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Valorization of *Citrus limon* residues for the recovery of antioxidants: Evaluation and optimization of microwave and ultrasound application to solvent extraction

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ABSTRACT

Ultrasound assisted extraction (UAE) and microwave-assisted extraction (MAE) were optimized (by response surface methodology, RSM) and compared for the recovery of total phenolic compounds (TPC expressed as gallic acid equivalents (GAE)) from *Citrus limon* peels. The optimized result for MAE was 48% ethanol as extraction solvent, 28:1 mL/g of solvent: solid ratio, 123 s and 400 W for irradiation time and power. The optimized result for UAE was 63.93% ethanol as extraction solvent, 40 mL/g of liquid/solid ratio, 15.05 min of holding time and 77.79% for amplitude. Maximum predicted TPC recoveries under the optimized conditions for MAE and UAE were 15.74 and 15.08 mg GAE/g respectively, which were close to the experimental values of 15.78 ± 0.8 and 15.22 ± 0.88 mg GAE/g, indicating suitability of the employed model and the success of RSM in optimizing the extraction conditions. The antioxidant activity determined by the DPPH and reducing power tests confirmed the suitability of MAE for the preparation of antioxidant-rich plant extracts.

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1. Introduction

Citrus is the most important fruit crop in the world. The production of citrus fruits has been increasing enormously in the last few decades, going from an average of 48 million tons a year in the period 1970–1979, to more than 100 million tons in 2004–2005 (Dugo and Di Giacomo, 2002; González-Molina et al., 2010). Citrus fruits are cultivated in more than 100 countries all over the world, mainly in tropical and subtropical areas, where favorable soil and climatic conditions prevail (Dugo and Di Giacomo, 2002).

Among fruit and vegetables, citrus fruits are reported to be a very rich source of health promoting substances (Artés-Hernández et al., 2007). Although their health-related properties have always been associated with their content of vitamin C, it has recently been shown that flavonoids also play a role in this respect. In ancient medicine *Citrus limon* (*C. limon* Burm) and melissa (*Melissa officinalis* L.) have long been used as natural insect repellents (Oshaghi et al., 2003).

Citrus is an important crop mainly used in food industries for fresh juice production. Peels, the main waste fraction of citrus fruits, represent roughly half of the fruit mass and have been widely studied because they contain numerous biologically active compounds including natural antioxidants such as phenolic acids and flavonoids (Hayat et al., 2009, 2010).

The first step for both analysis and exploitation of medicinal plant bioactive constituents is their extraction from the cellular matrix. The "ideal" extraction method should be quantitative, non-destructive, and time saving. Besides conventional solvent extraction processes commonly used for the recovery of phenolic compounds (Proestos et al., 2006), non-conventional, more rapid and automated methods have been recently used, e.g. supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and ultrasound assisted extraction (UAE) (Aybaster et al., 2013; Liazid et al., 2007). Actually, the state of the art in these fields has shown that ultrasound and microwave radiation could accelerate the extracting process improving bioactive compounds extraction, particularly phenolic compounds (Garofulić et al., 2013; Muñiz-Márquez et al., 2013; Morelli and Prado, 2012; Li et al., 2011).

MAE is attractive because it allows for rapid heating of aqueous samples and presents advantages over conventional extraction techniques, such as improved efficiency, reduced extraction time, lower solvent consumption, higher selectivity toward target

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molecules and higher level of automation (Han et al., 2011; Singh et al., 2011; Zhang et al., 2008). In addition of these advantages, a wider range of solvents can be used in MAE, as the technique is less dependent on solvent affinity (Yang and Zhai, 2010).

UAE is particularly attractive for its simplicity, low cost of equipment (Carrera et al., 2012), efficiency in extracting analytes from different matrices (Herrera and de Castro, 2005), low energy required and reduced solvent- and time-consuming method (Xia et al., 2011). The enhancement of the extraction process by ultrasounds is attributed to the disruption of the cell walls, reduction of the particle size and the increase of the mass transfer of the cell content to the solvent caused by the collapse of the bubbles produced by acoustic cavitation (Xia et al., 2011; Rodrigues et al., 2008).

To our best knowledge, no literature report exists on the optimization and comparison of MAE and UAE procedure for the extraction of total phenolic compounds (TPC) from *C. limon* peels. Therefore, the objectives of the current study were to:

- investigate the effects of different parameters on the extraction efficiency (in terms of recovery and antioxidant activity of total phenolic compounds) by MAE and UAE processes;
- optimize the MAE and UAE conditions by response surface methodology (RSM);
- compare the optimized MAE and UAE results with a reference Conventional Solvent Extraction procedure (CSE).

2. Materials and methods

2.1. Plant material

The fruit samples of *C. limon* were collected in the area of Sidi Aich (Bejaia, Algeria), washed with distilled water and peeled off with hands. Peels were dried for about 15 days at room temperature in a ventilated darkroom to protect the active compounds content from light oxidation. Dried peels were ground with an electrical grinder (IKA model A_{11} Basic, Germany), the powder was passed through standard 125 μm sieve and only the fraction with particle size <125 μm was used. The powder was stored in airtight bags until use. The water activity (a_w) was determined by HygroPalm AW and was 0.25 ± 0.02 at 23 °C.

2.2. Reagents

Sodium carbonate (Na_2Co_3), Folin–Ciocalteau's phenol reagent and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Prolabo (made in CE) and 1,1-diphenyl-2-picryl-hydrazil (DPPH) from Sigma Aldrich (Germany). Gallic acid, ferric chloride ($FeCl_3{\cdot}6H_2O$), potassium ferricyanide ($C_6N_6FeK_3$), trichloroacetic acid and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Biochem-chemopharma (UK). All solvents used were of analytical grade and purchased from Prolabo (CE).

2.3. Experimental work

For optimization of the MAE and UAE procedure, the influences of the process parameters were firstly separately investigated in single-factor experiments to limit the total experimental work (Tables 1 and 2). When one variable was not studied, it was kept constant. In the MAE trials, the constant values for irradiation time, solvent-to-solid ratio and ethanol concentration were 120 s, 20 mL/g and 50%, respectively. Microwave power was set at 500 W in the trials to investigate the influence of solvent type and ethanol concentration, and at 400 W in the trials to investigate the influence of solvent-to-solid ratio. In the UAE trials, the constant values of sonication time, solvent-to-solid ratio and ethanol concentration were 10 min, 40 mL/g and 50%. Radiation amplitude was set at 50% in the trials to investigate the influence of ethanol concentration and sonication time, and at 60% in the trials to investigate the influence of solvent-to-solid ratio.

On the basis of the single-factor experimental results, major influence factors were selected. Then, an RSM based on a Box–Behnken Design (BBD) for MAE and Central Composite Rotatable Design (CCRD) for UAE was conducted to optimize both processes (Tables 3 and 4) (Vázquez et al., 2012; Wu et al., 2013). Regression analysis of the data to fit a second-order polynomial equation (quadratic model) was carried out according to the following general equation (Eq. (1)) which was, then, used to predict the optimum conditions of extraction process.

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X^2 + \sum_{i>1}^{k} B_{ii} X_i X_j + E \quad (1)$$

where Y represents the response function (in our case the TPC yield); B_0 is a constant coefficient; B_i, B_{ii} and B_{ij} are the coefficients of the linear, quadratic and interactive terms, respectively, and X_i and X_j represent the coded independent variables. According to the analysis of variance, the regression coefficients of individual linear, quadratic and interaction terms were determined. In order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions, the regression coefficients were used to generate 3-D surface plots from the fitted polynomial equation. The factor levels were coded as −1 (low), 0 (central point or middle) and 1 (high), respectively. The variables were coded according to the following equation (Eq. (2)):

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (2)$$

where x_i is the (dimensionless) coded value of the variable X_i; X_0 is the value of X at the center point and ΔX is the step change.

Analysis of variance was performed for the response variable using the full model where P-values (partitioned into linear and interaction factors) indicated whether the terms were significant or not. To verify the adequacy of the models, additional extraction trials were carried out at the optimal conditions predicted with the RSM and the obtained experimental data were compared to the values predicted by the regression model.

Efficiency of the two non-conventional methods (MAE and UAE) was compared based on the TPC recovery and the quality of the recovered phenolic compounds in terms of antioxidant activity (according to DPPH assay and Fe reducing ability) which was measured only on the extracts obtained under the optimum conditions selected by RSM.

Optimized MAE and UAE conditions were then compared to a reference CSE procedure.

Finally, in order to investigate the influence of ultrasound, microwave and maceration on the microstructure of the samples powder, the peel powder samples (before and after the different extraction processes) were observed by scanning electron microscopy (SEM).

2.3.1. Microwave assisted extraction

A domestic microwave oven (NN-S674MF, Samsung, Malaysia) with cavity dimensions of 22.5 cm × 37.5 cm × 38.6 cm and 2450 kHz working frequency was used. The apparatus was equipped with a digital control system for irradiation time and microwave power (the latter linearly adjustable from 200 to 1000 W). The oven was modified in order to condensate into the sample the vapors generated during extraction giving a constant sample volume.

For the extraction, one gram of the peel powder was placed in a 250 mL volumetric flask containing the extraction solvent. The suspension was irradiated at regular intervals according to

Table 1
Results of single-factor experiments for microwave assisted extraction. Results are reported as means ± S.D. Same letters in the same column refer to means not statistically different according to ANOVA and Tukey's test. TPC, total phenols yield referred to dry weight (dw) of *C. limon* peels; GAE, gallic acid equivalents.

Solvent		Ethanol concentration		Irradiation time		Microwave power		Solvent-to-solid ratio	
Type	TPC yield (mg_{GAE}/g_{dw})	(% v/v)	TPC yield (mg_{GAE}/g_{dw})	(s)	TPC yield (mg_{GAE}/g_{dw})	(W)	TPC yield (mg_{GAE}/g_{dw})	(mL/g)	TPC yield (mg_{GAE}/g_{dw})
Water	7.38 ± 1.19^{b}	20	10.43 ± 1.28^{bc}	90	10.04 ± 0.95^{c}	300	10.65 ± 1.30^{b}	15	12.20 ± 0.46^{c}
50% MeOH	8.50 ± 0.89^{b}	40	12.11 ± 0.62^{ab}	120	13.68 ± 0.24^{a}	400	13.68 ± 0.34^{a}	20	13.68 ± 0.14^{b}
50% EtOH	12.30 ± 0.47^{a}	50	13.30 ± 0.47^{a}	150	12.88 ± 0.30^{ab}	500	12.30 ± 0.39^{ab}	25	14.72 ± 0.39^{a}
50% Acetone	9.18 ± 0.44^{b}	60	10.95 ± 0.16^{bc}	180	11.86 ± 0.81^{b}	600	12.09 ± 0.23^{ab}	30	13.50 ± 0.27^{b}
		80	9.95 ± 0.81^{c}	210	11.58 ± 0.27^{bc}				
				240	11.44 ± 0.90^{bc}				

Table 2
Results of single-factor experiments for ultrasound assisted extraction. Results are reported as means ± S.D. Same letters in the same column refer to means not statistically different according to ANOVA and Tukey's test. TPC: total phenols yield referred to dry weight (dw) of *C. limon* peels. GAE, gallic acid equivalents.

Ethanol concentration		Sonication time		Amplitude radiation		Solvent-to-solid ratio	
(%)	TPC yield (mg_{GAE}/g_{dw})	(min)	TPC yield (mg_{GAE}/g_{dw})	(%)	TPC yield (mg_{GAE}/g_{dw})	(mL/g)	TPC yield (mg_{GAE}/g_{dw})
30	12.02 ± 0.53^{b}	5	12.30 ± 0.60^{b}	20	12.03 ± 1.20^{c}	20	11.09 ± 1.20^{b}
50	14.10 ± 0.21^{a}	10	14.16 ± 0.42^{a}	40	13.16 ± 0.71^{bc}	30	12.11 ± 0.60^{b}
70	13.20 ± 0.41^{ab}	15	15.11 ± 0.31^{a}	60	14.90 ± 0.83^{a}	40	14.88 ± 0.30^{a}
100	12.11 ± 1.12^{b}	20	12.21 ± 0.36^{b}	80	14.51 ± 0.22^{ab}	50	15.02 ± 0.51^{a}
				100	14.00 ± 0.33^{abc}		

oven operation. Depending on the trial, a different solvent, irradiation time, microwave power and solvent-to-solid ratio were used (Tables 1 and 3). At the end of microwave irradiation, the volumetric flask was allowed to cool to room temperature.

After extraction, the extract was recovered by filtration in a Büchner funnel through Whatman No. 1 paper, and collected in a volumetric flask. The extract was stored at (4 °C) until use and analyzed for the TPC and, for the extract obtained under the optimum conditions by RSM, also for the antioxidant activity.

2.3.2. Ultrasound assisted extraction

An ultrasonic apparatus (SONICS Vibra cell, VCX 130 PB, Stepped microtips and probes, No. 630-0422) was used for UAE with working frequency fixed at 20 kHz.

For the extraction, one gram of the peel powder was placed in a 250 mL amber glass bottle containing the extraction solvent. The suspension was exposed to acoustic waves under variations of ethanol concentration, irradiation time, amplitude and liquid-to-solid ratio (Tables 2 and 4). The temperature was controlled

Table 3
Box–Behnken design with the observed responses and predicted values for yield of total phenolic compounds (TPC) referred to dry weight (dw) of *C. limon* peels using microwave assisted extraction. GAE, gallic acid equivalents.

Run	*X*1 – ethanol concentration (% v/v)	*X*2 – time (s)	*X*3 – power (W)	*X*4 – ratio (mL/g)	TPC yield (mg_{GAE}/g_{dw})	
					Experimental	Predicted
1	50	120	400	25	15.00 ± 0.83	14.97
2	60	150	400	25	13.49 ± 1.48	13.48
3	50	90	400	30	14.19 ± 0.90	14.23
4	50	120	300	20	13.72 ± 0.88	13.76
5	60	120	300	25	12.49 ± 0.99	12.70
6	50	120	500	20	14.05 ± 1.49	14.17
7	50	150	400	20	14.05 ± 1.41	14.22
8	50	120	400	25	14.98 ± 0.93	14.97
9	50	150	500	25	13.44 ± 0.99	13.57
10	40	120	400	20	14.33 ± 0.93	14.49
11	60	120	400	20	13.65 ± 0.95	13.75
12	50	90	500	25	13.49 ± 1.79	13.59
13	50	120	300	30	13.74 ± 1.11	13.98
14	40	90	400	25	14.30 ± 1.06	13.97
15	50	150	400	30	14.51 ± 0.99	14.76
16	60	90	400	25	12.91 ± 0.88	12.93
17	50	90	400	20	14.23 ± 1.32	14.36
18	50	90	300	25	12.92 ± 0.95	12.99
19	60	120	400	30	13.84 ± 1.25	14.01
20	50	150	300	25	13.25 ± 0.83	13.40
21	40	150	400	25	13.93 ± 1.25	13.82
22	40	120	400	30	14.40 ± 1.14	14.64
23	50	120	500	30	14.61 ± 1.11	14.35
24	40	120	500	25	13.58 ± 0.83	13.77
25	50	120	400	25	14.93 ± 0.88	14.97
26	40	120	300	25	13.40 ± 0.97	13.34
27	60	120	500	25	13.38 ± 1.20	12.99

Table 4
Central Composite Rotatable Design with the observed responses and predicted values for yield of total phenolic compounds (TPC) referred to dry weight (dw) of *C. limon* peels using ultrasound assisted extraction. GAE, gallic acid equivalents.

Run	*X*1 – time	*X*2 – amplitude	*X*3 – solvent	TPC yield (mg_{GAE}/g_{dw})	
				Experimental	Predicted
1	5	30	30	12.92 ± 1.20	13.21
2	15	30	30	11.09 ± 0.32	11.27
3	5	70	30	14.15 ± 0.53	14.18
4	15	70	30	12.49 ± 0.45	12.71
5	5	30	70	13.01 ± 0.15	12.72
6	15	30	70	13.69 ± 1.02	13.59
7	5	70	70	14.38 ± 0.36	14.13
8	15	70	70	15.82 ± 0.96	15.47
9	10	50	50	15.01 ± 0.89	14.91
10	10	50	50	14.51 ± 0.16	14.91
11	10	50	50	15.01 ± 1.60	14.91
12	10	50	50	14.91 ± 0.99	15.01
13	10	50	50	15.92 ± 1.23	14.57
14	17.35	50	50	14.58 ± 0.52	13.57
15	2.65	50	50	14.91 ± 0.86	15.67
16	10	79.4	50	15.48 ± 0.36	12.28
17	10	20.6	50	13.67 ± 0.45	13.95
18	10	50	79.4	13.32 ± 0.12	13.26
19	10	50	20.6	12.82 ± 1.22	13.25

continuously and kept at room temperature by circulating external cold water bath. After the extraction, the extract was recovered and analyzed as reported for MAE in Section 2.3.1.

2.3.3. Conventional solvent extraction

For the conventional extraction, one grams of sample powder were placed in a conical flask, and 50 mL of 50% (v/v) ethanol were added. The mixture was kept in a thermostatic water bath (mod. WNB22 Memmert, Germany) with shaking speed of 110 strokes per minute, at 60 °C for 2 h, according to the method recommended by Spigno et al. (2007). The extract was then recovered and analyzed as reported for MAE in Section 2.3.1.

2.4. Analytical determinations

2.4.1. Total phenolic content (TPC)

Total phenolic content of the extracts was determined according to the Folin–Ciocalteu assay (Jaramillo-Flores et al., 2003). Briefly, 100 μL of the extract (opportunely diluted in distilled water) was mixed with 750 μL of 10-fold diluted Folin–Ciocalteau reagent. The solution was mixed and incubated at room temperature for 5 min. After 5 min, 750 μL of 7.5% sodium carbonate (Na_2CO_3) were added. After incubation at 25 °C for 90 min, the absorbance of the sample was measured at 725 nm against a blank (made as reported for the sample but with 100 μL of distilled water) by using a UV–vis Spectrophotometer (SpectroScan 50, UK).

Gallic acid hydrate was used as standard for the calibration curve to express the TPC concentration of the sample as mg/L of gallic acid equivalents (GAE). TPC yield was then calculated based on the sample concentration, extract volume and peel powder dry weight, according to the following equation (Eq. (3)):

$$\text{TPC Yield} = \frac{mg_{GAE}/L \cdot L_{extract}}{g_{peel\ powder}} \quad (3)$$

2.4.2. Antioxidant activity by DPPH assay

The electron donation ability of the extracts (CSE samples and MAE and UAE samples obtained under optimized condition) was measured by bleaching of the purple-colored solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Dudonné et al. (2009).

DPPH radicals have an absorption maximum at 515 nm, which disappears with reduction by an antioxidant compound. A DPPH• solution in absolute methanol (60 μM) was prepared, and 3 mL of this solution were mixed with 0.1 mL of peels extract diluted in distilled water (dilutions were based on the TPC in order to have 100, 200, 300, 400 and 500 μg/mL). The samples were incubated for 20 min at 37 °C in a water bath and, then, the decrease in absorbance at 515 nm was measured.

The antioxidant capacity was expressed as percentage of inhibition of DPPH radical (%DPPH inhibition) calculated according to the following equation (Eq. (4)):

$$\%\text{DPPH inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \quad (4)$$

where $Abs_{control}$ is the absorbance of DPPH radical + distilled water; Abs_{sample} is the absorbance of DPPH radical + sample extract at 20 min. From data elaboration (% inhibition plotted versus total phenols concentration), the concentration of TPC required to reach 50% radical inhibition (IC_{50}) was calculated.

2.4.3. Antioxidant activity as iron reducing power

The reducing power of the extracts (CSE samples and MAE and UAE samples obtained under optimized condition) was evaluated as the capacity of reducing Fe^{3+} to Fe^{2+} according to the method of Shon (2003). To determine the reducing power, four concentrations of peel extract (50, 100, 150 and 200 μg GAE/mL obtained diluting with distilled water) were added separately to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After this time, the reaction was terminated by adding 2.5 mL of 10% trichloroacetic acid and the mixture was centrifuged at 650 × *g* for 10 min. The supernatant was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride ($FeCl_3$), and then absorbance was measured at 700 nm. High absorbance value shows high reducing activity (Pan et al., 2010).

2.4.4. Scanning electron microscopy (SEM) analysis

The powder before and after extraction was observed under SEM (Quanta 200, FEI company) for morphological characterization (Lou et al., 2010). Four samples of powders (untreated and residues after CSE and MAE and UAE carried out under optimized conditions)

were collected and dried until constant mass in oven at 60 °C before SEM analysis.

Sample particles were fixed on the specific carbon film support, and their shape and surface characters were observed by using GSED detector with environmental mode (ESEM).

2.5. Statistical analysis

Each extraction trial and all the analyses were carried out in triplicate and all the data in this paper have been reported as means ± S.D. Influence of each factor on the TPC yield in the single-factor experiment for both the MAE and UAE was statistically assessed by ANOVA and Tukey's post hoc test with 95% confidence level.

Data obtained from the BBD and CCRD trials for the MAE and UAE, respectively, were statistically analyzed using ANOVA for the response variable in order to test the model significance and suitability. $P < 0.05$ and $P < 0.01$ were taken as significant and highly significant level, respectively.

The JMP (Version 7.0, SAS) and Design-Expert (Trial version 8.0.7.1) software were used to construct the BBD and CCRD and to analyze all the results.

3. Results and discussion

3.1. Microwave assisted extraction

3.1.1. Single-factor experiments

Table 1 shows the results of the single-factor experiments carried out for preliminary optimization of MAE.

At the beginning of this study, an effect of solvent type was investigated. The investigated solvents are the most commonly employed solvents in phenolic extraction from botanical materials (Inglett et al., 2010; Spigno et al., 2007). Type of solvent significantly influenced the TPC yield, with aqueous ethanol being the best one and water, acetone and aqueous methanol giving comparable results which may be attributed to the difference in dielectric properties of the solvent. Water has the highest dielectric constant ($\varepsilon' = 80.4$) and the lowest dissipation factor ($\tan = 1570$), hence, the rate at which water absorbs microwave energy is higher than the rate at which the system can dissipate the heat, these phenomena account for the "superheating" effect which occurs when water is used (Proestos and Komaitis, 2008). Furthermore, since the Folin assay used to quantify TPC actually measures the reducing power of the compounds, the Folin results are influenced by the oxidative status of the sample, therefore lower values may suggest that intense heating has caused degradation of the bioactive compounds. However, mixtures of high and low microwave absorbing solvents can be exploited to produce optimum results. For the tested aqueous solvents, the ethanol/water system has the highest boiling point. This allowed carrying out extraction at higher temperatures resulting in improved extraction efficiencies due to increased mass transfer coefficients of TPC and reduced surface tension and solvent viscosity which improve sample wetting and matrix penetration. This is in agreement with results reported by Pan et al. (2003). Aqueous ethanol was then selected as the solvent for the RSM trials and for the next single-factor trials.

In agreement with other literature results (Spigno et al., 2007; Pan et al., 2003), TPC yield increased with increasing ethanol concentration up to 50% (v/v) and then decreased for higher concentrations. The maximum TPC yield was obtained with ethanol 50% (even though statistically equal to ethanol 40%) which was fixed for the next single-factor experiments, while, based on the statistical analysis reported in Table 1, the concentration range 40–60% was selected for the RSM trials.

Also irradiation time significantly influenced the TPC yield, with 120–150 s as the best values, with apparent thermal degradation of phenolic compounds at longer irradiation times (Proestos and Komaitis, 2008; Proestos et al., 2006). Based on these results, 120 s was selected for the next single-factor trials, while the range 90–150 s was selected for the RSM trials.

Microwave power significantly influenced the TPC yield in the tested conditions (Table 1). However, the yield increased with increasing microwave power from 300 to 400 W and then it remained constant or slightly decreased for higher powers (average values at 500 and 600 W were statistically not different from both values at 300 and 400 W). It must be underlined that the operating temperature could not be regulated in the used equipment and that for a constant sample size temperature increases with microwave power (Spigno and De Faveri, 2009). Since it has been reported that the main effect of microwaves, and in many cases the only, is the heating effect (Kappe et al., 2013), the obtained results were probably due to an enhancement of phenols recovery due to a heating effect, with consequent increase of mass transfer phenomena, up to a certain microwave power value and, then, to thermal degradation of bioactive compounds at higher powers.

The range 300–500 W was selected for the RSM study, while the 400 W power was used for the last single-factor trials.

The solvent-to-solid ratio significantly influenced the TPC yield, showing the same trend as reported by Spigno and De Faveri (2009). In fact, in the present study, the ratio was increased keeping constant the solid weight, therefore, the positive effect of an increased volume due to a larger concentration gradient to drive the mass transfer, is, at a certain point, compensated by a lower heating due to reduced microwave penetration in the sample. Furthermore, the solvent volume must be sufficient to ensure that the entire sample is immersed, especially when having a sample that will swell during the extraction process (Eskilsson and Björklund, 2000). Based on statistical analysis, the range 20–30 mL/g was selected for the RSM optimization.

3.1.2. Optimization by RSM

The TPC yields obtained in the trials of the BBD are reported in Table 3, while Table 5 reports the statistical analysis of the regression model (Eq. (1)). Neglecting the non-significant terms ($P > 0.01$) the following predictive equation was obtained:

$$Y = 14.91 - 0.35X1 - 0.23X3 + 0.13X4 + 0.23X1X2 + 0.22X3X4 - 0.72X1^2 - 0.6X2^2 - 0.97X3^2 \quad (5)$$

It can be seen that irradiation time ($X2$) did not influence the extraction yield but its interaction with ethanol did it. The interaction power and solvent-to-solid ratio was highly significant such as the quadratic terms for ethanol, time and microwave power.

Eq. (5) was used to obtain the response surfaces for all the possible variables interactions (Fig. 1), where maximal TPC yield could be obtained at optimal-values as combination of two variables, with the third and fourth variables kept constant at zero level. Consequently, the quadratic experimental model had a stationary point, and the predictive TPC yield was the maximal value in the stationary point (Pan et al., 2012). Confirming the results of the single-factor trials, the TPC yield reached a maximum level when irradiation time, ethanol concentration and extraction power were set at medium levels (0 coded values), while the solvent-to-solid ratio showed a very limited influence which was apparently in contrast with single-factor experiments. However, in the 20–30 mL/g solvent-to-solid ratio range selected for the RSM optimization, the single-factor results had shown a significant increase in TPC yield from the 20 to 25 ratio and, then, a significant reduction from the 25 to 30 ratio back to values statistically not different from that

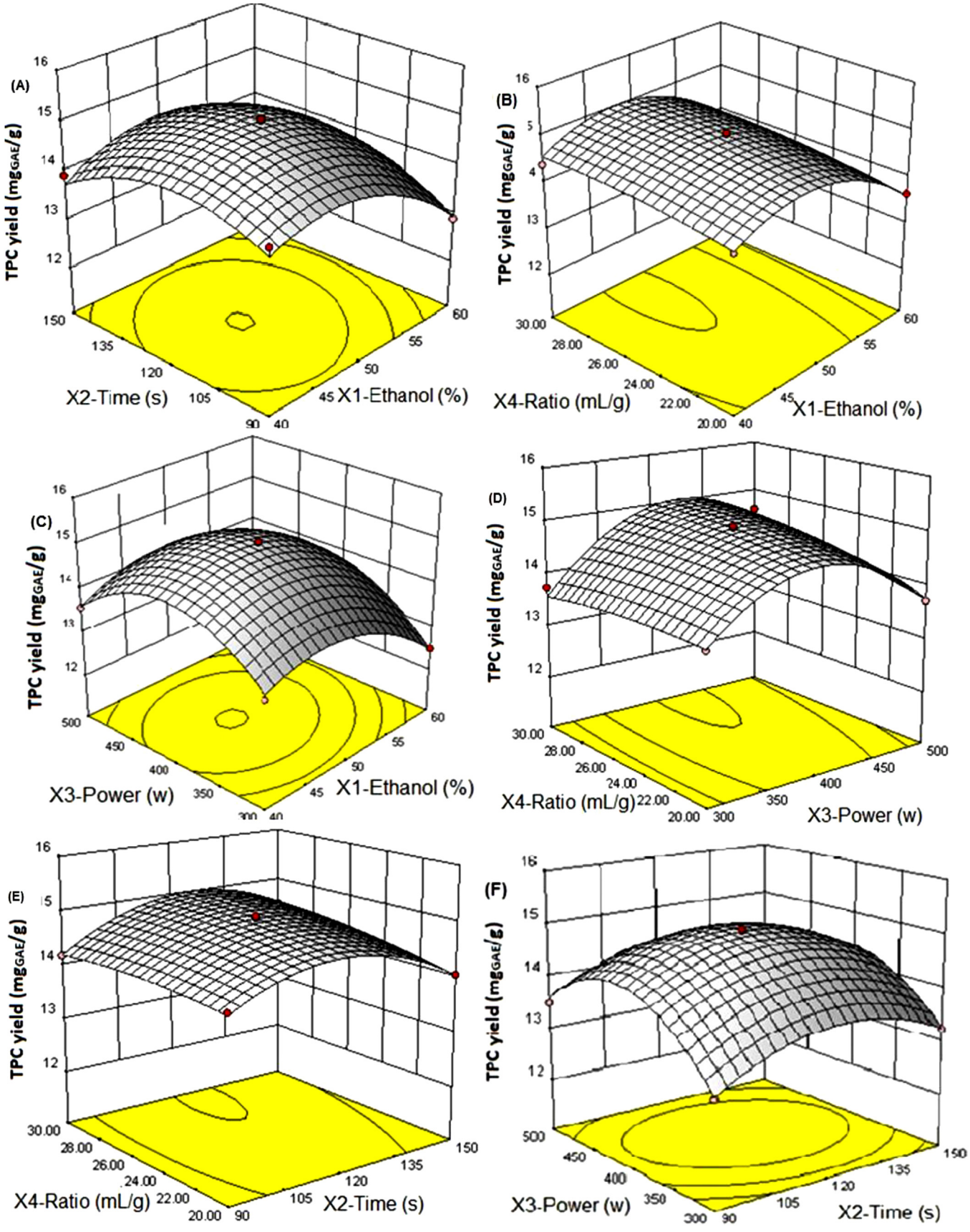

Fig. 1. Response surface analysis for the total phenolic yield from *C. limon* peels with microwave assisted extraction with respect to irradiation time and ethanol percentage (A); solvent-to-solid ratio and ethanol percentage (B); microwave power and ethanol percentage (C); solvent-to-solid ratio and microwave power (D); solvent-to-solid ratio and irradiation time (E); microwave power and irradiation time (F).

Table 5
Analysis of mean square deviation of the quadratic model terms (Eq. (1)) applied to the experimental values of total phenolic yields obtained with microwave assisted extraction. df, degrees of freedom.

Source	Sum of squares	df	*F*-value	*P*-value Prob >*F*	
Model	0.0550	14	39.7721	<0.0001	Significant
*X*1 – ethanol	0.0079	1	79.8241	<0.0001	
*X*2 – time	0.0001	1	2.0073	0.1820	
*X*3 – power	0.0034	1	34.0340	<0.0001	
*X*4 – ratio	0.0010	1	10.6458	0.0068	
*X*1*X*2	0.0012	1	12.4428	0.0042	
*X*1*X*3	0.0008	1	7.7829	0.0164	
*X*1*X*4	2.0028E−05	1	0.2025	0.6607	
*X*2*X*3	0.0002	1	2.1162	0.1714	
*X*2*X*4	0.0003	1	3.1018	0.1036	
*X*3*X*4	0.0009	1	9.5278	0.0094	
$X1^2$	0.0153	1	154.6386	<0.0001	
$X2^2$	0.0104	1	105.3051	<0.0001	
$X3^2$	0.0271	1	274.5798	<0.0001	
$X4^2$	0.0001	1	1.3568	0.2667	
Residual	0.0012	12			
Lack of fit	0.0012	10	20.2406	0.0580	Not significant
Pure error	1.16E−05	2			
Cor total	0.0550	26			

obtained with the 20 ratio, which can explain the limited influence observed in the RSM study. Optimal conditions resulted in irradiation time 120 s, extraction power 400 W, solvent-to-solid ratio 28:1 and 48% ethanol concentration with a predicted TPC yield of 15.74 mg GAE/g. MAE was carried out at these optimal conditions obtaining a TPC yield of 15.78 ± 0.80 mg GAE/g, very close to the value predicted by the model.

Results are in agreement with other literature works which underlined the influence of ethanol concentration, microwave power and irradiation time on the extraction of phenolic compounds from vegetable tissues (Song et al., 2011; Sutivisedsak et al., 2010).

3.2. Ultrasound assisted extraction

3.2.1. Single-factor experiments

Table 2 shows the results of the single-factor experiments carried out for preliminary optimization of UAE.

The effect of ethanol concentration was significant with a maximum TPC yield at 50% ethanol/water. This is in agreement with Wang et al. (2008) and with the results obtained with MAE.

The ethanol concentration range of 30–70% was selected for the RSM trials, while the 50% for the next single-factor trials.

With regard to extraction time, the TPC yield increased significantly up to 10–15 min. Results indicated that extraction of active compounds from the peels extract were accomplished after 10 min. Overmuch sonication time would increase energy supply which is needed to release the target compounds but can also accelerate degradation of phenolic compounds (Carrera et al., 2012). Similar observations were reported by Hossain et al. (2012) for the optimization of UAE of antioxidant compounds from marjoram (*Origanum majorana*). Sonication time range of 5–15 min was selected for the RSM trials, while time of 10 min was used for the next single-factor trials.

As it concerns the radiation amplitude, TPC yield was constant at 20 and 40% amplitude and, then, increased and remained constant for amplitude between 60 and 100%, as also reported by Hossain et al. (2012). Increasing radiation amplitude may favor the disruption of the plant cell wall due to cavitation phenomena, thereby increasing the contact surface area between solid and liquid phase, enhancing solvent penetration into the plant material and facilitating the release of solutes (Jerman et al., 2010; Ma et al., 2009). However, all reactions, including the formation of free radicals which can be scavenged by phenolic compounds (Jerman et al., 2010), are promoted when high amplitudes are used (Stanisavljević et al., 2009).

In this study, operating temperature during UAE was kept constant at room temperature; therefore any heating effect was excluded.

A 30–70% amplitude range was selected for the RSM trials, while the 60% was used for the last single-factor trials.

Finally, the TPC yield augmented significantly with the increase of liquid/solid ratio up to 40 mL/g which was, then, fixed for the RSM trials.

3.2.2. Optimization by RSM

The TPC yields obtained in the trials of the CCRD are reported in Table 4.

Regression coefficients and analysis of the regression model are summarized in Table 6 with indication that the model had adequately represented the real relationship between the chosen parameters (Liyanapathirana and Shahidi, 2005).

Neglecting the non-significant terms ($P > 0.01$), the final predictive equation for UAE was (Eq. (6)):

$$Y(\text{TPC}) = 14.75 - 0.71X2 + 0.57X3 + 0.7X1X3 - 1.02X3^2 \quad (6)$$

To investigate the interactive effects of operational parameters and determine optimal levels of the variables, three-dimensional surface plots were constructed according to Eq. (6) as for MAE (Fig. 2). The plots show clearly the great effect of ethanol concentration on TPC yield, as also observed by other authors who investigated and optimized by RSM the UAE of phenolic antioxidants from different plant materials (Morelli and Prado, 2012; Rodrigues et al., 2008).

Summarizing, ethanol concentration and extraction amplitude were two crucial factors in UAE, with significant effect of their corresponding linear terms and also of the quadratic term for ethanol concentration. Time did not influence the TPC yield as linear term, but it did as interactive factor with ethanol concentration. This was apparently in contrast with the single-factor experiments, according to which time was an influent factor. However, in the 5–15 min time range selected for the RSM optimization, the single-factor results had shown a significant increase in TPC yield only from 5 to 10 min, which can explain the limited influence observed in the RSM study.

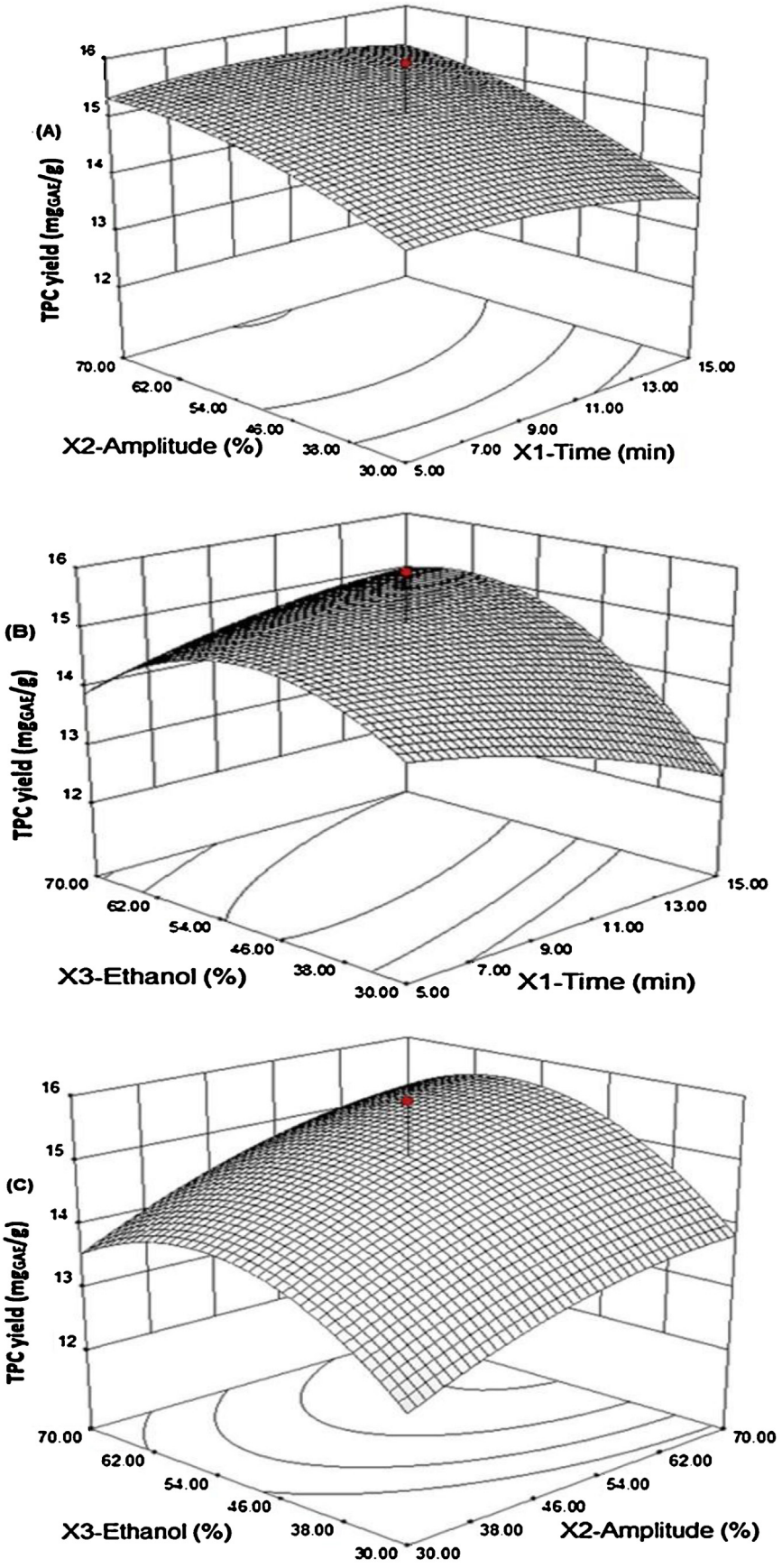

Fig. 2. Response surface analysis for the total phenolic yield from *C. limon* peels with ultrasound assisted extraction with respect to ultrasound amplitude and extraction time (A); ethanol percentage and extraction time (B); ethanol percentage and ultrasound amplitude (C).

Table 6
Analysis of mean square deviation of the quadratic model terms (Eq. (1)) applied to the experimental values of total phenolic yields obtained with ultrasound assisted extraction. df, degrees of freedom.

Source	Sum of squares	df	*F*-value	*P*-value Prob > *F*	
Model	24.7117	9	11.3956	0.0011	Significant
*X*1 – time	0.2792	1	1.1589	0.3131	
*X*2 – amplitude	6.2710	1	26.0264	0.0009	
*X*3 – ethanol	3.9580	1	16.4271	0.0037	
*X*1*X*2	0.1081	1	0.4486	0.5218	
*X*1*X*3	3.9340	1	16.4271	0.0037	
*X*2*X*3	0.0946	1	0.3926	0.5484	
$X1^2$	0.4289	1	1.7804	0.2188	
$X2^2$	0.7978	1	3.3114	0.1063	
$X3^2$	9.0160	1	37.4189	0.0003	
Residual	1.9275	8			
Lack of fit	1.2508	5	1.1090	0.4978	Not significant
Pure error	0.6767	3			
Cor total	28.4531	18			

From the model the optimal conditions of UAE were: 63.93% ethanol concentration, 15.05 min at 77.79% amplitude radiation with a predicted yield of 15.08 mg GAE/g. UAE was carried out under these conditions giving a real recovery of 15.22 ± 0.88 mg GAE/g (not statistically different from the MAE yield).

3.3. Comparison between MAE, UAE and CSE

The two alternative extraction technologies, MAE and UAE, were compared with each other and with CSE considering the TPC yield along with the antioxidant activity of the extracts. Selection of an extraction method would mainly depend on the advantages and disadvantages of the processes such as extraction yield, complexity, production cost, environmental friendliness and safety (Li et al., 2010).

The CSE gave a TPC yield of 15.03 ± 0.12 mg GAE/g_{dw} which was not statistically different than MAE and UAE yields. MAE should be preferred based on the lower solvent consumption and extraction time (Spigno and De Faveri, 2009; Li et al., 2011; Upadhyay et al., 2012). However, in this study, operating temperature in the UAE was kept constant at room temperature, excluding any heating effect wich might positevely or negatively influece the phenols recovery depending on the applied amplitude. Furthermore, a punctual energy cost analysis should be required to compare the two techniques from the energy consumption point of view.

From the point of view of the quality of the extracts, the DPPH test showed again MAE as the best technology (Fig. 3) due to the significantly lower IC_{50} (203.59 ± 5.59 μg GAE/mL) than UAE (268.24 ± 10.62 μg GAE/mL) which was, on its turn, significantly lower than CSE (298.82 ± 8.60 μg GAE/mL).

Also the FRAP tests showed a higher activity for the MAE extract (Fig. 4).

The lower activity of UAE and CSE extract could be resulted from extended extraction time, hence exposure to unfavorable conditions such as light and oxygen. Moreover, it is commonly known that ultrasonication could induce free radicals formation within the liquid medium, thus causing oxidation and degradation of the active compounds (Hayat et al., 2009).

SEM observation of the residue after MAE, UAE and CSE (Fig. 5) showed that, in comparison with the untreated sample, solvent extraction produced cell changes in all samples, although the extent of damage differed (Aspé and Fernández, 2011).

It is well known that ultrasounds disrupt plant cells via cavitation phenomena (Kong et al., 2010), but UAE did not seriously affect the sample in the present study if compared to CSE. This suggests that the dried *C. limon* peels particles are highly resistant to ultrasound energy (Aspé and Fernández, 2011). On the opposite, microwave heating caused a higher cellular damage helping the rapid release of solutes into the solvents and enhancing the well-known main heating effect of microwaves.

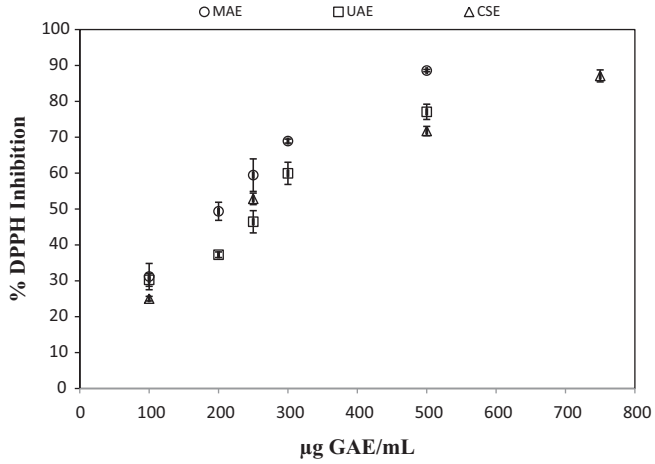

Fig. 3. DPPH activity (as % DPPH inhibition) as a function of total phenol concentration (as gallic acid equivalents (GAE)) of microwave assisted (MAE) and ultrasound assisted (UAE) extracts (obtained under RSM optimized conditions) compared to conventional solvent CSE extract. Error bars indicate ±S.D.

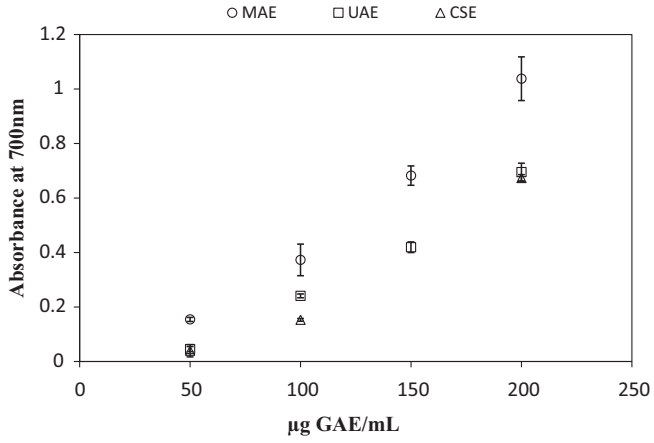

Fig. 4. Iron reducing power (absorbance at 700 nm) as a function of total phenol concentration (as gallic acid equivalents (GAE)) of microwave assisted (MAE) and ultrasound assisted (UAE) extracts (obtained under RSM optimized conditions) compared to conventional solvent (CSE) extract. Error bars indicate ±S.D.

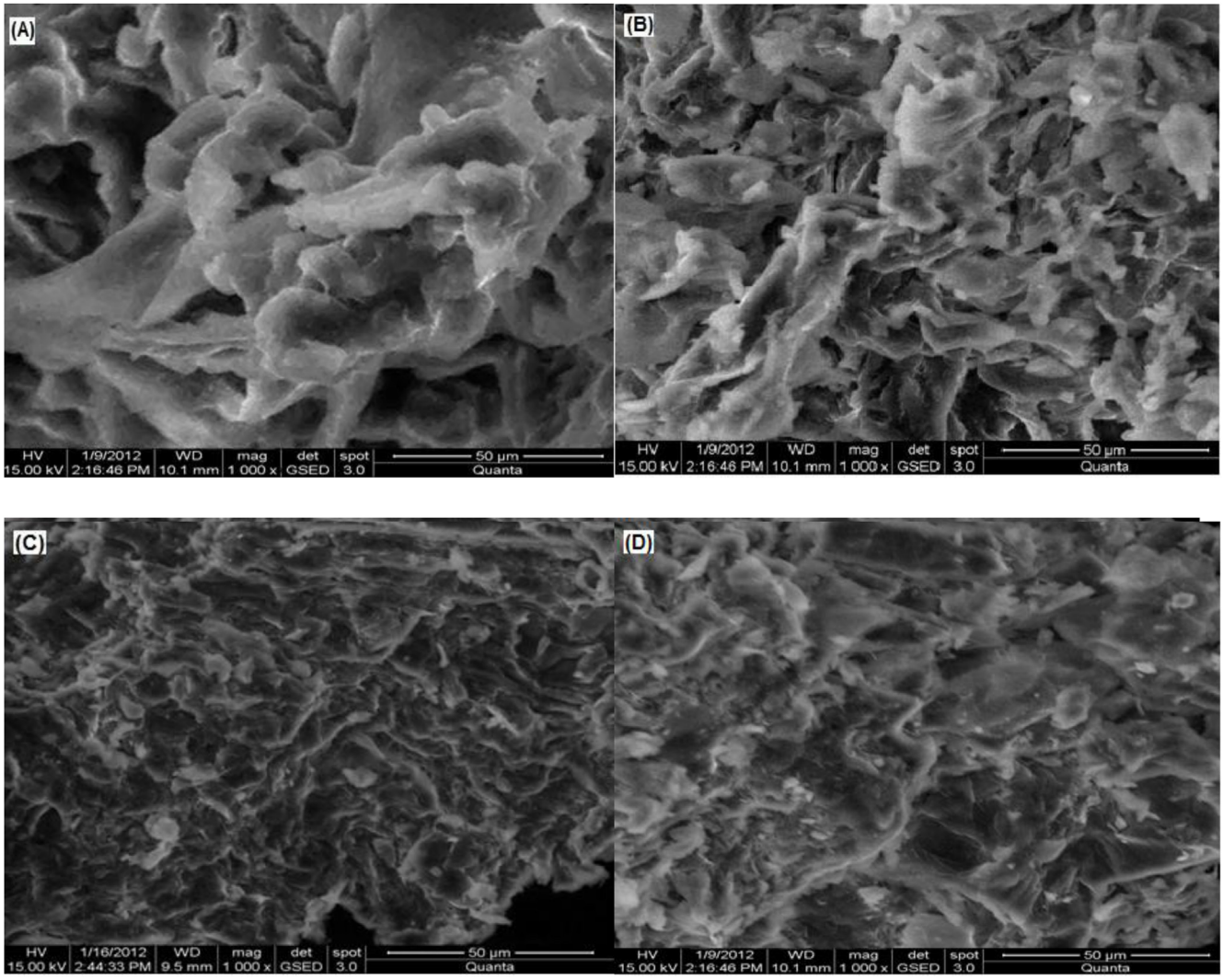

Fig. 5. Scanning electron microscope images of *C. limon* peels powder before (A) and after extraction by conventional solvent extraction (B), microwave assisted extraction (C) and ultrasound assisted extraction (D).

4. Conclusions

The response surface methodology was successfully employed to optimize total phenolic yield from dried *C. limon* peels, according to different non-conventional solvent extraction processes (MAE and UAE). The applied second-order polynomial model gave a satisfactory description of the experimental data showing that the TPC yield was most affected by ethanol concentration, extraction power and liquid–solid ratio for MAE, and by sonication amplitude and ethanol concentration for UAE. The proposed MAE method appeared to be better than both UAE and CSE allowing for higher recovery yield and specific antioxidant activity with a shorter working time and a lower solvent consumption. SEM observation suggested that these results were due to more intense tissue degradation under microwave irradiation.

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