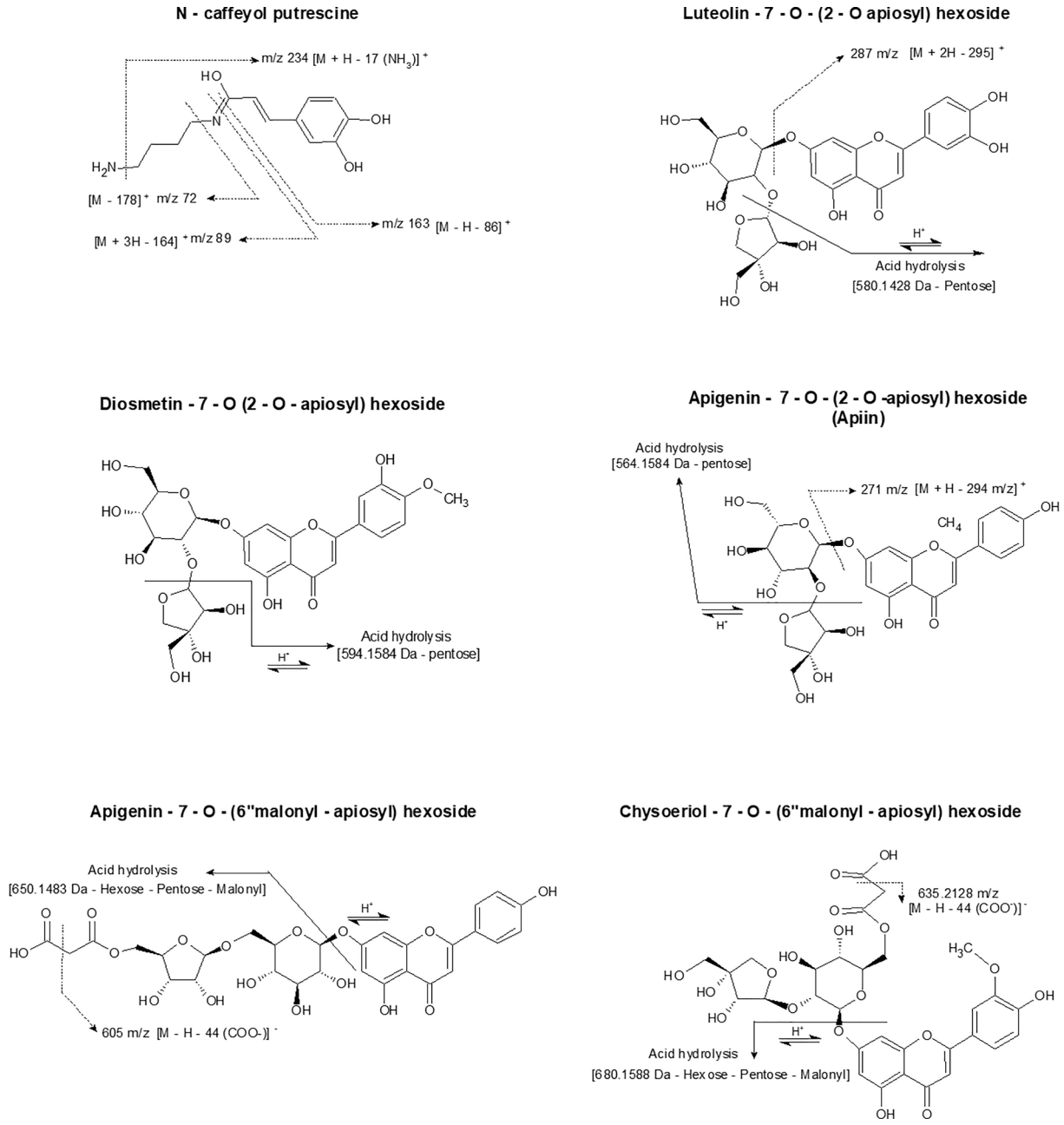

Fig. 4. Structure of principal phenolic compounds found in *C. chinense* leaves by UPLC-PDA-ESI-MS/MS.

the 7-O-glycosidic bond ([M – 2H – 163 (glycosyl)]$^-$), fragment at *m/z* 283 possible represented the loss of a hexose ([M – 179 (hexoside)]$^-$) and fragment at *m/z* 255 attributed to the rupture of the "C" ring of the flavonoid skeleton of the diosmetin and the loss of an OH $^-$ group ([M + H – 192]$^-$). In the positive ion mode, the fragment at *m/z* 301 ([M + 2H – 163]$^+$) corresponded to the protonated diosmetin (Fig. 3) was detected.

Compound 13 (R_T = 13.212 min; λ_{max}: 206, 253, 266, 348) showed molecular ions at *m/z* 665 ([M – H]$^-$) and *m/z* 667 ([M + H]$^+$) in negative and positive ion mode, respectively. It was identified as luteolin-7-O-(2-apiosyl-6-malonyl) hexoside (L-7-(2-Ap-Ma) H) [2,23,30,32,35]). For this compound, the fragment at *m/z* 621 was detected in negative ion mode corresponding to the loss of a carboxyl ion group ([M – H – 44 (COO^-]$^-$). The molecular ions at *m/z* 285 ([M – H]$^-$) and *m/z* 287 ([M + H]$^+$) in negative and positive ion mode, respectively, were found in the hydrolyzed extract that allows identifying the compound as luteolin (Table 3) after the loss of its substituents by acid hydrolysis.

Compounds 14 (R_T: 17.761; λ_{max}: 208, 266, 336 nm) showed molecular ions at *m/z* 649 ([M – H]$^-$) and *m/z* 651 ([M + H] $^+$) in negative and positive ion mode, respectively. It was identified as apigenin-7-O-(6-malonyl-apiosyl) hexoside (A-7-(6-Ma-Ap) H) [28]. For this compound, the fragment at *m/z* 605 correspondings to the loss of a carboxyl group ([M – H – COO $^-$] $^-$) was also observed. The molecular ion at *m/z* 269 ([M – H] $^-$) and *m/z* 271 ([M + H] $^+$) were detected in the hydrolyzed extract corresponding to the apigenin compound after the loss of all its substituents by acid hydrolysis. The fragments at *m/z* 246 were also found in positive ion mode that possibly corresponds to the loss of a C_2H_2 group ([M + 2H – 26 (C_2H_2)] $^+$) and fragment at *m/z* 117 in negative ion mode suggested the rupture of the "C" ring of the flavonoid skeleton ([M – H – 152] $^-$) (Table 4).

Compound 15 (R_T: 18.603 min; λ_{max}: 210, 251, 266, 347 nm) showed molecular ions at *m/z* 679 ([M – H] $^-$) and *m/z* 681 ([M + H] $^+$) in negative and positive ion mode, respectively. It was identified as chrysoeriol-7-O-(6-malonyl-apiosyl) hexoside (C-7-(6-Ma-Ap) H) [28]).

Table 4
Quantitation of phenolic compounds presents in *C. chinense* leaves extracts obtained by maceration (CE) and UAE using different pure solvents and aqueous binary mixtures.

CE										
Compound*	Solvent									
	W	1% AcOH	20% MeOH	50% MeOH	80% MeOH	80% Ace	MeOH	Ace	AcOEt	Hx
N-C p[A]	NQ	NQ	0.58 ± 0.01^{b}	0.85 ± 0.06^{a}	0.82 ± 0.00^{a}	NQ	0.89 ± 0.04^{a}	NQ	NQ	NA
L-7-(2-Ap) H[B]	NQ	NQ	NQ	5.05 ± 0.16^{c}	6.93 ± 0.16^{b}	8.69 ± 0.12^{a}	7.34 ± 0.05^{b}	1.73 ± 0.35^{d}	0.49 ± 0.00^{e}	NA
A-7-(2-Ap) H[C]	NQ	NQ	NQ	8.33 ± 0.36^{d}	11.24 ± 0.28^{c}	14.23 ± 0.03^{a}	12.41 ± 0.11^{b}	4.11 ± 0.78^{e}	1.06 ± 0.00^{f}	NA
D-7-(2-Ap) H[D]	NQ	NQ	NQ	13.21 ± 0.21^{c}	15.92 ± 0.43^{b}	18.81 ± 1.07^{a}	17.24 ± 0.11^{ab}	6.66 ± 0.24^{d}	1.59 ± 0.03^{e}	NA
A-7-(6-Ma-Ap) H[C]	NQ	NQ	NQ	0.55 ± 0.05^{c}	1.23 ± 0.01^{b}	1.91 ± 0.02^{a}	1.17 ± 0.03^{b}	0.26 ± 0.05^{d}	0.08 ± 0.00^{e}	NA
C-7-(6-Ma-Ap) H[D]	NQ	NQ	NQ	1.48 ± 0.23^{d}	2.75 ± 0.02^{b}	3.91 ± 0.03^{a}	2.39 ± 0.06^{c}	0.83 ± 0.12^{e}	0.36 ± 0.00^{f}	NA
TOTAL	0.00 ± 0.00	0.00 ± 0.00	0.58 ± 0.01^{f}	29.48 ± 0.04^{c}	38.89 ± 0.86^{b}	47.55 ± 1.26^{a}	41.43 ± 0.26^{b}	13.59 ± 1.54^{d}	3.57 ± 0.04^{e}	NA
UAE										
Compound*	Solvent									
	W	1% AcOH	20% MeOH	50% MeOH	80% MeOH	80% Ace	MeOH	Ace	AcOEt	Hx
N-C p[A]	0.10 ± 0.00^{d}	0.39 ± 0.00^{bc}	NQ	0.90 ± 0.08^{a}	0.83 ± 0.22^{a}	0.22 ± 0.02^{c}	0.43 ± 0.02^{b}	NQ	NQ	NA
L-7-(2-Ap) H[B]	NQ	NQ	NQ	7.25 ± 0.33^{b}	7.20 ± 0.08^{b}	8.01 ± 0.01^{a}	6.18 ± 0.05^{c}	1.33 ± 0.09^{d}	0.29 ± 0.04^{e}	NA
A-7-(2-Ap) H[C]	NQ	NQ	NQ	11.53 ± 0.64^{b}	11.79 ± 0.18^{ab}	12.74 ± 0.31^{a}	10.44 ± 0.01^{c}	2.58 ± 0.19^{d}	0.47 ± 0.18^{e}	NA
D-7-(2-Ap) H[D]	NQ	NQ	NQ	16.73 ± 0.89^{b}	16.50 ± 0.23^{b}	18.43 ± 0.64^{a}	14.90 ± 0.19^{c}	3.19 ± 0.31^{d}	0.83 ± 0.29^{e}	NA
A-7-(6-Ma-Ap) H[C]	NQ	NQ	NQ	1.21 ± 0.17^{c}	1.63 ± 0.02^{b}	1.89 ± 0.02^{a}	1.13 ± 0.07^{c}	0.23 ± 0.02^{d}	0.07 ± 0.02^{d}	NA
C-7-(6-Ma-Ap) H[D]	NQ	NQ	NQ	2.89 ± 0.33^{b}	3.70 ± 0.01^{a}	4.05 ± 0.04^{a}	2.63 ± 0.31^{b}	0.67 ± 0.02^{c}	0.40 ± 0.05^{c}	NA
TOTAL	0.10 ± 0.00 g	0.39 ± 0.00^{f}	0.00 ± 0.00	40.51 ± 2.43^{b}	41.65 ± 0.71^{ab}	45.34 ± 0.41^{a}	35.71 ± 0.64^{c}	7.89 ± 0.63^{d}	1.94 ± 0.55^{e}	NA

[A] Quantified as μmol Eq of caffeic acid g^{-1} dry wt; [B] Quantified as μmol Eq of luteolin g^{-1} dry wt; [C] Quantified as μmol Eq of apigenin g^{-1} dry wt; [D] Quantified as μmol Eq of quercetin g^{-1} dry wt.

* **N-C p**: N-Caffeyol putrescine; **L-7-(2-Ap) H**: Luteolin-7-O-(2-O-apiosyl) hexoside; **A-7-(2-Ap) H**: Apigenin-7-O-(2-O-apiosyl) hexoside; **D-7-(2-Ap) H**: Diosmetin-7-O-(2-O-apiosyl) hexoside; **A-7-(6-Ma-Ap) H**: Apigenin-7-O-(6-malonyl-apiosyl) hexoside; **C-7-(6-Ma-Ap) H**: Chrysoeriol-7-O-(6-malonyl-apiosyl) hexoside. **NQ**: Non-quantifiable; **NA**: Analysis of non-polar solvent hexane is not compatible with reverse phase chromatography.

The fragment at *m/z* 635 was also detected and its suggested that it corresponding to the loss of a carboxyl ion ([M – H – 44 (COO^-)$^-$) after ionization (Fig. 4). The molecular ions at *m/z* 299 ([M – H]$^-$) and *m/z* 301 ([M + H]$^+$) fragments were detected in the hydrolyzed extract in negative and positive ion mode, respectively. It was identified as chrysoeriol, the aglycone form of C-7-(6-Ma-Ap) H (Table 3). The fragment at *m/z* 284 corresponding to the loss of a methyl group ([M – H – 15 (CH_3)]$^-$), fragment at *m/z* 151 representing the "C" ring breakage of the flavonoid skeleton ([M – 149]$^-$) and fragment at *m/z* 107 representing a product of the "C" ring breaking were also observed.

3.6. Quantification of identified phenolic compounds from C. Chinense leaves extracts.

The best response for TPC was obtained using 50% MeOH as solvent, which can be selected as the best option to achieve the major recovery of phenolic compounds in *C. chinense* leaves. However, the spectrophotometric method for the determination of TPC is not specific, therefore, to a better understanding of the interactions of each identified compound with the solvent used for its extraction the quantification of each compound obtained by the different solvents is showed in table 4. The highest content of N-caffeoyl putrescine was 0.85 ± 0.06 and 0.90 ± 0.08 μmol Eq CA g^{-1} dry wt by CE and UAE respectively, using 50% MeOH as solvent, while the highest general content of identified flavonoids was obtained using 80% Ace independently of the extraction method employed. The flavonoids with the highest concentration were: L-7-(2-Ap) H, A-7-(2-Ap) H and D-7-(2-Ap) H containing 8.69 ± 0.12 μmol Eq luteolin g^{-1} dry wt, 14.23 + 0.03 μmol Eq of apigenin g^{-1} dry wt and 18.81 ± 1.07 μmol Eq of quercetin g^{-1} dry wt, respectively. In both cases, for phenolic acid (N-Cp) and flavonoids it was observed a similar behavior to that described for the correlation between TPC and "ε" of the evaluated solvents, an increase in the individual content of each phenolic compound as a function of polarity (ε), followed by a maximum recovery point and a decrease as the polarity of the solvent increases. However, the ideal values of "ε" for the recovery of each phenolic compound is different from each other. In this sense, for the recovery of N-Cp the values are between 46.99 and 52.09, while for flavonoids are between 20.62 and 22.45 that are obtained with solvents of MeOH at 50% and Ace at 80% respectively. The results also show similarities in the recovery of phenolic compounds between both methods, the UAE and CE. However, the advantages offered by the UAE such as the mechanism and time of extraction must be considered. For example, the results reported by Pacheco *et al.* [38] show that through UAE a higher recovery of rosmarinic acid can be achieved from *Cordia dodecandra* fruits than through extraction by maceration.

Regarding the biological activity of these compounds, Park et al. [39] observed the accumulation of chlorogenic acid and N-caffeoyl putrescine in *C. annuum* fruits at the local site of *C. coccodes* infection. The latter compound was reported as a "de novo" synthesis in *C. annuum* and is classified as a possible phytoalexin. The proposed function is that its accumulation is related to the formation of mechanical barriers to prevent the progression of the infection. The accumulation of flavonoids

glycosylated is observed in healthy leaves due to antiradical activity. León-Chan et al. [27] reported that in *C. annuum* leaves flavonoids as apigenin-7-O-glucoside (A-7-G) and luteolin-7-O-glucoside (L-7-G) are related to defending mechanism against UV-B radiation and low temperatures. Du et al. [20] reported that the accumulation of flavonoids as apigenin and luteolin in sorghum are related to inhibition of spore germination of *C. sublineolum*, while Mikulic-Petkovsek et al. [32] reported that accumulation of flavonoids is observed in tissues compromised to infection produced by *C. coccodes*. Secondly, glycosylation to flavonoids is an important step to their accumulation in different tissues. This process is realized by UDP-glycosyltransferases (UGT) and allow stored flavonoids in cellular vacuoles contributing to plant homeostasis and resistance mechanisms against abiotic and biotic stresses [26].

4. Conclusions

The results indicated a higher recovery of phenolic compounds with MeOH at 50% according to the response of TPC, besides an important correlation between TPC and antioxidant activity ($ABTS^{+}$, DPPH) was found. TPC showed relation with the "ε" that is a polarity parameter related to the molecular interaction between solvents and solutes. This relation was analyzed by the Weibull and Gaussian models presenting high regression values (0.93 to 0.95), suggesting that the highest phenolic compounds recovery is obtained using solvents with "ε" values between 35 and 52 by UAE. The chromatographic analysis showed that there are important differences in the recovery of specific phenolic compounds, while MeOH at 50% is adequate for a better recovery of phenolic acids (N-Cp), the use of 80% Ace turned out to be more favorable for the recovery of flavonoids. Finally, this study provides a key criterion for the selection of the extraction solvent to be used in UAE method for TPC recovery of *C. chinense* leaves that could be useful for the study of metabolic changes of different groups of phenolic compounds, which are one of the main mechanisms triggered by plants to interact with their environment.

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CRediT authorship contribution statement

Emanuel Herrera-Pool: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft. **Ana Luisa Ramos-Díaz:** Conceptualization, Resources, Writing - review & editing, Funding acquisition. **Manuel Alejandro Lizardi-Jiménez:** Validation, Writing - review & editing. **Soledad Pech-Cohuo:** Methodology, Writing - review & editing, Supervision. **Teresa Ayora-Talavera:** Conceptualization, Resources, Writing - review & editing, Supervision. **Juan C. Cuevas-Bernardino:** Writing - review & editing, Supervision. **Ulises García-Cruz:** Writing - review & editing, Supervision. **Neith Pacheco:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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