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PRICKLY PEAR (*OPUNTIA FICUS-INDICA*) SEEDS AS A SOURCE OF PHENOLIC COMPOUNDS: MICROWAVE-ASSISTED EXTRACTION OPTIMIZATION AND EFFECT ON FOOD LIPID OXIDATIONS

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This study was carried out to optimize Microwave-assisted extraction (MAE) process and assess the possibility of using *Opuntia ficus-indica* (*OFI*) seeds as natural antioxidant additives in food. Box–Behnken design was used to optimize total phenolic contents (TPC) and antioxidant activity DPPH• (AA) extraction conditions. The models were verified and validated and the interactions between the factors were studied. Under the optimal conditions, corresponding experimental values for TPC and AA were 5.22± 0.12 mg GAE/g DW and 2.30±0.27 mg GAE/g DW, respectively. Seven and eight compounds were identified in TPC and AA under optimal conditions, respectively, using High-Performance Liquid Chromatography (HPLC) coupled to Mass Spectrometry (MS) analysis. Ferulic acid glucoside was the most concentrated compounds in the *OFI* seed extract. Rancimat test showed that *OFI* seed extract gives the highest oxidative stability with 150 ppm. The presented data could be a reliable guideline for establishing full-scale, cost-effective and resource-effective food industry process.

Keywords: *Opuntia ficus-indica* seeds, antioxidant, microwave, Box-Behnken design, lipid oxidation control.

Introduction

Opuntia ficus-indica, commonly known as prickly pear, is widely distributed in the Mediterranean area, Mexico, and South Africa, and belongs to the Cactaceae family. *Opuntia ficus-indica* is a cactus well adapted to arid and semiarid conditions (Jorge *et al*., 2013). It is a crop plant that has been domesticated for a long time, which have an importance in agricultural economics (Bensadón *et al*., 2010).

The prickly pears can be consumed fresh or after desiccation under the sun. Their stems (cladodes) and fruits have a high nutritional value (Bensadón *et al*., 2010).

Therefore for a long time, people have used prickly pear for their medicinal benefits (El Kossori *et al*., 1998). The current research reveals that consumption of seeds, fruits and vegetables has a positive correlation with prevention of some diseases like atherosclerosis, cancer, diabetes, arthritis and also aging (Kaur and Kapoor, 2001). The nutritional and health benefits of cactus fruit are associated with their antioxidant properties (Yahia and Mondragon-Jacobo, 2011). A number of studies such as Chaalal *et al*. (2013) show that prickly pear seeds are a good source of phenolic compounds.

In order to study these molecules, the first step of processing is "extraction", which involves separation of phytochemicals from the cellular matrix. The "ideal" extraction method must provide high extraction rates and should be time saving and non-destructive. There are various methods for extracting phenolic compounds such as leaching-out extraction (Zhang *et al*., 2007) but, the time required and the concentration found are not enough. Microwave-assisted extraction (MAE) has received a great attention as a potential alternative to conventional extraction methods, mainly due to considerable savings in processing time, solvent consumption, and energy (Hayat *et al*., 2009).

The different structure of phenolic compounds can be affected by various extraction parameters such as extraction time, temperature, solvent composition, solid/ liquid ratio, and their interactions (Ilaiyaraja *et al*., 2015). Therefore, optimization of the extraction protocol is required. Response surface methodology (RSM) is a statistical method that uses quantitative data from an appropriate experimental design to determine or simultaneously solve a multivariate equation. RSM such as Box–Behnken design (BBD) can then generate a mathematical model and take into account the possible interrelationships among the test variables while minimizing the number of experiments (Spigno and De Faveri, 2009).

The margarine industry has several problems, the oxidation of the lipids is one of the major issue because oxidation is responsible for reducing the nutritional and sensory quality of food (Prior, 2003). A number of synthetic antioxidants, such as butylhydroxytoluene (BHT), butylhydroxyanisole (BHA) butylhydroxyquinone (BHQT) have been added to foodstuffs. However, the toxicity concerns regarding these antioxidants make their use questionable. There are reports showing that BHA and BHT are tumor promoters (Kahl and Kappus, 1993). The attention has therefore been directed towards natural antioxidants from botanical sources, thereby the use of cactus pear extracts as a source of antioxidants could be a promising alternative for the food industry (Sumaya-Martínez *et al*., 2011). The overall goal of this investigation was to study the use of prickly pear seed as a natural antioxidant additive in margarine. To reach this goal, the first step was to use the Box-Behnken design (BBD) to extract with MAE a maximum of antioxidants in seeds from *Opuntia ficus-indica*, and the second steps was to identify and quantify the phenolic compounds. Finally, the optimal extract was incorporated into margarine as food lipids sensitive to fatty acid oxidations, where the rancimat test was carried out to assess the oxidative stability of margarines.

Materials and methods

Plant material

The mature prickly pear (*Opuntia ficus-indica*, *OFI*) were harvested in Seddouk, from Bejaia region (Algeria). Fruits were washed carefully and peeled with a knife. The seeds were removed from the pulp, washed with distilled water, dried in the ventilated oven (Binder, Germany) at 40°C during 24 h, then grounded, and sieved (diameter of the sieve gate was 250 µm). The lipids were removed from the seeds powder using Soxhlet method (behr, Germany). The extraction took place for 4 hours using hexane as extraction solvent with a ratio of 20 g powder: 150 mL hexane, where the oil percentage yield was 8%. Then the *OFI* seed powder was dried again in the same condition and stored at 4 °C in airtight bags until further use.

Chemical reagents

Folin Ciocalteu reagent was purchased from Biochem, Chemopharma (Montreal, Quebec), sodium carbonate was from Biochem, Chemopharma (Georgia, USA), gallic acid was from Biochem-Chemopharma (UK), acetone, ethanol and methanol were from Prolabo (CE); all other chemicals were from Sigma Chemical (Sigma–Aldrich GmbH, Germany).

Microwave-assisted extraction (MAE)

The phenolic compounds extraction was carried out using a modified domestic microwave oven (Maxi power, China). The apparatus was equipped with a digital control system for irradiation time and microwave power. Refrigerating system was added to microwave to condense the solvent vapors in order to keep the volume of the sample constant during the extraction process. The phenolic compounds were extracted according to Mandal and Mandal (2010). One gram of *OFI* seed powder was mixed with 25 mL of ethanol. After a pre-leaching time of 5 min, the suspension was irradiated at different experimental conditions (Table 1). Then, 1 min was taken to cool suspension. The extract was centrifuged at 4500 rpm for 10 min (Nüve, Turkey) and filtered. Each experiment was carried out in triplicate.

Determination of total phenolic content (TPC)

The phenolic compound content of the extracts was determined according to Velioglu *et al*. (1998). *OFI* seed extracts (0.5 mL) was mixed with 1.5 mL Folin-Ciocalteu reagent, diluted ten times. After 5 min, 1.5 mL of Na_2CO_3 solution (6%) was added. The mixture was incubated in darkness for 1 hour and then the absorbance was measured at 750 nm against ethanol blank (Uvline9400, Secomam, Alès, France). Gallic acid was used as a standard for the calibration curve. The results were expressed as milligram gallic acid equivalents (GAE) per gram dry weight (DW).

Determination of antioxidant activity (AA, DPPH radical scavenging assay)

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the *OFI* seed extracts was measured according to the method of Molyneux (2004). The ethanolic test solution (200 µL) was added to 1 mL of methanolic DPPH solution

(60 µM). The decolorizing of purple color was recorded at 515 nm after 30 min. The antioxidant capacity was expressed as milligram gallic acid equivalents (GAE) per gram dry weight (DW).

Experimental Design and Statistical Analyses

The influence of the process parameters, i.e., X_1, X_2, and X_3 were investigated using surface methodology (RSM). The JMP software (Version 10, SAS) package was used to establish the mathematical models and to obtain the optimal conditions for the TPC extraction and the AA. In the present study, three-factorial at three-levels, Box–Behnken experimental design (BBD) were applied in order to investigate and validate the extraction process parameters affecting the extraction of phenolic compounds from *OFI* seeds.

The three factors chosen for the current study were designated as X_1 for ethanol concentration (40-80% V/V), X_2 for extraction time (40-140 s) and X_3 for microwave power (400-1000 W).

The experiments were performed according to the design of experiments shown in Table 1. The output results were fitted to a second-order polynomial equation, according to the model in Eq. (1).

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

Where Y represents the response function (TPC and AA); B_0, B_i, B_{ii}, and B_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j represent the actual independent variables. In order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions, the three-dimensional response surface plots of microwave-assisted extraction (MAE) were generated in Figure 1.

Analysis of variance (ANOVA) was performed for response variable using the full models where *p*-values (partitioned into linear and interaction factors) indicated whether the terms were significant or not; *p*-values< 0.05 were regarded as significant. Fischer's test was used to determine the type of the model equation. The coefficient of determination, R^2, and adjusted R^2 were also calculated (Table 2).

High-performance liquid chromatography-mass spectrometry (HPLC–ESI-MS) analysis

The *OFI* seed extracts obtained on the optimal conditions undergone a solvent evaporation step using a rotavapor (BÜCHI, Switzerland), lyophilization (Freeze Drier Alpha 1–4 LD; Christ, Osterode am Harz, Germany), and then the identification of the phenolic compounds was obtained by using ultrahigh-performance liquid chromatography–mass spectrometry with electrospray ionization. The equipment was Xevo G2 mass spectrometer consisting of a hexapole, a collision cell and a time of flight analyzer (QTOF) supplied by Waters (Milford, MA, USA). The electrospray probe was used in positive (ESI+) and negative (ESI−) modes as well as in sensitivity analyzer mode. The mass range considered was from 10 to 1,000 Da. The corona voltage was 2.5 kV for (ESI+)

and 0.5 kV for (ESI−). The sampling cone voltage was optimized between 20 and 50 V. Finally, 30 V was selected for the screening because more peaks were detected. Other MS parameters were as follows: the source temperature was 150 °C, the desolvation gas temperature 450 °C and the desolvation gas flow 650 Lh−1. MSE mode was selected for the acquisition, and collision ramp energy from 5 to 40 V was used. MassLynx v.4.1 software (Waters, Milford MA, USA) was used to analyse the samples and CromaLynx (Waters, Milford MA, USA) was used to deconvolve the spectra. Figure 2 shows the phenolic profile from *OFI* seeds in the optimum extract.

Margarines manufacture

Four margarines were manually produced at laboratory scale; these margarines are composed mainly of two phases: fatty phase (82%): palm oil, sunflower oil and equivalent hydrogenated soybean, emulsifier (lecithin) and β-carotenes (12 mg/kg); and aqueous phase (18%): milk, aroma (diacetyl: 25 ppm), salt (0.60%), lactic acid (0.5 mL/kg) and potassium sorbate (300 mg/kg).

The difference between these margarines was in the antioxidants composition, where the first margarine contained 100 ppm of vitamin E, the second, third and fourth contained 50 ppm, 100 and 150 ppm of lyophilized *OFI* seed extracts with optimum antioxidant activity. The weight of margarine tubs was 250 g. The final products were stored at 4 °C for 24 hours.

Determination of physicochemical properties of the developed margarines

In order to make sure of the final quality of the margarines and compare their oxidative stability, the following analyses were carried out: moisture content (ISO International Standard, 1998a); peroxide value determination (ISO International Standard, 1998b); determination of the oxidative stability of margarines (rancimat test) (ISO International Standard, 2006). The pH of the margarines was measured directly on the aqueous phase.

Results and discussion

Optimization of MAE

As shown in Table 1, the total phenolic compound content of prickly pear seeds varied from 3.96 to 5.54 mg GAE/g DW and the antioxidant activity ranged from 1.60 to 2.15 mg GAE/g DW. These results confirm the influence of the optimized parameters (ethanol concentration, irradiation time and microwave power). The same trend was reported by Dahmoune *et al*. (2013). Table 2 shows that the F values of both TPC and AA models were high, suggesting that the variations of response measured were due to the effects of the factors. This was confirmed by *p* values<0.05 ($p<0.0001$* and 0.0028*, for TPC and AA, respectively). Hence, both models were significant. Moreover, the lack of fit ($p>0.05$) was not significant for the two models indicating the good predictability of the responses by the models. The fit of the models was also checked by calculating the determination coefficients (R^2 and $R^2_{adjusted}$), i.e. R^2 was an indicator of the quality of the models. In the present study, the determination coefficients (R^2) of the models were equal

to 0.99 and 0.97, and the values of the adjusted determination coefficients ($R^2_{adjusted}$) were equal to 0.99 and 0.92, respectively, for total phenolic contents and the antioxidant activity. These values showed a close agreement between the experimental results and the theoretical values envisaged by the polynomial models.

Neglecting the non-significant terms ($p>0.05$), the final predicted second-order polynomial equation for TPC and AA are given in Eqs. 2 and 3, respectively.

$$TPC=4.917+0.273X_1+0.121X_2+0.404X_3-0.295X_1X_2+0.040X_1X_3-0.157X_2X_3+0.128X_1^2-0.055X_2^2-0.235X_3^2 \quad (2)$$

$$AA=2.110+0.124X_1+0.146X_2+0.073X_3-0.106X_1^2-0.151X_2^2 \quad (3)$$

The above mentioned mathematical equations demonstrate that the three linear factors were significant at $p<0.05$ level for both phenolic extraction and antioxidant activity. However, all TPC factor interactions were significant at $p<0.05$. As regards the quadratic effect, the three TPC quadratic factors were also significant at $p<0.05$, while only ethanol concentration and irradiation time quadratic factors were significant at $p<0.05$ level of antioxidant activity. Furthermore, the same equations show that the linear factors influence positively both TPC and AA responses, while quadratic factors X_2X_2 influence negatively both responses.

Table 1. Experimental data and predicted values for total phenolic contents and antioxidant activity in *OFI* extract using microwave-assisted extraction (MAE).

				TPC*		AA*	
Run	**Factors**			**Experimental data**	**Predicted**	**Experimental data**	**Predicted**
	X_1	**X_2**	**X_3**				
1	60	90	700	4.90	4.92	2.12	2.11
2	80	40	700	5.42	5.43	1.89	1.84
3	60	90	700	4.93	4.92	2.09	2.11
4	60	40	400	3.96	3.94	1.62	1.67
5	80	90	400	4.64	4.64	1.95	1.96
6	60	140	1000	4.98	5.01	2.15	2.10
7	40	90	400	4.16	4.17	1.80	1.78
8	60	90	700	4.92	4.92	2.12	2.11
9	60	40	1000	5.07	5.07	1.80	1.83
10	40	90	1000	4.90	4.90	1.86	1.85
11	40	40	700	4.30	4.30	1.60	1.58
12	40	140	700	5.15	5.13	1.83	1.88
13	80	140	700	5.09	5.09	2.09	2.12
14	80	90	1000	5.54	5.53	2.15	2.17
15	60	140	400	4.50	4.50	2.01	1.98

*mg GAE/g DW. X_1: Ethanol concentration (%V/V); X_2: Extraction time (s); X_3: Power (W).

The optimal conditions were determined by using the JMP prediction profiler. The results regarding the optimized conditions of microwave-assisted extraction (MAE) in the case of total phenolic contents were: ethanol 56.05%, 131.45 seconds,

869.63 Watt, with 5.05 predicted responses; the experimental value was 5.22±0.12 mg GAE/g DW. On the other hand, the predicted conditions in the case of DPPH antioxidant activity were 73.71%, 112.13 s and 916.64 W for (X_1, X_2, and X_3). The predicted and the experimental responses were 2.21 and 2.30±0.27 mg GAE/g DW respectively. These results show that the irradiation time of the predicted AA was shorter than that of predicted TPC, because longer irradiation duration without temperature control would result in a thermal decomposition of phenolics. The experimental and predicted responses were close. Hence, these results suggest that the models could function well for prevision of antioxidant extraction by MAE from *OFI* seeds.

Table 2. Analysis of variance (ANOVA) for the fitted quadratic polynomial models

	DF	Sum of Squares	F value	*p* value	Coefficients of determination
TPC	Model 9 Lack of fit 3 Pure error 2	Model 2.7600 Lack of fit 0.0014 Pure error 0.0005	Model 832.595 Lack of fit 1.9643	Model <0.0001* Lack of fit 0.3549	R^2 0.99 Adj. R^2 0.99
AA	Model 9 Lack of fit 3 Pure error 2	Model 0.4466 Lack of fit 0.014 Pure error 0.0006	Model 17.785 Lack of fit 15.528	Model 0.0028* Lack of fit 0.0611	R^2 0.97 Adj. R^2 0.92

Response surface plots

The effects of the independent variable and their mutual interactions on TPC and AA can be visualized on the generated three-dimensional response surface plots shown in Figure 1 (A, B and C) in the case of total phenolic contents, Figure 1 (A', B' and C') in the case of the antioxidant activity.

According to Figure 1 (A and A'), the TP content increased when ethanol concentration and the extraction time increased. This is in agreement with the results found by Liu *et al*. (2013). The same observation was found in the case of AA. Figure 1 (B and B') shows that there is interactive influence between the ethanol concentration and the extraction power. When ethanol concentration and the power increase, the TPC and AA increase too. The high power input facility extraction of bioavailable compounds become available quickly, due to the increase in the amount of energy transferred to the substrate, through the rapid heating of the solvent and the rupturing of the cell walls (Ahmad and Langrish, 2012). As regarding the interactive influence of time and power on microwave extraction (Figure 1 C and C'), the phenolic content increases proportionally with increasing microwave power. This result is in agreement with those found by Xiao *et al*. (2008). However, high microwave power might cause poor extraction due to

the thermal degradation of labile compounds. As power level alone does not give sufficient information, so overexposure to microwave radiation even at low operating power was found to decrease the extraction yield due to the loss of chemical structure of the active compounds and oxidation (Chan *et al*., 2011). The high microwave power for a short time may be the most effective way to extract phenolic compounds (Ballard *et al*., 2010).

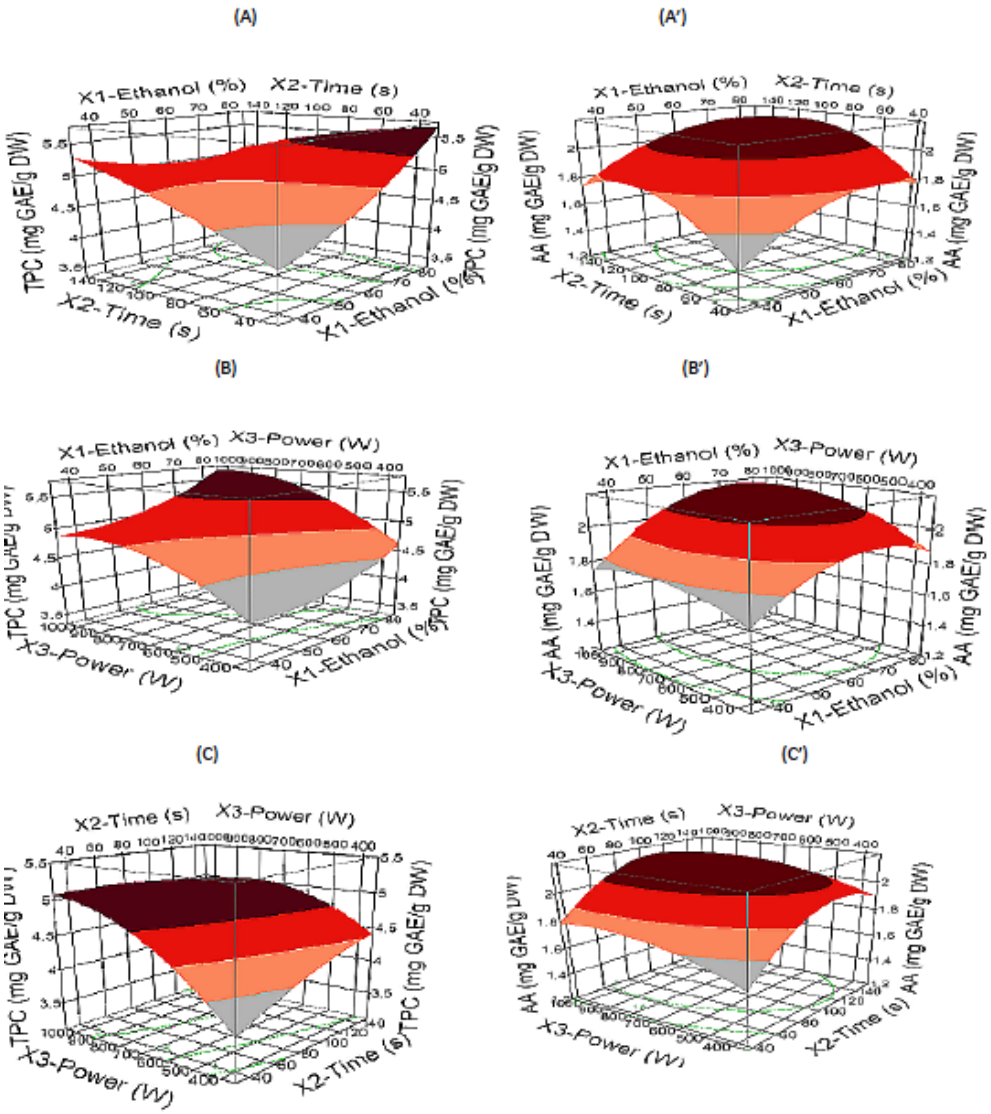
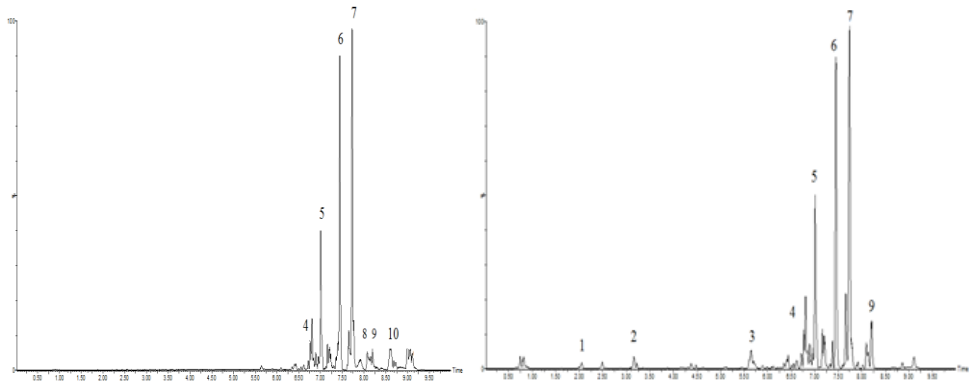


Figure 1. Response surface plots showing the effects of ethanol concentration, extraction time and microwave power on TPC extraction (A, B and C) and the antioxidant activity (A', B' and C') from prickly pear seeds.

Phenolic compound profile of the OFI seed optimized extracts

The phenolic profiles of the *OFI* seeds extracts used for assaying the TPC and AA were further compared as shown in Figure 2.

Figure 2 shows that the profile phenolic was influenced by the different MAE extraction condition, where seven compounds were identified in the case of TPC and eight compounds were identified in the case of AA under optimal conditions respectively. Coumaric acid, amentoflavone, and lupinisoflavone were only found in AA extract. On the other hand, kaempferol-3-O-rutinoside and 1,2 Di-O-sinapoyl glucose were only found in TPC extract. The following compounds: isorhamnetin, myricetin, chlorogenic acid, ferulic acid glucoside, ferulic acid 4-glucuronide were found in the two extracts, but in different amounts, except for myricetin, all other phenolic compounds were in higher amount in AA under optimal conditions than TPC extract. Ferulic acid glucoside was the prevalent compound found in *OFI* seed extracts.



A B

Figure 2. HPLC–ESI-MS chromatograms profile of OFI seed extracts for A: TPC, B: AA. (1) Coumaric acid; (2) Amentoflavone ;(3) Lupinisoflavone (4) Isorhamnetin ; (5) Myricetin ; (6) Chlorogenic acid ; (7) Ferulic acid glucoside; (8) Kaempferol-3-O-rutinoside ; (9) Ferulic acid 4-glucuronide; (10) 1,2 Di-O-sinapoyl glucose.

There are limited publications about the identification of compounds from OFI seeds. Chougui *et al*. (2013) have tried to identify them, but they found only feruloyl derivation and sinapoyl-diglucoside. Isorhamnetin was identified by Chahdoura *et al*. (2015) in *Opuntia microdasys* from Tunisia. It is the first time where the following compounds are identified in *OFI* seeds: coumaric acid, amentoflavone, lupinisoflavone, myricetin, chlorogenic acid, kaempferol-3-O-rutinoside. However, some of these compounds have been already identified by (Alimi *et al*., 2013; Guevara-Figueroa *et al.*, 2010); Moussa-Ayoub *et al*. (2011) in the juice, napal, and peel of prickly pear respectively.

Margarines developed with OFI seed extract

The humidity and the peroxide value contents for the four types of margarines M_0, M_1, M_2 and M_3 were very close, and all the values of the physicochemical

properties of margarines were within the standard range (Table 3). That means that these margarines were marked at same conditions (the proportion were respected). The pH values of the aqueous phase of these four margarines were also very close and in the standard range, because of the amount of lactic acid added.

Table 3. Physicochemical properties of developed margarines.

Parameter	Values	Standard ISO662 (1998-90-1)	Parameter	Values	Standard ISO662 (1998-90-1)
Humidity (%)			***pH***		
M_0	15.35±0.04[a]		***M_0***	4.50±0.12[a]	
M_1	15.33±0.06[a]	16	***M_1***	4.49±0.01[a]	4-5.5
M_2	15.21±0.04[a]		***M_2***	4.48±0.03[a]	
M_3	15.33±0.05[a]		***M_3***	4.48±0.02[a]	
Peroxide index (meq/kg)					
M_0	0.27±0.02[a]				
M_1	0.28±0.02[a]	10			
M_2	0.33±0.01[b]				
M_3	0.30±0.02[ab]				

M_0: margarine reference with vitamin E; M_1: margarine with 50 ppm of *OFI* seeds; M_2: margarine with 100 ppm of *OFI* seeds; M_3: margarine with 150 ppm of *OFI* seeds.

Results are reported as means ± S.D.

a-b: different letters indicate statistically significant differences at $p<0.05$ according to ANOVA and Tukey's post-hoc test.

Rancimat accelerates the aging process of the sample by exposing it to heat and air, and it measures the induction time or oxidation stability index. The induction time is determined by conductimetry, it corresponds to the time during which the fat has resisted to the oxidative stress. Thanks to the minimal labor requirement, the rancimat method is suitable not only for the determination of the oxidative stability of fats and oils but also for the investigations of antioxidants (Läubli and Bruttel, 1986).
The rancimat results were represented as a curve (conductivity as a function of time) in Figure 3.

The induction time obtained for the four margarines: margarine with vitamin E and with *OFI* seeds at different concentrations 50, 100 and 150 ppm, were respectively equivalent to 16.90±0.01, 16.87±0.02, 18.57±0.02 and 21.86±0.01 hours. It is noticeable that already 50 ppm of *OFI* seeds prickly pear has similar stability than margarine with synthetic antioxidant (M_0: with Vitamin E as synthetic antioxidant). The oxidative stability of margarine increases with the increasing *OFI* seeds concentration from 50 up to 150 ppm. The best stability was found using 150 ppm of *OFI* seed extracts. Chougui *et al.* (2015) have investigated the incorporation of prickly pear peels into the margarine, and the results showed that their margarine had a 1.16 hours oxidative stability compared to the margarine reference (vitamin E). These results were lower than the present investigation, where *OFI* seed extract have 4.96 hours oxidative stability compared to the reference margarine. Thereby

confirm that the antioxidants from *OFI* seeds are an alternative to the synthetic ones.

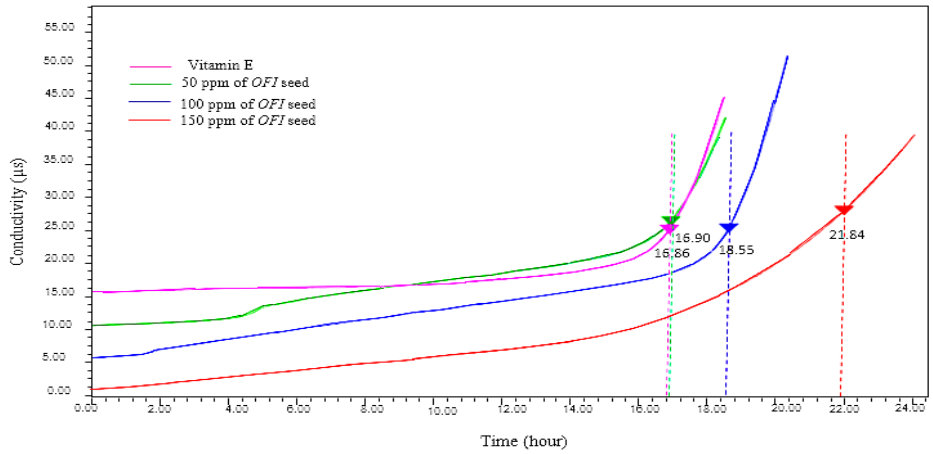


Figure 3. Rancimat curve results for margarines with vitamin E and *OFI* seeds.

Conclusions

The optimization of microwave-assisted extraction procedure for phenolic compounds and antioxidant activity of *Opuntia ficus-indica* seeds were successfully examined using response surface methodology. The optimized condition was validated and found to be fitted with the experimental values. The second-order polynomial models gave satisfactory results, showing that the models could function well for the forecast of antioxidant extraction by MAE from the prickly pear seeds. Ten compounds were found in *OFI* seeds, where six of them were identified for the first time in prickly pear seeds. Extraction process parameters influence the quality and quantity of phytochemical extraction compounds. *OFI* seed extracts incorporated in margarine showed higher oxidative stability than vitamin E, with an improvement of 4.96 hours according to rancimat measurements. Thereby confirm that *OFI* seeds are an excellent source of natural antioxidants that can be used as a natural additive in food sectors.

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